Expression of ActA, Ami, InlB, and Listeriolysin O in *Listeria monocytogenes* of Human and Food Origin

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Expression of proteins involved in the adhesion of *Listeria monocytogenes* to mammalian cells or in the intracellular life cycle of this bacterium, including listeriolysin O (LLO), ActA, Ami, and InlB, was used to compare two populations of *L. monocytogenes* strains. One of the populations comprised 300 clinical strains, and the other comprised 150 food strains. All strains expressed LLO, InlB, and ActA. No polymorphism was observed for LLO and InlB. Ami was detected in 283 of 300 human strains and in 149 of 150 food strains. The strains in which Ami was not detected were serovar 4b strains. Based on the molecular weights of the proteins detected, the strains were divided into two groups with Ami (groups Ami1 [75% of the strains] and Ami2 [21%]) and into four groups with ActA (groups ActA1 [52% of the strains], ActA2 [18%], ActA3 [30%], and ActA4 [one strain isolated from food]). Logistic regression showed that food strains were more likely to belong to group ActA3 than human strains (odds ratio [OR] = 2.90; $P = 1 \times 10^{-4}$). Of the strains isolated from patients with non-pregnancy-related cases of listeriosis, bacteremia was predominantly associated with group Ami1 strains (OR = 1.89; $P = 1 \times 10^{-2}$) and central nervous system infections were associated with group ActA2 strains (OR = 3.04; $P = 1 \times 10^{-3}$) and group ActA3 strains (OR = 3.91; $P = 1 \times 10^{-3}$).

Since the transmission of human listeriosis was first shown to be food borne in 1981, *Listeria monocytogenes* has been recognized as a major food-borne pathogen. The severity of listeriosis, which can cause abortions, bacteremia, and central nervous system infections (CNSI) and has a high mortality rate (20 to 30% [13, 36]), makes it a serious public health problem. In addition, the fact that various foods have been implicated in outbreaks of listeriosis (37) and the increase in product recalls (48) have led to serious economic problems associated with this bacterium.

Based on present knowledge, any strain of *L. monocytogenes* should be considered potentially pathogenic for humans. Nevertheless, a number of observations suggests that *L. monocytogenes* virulence is heterogeneous. Serotyping has revealed that strains are heterogeneously distributed; most major outbreaks of listeriosis (37) have been caused by strains belonging to serovar 4b, which has also been responsible for numerous sporadic cases (13, 36). However, a low percentage of food samples is contaminated with strains of this serovar (1, 25). Analysis of surface proteins, sequencing of virulence genes, and PCR-restriction enzyme analysis of virulence genes have revealed that differences are connected to the origin or typing characteristics of the strains (34, 38, 43, 49, 50, 51). The tests used to study *L. monocytogenes* pathogenicity include tissue culture assays and tests in which laboratory animals are used (5, 7, 9, 44, 46). These methods also have revealed heterogeneity in the virulence of strains. For example, counts of viable

bacteria in spleens revealed that virulence levels varied from less than 10^3 to 10^8 CFU (5). However, there was no clear correlation between strain characteristics and the degree of virulence. Therefore, there are currently no laboratory tests which can predict the danger associated with *L. monocytogenes* strains.

The mechanisms by which *L. monocytogenes* invades a host have been studied in detail (21). Listeriolysin O (LLO) is a 58- to 60-kDa secreted protein which allows the bacterium to escape from a phagocytosis vacuole (8, 15). LLO is a toxin which acts only on cholesterol-containing membranes in which cholesterol is considered the receptor. Nonhemolytic mutants are always avirulent in mice (15). However, the level of in vitro hemolysin production is not directly proportional to the virulence of a strain (18). InlB, which is a 67-kDa surface and secreted protein, is sufficient for entry of the bacterium into cells (4). InlB can promote the entry of noninvasive bacterial cells into mammalian cells and cause internalization of inert particles. Ami is detected exclusively on the bacterial surface and has a predicted molecular mass of 98 kDa. Ami is a bacteriolysin and may play a role in adhesion (3, 29). ActA, a 90-kDa surface protein, is required for actin polymerization and thus allows intracytoplasmic movement of *L. monocytogenes* (10, 20, 31, 45). Mutants unable to polymerize actin are avirulent in mice (6).

