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Rapid Enumeration of *Listeria monocytogenes* in Pork Meat Using Competitive PCR

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Abstract Competitive polymerase chain reaction (cPCR) was used to develop a direct enumeration method of *Listeria monocytogenes* in pork meat. Pork meat was artificially inoculated with *L. monocytogenes* and DNA was extracted using guanidine thiocyanate-phenol-chloroform and subjected to PCR amplification. Sixteen primer sets for *L. monocytogenes hlyA* gene were tested for sensitive detection and the DG69/DG74 primer set was selected. The detection limit achieved with this primer set was as low as 860 colony-forming units (cfu) per 0.1 g of pork meat. When the samples were cultured at 30°C for 16 hr in Brain Heart Infusion (BHI) medium, even a single bacterium could be detected with this primer set by PCR. For cPCR, the *hlyA* gene, which features a 148 bp-deletion, was cloned in the pGEM-4Z vector. A known amount of competitor DNA which has the same primer binding sites was co-amplified with *L. monocytogenes* total DNA from the artificially inoculated pork meat. The cell-number determined by cPCR was approximately equal to cfu from the Most Probable Number (MPN) method. The whole procedure took only 5 hr.

Keywords: pork meat, *Listeria monocytogenes*, *hlyA* gene, polymerase chain reaction, competitive PCR (cPCR)

Introduction

The incidence of listeriosis caused by *Listeria monocytogenes* has increased drastically in recent years and *L. monocytogenes* is becoming a health-threatening microorganism which has been isolated from a variety of foods (1-4). Preventive treatment such as Hazard Analysis and Critical Control Point (HACCP) requires rapid detection and quantification of the contaminated pathogen. Therefore, the development of rapid detection and counting methods for *L. monocytogenes* is essential for such treatment.

Although many different conventional testing methods have been developed for the detection and enumeration of *L. monocytogenes* from food, they are laborious, time-consuming and sometimes underestimate the numbers (5-8). To overcome these limitations, molecular biological, biochemical and immunological techniques have been applied, and found somewhat successful in reducing the time to detect *L. monocytogenes* (9-13). However, most of these techniques only detect the presence of the pathogens and do not enumerate them. Real-time PCR has been used for the enumeration of contaminated pathogens in food (14, 15), but it requires an expensive detection system and is not well suited for routine use in the practical field. However, an alternative exists: competitive polymerase chain reaction (cPCR). Recently, we found that cPCR can be applied to estimate directly the numbers of *L. monocytogenes* (16), *Salmonella enteritidis* (17) and *Yersinia enterocolitica* (18) in milk and that it can be completed within 5 hr without any sophisticated apparatus, isotope or fluorescent dye etc. Therefore, in this work, a similar approach was undertaken to develop a simple, semiquantitative method based on cPCR technology for the rapid enumeration of *L. monocytogenes* cells in pork meat as a model system. We chose the *hlyA* gene of *L. monocytogenes* encoding 58 kDa listeriolysin O which has been tested for the specific detection of *L. monocytogenes* in various foods (16, 19, 20), but modified it for the simple enumeration of *L. monocytogenes*. The procedure described in this paper provides a rapid and simple enumeration method for *L. monocytogenes* in pork meat.

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Materials and Methods

Listeria monocytogenes *L. monocytogenes* ATCC 19113 was obtained from the Korean National Veterinary Research and Quarantine Service and was grown in TSBY containing Tryptic Soy Broth (Becton Dickinson, Sparks, MD, USA) and 0.6% yeast extract (Becton Dickinson) or Brain Heart Infusion (BHI) medium (Becton Dickinson). The colony numbers were determined using the Most Probable Number (MPN) method by plating on TSBY agar at 30°C overnight.

