APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1991, p. 1609–1614
0099-2240/91/061609-06$02.00/0

Vol. 57, No. 6

Rapid Confirmation of *Listeria monocytogenes* Isolated from Foods by a Colony Blot Assay Using a Digoxigenin-Labeled Synthetic Oligonucleotide Probe

CHANGMIN KIM,† BALASUBRAMANIAN SWAMINATHAN,* PAMELA K. CASSADAY, LEONARD W. MAYER,‡ AND BRIAN P. HOLLOWAY§

Meningitis and Special Pathogens Branch, Division of Bacterial and Mycotic Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333

Received 8 November 1990/Accepted 12 March 1991

An oligodeoxyribonucleotide probe based on the sequence of a 321-bp internal fragment of the *msp* gene encoding a major secreted polypeptide of *Listeria monocytogenes* was labeled with digoxigenin by using terminal deoxynucleotidyl transferase. The specificity of the digoxigenin-labeled probe was determined by dot blot assays. The probe reacted with all strains of *L. monocytogenes* tested (12 of 12 strains representing five serotypes). The probe did not react with any other *Listeria* species or with other gram-positive bacteria (*Brochothrix*, *Erysipelothrix*, *Corynebacterium*, *Rhodococcus*, *Lactobacillus*, *Leuconostoc*, *Bacillus*, *Staphylococcus*, and *Streptococcus*). The probe was used to develop a colony blot assay for the rapid confirmation of *L. monocytogenes* on *Listeria*-selective agars which had been streaked with food enrichment cultures. Forty-eight food samples were tested by conventional culture and DNA colony blot assay. The sensitivity and specificity of the DNA colony blot were 100 and 97%, respectively.

Rapid isolation and confirmation methods for *Listeria monocytogenes* in foods are urgently needed because foods have been implicated in all of four listeriosis outbreaks investigated between 1981 and 1988 (1, 11, 26, 34). With the cold enrichment procedure, it may take as long as 6 months to isolate *L. monocytogenes* from contaminated food and environmental samples (13, 15, 19). The use of recently developed selective enrichment and plating methods (27, 29, 38) significantly reduces the time required for isolation and confirmation. However, the procedure still requires 2 to 3 days for presumptive positive results. After initial isolation, an additional 2 to 4 days are required to confirm suspect colonies on selective agar plates by biochemical and serologic tests (36). The long time required for confirmation of *L. monocytogenes* by traditional methods presents problems in the quality control of semiperishable food commodities.

Enzyme immunoassays based on monoclonal antibodies (4, 8) and nucleic acid hybridization assays using DNA probes (6, 21, 22) have been explored as alternatives for rapid detection and confirmation of *Listeria* spp. in foods. The monoclonal antibodies that have been developed so far are not specific for *L. monocytogenes*, but are *Listeria* genus specific. Currently available nucleic acid hybridization assays either are not species specific or use ^{32}P-labeled DNA probes which pose safety, disposal, and license requirement problems for the users (25).

Flamm (9) cloned a 5.2-kb fragment of the chromosomal DNA of *L. monocytogenes* serotype 1/2a, thought to contain the gene coding for beta-hemolysin, into pBR325. The constructed plasmid was designated pRF102. An internal *Hin*cII and *Hin*dIII fragment (reported by Flamm to be approximately 500 bp in size) of the *L. monocytogenes* DNA insert in pRF102 was cloned into pUC8, and this construction was designated pRF106. The pRF106 fragment was used as a DNA probe to detect *L. monocytogenes* in a Southern blot format (10) and in a colony blot format (5). Investigators in these studies reported that the probe was specific for *L. monocytogenes*. However, we found that the pRF106 fragment cross-hybridized to the DNA of a food isolate of *L. seeligeri* (20).

In this investigation, the pRF106 fragment was subcloned into M13 bacteriophage vectors and sequenced by the Sanger dideoxy sequencing technique. Oligonucleotides derived from the sequence information were used to develop an *L. monocytogenes*-specific nonisotopic colony hybridization assay to confirm rapidly the presence of *L. monocytogenes* on lithium chloride-phenylethanol-moxalactam (LPM) agar plates. Specificity and sensitivity of the digoxigenin-oligonucleotide colony hybridization assay were evaluated with naturally contaminated food samples.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. The strains were stored frozen in sheep blood at 70°C. They were revived by growing on blood agar (tryptic soy agar plus 5% sheep blood) at 35°C for 16 to 18 h and checked for purity before use. *Brochothrix thermosphacta* was grown at 25°C.

Isolation of plasmid pRF106. *Escherichia coli* LE392 containing plasmid pRF106 was kindly provided by R. K. Flamm, Washington State University. Plasmid DNA (pRF106) was isolated by the procedure of Ish-Horowicz and Burke (17) and purified by cesium chloride-ethidium bromide density gradient centrifugation (28).

