Detection of Multiple Virulence-Associated Genes of *Listeria monocytogenes* by PCR in Artificially Contaminated Milk Samples

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The inhibitory effect of milk in the PCR detection of *Listeria monocytogenes* could be overcome by washing the contaminated milk sample with phosphate-buffered saline and concentrating the bacteria to 1/10 of the original volume. In order to avoid a possible failure in the detection of virulent *L. monocytogenes*, a one-step procedure which enabled demonstration of three virulence-associated genes, *prfA*, *hlyA*, and *plcB*, simultaneously in a single PCR mixture was developed.

It has been widely accepted that listeriosis is a food-borne infection (4, 9, 15). Among various foodstuffs reported to harbor *Listeria* spp., milk is a major vehicle of infection (4, 12, 13). Surveys made in various countries have shown that 0 to 7.2% of milk samples are contaminated with *Listeria monocytogenes* (12). In comparison with the standard procedure of isolation and biochemical identification of *L. monocytogenes* in the food, which requires at least 5 days, the recent application of PCR has successfully shortened the detection time (1, 6, 7, 13, 18, 20, 21). One of the problems encountered in the direct detection by PCR may be the presence of a PCR-inhibitory factor in the food (6). Therefore, a preenrichment method (13, 18) or separation of bacteria using a monoclonal antibody (6) has been reported. In the present study, first we tried to find a simple procedure enabling the detection of *L. monocytogenes* in milk without enrichment culture of the samples.

A virulent strain of *L. monocytogenes* EGD was grown on tryptic soy agar and suspended in distilled water to give 50 units of optical density at 660 nm. To find an optimal condition for preparing DNA for PCR, various samples were prepared by changing the heating (5 min) temperature, concentrations of Tween 20 and $MgCl_2$, or culture period. All the samples were subjected to PCR using the primer pair of 3'-AATCGTA CAGGACGATGAACCC-5' and 5'-GGTATCACAAAGCT CACGAG-3', specific for *prfA* (GenBank accession number, X61210). PCR products were obtained after 25 thermal cycles of amplification consisting of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; separated by agarose (1.2%; low-melting-temperature agarose L) gel electrophoresis; stained with ethidium bromide; and visualized by a UV transilluminator. The predicted size of the amplified product from *prfA* was 571 bp. The presence of 0.05% Tween 20 in the lysis buffer appeared to be beneficial when a smaller amount of the DNA preparation was used in the PCR mixture (Fig. 1, lanes 1, 2, 7, and 8). Boiling for 5 min at 100°C was superior to heating at 95°C in that a smaller amount of sample DNA could be used for the detection of the *prfA*-specific PCR product (Fig. 1,

lanes 4, 5, 7, and 8). When bacteria cultured overnight were compared with those obtained from a 5-day-old culture on a tryptic soy agar plate, it appeared that an old culture was superior in the detection from the smaller amount of sample (Fig. 1, lanes 7, 8, 10, and 11); however, the detection from a fresh colony became more sensitive at 100°C. The increase of the Mg^{2+} concentration to 2 mM showed a significant effect in the improvement of detection (Fig. 1, lanes 7, 8, 13, and 14). On the basis of these results, the bacterial specimen was boiled at 100°C for 5 min in the presence of 0.05% Tween 20; then a 1-μl DNA sample was subjected to PCR in the presence of 2 mM $MgCl_2$ in the following experiment.

Suspensions of viable *L. monocytogenes* EGD were prepared ranging from 4×10^3 to 4×10^7 cells ml^{-1} in phosphate-buffered saline (PBS). One microliter of the DNA sample prepared from each suspension was subjected to PCR for various cycles of amplification. As shown in Table 1, it was possible to detect as few as 4×10^3 bacteria ml^{-1} at 34 cycles of amplification. Since only 1 μl was taken from the boiled specimen into the PCR mixture, it indicated that fewer than 10 cells could be detected. The use of a pair of primers (3'-CGTTAGTCACTTCCCTTTTA-5' and 5'-CACTCAGCATT GATTTGCCA-3') specific for *hlyA* (GenBank accession number, X12157) encoding listeriolysin O yielded a similar result with primers specific for *prfA*; therefore, the present level of sensitivity in the detection seemed to be applicable to the practical survey of contamination in the milk. When 500 CFU of bacteria ml^{-1} was subjected to PCR (0.5 CFU per PCR), a 276-bp PCR product specific for *hlyA* could be obtained in 3 of 10 repeated PCRs (Fig. 2). The 30% positive rate in the detection of (theoretically) 0.5 copy of DNA was consistent with the Poisson distribution, in which the calculated probability of having a negative result is about 60%.

