JOURNAL OF CLINICAL MICROBIOLOGY, May 1996, p. 1086–1090 Vol. 34, No. 5
0095-1137/96/$04.00+0

Ribotype Diversity of *Listeria monocytogenes* Strains Associated with Outbreaks of Listeriosis in Ruminants

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Received 18 September 1995/Returned for modification 6 December 1995/Accepted 12 February 1996

Ribotyping is a molecular method for the characterization, identification, and typing of bacterial isolates that has value in epidemiological studies. To demonstrate the utility of this technique for typing of *Listeria monocytogenes*, four outbreaks of epizootic listeriosis in ruminants were investigated through coordinated detection and characterization methods utilizing classical microbiology and nucleic acid-based techniques. *L. monocytogenes* strains isolated from clinical samples and the silage consumed by the affected animals were ribotyped to establish the causal relationship between feed and the disease outbreak. For all but one outbreak, we were able to isolate *L. monocytogenes* strains represented by the same ribotype from both clinical and silage samples. Additional *L. monocytogenes* strains with ribotypes different from those of the respective clinical samples were isolated from all silage samples. This indicates that a diverse population of *L. monocytogenes* strains exists in farm environments, of which some may be more likely than others to cause disease.

Epidemiological studies of bacterial diseases require methods to differentiate isolates beyond the species and subspecies levels. A variety of typing procedures have been applied to different bacterial species. Current typing methods include serotyping (18), fermentation patterns (biotyping), antibiotic resistance patterns (10), pyrolysis mass spectrometry (14), fatty acid analysis (2), phage typing (16), and multilocus enzyme electrophoresis (4). Increasingly, methods which differentiate bacterial strains on the basis of characteristic nucleic acid sequences (so-called fingerprints) are also being utilized. Examples include plasmid typing (15), restriction enzyme analysis (24), random amplified polymorphic DNA (RAPD) (27), arbitrarily primed PCR (23), and rRNA gene fingerprinting (ribotyping), which provides species identification and typing (5, 11, 12). Although several methods of bacterial species typing have been investigated, most of them are still labor intensive and therefore do not easily allow rapid screening of a large number of isolates (17) and some are limited to a single species. Also, many typing methods show only a limited ability to discriminate between isolates while others are not able to type all isolates of a given species (e.g., plasmid typing and phage typing) (1, 15). These limitations restrict the use of typing methods for bacterial strains. A recently described ribotyping system (5, 12), for which a large database of *Listeria monocytogenes* strains and other bacterial species has been established, was applied in the study described here to identify and type strains collected from listeriosis outbreaks. This typing method is based on *Eco*RI digestion of chromosomal DNA followed by Southern hybridization probing with the *Escherichia coli rrnB* rRNA operon (5, 12) and has been shown to differentiate serotypes 1/2b and 4b in 12 and 6 different types, respectively (22).

This ribotyping system was evaluated within a sequential coordinated detection, identification, and typing system for *L. monocytogenes* strains in four disease outbreaks. A listeriosis outbreak was defined as the identified occurrence of disease involving one or more animals on an individual farm (20). *L. monocytogenes* was chosen as a model organism because it is an important cause of animal and human foodborne disease. An effective and easy-to-use identification and typing method would be a valuable tool for following the infection chain from animal feed via infected animals to food of animal origin and, ultimately, human disease cases.

A variety of previous reports have indicated that improperly fermented silage (i.e., that with a pH of >5.0) is the predominant source for listeriosis outbreaks in ruminants (14, 26). In this investigation, *L. monocytogenes* isolates obtained from different silos, as well as from different locations within a silo, during or after confirmed listeriosis outbreaks in ruminants were used to determine the diversity of *L. monocytogenes* strains in silage and clinical samples to further understand the epidemiology and pathogenesis of animal listeriosis (14).

