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Specific Gene Probe for Detection of Biotyped and Serotyped *Listeria* Strains

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A total of 284 strains of *Listeria*, including all known serovars and biovars together with *Listeria grayi* and *Listeria murrayi*, were biotyped and serotyped. Biotyping and serotyping could be done in 2 days. A gene probe encoding a delayed hypersensitivity factor (DTH) was used in the detection of pathogenic biotypes and serotypes of the tested strains. The gene was found in all 117 tested *Listeria monocytogenes* strains of serogroups 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4c, 4d, 4e, 4ab, and 7. It was also present in *Listeria ivanovii*. Of 78 *L. monocytogenes* strains of serogroup 4b, 77 strains contained the gene, whereas it was absent in all 10 tested *L. monocytogenes* strains of serogroup 4a. Furthermore, the gene was absent in *Listeria seeligeri*, *L. grayi*, *L. murrayi*, and *L. innocua* of serogroups 3c, 4b, and 6a and in *L. welshimeri* of serogroups 1/2b, 3b, 6a, and 6b. Since *L. monocytogenes* and *L. ivanovii* are the only two biotypes of the genus *Listeria* considered pathogens, the data obtained indicate that the DNA probe tested may be a useful tool in the detection of virulent *Listeria* isolates in clinical, environmental, and food samples.

The genus *Listeria* consists of a heterogeneous group of microorganisms including *Listeria monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii*, *L. grayi*, and *L. murrayi*. The *Listeria* species are distinguished from each other on the basis of hemolytic activity, nitrate reduction, and fermentation of certain sugars (23). For epidemiological purposes, serological identification as described by Seeliger and Höhne (25) has been developed. Phage typing has also been suggested to be useful in elucidating epidemics of listeriosis (22). Only bacteria of the species *L. monocytogenes* and *L. ivanovii* are pathogenic for humans and animals, respectively. Pathogenicity can be tested by intraperitoneal injection of the test strain into mice (12, 17). In a recent study, Stelma et al. (26) have shown that immunocompromised mice are suitable for such testing, since a dose of 10^4 CFU of a pathogenic isolate per mouse causes death in 3 days.

In humans, bacterial meningitis is the most common form of listeriosis, while perinatal infections may result in abortion, stillbirth, and infant death. Abortion, encephalitis, septicemia, and mastitis are the main clinical manifestations in cattle and sheep (2, 4, 13, 14). Conditions predisposing for *Listeria* infections in nonpregnant adults include diabetes, alcoholism, cancer, and treatment with corticosteroids or other immunosuppressive drugs (4, 11, 14). However, Schlech et al. (24) have described an outbreak of listeriosis in which none of the patients had evidence of any underlying immunosuppressive condition. Direct transmission has been documented in veterinary surgeons, abattoir workers, and farmers (3, 21). Indirect transmission by food seems the most important link, and outbreaks of listeriosis have been traced to pasteurized milk (8), to cheese (13), and to coleslaw (24).

Current methods for the isolation and identification of *L. monocytogenes* are laborious and time consuming. The procedure includes (selective) enrichment in fluid media, subsequent plating of the enrichment fluid, and testing of suspected colonies by a number of biochemical tests followed by biotyping and serotyping (2). Alternative methods for rapid and reliable detection of *L. monocytogenes* in food, clinical, and environmental samples are required. A promising technique is the use of a DNA probe specific for *L. monocytogenes* in DNA hybridization. An ideal probe would be one that encodes a virulence factor. However, the mechanisms of pathogenesis of *L. monocytogenes* are not completely clear. Among the factors that contribute to the virulence of *L. monocytogenes* are hemolysins (10, 12, 15, 16), invasive factors (9), and factors which facilitate intracellular survival (5) and cause delayed hypersensitivity (1, 19). A gene encoding a delayed-hypersensitivity-inducing protein from a virulent *L. monocytogenes* serotype 1/2a strain has been cloned and sequenced (M. Leimeister-Wächter and T. Chakraborty, manuscript submitted). In this study, we used the *Listeria* DNA insert encoding the delayed-hypersensitivity-inducing protein (DTH-18) and probed for its presence in reference strains of all known *Listeria* species. Furthermore, we screened 284 *Listeria* strains for the presence of the gene. All strains were biotyped and serotyped by methods described in this study. This extended study, using *Listeria* of all known serotypes together with *L. grayi* and *L. murrayi*, showed that the presence of the DTH-18 gene is indeed highly correlated with virulent types of *Listeria*. This suggests its usefulness as a probe for detection of *L. monocytogenes* in food, clinical, and environmental samples.

