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Use of Polymerase Chain Reaction for Detection of *Listeria monocytogenes* in Food

C. NIEDERHAUSER,[1]* U. CANDRIAN,[1] C. HÖFELEIN,[1] M. JERMINI,[2] H.-P. BÜHLER,[3] AND J. LÜTHY[1]

Laboratory of Food Chemistry, Institute of Biochemistry, University of Bern, Freiestrasse 3,[1] and Kantonales Laboratorium,[3] 3012 Bern, and Laboratorio Cantonale d'Igiene, 6900 Lugano,[2] Switzerland

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A previously described polymerase chain reaction (PCR) assay (B. Furrer, U. Candrian, C. Höfelein, and J. Lüthy, J. Appl. Bacteriol. 70:372–379, 1991) was used to analyze food for the presence of *Listeria monocytogenes*. Food samples were artificially contaminated to develop two procedures to detect the organism following enrichment steps. Procedure A was based on dilution of the enrichment broth followed by lysis of the bacteria and direct analysis of the lysate with PCR. With procedure A and artificially contaminated food samples, it was possible to detect fewer than 10 bacteria per 10 g of food. In procedure B, centrifugation was used to concentrate bacteria before lysis and PCR. With procedure A, 330 naturally contaminated food samples of several types were analyzed. Twenty samples were found to be positive for *L. monocytogenes*, which was in agreement with the classical culture technique. By using procedure B on a subset of 100 food samples, 14 were found to be positive by PCR whereas the classical culture method detected only 13. Analysis times, including enrichment steps, were 56 and 32 h with procedures A and B, respectively.

Although *Listeria monocytogenes* has been recognized as a cause of disease in humans and animals for over 50 years, recognition of listeriosis as an important public health problem dates from the documentation of common-source foodborne outbreaks in the past decade (3, 5). Various reports showed that *Listeria* spp. can occur in dairy products (12), meat and poultry (6), and vegetables (2). Special risk groups are pregnant women, newborns, and immunocompromised patients. Evidence that the gastrointestinal tract is an important route of infection and that the epithelial cells of the intestine may be the primary site of entry for these bacteria has been provided by electron microscopic studies of tissues of infected guinea pigs (15) and the occurrence of foodborne infections. Current microbiological culture methods require a minimum of 5 days to declare a food product *Listeria* free and about 10 days to recognize *Listeria* spp. and identify *L. monocytogenes*. In view of the limited shelf lives of certain food products and the high cost of product storage, we decided to pursue the development of a rapid method for detection of *L. monocytogenes*. While cultivation of *Listeria* spp. is the standard method of detection, new methods based on polymerase chain reaction (PCR) technology for rapid detection of that organism have been developed (1, 4, 8, 9, 17). In these studies, DNA primers for enzymatic amplification were directed towards virulence genes for which species-specific probes have been described (7, 11, 13, 14).

In the analysis of food samples, we used a PCR test which detects the *hlyA* and *iap* genes of *L. monocytogenes* (9). *HlyA* encodes the well-recognized virulence factor listeriolysin O (13). The *iap* gene is a presumptive virulence gene which is thought to code for an invasion-associated protein (11).

MATERIALS AND METHODS

***Listeria* spp. strains and media.** The reference strains *L. ivanovii* serotype 5 (ATCC 19119) and *L. monocytogenes* serotypes 3a (NCTC 5105), 3b (SLCC 2540), 3c (SLCC 2479), 4a (ATCC 19114), 4c (ATCC 19116), 4d (NCTC 10888), 4e (ATCC 19118), 4ab (NCTC 10528), and 7 (SLCC 2482) used in this study were obtained from S. Notermans (14). All other *Listeria* spp. were human or environmental isolates, some of which were supplied by M. Tabouret (Institut National de la Recherche Agronomique, Nouzilly, France). All artificially contaminated food samples were inoculated with *L. monocytogenes* 1/2a. Strains were grown on tryptone soy agar or PALCAM agar (Oxoid, Basingstoke, England). Liquid media were prepared in accordance with the *Swiss Food Manual* (16). Listeria enrichment broth (LEB FDA) contained (per liter) 30 g of tryptone soy broth, 6 g of yeast extract, 15 mg of acriflavin, 50 mg of cycloheximide, and 40 mg of nalidixic acid. LEBI FSIS contained (per liter) 5 g of Proteose Peptone, 5 g of tryptone, 5 g of meat extract (Lab-Lemco Powder; Oxoid), 5 g of yeast extract, 20 g of NaCl, 1.35 g of KH_2PO_4, 12 g of $Na_2HPO_4 \cdot 2H_2O$, 1 g of esculin, 12 mg of acriflavin, and 20 mg of nalidixic acid. LEBII FSIS had the same composition as LEBI FSIS but with 24.8 mg of acriflavin.