Ligation and transformation. Plasmid pRF106 was di-

* Corresponding author.
† Present address: Division of Biotechnology, National Institute of Health, Seoul, Korea.
‡ Present address: Division of Vector-Borne Infectious Disease, Center for Infectious Diseases, Centers for Disease Control, Ft. Collins, CO 80522.
§ Present address: Biotechnology Resource Facility, Scientific Resources Program, Center for Infectious Diseases, Centers for Disease Control, Atlanta, GA 30333.

TABLE 1. Bacterial strains used

No.	Bacterial species	Serotype	EIL reference no.[a]	Source
1	*L. monocytogenes*	1/2a	F4246	Patient
2	*L. monocytogenes*	1/2a	F4263	Patient
3	*L. monocytogenes*	1/2a	F4259	Patient (blood)
4	*L. monocytogenes*	1/2b	F4245	Patient (lung)
5	*L. monocytogenes*	1/2b	F4260	Patient (blood)
6	*L. monocytogenes*	1/2b	F4398	Food (ice cream)
7	*L. monocytogenes*	3c	F1707	Patient
8	*L. monocytogenes*	4b	F4234	Patient
9	*L. monocytogenes*	4b	F4244	Patient (CSF[b])
10	*L. monocytogenes*	4b	F4262	Patient
11	*L. monocytogenes*	4b	F4264	Patient
12	*L. monocytogenes*	4b	F4393	Food (cheese)
13	*L. monocytogenes*	4ab	F1067	Food (milk)
14	*L. innocua*		F4247	Food isolate from FDA[c]
15	*L. innocua*		F4248	Food isolate from FDA
16	*L. murrayi*		F4076	ATCC[d] 25401
17	*L. murrayi*		F4077	ATCC 25402
18	*L. ivanovii*		F4081	ATCC 19119
19	*L. ivanovii*		F4084	Food (raw cream)
20	*L. seeligeri*		F4856	Food (ground beef)
21	*L. seeligeri*		F4882	Food (cheese)
22	*L. seeligeri*		F5761	Food (lunch meat)
23	*L. welshimeri*		F4082	H. P. R. Seeliger, SLCC 5355
24	*L. welshimeri*		F4083	ATCC 35897
25	*L. grayi*		F4085	ATCC 19120
26	*L. grayi*		F4086	ATCC 25400
27	*Jonesia denitrificans*		F4087	ATCC 14870
28	*Brochothrix thermosphacta*		F5950	ATCC 11509
29	*Erysipelothrix rhusiopathiae*		F5937	Patient (blood)
30	*Corynebacterium diphtheriae*		F5944	University of Louisville
31	*C. aquaticum*		F5942	Patient (CSF)
32	*C. striatum*		F5946	Patient (blood)
33	*C. minutissimum*		F5940	Patient
34	*C. ulcerans*		F5947	Patient
35	*Rhodococcus equi*		F5943	ATCC 6939
36	*Lactobacillus casei*		F5952	ATCC 393
37	*Leuconostoc dextranicum*		F5953	ATCC 19255
38	*Bacillus cereus*		F5939	ATCC 6051
39	*Stapylococcus aureus*		D1233	Patient
40	*Streptococcus agalactiae*		F5949	ATCC 13813
41	*Lactobacillus lactis*		F5948	ATCC 19435
42	*Enterococcus faecalis*		F4069	Patient
43	*Escherichia coli*		D0145	Patient

[a] EIL, Epidemic Investigations Laboratory, Meningitis and Special Pathogens Branch, Division of Bacterial and Mycotic Diseases, Center for Infectious Diseases, Centers for Disease Control.
[b] CSF, Cerebrospinal fluid.
[c] FDA, U.S. Food and Drug Administration.
[d] ATCC, American Type Culture Collection.

gested with *Hin*cII and *Hin*dIII (New England BioLabs, Beverly, Mass.) to isolate the *L. monocytogenes* DNA insert (pRF106 fragment). The pRF106 fragment was ligated to bacteriophage M13-mp8 and M13-mp9 replicative form DNA. *E. coli* 71-18 (7) was transformed with the ligated M13 DNA mixtures by the procedure of Hanahan (14).

Identification of recombinants. White plaques containing recombinants on B agar (28) plates with isopropylthio-β-galactoside and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were picked for further experiments. M13-mp8 and M13-mp9 recombinants were tested to check DNA complementarity of recombinant phages with each other by the c-test. A pair of M13-mp8 and M13-mp9 recombinant phages that migrated more slowly in the gel was used for sequencing analysis (12, 31).

Sequencing analysis. M13 recombinant phage DNA was isolated from M13 phages by the procedures of Schreier and Cortese (35) and sequenced by the dideoxynucleotide chain termination method (33) with [α-^{35}S]dATP and T7 polymerase, using a Sequenase kit (United States Biochemical Corp., Cleveland, Ohio) and following the manufacturer's protocols. The mixtures from sequencing reactions were electrophoresed on 8% (acrylamide–BIS [*N*,*N*′-methylenebisacrylamide], 20:1), 6% (20:1), and 6% (40:1) polyacrylamide gels containing 7 M urea. Electrophoresis was done at 1,700 V for 2 h, 1,300 V for 4 h, and 1,000 V for 14 h, respectively. After electrophoresis, gels were dried under vacuum and exposed to X-ray film at room temperature for 40, 20 to 24, and 24 h, respectively. Computer analyses of sequence data were performed with the DNASTAR software package (DNASTAR Inc., Madison, Wis.).