MATERIALS AND METHODS

Cultures. The reference strains used in this study are summarized in Table 1. All other *Listeria* strains used were chosen from a collection of about 3,000 strains at the National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands. From this collection,

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TABLE 1. Hybridization reactions of a 1.1-kb DNA probe encoding a delayed-type hypersensitivity factor with *Listeria* strains

Serogroup	Reference strains			Other strains		
	Strain	Species	Hybridization reaction	Species	No. tested	No. with hybridization reactions
1/2a	NCTC 7973	*L. monocytogenes*	+	*L. monocytogenes*	34	34
1/2b	SLCC 2755	*L. monocytogenes*	+	*L. monocytogenes*	37	37
	SLCC 3954	*L. seeligeri*	−	*L. seeligeri*	1	0
				L. welshimeri	1	0
1/2c	NCTC 5348	*L. monocytogenes*	+	*L. monocytogenes*	16	16
3a	NCTC 5105	*L. monocytogenes*	+	*L. monocytogenes*	5	5
3b	SLCC 2540	*L. monocytogenes*	+	*L. monocytogenes*	3	3
				L. welshimeri	1	0
				L. seeligeri	1	0
3c	SLCC 2479	*L. monocytogenes*	+	*L. monocytogenes*	1	1
				L. innocua	1	0
4a	NCTC 5214	*L. monocytogenes*	−	*L. monocytogenes*	9	0
4b	NCTC 10527	*L. monocytogenes*	+	*L. monocytogenes*	77	76
4c	ATCC 19116	*L. monocytogenes*	+	*L. seeligeri*	5	0
4d	NCTC 10888	*L. monocytogenes*	+	*L. monocytogenes*	2	2
4e	ATCC 19118	*L. monocytogenes*	+			
4ab	NCTC 10528	*L. monocytogenes*	+	*L. monocytogenes*	1	1
5	ATCC 19119	*L. ivanovii*	+			
6a	SLCC 5334	*L. welshimeri*	−	*L. innocua*	32	0
	NCTC 11288	*L. innocua*	−	*L. welshimeri*	2	0
6b	NCTC 11289	*L. innocua*	−	*L. innocua*	18	0
				L. welshimeri	5	0
7	SLCC 2482	*L. monocytogenes*	+	*L. monocytogenes*	7	7
	RIVM 1	*L. grayi*[a]	−	*L. grayi*	2	0
	RIVM 2	*L. murrayi*[a]	−	*L. murrayi*	3	0

[a] Not serologically classified (25).

all strains belonging to the rarely occurring serotypes 3a, 3b, 3c, 4a, 4c, 4d, 4e, 4ab, 5, and 7 were chosen. They were supplemented with strains belonging to serotypes 1/2a, 1/2b, 1/2c, 4b, 6a, and 6b. The strains tested included all 40 strains isolated from listeriosis patients as described by Kampelmacher and van Noorle-Jansen (14). Other human isolates originated from cerebrospinal fluid (28 strains), feces (40 strains), blood (10 strains), lochia (8 strains), autopsied brains (7 strains), autopsied livers (4 strains), abortions (10 strains), cervical swabs (3 strains), amniotic fluid (3 strains), and unknown sources (19 strains). Other strains tested originated from food (50 strains), grass-silage (10 strains), and animals (32 strains). In addition to *Listeria* strains, 10 randomly selected isolates of *Escherichia coli*, *Yersinia* spp., *Staphylococcus aureus*, *Vibrio* spp., *Salmonella typhimurium*, *Bacillus cereus*, and *Enterococcus faecalis* were used. All randomly selected strains were isolated from food by routine tests.

Serotyping. Serotyping was based on the antigenic scheme of Seeliger and Höhne (25). Standard strains and methods for the preparation and absorption of antisera raised in rabbits were kindly supplied by H. P. R. Seeliger (University of Würzburg, Federal Republic of Germany). For serotyping, the strains were inoculated into 4 ml of tryptone phosphate broth supplemented with 1% glucose and incubated in a shaking water bath at 37°C for 6 to 7 h. These cultures were used to inoculate tryptose agar plates and incubated overnight at 37°C. A second tube of tryptose phosphate broth plus 1% glucose was inoculated and incubated with vigorous aeration at 25°C overnight. The tryptose phosphate plate was harvested in 5 ml of phosphate-buffered saline (pH 7.4), heated for 1 h in a boiling-water bath, and centrifuged. The cells were suspended and adjusted to 5×10^8 cells per ml on the basis of the observation that a suspension of 1×10^9 cells per ml has an optical density of 1.0 at 600 nm. Of this suspension, 0.2 ml was added in glass tubes (45 mm; diameter, 8 mm) to 0.2 ml of antiserum which had been diluted 75 times and which was directed against O-factor complexes I-II; V-VI, and XIII-XIV. The mixture was then incubated at 45°C for 2 h and left overnight at 4°C before the