Naturally contaminated food samples. Food samples were collected at local food outlets by the two official laboratories (Laboratorio Cantonale d'Igiene, Lugano, and Kantonales Laboratorium, Bern). Samples found to be positive by the classical culture method (see below) were considered to be naturally contaminated. Enumeration was by direct plating of dilutions of the food homogenate (see below) on PALCAM agar (Oxoid).

Artificially contaminated food samples. Samples which were negative by classical culture were used for artificial contamination. An environmental *L. monocytogenes* 1/2a strain was grown overnight in tryptone soy broth (Oxoid), and dilutions ranging from 1 CFU/10 g to 1.8×10^6 CFU/10 g of food sample were added to the food homogenate (see below).

Procedure A for analysis of food samples. Primary enrichment (PE) and secondary enrichment (SE) steps were performed in accordance with the *Swiss Food Manual* (16). Briefly, for PE 10 g (wet weight) of meat or other food samples was homogenized in a Colworth Stomacher 400

* Corresponding author.

(Seward, London, England) with 90 ml of LEBI FSIS or 390 ml of LEB FDA, respectively, for 60 s and incubated for 20 to 24 h at 30°C. For SE, PE was diluted 100-fold in LEBII FSIS and LEB FDA for meat and other food samples, respectively, and then incubated for 20 to 24 h at 30°C. PE and SE were diluted 100-fold in PCR buffer (50 mM KCl, 10 mM Tris hydrochloride [pH 8.4], 2.0 mM $MgCl_2$, gelatin at 100 μg/ml) containing 2 mg of lysozyme (Sigma, St. Louis, Mo.) per ml and incubated for 15 min at room temperature. The samples were then digested with proteinase K (Merck, Darmstadt, Germany) at 200 μg/ml for 60 min at 60°C and finally boiled for 15 min. A 25-μl volume of this lysate, representing a weight fraction of 2.5×10^{-8} (meat) or 6.25×10^{-9} (other food types) of the original food sample, was subjected to PCR analysis without further purification.

Procedure B for analysis of food samples. PE and SE were as in procedure A. To increase sensitivity compared with procedure A, PE and SE were concentrated fivefold by centrifugation before lysis. The centrifugal forces used were $100 \times g$ to eliminate food particles and $3,000 \times g$ to collect bacteria. Afterwards, the samples were lysed as described in procedure A. The lysate was subjected to PCR undiluted and diluted 10- or 100-fold.

Classical culture method. PE and SE were as in procedure A. Three loopsful (~30 μl) of SE was then plated onto PALCAM agar plates (Merck) and incubated under microaerophilic conditions (5% O_2) at 37°C for 2 to 3 days. Colonies thought to be *Listeria* spp. were tested for hemolysis on Columbia agar plates containing 5% sheep blood (bioMérieux, Geneva, Switzerland). Further microbiological and biochemical tests (cell morphology, Gram staining, catalase, motility, CAMP test, and assimilation of rhamnose, mannitol, and xylose) were carried out in accordance with the *Swiss Food Manual* (16). For serotyping, strains were sent to the Centre Nationale de Listeria, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland.

Lysis of bacteria. Bacteria were lysed as described previously (9).