Selection of nucleotide sequences for oligonucleotide synthesis. Four different oligonucleotide sequences were chosen for evaluation as *L. monocytogenes*-specific probes from the pRF106 fragment sequence. The oligonucleotides were synthesized by beta-cyanoethyl phosphoramidite chemistry, using an Applied Biosystems DNA synthesizer 380B (Applied Biosystems, Foster City, Calif.). The sequences of the four oligonucleotides and their relative position on the pRF106 fragment nucleotide sequence are shown in Table 2.

Extraction of chromosomal DNA. Chromosomal DNA was extracted from *L. monocytogenes*, other *Listeria* spp., and other gram-positive bacteria by a method developed in our laboratory (20).

Preparation of filters for dot blots. The protocol of Kafatos et al. (18) was used to denature DNA and to immobilize denatured DNA on 0.45-μm nylon membranes (Magnagraph; Micron Separations, Westboro, Mass.). The denatured DNA was loaded on the nylon membrane by using a Bio-Dot microfiltration apparatus (Bio-Rad Laboratories, Richmond, Calif.). The membrane was dried at room temperature and baked at 80°C for 1 h. Nylon membranes with immobilized DNA were stored at 4°C until use.

Preparation of food enrichment broth. Food enrichment broths were obtained from the Epidemic Investigations and Support Laboratory, Centers for Disease Control. Foods were routinely analyzed for *L. monocytogenes* by conventional culture methods as part of an active surveillance program to determine the role of foods in sporadic cases of human listeriosis. The U.S. Department of Agriculture primary and secondary enrichment procedures were used to isolate *L. monocytogenes* in foods (29). After incubation, 0.1 ml of each broth was streaked to LPM agar plates. The LPM plates were incubated at 35°C for 48 h and examined for the presence of typical *Listeria* colonies by the Henry method of oblique lighting (16). Suspicious colonies were picked and streaked onto sheep blood agar plates to check for beta-hemolysis and biochemical tests (36).

Preparation of membranes for colony blots. Bacterial colonies grown on LPM agar were transferred, denatured, and

TABLE 2. Synthetic oligonucleotide sequences

Oligonucleotide	Sequence (5′ to 3′)	Location on pRF106 fragment[a]
Msp81	CGGATAAAGCCCAAATAGTGTCACCGCTTTTGACAG	81–116
Msp110	TTGACAGCGTGTGTAGTAGCA	110–130
Msp162	CTGCTACTTTAGGCGCAGGTGTAG	162–185
Msp297[b]	GCAGTAAGCACTCCAGTTGCACCAACACAAGAAGTG	297–262

[a] Sequence of pRF106 fragment is shown in Fig. 1.
[b] Complementary sequence.

fixed on the membrane by microwave procedures (3). Briefly, bacterial colonies on LPM agar were lifted to a nylon membrane (Magnagraph, 0.45 μm; Micron Separations) by contacting for 3 min. The membrane was laid on two sheets of Whatman 3 MM chromatography paper soaked with 2× SSC–5% sodium dodecyl sulfate (SDS) (1× SSC contains 0.15 M sodium chloride and 0.015 M sodium citrate) and left for 2 min in a glass dish. The glass dish with the membrane was transferred to a 700-W Kenmore microwave oven (Sears, Roebuck and Co., Chicago, Ill.) and microwaved for 2.5 min to lyse the cells, denature, and fix the bacterial DNA to the membrane. The membrane was used for hybridization or stored at 4°C until used.

Labeling of oligonucleotide Msp110. The four oligonucleotides were end labeled with [γ-^{32}P]ATP, using T4 polynucleotide kinase. The ^{32}P-labeled oligonucleotides were evaluated for their specificity and sensitivity by using them to probe genomic DNA from several strains of *L. monocytogenes*, other *Listeria* species, and other gram-positive bacteria. These evaluations were done with radioactively labeled probes because it was easier to adjust the stringencies of hybridization and to determine the optimum posthybridization washing conditions. After an oligonucleotide probe was selected based on the specificity and sensitivity evaluations, further experiments were done with the selected oligonucleotide (Msp110) by using a nonisotopic method. Oligonucleotide Msp110 was end labeled with digoxigenin, using a DNA tailing kit according to the manufacturer's recommendations (Boehringer Mannheim, Indianapolis, Ind.).

Hybridization. Hybridization of ^{32}P-labeled oligonucleotides (200 ng of 10^8 dpm/μg) with target DNA was done at 37°C for 16 to 18 h in 5× SSC–5× Denhardt solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin)–0.5% SDS. Hybridization of digoxigenin-labeled Msp110 (200 ng) with target DNA was done at 50°C in 5× SSC–0.1% *N*-lauroylsarcosine–2% SDS–0.5% blocking agent (Boehringer Mannheim). Two hybridization times (2 h and overnight) were evaluated for digoxigenin-labeled Msp110. After hybridization, the membranes were washed in 2× SSC–0.1% SDS for 20 min at 50°C, which corresponded to a stringency of T_m − 14°C (32). Immunologic detection was done with anti-digoxigenin antibodies conjugated to alkaline phosphatase (Boehringer Mannheim). The substrates for alkaline phosphatase were nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt.

