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Comparative genetic characterization of *Listeria monocytogenes* isolates from human and animal listeriosis cases

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Listeria monocytogenes **isolates from human sporadic and epidemic cases (*n* = 119) and from animal cases (*n* = 76) were characterized by automated ribotyping and PCR-restriction fragment length polymorphism (PCR-RFLP) typing of the virulence genes *actA* and *hly*. This combination of typing methods differentiated 39 distinctive strains, each reflecting a unique combination of ribotypes, *hly* and *actA* alleles. Simpson's index of discrimination indicated a high discriminatory ability of ribotyping for both animal (0·867) and human isolates (0·857), which was further increased by the addition of *hly* and *actA* typing (0·916 and 0·904, respectively). Ribotype and *hly* allele data were further used to group isolates into three genetically distinct lineages. Each lineage is composed of several ribotype fragment subsets, each of which contains multiple ribotypes characterized by common ribotype fragments. To determine whether certain clones of *L. monocytogenes* show indications for unique pathogenic potential or host specificity, frequency distributions for five genetic characteristics (i.e. lineage, ribotype, ribotype fragment subset and *hly* and *actA* allele) were calculated for isolates from animal cases, human epidemic cases and human sporadic cases. Lineage III isolates were found less frequently in human cases (1 of 119 isolates) than in animal cases (8 of 76 isolates; *P* = 0·003). These results suggest the possibility of host specificity for non-primate mammals among lineage III strains. In addition, lineage I strains were found more frequently among human cases than among animal cases (*P* < 0·001). Among the eight *hly* alleles observed, *hly* allele 1 was more common among human isolates as compared to animal isolates (*P* = 0·002). We also identified one ribotype (DUP-1030) which was significantly more common among animal isolates (*P* = 0·005) and one ribotype (DUP-1038; lineage I) which was significantly more common among human epidemic isolates as compared to human sporadic isolates (*P* < 0·001). These findings confirm the presence of clonal groups of *L. monocytogenes*, which appear to be characterized by unique virulence or host specificity patterns. This study also establishes baseline data describing the genetic diversity of human and animal *L. monocytogenes* isolates which can be utilized in future surveillance programmes to track the emergence of new strains.**

Keywords: ribotyping, lineages, subtyping, pathogenic potential

INTRODUCTION

Listeria monocytogenes is a Gram-positive, facultative intracellular bacterium, which is considered a zoonotic pathogen. The organism is predominantly transmitted to humans by the food-borne route (Farber & Peterkin, 1991). Recent estimates suggest that approximately 2500 human listeriosis cases occur annually in the US alone; these cases lead to about 500 deaths (Mead *et al.*, 1999).

Abbreviation: SID, Simpson's index of discrimination.

Table 1. Human epidemic isolates

Outbreak (source)	Year	Isolate designation	Ribotype	Serotype	Allele for:		Lineage	Reference
					hly	*actA*		
Anjou, France	1976	FSL-J1-112/TS31/L4491a	1038	4b	1	3	I	Carbonelle *et al.* (1978)
Boston, USA (raw vegetables)	1979	FSL-J1-220	1042	4b	1	4	I	Ho *et al.* (1986)
Carlisle, England	1981	FSL-J1-105/TS23/L745	1030	1/2a	2	4	II	McLauchlin *et al.* (1986)
Nova Scotia, Canada (coleslaw)	1981	FSL-J1-003	1038	4b	1	3	I	Schlech *et al.* (1983)
		FSL-J1-107/TS26/L4742	1038	4d	1	3	I	
		FSL-J1-108/TS27/L4738	1038	4b	1b	3	I	
Massachusetts, USA (pasteurized milk)	1983	ScottA	1042	4b	1	4	I	Fleming *et al.* (1985)
United Kingdom (paté)	1988–1990	FSL-J1-116/TS38/L3306	1042	4b	1	4	I	McLauchlin *et al.* (1991)
		FSL-J1-129/TS65/L3238	1042	4bx	1	4	I	
Los Angeles, USA (soft cheese)	1985	FSL-J1-110/TS29/F2365	1038	4b	1	3	I	Linnan *et al.* (1988)
		FSL-J1-002	1038	4b	1	4	I	
Switzerland (soft smear cheese)	1983–1987	FSL-J1-123/TS55/L4486a	1038	4b	1	3	I	Bille (1990)
Philadelphia, USA (ice cream, salami)	1987	FSL-J1-012	1038		1	3	I	Schwartz *et al.* (1989)
		FSL-J1-020	1042		1	4	I	

Listeriosis symptoms in humans and animals include encephalitis, meningitis, septicaemia and abortion. Despite the fact that not all strains of *L. monocytogenes* are thought to be pathogenic to humans (Hof & Rocourt, 1992), host specificity has yet to be demonstrated for different *L. monocytogenes* strains. Host specificities have been identified in other bacterial pathogens that affect both animals and humans. For example, host specificity has been suggested for either humans or cattle among different clones of *Staphylococcus aureus* (Kapur *et al.*, 1995; Musser & Selander, 1990). Different strains of *Pasteurella haemolytica*, the aetiological agent of pneumonic pasteurellosis, also appear to display host specificity for either cattle or sheep (Davies *et al.*, 1997).