Cell cultures and laboratory animal tests cannot be used to ascertain the virulence or lack of virulence of *L. monocytogenes* strains in humans. The approach used in the present study was to compare two bacterial populations, one comprising human isolates (a priori pathogenic strains) and the other one comprising strains isolated from foods, which may contain nonvirulent or attenuated strains. Expression of the *L. monocytogenes* proteins ActA, Ami, InlB, and LLO was studied in order to

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TABLE 1. Distribution of strains according to origin, main serovar, serogroup, Ami group, and ActA group

Serovar or serogroup	Origin	Total no. of strains	Distribution											
			Ami groups						ActA groups ^a					
			Group Ami1		Group Ami2		Group Ami0		Group ActA1		Group ActA2		Group ActA3	
			No. of strains	%	No. of strains	%	No. of strains	%	No. of strains	%	No. of strains	%	No. of strains	%
1/2a	Human	132	132	100	0	0	0	0	87	66	34	26	11	8
	Food ^b	89	89	100	0	0	0	0	48	54	10	11	30	34
	All	221	221	100	0	0	0	0	135	61	44	20	41	19
1/2b	Human	57	57	100	0	0	0	0	25	44	0	0	32	56
	Food	26	26	100	0	0	0	0	7	27	0	0	19	73
	All	83	83	100	0	0	0	0	32	39	0	0	51	61
1/2c	Human	8	8	100	0	0	0	0	8	100	0	0	0	0
	Food	17	17	100	0	0	0	0	17	100	0	0	0	0
	All	25	25	100	0	0	0	0	25	100	0	0	0	0
4b	Human	98	0	0	81	83	17	17	28	28	33	34	37	38
	Food	12	0	0	11	92	1	8	7	58	3	25	2	17
	All	110	0	0	92	84	18	16	35	32	36	33	39	35
1/2 ^c	Human	197	197	100	0	0	0	0	120	61	34	17	43	22
	Food ^b	132	132	100	0	0	0	0	72	55	10	8	49	37
	All ^b	329	329	100	0	0	0	0	192	59	44	13	92	28
4 ^c	Human	98	0	0	81	83	17	17	28	28	33	34	37	38
	Food	13	0	0	12	92	1	8	7	54	4	31	2	15
	All	111	0	0	93	84	18	16	35	32	37	33	39	35
All	Human	300	201	67	82	27	17	6	152	50	68	23	80	27
	Food ^b	150	136	90	13	9	1	1	80	53	14	9	55	37
	All ^b	450	337	75	95	21	18	4	232	52	82	18	135	30

^a The ActA groups contained 449 strains.

^b One strain was in group ActA4.

^c Serogroup 1/2 contained serovars 1/2a, 1/2b, and 1/2c. Serogroup 4 contained serovars 4b and 4c.

detect differences between the two populations of strains and thus detect potential markers of *L. monocytogenes* virulence in humans.

MATERIALS AND METHODS

Bacterial strains. A total of 450 strains of *L. monocytogenes* were studied. All of the strains isolated from human sporadic cases of listeriosis in France in 1995 (300 strains) and collected by the National Reference Center (NRC) for surveillance of listeriosis were included (35). Basic demographic and clinical data were available for each patient. Sixty-one (20%) of these strains were responsible for pregnancy-related cases (PRC) of listeriosis, and 239 (80%) were responsible for non-pregnancy-related cases (NPRC). The 239 NPRC included 162 (68%) cases of bacteremia, 56 (23%) cases of CNSI, and 21 (9%) cases of other clinical syndromes (8 ascitic cases, 3 aortic aneurysm cases, 2 pleurisy cases, 2 urinary infection cases, 1 peritonitis case, 1 endocarditis case, 1 panophthalmia case, 1 adenophlegmon case, 1 polyarteritis nodosa case, and 1 cervical abscess case). A total of 150 strains from various types of food were randomly selected (by using a table of randomly assorted digits) from the 4,995 food strains isolated by French laboratories and received by the NRC in 1995. Sixty of the strains were isolated from milk or dairy products, 50 strains were isolated from meat or meat products, 23 strains were isolated from seafoods, 15 strains were isolated from other foods, and 2 strains were isolated from unknown sources. The foods included raw foods and ready-to-eat foods. The stage of the food chain at which foods were sampled was unknown. All strains were identified and serotyped by standard methods (2, 40). The distribution of the serovars is shown in Tables 1 and 2. Strains EGD (serovar 1/2a from the Trudeau Institute) and LO28 (serovar 1/2c from a stool culture of a pregnant woman) were included as reference strains.