Optimization of the colony hybridization time. Hybridizations of 2 and 16 to 18 h were tested to determine whether the hybridization period could be reduced without affecting sensitivity. *L. monocytogenes* cells were serially diluted with 0.01 M phosphate-buffered saline (pH 7.2), and approximately 300 cells were plated on LPM agar and incubated at 35°C for 40 h. The colonies were then transferred to nylon membranes, microwaved, and hybridized with digoxigenin-labeled Msp110.

Nucleotide sequence accession number. The sequence of the pRF106 fragment has been submitted to GenBank. The accession number is M62749.

RESULTS

Nucleotide sequence of the pRF106 fragment. The pRF106 fragment contained 321 bp and had a guanine-plus-cytosine content of 37%. The nucleotide sequence of the 321-bp pRF106 fragment is shown in Fig. 1. Computer analysis of the sequence with the open reading frame program of DNASTAR indicated that the pRF106 sequence contained an open reading frame from base 118 to 309 (63 amino acids), but no start or stop codons were found. The reverse complement of the pRF106 fragment sequence contained an open reading frame from base 1 to 321, suggesting that pRF106 could be the internal fragment of a coding sequence. When the direct strand (pRF106) and reverse strand (~pRF106) sequences were compared with the published *iap* sequence (23; GenBank accession no. X52268), using DNA Align ver.5.87 of DNASTAR, there was 98.7% homology between the *iap* sequence (nucleotides 842 to 1168) and the ~pRF106

```
        10         20         30         40         50         60
AAGCTTTTGACCTACATAAATAGAAGAAGAAGATAAATTATTCCATGACATAATGTCTTG

        70         80         90        100        110        120
AACAGAAACACCGTATTTTACGGATAAAGCCCAAATAGTGTCACCGCTTTTGACAGCGTG

       130        140        150        160        170        180
TGTAGTAGCATTTTGATCTATTACTGGAGTTTCTTTCGTTTCTGCTACTTTAGGCGCAGG

       190        200        210        220        230        240
TGTAGTTGCTTGTGTAGTTTGTTTTACTTCAGTTTTTGTTTCTGCAACAGGTGCAGCTTG

       250        260        270        280        290        300
TTGAGTAGTAGTTTCTTTTTTCACTTCTTGTGTTGGTGCAACTGGAGTGCTTACTGCTTT

       310        320
GTCAGTTAAGTATTTACCGTT
```

FIG. 1. Sequence of the 321-bp internal fragment of the *msp* gene of *L. monocytogenes*.

TABLE 3. Difference in the nucleotide sequences of pRF106 and an internal fragment (nucleotides 825 to 1137) of the *iap* gene of *L. monocytogenes*

Location[a] on ~pRF106[b] sequence	Nucleotide	Location on *iap*[c] gene sequence	Nucleotide
95	T	942	C
181	A	1028	G
208	G	1055	C
210	C	1057	T

[a] All locations are given from the 5′ end of the sequence.
[b] Reverse complement of pRF106.
[c] Sequence of the *iap* gene is from reference 23 (GenBank accession no. X52268).

sequence. Four differences were found between the two sequences (Table 3).

Evaluation of oligonucleotides derived from the pRF106 fragment. Oligonucleotides Msp81, Msp110, Msp162, and Msp297 reacted with all five test strains of *L. monocytogenes*. Oligonucleotides Msp81, Msp162, and Msp287 reacted with other *Listeria* species, and oligonucleotides Msp81 and Msp297 cross-reacted with other gram-positive bacteria. Based on these results, Msp110 was selected for the development of a nonisotopic colony hybridization assay.

Evaluation of the digoxigenin-labeled Msp110. Digoxigenin-labeled Msp110 was evaluated for specificity and sensitivity by dot blot assays. Figure 2 shows the specificity of digoxigenin-labeled Msp110. Its sensitivity was 100% (reacted with 12 of 12 tested strains of *L. monocytogenes* representing five serotypes). Its specificity was 100% because it did not react with other *Listeria* strains or other gram-positive bacteria (nine genera). An evaluation of two hybridization times for digoxigenin-labeled Msp110 indicated no significant differences between the 2- and 24-h hybridization times (data not shown). Therefore, a shorter hybridization time (2 h) was used for further colony blot assays.

