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Diagnosis and Epidemiological Association of *Listeria monocytogenes* Strains in Two Outbreaks of Listerial Encephalitis in Small Ruminants

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Two outbreaks of epizootic listerial encephalitis, one in sheep and one in goats, were investigated through pathology, microbiology, and DNA amplification-based techniques. Efforts were made to survey the diversity of *Listeria monocytogenes* strains in the silage consumed by affected animals and to verify the causal relationship between silage and disease outbreak. In both outbreaks, *L. monocytogenes* was isolated from silage and brain tissue samples. Random amplified polymorphic DNA patterns revealed two distinct *L. monocytogenes* strains, one of which was identical to the sheep brain isolate, in the silage associated with the outbreak in sheep. Three brain isolates and one silage isolate, all of which had different random amplified polymorphic DNA patterns, were found in the outbreak involving goats. All isolates from both outbreaks were indistinguishable in an in vitro assay for cell-to-cell spread and growth in macrophages. All brain isolates from the goat outbreak had identical intracellular ActA patterns, which were different from the pattern for the silage isolate. While the sheep brain isolate had an ActA pattern different from that of the corresponding silage isolate, the patterns for the brain isolates from the two outbreaks were not identical. This survey demonstrates the diversity of *L. monocytogenes* in silage and suggests the existence of one or more selective processes by which certain strains are more prone to give rise to disease.

Listeria monocytogenes is an important pathogen in humans as well as in animals (8, 13). The incidence of listeriosis in ruminants has markedly increased over the last few decades (1, 17, 22), and feeding of poor-quality silage seems to be a key factor in disease transmission (9, 25). Epidemiological surveys of listeriosis outbreaks, mainly in sheep, were conducted by using a variety of typing procedures, including phage typing (25), pyrolysis mass spectrometry (17), and multilocus enzyme electrophoresis (2). Using these techniques, only a few groups were able to isolate matching *L. monocytogenes* strains from clinical samples (usually brain tissue, cerebrospinal fluid [CSF], or aborted material) and from samples of silage implicated as the outbreak source (17, 25); in other cases, only distinct *L. monocytogenes* strains were isolated from these sources (10, 17, 18). These findings gave rise to speculation about the origin of these disease-producing strains. Some authors assumed that there is often no relationship between silage containing *L. monocytogenes* and outbreaks of listeriosis (12, 18), while others suspected preferential growth of some strains during enrichment (25). We investigated two outbreaks of listeriosis in small ruminants by performing genetic and biochemical characterization of *L. monocytogenes* strains isolated from clinical and feed samples by random amplified polymorphic DNA (RAPD) patterns (6), in vitro cell culture assays, and examination of ActA species and their intracellular phosphorylation patterns.

In addition, we utilized a nested PCR targeting the listeriolysin O gene (*hly*) (4) and a PCR-coupled ligase chain reaction (LCR) targeting *L. monocytogenes*-specific sequences in the 16S rRNA gene (26, 27) for specific detection of *L. monocytogenes* from CSF, brain tissue, environmental, and feed samples. These methodologies helped in the rapid identification of the source of *L. monocytogenes*, which when eliminated resulted in cessation of the respective outbreaks.

MATERIALS AND METHODS

Cases and animals. Two outbreaks of listeriosis occurred during the spring of 1993, which was preceded by a very wet summer and fall in 1992. An outbreak in sheep involved a herd of 48 Dorset sheep, 4 to 5 years old, of which two mature ewes were admitted to the ambulatory clinic at Cornell University Veterinary College. These animals showed symptoms of central nervous system complication such as circling, decreased tongue tone, diminished facial sensation (involving nerve V), facial nerve paralysis, and depression. Both ewes were hospitalized and treated with procaine penicillin G, rifampin, and thiamine but showed no improvement after initiation of the treatment. All of the sheep on the farm were fed grass hay supplemented with grass haylage throughout the preceding three months. Approximately 5 ml of CSF was obtained from both sheep antemortem (after initiation of treatment) for diagnostic purposes. Sheep 1 died in the clinic after 24 h, and sheep 2 was euthanatized 48 h after hospitalization. To confirm the outbreak source, two silage samples were obtained. Extensive fungal growth was found, and the pH was between 7.5 and 8.0, which demonstrated the poor quality of this feed. Silage feeding was discontinued immediately, and no other sheep were affected.

