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Subtyping *Listeria monocytogenes* Isolates Genetically Related to the Swiss Epidemic Clone

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Macrorestriction analysis by pulsed-field gel electrophoresis was used to assess the diversity of strains within the epidemic-associated electrophoretic type 1 (ET1) clone of *Listeria monocytogenes*. For this purpose, a total of 144 isolates from Switzerland shown by multilocus enzyme electrophoresis to belong to the ET1 were examined. These isolates were subtyped by macrorestriction analysis using the enzymes *Apa*I and *Sma*I and field inversion gel electrophoresis. Among these 144 isolates, 45 were isolated in human listeriosis cases of the postepidemic period of 1988 to 1993 and 44 were isolated in animal listeriosis cases of the same period. Forty-seven isolates were from the epidemic period of 1983 to 1987, and eight additional isolates were from cattle from two different farms. Twenty-nine different subtypes could be identified among the 144 isolates tested. Five major subtypes were found more frequently than the others during the postepidemic period, both in humans and in animals. Two of these subtypes had been previously implicated in outbreaks of listeriosis, thus suggesting that particular pulsed-field gel electrophoresis subtypes may be frequently associated with disease in humans and animals. Two of these frequent subtypes were also suspected to be related to small clusters of listeriosis cases during the postepidemic period. The results obtained by typing epidemiologically related isolates from different animals within the same farms and from different body sites of a given patient confirmed the potential of macrorestriction analysis for epidemiological studies restricted to short periods of time and to small number of isolates. The analysis of 47 isolates related to the Swiss listeriosis epidemic period of 1983 to 1987 and the use of Southern blotting and hybridization experiments show that the interpretation of relatedness between isolates presenting slightly different macrorestriction patterns may be more complex than commonly accepted. In such cases, careful interpretation of the potential molecular mechanisms leading to the differences observed between patterns is necessary.

Listeria monocytogenes is a facultative human and animal pathogen responsible mainly for abortion, stillbirth, meningitis, encephalitis, and septicemia. *L. monocytogenes* is widespread in the environment and often contaminates food, which is considered to be the main source of infection. Listeriosis occurs usually as sporadic cases (2 to 15 cases per million people per year [11]). However, listeriosis epidemics have been repeatedly described in the past years (11, 13) and have caused great concern in the medical community and the food industry. Several typing methods have been applied to the epidemiological surveillance and tracing of *L. monocytogenes*. Multilocus enzyme electrophoresis (MEE) has shown that several of the recent major epidemics over the world were caused by a group of genetically closely related serovar 4b strains of *L. monocytogenes* (18), designated electrophoretic type 1 (ET1) clone in the present study and in several previous studies (5, 17, 18, 23). Other groups have confirmed these results by using different typing methods (9, 13). This clone has also been shown to be responsible for a significant proportion of the sporadic human and animal listeriosis cases (5, 17, 23).

The recently developed method of DNA macrorestriction analysis by pulsed-field gel electrophoresis (PFGE) has been shown to be highly discriminatory. PFGE often facilitates the identification of subtypes within isolates indistinguishable by other methods (7–9, 13, 14). In the past few years, several authors have successfully tested and applied this technique to epidemiological investigations of listeriosis (7–10, 13–15, 19). However, the instability of the PFGE subtypes may be problematic. Single genetic events such as insertions, deletions, or point mutations have been shown to be responsible for changes in PFGE patterns of epidemiologically related isolates (1, 22). This instability and the related difficulties in the epidemiological interpretation of PFGE patterns have been and are still the object of many discussions in the scientific and medical communities. The stability of macrorestriction patterns of *L. monocytogenes* has not been tested or examined in detail.

In the present work, we first assessed the diversity within the ET1 clone by means of macrorestriction analysis and subsequently compared human and animal isolates of the ET1 clone to see if they form different and separated subpopulations. In the second step, we examined the ET1 subtypes found during the Swiss listeriosis epidemic of 1983 to 1987 and compared them with those of ET1 isolates recovered during the postepidemic period.

