APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1995, p. 3872–3874 Vol. 61, No. 11
0099-2240/95/$04.00+0

Division of *Listeria monocytogenes* Serovar 4b Strains into Two Groups by PCR and Restriction Enzyme Analysis

H. ERICSSON,[1]* P. STÅLHANDSKE,[1] M.-L. DANIELSSON-THAM,[1] E. BANNERMAN,[2] J. BILLE,[2] C. JACQUET,[3] J. ROCOURT,[3] AND W. THAM[1]

Department of Food Hygiene, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, Uppsala, Sweden[1]; Medical Bacteriology Laboratory, University Hospital, Lausanne, Switzerland[2]; and Centre National de Référence des Listeria, World Health Organization Collaborating Center for Foodborne Listeriosis, Institut Pasteur, Paris, France[3]

Received 15 May 1995/Accepted 30 August 1995

Altogether, 133 strains of *Listeria monocytogenes* serovar 4b were investigated. A segment of 2,916 bp containing parts of the two genes *inlA* and *inlB* in *L. monocytogenes* was amplified by the PCR technique. The PCR product obtained was cleaved with the restriction enzyme *Alu*I, and the fragments generated were separated by gel electrophoresis, leading to two distinct groups: PCR-restriction enzyme analysis groups I and II, containing 37 and 96 strains, respectively. The PCR-restriction enzyme analysis method described in this paper could be a useful tool for the subtyping of *L. monocytogenes* serovar 4b strains.

During the last decade, several outbreaks and single cases of listeriosis among humans have demonstrated that listeriosis is often transmitted by contaminated foods. The most common manifestations of the disease are abortion, septicemia, and meningitis in immunocompromised individuals (15). The majority of the human cases of listeriosis during the last decade have been caused by *Listeria monocytogenes* serovar 4b strains (2). Methods for the subtyping of such strains are, therefore, especially urgent.

L. monocytogenes has a number of virulence factors. One of these is the protein listeriolysin O, encoded by the gene *hly*. It has been suggested by Geoffroy et al. (4) that listeriolysin O acts by disrupting the macrophage phagosomes and thereby makes it possible for the bacteria to grow intracellularly. Rasmussen et al. (9) found that strains belonging to different serovars of the species *L. monocytogenes* could be divided into two major groups according to part of the sequence of the *hly* gene. Also, Vines et al. (16) presented two groups of strains within the species. Their division was based on PCR combined with restriction enzyme analysis (REA) on *hly* and three other virulence-associated genes.

Another virulence factor is internalin (encoded by the gene *inlA*), a cell wall surface protein which is essential for *L. monocytogenes* to invade epithelial cells (3). The aim of this study was to investigate the potential differentiation of *L. monocytogenes* serovar 4b strains by PCR-REA of a gene segment containing part of *inlA* and part of another gene, *inlB*, belonging to the same gene family. The segment studied is 2,916 bp long and includes the downstream end of *inlA* (955 bp), the space between *inlA* and *inlB* (85 bp) and 1,876 bp of *inlB*. *inlA* encodes internalin, and *inlB*, located downstream of *inlA*, encodes a protein containing a possible signal peptide (for sequence data, see the work of Gaillard et al. [3]).

* Corresponding author. Mailing address: Box 7009, Department of Food Hygiene, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, S-750 07 Uppsala, Sweden. Phone: 46 (18) 672384. Fax: 46 (18) 673334.

MATERIALS AND METHODS

Bacterial strains. One hundred thirty-three strains of *L. monocytogenes* serovar 4b, of different phagovars, isolated during the 1970s and 1980s from food, animals, humans, and the environment in Sweden, were studied. In addition, six strains of *L. monocytogenes* serovar 1/2a, five strains of serovar 1/2b, five strains of serovar 1/2c, and six strains of serovar 3b were included. The strains had previously been serotyped and phage typed according to reference methods (10, 14) (see Tables 1 and 2).