Several studies have suggested that *L. monocytogenes* may be characterized by a clonal structure and that pathogenic potentials may differ among clonal groups (Piffaretti *et al.*, 1989; Wiedmann *et al.*, 1997). Rasmussen *et al.* (1995) demonstrated that *L. monocytogenes* can be divided into three lineages based on sequence determination of the virulence genes *hly*, *iap* and the gene *flaA* encoding flagellin. Wiedmann *et al.* (1997) confirmed classification of *L. monocytogenes* into three genetically distinct lineages through ribotyping and PCR-RFLP of the virulence gene *hly*. Notably, all human epidemic isolates clustered into lineage I and none of the 21 human isolates examined fell into lineage III (Wiedmann *et al.*, 1997). Isolates from human sporadic cases were distributed between lineage I and lineage II (65 and 35%, respectively), while animal isolates appeared to encompass a more diverse population which spanned all three lineages (Wiedmann *et al.*, 1997).

At the outset of this study we hypothesized (i) that the three genetic lineages or specific clonal groups of *L. monocytogenes* show unique pathogenic potentials for different hosts, namely humans and animals; (ii) that all lineage III strains, or a subset thereof, show attenuated virulence in humans; and (iii) that all lineage I strains, or a subset thereof, show unique pathogenic potential towards humans. This current study was designed to probe the clonal structure of *L. monocytogenes* and to evaluate the distribution of different clonal groups and virulence gene alleles among human and animal *L. monocytogenes* isolates to identify possible differences in pathogenic potentials and host specificities within this species. To that end, we have assembled a collection of 119 human and 76 animal *L. monocytogenes* isolates and have characterized these strains using automated ribotyping and PCR-RFLP typing of the virulence genes *actA* and *hly*. These data were analysed for the existence of distribution differences for specific genetic types and strains among human epidemic, human sporadic and animal listeriosis isolates. Specific clones or strains that differ in distribution to these groups are likely to differ from each other in their pathogenic potentials. Furthermore, our data were utilized to determine the discriminatory power of the typing methods used (i.e. automated ribotyping and PCR-RFLP of *actA* and *hly*).

METHODS

Strains and isolates. A total of 119 human and 76 animal *L. monocytogenes* isolates from various sources were used in this study. Human isolates included those from sporadic cases as well as those from nine different epidemic outbreaks defined as such in the World Health Organization's (WHO) International Multicentre *L. monocytogenes* subtyping study (Table 1) (Bille & Rocourt, 1996). Human isolates from sporadic cases were also obtained from the Sunnybrook Health Science Centre (University of Toronto, Ontario, Canada) as described by Louie *et al.* (1996), the Centers for Disease Control (Atlanta, Georgia), the Department of Health Services (Los Angeles, California) and Qualicon Inc. (Wilmington, Delaware). The majority of these human isolates were collected between 1982 and 1995. The animal isolate collection, previously described by Wiedmann *et al.* (1997), was obtained from the Diagnostic Laboratory, College of Veterinary Medicine, Cornell University. Sixty-four isolates were from ruminants (cattle, sheep and goats) and 10 were from non-ruminants (horses, primates and birds) (Wiedmann *et al.*, 1997).

All isolates were characterized by automated ribotyping and PCR-RFLP analysis of the virulence genes *actA* and *hly*. These data were used to classify isolates on the basis of the following five genetic characteristics: lineage, ribotype, ribotype fragment subset, *hly* allele and *actA* allele. Isolate classification to lineage was designated based on ribotype and *hly* allele data as described by Wiedmann *et al*. (1997). Isolates were clustered into ribotype fragment subsets as described by Bruce *et al*. (1995).

Virulence gene allele characterization. Virulence gene alleles for *actA* and *hly* were determined by PCR-RFLP as described previously (Wiedmann *et al*., 1997).

Ribotyping. A subset of isolates was manually ribotyped using the procedure described by Bruce *et al*. (1995) and reported by Wiedmann *et al*. (1997). The remaining samples and representatives from each of the reported ribotypes were then processed using the RiboPrinter Microbial Characterization System (Qualicon). This procedure was carried out to enable the use of standardized data for future reference. The RiboPrinter system process has been described by Bruce (1996). In brief, this automated process includes bacterial cell lysis and cleavage of the DNA using the restriction enzyme *Eco*RI. DNA fragments are separated by size using electrophoresis and analysed using a modified Southern hybridization blotting technique. The DNA is hybridized with a labelled rRNA operon probe derived from *E. coli* and the bands are detected using a chemiluminescent substrate. An image is captured using a customized charge-coupled device camera and is then electronically transferred to the system's computer. Each sample lane of data is normalized to a standard marker set, characterized and identified using similarity measurements to previously run isolates and reference patterns.

Simpson's index of discrimination (SID). The suitability of typing methods for differentiation of strains was determined using Simpson's numerical index as described by Hunter & Gaston (1988).

Statistical methods. Isolates were separated into three categories (i.e. human epidemic, human sporadic and animal isolates), then frequency distributions were calculated separately for each genetic characteristic. Human epidemic and human sporadic isolates were also pooled to allow a comparison of human isolates versus animal isolates. The frequency distributions of each genetic characteristic between animal versus human and human epidemic versus human sporadic isolates were compared using a χ^2 of independence test. For comparisons where one or more of the expected values were <5, Fisher's exact test was used. P values $\leqslant 0{\cdot}05$ were considered statistically significant. The significance level was left at $\leqslant 0{\cdot}05$ and was not adjusted for the fact that multiple comparisons were made. We chose this approach to avoid missing possible associations of genetic characteristics (caused by a very conservative P value) and provided observed P values instead. With a Bonferroni correction for multiple comparisons ($n = 83$), a significant P value would be approximately $P \leqslant 0{\cdot}0006$. Readers can evaluate the significance according to their preferred criteria.