tubes were examined for the presence of visible agglutination. If O-factor complex I-II was positive, then factor complex I-II and factors I and II were titrated starting from a 1:20 dilution in decimal dilutions and incubated in the same way as for the factor complex. The same was done if O-factor complex V-VI was positive for factor complex V-VI and for factors VI, VII, VIII, IX, X, XI, and XV. If O-factor complex XIII-XIV was positive, the same was done for factor XIII and XIV, starting from a 1:20 dilution in decimal dilutions and incubated in the same way as for the factor complexes. For H-antigen typing, Formalin was added to the second tube of tryptose phosphate broth plus 1% glucose to a final concentration of 0.5% and the culture was adjusted to 5×10^8 cells per ml. A volume of 0.2 ml of the Formalin-killed bacteria was added to 0.2 ml of H antiserum which had been diluted 100 times and which was directed against AB, A, C, D, and E. The mixture was then incubated at 45°C for 2 h and stored overnight at 4°C before the tubes were inspected for agglutination.

Biotyping. Biotyping was based on the system described by Rocourt et al. (23). Strains were inoculated in tubes containing 4 ml of semisolid peptone agar medium to which 1% of either D-xylose or L-rhamnose was added, and tubes were incubated at 37°C for 2 days. The peptone agar medium consisted of 10 g of Bacto-Peptone, 5 g of NaCl, 5.5 g of Oxoid agar L28, and 0.08 g of bromothymol blue dissolved in 1,000 ml of distilled water (pH 7.6) and autoclaved at 120°C for 15 min. After autoclaving, filter-sterilized sugar solution was added to a final concentration of 1%. Hemolysin production was checked by growing the strains overnight at 37°C in brain heart infusion broth. From this culture, 0.2 ml was added to 0.2 ml of 2% sheep erythrocytes (washed three times) in phosphate-buffered saline in glass tubes. After 2 h the tubes were checked for hemolysis. *L. monocytogenes* NCTC 7973 and *L. innocua* NCTC 11289 served as positive and negative controls, respectively.

DNA probe. Plasmid pLM10 is a pUC18 clone containing a 1.1-kilobase (kb) *L. monocytogenes* DNA insert. This insert, which encodes a delayed-type hypersensitivity factor (DTH-18), was excised and purified by two successive runs of agarose gel electrophoresis and electroelution. To serve as a probe, 200 ng of this DNA was ^{32}P labeled by the method of Feinberg and Vogelstein (7).

Colony hybridization. A colony hybridization procedure was used for testing hybridization of the DNA probe with the strains. All strains were grown on brain heart infusion agarose plates for 18 h at 37°C. Colonies were lifted from the plates onto GeneScreen Plus membranes (Du Pont Co.). The cells were lysed by an alkaline steaming procedure. The membranes were placed on filter paper soaked in 0.5 M NaOH and incubated for 5 min just above the water level in a boiling-water bath. They were then placed on 1 ml of fresh 0.5 M NaOH and neutralized with 1 ml of 1 M Tris solution (pH 7.5). This neutralization step was repeated once. The membranes were subsequently immersed in 100 ml of 5× SSC (1× SSC is 0.15 M NaCl and 0.0015 M sodium citrate). While immersed, they were rubbed thoroughly with a tissue to remove cell debris. They were then air dried to fix the DNA onto the membranes. The membranes were prehybridized for 6 to 16 h at 40°C in 15 ml of a solution containing 50 mM Tris (pH 7.5), 10 mM EDTA, 1 M NaCl, 10× Denhardt solution (1× Denhardt solution contains 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 100 μg of denatured herring sperm DNA per ml, 0.1% sodium PP_i, and 1% sodium dodecyl sulfate. The DNA probe was then added and allowed to hybridize for 18 h. The membranes were washed for 15 min.

Hybridization was carried out at 60°C, and washing was done in a 0.2× SSC buffer containing 1% sodium dodecyl sulfate and 0.1% sodium PP_i at 65°C. The membranes were wrapped in plastic foil and exposed to Konica A_2 film with an intensifying screen at −70°C for approximately 18 h.