PCR analysis. One colony of each strain was inoculated into 50 ml of brain heart infusion broth and incubated at 37°C for 24 h. After incubation, 15 μl of each culture was mixed with 140 μl of sterile water and denatured with 14 μl of 0.8 M NaOH in an Eppendorf tube. The tubes were put into a heating block (70°C) for 10 min, cooled on ice, and further supplied with 18 μl of 1 M Tris (pH 8.0) and 12 μl of 0.8 M HCl. The pH of the suspension was checked and considered acceptable if it was between 7 and 9. Five microliters (approximately 10,000 bacteria) was used for PCR.

The primers were LIP 32 (5′ AACGACAACATTTAGTGGAACCGTGACG 3′, positions 2977 to 3004) and LIP 23 (5′ ATTAGCTGCTTTCGTCCAAC CAATGAAAG 3′, positions 5893 to 5865). Sequence data used for construction of the primers were those previously published by Gaillard et al. (3). The PCR was essentially performed as described by Saiki et al. (11). The PCR mixture (50 μl) contained 30 mM Tricine, pH 8.4 (Sigma, St. Louis, Mo.), 2.0 mM $MgCl_2$, 0.1% Thesit (Sigma), 200 μmol of each deoxynucleoside triphosphate (dATP, dTTP, dCTP, dGTP; Boehringer Mannheim), 2 μmol of each primer, and 2.0 U of *Taq* polymerase (Perkin Elmer). PCR was carried out in a Perkin Elmer thermocycler (P13480) and run for 40 cycles (1 min, 15 s, 94°C; 1 min, 15 s, 50°C; 6 min 72°C).

Four microliters of the reaction mixture was mixed with 2 μl of gel loading buffer, type IV (12), fractionated on a 2% agarose gel (SeaKem, LE; FMC) in 0.5× TBE (45 mM Tris-borate, 1 mM EDTA) at 8 V/cm for 30 min. The PCR product was visualized by ethidium bromide staining (1.5 μg/ml for 15 min) and photographed with a Polaroid MP-3 camera. When the PCR product showed up as a single, clean, and right-sized fragment, it was confirmed by dot blot hybridization.

Dot blot hybridization. Twenty microliters of the PCR product was mixed with 5 μl of 1.6 M NaOH, vortexed, and left at room temperature for 15 min. Afterwards, 5 μl of 6 M ammonium acetate was added, and the mixture was put on a nylon membrane (Hybond N+) in a mini dot blot chamber (Ubitech AB, Uppsala, Sweden). After the membrane was baked at 80°C for 2 h, 100 pmol of the probe (5′ TTAGGTGCAGTTATCCGCGTCCCT 3′, positions 5061 to 5038; Innovagen, Lund, Sweden), biotinylated in both the 5′ and 3′ positions, was added. Hybridization was carried out at 50°C for 15 min; this was followed by 5 min of incubation at 50°C with a filter-blocking solution containing yeast RNA. Finally, 2.0 U of streptavidin alkaline phosphate conjugate (Boehringer Mannheim) was added, and the hybrid was visualized by using a stabilized substrate for alkaline phosphatase (Western Blue; Promega).

REA. Twenty microliters of the PCR product was cleaved with 20 U of the restriction enzyme *Alu*I (Promega) according to the manufacturer's instructions and subsequently precipitated in 2.0 M ammonium acetate and 2.5 volumes of ethanol (99.8%, vol/vol). The pellet was washed twice with 2 ml of ethanol (75%, vol/vol) and dried for 1 h under a table lamp. The pellet was dissolved in 12 μl of 0.5× TBE, mixed with gel loading buffer, and separated on a 3% agarose gel