MATERIALS AND METHODS

***L. monocytogenes* isolates related to the Swiss epidemic of 1983 to 1987.** Forty-seven isolates originating from human listeriosis cases ($n = 31$) and from implicated cheese and its production environment ($n = 16$) were examined (Table 1). Twenty-five isolates were of the major Swiss epidemic lysovar 47/108/340/2389/2425/2671/3274, and 22 were of the minor Swiss epidemic lysovar 47/108/340/2389.

***L. monocytogenes* isolates from the postepidemic period of 1988 to 1993.** One hundred and forty-nine *L. monocytogenes* isolates representing the 145 human listeriosis cases registered in Switzerland during the period of 1988 to 1993 were screened by MEE for the presence of the epidemic-associated ET1 clone. One hundred and seventeen isolates from animal listeriosis cases which occurred during the same period in 116 different locations were examined in the same way.

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TABLE 1. Characteristics and PFGE subtypes of 144 ET1 *L. monocytogenes* isolates from Switzerland[a]

PFGE type	*Apa*I type	*Sma*I type	Lysovar	Strains (origin and yr of isolation)
I	1	1	47/108/340/2389/2425/2671/3274	LL15, LL18 (human, 1983); LL27, LL28, LL29, LL52, LL94 (human, 1984); LL128 (human, 1985); LL127, LL129, LL159, LL165 (human, 1986); PF69, PF70, PF110 (cheese, 1986); PF23, PF29 (cheese environment, 1986); LL195, LL393, LL398, LL420 (human, 1987); BE53, BE61 (cheese, 1987); PF492 (cheese environment, 1987); 636, LL474 (human 1988); 1989 (human, 1989); LL513 (animal, 1990); 6752, LL533, LL536 (animal, 1991); 6924 (human, 1992); 7015 (animal, 1992); 8494 (human, 1993)
I	1	1	NT	3553 (human, 1989)
I	1	1	47	8623 (animal, 1993)
II	2	2	47/108/340/2389	LL20 (human, 1983); LL105 (human, 1984); LL140, LL146, LL155 (human, 1985); LL161, LL179, LL181, LL186 (human, 1986); LL201, LL215, LL352, LL423 (human, 1987); PF2, PF48, PF49 (cheese, 1986); PF8 (cheese environment, 1986); LL448, LL473 (human, 1988); 4428 (animal, 1990); 6836, LL539, LL540 (animal, 1991)
II	2	2	47/340/2389	1879 (human, 1989)
II	2	2	47/52	4856 (human, 1990)
II	2	2	47/340	6350 (human 1991)
II	2	2	47/52/108/312/340/1444/2389/3552	8558, 8735, 8769, 8798, 8844 (human, 1993)
II	2	2	47/52/108/312/340/2389/3552	9090 (human, 1993)
II	2	2	ND	A4, A13, A14 (animal, farm 3, 1986); A31, A34 (animal, 1988); A43 (animal, 1989)
II	2	2	47/52/108/340/1444/2389	8319 (animal, 1993)
II	2	2	47/52/108/312/340/1444/2389/2425/2671/3552	8796 (animal, 1993)
II	2	2	47/108/340/2389/2425/2671/3274	8964 (animal, 1993)
III	3	2	47/2425	6073 (animal, 1991)
III	3	2	NT	2352, 3193*, 3194*, 3195*, 3196*, 3204*, 3415, LL493 (human, 1989); 7278 (animal, 1992)
III	3	2	312/1444	2392 (human, 1989)
III	3	2	1444	LL510 (human, 1991)
III	3	2	47/108/340/2389/2425/2671/3274	LL512 (human, 1991)
IV	4	12	ND	A29, A36 (animal, 1988)
IV	4	12	47/108/340/2389/2425/3274	LL541 (animal, 1991)
IV	4	12	47/108/340/2389	LL400 (human, 1987); PF104 (cheese, 1986); BE56, BE62 (cheese, 1987); LL472 (human, 1988); 7110 (animal, 1992)
IV	4	12	NT	2337 (human, 1989)
IV	4	12	47/108/340/1444/2389	2537 (human, 1989)
V	5	2	47/108/340/2389/2425/2671/3274	2355 (human, 1989); 6445 (human, 1991); 7349 (human, 1992); 6046 (animal, 1991); 7133 (animal, 1992)
V	5	2	47/52/107/108/340/1444/2389/2425/2671/3274/3552	8625, 8699 (animal, 1993)
V	5	2	47/108/340/2389/2425/2671	9059 (human, 1993)
VI	1	14	ND	8493, 8499 (human, 1993)
VII	10	3	ND	LL481, LL487 (human, 1989)
IX	18	10	ND	A1, A7, A8, A9, A20 (animal, farm 2, 1986); 8396 (animal, 1993)
X	13	2	ND	A38 (animal, 1988); 6925 (animal, 1992)
XI	11	2	ND	LL535 (animal, 1991); 7355 (animal, 1992)
XII	7	3	ND	683 (human, 1988)
XIII	17	3	ND	495 (human, 1988)
XV	12	1	ND	LL478 (human, 1989)
XVI	8	6	ND	2521 (human, 1989)
XVII	24	8	ND	8500 (human, 1993)
XVIII	6	9	ND	6294 (human, 1991)
XIX	14	2	ND	LL538 (animal, 1991)
XX	15	2	ND	6074 (animal, 1991)
XX	15	2	47/108/340/2389	PF654 (cheese, 1988)
XXI	16	2	ND	4416 (animal, 1990)
XXII	17	2	ND	A45, A46 (animal, farm 1, 1989)
XXIII	21	2	ND	LL530 (animal, 1991)
XXIV	22	2	ND	A42 (animal, 1989)
XXV	23	2	ND	LL529 (animal, 1991)
XXVI	4	4	ND	A40 (animal, 1988)
XXVII	2	5	ND	A37 (animal, 1988)
XXVIII	20	7	ND	A41 (animal, 1988)
XXIX	5	11	ND	6103 (animal, 1991)
XXX	19	13	ND	A28 (animal, 1988)
XXXI	25	15	47/108/340/2389/2425/2671/3274	LL143 (human, 1985)