An outbreak in goats occurred in a commercial dairy goat herd of approximately 180 animals. About 1 week before the occurrence of the first case, the owner started feeding clover baylage (haylage baled in plastic foil) purchased from another

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farm. Over a 3-month period, eight adult goats developed typical signs of listeriosis, and seven (designated goats 1 to 7) died or were euthanatized while one (designated goat 8) survived after being treated subcutaneously with procaine penicillin G. The clinical signs in all affected animals included unilateral facial paralysis; some animals exhibited circling. After a change in the silage fed, no listeriosis cases were observed for approximately 2 months, after which a second episode of four presumptive listeriosis cases (goats 9 to 12) occurred over a 6-week period. Starting approximately 3 weeks before this second episode, the goats were fed only grain and hay but no silage. To track the source and spread of *L. monocytogenes*, 12 feed (baylage and grain) and environmental samples (samples 1 to 12) as well as 2 milk samples (samples 13 and 14) from acutely infected animals were collected during the first episode and 9 feed (grain and hay) and environmental samples (samples 15 to 23) and one bulk milk sample (sample 24) were collected during the second episode.

In the pathological examinations, gross lesions were found in the brain of only one goat, while histological examination revealed signs typical for listerial encephalitis in all investigated animals from both outbreaks.

Isolation procedures for *L. monocytogenes*. For the two sheep with clinical signs, 100 μl of CSF was cultured by either nonselective preenrichment in tryptic soy broth (TSB) (Sigma, St. Louis, Mo.) followed by selective enrichment in listeria enrichment broth (LEB) (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.) and subsequent plating on Oxford agar (Unipath Ltd., Basingstoke, Hampshire, United Kingdom) or cold enrichment at 4°C in TSB with subsequent plating on Oxford agar once every 2 weeks for 6 weeks. Between three and five single colonies typical for *Listeria* spp. (on the basis of esculin hydrolysis and colony morphology) were subcultured in TSB, and fermentation tests using xylose, mannitol, and rhamnose (16) were performed. Hemolysin production was determined by using a microtiter plate format (7).

In most cases, samples from the medulla oblongata were obtained postmortem for bacteriological diagnosis (see Table 1). The isolation was performed as described above for the CSF samples; at least two different areas from the brain were selected for separate enrichments.

To clarify the origin and distribution of *L. monocytogenes*, isolation of listeriae from the feed and environmental samples was attempted by using an initial selective enrichment of 10 g in 90 ml of LEB, followed by subculturing of a 0.01% aliquot in 9 ml of LEB and subsequent plating on Oxford agar. *L. monocytogenes* was also isolated from silage samples by direct plating on modified *Listeria*-selective agar medium except that the blood agar overlay step was omitted (11, 25). In both procedures, 10 to 20 independent suspect *Listeria* colonies per sample were isolated and fermentation patterns and hemolysis were determined.

PCR and PCR-coupled LCR. For the specific detection of *L. monocytogenes*, a nested PCR-reverse dot blot (PCR-RDB) assay targeting *hly* was performed with the internal primers α1 and β1 and the external primers αn and βn (4). CSF samples were prepared for PCR-RDB as described by Jaton et al. (14). Suspect *Listeria* colonies from Oxford agar were confirmed as *L. monocytogenes* by the PCR-RDB assay and a PCR-coupled LCR assay specific for *L. monocytogenes* (26, 27).

To quantify the *L. monocytogenes* present in representative silage samples, a modified three-tube most-probable-number technique was used. For this purpose, 10 g of silage was homogenized in 90 ml of LEB, and 1 ml of serial dilutions was inoculated in triplicate in 9 ml of LEB. After 24 h of incubation at 37°C, 1 ml of this enrichment was used to prepare a crude lysate for the PCR-RDB assay.

To allow fast detection of *L. monocytogenes* in the feed and environmental samples from the outbreak in goats, a PCR-RDB assay on a crude lysate from a 1-ml aliquot of the second enrichment in LEB was performed.

