Typing of Human, Animal, Food, and Environmental Isolates of *Listeria monocytogenes* by Multilocus Enzyme Electrophoresis

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In order to elucidate some aspects of the epidemiology of listeriosis in Switzerland, 181 strains of *Listeria monocytogenes* isolated from humans, animals, food, and the environment have been analyzed by multilocus enzyme electrophoresis at 21 enzyme loci. The clone responsible for several recent food-borne outbreaks in Switzerland and in North America (marked by electrophoretic type 1 and serovar 4b) has been found frequently among strains isolated from animals. Thus, animals may represent a major source of diffusion of this clone in the environment and in food, in which it has been found only sporadically, however. Two other unrelated clones (including strains belonging to serovars 1/2b and 1/2c) have often been isolated from meat but not from animals. These findings indicate that contamination of meat with *L. monocytogenes* might originate mainly from the environment in which it is processed rather than from animals themselves. This could explain the differences in the distribution of *L. monocytogenes* serovars isolated from meat and from animals.

Listeriosis in humans and animals is caused by the opportunistic bacterium *Listeria monocytogenes*. The most frequent clinical consequences of this severe disease are meningitis, encephalitis, septicemia, abortion, and stillbirth (11). The incidence of listeriosis has been low but seems to be rising (18), and the repeated outbreaks in the 1980s have increased the interest in the epidemiology of this disease.

L. monocytogenes is widely distributed in the environment (22, 29), and healthy carriers are found among humans and animals (11, 15, 27, 28). This microorganism has also been isolated in numerous foodstuffs, in which it can survive and multiply (30). Some of these isolates (from milk, cheese, chicken, sausages, seafood, and vegetables) have been suspected, or proven, to be the source of infection (2, 3, 6–8, 10, 13, 14, 16, 17, 20, 23, 24). Thus, both in sporadic cases and in epidemics, listeriosis is presently considered to be mainly a food-borne disease.

The ubiquity of *L. monocytogenes* makes typing methods essential for the study of its epidemiology. One typing method is multilocus enzyme electrophoresis (MEE) (26). By this powerful method, bacterial isolates are differentiated according to the variation in electrophoretic mobility of a large number of metabolic enzymes. Different electromorphs of each enzyme are presumed to reflect alleles at the corresponding gene locus, and the electromorph profiles for a number of enzymes are interpreted as multilocus phenotypes (electrophoretic types [ETs]) reflecting the chromosomal genotype. MEE has already been successfully applied to *L. monocytogenes* and has been shown to be much more discriminatory than the commonly used serotyping (4, 5, 21). Furthermore, by MEE, all strains of *L. monocytogenes* can be typed, whereas this is often not the case when phage typing is used (1). Another significant advantage of MEE is its ability to estimate the genetic relatedness among strains.

An epidemic of listeriosis due to contamination of a regional soft cheese occurred in Switzerland in the years 1983 to 1987 (6). Using MEE, we have shown that despite the high genetic diversity in natural populations of *L. monocytogenes*, only two clones were responsible for this and

four other outbreaks (10, 13, 17, 21, 23). These results were recently confirmed by Bibb et al. (4) and led to the hypothesis that, as in many other bacterial species, most of the clinical cases are caused by only a few clones which either are particularly common in the environment or have an unusually high level of pathogenicity (19, 21).

In this report, we describe the analysis with MEE of Swiss *L. monocytogenes* isolates of different origins (humans, animals, the environment, meat products, milk, and cheese). The results of this study provide information about the distribution of the clone responsible for the Swiss epidemic of 1983 to 1987 (6) and other clones.

MATERIALS AND METHODS

Bacterial isolates. We analyzed 181 strains of *L. monocytogenes* isolated from different parts of Switzerland during the period from 1981 to 1989. Of these, 43 isolates were of human origin, 49 were from animals, 40 were from meat and meat products, 19 were from milk and cheese, and 30 were from the environment (silage, salad, grass, soil, dairy, material used for the production of cheese, and sewage).

Serotyping. Serotyping was performed according to the method of Seeliger and Höhne (25).