Preparation of protein extracts. Bacteria were grown in 5 ml of brain heart infusion medium. One milliliter of a bacterial suspension ($1.2 < \text{optical density at } 600 \text{ nm} < 1.6$) was centrifuged at $1,000 \times g$ for 5 min, washed three times in phosphate-buffered saline (PBS), resuspended in $5 \times \text{Laemmli}$ sample buffer, and boiled for 5 min. Supernatants were used directly, or proteins were precipitated from culture supernatants by adding 10% trichloroacetic acid; precipitates

were washed with cold acetone and suspended in $5 \times \text{Laemmli}$ sample buffer. Protein extracts were stored at -20°C until they were used.

Immunoblotting. Western blotting was carried out with total bacterial extracts for ActA, InlB, and Ami, with culture supernatants for LLO, and with trichloroacetic acid precipitates for InlB and Ami. Total bacterial extracts, culture supernatants, or trichloroacetic acid precipitates, as well as a biotinylated molecular weight marker (New England Biolabs), were boiled in $5 \times \text{Laemmli}$ sample buffer, separated on sodium dodecyl sulfate-8% polyacrylamide gel electrophoresis minigels, and transferred onto nitrocellulose filters by using a semi-dry transfer system. The filters were blocked for 1 h in PBS containing 0.2% Tween 20 and 5% dried skim milk and were then incubated for 1 h with the primary antibody in the same buffer (14). They were washed several times in PBS containing 0.2% Tween 20 and subsequently were incubated for 1 h with the secondary antibody and washed again. A phototope-horse radish peroxidase (HRP) Western blot detection kit (New England Biolabs) was used for chemiluminescent detection of the proteins with anti-rabbit or anti-mouse immunoglobulin G HRP-linked secondary antibody, depending on the protein to be tested, and HRP-linked anti-biotin antibody. LumiGLO reagent was added for detection. The light emitted by the destabilized LumiGLO reagent was subsequently captured on X-ray film. The following antigens and antibodies were used in this study: for LLO, LLO antigen and mouse monoclonal D21.1.4 primary antibody (32); for ActA, Y21T antigen and rabbit polyclonal R1 primary antibody (19); for InlB and Ami, InlB-6 $\times$ His antigen and rabbit polyclonal R24 primary antibody, which recognizes both InlB and Ami (11); and for Ami, *L. monocytogenes* sodium dodecyl sulfate surface protein extract antigen and rabbit polyclonal R4 primary antibody (3).

Data analysis. The expression and apparent molecular weight of each protein were compared to the expression and apparent molecular weight of the same protein detected in strain EGD or LO28. Because the ActA and LLO antibodies were from strain LO28, the ActA and LLO results were compared with the results obtained for LO28. For the same reason, the Ami and InlB results were compared with the results obtained for EGD. The EpiInfo software (version 6.04) was used for descriptive analysis. Percentages were calculated for each group of strains and compared by the chi-square test. Odds ratios (OR) were calculated when a statistical difference in protein expression was detected. Stepwise logistic regressions were performed by using SAS software (version 6.12) to

TABLE 2. Distribution of human strains according to clinical presentation, serovar, Ami group, and ActA group