Southern hybridization. Isolation of DNA and Southern hybridization were performed according to standard protocols (20, 24). Probes were labeled during PCR with 2.5 μM digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, Ind.) by using *hly* primers αn and βn. The Southern blot was developed with antibodies against digoxigenin and the chemiluminescent substrate Lumi-Phos 530 (Boehringer Mannheim) according to the manufacturer's recommendations for the Genius kit (Boehringer Mannheim).

RAPD. RAPD patterns were generated to clarify the relationship among isolates from both outbreaks. A total of 10 different RAPD primers (OPA-1 to OPA-10; Operon Technologies, Alameda, Calif.) were tested on crude cell lysates (6). Patterns that differed consistently in at least one major band for at least one of the primers tested were classified as a different RAPD type (each type was denoted by a different letter [see Tables 1 and 2]).

Cell culture conditions and intracellular protein expression assay. Monolayers of J774 cells were infected with selected *L. monocytogenes* isolates as previously described (21), and their behavior was visually examined.

Intracellular protein expression in J774 cells was determined by using protein synthesis inhibitors and [^{35}S]methionine (3). Labeled proteins were electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (8% polyacrylamide), followed by fluorography (En^{3}Hance; Du Pont, Wilmington, Del.).

RESULTS

Isolation of *L. monocytogenes*, PCR, and PCR-coupled LCR. *L. monocytogenes* was not detected in the two CSF samples from the sheep by culturing in TSB and LEB or by cold enrichment. However, the CSF sample from sheep 1 gave a positive result in the PCR-RDB assay (Table 1). *L. monocytogenes* cultured on Oxford agar (after enrichment in TSB and LEB) and confirmed by PCR-RDB and PCR-coupled LCR was isolated from the brain tissue from sheep 1 (Table 1) and from three of four brain tissue samples from goats (Table 1). *L. monocytogenes* was isolated from the CSF as well as from the brain tissue of goat 12 (Table 1).

By the modified most-probable-number technique, 4.8 × 10^5 and 4.6 × 10^4 *L. monocytogenes* cells per g were found in the silage consumed by the sheep and goats, respectively. Seventeen PCR-RDB- and PCR-coupled LCR-positive *L. monocytogenes* isolates were obtained from the silage samples from each of the two outbreaks by screening of the colonies obtained by either direct plating, cold enrichment, or enrichment in LEB (Table 2). *Listeria innocua* was identified by hemolysis and fermentation tests on *Listeria*-like esculin-positive colonies in the silage consumed by the sheep.

To rapidly identify the source of *L. monocytogenes*, the nested PCR-RDB assay was used to screen crude lysates from the second preenrichment of feed and environmental samples from the outbreak in goats. Six of seven silage samples were positive for *L. monocytogenes*, while all other feed and environmental samples were negative (Table 2). Spiked PCR samples confirmed that the PCR was not inhibited. Two milk samples from goats showing signs of listeriosis and one bulk milk sample, which were tested to investigate possible shedding, were negative for *L. monocytogenes*.

TABLE 1. Recovery and characterization of *L. monocytogenes* strains from animals with clinical signs of listeriosis

Outbreak and animal no.	PCR-RDB result (CSF sample)	*L. monocytogenes* isolate(s) recovered from:		Serotype	RAPD pattern(s) for isolate(s)[a]
		CSF	Brain tissue		
Sheep					
1	Positive	None	CU-BR1/93 and CU-BR2/93	1/2a	A
2	Negative	None	NT[b]	NT	NT
Goat[c]					
1	NT	NT	CU-BR3/93 to CU-BR8/93	1/2b	C
2	NT	NT	None	NT	NT
3	NT	NT	CU-BR27/93	ND[d]	D
12	NT	CU-BR47/93 to CU-BR49/93	CU-BR32/93 to CU-BR46/93	ND[e]	F, D[f]

[a] Distinctive RAPD patterns were designated by consecutive letters.
[b] Not tested (sample not available).
[c] Only 4 of 12 affected animals were examined.
[d] Not determined.
[e] Applies to both CSF and brain tissue isolates.
[f] The CSF isolates gave pattern D, and the brain tissue isolates gave pattern F.