MATERIALS AND METHODS

Cases and animals. Two outbreaks of listeriosis in sheep and goats were previously reported (26) (designated outbreaks 1 and 2; Table 1). Two additional outbreaks of listeriosis which occurred during 1994 are described below. On all of these farms, either no cases or only isolated single cases of listeriosis or suspected listeriosis had been reported for at least 2 years previous to the described outbreaks.

In one outbreak (designated outbreak 3), three animals in a herd of 250 Holstein-Friesian milking cattle and 150 heifers showed clinical signs compatible with listerial encephalitis. The milking cows were housed in two free-stall barns and fed haylage, corn silage, wet brewer's grain, corn meal, and a commercial protein supplement. Case 1 was observed at the end of February 1994, and cases 2 and 3 occurred in late April and the beginning of June 1994, respectively. Initial clinical signs in all three affected animals included rapid decline of food intake (as determined by an on-farm computerized feeding system) and decreased milk yields. Symptoms in the first two animals included hyperesthetic sensations, incoordination, facial muscle fasciculations, and loss of balance. The animals progressed to lateral recumbency and showed paddling behavior, at which time they were euthanized. Material for microbiological diagnosis was only available for case 2; isolate DL 773014 was obtained from a brain sample from this animal. Five 500-g samples of hay silage (designated F3/1 to F3/5; Table 2) were taken from a single bunker silo on 17 May 1994. Additionally, one 500-g sample of freshly prepared grass silage was taken from another bunker silo on 13 June 1994 (F3/11; Table 2).

Another outbreak (designated outbreak 4) involved at least four cases of suspected listerial abortion between 1 June and 2 July 1994 in a 180-head Holstein-Friesian dairy herd kept in free stalls. The affected animals aborted

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TABLE 1. Sources and ribotypes of isolated *L. monocytogenes* strains

Outbreak no., sample source, and sample no.[a]	*L. monocytogenes* isolate(s)	Ribotype
1, Sheep[b]		
Brain, C1/1	CU-BR1/93	dd 1067
Silage, F1/1	CU-SI10/93	dd 1151
Silage, F1/2	CU-SIMCII-1	dd 1151
	CU-SIDII-7	dd 1067
2, Goat[b]		
Brain, C2/1	CU-BR3/93	dd 0653
Brain, C2/3	CU-BR27/93	dd 0566
Brain, C2/12	CU-BR32/93	dd 1153
CSF,[c] C2/12	CU-BR47/93	dd 0566
Silage, F2/1	CU-SIK17/93	dd 0566
3, Bovine encephalitis		
Brain, C3/1	DL 773014	dd 0647
Silage, F3/1	CU-SI133/94	dd 1962
	CU-SI135/94	dd 1962
	CU-SI137/94	dd 1962
	CU-SI153/94	dd 0653
	CU-SI154/94	dd 0566
Silage, F3/2	CU-SI109/94	dd 1962
	CU-SI111/94–CU-SI115/94	dd 1962
	CU-SI120/94	dd 1151
Silage, F3/5	CU-SI122/94	dd 1153
	CU-SI129/94–CU-SI131/94	dd 1151
	CU-SI132/94	dd 1153
Silage, F3/11[d]	CU-SI161/94	dd 0566
4, Bovine abortion		
Fetus, C4/1	DL 872779-1	dd 0566
Silage, F4/9	CU-SI162/94	dd 1962
	CU-SI163/94–CU-SI164/94	dd 0566
	CU-SI165/94	dd 3581

[a] Clinical samples carry the prefix C; feed samples carry the prefix F.
[b] These outbreaks and isolates were previously described (26).
[c] CSF, cerebrospinal fluid.
[d] This sample was taken from fresh silage after the outbreak occurred.

between days 120 and 165 of gestation without further clinical signs. Additional terminated pregnancies were observed by rectal pregnancy examination during this time period; however, the aborted fetuses were not found. The cows were fed corn and grass silage supplemented with dry hay, corn meal, dried shell corn, roasted soybeans, and distiller's grains. Corn and hay silage samples (F4/1 to F4/9; Table 2; approximately 500 g per sample) were collected on 21 July, i.e., about 7 weeks after the last case was diagnosed. All corn silage samples (F4/2 to F4/8) were obtained from different locations within the same bunker silo. Isolate DL 872779-1 was recovered from the aborted fetus of the third infected cow.