Using the above-mentioned PCR setting, we tried to detect *L. monocytogenes* suspended at serial concentrations ranging from 10^2 to 10^8 cells ml^{-1} in commercially available cow milk pasteurized at an ultrahigh temperature. Compared with the detection limit for bacteria suspended in PBS, the detection limit for bacteria in milk was over 100-fold lower, suggesting a possible presence of interference by the milk (Fig. 3A and B). Such an interference could be successfully eliminated by washing the artificially contaminated milk with PBS three

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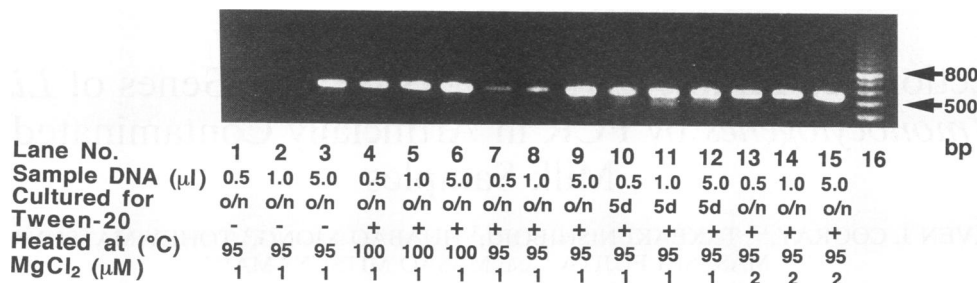
Lane No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	bp
Sample DNA (µl)	0.5	1.0	5.0	0.5	1.0	5.0	0.5	1.0	5.0	0.5	1.0	5.0	0.5	1.0	5.0		
Cultured for	o/n	o/n	o/n	o/n	o/n	o/n	o/n	o/n	o/n	5d	5d	5d	o/n	o/n	o/n		
Tween-20	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+		
Heated at (°C)	95	95	95	100	100	100	95	95	95	95	95	95	95	95	95		
MgCl$_2$ (µM)	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2		

FIG. 1. Agarose gel electrophoresis of the amplification products of the *prfA* gene of *L. monocytogenes* EGD prepared under various conditions. Factors tested to find out the best condition for sample preparation were the amount of sample DNA, period of bacterial culture, presence (+) or absence (−) of 0.05% Tween 20, heating temperature, and the concentration of MgCl$_2$ in the PCR mixture. PCR was carried out for 25 cycles of amplification, and 10 µl of the PCR product was run on 1.2% low-melting-temperature agarose. The gel was stained with ethidium bromide. Lane 16, 100-bp ladder. o/n, overnight; 5d, 5 days.

times at 5,000 × *g* for 5 min and then preparing a final suspension to 1/10 of the original volume of PBS. This washing procedure resulted in a significant increase of the detection level (Fig. 3C). The possible loss of bacteria by this washing step was negligible, since pour plate determination showed that 99% of bacteria suspended at 50 CFU ml of milk^{-1} could be recovered after the washing step (51 ± 2 CFU before washing versus 50 ± 6 CFU after washing).

Both *prfA* and *hlyA* are regarded as the genes critically involved in virulence (2, 10, 11) and also unique to the species *L. monocytogenes* (3, 21). In this respect, the detection of either *prfA* or *hlyA* appears to be sufficient to determine the presence of *L. monocytogenes*. However, it is plausible that some *L. monocytogenes* strain may lack one or more virulence determinants because of some mutation. Though the frequency of spontaneous mutation or deletion in these virulence-associated genes is not known, we cannot deny the possibility that some mutants like those produced by transposon insertion (10, 17) may exist in nature. Therefore, it seemed useful to develop a procedure in which various virulence-associated genes could be detected simultaneously in a simple step. To this end, we have tried to develop a simultaneous PCR detection of multiple genes in one reaction. In addition to *prfA* and *hlyA* genes, *plcB* (19), which encodes a 29-kDa phospholipase C (8), was employed for a PCR target because these three genes are not only involved in virulence but also located in a similar region of the chromosome, separated by *plcA* (between *prfA* and *hlyA*) and *mpl* (between *hlyA* and *plcB*) (14). The expected size of the *plcB* (GenBank accession number, X59723) product amplified with the primers used (oligonucleotides 3'-AGTGTCGTTT GAAACCGTCCA-5' and 5'-GCAAGTGTTCTAGTCTTTC CGG-3') was 795 bp. Preliminary experiments showed that *Taq* DNA polymerase needed to be increased to 3 U when the

three pairs of primers were placed in the same reaction mixture. As the test sample, *L. monocytogenes* ATCC 15313, which is a known non-hemolysin-producing strain, and non-virulent *Listeria innocua* 2740 and 2769 were used in addition to fully virulent *L. monocytogenes* EGD. After 34 amplification cycles, it was possible to detect the PCR products of all three genes of strain EGD in a single step (Fig. 4). This simultaneous detection of three genes was possible for at least 10 bacteria in the sample. In contrast to *L. monocytogenes* EGD, it was clear that ATCC 15313 lacks the intact gene encoding listeriolysin O while the presence of the other two genes, *prfA* and *plcB*, was confirmed by two corresponding bands of 571 and 795 bp, respectively. None of the PCR products of virulence-associated genes was observed in DNA samples prepared from the same concentration of *L. innocua*. This result indicated that there is a possibility of failure in the detection of some *L. monocytogenes* strains if only one target gene is employed for the detection by PCR.