[a] Isolates isolated from humans in the years 1983 to 1987 have been previously considered related to the Swiss epidemic of 1983 to 1987 on the basis of their serotype, phage type, MEE type, and microrestriction type. Isolates from cheese and cheese environment are from the cheese implicated in the Swiss epidemic of 1983 to 1987 or from its production environment. Isolates from human and cheese related to the Swiss epidemic (1983 to 1987) are always the first listed for each corresponding subtype, ND, not determined; NT, not typeable by phage typing. Asterisks indicate isolates from the same patient.

Eight additional isolates previously shown to belong to ET1 (5) and originating from animal listeriosis cases ($n = 2$) or healthy shedders ($n = 6$) on two different farms were also used for the present study.

Serotyping. All of the *L. monocytogenes* strains mentioned above were serotyped by the method of Seeliger and Höhne (20).

MEE. The 22 enzymes examined and the buffer systems used for electrophoresis are as follows (21). Buffer system A included aconitase, alanine dehydrogenase, α-naphthyl-propionate esterase, β-naphthyl-propionate esterase, glutamate-oxalate transaminase, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, mannose-phosphate isomerase, nucleoside phosphorylase, phenylalanyl-leucine peptidase, leucyl-glycyl-glycine peptidase, phosphoglucose isomerase, and 6-Phosphogluconate dehydrogenase. Buffer system F included acid phosphatase, adenylate kinase, catalase, fumarase, glutamate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, indophenyl oxidase, lactate dehydrogenase, and phosphoglyceromutase. Strains from the Swiss epidemic of 1983 to 1987 previously shown to be ET1 were used as a reference (18). Sample preparation and electrophoresis were performed as described previously (5, 6). Staining procedures were the same as those previously described (5).

Macrorestriction analysis. The ET1 isolates found in the previous steps (Table 1) were examined by macrorestriction analysis as follows. The preparation of the DNA and the restriction with the enzymes *Apa*I and *Sma*I were performed as described by Brosch et al. (7). Field inversion gel electrophoresis with 1.1% Fast Lane agarose gels (FMC BioProducts, Rockland, Maine) was used to separate the DNA fragments after restriction. The gels were run in recirculated 0.5× Tris-borate-EDTA buffer at 10°C with a voltage of 10 V/cm. Switchback pulse controller PC500 (Hoefer Scientific Instruments, San Francisco, Calif.) was used for generating the pulsations. For the fragments obtained with *Apa*I 20-h electrophoresis with pulsations from 1 to 15 s (linear ramp; forward to reverse ratio, 3:1) was followed by 5 h with pulsations from 0.5 to 5 s (linear ramp; forward to reverse ratio, 3:1). For the fragments obtained with *Sma*I, 22-h electrophoresis with pulsations from 0.9 to 7 s (linear ramp; forward to reverse ratio, 3:1) was used.