RESULTS

Genetic characterization of human and animal isolates

Ribotypes and PCR-RFLP alleles for *actA* and *hly* were determined for 119 human and 76 animal isolates (Table 2). These analyses designated 39 subtypes (A–AN), which are characterized by unique combinations of ribotypes, *actA* and *hly* alleles. Human and animal isolates comprised 28 and 24 subtypes, respectively. Fifteen and 11 subtypes were unique to either humans or animals, respectively. Eighteen ribotypes were found among the 119 human isolates, while 17 ribotypes were found among the 76 animal isolates.

The genetic characteristics of 14 human epidemic

Table 2. Human and animal isolates and their genetic characteristics

Strain type	Ribotype	Allele for:		No. of isolates for each strain type obtained from:	
		actA	*hly*	Humans ($n = 119$)	Animals ($n = 76$)
A	DUP-1024	3	1	4	0
B	DUP-1024	4	1	1	0
C	DUP-1026	3	1	1	0
D	DUP-1027	3	1	3	0
E	DUP-1027	3	1b	1	0
F	DUP-1029	4	2	2	0
G	DUP-1030	3	2	1	3
H	DUP-1030	4	2	12	17
I	DUP-1030	4	2c	0	1
J	DUP-1035	4	2	1	0
K	DUP-1038	3	1	17	9
L	DUP-1038	3	1b	1	0
M	DUP-1038	4	1	1	0
N	DUP-1039	3	2	1	0
O	DUP-1039	4	2	4	1
P	DUP-1039	4	2b	0	1
Q	DUP-1040	4	1	0	1
R	DUP-1042	3	1	9	9
T	DUP-1042	4	1	28	6
U	DUP-1043	3	1	1	0
V	DUP-1044	3	1	2	4
W	DUP-1044	4	1	5	1
X	DUP-1045	3	2	0	1
Y	DUP-1045	4	2	4	6
Z	DUP-1047	4	2	1	3
AA	DUP-1052	3	1	4	2
AB	DUP-1052	4	1	1	0
AC	DUP-1053	4	2	4	0
AD	DUP-1054	4	2	0	1
AE	DUP-1056	4	2	2	0
AF	DUP-1059	4	1b	0	3
AG	DUP-1059	4	4b	1	1
AH	DUP-1061	4	4b	0	1
AI	DUP-1062	4	2	6	1
AJ	DUP-1062	4	2b	1	0
AK	DUP-10146	4	1c	0	1
AL	DUP-10147	4	4a	0	1
AM	DUP-10144	4	2	0	1
AN	DUP-10145	4	4a	0	1

Table 3. Frequency distribution of *Listeria monocytogenes* types among animal, human epidemic and human sporadic isolates

Genetic strain types	Number of isolates (%) from:			*P* value of χ^2 test for:*	
	Animal cases ($n = 76$)	Human epidemic cases ($n = 14$)	Human sporadic cases ($n = 105$)	Animal cases vs human cases	Human epidemic cases vs human sporadic cases
***hly* alleles**					
1	32 (42·1)	12 (85·7)	65 (61·9)	0·002†	0·134
1b	3 (3·9)	1 (7·1)	1 (1·0)	0·380	0·222
1c	1 (1·3)	0	0	0·390	NA
2	34 (44·7)	1 (7·1)	37 (35·2)	0·071	0·063
2b	1 (1·3)	0	1 (1·0)	1·000	1·000
2c	1 (1·3)	0	0	0·390	NA
4a	2 (2·6)	0	0	0·151	NA
4b	2 (2·6)	0	1 (1·0)	0·561	1·000
***actA* alleles**					
3	28 (36·8)	7 (50·0)	38 (36·2)	0·891	0·317
4	48 (63·2)	7 (50·0)	67 (63·8)	0·891	0·317
Lineages					
I	32 (42·1)	13 (92·9)	66 (62·9)	8×10^{-4}†	0·033‡
II	36 (47·4)	1 (7·1)	38 (36·2)	0·041‡	0·034‡
III	8 (10·5)	0	1 (1·0)	0·003†	1·000
Ribotype fragment subsets					
E 11.2	11(14·5)	8 (57·1)	20 (19·0)	0·123	0·004†
E 5.2	28 (36·8)	1 (7·1)	31 (29·5)	0·142	0·109
E 5.3	2 (2·6)	0	5 (4·8)	0·707	0·634
E 5.3, -D	4 (5·3)	0	8 (7·6)	0·768	0·594
E 9.2	18 (23·7)	5 (35·7)	38 (36·2)	0·067	0·972
G 6.2, H 9.0	1 (1·3)	0	1 (1·0)	1·000	1·000
G 8.1, H 7.1	8 (10·5)	0	1 (1·0)	0·003†	1·000
Temperature-controlled	4 (5·3)	0	1 (1·0)	0·077	1·000
Ribotype					
1024	0	0	5 (4·8)	0·159	0·634
1026	0	0	1 (1·0)	1·000	1·000
1027	0	0	4 (3·8)	0·158	1·000
1029	0	0	2 (1·9)	0·522	1·000
1030	20 (26·3)	1 (7·1)	12 (11·4)	0·005†	0·710
1035	0	0	1 (1·0)	1·000	1·000
1038	9 (11·8)	8 (57·1)	11 (10·5)	0·423	$2{\cdot}0 \times 10^{-6}$†
1039	2 (2·6)	0	5 (4·8)	0·707	0·634
1040	1 (1·3)	0	0	0·390	NA
1042	15 (19·7)	5 (35·7)	32 (30·5)	0·080	0·761
1043	0	0	1 (1·0)	1·000	1·000
1044	6 (7·9)	0	7 (6·7)	0·583	0·598
1045	7 (9·2)	0	4 (3·8)	0·113	1·000
1047	3 (3·9)	0	1 (1·0)	0·301	1·000
1052	2 (2·6)	0	5 (4·8)	0·707	0·634
1053	0	0	4 (3·8)	0·158	1·000
1054	1 (1·3)	0	0	0·390	NA
1056	0	0	2 (1·9)	0·522	1·000
1059	4 (5·3)	0	1 (1·0)	0·077	1·000
1061	1 (1·3)	0	0	0·390	NA
1062	1 (1·3)	0	7 (6·7)	0·153	0·598
dd11696	1 (1·3)	0	0	0·390	NA
dd11698	1 (1·3)	0	0	0·390	NA
dd11900	1 (1·3)	0	0	0·390	NA
dd11903	1 (1·3)	0	0	0·390	NA