PCR. Synthesis and purification of oligonucleotides and PCR were carried out as described by Furrer et al. (9). In this protocol, two pairs of amplification primers were used in the same reaction tube, yielding a 234-bp fragment from the *hlyA* gene (oligonucleotides 5'-CGGAGGTTCCGCA AAAGATG-3' and 5'-CCTCCAGAGTGATCGATGTT-3') and a 131-bp fragment from the *iap* gene (oligonucleotides 5'-ACAAGCTGCACCTGTTGCAG-3' and 5'-TGACAGC GTGTGTAGTAGCA-3').

DNA analysis. A 20-μl portion of the 100-μl total PCR product was analyzed by gel electrophoresis in a 1.5% agarose gel. DNA was made visible by ethidium bromide staining and UV transillumination.

RESULTS

Specificity and sensitivity of PCR. The multiplex PCR assay (*hlyA-iap* PCR) used in this study was described by Furrer et al. (9). However, additional *Listeria* spp. and serotypes were tested to validate this PCR system further (Table 1). The test allows specific identification of *L. monocytogenes* except for the rare serotypes 4a and 4c, which are not recognized by the *iap* primers. The detection limit was 250 bacteria per amplification reaction (data not shown). Since 25 μl of a 100-fold dilution of PE or SE was used in procedure A, the detection limit with this protocol was 10^6 bacteria per ml of enrichment. In procedure B, PE or SE was concentrated fivefold by centrifugation, resulting in a detection limit of 2,000 bacteria per ml of enrichment.

TABLE 1. *Listeria* spp. tested for the presence of *hlyA* and *iap* sequences by PCR

Species	Serotype	No. of isolates tested[a]	Presence of gene fragment	
			hlyA	*iap*
L. monocytogenes	1/2a	15	+	+
	1/2b	4	+	+
	1/2c	19	+	+
	3a	7	+	+
	3b	4	+	+
	3c	2	+	+
	4a	10	+	−
	4b	6	+	+
	4c	1	+	−
	4d	1	+	+
	4e	1	+	+
	4ab	2	+	+
	7	8	+	+
	ND[b]	18	+	+
L. ivanovii	5	4	−	−
L. innocua	6a	4	−	−
	6b	8	−	−
	NT	3	−	−
L. seeligeri	1/2a	1	−	−
	1/2b	3	−	−
L. welshimeri	6a	1	−	−
	NT	2	−	−

[a] Reference strains of the following serotypes were included: *L. ivanovii* 5 (ATCC 19119) and *L. monocytogenes* 3a (NCTC 5105), 3b (SLCC 2540), 3c (SLCC 2479), 4a (ATCC 19114), 4c (ATCC 19116), 4d (NCTC 10888), 4e (ATCC 19118), 4ab (NCTC 10528), and 7 (SLCC 2482).
[b] ND, not determined.

Artificially contaminated food samples. The feasibility of procedure A was evaluated with various types of food samples artificially contaminated with different levels of *L. monocytogenes* 1/2a (Table 2). The contamination ranged from 1 CFU/10 g to 1.8×10^6 CFU/10 g of food sample. Using either PE or SE, the detection limit for initial contamination of all samples was less than 10 CFU/10 g of food, except for raw meat, for which the lowest contamination level in PE could not be detected (Table 2).

As an example, the PCR analysis results of a precooked meat product sample contaminated with serial 10-fold dilutions of *L. monocytogenes* 1/2a are shown in Fig. 1. In this case, the sample was inoculated with the following numbers of *L. monocytogenes* 1/2a: 0, 0.2, 2.4, 24, 239, and 2,387 CFU/10 g. As shown in lanes 1 to 6 and 7 to 12, PCR analysis yielded identical results for PE and SE, respectively. In both enrichment broths, the lowest contamination levels were negative (lanes 1 and 2 and 7 and 8, respectively). These results were in perfect accordance with those of the classical culture method.