Isolation of *L. monocytogenes*. *L. monocytogenes* was isolated from clinical samples (brain and aborted fetal tissues) by cold enrichment in Trypticase soy broth (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.) and weekly subculturing on Columbia colistin nalidixic acid agar plates with 5% sheep blood (BBL). Listeriae were isolated from the feed samples by direct plating of 10-fold dilutions of the blended samples on modified *Listeria* selective agar medium as described previously (8), without a blood agar overlay (26). Between 8 and 12 esculin-positive *Listeria*-suspected colonies were picked and confirmed as *L. monocytogenes* by a nested PCR assay (6). For this purpose, bacterial DNA was prepared from a single colony by using InstaGene Purification Matrix (Bio-Rad, Hercules, Calif.) in accordance with the instructions of the manufacturer. Nested PCR was performed simultaneously with four primers, the internal one of which amplifies an 858-bp fragment of the listeriolysin gene (6). The presence of PCR products was determined by agarose gel electrophoresis. Isolates positive in the *hly* PCR were further confirmed as *L. monocytogenes* by ribotyping as described below. Total numbers of *L. monocytogenes* bacteria per gram of silage were estimated after determining the frequency of *L. monocytogenes*. Additionally, the pH of each silage sample was determined as previously described (8).

Direct PCR detection of *L. monocytogenes* from silage. Feed samples containing *L. monocytogenes* were identified by nested PCR (6) with a crude cell lysate from an 18-h *Listeria* enrichment broth (LEB; Difco Laboratories, Detroit, Mich.) culture. Ten grams of silage was mixed with 90 ml of LEB and stomached for 30 s (Stomacher 400; Seward Medical Ltd., London, United Kingdom). Tenfold serial dilutions (10^0 to 10^2) in LEB were incubated at 37°C for 18 h, and crude cell lysates were prepared from a 0.5-ml aliquot by using either a method described by Furrer et al. (9) (method 1) or InstaGene Purification Matrix (method 2). For method 2, DNA purification was performed as recommended by the manufacturer for DNA preparation from single bacterial colonies.

Ribotyping. Ribotyping with normalized data for identification and characterization of *L. monocytogenes* was performed as previously described (5, 12). Briefly, *L. monocytogenes* DNA was purified from pure cultures, digested with *Eco*RI, separated by agarose gel electrophoresis, and electroblotted onto a nylon membrane. Hybridization of a sulfonated *E. coli rrnB* rRNA operon probe was detected with alkaline phosphatase-labelled anti-sulfonated-DNA antibodies and a chemiluminescent substrate. Images were acquired with a charge coupled device camera and processed by using custom software as previously described (5).

RESULTS

Cases and ribotyping. Ribotype patterns for all isolates are shown in Fig. 1. Initially, nine *L. monocytogenes* isolates from two previous listerial encephalitis outbreaks (26) were ribotyped (Table 1), and these results were compared with their RAPD types (described in reference 26). The ribotypes and RAPD types of all isolates corresponded, with the exception of those of silage isolate CU-SIK17/93. Two different ribotypes (dd 1067 and dd 1151) were observed for outbreak 1, while three different ribotypes (dd 0566, dd 0653, and dd 1153) were observed for outbreak 2. Isolate CU-SIK17/93 and two clinical isolates (CU-BR27/93 and CU-BR47/93) from the same outbreak had the same ribotype. While the former isolate was RAPD type E, the latter were RAPD type D (26).

L. monocytogenes strains isolated during two additional outbreaks also were characterized by ribotyping. For outbreak 3, a total of five different ribotypes were isolated from hay silage samples taken from bunker silo I, but none of these isolates matched the ribotype of the clinical isolate. Interestingly, two or three different ribotypes were found in individual silage samples from this outbreak (F3/1, F3/2, and F3/5; Table 1).