Multiple esculin-positive silage isolates from both outbreaks gave rise to two spurious PCR products of 750 and 600 bp upon amplification with the external *hly* primers αn and βn. These amplicons are smaller than the 963-bp product expected for *L. monocytogenes hly* with these primers. These isolates showed no hemolysis, had fermentation patterns compatible with *L. innocua* (mannitol negative, xylose negative, rhamnose positive), and were positive for the initial *Listeria*-specific 16S rRNA gene PCR but negative in the *L. monocytogenes*-specific LCR step which followed (27). When a representative (CU-SI9/93) of these isolates was tested with other *hly* primers internal to αn and βn, amplification products slightly larger than expected for the *L. monocytogenes hly* were observed (data not shown). This isolate also gave a weak background reaction in the PCR-RDB assay. To further clarify the origin of these spurious amplicons, two representatives (CU-SI9/93 and

TABLE 2. Recovery and characterization of *L. monocytogenes* strains from silage and environmental samples

Outbreak and sample no.	Origin of sample	PCR-RDB result[a]	*L. monocytogenes* isolate(s) recovered	RAPD pattern for isolate(s)[b]
Sheep				
1[c]	Silage	ND	CU-SI10/93	B
			CU-SI15/93	B
			CU-SIMC21/93 to CU-SIMC24/93	B
			CU-SIMC31/93 to CU-SIMC37/93	B
			CU-SIDI-5	B
2	Silage	ND	CU-SIMCII-1	B
			CU-SIDII-1	B
			CU-SIDII-7	A
Goat				
1[d]	Baylage	+	CU-SIK17/93	E
			CU-SIK18/93	E
			CU-SIK35/93	E
			CU-SIK50/93	E
2	Baylage	+	CU-SIK19/93	E
			CU-SIK20/93	E
3	Baylage	+	CU-SIK97/93 to CU-SIK107/93	E
4	Baylage	+	None	NT[e]
5	Baylage	−	None	NT
6	Floor	−	None	NT
7	Straw	−	None	NT
8	Baylage	+	None	NT
9	Baylage	+	None	NT
10 to 12	Grain	−	None	NT
13 to 14	Milk[f]	−	None	NT
15 to 18	Grain	−	None	NT
19 to 20	Water supply	−	None	NT
21 to 23	Floor	−	None	NT
24	Bulk milk	−	None	NT

[a] ND, not determined; +, positive result; −, negative result.
[b] Distinctive RAPD patterns were designated with consecutive letters.
[c] By the modified most-probable-number technique, 4.8×10^5 *L. monocytogenes* cells per g of silage were found (see Materials and Methods).
[d] By the modified most-probable-number technique, 4.6×10^4 *L. monocytogenes* cells per g of silage were found (see Materials and Methods).
[e] Not tested (sample not available).
[f] From goats 4 and 8.

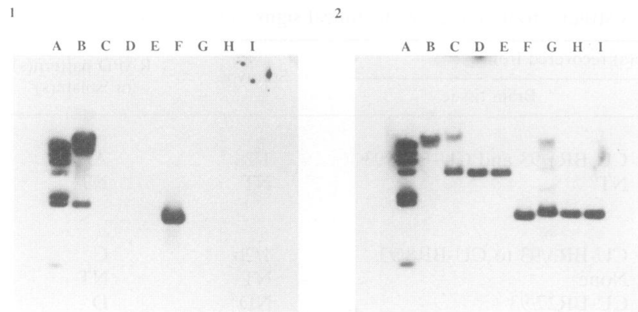

FIG. 1. Southern hybridization of *L. monocytogenes* Scott A, *L. innocua* P5V5, and silage isolates CU-SIMC29/93 and CU-SI9/93. (Panel 1) Blots were probed with a digoxigenin-labeled 963-bp αn- and βn-generated authentic *hly* fragment from Scott A. Lanes: A, digoxigenin-labeled λ phage DNA, *Hin*dIII digested, with molecular weights of 23,130, 9,416, 6,557, 4,361, 2,322, 2,027, 564, and 125; B, Scott A, *Eco*RI digested; C, *L. innocua* P5V5, *Eco*RI digested; D, CU-SIMC29/93, *Eco*RI digested; E, CU-SI9/93, *Eco*RI digested; F, Scott A, *Ssp*I digested; G, *L. innocua* P5V5, *Ssp*I digested; H, CU-SIMC29/93, *Ssp*I digested; I, CU-SI9/93, *Ssp*I digested. (Panel 2) Blots were probed with a digoxigenin-labeled 600-bp αn- and βn-generated PCR fragment from CU-SIMC29/93. Lanes are as described for panel 1.

