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Rapid enumeration of *Listeria monocytogenes* in milk using competitive PCR

Weon Sang Choi[a,*], Chong-Hae Hong[b]

[a] *Department of Biotechnology, College of Natural Sciences, Dongguk University, Gyeongju 780-714, Republic of Korea*
[b] *Department of Veterinary Medicine, Kangwon National University, 192-1 Hyoja 2 dong, Chunchon, Kangwon-do 200-701, Republic of Korea*

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Abstract

Competitive polymerase chain reaction (cPCR) was used to develop a direct enumeration method of *Listeria monocytogenes* in milk. Sterile milk was artificially inoculated with *L. monocytogenes* and DNA was extracted using guanidine thiocyanate/phenol/chloroform, followed by PCR. Several primers for *L. monocytogenes hlyA* gene were tested for specific detection and DG69/DG74 primer set was selected. The primer set produced a 636-bp band from *L. monocytogenes*, but no band appeared from the other six *Listeria* spp. tested. A detection limit was as few as 10^3 colony-forming unit (cfu) per 0.5 ml of milk with this primer set. When the samples were cultured at 25 °C for 15 h in a TSBY medium, even a single bacterium could be detected with this primer set by PCR. For the cPCR, *hlyA* gene segment was cloned in pGem-4Z vector and was modified to produce competitor DNA. The competitor DNA has the same primer binding sites and sequences as the target DNA except *Eco*RI site. Known amount of competitor DNA was coamplified with *L. monocytogenes* total DNA isolated from artificially inoculated milk. The target DNA and competitor DNA were distinguished by *Eco*RI digestion after cPCR. The cell number determined by cPCR was approximately equal to the colony-forming unit from conventional plate counting method. For the whole procedure, it took only 5 h.

Keywords: *Listeria monocytogenes*; Competitive PCR (cPCR)

1. Introduction

Listeria monocytogenes is one of the important etiologic microorganisms of food poisoning. Listeriosis caused by *L. monocytogenes* has increased drastically in recent years. *L. monocytogenes* has been isolated from a variety of foods (Norrung et al., 1999; Inoue et al., 2000; Rocourt et al., 2000; Maijala et al., 2001). The preventive activity such as HACCP against *L. monocytogenes* contamination in food processing has been regarded as an effective measure for the prevention of listeriosis outbreaks. It requires rapid detection and quantification of contaminated pathogen for microbiological testing in verifying food safety. The development of rapid detection and rapid counting methods for the *L. monocytogenes* will play an important role for this goal.

* Corresponding author. Tel.: +82-54-770-2227; fax: +82-54-770-2210.
E-mail address: weonsang@mail.dongguk.ac.kr (W.S. Choi).

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Although many different conventional testing methods have been developed for the detection and enumeration of *L. monocytogenes* from food, these have relied almost exclusively on the use of specific culture media followed by a series of tests for confirmation. Conventional plate counting methods are laborious, time-consuming and sometimes underestimate the numbers (Johansson, 1998; Donnelly, 1999; Besnard et al., 2000; Scotter et al., 2001). To overcome these limitations, molecular biological, biochemical and immunological techniques have been applied for the rapid and specific detection of *L. monocytogenes* (Klein and Juneja, 1997; Manzano et al., 1998; Wang and Hong., 1999; Almeida and Almeida, 2000; Solve et al., 2000). Although these techniques have been somewhat successful at reducing the time it takes to detect *L. monocytogenes*, most of the techniques just detect the existence of the pathogen. Although one of them (Nogva and Lillehaug, 1999; Nogva et al., 2000) tried to enumerate the contaminated pathogen in food using 5′ nuclease PCR, this method requires fluorescence detection system and is not well suited for routine use in practical field. Competitive polymerase chain reaction (cPCR) can be an alternative. To perform cPCR, a series of diluted competitor DNA and constant amount of sample DNA are coamplified in the same tube by the thermocycler. Following PCR, the amplification products are quantified by analyzing gel electrophoresis pattern (Mullis et al., 1994). Recently, we found that cPCR can be applied to directly estimate the amount of *Salmonella enteritidis* and *Yersinia enterocolitica* in milk (Choi, 2000, 2001) and can be completed within 5 h without using any sophisticated apparatus, isotope or fluorescence dye, etc. Therefore, in this paper, a similar approach was undertaken to develop a simple semiquantitative method based on cPCR technology for the rapid enumeration of *L. monocytogenes* cells in milk as a model system. The *hlyA* gene of *L. monocytogenes* encoding 58 kDa listeriolysin O (Mengaud et al., 1988) was chosen as a target for PCR amplification because this gene is a virulence determinant and has been tested for the specific detection of *L. monocytogenes* in various foodstuffs (Bessesen et al., 1990; Deneer and Boychuk, 1991; Furrer et al., 1991; Golsteyn Thomas et al., 1991; Rossen et al., 1991; Herman and De Ridder, 1993; Klein and Juneja, 1997). The procedure described in this paper provides a rapid and simple enumeration method to be employed in microbial qualitative risk assessment.