Probe preparation, blotting, and hybridization. After restriction with the enzyme *Apa*I and electrophoresis as described above in 1.1% ultrapure LMP agarose (Gibco BRL, Gaithersburg, Md.), the desired bands were cut out of the gel and the corresponding DNA was purified with the Geneclean kit (Bio 101, Vista, Calif.). The purified DNA was digested with *Eco*RI, the restriction enzyme was inactivated at 65°C, and the DNA was nonradioactively labelled with fluorescein by random priming with the Renaissance kit (DuPont Biotechnology Systems, Boston, Mass.).

After restriction with either *Apa*I or *Sma*I and electrophoresis under the respective conditions described above, the DNA in the gels was nicked by UV light treatment in a GS Gene Linker (Bio-Rad, Hercules, Calif.) according to the instructions of the manufacturer. Transfer onto GeneScreen Plus membranes (DuPont Biotechnology Systems) was made by vacuum blotting using 0.4 M NaOH. Hybridization was done overnight at 65°C and was followed by washing at 65°C in 2× and 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate) for 30 min each. The hybrid patterns were visualized as described by the manufacturer of the Renaissance detection kit.

RESULTS

Frequency of ET1 in human and animal listeriosis during the postepidemic period of 1988 to 1993. Forty-five (28%) of the 145 human clinical cases registered at our center between 1988 and 1993 were related to the epidemic-associated ET1 clone (Table 1). The highest prevalence of ET1 in human cases was in the years 1989 (45%) and 1993 (37%). Forty-four (37%) of the 116 unrelated animal isolates received at our center between 1988 and 1993 (Table 1) were ET1.

PFGE subtypes within the ET1. A total of 144 ET1 isolates were subtyped by macrorestriction analysis (Table 1). In addition to the 45 human and 44 animal ET1 isolates from the postepidemic period mentioned above, 47 ET1 isolates from the epidemic period (31 human and 16 cheese and environment isolates) were examined. Finally, eight ET1 isolates from cattle recovered in 1986 from two different farms were also included. These 144 ET1 isolates yielded a total of 24 *Apa*I patterns and 15 *Sma*I patterns, resulting in 29 subtypes when the results obtained with the two enzymes were combined (Table 1). Five profiles were clearly more frequent than the others (PFGE types I to V [Table 1]). The distribution of the human isolates of these five subtypes during the postepidemic period of 1988 to 1993 is shown in Fig. 1. One cluster also confirmed by phage typing (Table 1) appears for subtype II in 1993, and another cluster appears for subtype III in 1989.

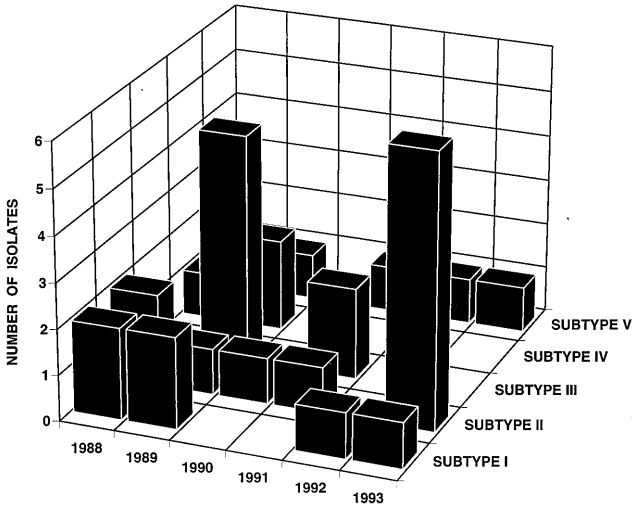

FIG. 1. Occurrence among human listeriosis cases of the five most frequent PFGE subtypes of the ET1 clone during the years 1988 to 1993. Each isolate represents one listeriosis case. Two clusters of cases can be observed for subtypes II and III in 1993 and 1989, respectively.