CU-SIMC29/93) of these isolates, *L. monocytogenes* Scott A and *L. innocua* P5V5, were examined by Southern hybridization using either the authentic amplified 963-bp *hly* fragment (from Scott A) or the 600-bp spurious PCR fragment (amplified with primers αn and βn from CU-SIMC29/93) as probes (Fig. 1). The Southern blot results show that isolates CU-SIMC29/93 and CU-SI9/93, presumptive *L. innocua*, do not contain sequences with overall homology to the *L. monocytogenes hly*.

RAPD. All silage, brain, and CSF isolates from both outbreaks were typed by their RAPD patterns. After an initial screening of 10 primers, the primers OPA-2, OPA-3, OPA-7, OPA-8, and OPA-10 were selected for further use, on the basis of their ability to generate diverse yet clear RAPD patterns. Multiple brain isolates from individual animals always had the same RAPD patterns (Table 1).

Of the 17 silage isolates from the outbreak in sheep, only 1 (designated CU-SIDII-7) had the same RAPD pattern (pattern A) as the corresponding brain isolates (CU-BR1/93 and CU-BR2/93). The other 16 isolates were all identical to each other (RAPD pattern B) yet different from the brain isolates. Representative RAPD patterns for one brain isolate and the silage isolates are shown in Fig. 2, lanes B to I.

On the basis of their RAPD patterns, the *L. monocytogenes* isolates from brain tissue of the three goats were different for each animal as well as distinct from all silage isolates recovered from this outbreak (Tables 1 and 2). Representative RAPD results for silage isolates and brain isolates from goats 1 and 3 are shown in Fig. 2, lanes K to V. Surprisingly, RAPD patterns for brain isolates (CU-BR32/93 to CU-BR46/93) from goat 12 were different from the patterns for the CSF isolates (CU-BR47/93 to CU-BR49/93) from the same animal. Furthermore, these CSF isolates from goat 12 had RAPD patterns identical to those of the brain isolate from goat 3 (CU-BR27/93). Despite numerous attempts with cold enrichment, direct plating, or selective enrichment in LEB, an *L. monocytogenes* isolate with a RAPD pattern identical to that of either of the goat brain or CSF isolates could not be recovered from the available silage samples.

In vitro pathogenicity of isolated strains. To screen for differences in virulence, one representative of each brain and

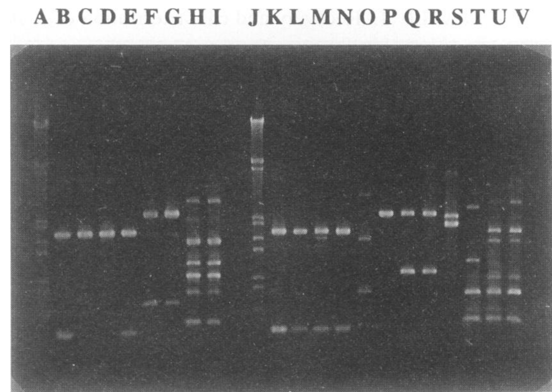

FIG. 2. Representative RAPD patterns for brain and silage isolates from the outbreak in sheep (lanes B to I) and the outbreak in goats (lanes K to V). Reactions in lanes B to E and K to N were performed with primer OPA-10; those in lanes F and G and O to R were performed with primer OPA-2; and those in lanes H and I and S to V were performed with primer OPA-3. The isolates tested were CU-BR1/93 (lanes B, F, and H), CU-SIMC31/93 (lane C), CU-SIMC33/93 (lane D), CU-SIDII-7 (lanes E, G, and I), CU-BR3/93 (lanes K, O, and S), CU-BR27/93 (lanes L, P, and T), CU-SIK17/93 (lanes M, Q, and U), and CU-SIK19/93 (lanes N, R, and V). Lanes A and J contain λ phage digested with *Hin*dIII and *Eco*RI, with molecular weights of 21,227, 5,148, 4,973, 4,268, 3,530, 2,027, 1,904, 1,584, 1,375, 947, 831, and 125.