* NA, χ^2 test not applicable.

† *P* values $\leqslant 0{\cdot}05$.

‡ *P* values $\leqslant 0{\cdot}05$ which were $> 0{\cdot}05$ when calculations were repeated counting only one single isolate of each genetic type for each of the outbreaks represented by multiple isolates (see Results).

isolates from nine different outbreaks are presented separately in Table 1. Multiple epidemic isolates were available for subtyping for four outbreaks (Nova Scotia, Canada, 1981; Los Angeles, USA, 1985; United Kingdom, 1988–1990; Philadelphia, USA, 1987). Multiple isolates from each of the four outbreaks differed by at least one genetic characteristic or by their serotype, suggesting that each represented a distinctive strain (Table 1). For example, one of the three isolates from the 1991 Nova Scotia outbreak was characterized by *hly* allele 1b while the other two carried *hly* allele 1. The two isolates from the 1985 Los Angeles outbreak in our collection carried different *actA* alleles (Table 1). Since only two isolates from this outbreak were available for subtyping in our laboratory, the frequency of these two subtypes among all isolates from this outbreak is not known. For the outbreaks in the United Kingdom (1988–1990) and in Philadelphia (1987), different subtypes were shown to cause a significant number of cases (Schwartz *et al*., 1989; McLauchlin *et al*., 1991). The serotype 4d strain isolated during the Nova Scotia outbreak (Table 1) appears to have caused only a few cases or a single case in this outbreak (Schlech *et al*., 1983). For the other outbreaks included in this study, a single *L. monocytogenes* subtype appears to have been responsible for all cases (see references in Table 1).

Frequency distribution of *L. monocytogenes* genetic characteristics among human and animal isolates

The information in Table 2 was used to determine the frequency distribution in each category (human epidemic, human sporadic and animal) of the five genetic characteristics (*hly* alleles, *actA* alleles, lineage, ribotype and ribotype fragment subset) determined for each isolate (Table 3). We chose to include each of the multiple isolates from a given outbreak in calculating the frequency distribution of the human epidemic isolates since each isolate appeared to represent a distinct strain (see above and Table 1). To account for this possible sampling bias, calculations were also repeated counting only one single isolate of each genetic type for each of the outbreaks represented by multiple isolates. For example, in these calculations, only one isolate from the Nova Scotia outbreak was included in the frequency distribution of lineages (as all three isolates belong to lineage I), while one *hly* allele 1 and one *hly* allele 1b isolate were included in the frequency distribution of *hly* alleles. In the remainder of this paper, we will refer to these calculations as the 'conservative calculation with epidemic duplicates removed'. Using these conservative calculations with epidemic duplicates removed, five *P* values which previously indicated significant differences with *P* values between 0·02 and 0·05 changed to non-significant levels ($P > 0{\cdot}05$) (Table 3). These *P* values are indicated by ‡ in Table 3, while *P* values with significant differences in both calculations are indicated by †. Only frequency distributions that showed significant differences in both calculations were considered to reflect robust differences in frequency of the respective genetic types.

Lineage III strains were isolated less frequently from human cases (0·8%) than from animal cases (10·5%). Lineage I strains represented 92·9% of human epidemic and 62·9% of human sporadic isolates and were significantly more frequent among human cases as compared to animal cases (42·1%). Animal isolates were classified into lineages I and II with similar frequencies (42·1 and 47·4%, respectively).

Ribotype fragment subsets E 11.2, E 5.2 and E 9.2 represent the most frequently isolated ribotype fragment subsets from humans and animals (82·1% of all isolates). Ribotype fragment subset G 8.1, H 7.1, one of three subsets constituting lineage III (Wiedmann *et al*., 1997), was significantly less frequently isolated from humans (0·8%) than from animals (10·5%). Subset E 11.2 was significantly more common among the epidemic isolates (57·1%) than among the human sporadic isolates (19·0%; $P = 0{\cdot}004$). The *P* value for the comparison of human epidemic and sporadic isolates increased to $P = 0{\cdot}038$ in the conservative calculation with epidemic duplicates removed.