Reagents Restriction enzymes, DNA polymerase I Klenow Fragment, pGEM-4Z DNA and Wizard Genomic DNA purification kit were purchased from Promega (USA). Ligase was obtained from Roche (Germany). The DNA polymerase, reagents for the PCR, PCRquick-spin™ PCR Product Purification kit and G-spinTM Genomic DNA extraction kit (for bacteria) were purchased from Intron Biotechnology (Korea). Gene ALL™ plasmid SV mini was obtained from General Biosystem (Korea). Pork meat (for cutlet) was purchased at a supermarket (Samsung TESCO Home Plus, Gyeongju, Korea) and was tested before use as negative for the *L. monocytogenes* with PCR. All chemicals, unless otherwise noted, were purchased from Sigma (USA).

Primer design The DNA primers used in this study are listed in Table 1. All the primers were purchased from GenoTech (Daejon, Korea). The *hlyA* gene (GenBank accession number X15127) was chosen because this gene has been tested for specific detection of *L. monocytogenes* (16). The specificity of the primers was checked by Basic Local Alignment Search Tool (BLAST).

Construction of the plasmid encoding *L. monocytogenes hlyA* gene *Eco*R V site in the *hlyA* gene was created by overlap extension. The DG191/DG192 primers pair was used to amplify the DNA segment that contains the *Eco*R V site together with upstream *Sac* I sequences for subcloning. The DG193/DG194 primers pair was used to amplify the DNA segment that contains the *Eco*R V site together with downstream *Bam*H I sequences for subcloning. The amplified DNAs were purified with PCRquick-spin™ PCR Product Purification kit and were used for amplification with the DG191/DG194 pair. The amplified DNA segment was cleaved with *Sac* I/*Bam*H I, and was cloned into the pGEM-4Z plasmid, which does not have an *Eco*R I site because the *Eco*R I site had been removed for our purpose. The resulting plasmid (pGEM-4Z LM) DNA was cleaved with *Eco*R I/*Eco*R V, gap-filled with Klenow fragment and introduced into *Escherichia coli* DH5α. The colonies were screened for the presence of *Eco*R I and *Eco*R V sites by restriction enzyme digestion. After sequencing using DG69 as a primer, the final clone was designated as pGEM-4Z LMΔRI-RV (Fig. 1).

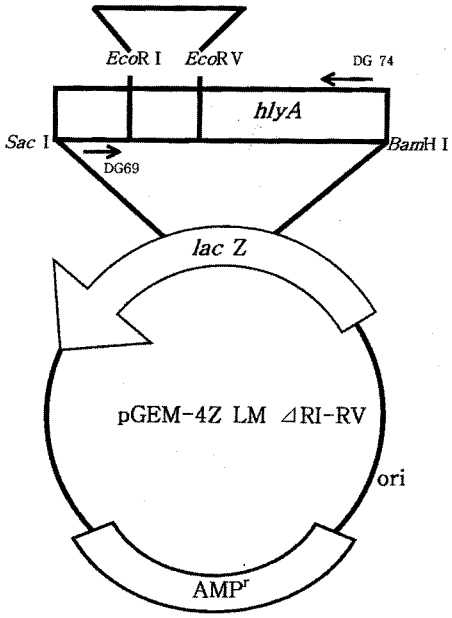

Fig. 1. Construction of pGEM-4Z LMΔRI-RV encoding *L. monocytogenes hlyA* gene. A DNA segment containing the *hlyA* gene sequence was amplified by overlap extension to create the *Eco*R V site and was cloned into a pGEM-4Z vector in which the *Eco*R I site had been removed. The resulting plasmid was cleaved with *Eco*R I/*Eco*R V and was gap-filled. The final clone was designated pGEM-4Z LMΔRI-RV. The deleted portion (EcoR I-EcoR V) is marked in the Figure. PCR with primers DG69/DG74 amplified *L. monocytogenes* specific 636 base sequences from bacteria, but amplified 488 base sequences from pGEM-4Z LMΔRI-RV.