These two clusters of isolates probably correspond to listeriosis cases related to undetected common sources of infections. This hypothesis is supported for the subtype II cluster by a specific lysovar found in all these isolates. When these probably related cases were excluded from the analysis, the relative frequencies of the five major subtypes remained the highest within ET1 isolates of human origin and were not significantly different from those in ET1 isolates of animal origin ($P > 0.9$ [chi-square test]). ET1 isolates of PFGE subtype I and subtype II, which were also the main subtypes associated with the Swiss epidemic of 1983 to 1987 were found in 5 and 8%, respectively, of the 261 cases of human and animal listeriosis during the postepidemic period of 1988 to 1993.

Phage typing was in broad agreement with the grouping of the five major PFGE subgroups evidenced by macrorestriction analysis (subtypes I to V [Table 1]). Among the 104 isolates of these five subgroups found in sporadic, epidemic, and epidemiologically related cases (Table 1), only 21 differed significantly (i.e., for more than one phage reaction) from the other members of their respective PFGE subgroup. A majority of subtype IV isolates were of the same lysovar as subtype II isolates.

Multiple epidemiologically related isolates. No differences were observed for PFGE patterns within the ET1 isolates of each of the three farms studied (Table 1). Five isolates originating from different body sites of one patient were also indistinguishable by macrorestriction analysis (Table 1).

Subtypes of isolates related to the Swiss epidemic of 1983 to 1987. Three different PFGE subtypes (subtypes II, IV, and XX [Table 1]) were found among the eight ET1 isolates of lysovar 47/108/340/2389 from the cheese implicated in the Swiss outbreak and from its production environment. All the ET1 human isolates of this phage type from the epidemic period also belonged to the PFGE subtypes II and IV. The subtype IV restriction patterns (lane 2 in Fig. 2a) differed from subtype II patterns (lane 1 in Fig. 2a) by a barely visible shift of a single band toward slightly higher molecular size with both enzymes *Apa*I and *Sma*I.

Eight isolates from the implicated cheese and from its production environment belonging to lysovar 47/108/340/2389/

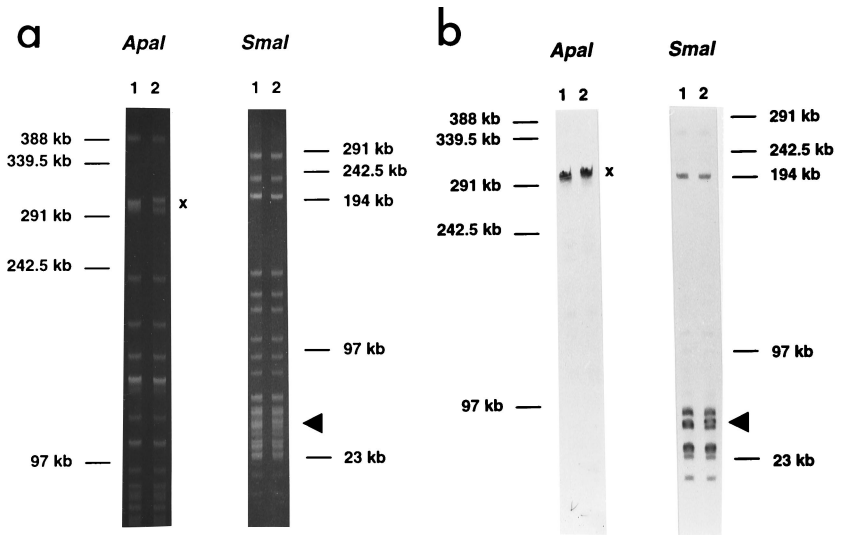

FIG. 2. PFGE (a) and Southern hybridization (b) patterns obtained with the two closely related *L. monocytogenes* ET1 isolates LL140 (subtype II) (lanes 1) and LL400 (subtype IV) (lanes 2) of lysovar 47/108/340/2389. The band with a slight shift (mentioned in the text) obtained with the enzyme *Apa*I is indicated (x). Arrowhead, LL400 band shift compared with LL140. A probe made with fragment x of panel a was used for Southern hybridization of the same gels (b).