Ribotypes DUP-1030 and DUP-1038 are the only ribotypes that showed significant differences in their frequencies among human epidemic, human sporadic and animal cases. DUP-1030 was more frequently associated with animal cases (26·3%) than with human cases (11·4%). DUP-1038 was more frequent among human epidemic isolates (57·1%) than among human sporadic isolates (10·5%; $P = 2{\cdot}0 \times 10^{-6}$). This *P* value increased to $P = 0{\cdot}0046$ in the conservative calculation with epidemic duplicates removed. DUP-1042 is the most common ribotype accounting for 35·7% of the human epidemic isolates and for 30·5% of the sporadic isolates, but for only 19·7% of all animal isolates.

PCR-RFLP analysis of the *hly* gene allowed us to differentiate eight *hly* alleles among the 195 isolates. *hly* alleles 1 and 2 were the two most common *hly* alleles. *hly* allele 1 represented 64·7% of all human isolates (epidemic and sporadic) and was significantly more common among human as compared to animal isolates (42·1%). *hly* allele 1b was found in two lineage I human isolates as well as in three lineage III animal isolates. *Hly* alleles 1c, 4a and 4b were unique to lineage III isolates and accounted for 5 of the 76 animal isolates (6·6%) but only 1 of the 119 human isolates.

The *actA* PCR-RFLP assay used in this study differentiates two alleles, which are characterized by the presence of either three or four proline-rich repeats for *actA* allele 3 or 4, respectively. No significant differences in *actA* allele distributions were found among the three categories of isolates (human epidemic, human sporadic and animal) (Table 3). However, the frequencies of the two *actA* alleles differed significantly among the three lineages ($P < 0{\cdot}0001$; 3×2 χ^2 test). *actA* allele 4 was

Table 4. SID for various typing methods

Typing method	SID	
	Animal isolates	Human isolates
Ribotyping only	0·867	0·857
Ribotyping and *hly*	0·877	0·860
Ribotyping and *actA*	0·909	0·900
Ribotyping, *hly* and *actA*	0·916	0·904
hly and *actA*	0·737	0·703

found in 39·6, 92 and 100% of the clinical isolates in lineages I, II and III, respectively.

SID

SID was calculated for each typing method, as well as for all possible combinations of different typing methods used (Table 4). The numerical value of this index (D) indicates the suitability of a given method for differentiating strains by estimating the probability that two unrelated strains are differentiated by a given typing method (Hunter & Gaston, 1988). As the numerical index approaches the maximum value of $D = 1$ (representing 100% discriminatory ability of a method), the higher the probability that a given method will be able to discriminate between two unrelated strains. SID for automated ribotyping of either human or animal isolates was very similar (0·867 and 0·857, respectively). When ribotyping was combined with allelic typing of both *actA* and *hly*, the index of discrimination increased to 0·916 and 0·904 for animal or human isolates, respectively (Table 4).

DISCUSSION

We have defined specific subsets and clonal groups of *L. monocytogenes* that differ in their associations with animal and human listeriosis cases and outbreaks, and thus may show host specificities or unique pathogenic potentials. We have also demonstrated that ribotyping, particularly in conjunction with PCR-RFLP of *hly* and *actA* virulence genes, provides an effective means for differentiating and characterizing *L. monocytogenes* isolates. The strains used in this study compose a collection of genetically well characterized human and animal *L. monocytogenes* isolates which should provide a valuable resource for further evaluation of typing and detection methods and for additional investigations of the role of strain diversity in *L. monocytogenes* pathogenesis and virulence.

Clonal structure of *L. monocytogenes*

The combination of PCR-RFLP and ribotyping defined 39 distinctive *L. monocytogenes* strains (Table 2). We differentiated 8 *hly* alleles, 2 *actA* alleles, 25 ribotypes, 8 ribotype fragment subsets and 3 lineages, thus illustrating this organism's diversity among animal and human isolates. Separation of *L. monocytogenes* into three lineages representing genetically distinctive subsets of this species is consistent with a variety of previous studies. Phylogenetic classification methodologies have categorized *L. monocytogenes* strains initially into two, then more recently, three, distinct lineages (Brosch *et al*., 1994; Graves *et al*., 1994; Gutekunst *et al*., 1992; Piffaretti *et al*., 1989; Rasmussen *et al*., 1991; Vines *et al*., 1992). The different methodologies have resulted in notably similar strain groupings based on serotype distribution to distinct lineages, with *a* and *c* serotypes falling into one subgroup and *b* serotypes into another (Gutekunst *et al*., 1992). Rasmussen *et al*. (1995) first suggested the existence of a third lineage based on sequence comparisons for *hlyA*, *iap* and the gene encoding flagellin (*flaA*). The strains in this group correspond with those classified into our lineage III by ribotyping and virulence gene alleles (Wiedmann *et al*., 1997). The clonal structure of the *L. monocytogenes* strains examined in this study is illustrated in Fig. 1. Each of the three lineages is divided into multiple ribotype fragment subsets, which represent groups sharing common ribotype fragments (Bruce *et al*., 1995). The number of ribotypes found in each subset and the positions of the three specific ribotypes with frequencies >10% among our isolates are also indicated in Fig. 1. DUP-1042, the most common ribotype found among all isolate groups in this study, was responsible for four listeriosis epidemics (Table 1). DUP-1038, which was significantly more common in human epidemic cases than in animal or human sporadic cases, was responsible for five listeriosis epidemics (Table 1). While DUP-1030 was significantly more common among animal cases than among human cases, it was responsible for a human listeriosis outbreak in Carlisle in 1981.