Preparation of DNA in artificially inoculated pork meat Pork meat was homogenized with a Stomacher (AES Laboratoire, France) in distilled water (1:1, w/v), dispensed into bags, and stored at -70°C until use. The guanidine thiocyanate-phenol-chloroform method (16) was modified for the preparation of PCR template of *L. monocytogenes*. Briefly, the artificially inoculated 0.1 g pork meat was extracted with 0.25 mL solution D (4 M guanidine thiocyanate, 0.025 M sodium citrate, 0.5% N-Lauroylsarcosine) and 0.5 mL phenol-chloroform (1:1). The aqueous phase was extracted with 400 μL chloroform and the DNA was precipitated with isopropanol and sodium acetate. The resulting pellet was washed once with 70% ethanol, dried and used for the PCR.

Polymerase chain reaction (PCR) The PCR mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, 2.5 units of iMax*Taq* DNA polymerase, 100 pmol of each primer and varying amounts of template DNA. Samples were denatured at 94°C for 5 min and subjected to amplification cycles in a thermocycler (Perkin-Elmer 2400, Foster, CA, USA). Each cycle consisted

Table 1. Primers used in this study

primers	Sequences (5'-3')	Polarity	T_m value (°C)	Reference
DG69	GTGCCGCCAAGAAAAGGTTA	sense	57.3	16
DG74	CGCCACACTTGAGATAT	antisense	50.4	16, 20
DG164	CACGCGGATGAAATCGATAAGT	sense	58.4	
DG165	AGTATACCACGGAGATGCAGTG	sense	60.3	
DG166	AAAGATGGAAATGAATATAT	sense	45.0	
DG167	TCTGGAAGGTCTTGTAGGTTCA	antisense	58.4	
DG168	GCAACTGCTCTTTAGTAACAGC	antisense	58.4	
DG169	CACTTGAGATATATGCAGGAGG	antisense	58.4	
DG170	ATTTCAAATAAACTTGACGGCC	antisense	54.7	
DG171	TTACGGCTTTGAAGGAAGAATT	antisense	54.7	
DG191	CTCCGCCTGCAA**GAGCTC**AGACGCCAATCG	sense	73.6	
DG192	CATCTTTCCACT**GATATC**TTTACTGCGTTG	antisense	64.0	
DG193	CAACGCAGTAAA**GATATC**AGTGGAAAGATG	sense	64.0	
DG194	GGGAACTCCTGG**GGATCC**TCGATTAAAAGT	antisense	68.1	

of a 45-sec denaturation step (94°C), 45-sec annealing step (55°C), and 45-sec extension step (72°C). Finally, the products were extended for 7 min at the completion of 30 amplification cycles.

Results and Discussion

Detection of *L. monocytogenes* in pork meat According to the published sequence of the listeriolysin O (*hlyA*) gene (21)(GenBank accession number, X15127), 10 primers were designed. Primer set DG69/DG74 (16) has been published previously and amplifies the 636 bp DNA fragment. To find a better primer set for PCR, the band intensity of the PCR products using the 16 primer sets was compared. The band intensity varied significantly among the primer sets. DG69/DG74, DG164/DG168, and DG165/DG171 produced more DNA copies than the other primer sets, and thicker bands were observed (Fig. 2). Since we couldn't find any differences between the three primer sets after comparing sensitivities (data not shown), and as this primer set yielded a specific 636 bp band from six *L. monocytogenes*, but no band from the other *Listeria* spp. (16), the following experiments were performed using DG69/DG74.