CSF strain as well as representative silage isolates (CU-SI10/93, CU-SIMC22/93, CU-SIDII-7, and CU-SIK17/93) were examined for their behavior in a macrophage cell line (J774). All isolates, as well as the laboratory strain *L. monocytogenes* 10403S (21), showed by visual examination identical characteristics in growth and cell-to-cell spread in J774 cells (data not shown).

Intracellular protein expression. Representative results from the assay for ActA species and their intracellular phosphorylation inside J774 cells are shown in Fig. 3. As previously demonstrated (3), the prominent bands with a molecular mass around 90 kDa represent multiple bands of ActA, due to its phosphorylation. The brain isolate CU-BR1/93 (Fig. 3, lane A) showed three ActA bands of slightly lower molecular weight than the ActA bands in the silage isolates (lanes B and C) and *L. monocytogenes* 10403S (lane D). CU-BR1/93 (Fig. 3, lane A) and the silage isolate CU-SIDII-7 (not shown) had identical ActA patterns, which differed from those of the two silage isolates (CU-SI10/93 and CU-SIMC22/93) from the outbreak in sheep. From the outbreak in goats, all brain and CSF isolates had ActA patterns identical to those of strain 10403S, while a representative silage isolate had the same ActA pattern as CU-BR1/93.

DISCUSSION

This report describes two epizootic outbreaks of encephalitic listeriosis in small ruminants which were diagnosed by pathology as well as classical and molecular bacteriology techniques. The molecular biology techniques used were particularly helpful for rapid detection of *L. monocytogenes* in preenrichment cultures from brain tissue and feed samples. The PCR-RDB assay gave positive results for *L. monocytogenes* on one CSF sample which was negative by culturing procedures. This positive result was supported by isolation of *L. monocytogenes* from a brain sample from the same animal. The discrepancy between culturing and PCR-RDB results for the CSF sample could be explained by the presence of nonviable bacteria (due

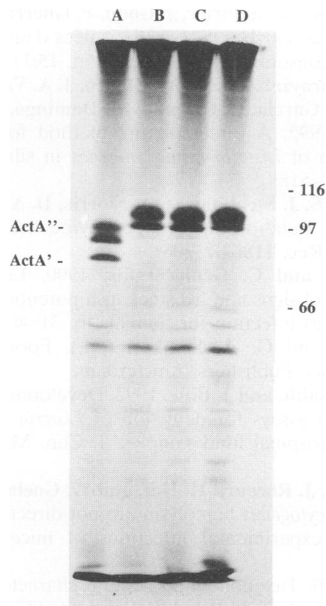

FIG. 3. Intracellular ActA expression in J774 cells, determined by fluorography of an SDS–8% PAGE gel (for details, see Materials and Methods). Lanes: A, CU-BR1/93; B, CU-SI10/93; C, CU-SIMC22/93; D, 10403S. Molecular masses (in kilodaltons) are marked on the right. The locations of the unphosphorylated ActA are indicated by ActA′ (for lane A) and ActA″ (for lanes B to D).

to freezing of the CSF sample), which would still give a positive result in the PCR-RDB.

The DNA amplification techniques also assisted in the rapid identification of the outbreak source, preventing further exposure of the animals to contaminated feed. The nested *hly* PCR-RDB showed a weak background reaction with a single presumptive *L. innocua* strain isolated from silage, while a PCR-coupled LCR (27) allowed correct identification of this strain. In contrast, this PCR-RDB assay correctly differentiated over 65 different *L. monocytogenes* isolates representing five serotypes from all other listeriae, including five *L. innocua* strains (4, 5). The reason why this particular *L. innocua* isolate gave spurious results is not clear. This, however, demonstrates the potential problems when restricted characteristics are used for the identification of bacterial species.

In order to determine the outbreak sources and the distribution of *L. monocytogenes*, feed and environmental samples from the two farms were investigated. In silage samples from both outbreaks, large numbers of *L. monocytogenes* (4.6×10^4 to 4.8×10^5 cells per g) were found. Silage supplies with more than 1.2×10^4 *L. monocytogenes* cells per g were previously implicated as sources of other listeriosis outbreaks (9, 11, 25), while silage containing approximately 1.5×10^3 *L. monocytogenes* cells per g did not cause listeriosis in animals consuming it (11). None of the other feed or environmental samples were positive for *L. monocytogenes*, suggesting that dissemination of this bacterium by environmental contamination from infected animals played no role in the spread of the disease at the times samples were taken. Previous reports, on the other hand, described evidence for spread of *L. monocytogenes* by aerosol or feces and other excretions from symptomatic and asymptomatic animals (reviewed in reference 13).