Clinical form	Serovar	Total no. of strains	Distribution											
			Ami groups						ActA groups					
			Group Ami1		Group Ami2		Group Ami0		Group ActA1		Group ActA2		Group ActA3	
			No. of strains	%	No. of strains	%	No. of strains	%	No. of strains	%	No. of strains	%	No. of strains	%
PRC	All	61	38	62	19	31	4	7	33	54	13	21	15	25
	1/2a	24	24	100	0	0	0	0	15	63	9	37	0	0
	1/2b	14	14	100	0	0	0	0	8	57	0	0	6	43
	4b	23	0	0	19	83	4	17	10	44	4	17	9	39
NPRC	All	239	163	68	63	26	13	6	119	50	55	23	65	27
CNSI	All	56	30	54	23	41	3	5	15	27	17	30	24	43
	1/2a	19	19	100	0	0	0	0	13	68	4	21	2	11
	1/2b	10	10	100	0	0	0	0	1	10	0	0	9	90
	1/2c	1	1	100	0	0	0	0	1	100	0	0	0	0
	4b	25	0	0	22	88	3	12	0	0	12	48	13	52
	US ^a	1	0	0	1	100	0	0	0	0	1	100	0	0
	All	162	119	74	33	20	10	6	90	56	32	20	40	24
Bacteremia	1/2a	76	76	100	0	0	0	0	49	64	19	25	8	11
	1/2b	32	32	100	0	0	0	0	15	47	0	0	17	53
	1/2c	7	7	100	0	0	0	0	7	100	0	0	0	0
	3c	2	2	100	0	0	0	0	2	100	0	0	0	0
	4b	43	0	0	33	77	10	23	15	35	13	30	15	35
	7	1	1	100	0	0	0	0	1	100	0	0	0	0
	US	1	1	100	0	0	0	0	1	100	0	0	0	0
Others	All	21	14	67	7	33	0	0	14	67	6	29	1	4
	1/2a	13	13	100	0	0	0	0	10	77	2	15	1	8
	1/2b	1	1	100	0	0	0	0	1	100	0	0	0	0
	4b	7	0	0	7	100	0	0	3	43	4	57	0	0
Total	All	300	201	67	82	27	17	6	152	50	68	23	80	27

^a US, undesignated serovar.

select independent markers associated with human strains but not food strains or associated with one clinical form but not the other clinical forms.

RESULTS

Protein analysis. LLO was detected in all 150 strains tested (75 human strains and 75 food strains that were randomly selected) and had the same apparent molecular weight as the LLO in EGD and LO28. Thus, the study of LLO was not extended to all strains.

All strains expressed InlB, and no polymorphism was detected (the molecular weight was the same as the molecular weight of EGD and LO28 InlB).

Ami was detected in 432 strains. It was not detected in 17 human strains (serovar 4b) and one food strain (serovar 4b, from meat or a meat product) after 7 to 10 attempts with R24 and R4 antibodies, either in total extracts or in trichloroacetic acid precipitates. *ami* was detected by PCR in these 18 strains (E. Milohanic, R. Jonquière, P. Berche, P. Cossart, and J.-L. Gaillard, unpublished results). Two groups were identified based on the apparent molecular weight of the protein detected; group Ami1 contained 337 (75%) strains in which Ami had a molecular size of 98 kDa, as it did in EGD and LO28; and group Ami2 contained 95 (21%) strains in which the molecular size of the Ami protein was approximately 80 kDa (Tables 1 and 2 and Fig. 1).

ActA was expressed in all strains. Four groups were identified based on the molecular weight of the protein detected (Tables 1 and 2 and Fig. 2). Group ActA1 contained 232 (52%) strains in which the molecular weight of ActA was the same as

the molecular weight of ActA in LO28; group ActA2 contained 82 (18%) strains in which the molecular weight of ActA was the same as the molecular weight of ActA in EGD; group ActA3 contained 135 (30%) in which the molecular weight of ActA was lower than the molecular weight of LO28 ActA; and group ActA4 contained one food strain (serovar 1/2a from milk or dairy product) in which the molecular weight of ActA was lower than the molecular weight of ActA in group ActA3 strains (approximately 75 kDa). Due to the small differences in the apparent molecular weights of the proteins, the molecular

FIG. 1. Autoradiogram for Ami. Lane 1, group Ami2; lane 2, group Ami1; lane 3, broad-range biotinylated protein marker (New England Biolabs).

FIG. 2. Autoradiogram for ActA. Lane 1, group ActA2; lane 2, broad-range biotinylated protein marker (New England Biolabs); lane 3, group ActA1; lane 4, group ActA3; lane 5, group ActA4.

weights of ActA in group ActA2 and ActA3 strains cannot be estimated.

Univariate analysis. (i) Serovars. Significant differences in the distribution of serovars were found depending on the origin of the strains. Serogroup 1/2 was the predominant serogroup of food strains ($OR = 5.05$; $P < 1 \times 10^{-7}$); 59% of the food strains were serovar 1/2a, 17% were serovar 1/2b, and 11% were serovar 1/2c. (In this study, serogroup 1/2 contained strains belonging to serovars 1/2a, 1/2b, and 1/2c, and serogroup 4 contained strains belonging to serovars 4b and 4e.) Serovar 1/2a ($OR = 1.86$; $P = 2 \times 10^{-3}$) and serovar 1/2c ($OR = 4.67$; $P = 1 \times 10^{-4}$) were particularly predominant. In contrast, human strains were more frequently serovar 4b strains ($OR = 5.58$; $P < 1 \times 10^{-7}$). No differences were observed between PRC human strains and NPRC human strains. Of the strains isolated from NPRC patients, a higher proportion of serovar 4b strains was related to CNSI than to bacteremia or other clinical forms ($OR = 2.15$; $P = 1 \times 10^{-2}$). Of the food strains, serovar 4b strains were more often associated with milk or dairy products than with other types of food ($OR = 3.31$; $P = 5 \times 10^{-2}$).