Naturally contaminated food samples analyzed by procedure A. A total of 330 natural food samples were tested for the presence of *L. monocytogenes* by the classical culture method and by PCR analysis of PE and SE. Twenty samples were found to be positive with both methods (Table 3), indicating good agreement. *L. monocytogenes* was found in

TABLE 2. Food samples artificially contaminated with serial 10-fold dilutions of *L. monocytogenes* 1/2a

Type of food sample	PCR with:		Culture method	Range of initial contamination tested (CFU/10 g)
	PE	SE		
Milk and dairy products				
Pasteurized milk	+	+	+	18,000 – 1.8×10^9
Vanilla ice cream	+	+	+	3 – 3.0×10^5
Chocolate ice cream	+	+	+	3 – 3.0×10^5
Soft cheese I	+	+	+	9 – 9.0×10^5
Soft cheese II	+	+	+	2 – 2.0×10^5
Semisoft cheese	+	+	+	3 – 3.0×10^5
Raw meats				
Minced beef	(+)[a]	+	+	4 – 4.0×10^5
Minced pork	(+)[a]	+	+	4 – 4.0×10^5
Fermented sausage (Landjäger)	+	+	+	9 – 9.0×10^5
Cooked meats				
Bratwurst	+	+	+	2 – 2.0×10^5
Fleischkäse	+	+	+	18,000 – 1.8×10^9
Cervalat	+	+	+	3 – 3.0×10^5
Pâté	+	+	+	64 – 6.4×10^6
Smoked salmon	+	+	+	1 – 1.0×10^5
Salad	+	+	+	64 – 6.4×10^6

[a] The lowest contamination level was negative.

raw meat, ready-to-eat (cooked) meat products, and seafood. PCR results of some samples are shown in Fig. 2. In lanes 1 to 8, results obtained with procedure A from PE and SE are shown. Among the 20 positive samples, 3 were already positive in PE (Fig. 2, lanes 1 and 3). The *Listeria* strains isolated were of serotypes 1/2a, 1/2b, 1/2c, and 4b.

TABLE 3. Naturally contaminated samples analyzed with procedure A

Type of food sample	No. of samples positive/no. tested by:		
	PCR[a] with PE	PCR with SE	Culture method
Dairy products	0/38	0/38	0/38
Raw meat[b]	0/10	2/10	2/10
Cooked meat	3/243	17/243	17/243
Poultry	0/15	0/15	0/15
Vegetables	0/16	0/16	0/16
Seafood	0/8	1/8	1/8
Totals	3/330	20/330	20/330

[a] *hlyA-iap* PCR.
[b] Included fermented sausages such as salami.

Eight samples of cooked ready-to-eat meat products containing *L. monocytogenes* 1/2c were collected from the same production plant. Numbers of *L. monocytogenes* organisms determined by direct plating were 100 or fewer CFU/g. Additional *L. monocytogenes* 1/2c organisms were found in one sample of raw meat and three samples of cooked meat products. One sample of salami contained, as well as *L. monocytogenes* 1/2c, nontypeable (NT) *L. innocua*. Two cooked meat product samples containing serotype 4b were from the same butcher shop. One of the samples was also contaminated with *L. seeligeri* 1/2b. Another, unrelated sample of a cooked meat product and a sample of salmon were contaminated with *L. monocytogenes* 4b. Furthermore, one *L. monocytogenes* 1/2b and two *L. monocytogenes* 1/2a isolates came from three samples of cooked meat products. One serotype 1/2a isolate was coisolated with NT *L. welshimeri*. In addition, we found 13 samples contaminated with *Listeria* spp. other than *L. monocytogenes*. *L. innocua* 6a was detected in a sample of seafood salad and in two samples of cooked meat products. *L. innocua* 6b was