Electrophoresis of enzymes. Lysate preparation, electrophoresis, and the following enzyme selective stainings were done as described by Selander et al. (26): aconitase, acid phosphatase, adenylate kinase, alanine dehydrogenase, catalase, α-esterase (developed with α-naphthyl propionate as a substrate), β-esterase (developed with β-naphthyl propionate as a substrate), fumarase, NADP-dependent glutamate dehydrogenase, glutamic-oxalacetic transaminase, NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (GP1), glucose-6-phosphate dehydrogenase (G6P), isocitrate dehydrogenase, indophenol oxidase, lactate dehydrogenase, mannose phosphate isomerase, nucleoside phosphorylase, L-phenylalanyl-L-leucine peptidase, 6-phosphogluconate dehydrogenase, phosphoglucose isomerase, and phosphoglucomutase. The following modifications were made: electrophoreses for catalase, α-esterase, β-esterase, and adenylate kinase were made in buffer F, and those for G6P and mannose phosphate isomerase were made in buffer B (26).

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TABLE 1. Number of alleles and genetic diversities at 21 enzyme loci among ETs of *L. monocytogenes*

Enzyme locus[a]	No. of alleles	Genetic diversity
ACO	3	0.215
ACP	5	0.581
ADK	3	0.583
ALDH	4	0.307
CAT	7	0.640
ESTα	5	0.658
ESTβ	10	0.780
FUM	6	0.684
GD2	3	0.078
GOT	2	0.180
GP1	2	0.497
G6P	3	0.609
IDH	4	0.284
IPO	1	0.000
LDH	6	0.350
MPI	3	0.530
NSP	1	0.000
PEP	6	0.608
6PG	2	0.487
PGI	2	0.039
PGM	6	0.613
Mean	4.0	0.415

[a] ACO, aconitase; ACP, acid phosphatase; ADK, adenylate kinase; ALDH, alanine dehydrogenase; CAT, catalase; ESTα, α-naphthyl propionate esterase; ESTβ, β-naphthyl propionate esterase; FUM, fumarase; GD2, NADP-dependent glutamate dehydrogenase; GOT, glutamic-oxalacetic transaminase; GP1, NAD-dependent glyceraldehyde-3-phosphate dehydrogenase; G6P, glucose-6-phosphate dehydrogenase; IDH, isocitrate dehydrogenase; IPO, indophenol oxidase; LDH, lactate dehydrogenase; MPI, mannose phosphate isomerase; NSP, nucleoside phosphorylase; PEP, L-phenylalanyl-L-leucine peptidase; 6PG, 6-phosphogluconate dehydrogenase; PGI, phosphoglucose isomerase; PGM, phosphoglucomutase.

For α-esterase, only the lowest band appearing on the gel was considered. Specific stainings for catalase and glutamic-oxalacetic transaminase were performed according to the method of Harris and Hopkinson (12).

Statistical analysis. Statistical analysis of the data was made with a computer program designed by T. S. Whittam and R. K. Selander. Briefly, the genetic diversity for each enzyme locus among ETs was calculated as $h = (1 - \Sigma x_i^2) [n/(n - 1)]$, where x_i is the frequency of the ith allele and n is the number of ETs. The genetic distance between ETs was expressed as the proportion of loci at which dissimilar alleles occurred with the contribution of each locus inversely weighted by the genetic diversity (h) at the locus (26). The clustering of ETs was made by the average-linkage method from a matrix of coefficients of pairwise genetic distances.

RESULTS

For the 21 enzymes analyzed, the number of alleles per locus ranged from 1 (indophenol oxidase and nucleoside phosphorylase) to 10 (β-esterase), with an average of 4.0. The genetic diversities at each locus were between 0.000 and 0.780 (Table 1). Fifty ETs could be identified for the 181 strains of *L. monocytogenes* examined. The genetic relatedness and the source of the isolates are represented in the dendrogram of Fig. 1. The population of *L. monocytogenes* studied is composed of two main clusters of ETs separated at a genetic distance of 0.47 (clusters I and II of Fig. 1). Specific association of alleles for acid phosphatase, fumarase, GP1, and G6P was found in these main clusters.