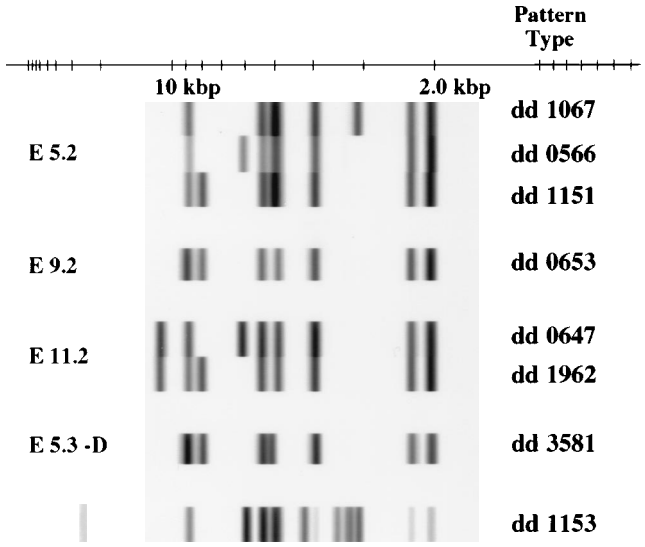

FIG. 1. Visually arranged pattern types (*Eco*RI) of the isolated *L. monocytogenes* strains. Ribotypes are indicated on the right, and the *L. monocytogenes* *Eco*RI subsets defining a group of ribotypes with identical *Eco*RI rRNA operon restriction fragments (5) are indicated on the left. The running direction of the gels is from left to right, and fragment sizes are indicated at the top. Images were acquired with a charge coupled device camera (Star One Camera; Photometrics, Tuscon, Ariz.) and processed by using custom software as previously described (12). Additional methodological details are described in references 5 and 12.

TABLE 2. Locations, pHs, and *L. monocytogenes* loads of silage samples obtained during and after outbreaks 3 and 4

Outbreak and sample no.	Location	pH	No. of *L. monocytogenes* bacteria/g	PCR
Outbreak 3				
F3/1	Hay silage, top of bunker silo I, left side	8.2	1×10^8	+
F3/2	Hay silage, top of bunker silo I, right side	8.1	1×10^4	+
F3/3	Hay silage, top of bunker silo I, middle	5.1	None[a]	−
F3/4	Hay silage, bottom of bunker silo I, left side	7.3	None	+
F3/5	Hay silage, bottom of bunker silo I, right side	6.3	5×10^3	+
F3/11[b]	Hay silage, top of bunker silo II, right side	8.3	1×10^5	+
Outbreak 4				
F4/1	Grass silage, tower silo I	5.1	NT[c]	−
F4/2	Corn silage, middle of bunker silo, right side	7.7	NT	−
F4/3	Corn silage, 1 ft (30.48 cm) below top of bunker silo, left side	5.4	NT	−
F4/4	Corn silage, top of bunker silo, left side	7.1	NT	−
F4/5	Corn silage, top of bunker silo, middle	3.8	NT	−
F4/6	Corn silage, top of bunker silo, right side	6.9	NT	−
F4/7	Corn silage, bottom of bunker silo, middle	4.4	NT	+
F4/8	Corn silage, bottom of bunker silo, right side	4.9	NT	−
F4/9	Grass silage, tower silo II	6.0	2×10^3	+

[a] None, below detection limit for direct plating.
[b] Sample F3/11 was from fresh silage prepared after the outbreak occurred.
[c] NT, not tested.

In outbreak 4, two of four isolates from the same grass silage sample (Table 1) had a ribotype (dd 0566) identical to that of the clinical isolate while the two other silage isolates had different ribotypes. Although the pH of three of the corn silage samples was >6.0, they were negative for *L. monocytogenes* (Table 2). These results incriminate the grass silage as the source of the disease outbreak. This grass silage was obtained from a silo which was last used for feeding in the middle of January 1994, while the first abortion was observed on 1 June 1994.