Indications for host specificities and differences in pathogenic potential

The pathogenic potential of bacterial pathogens in various hosts has been demonstrated to be non-randomly distributed along phylogenetic lines in many species (Musser *et al*., 1989; Selander & Musser, 1990; Selander *et al*., 1987). For example, host specificities among clonal subsets have been described for humans and for different animal species in *Staphylococcus aureus* (Musser & Selander, 1990), for different animal species in *Bordetella* spp. (Musser *et al*., 1987) and for cattle and sheep in *P. haemolytica* (Davies *et al*., 1997). Clonal groups with unique virulence potentials have also been reported for a variety of bacterial pathogens including *Haemophilus influenzae* (Quentin *et al*., 1990) and *Streptococcus agalactiae* (Helmig *et al*., 1993; Musser *et al*., 1989; Quentin *et al*., 1995).

We evaluated frequency distributions of five different genetic characteristics (*hly* allele, *actA* allele, ribotype, ribotype fragment subset and lineage) among three categories of *L. monocytogenes* isolates (human spor-

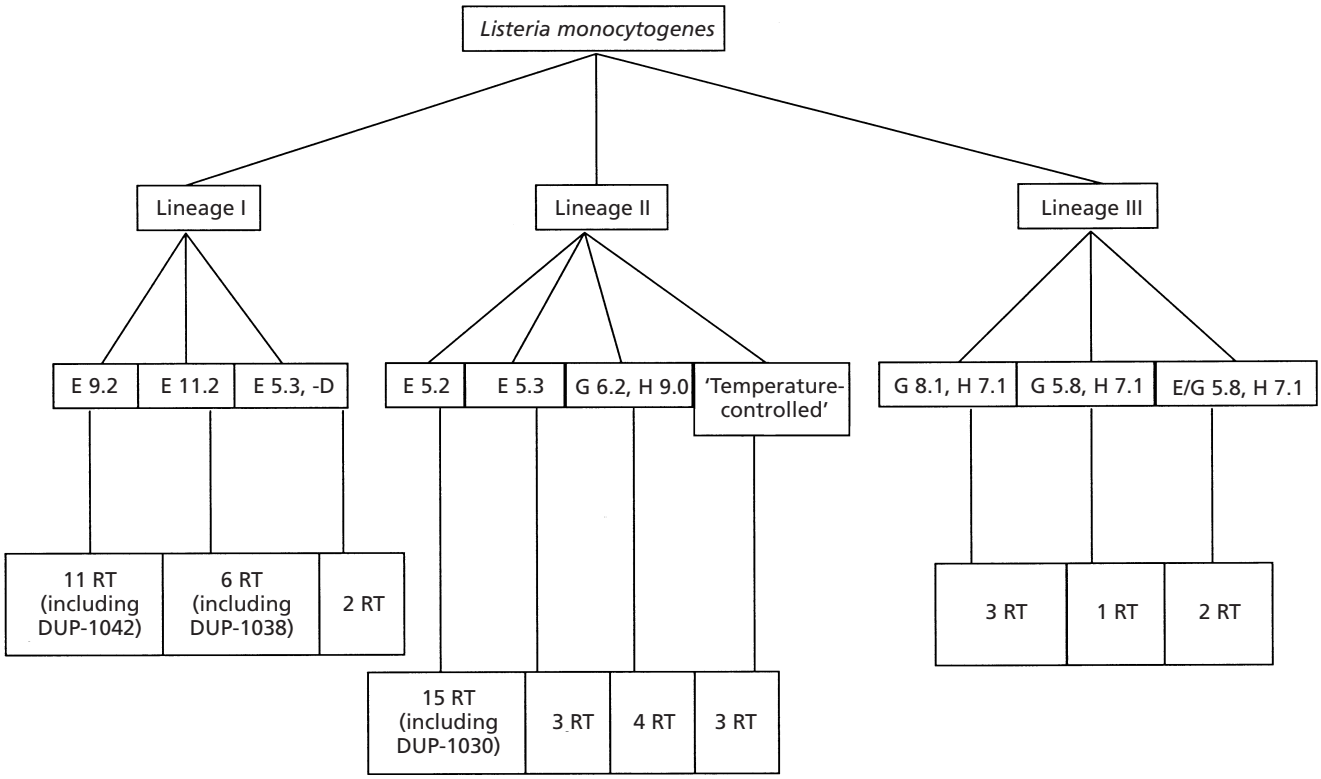

Fig. 1. Clonal structure of *L. monocytogenes*. *L. monocytogenes* consists of three lineages (Wiedmann *et al.*, 1997), each of which is divided into multiple ribotype fragment subsets. Ribotype (RT) fragment subsets represent groups of ribotypes which share common ribotype fragments (Bruce *et al.*, 1995). The number of ribotypes found in each subset is also indicated. The positions of the three specific ribotypes (DUP-1042, DUP-1038 and DUP-1030) with frequencies $>10\,\%$ among our isolates are also indicated.

adic, human epidemic and animal sources) to assess the possibilities of host specificities or unique pathogenic potentials among specific subsets or clonal groups of this organism. The separation into human sporadic and epidemic isolates may not always be accurate since some apparent sporadic cases may actually represent unrecognized epidemics. We thus focused our initial analyses on comparing the frequency of animal isolates to all human isolates (sporadic and epidemic). Our results suggest that *L. monocytogenes* is a bacterial pathogen with non-random distributions of host specificities and pathogenic potentials among different clonal groups. Ribotypes or specific ribotype fragments that differentiate ribotype fragment subsets, appear to be good markers for *L. monocytogenes* clones that vary in virulence for particular host species.