Strains of ET 1 (which was responsible for the Swiss epidemic of 1983 to 1987) were the most frequent in our collection (26.5%). Their characteristics are listed in Table 2. ET 1 was particularly common among *L. monocytogenes* of animal origin (49.0%). It was found mainly in clinical cases but also in a few healthy carriers, most of whom were associated with farms where clinical animal cases caused by ET 1 occurred. *L. monocytogenes* of animal origin marked by this ET was widely distributed throughout Switzerland and not limited to the region of production (Canton Vaud) of the soft cheese responsible for the Swiss epidemic. ET 1 represented also 28.6% of the human strains of our sample, but some of them might be related to the epidemic of 1983 to 1987. Considering only the postepidemic period (1988 to 1989), they still represented 24.2% of the human isolates we studied. Finally, strains of ET 1 were found sporadically in foodstuffs, silage, soil, and the dairy environment (Table 2). Unfortunately, no data on the frequency of the clone marked by ET 1 prior to the epidemic period in Switzerland are available.

The clones marked by ET 19 and ET 20 were also well represented in our sample of strains (11.6 and 8.3%, respectively), particularly in strains obtained from meat and meat products (40.0 and 22.5%, respectively). Their characteristics are listed in Table 3.

No obvious correlations between a particular ET and its source were observed.

DISCUSSION

Previous studies on *L. monocytogenes* with MEE have shown that despite the significant number of ETs encountered in the species *L. monocytogenes*, only a very few clones seem to be at the source of the outbreaks which occurred during the 1980s. The clone marked by ET 1 was responsible for an epidemic of listeriosis in Switzerland during the years 1983 to 1987 (21): a contaminated regional soft cheese was the source of infection (6). The same ET was responsible in 1985 for another outbreak in California associated with the consumption of a contaminated Mexican-style cheese (17, 21). A strain of the same ET (4, 21) was also linked to an epidemic due to coleslaw in the maritime provinces of Canada in 1981 (23). Further studies are necessary to explain the repeated occurrence of the clone marked by ET 1 in epidemics and to establish whether this ET has an unusually high level of pathogenicity or is simply particularly frequent in the environment and in food. This is the reason we have performed this study on the distribution in Switzerland of the clone marked by ET 1 and of other clones.

Although the *L. monocytogenes* isolates included in this study were chosen randomly, the collection we examined represents only a limited sample of the strains isolated in Switzerland during the period considered; moreover, the few environmental strains analyzed may not be representative. Nevertheless, the results of this study show clear trends in the distribution of some clones of *L. monocytogenes*.

The mean genetic diversity of 0.415 found at the 21 enzyme loci analyzed is comparable to that found in other studies on *L. monocytogenes* but with fewer enzyme loci (4, 5, 21). The genetic structure of the population with two main clusters of ETs separated at a genetic distance of 0.47 is similar to that found previously (4, 6a, 21). The monomorphic enzyme loci we have observed (indophenol oxidase and nucleoside phosphorylase) differ from that described by Bibb et al. (aconitase) (4). The loci where association between alleles and main clusters occurred (acid phosphatase,

ET	A	M	H	C	E	T
1	24	1	13	4	6	48
13	1	-	-	-	-	1
22	-	2	1	-	1	4
8	1	-	-	-	-	1
9	1	-	-	-	-	1
12	1	-	-	-	-	1
37	-	-	4	1	1	6
50	-	-	-	-	1	1
34	-	-	1	-	-	1
43	-	-	-	1	-	1
49	-	-	-	-	1	1
10	1	-	-	-	-	1
15	1	-	-	-	-	1
20	-	9	1	1	4	15
38	-	-	1	-	-	1
28	-	1	3	1	2	7
36	-	-	2	-	-	2
42	-	-	1	-	-	1
33	-	-	1	-	-	1
4	1	-	-	1	-	2
5	4	1	2	2	-	9
27	-	1	2	-	-	3
18	-	1	-	-	-	1
2	3	-	3	-	5	11
6	1	-	-	-	-	1
32	-	-	1	-	-	1
24	-	1	1	-	2	4
47	-	-	-	1	-	1
23	-	1	-	-	-	1
29	-	1	-	-	-	1
26	-	1	-	-	1	2
41	-	-	1	-	-	1
11	1	-	-	-	-	1
40	-	-	1	1	-	2
48	-	-	-	-	1	1
25	-	2	-	-	-	2
39	-	-	1	-	-	1
31	-	-	1	-	-	1
3	5	-	-	2	1	8
35	-	-	1	-	-	1
19	-	16	1	1	3	21
21	-	1	-	-	-	1
30	-	1	-	-	-	1
7	1	-	-	-	-	1
16	1	-	-	-	-	1
44	-	-	-	1	1	2
46	-	-	-	1	-	1
14	1	-	-	-	-	1
45	-	-	-	1	-	1
17	1	-	-	-	-	1