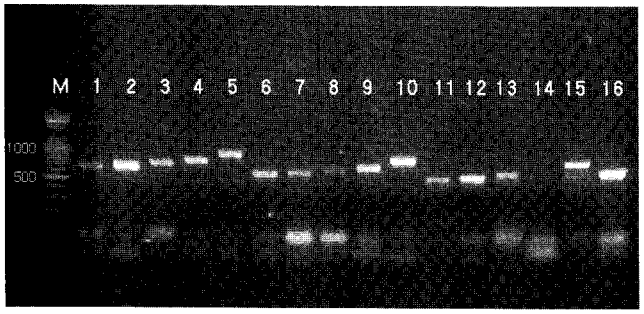

Fig. 2. Gel electrophoresis profiles obtained with 16 primer sets. For the PCR, the pGEM-4Z LMRI(-) (16) plasmid DNA was used. Lane M, 100-bp DNA ladder; lane 1, DG164/DG167 (636 bp); lane 2, DG164/DG168 (670 bp); lane 3, DG164/DG169 (718 bp); lane 4, DG164/DG170 (748 bp); lane 5, DG164/DG171 (871 bp); lane 6, DG165/DG167 (577 bp); lane 7, DG165/DG168 (611 bp); lane 8, DG165/DG169 (659 bp); lane 9, DG165/DG170 (689 bp); lane 10, DG165/DG171 (812 bp); lane 11, DG166/DG167 (528 bp); lane 12, DG166/DG168 (562 bp); lane 13, DG166/DG169 (610 bp); lane 14, DG166/DG170 (640 bp); lane 15, DG166/DG171 (763 bp); lane 16, DG69/DG74 (636 bp).

To find a better DNA extraction method, we compared the modified guanidine thiocyanate-phenol-chloroform method with two commercial kits (G-spinTM Genomic DNA extraction kit and Wizard Genomic DNA purification system) for detecting bacteria. However, we found no differences in the detection threshold (172-430 copies) between the three methods (Fig. 3). Considering the sample size and the difficulty in the application to pork meat, the following experiments were performed using the modified guanidine thiocyanate-phenol-chloroform method.

To measure the sensitivity, homogenized pork meat samples were artificially inoculated with $8.6\text{-}8.6 \times 10^6$ colony-forming units (cfu) of *L. monocytogenes* per 0.1 g of meat. DNAs were directly extracted and subjected to PCR amplification. The limit of detection was 860 cfu/0.1 g of pork meat (Fig. 4), which was similar to that observed previously (16, 22). The experiments were performed in triplicate with good reproducibility being demonstrated. The cycle extension up to 50 cycles or another 30 cycles of PCR did not increase the sensitivity. After 20-fold dilution with BHI medium and incubation at 30°C for 15 hr, improved sensitivity could be achieved. Five cfu of *L. monocytogenes* per 0.1 g meat was detected (Fig. 5) and even a single bacterium could be detected in 2 of 3 trials by PCR (data not shown). The same result was obtained with TSBY medium (data not shown).

Competitive PCR for the direct enumeration of *L. monocytogenes* in pork meat Previously we have shown that amplification of radioisotope-labeled, *hlyA* specific sequences using DG69/DG74 was still in the exponential range after 30 cycles (16). Therefore, we used the same condition for cPCR. For the quantification of *L. monocytogenes* DNA, DNA from artificially inoculated pork meat was co-amplified in the presence of known copy numbers of a competitor plasmid DNA, pGEM-4Z LMΔRI-RV, that binds the same primers (DG69/DG74) but that has been deleted by 148 bp in order to distinguish