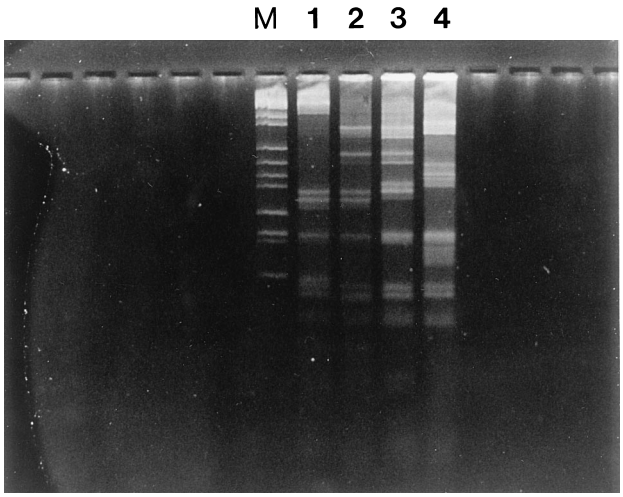

FIG. 1. PCR-REA profiles of *L. monocytogenes* serovars produced by PCR amplification of the *inlA* and *inlB* regions followed by cleavage with *Alu*I. Lane M, pBR328 *Bgl*I plus pBR328 *Hin*fI markers; lane 1, profile I (serovar 4b); lane 2, profile II (serovar 4b); lane 3, profile III (serovars 1/2b and 3b); lane 4, profile IV (serovars 1/2a and 1/2c).

(SeaKem, LE; FMC) in 0.5× TBE, at 8 V/cm for 30 min. The gel was stained and photographed as described above. The strains were divided into different groups according to the restriction profiles obtained.

In the preliminary experiments we also used the restriction enzyme *Taq*I, but it was abandoned since it generated too few fragments (data not shown).

RESULTS AND DISCUSSION

By using enzyme *Alu*I, four different cleavage profiles were obtained (Fig. 1). Strains of serovar 4b were divided into two groups (profiles I and II). The strains of serovars 1/2b and 3b yielded the same profile (III), as did the strains of serovars 1/2a and 1/2c (profile IV). The results from PCR-REA of strains sharing serovar 4b are shown in Tables 1 and 2. It was interesting to note that none of the strains sharing profile II was sensitive to phage 1317. Another interesting finding is that all 74 4b strains belonging to phagovar 2389:2425:3274:2671:47:108:340 yielded the same profile (profile II). Phagovar 2389:2425:3274:2671:47:108:340 has been recognized in some large outbreaks of listeriosis during the recent years: Switzerland 1983 to 1987 (soft cheese), Denmark 1985 to 1987 (unknown origin) and 1989 to 1990 (blue mold cheese), and France 1992 (pig tongue in jelly) (1, 5, 6, 13). Furthermore, more than 25% of all human isolates of *L. monocytogenes* in Sweden serotyped and phage typed so far belong to serovar 4b and phagovar 2389:2425:3274:2671:47:108:340. Therefore, it would be essential to look particularly at strains sharing this phagovar when found in food and environmental samples. Phage typing, however, is an exclusive technique which is performed in only a few laboratories in the world. A rapid method for determining whether or not an isolated *L. monocytogenes* strain belongs to this phagovar is of value. Such a method might be the PCR-REA method described in this paper. Indeed, this would constitute a first-step subtyping procedure, since strains of some other phagovars also share profile II. Thus, phage typing is required for a final diagnosis.

In the present study, only four different cleavage patterns (two for serovar 4b) were obtained (Fig. 1). This indicates a low degree of polymorphism in the *inlA* and *inlB* regions studied. Also, other gene regions associated with virulence in *L. monocytogenes* show a low degree of polymorphism. Rasmussen et al. (9) sequenced a 160-bp region in the center of the *hly* gene of different *L. monocytogenes* strains, finding only two sequence groups. Strains belonging to serovars 4b and 1/2b were assigned to sequence group 1, and strains belonging to serovar 1/2a were assigned to sequence group 2. In a study performed by Vines et al. (16), restriction fragment length polymorphism in four virulence-associated genes in *L. monocytogenes* was analyzed. These genes were *iap*, coding for a major extracellular protein (p60) which is an invasion-associated factor; *mpl*, coding for a metalloprotease; and *prfA*, a positive regulator of *hly*, the fourth virulence-associated gene. Vines et al. (16) showed that 29 strains representing different serovars could be divided into two groups on the basis of results from all the restriction fragment length polymorphism analyses. In one group, 10 strains of serovars 1/2a, 1/2c, and 3a gathered together, and in the other group, 19 strains of serovars 1/2b, 3b, and 4b clustered. The authors also performed a