Previous studies have shown that human epidemic *L. monocytogenes* isolates represent two unique clones of this species (Piffaretti *et al.*, 1989), which are equivalent to ribotypes DUP-1038 and DUP-1042. In our study, human epidemic isolates showed a significant predominance of ribotype DUP-1038. The significant predominance of ribotype fragment subset E 11.2 among human epidemic isolates can be explained by the fact that DUP-1038 classifies into this subset. Our work confirms the unique position of human epidemic isolates and expands upon the findings of previous studies by showing that ribotype DUP-1038 is indeed significantly more common among human epidemic isolates than among human sporadic isolates. DUP-1038 occurs less frequently, but with an approximately equal frequency, among human sporadic cases (10·5 %) and animal cases (11·8 %). With one exception, all DUP-1038 and DUP-1042 isolates were found to carry *hly* allele 1, which is unique to lineage I strains and occurs in 98 % of lineage I isolates. Ribotypes DUP-1038 and DUP-1042 include isolates from eight of the nine epidemic outbreaks listed in Table 1. These two epidemic clones also account for a large proportion (41·0 %) of human sporadic listeriosis cases.

The human epidemic clone DUP-1042 is not significantly more common among human isolates as compared to animal isolates. Therefore, the occurrence of this clone in three human listeriosis outbreaks might not be indicative of a unique pathogenic potential, but might rather reflect the widespread distribution of this common *L. monocytogenes* ribotype. On the other hand, our data suggest that the predominance of DUP-1038 in human epidemics is not a consequence of the common occurrence of this *L. monocytogenes* strain. This strain is thus likely to be characterized by a unique potential to cause human food-borne listeriosis, possibly as a result of increased human virulence or an increased ability to

survive and multiply in contaminated foods (Piffaretti *et al*., 1989). All epidemic DUP-1038 isolates have been characterized by the presence of *actA* allele 3, with the exception of one of two isolates from the 1985 Los Angeles outbreak. Representatives of this clone have been characterized by the presence of a unique form of *actA* allele 3 which is not phosphorylated inside host cells (Wiedmann *et al*., 1997). Based on this observation and the fact that DUP-1038 shows only minimal allelic variation in *actA* and *hly*, we hypothesize that this ribotype represents a newly evolved clone with a unique ability to cause human listeriosis epidemics. This situation is reminiscent of *Streptococcus agalactiae*, where a single multilocus enzyme electrophoresis type (ET) is responsible for most of the serious human diseases caused by strains expressing the type III polysaccharide antigen. This ET is also characterized by the absence of allelic variations, thus suggesting that it represents a recently evolved clone (Musser *et al*., 1989).

Among the three major genetic lineages of *L. monocytogenes*, human isolates were significantly more frequently classified into lineage I than animal isolates. This lineage also contains the human epidemic clones. Thus, strains in this lineage appear more likely to cause human epidemic and sporadic listeriosis than strains classified into the other two lineages. Our results are consistent with previous studies, which indicate that certain *L. monocytogenes* subtypes are more commonly responsible for human listeriosis cases and outbreaks. For example, of 144 human isolates from sporadic cases serotyped by the CDC in 1986, 30% were 1/2a, 32% were 1/2b and 34% were 4b (Schwartz *et al*., 1989). Of 1363 human isolates collected in the UK, 15% were 1/2a, 10% were 1/2b and 64% were 4b (McLauchlin, 1990). Serotypes 1/2b and 4b, which are classified into lineage I, thus represent the majority of human isolates. Furthermore, Vines *et al*. (1992) reported that perinatal listeriosis is caused more frequently by strains equivalent to those in our lineage I than by strains in lineage II.

Classification into lineage III was significantly more common for animal isolates (10·5%) than for human isolates (0·8%). While animal isolates were found in all three lineages, human isolates were confined almost entirely to lineages I and II. We interpret these data as an indication that lineage III strains represent a clonal group of *L. monocytogenes* with possible host specificity for non-primate mammals and with limited virulence in humans. Host factors play a key role in virulence of bacterial pathogens. The single human case caused by a lineage III strain may represent an infection of a highly susceptible individual and may not reflect the virulence characteristics of the bacterial isolate. Unfortunately, no detailed patient information was available for this isolate.

Lineage III strains were previously shown to be genetically distinct from lineage I and II strains as well as from the non-pathogenic species *Listeria innocua* (Bruce *et al*., 1995; Wiedmann *et al*., 1997) and thus this lineage may represent a distinct subset, and perhaps subspecies, of *L. monocytogenes*. Specifically, lineage III strains have unique 16S rRNA sequences and have a distinctive ribotype fragment of 7·1 kb (designated H 7.1), which is not present in *L. monocytogenes* strains of lineages I and II, nor in *L. innocua* (Bruce *et al*., 1995; Wiedmann *et al*., 1997). Strains in two of the ribotype fragment subsets found in lineage III are rhamnose-negative, although *L. monocytogenes* is characteristically rhamnose-positive.