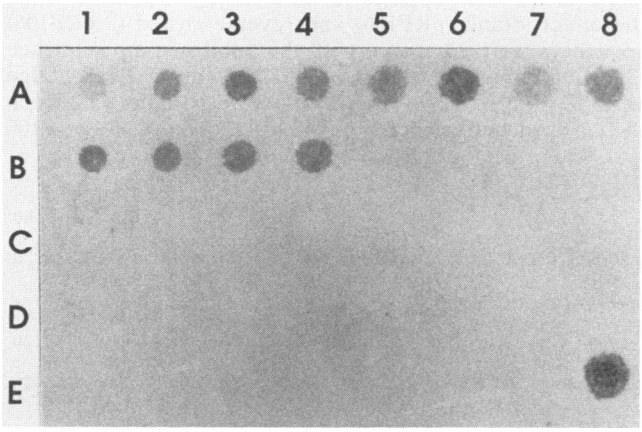

FIG. 2. Dot blots of *Listeria* species and other gram-positive bacteria probed with digoxigenin-labeled Msp110. A-1, *L. monocytogenes* (1/2a) F4246; A-2, *L. monocytogenes* (1/2a) F4263; A-3, *L. monocytogenes* (1/2a) F4259; A-4, *L. monocytogenes* (1/2b) F4245; A-5, *L. monocytogenes* (1/2b) F4260; A-6, *L. monocytogenes* (1/2b) F4398; A-7, *L. monocytogenes* (3c) F1707; A-8, *L. monocytogenes* (4b) F4234; B-1, *L. monocytogenes* (4b) F4244; B-2, *L. monocytogenes* (4b) F4264; B-3, *L. monocytogenes* (4b) F4393; B-4, *L. monocytogenes* (4ab) F1067; B-5, *L. innocua* F4247; B-6, *L. innocua* F4248; B-7, *L. murrayi* F4076; B-8, *L. murrayi* F4077; C-1, *L. ivanovii* F4081; C-2, *L. ivanovii* F4084; C-3, *L. seeligeri* F4856; C-4, *L. seeligeri* F4882; C-5, *L. welshimeri* F4082; C-6, *L. welshimeri* F4083; C-7, *L. grayi* F4085; C-8, *L. grayi* F4086; D-1, *Jonesia denitrificans* F4087; D-2, *Brochothrix thermosphacta* F5950; D-3, *Erysipelothrix rhusiopathiae* F5937; D-4, *Corynebacterium diphtheriae* F5944; D-5, *C. aquaticum* F5942; D-6, *C. striatum* F5946; D-7, *C. minutissimum* F5940; D-8, *C. ulcerans* F5947; E-1, *Rhodococcus equi* F5943; E-2, *Lactobacillus casei* F5952; E-3; *Leuconostoc dextranicum* F5953; E-4, *Bacillus cereus* F5939; E-5, *Staphylococcus aureus* D1233; E-6, *Streptococcus agalactiae* F5949; E-7, *Lactobacillus lactis* F5948; E-8, pRF106.

Application of digoxigenin-labeled Msp110 for the confirmation of *L. monocytogenes* in naturally contaminated food samples. *L. monocytogenes* colonies of 66 LPM plates prepared from 48 food samples were tested in parallel by colony hybridization with digoxigenin-labeled Msp110 and by biochemical tests. Table 4 shows the results of this study. Twenty-six food samples gave positive reactions by colony blot assays. Of these, 25 were positive by the culture method. One sample of cheddar cheese, which was positive by the colony hybridization, was negative by the culture method. The frozen selective enrichment broth of the cheddar cheese was negative for *L. monocytogenes* by reculture. Background problems were not encountered with the digoxigenin-labeled oligonucleotide probes (Fig. 3).

DISCUSSION

The guanine-plus-cytosine content of the pRF106 fragment compares well with that for *L. monocytogenes*, which has been determined to be 36 to 38% (36). The sequence of the pRF106 fragment had an open reading frame, indicating that it could be the internal fragment of the coding sequence for a regulatory or structural gene. Initially, Flamm (9) thought that the pRF102 insert DNA (5.2-kbp) contained a gene that encoded a 60-kDa beta-hemolysin of *L. monocytogenes*. However, after Mengaud et al. (30) cloned and sequenced the *hlyA* gene of the beta-hemolysin of *L. monocytogenes*, Flamm et al. (10) suggested that the *L. monocytogenes* DNA insert (5.2 kb) in pRF102 codes for a 60-kDa major secreted polypeptide and termed the gene *msp*. Recently, Kuhn and Goebel (24) reported that a 60-kDa protein is involved in the invasion of nonprofessional phagocytic cells by *L. monocytogenes*. Kohler et al. (23) sequenced an invasion-associated protein gene (*iap*) which codes for a 60-kDa protein of *L. monocytogenes*, compared it with the sequence of oligonucleotides derived from the pRF106 sequence (6), and reported that the pRF106 fragment may be an internal fragment of the *iap* gene. Our data show that 98.7% sequence homology exists between the nucleotide sequence of the pRF106 fragment and the *iap* gene, indicating that the pRF106 fragment is an internal fragment of the *iap* gene. The four differences observed between the two sequences could be attributed to either errors in reading the sequences or perhaps differences between the strains. The *iap* sequence was determined for *L. monocytogenes* EGD serotype 1/2a, whereas the pRF sequence was determined for *L. monocytogenes* 10403 serotype 1/2a.