2425/2671/3274 were indistinguishable from each other by PFGE (subtype I [Table 1]). From 17 ET1 isolates recovered in human listeriosis cases during the epidemic period and belonging to the same major lysovar, 16 had PFGE patterns indistinguishable from those of the cheese isolates (subtype I). The 17th isolate (LL143) had a pattern differing from the 16 other isolates for only two bands with *Apa*I (bands y and z in Fig. 3a) and two bands with *Sma*I (asterisk in Fig. 3a). This subtype was not found among any of the other isolates examined in the present study. The fragments obtained with the isolates LL143 and LL195 by restriction with *Sma*I were electrophoresed under different conditions in order to avoid compression or inversion zones known to occur in field inversion gel electrophoresis and to visualize the small-size fragments not visible under standard field inversion gel electrophoresis conditions. No differences could be demonstrated other than the shift of one band toward the higher-molecular-weight range for LL143 with *Apa*I digestion and the slight shift of one band toward a lower molecular weight with *Sma*I digestion (Fig. 3a). This suggests that the difference between LL143 and LL195 relies on more than one single genetic event.

The two major PFGE subtypes associated with the epidemic were also the two most frequently encountered subtypes during the postepidemic period among ET1 isolates (Table 1; Fig. 1).

Southern blotting and hybridization. Using the *Apa*I fragment x of strain LL400 (subtype IV [Fig. 2a]) as a probe for hybridization with DNA of strain LL140 (subtype II) shows that the slightly shifted corresponding fragment of strain LL140 contains sequences homologous to fragment x (Fig. 2b). A single insertion of a few hundred base pairs within this fragment could lead to the appearance of subtype IV from

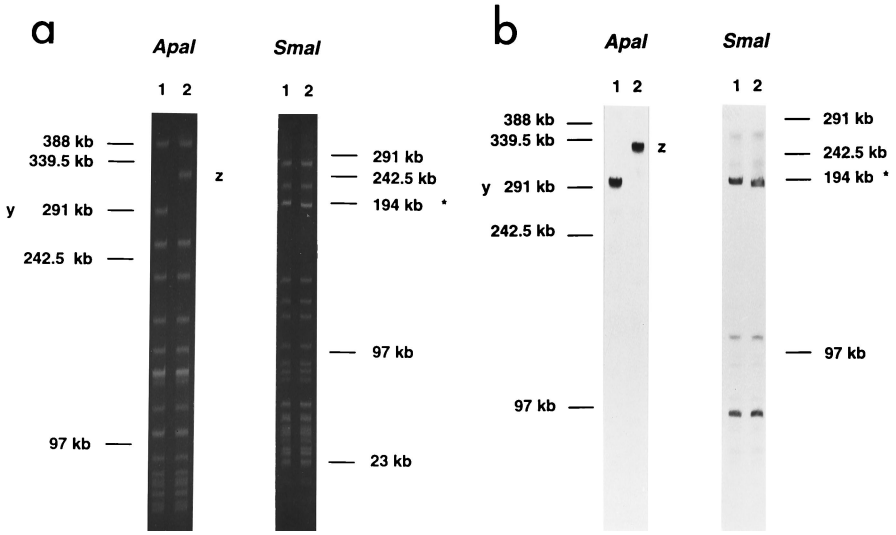

FIG. 3. PFGE (a) and Southern hybridization (b) patterns obtained with the two *L. monocytogenes* ET1 isolates LL195 (subtype I) (lanes 1) and LL143 (subtype XXXI) (lanes 2) of lysovar 47/108/340/2389/2425/2671/3274. y and z, LL195 and LL143 band shifts, respectively. Asterisk, LL143 band shift compared with LL195. A probe made with fragment z of panel a was used for Southern hybridization of the same gels (b).

subtype II. The hybridization of the same probe with the fragments obtained with *Sma*I also shows a shift of a few hundred base pairs in one fragment (Fig. 2b), thus confirming the hypothesis of an insertion.