2. Materials and methods

2.1. Bacterial strains

The bacterial strains used in this study (Table 1) were obtained from National Veterinary Research and Quarantine Service (Republic of Korea) and were grown in TSBY containing Tryptic soy broth (Difco, USA) and 0.6% yeast extract (Difco). The colony numbers were determined by plating on TSBY agar at 30 °C overnight.

2.2. Reagents

Restriction enzymes were purchased from Promega (USA). Sequenase was purchased from United States Biochemical (Cleveland, OH, USA). The *Taq* DNA polymerase and reagents for the PCR were obtained from Takara (Japan). Tryptic soy broth and yeast extract were purchased from Difco. Milk was purchased at the supermarket in Korea and was tested before use as negative for the *L. monocytogenes* with

Table 1
Listeria spp. strains used in this study

Species	Strains	Serotypes[a]	Isolation[b]
L. monocytogenes	ATCC19113	3	human
L. monocytogenes	ATCC19114	4a	
L. monocytogenes	ATCC19115	4b	human
L. monocytogenes	ATCC19117	4d	sheep
L. monocytogenes	ATCC19118	4e	chicken
L. monocytogenes	HPB410	1/2a	
L. monocytogenes	ATCC35152		guinea pig
L. ivanovii	ATCC19119		sheep
L. innocua	ATCC33090	6a	cow brain
L. welshimeri	ATCC35897	6b	decaying plant
L. seeligeri	ATCC35967		soil
L. grayi	ATCC19120		chinchilla feces
L. murrayi	ATCC25401		corn stalks and leaves

[a] source: http://www.atcc.org/ except *L. monocytogenes* HPB410.
[b] source: http://www.atcc.org/.

PCR. All chemicals, unless otherwise noted, were obtained from Sigma (USA).

2.3. Primer design

The DNA primers used in this study are listed in Table 2. All the primers were purchased from Geno-Tech (Daejon, Korea). The *hlyA* gene (GenBank accession number X15127) was chosen because this gene is virulence-associated and has been tested for specific detection of *L. monocytogenes*. Specificity of the primers was checked by GenBank database BLAST program and was confirmed by PCR (Fig. 3A).

2.4. Construction of the plasmid encoding L. monocytogenes hlyA gene

All DNA manipulations were performed according to standard procedures (Sambrook and Russel, 2001). A DNA segment containing *hlyA* gene sequences was amplified by PCR (Perkin-Elmer 2400, Foster, CA) with synthetic DNA primers DG67 and DG68. For the PCR, *L. monocytogenes* total DNA was used as a template. After PCR, the amplified DNA segment was cleaved with *Sac*I/*Bam*HI, cloned into the pGem-4Z plasmid (Promega) and resulted in the clone pGem-4Z LM. The pGem-4Z LM DNA was cleaved with *Eco*RI and gap-filled with Klenow and was introduced into *E. coli* DH5α. The colonies were screened for the presence of *Eco*RI site by restriction enzyme digestion. After sequencing using DG69 as a primer, the final clone was designated pGem-4Z LMRI(−) (Fig. 1).