Although the incidence of *L. monocytogenes* in milk so far reported is not so high (12, 15), the survey of this virulent bacterium in milk appears to be critically important from the viewpoint of public health, since even pasteurized milk is reported to be a vehicle of infection (5). Therefore, development of a feasible, time-saving method of PCR detection of virulent *L. monocytogenes* has a practical importance. The present method of PCR detection including washing of the milk sample could be completed in 5 h without preenrichment. With listeria enrichment broth and listeria plating medium, it was found that as little as 0.1 CFU of *L. monocytogenes* ml of milk^{-1} could be detected as starting material (18); however, an overnight culture was required, and the actual detection limit at the time of PCR has not been indicated. One of the

TABLE 1. Detection of PCR products from *L. monocytogenes* suspensions of different concentrations and the effect of changing the number of amplification cycles

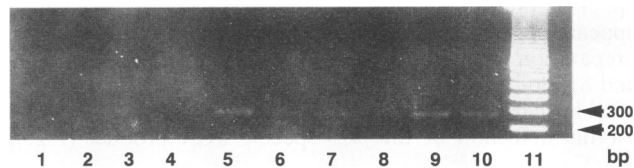| No. of PCR cycles	Band intensity at the following concn of bacteria (CFU/ml):				
	4 × 10^3	4 × 10^4	4 × 10^5	4 × 10^6	4 × 10^7
25	−	−	−	+	++
28	−	−	Trace	++	++
31	−	−	+	+++	+++
34	+	+	++	+++	+++
37	+	++	+++	+++	+++

FIG. 2. Agarose gel electrophoresis of the amplification products of the *hlyA* gene of *L. monocytogenes* EGD originally suspended at the concentration of 500 cells ml^{-1}. The gel shows products of 10 repeated PCRs using 1 µl of sample DNA. The PCR products can be seen in lanes 5, 9, and 10. Lane 11, 100-bp ladder.
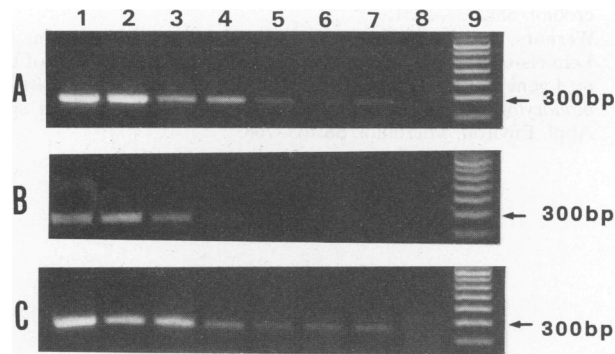
FIG. 3. Agarose gel electrophoresis of the amplification products of the *hlyA* gene of *L. monocytogenes* EGD suspended at 10^8 cells ml^{-1} (lanes 1) to 10^2 cells ml^{-1} (lanes 7) by serial 10-fold dilutions. Lane 8, a negative sample without bacteria; lane 9, 100-bp ladder. (A) Sample DNA was prepared from bacteria suspended in PBS. (B) Bacteria were suspended in milk, and sample DNA was prepared. (C) Bacteria suspended in milk as for panel B were centrifuged, washed in PBS, and finally suspended in PBS at 1/10 the volume of the original specimen; then, sample DNA was prepared.

advantages of enrichment before PCR may be the elimination of false-positive results due to the detection of DNA from nonviable bacteria in milk samples.

In the PCR detection, the choice of the target gene is of importance. The use of primers specific for *prfA* or *hlyA* seems to be reasonable because these are virulence-associated genes unique to *L. monocytogenes*. However, there can be exceptions like *L. monocytogenes* ATCC 15313 as shown in this study. This strain has been a type strain of *L. monocytogenes* for a long time but is known as a peculiar strain which neither is beta-hemolytic nor exhibits hemolysis in the CAMP test with *Staphylococcus aureus* (16). It is plausible that in nature there are some strains of *L. monocytogenes* defective in the intact *hlyA* gene or other virulence genes like ATCC 15313; therefore, it appears that the amplification of only one gene may result in an incorrect diagnosis. In this regard, simultaneous detection of multiple genes in a single step without using a separate reaction mixture will be desirable especially in a large-scale survey aiming at the detection of a virulent strain of *L. monocytogenes* without failure.
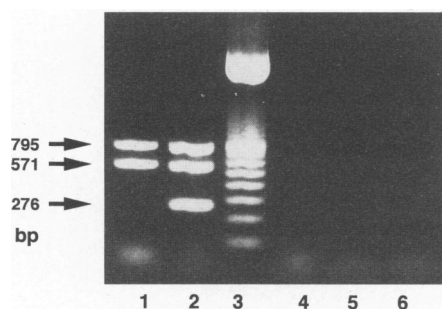

FIG. 4. Agarose gel electrophoresis of the amplification products of three virulence-associated genes, *hlyA*, *prfA*, and *plcB* (276, 571, and 795 bp, respectively), obtained in a single PCR. Lanes: 1, *L. monocytogenes* ATCC 15313; 2, *L. monocytogenes* EGD; 3, 100-bp ladder; 4 and 5, *L. innocua* 2740 and 2769, respectively; 6, a negative control without DNA.

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