Fragment z of LL143 (subtype XXXI [Fig. 3a]) was labelled and used as a probe for hybridization with Southern blots of strain LL195 (subtype I [lane 1 in Fig. 3b]) after digestion with the enzyme *Apa*I. The probe hybridized with both the z and the y fragments of the strains LL143 and LL195 (Fig. 3b). This shows that fragment z clearly contains sequences homologous to fragment y. When a membrane obtained after *Sma*I digestion of the same strains was probed, several fragments hybridized with the probe, including the fragments with a different size in LL143 and LL195 (Fig. 3b). This shows that the differences in fragment sizes between strains LL195 and LL143 observed with both restriction enzymes are related to the same region of the chromosome. With the enzyme *Apa*I, the difference seems to rely on an insertion in the genome of a type I strain. However, this is in contradiction with the results obtained with the enzyme *Sma*I, which apparently shows a short deletion in the genome of the LL143 strain.

DISCUSSION

Many methods have been used with success for typing and tracing *L. monocytogenes* in the environment. However, several of them have been shown to be of less value for examining isolates related to clinical listeriosis cases (2). Most cases are in fact caused by only three serovars (4b, 1/2a, and 1/2b). The genetic diversity within the clinically and epidemiologically predominant serovars 1/2b and 4b has been shown to be quite low (3, 12, 18). The majority of the typing methods available are consequently less effective in discriminating unrelated isolates within this group of strains (2, 4). This represents a clear obstacle to the tracing of these organisms and the differentiation between epidemiologically related and unrelated isolates belonging to this important serovar. The presence within serovar 4b strains of one major clone more frequently associated with clinical listeriosis (5, 17, 18), makes this problem even more acute.

Macrorestriction analysis of genomic DNA is becoming a major typing method for the epidemiological study of many microorganisms. Its very high discriminatory power is of particular interest for *L. monocytogenes* and could allow differentiation of strains in a more effective way than other methods, particularly for 4b strains (7, 9). However, the characteristics used to differentiate isolates with such a high discriminatory method may be evolving rapidly and may not be stable enough for practical use as reliable epidemiological markers. Thus, in the present work, we assessed the diversity evidenced by macrorestriction analysis within a widespread 4b clone and also examined the diversity of PFGE patterns found during a long-lasting epidemic of human listeriosis.

Our MEE results clearly confirm the already-observed high prevalence of the ET1 clone in sporadic listeriosis cases (5, 17, 23). More than one-fourth of the human cases registered in Switzerland during the postepidemic period of 1988 to 1993 and one-third of unrelated animal listeriosis cases examined were caused by the ET1 clone. Macrorestriction analysis showed, however, that the frequencies of two particular subtypes (subtypes II and III) were clearly increased among human isolates during 8 and 6 months in the years 1993 and 1989, respectively (Fig. 1). If one considers these cases as epidemiologically linked, the remaining sporadic cases still constitute more than 20% of ET1 isolates among cases of sporadic human listeriosis in the postepidemic period.

Our results showing the presence of at least 29 PFGE subtypes within the ET1 clone confirm the very high discriminatory power of PFGE (7, 14) and give a first picture of the diversity of the ET1 "endemic" population associated with clinical listeriosis in animals and humans. The frequencies of the main subtypes within ET1 isolates of human and animal origins were not significantly different. Thus, our results do not suggest the presence of two clearly distinct subpopulations of ET1 strains in the human and animal *Listeria* populations. We found that, in spite of the large number of subtypes evidenced, only five (subtypes I to V) represented the majority of the isolates examined (64% of the ET1 isolates of animal and human origins during the postepidemic period), even when the probably related cases mentioned above were subtracted. The two most frequent subtypes were those found during the Swiss epidemic of 1983 to 1987. One of these two subtypes has also been implicated in several other epidemics (9). Thus, these data suggest that not only the ET1 clone as a whole but also some very specific PFGE subtypes within this clone are frequently associated with clinical listeriosis cases, both in sporadic cases and in outbreaks of diverse magnitude (9; this study). It is, however, still not clear whether this high frequency relies on particular characteristics related to virulence or to a more frequent exposure of humans and animals to these strains.