A summary of the different ribotypes and their isolation frequencies in silage and clinical samples is shown in Fig. 2. *L. monocytogenes* ribotypes isolated from 42 more listeriosis cases in ruminants (25) are included in this figure, giving a total of 49 clinical isolates.

Of a total of eight individual *L. monocytogenes*-positive silage samples, three yielded only a single ribotype, another three samples yielded two different ribotypes, and two yielded three different ribotypes. Two to six different ribotypes per outbreak were isolated from clinical and silage samples. The strains isolated from clinical samples during these outbreaks are represented by pattern types dd 1067, dd 0653, dd 0566, dd 0647, and dd 1153. All clinical pattern types were found in *Eco*RI ribotypes reported to contain only *L. monocytogenes* (5). Outbreak 3 showed the most diversity, having pattern types dd 0566, dd 0647, dd 0653, dd 1151, dd 1153, and dd 1962, representing three of the four core subsets.

Detection of *L. monocytogenes* from silage. A nested PCR assay was adapted for detection of *L. monocytogenes* in crude lysates after an 18-h enrichment of feed samples. PCR inhibition by humic acids or other compounds present in the lysates can be eliminated by two rounds of culture enrichment in LEB before PCR (26), perhaps simply because of the 100-fold dilution. To avoid false-negative results due to PCR inhibition, a duplicate of each sample was spiked with purified *L. monocytogenes* DNA prior to PCR. Two different DNA preparation methods were compared for the ability to remove inhibitors. The spiked PCR on lysates prepared by method 1 or 2 from the enrichment culture inoculated with 10% silage failed in two of six and one of three cases, respectively. The enrichments from 1 and 0.1% inocula did not inhibit the PCR. Method 2 was faster, requiring a total of 30 min, compared with 90 min for method 1.

Confirmation by PCR of putative *Listeria* colonies on *Listeria* selective agar medium revealed that among the five silage samples, between 10 and 65% of the colonies were *L. monocytogenes*. This procedure is 12 h shorter than the method previously reported from our laboratory (26).

Table 2 describes the silage samples from outbreaks 3 and 4. In outbreak 3, most of the samples from the edges of the bunker silo top (samples F3/1, F3/2, and F3/11) had a pH of ≥8.1 and the highest *L. monocytogenes* counts (10^4 to 10^8 CFU/g). PCR screening of 18-h enrichment cultures was positive for five of the six samples tested. Only one of the PCR-positive samples (sample F3/4) was negative for *L. monocytogenes* by direct plating on *Listeria* selective agar medium. This discrepancy between PCR and microbiological analysis could be explained by low levels of *L. monocytogenes* which gave a positive PCR but no colonies upon direct plating. The theoretical detection limit for the PCR after 18 h of LEB enrichment of a 1:10 dilution is 10 *L. monocytogenes* cells per g of silage, while it is 10^2 *L. monocytogenes* cells per g of silage for direct plating.

DISCUSSION

This report documents the applicability of ribotyping for identification, typing, and tracking of *L. monocytogenes* strains. We coupled this typing method as a follow-up to a PCR-based *L. monocytogenes* detection system. The detection system features screening of feed and environmental samples by PCR, followed by isolation of *L. monocytogenes* strains by direct plating. Feed containing *L. monocytogenes* is identified within 24 h by PCR on an 18-h LEB enrichment. Quantitative data on the *L. monocytogenes* load are obtained after an additional 48 h by direct plating and PCR confirmation. Localization of putative outbreak sources is therefore possible within 24 h, and unequivocal source assignment requires less than 5 days after