(ii) Ami groups. There were statistically significant differences between serogroups depending on the Ami group; all of the serogroup 1/2 strains belonged to group Ami1, whereas group Ami2 consisted of 83% of the serogroup 4 strains and no Ami was detected for only 18 serovar 4b strains from humans and food ($P < 1 \times 10^{-7}$). Food strains were strongly associated with group Ami1 ($OR = 4.78$; $P < 1 \times 10^{-7}$), which is explained by the higher proportion of serogroup 1/2 strains obtained from food. There were no differences based on type of food. This may have been due to the lack of statistical power because of the low number of strains in each category. There was no statistical difference between PRC human strains and NPRC human strains. Among the strains obtained from NPRC patients, strains obtained from patients with bacteremia were more often associated with group Ami1 ($OR = 2.08$; $P = 1 \times 10^{-2}$), whereas strains obtained from patients with CNSI were associated with group Ami2 ($OR = 2.49$; $P = 4 \times 10^{-3}$), which is explained by the higher proportion of serovar 4b strains obtained from this clinical form.

(iii) ActA groups. There was a significant difference between serogroup 4 strains and serogroup 1/2 strains ($P < 1 \times 10^{-7}$).

TABLE 3. Selection of markers independently associated with the origins of the strains by stepwise logistic regression

Marker	Human strains		Food strains	
	OR	P	OR ^a	P
Serovar 1/2a	1		1	
Serovar 1/2b	2.62	1×10^{-2}	0.40	1×10^{-2}
Serovar 1/2c	0.29	1×10^{-3}	3.79	1×10^{-3}
Serovar 4b	7.11	1×10^{-4}	0.13	1×10^{-4}
Group ActA1	2.66	1×10^{-3}		
Group ActA2	4.60	1×10^{-4}		
Group ActA3	1		2.90	1×10^{-4}

^a The OR value for groups ActA1 and ActA2 was 1.

Serogroup 1/2 strains were more frequently associated with group ActA1 ($OR = 3.04$; $P = 1 \times 10^{-6}$). Serovar 1/2a strains were associated with groups ActA1, ActA2, and ActA3, but they were more often associated with group ActA1 ($OR = 2.18$; $P = 5 \times 10^{-5}$); serovar 1/2b strains were found in groups ActA1 and ActA3, but they were predominantly associated with group ActA3 ($OR = 5.37$; $P < 1 \times 10^{-7}$); serovar 1/2c strains were associated only with group ActA1; and similar proportions of serovar 4b strains were found in all three groups (32% were group ActA1 strains, 33% were group ActA2 strains, and 35% were group ActA3 strains). The distributions of food and human strains were significantly different in the different ActA groups ($P = 1 \times 10^{-3}$). There was no difference in group ActA1 ($P = 6 \times 10^{-1}$); there was a higher proportion of human strains in group ActA2 ($OR = 2.85$; $P = 6 \times 10^{-4}$); and there was a higher proportion of food strains in group ActA3 ($OR = 1.59$; $P = 3 \times 10^{-2}$). No difference based on type of food was observed. This may have been due to the lack of statistical power because of the low number of strains in each category. No statistical differences were found between human strains isolated from patients with PRC and human strains isolated from patients with NPRC. In NPRC, bacteremia strains were associated with group ActA1 ($OR = 2.07$; $P = 1 \times 10^{-2}$) and CNSI strains were associated with group ActA3 ($OR = 2.60$; $P = 3 \times 10^{-3}$).