TABLE 1. Phagovars of 37 *L. monocytogenes* serovar 4b strains clustering in PCR-REA group I (profile I)

Strain(s)	Phagovar												
501	1444	312											
520	1444	312		340								2425	
121	1444	312	108						3274	2671			
122, 123	1444	312	108	340					3274	2671			
560	1444	312	108	340			52			2671	1317		
439	1444		108	340	3552	2389	52	107					
253, 281, 285, 295	1444	312	108	340	3552	2389		107			1317		
562, 596	1444		108	340	3552	2389	52	107					47
267	1444	312	108	340	3552	2389	52	107					
74, 75		312	108	340	3552	2389	52	107					47
147, 149	1444	312	108	340	3552	2389	52		3274		1317		
540, 543	1444	312	108	340	3552	2389	52		3274				47
115	1444	312	108	340	3552		52	107	3274	2671			
280	1444	312	108	340	3552	2389		107	3274	2671	1317		
158	1444	312	108	340	3552	2389	52	107		2671	1317		
564	1444	312	108	340	3552	2389	52	107				2425	47
84	1444	312	108	340	3552		52	107	3274	2671		2425	
306, 311	1444	312	108	340	3552	2389		107	3274	2671	1317	2425	
143, 144	1444	312	108	340	3552	2389	52	107	3274	2671	1317		47
249, 260, 270, 312, 362, 404	1444	312	108	340	3552	2389	52	107	3274	2671	1317	2425	
500, 504	1444	312	108	340	3552	2389	52	107	3274	2671	1317	2425	47

TABLE 2. Phagovars of 96 *L. monocytogenes* serovar 4b strains clustering in PCR-REA group II (profile II)

Strain(s)	Phagovar											
7, 22												
26					3552							
28		312										
559		312	108									
183				340			52		3274			
128, 129			108	340		2389						47
94			108	340		2389	52				2425	47
497		312	108	340					3274	2671		
799	1444		108	340	3552	2389	52					47
74 strains of different origin			108	340		2389			3274	2671	2425	47
263	1444	312	108	340	3552	2389		107				
502, 1404, 1877, 1909, 1910			108	340	3552	2389	52		3274	2671	2425	47
503, 1815	1444		108	340	3552	2389	52		3274	2671	2425	47
764	1444	312	108	340	3552	2389	52		3274			47
542	1444		108	340	3552	2389	52		3274		2425	47
546		312	108	340	3552	2389	52	107	3274	2671	2425	

multilocus enzyme electrophoretic analysis of the same strains. That mapping provided results identical to those from the restriction fragment length polymorphism analyses. Also, in a study by Piffaretti et al. (8) using multilocus enzyme electrophoretic analysis at 16 genetic loci encoding metabolic enzymes, two groups were obtained. Twenty-three strains of serovars 1/2a and 1/2c clustered in one of the two groups, while the other group contained 22 strains of serovars 1/2b, 4a, and 4b.

It should be observed that in the last two studies mentioned, as well as in the present investigation, strains of serovars 1/2c and 1/2a group together. This is well in line with the comments made by Vines et al. (16) that "structural diversity in virulence associated genes occurred at about the same rate as changes in genes coding for essential cytoplasmic enzymes and the immunogenic surface antigens that are utilized in serotyping."

The use of monoclonal antibodies for the subtyping of *L. monocytogenes* serovar 4b has recently been demonstrated by Kathariou et al. (7). To our knowledge, however, the present study is the first to be reported that has shown that it is possible to divide *L. monocytogenes* serovar 4b strains into PCR-REA groups. The results, particularly the fact that all strains sharing phagovar 2389:2425:3274:2671:47:108:340 seem to yield the same *Alu*I cleavage pattern, might be useful in the epidemiological investigations of listeriosis.