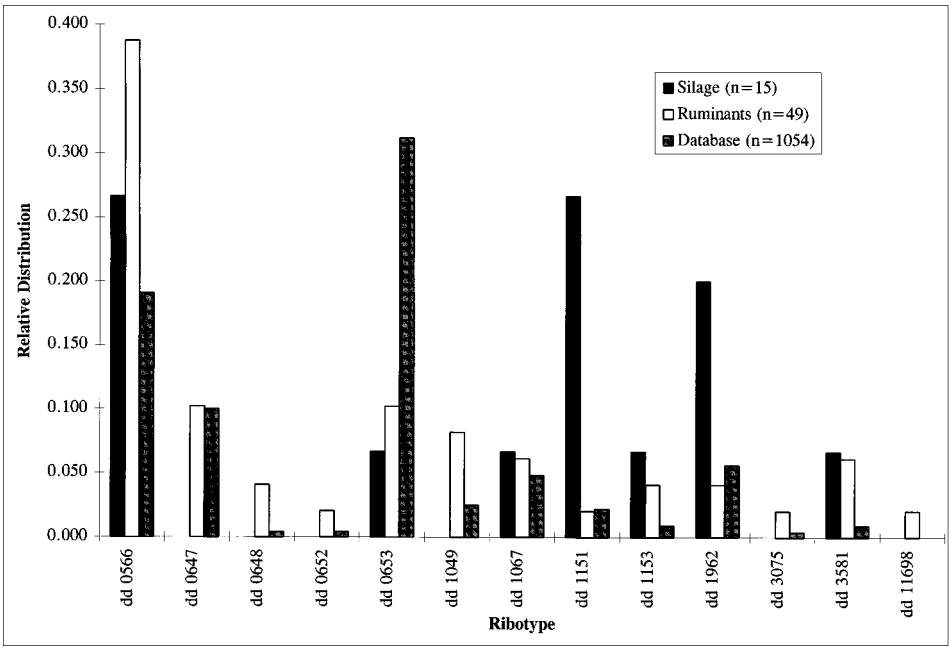

FIG. 2. Relative distribution of the different ribotypes in the Du Pont database, silage samples, and 49 clinical isolates from ruminants (25). For silage samples and clinical isolates from ruminants, the relative distribution of a given ribotype was calculated as the number of samples in which the ribotype was found as a fraction of the number of samples containing at least one *L. monocytogenes* strain of any ribotype. The relative frequency of *L. monocytogenes* strains in the DuPont database was calculated as the number of isolates with a particular ribotype divided by the total number of isolates (i.e., 1,346).

diagnosis of a listeriosis outbreak. Application of this system for the examination of listeriosis cases should limit the scope of an outbreak, especially where it is a chronic health problem.

In three of the four outbreaks, matching ribotypes were found between silage and clinical isolates, further confirming the importance of silage as a source for listeriosis outbreaks in ruminants (14, 26). Generally, silage samples were collected after a listeriosis case or outbreak was diagnosed. Therefore, the actual feed sampled might not be the same as that being consumed at the time of infection. Nevertheless, it seemed reasonable that the causative *L. monocytogenes* strain can be found as long as silage of the same origin as that present at the suspected infection time can be sampled. These limitations are inherent to all field investigations of listeriosis outbreaks.

L. monocytogenes was not isolated from any silage samples with a pH of <6.0, supporting previous findings that properly fermented silage does not allow survival or growth of *L. monocytogenes* (7, 8). However, three corn silage samples with a pH of >6.0 showed no *L. monocytogenes* contamination, which is also in agreement with previous reports (7, 8).

In outbreak 4, two silage isolates matching the clinical isolate from the aborted fetus were obtained from silage which supposedly had not been used as feed for more than 4 months, while no *L. monocytogenes* was isolated from the corn silage used as feed at the time of the outbreak. This suggests that the isolation of the same ribotype in the feed and the clinical sample reflects pure coincidence rather than a causal link. There are no data available on the incubation period for listerial abortions. Since this cow aborted at a gestational age of 4 to 5 months, there is a possibility that the silage was a source of infection early in pregnancy. An additional concern is the question of whether *L. monocytogenes* would survive for 4 months in silage. Although the survival time of *L. monocytogenes* in silage is not known, it has been shown that *L. monocytogenes* can survive for at least 4 months in cheese at pHs between 5.5 and 6.2 (19).