TABLE 2. Identification of species of *Listeria* strains unable to reduce nitrate as described by Rocourt et al. (23)

Species	Result[a] for:		
	β-Hemolysin production	Fermentation of:	
		D-Xylose	L-Rhamnose
L. monocytogenes	+	−	+
L. ivanovii	+++	+	−
L. seeligeri	+	+	−
L. innocua	−	−	±
L. welshimeri	−	+	±

[a] +, Positive; ±, positive or negative; −, negative; +++, strongly positive.

RESULTS

Biotyping. All 284 strains tested for hybridization with the DTH-18 factor probe were first biotyped. Biotyping of strains involved testing for fermentation of D-xylose and L-rhamnose and production of hemolysin. The test tube method for testing sugar fermentation yielded unequivocal results. Also, the test for hemolysin production using washed sheep erythrocytes in glass tubes gave clear reactions. Therefore, strains could be easily classified into species by the system of Rocourt et al. (23) (Table 2).

Serotyping. All strains tested could be serotyped, including *L. grayi* and *L. murrayi*. It was observed that *L. monocytogenes* was represented in serogroups 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4e, 4ab, and 7 (Table 1). *L. seeligeri* was found in serogroups 1/2b, 3b, and 4c. The strain of serogroup 1/2b was recently isolated from raw fish. The strain of serogroup 3b had been isolated from human feces, while all strains of serogroup 4c were recently isolated from the same lot of pork. *L. welshimeri* was found in serogroups 1/2b, 3b, 6a, and 6b. *L. welshimeri* of serogroup 1/2b had been isolated from human feces in 1967, and this strain did not ferment L-rhamnose. Also, *L. welshimeri* of serogroup 3b had been isolated from human feces and did not ferment L-rhamnose. *L. innocua* was represented in serogroups 3c, 6a, and 6b. The *L. innocua* strain of serogroup 3c fermented L-rhamnose and had been isolated from human cerebrospinal fluid in 1967.

Hybridization. With the 1.1-kb DNA probe (DTH-18), optimal hybridization signals were obtained after the membranes were washed in 0.2× SSC for 30 min at 65°C. Washing in 0.2× SSC for 30 min at 70°C reduced hybridization to the background level. An example of the hybridization results obtained is presented in Fig. 1. The results demonstrate that the protocol used for preparing the GeneScreen Plus membranes gave good hybridization signals and a negligible background. The 1.1-kb DNA probe reacted with the reference strain of *L. ivanovii* as well as with all *L. monocytogenes* strains except strain NCTC 5214, serotype 4a (Table 1). The reference strains *L. seeligeri* SLCC 3954, *L. welshimeri* SLCC 5334, and *L. innocua* NCTC 11288 and NCTC 11289 as well as *L. grayi* RIVM 1 and *L. murrayi* RIVM 2 did not give hybridization signals. These results

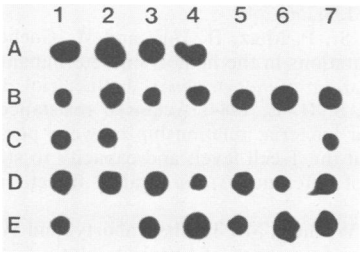

FIG. 1. DNA-DNA hybridization results obtained by colony blotting of 35 *Listeria* strains. Five rows of seven colonies each were inoculated. Hybridization experiments were carried out with a 1.1-kb DNA probe encoding a delayed-type hypersensitivity factor. By using the hybridization procedure described in Materials and Methods, positive results were obtained for *L. ivanovii* ATCC 19119 (B1) and *L. monocytogenes* strains (A1, strain NCTC 7973; A2, strain SLCC 2755; other positive signals are of wild-type strains of *L. monocytogenes*). Negative results were obtained by wild-type strains of *L. seeligeri* (A5, A6), *L. welshimeri* (A7), *L. innocua* (C3, C4), and *L. grayi* (C5, C6) and by *L. murrayi* (E2).

showed that the DNA probe used was specific for the detection of the pathogenic species, *L. monocytogenes* and *L. ivanovii*.

The efficacy of the gene probe in detecting virulent *Listeria* isolates was tested by using all the biotyped and serotyped *Listeria* strains described above. The hybridization reactions with the 1.1-kb DNA probe of all other strains are summarized in Table 1. Almost all *L. monocytogenes* strains tested were positive. There were a few exceptions. None of the nine *L. monocytogenes* strains of serogroup 4a in our collection gave a hybridization signal. These nine strains had been isolated from men between 1965 and 1973.