ACKNOWLEDGMENTS

This project was supported financially by the Swedish Council for Forestry and Agricultural Research and by the research foundation of Ivar and Elsa Sandberg, to whom we express our gratitude.

REFERENCES

1. **Bille, J., and M. P. Glauser.** 1988. Zur Listeriose-Situation in der Schweiz (Listeriosis in Switzerland). Bull. Bundesamtes Gesundheitswesen **3:**28–29.
2. **Buchrieser, C., R. Brosch, and J. Rocourt.** 1991. Use of pulsed field gel electrophoresis to compare large DNA-restriction fragments of *Listeria monocytogenes* strains belonging to serogroups 1/2 and 3. Int. J. Food Microbiol. **14:**297–304.
3. **Gaillard, J. L., P. Berche, C. Frehel, E. Gouin, and P. Cossart.** 1991. Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. Cell **65:**1127–1141.
4. **Geoffroy, C., J.-L. Gaillard, J. E. Alouf, and P. Berche.** 1987. Purification, characterization, and toxicity of the sulfhydryl-activated hemolysin listeriolysin O from *Listeria monocytogenes*. Infect. Immun. **55:**1641–1646.
5. **Goulet, V., A. Lepoutre, J. Rocourt, A. L. Courtieu, P. Dehaumont, and P. Veit.** 1993. Epidemie de listeriose en France. Bilan final et resultats de l'enquete epidemiologique. Bull. Epidemiol. Hebdomadaire **4:**13.
6. **Jensen, A., W. Fredriksen, and P. Gerner-Smidt.** 1994. Risk factors for listeriosis in Denmark, 1989–1990. Scand. J. Infect. Dis. **26:**171–178.
7. **Kathariou, S., C. Mizumoto, R. D. Allen, A. K. Fok, and A. A. Benedict.** 1994. Monoclonal antibodies with a high degree of specificity for *Listeria monocytogenes* serotype 4b. Appl. Environ. Microbiol. **60:**3548–3552.
8. **Piffaretti, J.-C., H. Kressbuch, M. Aeschbacher, J. Bille, E. Bannerman, J. M. Musser, R. K. Selander, and J. Rocourt.** 1989. Genetic characterization of clones of the bacterium *Listeria monocytogenes* causing epidemic disease. Proc. Natl. Acad. Sci. USA **86:**3818–3822.
9. **Rasmussen, O. F., T. Beck, J. E. Olsen, L. Dons, and L. Rossen.** 1991. *Listeria monocytogenes* isolates can be classified into two major types according to the sequence of the listeriolysin gene. Infect. Immun. **59:**3945–3951.
10. **Rocourt, J., A. Audurier, A. L. Courtieu, J. Durst, S. Ortel, A. Schrettenbrunner, and A. G. Taylor.** 1985. A multi-centre study on the phage typing of *Listeria monocytogenes*. Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A **259:** 489–497.
11. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
12. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., p. 6.12–6.13. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
13. **Samuelsson, S., N. P. Rothgardt, A. Carvajal, and W. Frederiksen.** 1990. Human listeriosis in Denmark 1981–1987 including an outbreak November 1985–March 1987. J. Infect. **20:**251–259.
14. **Seeliger, H. P. R., and K. Höhne.** 1979. Serotyping of *Listeria monocytogenes* and related species. Methods Microbiol. **11:**33–46.
15. **Seeliger, H. P. R., and D. Jones.** 1986. Genus *Listeria* Pirie 1940, 383^{AL}, p. 1235–1245. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. Williams & Wilkins, Baltimore.
16. **Vines, A., M. W. Reeves, S. Hunter, and B. Swaminathan.** 1992. Restriction fragment length polymorphism in four virulence-associated genes of *Listeria monocytogenes*. Res. Microbiol. **143:**281–294.