49 40 43 19 30 181

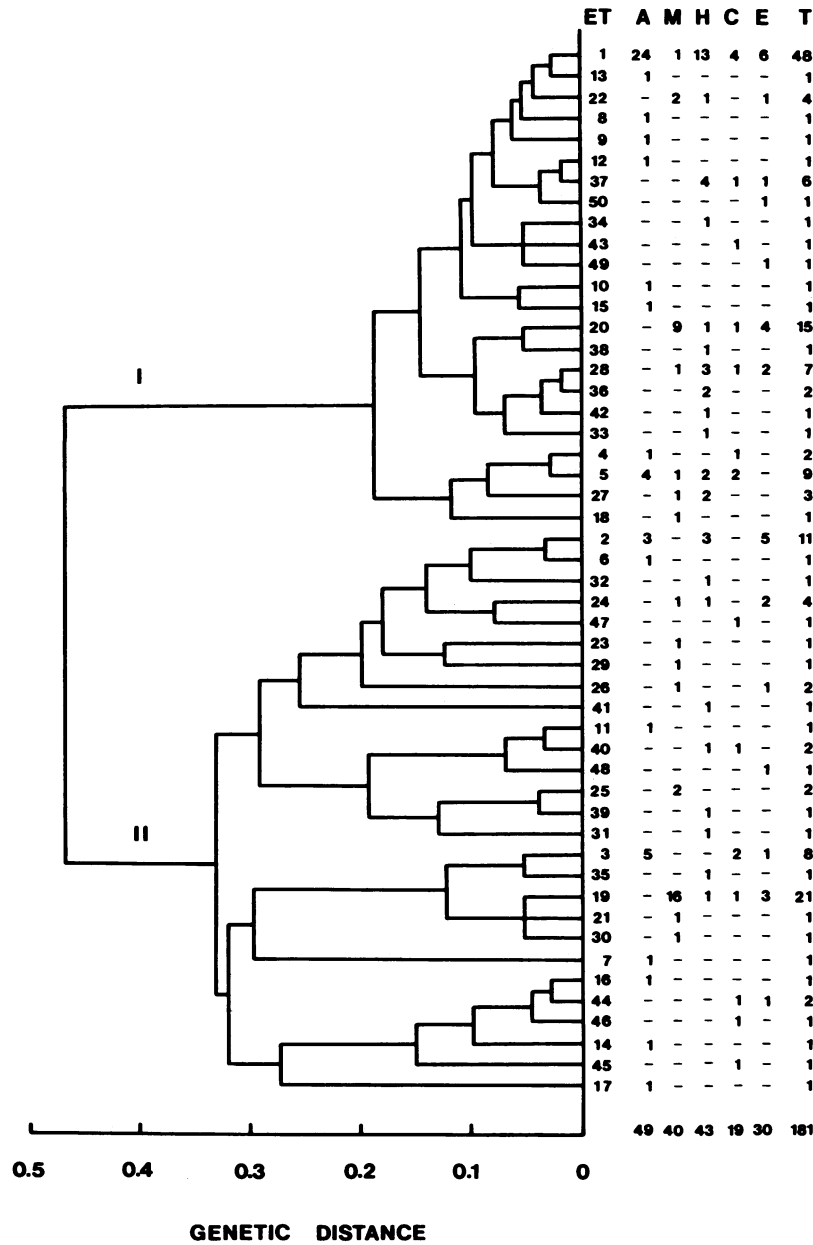0.5 0.4 0.3 0.2 0.1 0

GENETIC DISTANCE

FIG. 1. Genetic relationships among 50 ETs of *L. monocytogenes*. The dendrogram was generated by the average-linkage method of clustering from a matrix of pairwise coefficients of weighted genetic distances, based on electrophoretically demonstrable allelic variation at 21 enzyme loci. Note that except for ET 1, the ETs in this study do not correspond to those we published elsewhere (21). Strains were from animals (A), meat and meat products (M), humans (H), milk and cheese (C), and the environment (E). T, total.

fumarase, GP1, and G6P) also differ from those reported by these authors (G6P and 6-phosphogluconate dehydrogenase). This may be due to the different geographical origins of the strains examined. Alternatively, the different buffer systems used for electrophoresis, which can change the relative mobilities of enzymes and the number of distinguishable electromorphs, may also be the cause of these discrepancies.

Our results show that even if the incidence of listeriosis in Switzerland has dramatically dropped after withdrawal of the contaminated cheese (6), the proportion of human cases caused by ET 1 has remained high (24.2%). Thus, the clone

marked by ET 1 may still be responsible for a significant part of the sporadic cases in Switzerland. It is possible that an unrecognized source of infection is still present. It is interesting to note that lysotyping performed on a limited sample of strains isolated before 1983 in western Switzerland (the region where the infected cheese was produced) showed that the phage types associated with the epidemic clone were already present among these isolates (5a).