Of the 77 *L. moncytogenes* strains of serogroup 4b, 1 did not show any hybridization. This negative strain was recently isolated from cheese.

None of the wild-type isolates of *L. seeligeri*, *L. grayi*, *L. murrayi*, or *L. innocua* reacted with the DTH-18 probe.

All 40 *Listeria* strains isolated from patients with listeriosis during 1958 to 1977 as summarized by Kampelmacher and van Noorle-Jansen (14) were reactive with the DTH-18 probe.

All strains not belonging to the genus *Listeria*, such as *S. typhimurium*, *E. coli*, *Yersinia* spp., *S. aureus*, *Vibrio* spp., *B. cereus*, and *Enterococcus faecalis*, did not show hybridization signals with the DTH-18 probe.

DISCUSSION

Biotyping and serotyping of *Listeria* strains showed that *L. welshimeri*, *L. innocua*, and *L. seeligeri* occur in more serotypes than previously described by Rocourt et al. (22, 23). *L. welshimeri* was also found in serogroup 1/2a and 3b strains. *L. innocua* was now also represented in serogroup 3c, while one *L. seeligeri* strain was found in serotype 3b. These findings can be simply explained by the fact that in our study 284 strains were involved, while in the study by Rocourt et al. (22, 23) only 66 strains were tested.

In hybridization experiments with the 1.1-kb DNA probe, which encodes a DTH factor, and the reference strains, only the pathogenic species, *L. monocytogenes* (except serotype 4a) and *L. ivanovii*, gave positive hybridization reactions. Of the 284 *Listeria* strains tested, positive results were obtained with all *L. monocytogenes* strains of serogroups 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4d, 4ab, and 7. Of *L. monocytogenes* serotype 4b strains, a single strain showed no hybridization signal. This strain was isolated recently from cheese.

All strains of the rarely occurring serogroup 4a were negative for hybridization with the 1.1-kb DNA probe. No strain of *L. seeligeri*, *L. innocua*, *L. welshimeri*, *L. grayi*, or *L. murrayi* showed hybridization reactions. Also, no reactions were observed with randomly selected strains of *E. coli*, *Yersinia* spp., *S. aureus*, *Vibrio* spp., *S. typhimurium*, *B. cereus*, or *Enterococcus faecalis*. In a previous study, using a small number of strains, Datta et al. (6) tested a hemolysin gene probe. However, in the absence of data relating to biotyping, serotype, and hemolytic activity, it is not possible to compare their results and ours. Since homology exists between listeriolysin, streptolysin O, and pneumolysin (18, 20), it might be expected that a DNA probe encoding listeriolysin would exhibit cross hybridization to related toxins when samples (food, clinical material, etc.) are tested for the presence of *L. monocytogenes*. Indeed, the presence of the listeriolysin gene in *L. seeligeri* has been detected by using an internal DNA fragment from the cloned gene (M. Leimeister-Wächter, W. Goebel, and T. Chakraborty, manuscript submitted).

The work reported here is a study of the efficacy of a gene encoding a delayed-hypersensitivity-inducing protein (DTH-18) cloned from a virulent strain of *L. monocytogenes* serotype 1/2a. We have biotyped and serotyped all *Listeria* strains in this study by refined techniques that allowed unambiguous differentiation of *Listeria* species. On several occasions we have been guided by the DNA probe. For example, a serotype 1/2b strain was classified as *L. seeligeri* after showing no reaction to the DNA probe. This was confirmed by reexamining the biotyping data. With all strains tested, the detection efficacy of *L. monocytogenes* was 95%. The probe detected all 40 strains isolated from patients with listeriosis. We emphasize that the strains used in this communication were selected mainly on the basis of their serotype and are not representative of the distribution of serotypes in field isolates. For instance, in our entire collection of over 3,000 *Listeria* isolates maintained since 1959, only 9 strains are serotype 4a.

The data make it clear that the DNA probe tested is a useful marker in the detection of virulent *Listeria* strains in clinical, environmental, and food samples. The gene appears to be highly conserved in the *Listeria* species in which it is present, since increasing the washing temperature abolished hybridization signals of all positive isolates simultaneously. Our current studies are focused on understanding the role of this protein in the pathogenesis of *Listeria* infection. The lack of the gene in *L. monocytogenes* serotype 4a detected in this study offers a unique opportunity to study molecular mechanisms that lead to the loss or acquisition of the gene in this genus.

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