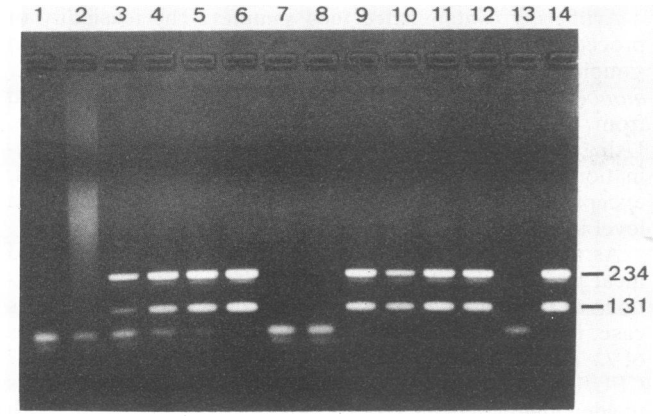

FIG. 1. Agarose gel electrophoresis of amplification products obtained from a cooked meat sample (Bratwurst) contaminated with serial 10-fold dilutions of *L. monocytogenes* 1/2a. PCR with α-hemolysin (*hlyA*) and *iap* gene primers. Lanes: 1 to 6, PCR products obtained from PE, with the following contamination levels of *L. monocytogenes* per 10 g of food: 0, 0.2, 2.4, 24, 239, and 2,387 CFU; 7 to 12, PCR products from SE with the same increasing contamination levels as above; 13, negative control (no DNA); 14, positive control (*L. monocytogenes* 4b). The numbers to the right are base pairs. Low-molecular-weight fragments smaller than the fragments of interest are primer-dimer artifacts caused by excess primers.

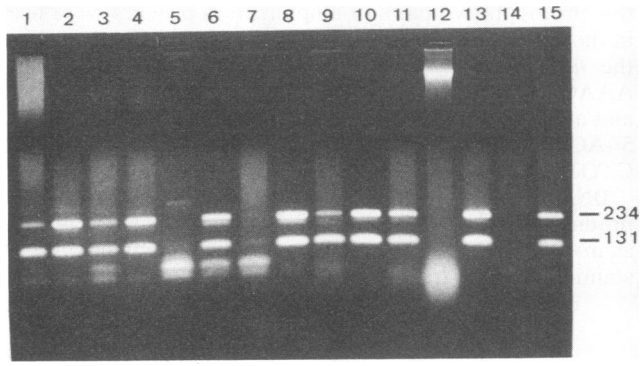

FIG. 2. Agarose gel electrophoresis of *L. monocytogenes* amplification products (*hlyA-iap* PCR) from four naturally contaminated food samples found to be positive by in vitro culture. Samples in lanes 1 to 8 and 9 to 13 were analyzed with procedures A and B, respectively. Lanes: 1 and 2, cooked meat sample no. 44275 PE and SE; 3 and 4, cooked meat sample no. 44276 PE and SE; 5 and 6, salmon sample no. 381 PE and SE; 7 and 8, cooked meat sample no. 54056 PE and SE; 9 and 10, salmon sample no. 381 PE and SE; 11 to 13, cooked meat sample no. 54056 PE, SE, and SE lysate diluted 100-fold; 14, negative control (no DNA); 15, positive control (*L. monocytogenes* 4b). The numbers to the right are base pairs. The bands below the fragments of interest are primer-dimer artifacts caused by excess primers.

TABLE 4. Naturally contaminated samples analyzed with procedure B

Type of food sample	No. of samples positive/no. tested by:		
	PCR[a] with PE	PCR with SE	Culture method and procedure A with SE
Raw meat	0/3	0/3	0/3
Cooked meat	14/97	13/97	13/97
Totals	14/100	13/100	13/100

[a] *hlyA-iap* PCR.

present in two samples of cooked meat products and three samples of raw meat. NT *L. innocua* was found in two samples of soft cheese. *L. welshimeri* 6a was isolated from one cooked meat product sample, and two NT *L. welshimeri* strains were found in other samples of cooked meat products.