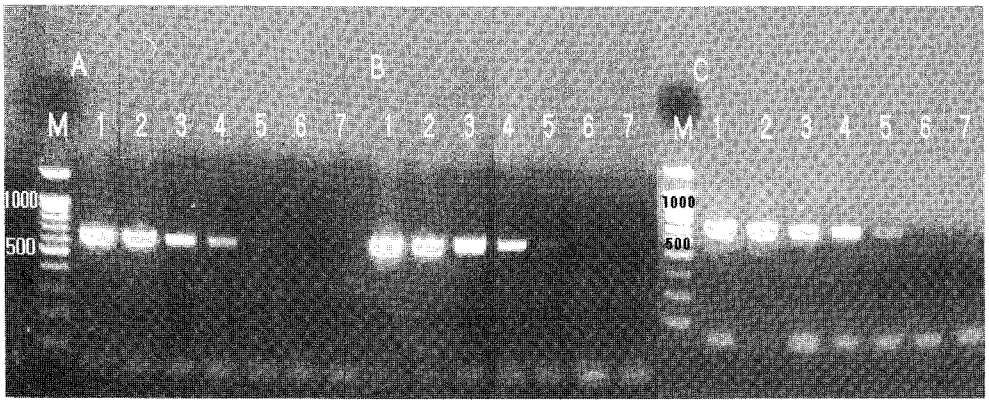

Fig. 3. Gel electrophoresis profiles with three template DNA isolation methods. For the PCR, the DG69/DG74 primer set was used. The colony-forming units (cfu) of *L. monocytogenes* were determined by MPN. (A) Modified guanidine thiocyanate-phenol-chloroform (B) Wizard Genomic DNA purification system (C) G-spin^{TM} Genomic DNA extraction kit (for bacteria). (A) and (B): Lane M, 100-bp DNA ladder; lane 1, 1.72×10^6 cfu; lane 2, 1.72×10^5 cfu; lane 3, 1.71×10^4 cfu; lane 4, 1.72×10^3 cfu; lane 5, 1.72×10^2 cfu; lane 6, 1.72×10 cfu; lane 7, 1.72 cfu (C): Lane M, 100-bp DNA ladder; lane 1, 4.30×10^6 cfu; lane 2, 4.30×10^5 cfu; lane 3, 4.30×10^4 cfu; lane 4, 4.30×10^3 cfu; lane 5, 4.30×10^2 cfu; lane 6, 4.30×10 cfu; lane 7, 4.30 cfu.

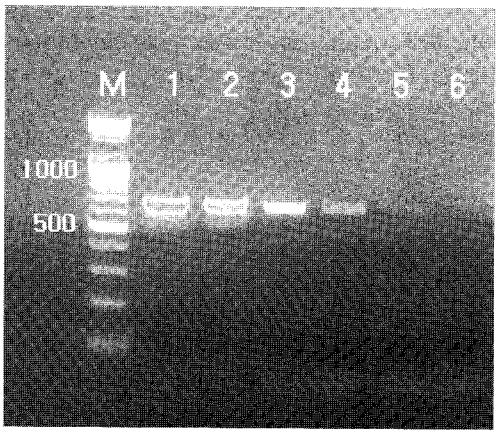

Fig. 4. Detection limit of *L. monocytogenes* in pork meat. 636-bp PCR products were detected from 8.6×10^6, 8.6×10^5, 8.6×10^4, 8.6×10^3 and 8.6×10^2 cfu of *L. monocytogenes* per 0.1 g pork meat (lanes 1-5). No band was detected from 8.6×10 and 8.6 cfu of *L. monocytogenes* per 0.1 g pork meat.

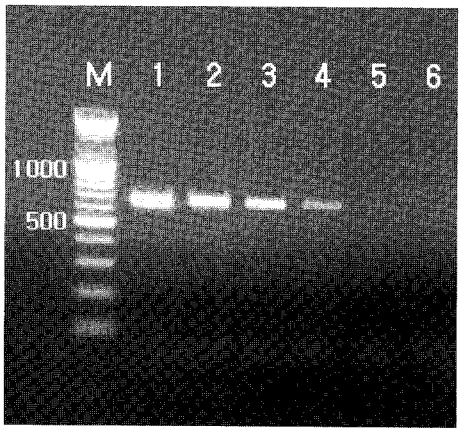

Fig. 5. Detection limit after growing in Brain Heart Infusion (BHI). Artificially inoculated pork meat was diluted 20-fold with BHI and incubated at 30°C for 16 hr at 150 rpm. Cultured broth (0.2 mL) was taken, and the DNA was extracted and subjected to PCR amplification. The inoculum size was 5×10^4 (lane 1), 5×10^3 (lane 2), 5×10^2 (lane 3), 5×10 (lane 4), 5 (lane 5) and 0.5 (lane 6) cfu of *L. monocytogenes*. Lane M shows 100-bp DNA ladder as a size standard.