We utilized the sequence information of the pRF106 fragment to synthesize oligonucleotide probes that could be used for the rapid and specific detection of *L. monocytogenes*. The use of oligonucleotides as DNA probes has several advantages. Because the probes are shorter (typically 10 to 50 bases), hybridization to target DNA occurs at a faster rate than with cloned probes, which are usually 200 bp or more. Very small (nanogram) quantities of the oligonu-

TABLE 4. Evaluation of the nonisotopic colony hybridization method with colonies isolated from foods

Sample identification	Type sample	Culture result	Probe result	Serotype
703	Beef wieners	+	+	1/2a
741	Chicken pot pie	+	+	1/2b
865	Chicken drumsticks	+	+	Not 4b[a]
891	Turkey bologna	+	+	Not 1/2b
896	Honey baked ham	+	+	3b
1117	Ground chuck	+	+	1/2b
1120	Frozen okra	+	+	Not 1/2
1204	Cheddar cheese	−	+	
1271	Celery	+	+	4b
1279	Black bean	+	+	4b
1442	Wieners	+	+	Not 4b
1447	Chorizo	+	+	Not 1/2a
1474	Plum/peach	+	+	1/2b
1489	Celery	−	−	
1497	Beef franks	+	+	1/2a
1499	Wieners	+	+	3b
1501	Boudin link no. 1	+	+	4b
1502	Boudin link no. 2	+	+	4b
1503	Boudin link no. 3	+	+	4b
1504	Boudin link no. 4	+	+	4b
1513	Spare ribs	+	+	1/2a
1516	Ice cream	+	+	1/2b
1520	Frozen vegetables	+	+	Not 4b
1530	Scallions	+	+	1/2b
1538	Pork/chorizo	+	+	4
1539	American cheese	−	−	
1540	Frozen turkey	−	−	
1541	Half-and-half	−	−	
1542	Szechwan beef	−	−	
1543	Tuna noodle casserole	−	−	
1544	Sirloin tips and vegetables	−	−	
1545	Seafood newberg	−	−	
1546	Vitamin D milk	−	−	
1547	Vitamin D milk	−	−	
1548	Ice cream	−	−	
1549	Frozen corn	−	−	
1550	Frozen corn	−	−	
1551	Corn on the cob	−	−	
1552	Corn on the cob	−	−	
1553	Cheddar cheese	−	−	
1554	Peas/carrots	−	−	
1555	Cooked squash	−	−	
1556	Lima beans	+	+	1/2a
1557	Lettuce	−	−	
1558	Lettuce	−	−	
1559	Refrigerator swab	−	−	
1560	Refrigerator swab	−	−	
CS1	Chicken salad	+	+	1/2b

[a] Did not type further.

cleotide probe are required for a hybridization experiment; therefore, one batch (1 to 2 mg) of an oligonucleotide probe may last for several hundred hybridization experiments (37). Also, because synthetic oligonucleotide probes are single stranded, there is no need to denature the probe before use, and competition from the complementary strand of the probe during hybridization to target is avoided. By using Msp110 as the probe, it was possible to reduce the hybridization time from 18 to 2 h.

Another advantage of oligonucleotide probes is their extreme specificity. By controlling the stringency of hybridization, it is possible to detect single-base-pair changes in the DNA or RNA target sequences (37). The pRF106 fragment was shown previously to react with DNA from one strain of *L. seeligeri* even when stringent hybridization conditions

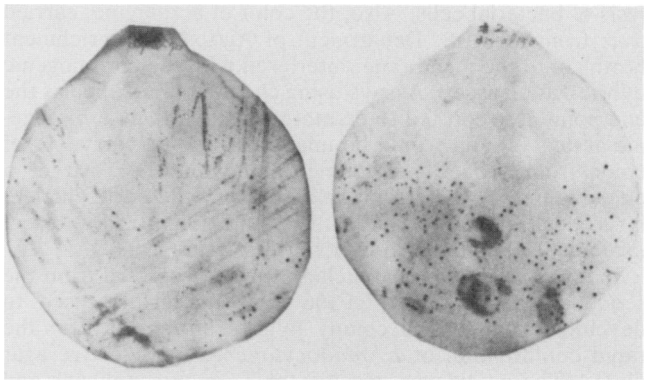

FIG. 3. Representative filters showing colonies isolated from food samples probed by the nonisotopic hybridization procedure, using digoxigenin-labeled Msp110.

(T_m − 5°C) were used (20). Although Msp110 was derived from the pRF106 fragment sequence, Msp110 did not hybridize to the same strain of *L. seeligeri* under stringent hybridization conditions.

Datta et al. (6) used an oligonucleotide derived from the pRF106 fragment sequence to develop a colony hybridization assay for *L. monocytogenes*. Interestingly, their probe (20-mer) and Msp110 (21-mer) differed in only one nucleotide. However, their probe hybridized to *L. seeligeri*, while Msp110 did not. The hybridization conditions used by Datta et al. (6) were less stringent than the conditions used in this study. This may have also contributed to the discrepancies between our results.