Allelic variation in *actA* and *hly*

Acquisition or loss of genes encoding virulence determinants or mutations leading to new virulence gene alleles may alter host specificities and/or pathogenic potentials among clonal subsets of bacterial pathogens. For example, in *Staphylococcus aureus*, strains bearing distinct allelic variants of the gene encoding enterotoxin C differ in their ability to stimulate ovine, bovine and human T-cells (Marr *et al*., 1993) and allelic variants of the toxic shock syndrome toxin also differ in their virulence properties (Lee *et al*., 1992).

In a previous study (Wiedmann *et al*., 1997), we hypothesized that allelic variation in virulence genes, and specifically in *actA*, may play a role in determining the unique pathogenic potential of the epidemic *L. monocytogenes* clone DUP-1038. It is also possible that these unique alleles are not solely responsible for virulence differences among strains and that they may instead be markers for the presence or absence of yet uncharacterized virulence genes responsible for host specificity or unique pathogenic potential among *L. monocytogenes* strains. In this study, we probed correlations between *hly* and *actA* allele frequencies and clinical histories (i.e. human or animal source) of *L. monocytogenes* isolates.

We did not find any significant differences in the distribution of *actA* alleles 3 and 4 among human epidemic, human sporadic and animal isolates. Significant differences existed for the frequencies of *actA* alleles 3 and 4 among the three lineages, however (data not shown; $P < 0{\cdot}001$). All (100%) lineage III clinical isolates and 92% of lineage II isolates carried *actA* allele 4. Interestingly, of 12 non-clinical lineage III isolates, 11 carried *actA* allele 3 (data not shown). We therefore hypothesize that lineage III strains carrying *actA* allele 3 are virulence-attenuated for both humans and animals. Indeed, previous work has shown that at least two lineage III isolates with *actA* allele 3 are virulence-attenuated (Chakraborty *et al*., 1994; Wiedmann *et al*., 1997). DNA sequencing further revealed that the *actA* alleles from these two isolates and from an attenuated lineage II isolate share an identical signature sequence with glutamic acid at position 375 and three proline-rich repeats (i.e. *actA* allele 3). This combination was unique in comparison with 11 other *actA* allele 3 genes sequenced (Wiedmann *et al*., 1997). Since previous DNA sequencing studies have indicated significant heterogeneity within each of the *actA* alleles (Wiedmann *et al*., 1997), it is important to note that the PCR-RFLP method

used in this study only differentiates two *actA* alleles, indicating the presence of either three or four proline-rich repeats. Since the presence of the unphosphorylated ActA protein unique to the epidemic clone DUP-1038 can only be detected by *actA* DNA sequencing or allele-specific PCR (Wiedmann *et al*., 1997), this study did not investigate the occurrence of this unique allele among clinical isolates. However, our data provide indirect evidence suggesting that unphosphorylated ActA, which appears to be unique to all DUP-1038 isolates (previously designated dd0647), might play an important role in human virulence since DUP-1038 shows significant predominance in human epidemic isolates (Table 3).

Discriminatory ability of automated ribotyping and PCR-RFLP of *actA* and *hly*

Reliable and discriminatory typing methods are crucial for studies of population genetics as well as for epidemiological investigations. We therefore evaluated the ability of the different typing methods used in this study to permit sensitive discrimination of clinical *L. monocytogenes* isolates. SID (Hunter & Gaston, 1988), a quantitative measure of the discriminatory ability of a given typing method, showed a high discriminatory power for the combination of ribotyping and PCR-RFLP of *actA* and *hly* (SID of 0·92 and 0·90 for animal and human isolates, respectively). As a single typing method from among these three approaches, ribotyping showed the highest discrimination (SID of 0·87 and 0·86 for animal and human isolates, respectively). In comparison, in the WHO International Multicentre *L. monocytogenes* subtyping study, the SID values achieved by various methods were as follows: ribotyping, 0·83–0·88; λ RFLP, 0·81–0·91 (Swaminathan *et al*., 1996); serotyping, 0·70; multilocus enzyme electrophoresis, 0·83–0·93 (Caugant *et al*., 1996). We conclude that automated ribotyping provides a high level of discrimination for both human and animal *L. monocytogenes* isolates. Strain discrimination by ribotyping is further enhanced by the addition of PCR-RFLP analyses of *actA* and *hly*.

Conclusions

Our data indicate the existence of distinct *L. monocytogenes* clonal groups, which appear to differ in their pathogenic potential and their host specificity. Furthermore, these different clonal groups may also differ in their adaptation to different ecological niches (Boerlin & Piffaretti, 1991). These findings may have major implications for the development of rational regulations and effective control measures for food-borne listeriosis, which should be specifically directed against pathogenic bacterial clones responsible for human infections. Our database of genetically well characterized clinical *L. monocytogenes* isolates also provides a basis for future surveillance programmes for monitoring the occurrence of listerial clones and their frequencies in human and animal listeriosis. Such database development can contribute to comprehensive public health programmes since the information may be used to help predict and detect new clones with unique host ranges and pathogenic potentials.

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