it from natural sequences. The efficiency of the amplification of the two different kinds of DNA should have been almost equal because the same primers bind the same sequences for amplification. The difference between the *hlyA* gene of the bacterial chromosome and the modified gene of pGEM-4Z LMΔRI-RV was only the deleted sequences (148 bp). Since the relative amounts of the cPCR products generated from the amplification reflected the relative initial levels of the two different kinds of DNA, cfu of *L. monocytogenes* was easily estimated by comparing the intensity of the two bands after electrophoresis. In fact, the calculated DNA copy number of the standard DNA and the cfu result from MPN were almost equal in this procedure (Fig. 6). Even though a recent paper has described an automated quantitative detection of *L. monocytogenes* using real-time PCR (15), the procedure still requires an expensive, special machine and reagents. Depending on the purpose, sometimes detection and rough estimation of cfu are enough for routine control. The procedure described in this study does not provide accurate numbers of contaminated *L. monocytogenes* in pork meat. Nevertheless, it will be useful for rapid enumeration of *L. monocytogenes* under conditions when real-time PCR is not available.

Since the procedure described in this paper is basically PCR-based, it does not differentiate dead cells from viable ones and can therefore yield exaggerated results. A recent paper has described the specific detection of *L. monocytogenes* using RT-PCR in food (9). Experiments using RT-cPCR to determine viable *L. monocytogenes* in pork meat are ongoing.

In summary, we have cloned and modified the *hlyA* gene and have successfully used it with cPCR for the rapid enumeration of *L. monocytogenes* contaminated in pork meat. The results of this study provide the basis for PCR as a rapid, inexpensive and convenient method for the detection and enumeration of *L. monocytogenes* in a practical field such as HACCP.

Acknowledgments

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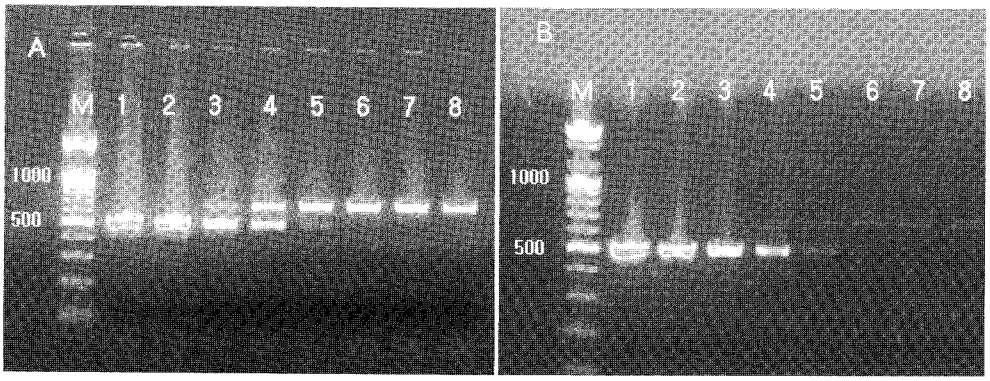

Fig. 6. Agarose gel electrophoresis after cPCR. The upper band (636 bp) is derived from artificially inoculated *L. monocytogenes* (ATCC 19113) DNA and the lower band (488 bp) is from pGEM-4Z LMΔRI-RV standard DNA. (A) 4.0×10^4 cfu *L. monocytogenes* (B) 4.0×10^3 cfu *L. monocytogenes*. Lane M, 100-bp DNA ladder as a size marker; lane 1, 4.0×10^7 copies of standard DNA (pGEM-4Z LMΔRI-RV); lane 2, 4.0×10^6 copies of standard DNA; lane 3, 4.0×10^5 copies of standard DNA; lane 4, 4.0×10^4 copies of standard DNA; lane 5, 4.0×10^3 copies of standard DNA; lane 6, 4.0×10^2 copies of standard DNA; lane 7, 4.0×10 copies of standard DNA; lane 8, 4.0 copies of standard DNA.

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