A large proportion (49.0%) of *L. monocytogenes* strains isolated from animals in different parts of Switzerland are also ET 1. This result has been confirmed by phage typing (data not shown): among 21 animal strains of ET 1 tested, 19

TABLE 2. *L. monocytogenes* strains marked by ET1

Strain designation	Serovar	Origin[a]	Source	Yr
A1	4b	JU[b]	Bovine (abortion)	1986
A7	4b	JU[b]	Bovine (milk)	1986
A20	4b	JU[b]	Bovine (milk)	1986
A8	4b	JU[b]	Bovine (feces)	1986
A9	4b	JU[b]	Bovine (feces)	1986
A4	4b	BE[c]	Bovine (feces)	1986
A13	4b	BE[c]	Bovine (feces)	1986
A14	4b	BE[c]	Bovine (feces)	1986
A3	4b	FR	Bovine (tonsils)	1986
A5	4b	BE	Sheep (brain)	1987
A26	4b	AG	Sheep (brain)[d]	1987
A28	4b	VD	Bovine (brain)	1988
A29	4b	VD	Goat (brain)	1988
A31	4b	VD	Bovine (brain)	1988
A34	4b	VD	Bovine (brain)	1988
A36	4b	VD	Bovine (brain)[e]	1988
A37	4b	VD	Bovine (brain)[e]	1988
A38	4b	VD	Bovine (brain)[e]	1988
A40	4b	TG	Sheep (brain)	1988
A41	4b	SG	Rabbit (sepsis)	1988
A42	4b	ZH	Hen (sepsis)	1989
A43	4b	SZ	Bovine (brain)	1989
A45	4b	ZH[f]	Bovine (abortion)	1989
A46	4b	ZH[f]	Bovine (abortion)	1989
H4	4b	SG	Human (neonatal sepsis)	1984
2	4b		Human (meningitis)	1985
H10	4b	AG	Human (meningitis/sepsis)	1987
H11	4b	AG	Human (meningitis)	1987
H21	4b	FR	Human (sepsis)	1988
H28	4b	GE	Human (sepsis)	1988
H32	4b	AG	Human (meningitis)	1988
H34	4b	GE	Human (meconium, premature baby)	1988
H40	4b	VD	Human (abortion/sepsis)	1988
H41	4b	NE	Human (abortion)	1988
E32	4b	VD	Human (feces)	1988
H18	4b	TI	Human (sepsis)[g]	1989
H19	4b	TI	Human (feces)[g]	1989
L11	4b	GE	Soft cheese	1986
L43	4b	ZG	Cheese mixture	1988
L58	4b	VD	Raw milk	1986
L60	4b	FR	Raw milk	1986
E24	4b	BE	Silage	1986
V78	4b		Minced meat	1987
E6	4b	SG	Salad	1986
E11	4b	BE	Soil (near a farm)	1987
E14	4b	VD	Scraps of meat[h]	1987
E19	4b	ZG	Cheese box	1988
E37	4b		Cheese cellar	1989