Multivariate analysis. Strains belonging to rare serovars (serovars 3a, 3b, 3c, 4e, and 7), undesigned serovar strains, and strains obtained from patients with unusual clinical forms of NPRC were not included in the multivariate analysis. Stepwise logistic regression showed that food strains were independently associated with group ActA3 ($OR = 2.90$; $P = 1 \times 10^{-4}$) and serovar 1/2c ($OR = 3.79$; $P = 1 \times 10^{-2}$), whereas human strains were associated with serovar 1/2b ($OR = 2.62$; $P = 1 \times 10^{-2}$), serovar 4b ($OR = 7.11$; $P = 1 \times 10^{-4}$), group ActA1 ($OR = 2.66$; $P = 1 \times 10^{-3}$), and group ActA2 ($OR = 4.60$; $P = 1 \times 10^{-4}$) (Table 3). No difference was found between strains from patients with PRC and strains from patients with NPRC. Among the strains from patients with NPRC, bacteremia strains were significantly more likely to belong to group Ami1 ($OR = 1.89$; $P = 1 \times 10^{-2}$) and CNSI strains were more likely to belong to group ActA2 ($OR = 3.04$; $P = 1 \times 10^{-2}$) or group ActA3 ($OR = 3.91$; $P = 1 \times 10^{-3}$) (Tables 4 and 5).

TABLE 4. Selection of the marker independently associated with bacteremia in human strains by stepwise logistic regression

Marker	OR	P
Group Ami2	1	
Group Ami2	1.89	1×10^{-2}

DISCUSSION

All human strains sent to the NRC in 1995 were included in this study. Listeriosis surveillance was essentially laboratory based (mandatory notification was established in 1998). Due to the passive nature of this process (strains are sent to the NRC voluntarily by clinical microbiologists), the number of strains collected is probably an underestimate of the true number of cases of listeriosis and its clinical forms. However, this is the only representative set of human listeriosis strains in France. Food strains were randomly selected from strains voluntarily sent by public and private food hygiene laboratories during the same period. Little information concerning these strains was provided. This set of strains was not representative of *L. monocytogenes* contamination of food at each step of the food chain and consumer exposure. Nevertheless, the variety of foods and the large number of manufacturers in industrialized countries make it impossible to compile a set of *L. monocytogenes* strains that is fully representative of the population present at each step of the food chain.

It is known that more serogroup 1/2 strains are recovered from foods than from humans (1, 24). This unequal distribution was observed in the set of strains which was used (OR = 5.05; $P < 1 \times 10^{-7}$), which explains why most food strains were in group Ami1. Food may be contaminated by virulent strains, as shown by food-borne outbreaks. However, several studies have indicated that attenuated or nonvirulent populations of *L. monocytogenes* may also be found among food isolates. These strains mainly belong to serogroup 1/2 (7, 28, 44, 46). The statistical comparison of food-borne and human strains in this study suggests that if strains that are attenuated or nonvirulent for humans exist, they are predominantly associated with serovar 1/2c.

All of the virulence determinants in *L. monocytogenes* identified to date are chromosome encoded. As in other pathogenic bacteria, virulence genes are organized in genetic islands; *hly* and *actA*, encoding LLO and ActA, respectively, are physically linked in a PrfA-dependent virulence gene cluster, *inlB* is in the internalin loci, and *ami* is in the autolysin gene region (27, 47). For a long time LLO was assumed to be a major virulence determinant, and spontaneous nonhemolytic strains, such as transposon-induced mutants, are avirulent in mice (15). Thus, the lack of polymorphism in LLO apparent molecular weight observed in this study was unexpected. Sequence determina-

tion, PCR-restriction fragment polymorphism analysis, mismatch amplification mutation assay-PCR, PCR-single-strand conformation polymorphism analysis, and deducing the amino acid sequence encoded by the *hly* gene have revealed some differences between *L. monocytogenes* strains (17, 24, 34, 49, 50). A comparison of four strains revealed one to five nucleotide differences in the *hly* genes (50). Furthermore, the *hly* genes of serotypes 1/2b and 4b form a cluster, which is not found in serotypes 1/2a and 1/2c. Due to the large number of strains tested in this study (75 clinical isolates and 75 food isolates) strains with such mutations were certainly included. However, slight changes in the LLO gene do not alter the molecular weight of LLO. These results confirm those of Matar et al. (26), who found no significant difference between *L. monocytogenes* serotypes 1/2a and 4b by using immunoaffinity-purified LLO.