PCR testing of an 18-h selective enrichment broth from feed and environmental samples seems reliable and rapid for *L. monocytogenes* screening. While a direct PCR might provide faster test results, this method is limited by its detection of nonviable or dead bacteria (21). Furthermore, removal of PCR inhibitors from environmental samples without compromising sensitivity is often difficult.

A rapid and robust typing system for isolated strains is an integral part of an effective on-farm bacteriological surveillance program performed by diagnostic centers. Preliminary data obtained during our study indicate that ribotyping might be slightly less discriminatory than RAPD typing. This relatively lower level of discrimination is not necessarily a disadvantage, since too sensitive a typing system might detect epidemiologically irrelevant differences, such as point mutations or DNA rearrangements (3). Also, some typing methods (e.g., RAPD and restriction enzyme analysis) might reveal plasmid-borne differences between two isolates. This is of particular concern with regard to the typing of *L. monocytogenes*, since approximately 78% of the *Listeria* strains examined contain extrachromosomal DNA (13). Acriflavine, a plasmid-curing agent, is frequently used in selective media for *Listeria* spp., potentially leading to plasmid losses. Nevertheless, the use of other typing methods (such as pulsed-field gel electrophoresis) in conjunction with ribotyping might improve strain differentiation if plasmid-borne differences can be excluded.

An unanticipated result of our investigations was the variety of ribotypes found in a single silage sample, as well as within an individual outbreak. Only one previous report describes the isolation of different *L. monocytogenes* strains from silage samples implicated in a listeriosis outbreak (14). There, distinct strains were isolated from separate silage bales on the same farm but not from the same silage sample or the same silo. Although we do not understand the importance or implication of the diversity we have found, our observations might be of general interest with regard to the ecology of pathogenic mi-

croorganisms. The high frequency of ribotype dd 1151 in silage samples is surprising considering that only 2.3% of the isolates in the Du Pont database of 1,346 *L. monocytogenes* strains belong to this ribotype (5). These results suggest that while this database includes a diverse sample of food isolates and some clinical isolates, ribotype dd 1151 occurs more frequently in specific ecological niches, such as silage. Also, ribotype dd 0566 was isolated from 38.8% of the clinical isolates from ruminants, while it is present in 19.1% of the strains in the Du Pont database. This could indicate that this strain has greater virulence for ruminants than do other ribotypes. However, a larger number of veterinary clinical and silage isolates needs to be typed to draw statistically valid conclusions regarding the frequency of various ribotypes.

The ribotyping system applied in our study has the advantage of providing typing results which are normalized, thus allowing data to be compared between different laboratories with no time restriction. Therefore, a database of ribotype patterns with a common nomenclature can be utilized to determine the significance of a given ribotype in clinical and epidemiologically implicated feed or environmental samples. Typing results which are comparable between laboratories would also greatly facilitate epidemiological investigations, as well as determination of the prevalence of certain *L. monocytogenes* ribotypes in disease cases. Many other typing methods (e.g., multilocus enzyme electrophoresis or RAPD analysis) lack comparability between laboratories, probably because of problems in standardization of reaction conditions and normalization of data.

ACKNOWLEDGMENTS

The help of veterinarians (R. Meyers and F. Welcome) and farmers in providing samples and clinical diagnosis in the two outbreaks in cattle is gratefully acknowledged. We are indebted to T. Divers and R. de la Rua-Domenech for helpful discussions.

This work was supported by the Northeast Dairy Foods Research Center (C.A.B.) and a grant from the Cornell Center for Advanced Technology (CAT) in Biotechnology, which is sponsored by the New York State Science and Technology Foundation (P.L.M.). M.W. is supported by a Spencer T. and Ann W. Olin Fellowship. A gift of ceftazidime (used in the *Listeria* selective agar medium formulation) from Glaxo is gratefully acknowledged.

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