The *L. monocytogenes* isolates from both outbreaks were initially typed by RAPD analysis. RAPD patterns revealed the presence of two different *L. monocytogenes* strains, one of which was identical to the isolates from the sheep brain, in the silage implicated in the outbreak in sheep. This strain was recovered from a silage sample only by direct plating on *Listeria*-selective agar medium. The brain strain (CU-BR1/93) and a representative of the additional silage strains (CU-SI10/93) from the outbreak in sheep both belong to serotype 1/2a. This exemplifies the greater discrimination of RAPD typing in comparison with serotyping.

The presence of two distinct strains in the silage, the suspected origin of the infection with *L. monocytogenes*, could be explained by two theories: (i) infection with the strain recovered from the brain occurred randomly, either with the two strains being present concurrently in the silage or with each strain being locally concentrated, or (ii) the strain recovered from the brain is more virulent than the other strain present in the silage in encephalic infections of sheep. The hypothesis of local concentration of the strains is unlikely, since both strains were isolated from the same small silage sample. In order to characterize the virulence of the different isolates, their behavior was assessed in a macrophage cell line. All tested strains were indistinguishable and showed behavior typical for *L. monocytogenes*, including cell-to-cell spread and growth characteristics. To our knowledge, there is only one report of distinct *L. monocytogenes* strains in silage samples implicated in a disease outbreak (17); other reports identified all isolates from one silage sample as identical by phage typing (11, 25).

The inability to find matching *L. monocytogenes* isolates in feed and brain samples from the outbreak in goats is consistent with some previous observations (10, 17, 18). Possible explanations for this phenomenon include the following: (i) the sampled silage was not directly responsible for the listeriosis outbreak (12, 18); (ii) there was preferential growth of one strain over the other during enrichment (25); (iii) two or more different strains, one of which was more virulent in goats, were present in the silage; and (iv) there were multiple sources of *L. monocytogenes* infection. Preferential growth during enrichment procedures can be excluded, since 15 of the isolates were isolated by direct plating (25). A higher degree of virulence would allow one strain to cause disease, even if it were present as only a small percentage of the total population of *L. monocytogenes*. Such a strain could therefore be overlooked by most isolation procedures. It is also possible that different *L. monocytogenes* strains were present at separate locations within the silage, which is likely when the silage is stored in separate bales. Therefore, the strain responsible for the disease outbreak may not be isolated since appropriate samples are no longer available. A second episode of listeriosis on this goat farm, 2 months later, could not be connected to feeding of contaminated silage. It might have been caused by spread of *L. monocytogenes* from infected animals or could be due to unusually long incubation periods. Likewise, both episodes could have been caused by an unidentifiable source other than the silage.

The isolation of two different *L. monocytogenes* strains from clinical samples from the same animal is rather surprising. Coinfection with two different *L. monocytogenes* strains seems rather unlikely, since in such a case both strains would be expected to be present in the brain. Confusion of strains is another possible, though unlikely, explanation for this observation.

actA encodes an approximately 90-kDa protein that is a key component in the interaction between *L. monocytogenes* and host cell microfilaments (19). Microheterogenicity in the molecular weights of ActA from different strains (19) and intracellular phosphorylation of the ActA polypeptide (3) have

been described previously. While all brain isolates from the outbreak in goats had identical ActA patterns, the pattern from the sheep brain isolate was different. Nevertheless, differences from the patterns of the corresponding silage isolates show that ActA patterns might be useful as an additional epidemiological marker.

Virulence differences between *L. monocytogenes* strains might play an important role with regard to the predominance of certain clusters in listeriosis outbreaks. Up to now, investigations of strain differences have focused on listeriolysin O; but no correlation of expression levels (15) or amino acid sequence differences (23) with virulence was observed. While it has been speculated that microheterogenicity in ActA molecular weights could be connected to virulence differences (19), our study could not find any support for this speculation. Characterization of additional clinical and food isolates with regard to virulence-related factors might give further insight into possible differences between silage and brain isolates and into the pathogenesis of encephalitic listeriosis.

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