Digoxigenin labeling of Msp110 with terminal transferase is a simple procedure and can be completed in 5 min. The labeled probe can be stored at −20°C for several months. No background problems were encountered. After the addition of the substrate reagent for the detection of hybridization, positive reactions were visible within 1 to 2 h and were complete in 18 h. Blots that had been probed with digoxigenin-labeled oligonucleotide retained their color after several months of storage at room temperature. Further, digoxigenin labeling did not alter the specificity of the probe because the specificities of ^{32}P-labeled and digoxigenin-labeled Msp110 were identical.

The results of the colony hybridization assay using digoxigenin-labeled Msp110 to confirm the presence of *L. monocytogenes* in naturally contaminated foods showed that the colony hybridization procedure has excellent sensitivity and specificity. There was only one disagreement between the colony hybridization assay and conventional culture. One cheese sample was positive by the colony hybridization but negative by culture. Attempts to reculture the enrichment broth (stored frozen at −70°C) of the cheese to determine whether the discrepancy was due to a failure of the cultural procedure were unsuccessful. No growth was observed on LPM plates upon reculture of the enrichment broth. It is possible that *L. monocytogenes*, even if originally present in the enrichment broth in small numbers, may have been killed during freezing and thawing.

Attempts were made to apply the digoxigenin-oligonucleotide hybridization assay directly to U.S. Department of Agriculture primary and secondary broth cultures. However, these attempts were unsuccessful. Possible reasons for the failure include the presence of inadequate numbers of bacteria in the selective enrichment broths and incomplete

lysis of bacterial cells. Also, the color of acriflavine, carried over from the U.S. Department of Agriculture enrichment broths onto the membrane, interfered with the chromogenic hybridization assay. Assays using chemiluminescence as the end point are reported to be more sensitive than chromogenic assays (2) and would be unaffected by the carryover of color from food or enrichment medium. The use of a chemiluminescent DNA probe assay combined with efficient lysis of cells may offer the required sensitivity to allow direct examination of selective enrichment cultures.

In conclusion, an oligonucleotide probe derived from the sequence of fragment pRF106 was successfully used to develop a nonisotopic colony hybridization assay for the rapid confirmation of *L. monocytogenes* on selective agar plates. The assay was specific for *L. monocytogenes* and could be completed in 24 h.