The exact significance of the PFGE subtypes described here has to be questioned, however. The stability of the PFGE subtypes over long periods of time may not be very high, and variants of an original strain may develop, even within an epidemic (1, 22). The multiple isolates from different body sites of one patient examined here showed all the same PFGE patterns. Similarly, the multiple ET1 isolates from animals on three different farms (Table 1) showed that the same PFGE profiles could be obtained within each farm. However, these isolates were obtained within short periods of time after a first index case (1 day to 6 months). This confirms the validity of macrorestriction analysis for short-term epidemiological tracing of *L. monocytogenes* and for limited numbers of isolates (22). The Swiss listeriosis epidemic spread over a much longer period of time, and the interpretation of the results is more complex to analyze. With phage typing, microrestriction analysis (16), and random amplification of polymorphic DNA (4), two clearly distinct subtypes corresponding to two different strains have been described for the ET1 isolates from the implicated cheese. The two subtypes have also been found in the majority of the patients in the epidemic (16). However, macrorestriction analysis allowed discrimination of two additional ET1 subtypes on the implicated cheese (subtypes IV and XX [Table 1]). This raises the question of whether the cheese and its production environment had been contaminated at the origin by several sources or by only two ET1 strains which evolved to produce other PFGE subtypes during the long-lasting colonization by *L. monocytogenes*. The fact that all four subtypes found on the cheese were also found in animals (three of them very frequently) and the presence of *L. monocytogenes* serovars other than 4b on the cheese simultaneously with the two main epidemic strains (data not shown) support the former hypothesis. However, our PFGE data using two different restriction enzymes as well as hybridization experiments suggest that the appearance of subtype IV could be due to a single insertion in the genome of a subtype II isolate. Furthermore, the close relationship between subtype II and subtype IV is also supported by the identity of the main phage type in both PFGE subtypes. Thus, the presence of subtype IV for example may not necessarily be due to a contamination of the cheese with a new strain but could be the consequence of the insta-

bility of the PFGE subtypes. The case of subtype XX, which was found only once on the cheese is less clear. The macrorestriction pattern differences between subtype XX and the other subtypes found on the cheese cannot be explained as easily by a single genetic event as could the difference between subtypes II and IV (data not shown). Thus, the subtype XX isolate may really represent a different and less closely related ET1 strain.

Four subtypes could be found among ET1 isolates from human patients during the epidemic period. The major subtypes found on the cheese (subtypes I, II, and IV) were also found in these human patients. However, one case of human listeriosis during the epidemic period (1985) was due to a PFGE subtype unique to this patient (subtype XXXI) but showing some similarities with the main subtype I. Separate analysis of the *Apa*I and the *Sma*I restriction patterns of this isolates could not explain by a single genetic event how a subtype XXXI isolate could derive from a subtype I isolate. Our results show that the difference between these two subtypes may be more complex than a single insertion and suggest that the corresponding isolates are not as closely related as could be thought on the basis of the results obtained with a single restriction enzyme. These data suggest that the listeriosis case due to the subtype XXXI strain was not related to the epidemic. Furthermore, no data demonstrating the exposure of this particular patient to the implicated cheese are available. The fact that the corresponding subtype was found neither among the cheese isolates nor among any of the other 143 ET1 isolates examined here also supports this hypothesis. Our results with subtype XXXI show that the relationships between PFGE patterns and possible mechanisms leading to the emergence of different patterns may be more complex than usually accepted. One should not just rely on the number of band differences between patterns obtained with only one enzyme to assess genetic relationships between isolates, but each suspected close relationship should be confirmed by an analysis with additional restriction enzymes. This is particularly important for examining a large number of isolates or potentially related isolates spread over a long period of time.

In conclusion, our results show that a few PFGE subtypes within the predominant ET1 clone are more frequently encountered than others in clinical listeriosis cases. The present study confirms that macrorestriction analysis represents a valid epidemiological tool for tracing *L. monocytogenes*, at least for short-term studies and for limited numbers of isolates. However, the interpretation of PFGE results in long-term studies may be more problematic. When an epidemiological link and close genetic relatedness between isolates are suspected on the basis of a small number of band differences in PFGE patterns, this relatedness should be examined by a more detailed analysis and should be well supported by solid epidemiological data.

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