[a] Cantons of Switzerland where the strains were isolated. JU, Jura; BE, Bern; FR, Fribourg; AG, Aargau; VD, Vaud, TG, Thurgau; SG, St. Gallen; ZH, Zürich; SZ; Schwyz; GE, Geneva; NE, Neuchâtel; TI, Ticino; ZG, Zug.
[b] Animals originating from the same farm.
[c] Animals originating from the same farm.
[d] Twenty sheep died of gastroenteritis in a flock of 200 animals on this farm, and *L. monocytogenes* was isolated in nine cases in spleen and liver.
[e] Consumption of silage mentioned in anamnesis.
[f] Animals originating from the same farm.
[g] Both strains were isolated simultaneously from the same patient.
[h] Cross-contamination with the soft cheese responsible for the Swiss epidemic of 1983 to 1987.

TABLE 3. *L. monocytogenes* strains marked by ET 19 and ET 20

Strain designation	Serovar	ET	Origin[a]	Source	Manufacturer	Yr
V2	1/2c	19		Chicken meat		
V3	1/2c	19		Chicken meat		1986
V4	1/2c	19		Chicken meat		1987
V6	1/2c	19	SO	Dry sausage[b]	A	1988
V12	1/2c	19	GE	Raw beef	B	1988
V13	1/2c	19	VD	Dry sausage	C	1988
V14	1/2c	19	VD	Dry sausage[b]	C	1988
V18	1/2c	19	VD	Dry sausage[b]	C	1988
V71	1/2c	19	SG	Dry sausage[c]		1987
V73	1/2c	19	SG	Dry sausage[c]	D	1987
V74	1/2c	19	SG	Dry sausage[c]	E	1988
V75	1/2c	19	SZ	Dry sausage	F	1988
V79	1/2c	19	SG	Minced beef		1987
V82	1/2c	19	SG	Minced meat		1988
V88	1/2c	19	SG	Chicken meat		1988
V91	1/2c	19	BE	Dry sausage[c]	G	1989
H13	1/2c	19	TI	Human (meningitis)		1988
L61	1/2c	19	TI	Raw milk		1986
E30	1/2c	19	BE	Sewage		
E35	1/2c	19	AG	Sewage		1988
E36	1/2c	19	AG	Sewage		1988
V5	1/2b	20		Chicken meat		1987
V17	1/2b	20	GR	Cured and dry meat	H	1988
V19	1/2b	20	GR	Cured and dry meat[c]	H	1988
V69	1/2b	20	TI	Cured and dry meat	I	1987
V70	1/2b	20	SG	Dry sausage	J	1987
V76	1/2b	20	TI	Dry sausage	I	1988
V77	1/2b	20	VD	Dry sausage	C	1988
V81	1/2b	20	AR	Minced meat	K	1988
V85	1/2b	20	SG	Cured and cooked sausage[b]	L	1988
H22	1/2b	20	FR	Human (sepsis)		1988
L59	1/2b	20	FR	Raw milk		1986
E16	1/2b	20	AI	Rinse water for cheese		1988
E20	1/2b	20	LU	Rinse water for cheese		1988
E22	1/2b	20	LU	Machine to wash cheese		1988
E29	1/2b	20	BE	Silage		

[a] Cantons of Switzerland where the strains were isolated. SO, Solothurn; GE, Geneva; VD, Vaud; SG, St. Gallen; SZ, Schwyz; BE, Bern; TI, Ticino; AG, Aargau; GR, Graubünden; FR, Fribourg; AI, Appenzell; LU, Luzern.
[b] Sampling during manufacture.
[c] To be eaten fresh.

were of the epidemic and related phage types mentioned by Piffaretti et al. (21). The ET 1 animal strains could not be associated with any particular clinical pattern or host species (Table 2). Our results show that ET 1 can be found in the feces or milk of animals of the same farm where a clinical

animal case occurred (Table 2). Thus, animals might play a major role in the dissemination of this strain, particularly in the environment. We have also found ET 1 strains in silage and in the soil near a farm (Table 2).