REFERENCES

1. **Anonymous.** 1988. Listeriose en Suisse. Bull. Bundesamt. Gesundheitswes. **3**:28–29.
2. **Bronstein, I., and P. McGrath.** 1989. Chemiluminescence lights up. Nature (London) **338**:599–600.
3. **Buluwela, L., A. Forster, T. Boehm, and T. H. Rabbitts.** 1989. A rapid procedure for colony screening using nylon filters. Nucleic Acids Res. **17**:452.
4. **Butman, B. T., M. C. Plank, R. J. Durham, and J. A. Mattingly.** 1988. Monoclonal antibodies which identify a genus-specific *Listeria* antigen. Appl. Environ. Microbiol. **54**:1564–1569.
5. **Datta, A. R., B. A. Wentz, and W. E. Hill.** 1987. Detection of hemolytic *Listeria monocytogenes* by using DNA colony hybridization. Appl. Environ. Microbiol. **53**:2256–2259.
6. **Datta, A. R., B. A. Wentz, D. Shook, and M. W. Trucksess.** 1988. Synthetic oligodeoxyribonucleotide probes for detection of *Listeria monocytogenes*. Appl. Environ. Microbiol. **54**:2933–2937.
7. **Dente, L., G. Cesareni, and R. Cortese.** 1983. pEMBL: a new family of single stranded plasmids. Nucleic Acids Res. **11**:1645–1655.
8. **Farber, J. M., and J. I. Speirs.** 1987. Monoclonal antibodies directed against the flagella antigens of *Listeria* species and their potential in EIA-based methods. J. Food Prot. **50**:479–484.
9. **Flamm, R. K.** 1986. Ph.D. dissertation, Washington State University, Pullman.
10. **Flamm, R. K., D. J. Hinrichs, and M. F. Thomashow.** 1989. Cloning of a gene encoding a major secreted polypeptide of *Listeria monocytogenes* and its potential use as a species-specific probe. Appl. Environ. Microbiol. **55**:2251–2256.
11. **Fleming, D. W., S. L. Cochi, K. L. MacDonald, J. Brondum, P. S. Hayes, B. D. Plikaytis, M. B. Holmes, A. Audurier, C. V. Broome, and A. L. Reingold.** 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. N. Engl. J. Med. **312**:404–407.
12. **Gardner, R. C., A. J. Howarth, P. Hahn, M. Brown-Luedi, J. Robert, R. J. Shepherd, and J. Messing.** 1981. The complete nucleotide sequence of an infectious clone of cauliflower mosaic virus by M13mp7 shotgun sequencing. Nucleic Acids Res. **9**:2871–2888.
13. **Gray, M. L., H. J. Stafseth, F. Thorp, Jr., L. B. Sholl, and W. F. Riley.** 1948. A new technique for isolating *Listerellae* from the bovine brain. J. Bacteriol. **55**:471–476.
14. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
15. **Hayes, P. S., J. C. Feeley, L. M. Graves, G. W. Ajello, and D. W. Fleming.** 1986. Isolation of *Listeria monocytogenes* from raw milk. Appl. Environ. Microbiol. **51**:438–440.
16. **Henry, B. S.** 1933. Dissociation in the genus *Brucella*. J. Infect. Dis. **52**:374–402.
17. **Ish-Horowicz, D., and J. F. Burke.** 1981. Rapid and efficient cosmid cloning. Nucleic Acids Res. **9**:2989–2998.
18. **Kafatos, F. C., C. W. Jones, and A. Efstratiadis.** 1979. Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. Nucleic Acids Res. **7**:1541–1552.
19. **Kampelmacher, E. H., and L. M. van Noorle Jansen.** 1969. Isolation of *Listeria monocytogenes* from faeces of clinically healthy humans and animals. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **211**:353–359.
20. **Kim, C., L. M. Graves, B. Swaminathan, L. W. Mayer, and R. E. Weaver.** 1991. Evaluation of hybridization characteristics of a cloned pRF106 probe for *Listeria monocytogenes* detection and development of a nonisotopic colony hybridization assay. Appl. Environ. Microbiol. **57**:289–294.
21. **King, W., S. Raposa, J. Warshaw, A. Johnson, D. Halbert, and J. D. Klinger.** 1989. A new colorimetric nucleic acid hybridization assay for *Listeria* in foods. Int. J. Food Microbiol. **8**:225–232.
22. **Klinger, J. D., A. Johnson, D. Croan, P. Flynn, D. Whippie, A. Kimball, J. Lawrie, and M. Curiale.** 1988. Comparative studies of nucleic acid hybridization assay for *Listeria* in foods. J. Assoc. Off. Anal. Chem. **71**:669–673.
23. **Kohler, S., M. Leimeister-Wachter, T. Chakraborty, F. Lottspeich, and W. Goebel.** 1990. The gene coding for protein p60 of *Listeria monocytogenes* and its use as a specific probe for *Listeria monocytogenes*. Infect. Immun. **58**:1943–1950.
24. **Kuhn, M., and W. Goebel.** 1989. Identification of an extracellular protein of *Listeria monocytogenes* possibly involved in intracellular uptake by mammalian cells. Infect. Immun. **57**:55–61.
25. **Leary, J. J., and J. L. Ruth.** 1989. Nonradioactive labeling of nucleic acid probes, p. 33–57. *In* B. Swaminathan and G. Prakash (ed.), Nucleic acid and monoclonal antibody probes. Marcel Dekker, Inc., New York.
26. **Linnan, M. J., L. Mascola, X. D. Lou, V. Goulet, S. May, C. Salminen, D. W. Hird, L. Yonekura, P. Hayes, R. Weaver, A. Audurier, B. D. Plikaytis, S. L. Fannin, A. Kleks, and C. V. Broome.** 1988. Epidemic listeriosis associated with Mexican-style cheese. N. Engl. J. Med. **319**:823–828.
27. **Lovett, J., D. W. Francis, and J. M. Hunt.** 1987. *Listeria monocytogenes* in raw milk: detection, incidence, and pathogenicity. J. Food Prot. **50**:188–192.
28. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **McClain, D., and W. H. Lee.** 1988. Development of USDA-FSIS method for the isolation of *Listeria monocytogenes* from raw meat. J. Assoc. Off. Anal. Chem. **71**:876–879; 892.
30. **Mengaud, J., M. F. Vincente, J. Chenevert, J. M. Pereira, C. Geoffroy, B. Gicquel-Sanzey, F. Baquero, J. C. Perez-Diaz, and P. Cossart.** 1988. Expression in *Escherichia coli* and sequence analysis of the listeriolysin O determinant of *Listeria monocytogenes*. Infect. Immun. **56**:766–772.
31. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–78.
32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., sect. 11.46. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Sanger, F., S. Nicklen, and A. E. Coulson.** 1977. DNA sequencing with chain-termination inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
34. **Schlech, W. F., P. M. Lavigne, R. A. Bortolussi, A. C. Allen, A. J. Wort, A. W. Hightower, S. E. Johnson, S. H. King, E. S. Nicholls, and C. V. Broome.** 1983. Epidemic listeriosis—evidence for transmission by food. N. Engl. J. Med. **308**:203–206.
35. **Schreier, P. E., and R. Cortese.** 1979. A fast and simple method for sequencing DNA cloned in the single-stranded bacteriophage M13. J. Mol. Biol. **129**:169–172.
36. **Seeliger, H. P. R., and D. Jones.** 1986. Genus *Listeria*, 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. The Williams & Wilkins Co., Baltimore.
37. **Tenover, F. C.** 1988. Diagnostic deoxyribonucleic acid probes for infectious disease. Clin. Microbiol. Rev. **1**:82–101.
38. **Van Netten, P., I. Perales, A. Van de Moosdik, G. D. W. Curtis, and D. A. A. Mossel.** 1989. Liquid and solid selective differential media for the detection and enumeration of *L. monocytogenes* and other *Listeria* spp. Int. J. Food Microbiol. **8**:299–316.