Naturally contaminated food samples analyzed by procedure B. Procedure B was used to analyze a total of 100 natural food samples (a subset of the 330 samples tested with procedure A) for the presence of *L. monocytogenes* and compared with procedure A and the classical culture method (Table 4). All food samples positive for *L. monocytogenes* with procedure A were detected with procedure B as well. In addition, procedure B already allowed reliable recognition of positive samples in PE (Fig. 2, lanes 9 and 11 compared with lanes 5 and 7). However, high levels of bacteria in some SE led to false-negative results (e.g., Fig. 2, lane 12). This problem was solved by diluting the lysate by a factor of 10 or 100 before PCR (Fig. 2, lane 13). In most cases, a 100-fold dilution yielded a stronger signal (data not shown). Moreover, by using procedure B we were able to detect *L. monocytogenes* in an additional food sample not recognized as positive with procedure A or in vitro culture (Table 4). This cooked meat product sample was from the same production plant as the cluster of samples contaminated with *L. monocytogenes* 1/2c (see above).

DISCUSSION

Different listeriosis outbreaks in the United States, Canada, and Europe have been observed in the past 10 years (3, 5). Detection of the human pathogen *L. monocytogenes* in food products by the existing culture method is a time-consuming procedure. In this study, we showed that a PCR-based method detects *L. monocytogenes* directly in PE and SE, leading to a substantial reduction in analysis time.

Two important steps in achieving this time reduction were the use of in vitro DNA amplification and the amplification of two independent virulence gene fragments in a single multiplex PCR analysis. This renders the application of fragment identification by restriction endonuclease analysis or oligonucleotide hybridization unnecessary since simple size determination of the amplification fragment doublet proved to be sufficient (this study; 9). However, to obtain sufficient PCR signal strength it is necessary to use fresh cultures. Experiments indicate that prolonged storage (more than a week) of PE or SE at 4°C or freezing may lead to false-negative results (data not shown). This is probably due to cell lysis and subsequent DNA degradation.

Comparison of methods shows that procedure A yields results which are in perfect agreement with in vitro culture. With procedure A, PCR analysis of SE requiring about 56 h, including enrichment steps, was in most cases (17 of 20; Table 3) necessary to recognize naturally contaminated food samples. Assuming several days of postenrichment analysis time in the case of in vitro culture (selective agar, determination of hemolysis, microbiological and biochemical tests, and serotyping), compared with several hours for DNA amplification, this PCR assay will gain valuable time for regulatory actions. An additional advantage of the two-step enrichment, which involves significant dilution of the original food sample, is elimination of potential inhibition of PCR by food components. Therefore, with procedure A it is possible to analyze virtually any type of food. Analysis of artificially contaminated samples originally indicated that procedure A allows reliable analysis of PE, resulting in an additional time saving of 20 to 24 h compared with PCR analysis of SE. However, this could not be confirmed with naturally contaminated food samples. We conclude that in contrast to fully viable *L. monocytogenes* used for contamination, bacteria present in natural food samples have reduced viability. It has been demonstrated that *L. monocytogenes* can be sublethally stressed by environmental challenges (10). In this state, the microorganisms require a period of recovery before they regain full growth potential. Nevertheless, to create a 32-h protocol we developed procedure B. With this procedure, reliable results are obtained with PE. It was even possible to detect *L. monocytogenes* in an additional sample of cooked meat. This sample was negative for *L. monocytogenes* when analyzed with procedure A and in in vitro culture. However, SE obtained from this sample was negative with procedure B as well. We conclude that this food sample was contaminated with *L. monocytogenes* not culturable under the conditions used. However, that does not exclude a potential human hazard. With procedure B, some assays from positive food samples yielded a high-molecular-weight band instead of the expected 234- and 131-bp fragments (Fig. 2, lane 12). This was probably due to high concentrations of bacterial DNA which led to inhibition of enzymatic amplification. This problem could be solved by diluting the lysate before PCR amplification.

Successful application of PCR technology to an assay which definitively identifies *L. monocytogenes* within 2 days after sample receipt offers a unique technology alternative to conventional culture methods. However, to facilitate introduction into routine laboratories significant automation of pre- and post-PCR handling is required. A step in this direction would be to replace agarose gel electrophoresis by a simple method open to automation, such as hybridization of the PCR product to a DNA probe immobilized on a solid phase (e.g., a microtiter plate).

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