Ericsson et al. (12) showed that the levels of sequence similarity of the *inlB* genes of 24 strains belonging to different *L. monocytogenes* serovars were between 89.2 to 100%, corresponding to levels of amino acid sequence similarity of 91.9 to 100%. Due to the large number of strains tested in this study (300 clinical isolates and 150 food isolates), strains with these variations were certainly included. Consequently, the lack of polymorphism for *InlB* in this study was unexpected. Results of the present study showed that there was no difference between strains responsible for CNSI and strains involved in the other clinical forms of disease even though *InlB* is not an important virulence factor for cerebral listeriosis (39). The fact that *InlB* proteins with the same molecular weight are expressed in all strains may be because *InlB* interacts quite specifically with its receptor (16). Alternatively, slight differences in *inlB* may have no effect on the molecular weight of *InlB*.

The role of *Ami* in *L. monocytogenes* virulence has been investigated recently (29). *Ami* plays a role in adhesion. In this study, strains involved in bacteremia were twice as likely to be associated with group Ami1 as NPRC strains. Thus, it would be interesting to understand the significance of the differences between strains. *Ami* was not detected in 6% of the human strains examined (serovar 4b). In these strains, *ami* is present, as indicated by PCR results, but further experiments are required to determine if the gene is defective or unexpressed.

Niebuhr et al. (33) found small but clear differences in the molecular weight of ActA in strains belonging to different serovars. In addition, ActA was detected in most strains of *L. monocytogenes* tested; the only exceptions were one serovar 3a strain and one serovar 4ab strain. Moriishi et al. (30) sequenced the *actA* gene of 24 strains isolated from healthy humans and patients and found two groups, one of which was characterized by deletion of one proline-rich repeat. No correlation between this classification and serovars was found. In the present study, four groups of strains were identified based on the molecular weight of ActA. These groups were not strictly correlated with serovars. The multivariate analysis used to compare human and food strains indicated that group ActA3 strains were found three times more frequently in food strains than in human strains, after adjustment for other significant markers (OR = 2.90; $P = 1 \times 10^{-4}$). This could suggest that attenuated or nonvirulent strains are associated with this marker. However, strains responsible for CNSI are also more likely to be characterized by this marker, which

TABLE 5. Selection of the marker independently associated with CNSI in human strains by stepwise logistic regression

Marker	OR	P
Group ActA1	1	
Group ActA2	3.04	1×10^{-2}
Group ActA3	3.91	1×10^{-3}

shows that additional markers are required to identify attenuated or nonvirulent strains. The molecular mass of ActA in group ActA3 strains was lower than the molecular mass of LO28, due to a deletion in *actA*. As this deletion is detected in human strains, (i.e., pathogenic strains), it is probably not in the amino-terminal region, which is essential for F-actin assembly and movement. This deletion is more likely in the internal proline-rich repeats or the carboxy-terminal domain, which are not essential (22, 23). This hypothesis is supported by the findings of Moriishi et al. (30), who located polymorphisms in the proline-rich region, and by the findings of Sokolovic et al. (42), who found that the lower molecular masses of the serovar 4a and 4e ActA proteins were due to a deletion in the same region. In vitro motility assays showed that this deletion decreases the motility of the bacteria (22). In the mouse model, a mutant with a mutation in the proline-rich region was less virulent than the wild type (41). Strains responsible for CNSI were also more likely to be associated with group ActA2. Further studies are required to understand these data and to determine whether the diversity of CNSI strains is correlated with other characteristics, such as phosphatidylcholine-specific phospholipase C, which is known to be a major virulence factor for this clinical form (39).

In conclusion, this study was the first step in determining the virulence marker heterogeneity in *L. monocytogenes* by statistically comparing human strains and food strains. Present results show that there are clear differences in the proteins expressed by different serovars, by strains having different origins (human versus food), and by organisms that cause different clinical forms of listeriosis. However, the markers identified in this study cannot be used to unambiguously identify food-borne strains which are attenuated or nonvirulent in humans. Further studies are required to analyze the group of strains belonging to group ActA3. Further investigations are also required to understand how differences in the expression of Ami and ActA affect the infectious process of the bacterium. It would be interesting to determine whether the observed differences are silent or influence or modulate pathogenicity or pathogenic mechanisms. In addition, more *L. monocytogenes* virulence proteins should be tested in order to elucidate the pathogenicity of *L. monocytogenes* strains depending on the human clinical manifestations, as well as the ecology of *L. monocytogenes* in food.

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