ET 1 strains were found twice in raw milk, twice in cheese other than that responsible for the epidemic of 1983 to 1987, and twice in the environment in which cheese is produced (Table 2). These findings, together with the role of this ET in two outbreaks caused by cheese, might indicate an adaptation of this clone to an ecological niche comprising milk, cheese, and their production sites. A higher frequency of contamination of milk by ET 1 than by other ETs through infected animals could also explain these findings.

Contamination of salad greens with ET 1 could also originate from animal sources. The use of infected animal

manure as fertilizer for vegetables was pointed out as a danger by Schlech et al. (23) in a report on an epidemic related to consumption of coleslaw. The cabbages used were likely contaminated by sheep manure from a flock in which listeriosis occurred. Furthermore, the clone responsible for this outbreak was ET 1 (4, 21). Thus, our results on the frequency of this ET in animals, its excretion in feces, and its presence in a salad support the hypothesis on the contamination route of the cabbage.

The high frequency of ET 1 we have found among *L. monocytogenes* strains isolated from both humans and animals, as well as its occurrence in three epidemics, supports the hypothesis that the clone marked by this ET is associated with particularly high level of pathogenicity. However, further studies are necessary to confirm this view, since our *L. monocytogenes* collection is not sufficiently representative to allow a reliable estimation of its frequency among environmental strains and their role as a source of infection in humans.

Two other distinct clones, marked by ET 19 (serovar 1/2c) and ET 20 (serovar 1/2b), were frequently found. These two clones formed together 62.5% of the strains isolated from meat. Both were found in various products (during and after processing) from several producers in different parts of Switzerland (Table 3). These results suggest that ET 19 and ET 20 represent a significant part of the strains isolated from meat and meat products. This hypothesis is supported by the observation from others that serovar 1/2b and 1/2c are the most frequent in this type of product in Switzerland (9). However, these two clones were found only sporadically in humans and milk but never in animals. Conversely, ET 1, which is frequent among isolates of animal origin, was found only once in meat (Fig. 1). Furthermore, among the 30 ETs discovered in animals or meat, only two (ET 1 and ET 5) were found in both (Fig. 1). These observations suggest that animal strains do not easily contaminate meat or survive and multiply during its processing. They are replaced by other strains which are presumably better adapted for survival and possibly multiplication in the processing plant and thereafter in meat and its by-products. Strains marked by ET 19 and ET 20 would constitute a good example for such a phenomenon. This hypothesis is supported by the results from another study performed by our laboratory in which we observed that when ET 1 strains were present on the skin of cattle at the beginning of slaughtering, they were not found elsewhere on the carcasses or in the environment at the end of the processing. In contrast, ET 19 strains were found several times on the carcasses of pigs at the end of processing in two slaughterhouses and in the environment of one of them, but not on living animals or at the beginning of the slaughtering procedure (unpublished results).

In conclusion, our results show that MEE is a powerful tool for studies on the epidemiology of listeriosis. The distribution of *L. monocytogenes* clones in the environment can be assessed with this method, and the possible contamination pathways of foodstuffs can be defined. We have shown that living animals might play a major role in the dissemination in Switzerland of the epidemical clone marked by ET 1. However, contamination of meat and meat products by other ETs, such as ET 19 and ET 20, seems to occur mainly by strains present in the slaughterhouse environment during the processing of the carcasses, rather than with strains originating from animals. The differences observed in the distribution of *L. monocytogenes* clones (animals versus meat) could also reflect important differences between strains and their adaptations to diverse ecological niches, as

well as variation in the pathogenicity level among *L. monocytogenes* clones.

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