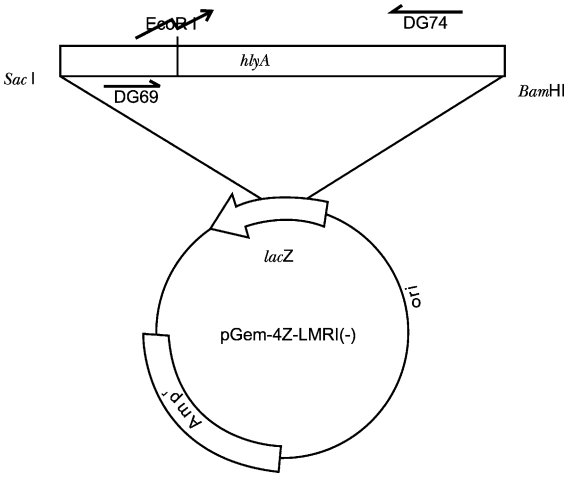

Fig. 1. Construction of pGem-4Z LMRI(−) encoding *L. monocytogenes hlyA* gene. A DNA segment containing *hlyA* gene sequences was amplified by PCR and cloned into the pGem-4Z vector to get pGem-4Z LM. The pGem-4Z LM was cleaved with *Eco*RI and gap-filled with Klenow. The final clone was designated pGem-4Z LMRI(−). PCR with primers DG69/74 amplifies *L. monocytogenes* specific 636 base sequences.

2.5. Preparation of DNA in artificially inoculated milk

The guanidine thiocyanate/phenol/chloroform method, which was used for the direct preparation of *S. enteritidis* (Choi, 2000) and *Y. enterocolitica* (Choi, 2001), was used for the preparation of PCR templates of *L. monocytogenes*. Briefly, the artificially inoculated 0.5 ml milk or bacterial culture was extracted with 0.25 ml solution D (4 M guanidine

Table 2
Primers used in this study

Primers	Sequences (5′-3′)	Polarity	T_m value[a] (°C)	Reference
DG67	CCTGCAA**GAGCTC**AGACGCC	sense	66	
DG68	CGTAA**GGATCC**GAGGTTGCC	antisense	64	
DG69	GTGCCGCCAAGAAAAGGTTA	sense	60	
DG70	CCTTCACTGATTGCGCCGAA	antisense	62	
DG71	CGGAGGTTCCGCAAAAGATG	sense	62	Furrer et al. (1991)
DG72	CCTCCAGAGTGATCGATGTT	antisense	60	Furrer et al. (1991)
DG73	GACATTCAAGTTGTGAA	sense	46	Golsteyn Thomas et al. (1991)
DG74	CGCCACACTTGAGATAT	antisense	50	Golsteyn Thomas et al. (1991)
DG75	GACCGCAAGGTTGAAACTCA	sense	60	
DG76	CAGCCTACAATCCGAACTGA	antisense	60	

[a] T_m values were calculated based on the equation T_m (in °C) = 2(A + T) + 4(G + C) (Sambrook and Russel, 2001).

thiocyanate, 0.025 M sodium citrate, 0.5% sarcosyl) and 0.5 ml phenol–chloroform (1:1). The aqueous phase was precipitated with isopropanol and sodium acetate. The pellet was washed once with 70% ethanol, dried and used for the PCR.

2.6. Polymerase chain reaction

Polymerase chain reaction (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 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. Each cycle consisted of a 45-s denaturation step (94 °C), a 45-s annealing step (55 °C), and a 45-s extension step (72 °C). Finally, products were extended for 7 min at the completion of 30 amplification cycles.

2.7. Kinetic analysis to find conditions for exponential amplification of hlyA specific sequences

DG69 primer was end-labeled with adenosine 5′-[γ-^{32}P]triphosphate (Amersham Pharmacia Biotech, NJ, USA) by using T4 polynucleotide kinase (Amersham Pharmacia Biotech). PCR products were analyzed by 8% polyacrylamide gel electrophoresis and stained with ethidium bromide. Bands corresponding to each specific product were excised from the gels, and the amount of incorporated radioactivity was determined by scintillation counting.

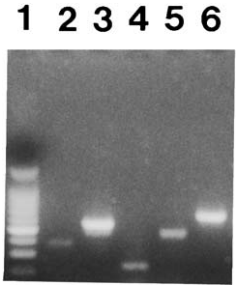

Fig. 2. Gel electrophoresis profiles obtained with five different primer sets. For the PCR, the same amount of template DNA was used except primer sets. Lane 1 shows 100-bp DNA ladder as a size standard. Lane 2, DG70/DG73 (386 bp); lane 3, DG73/DG74 (560 bp); lane 4, DG71/DG72 (234 bp); lane 5, DG69/DG70 (470 bp) and lane 6, DG69/DG74 (636 bp).

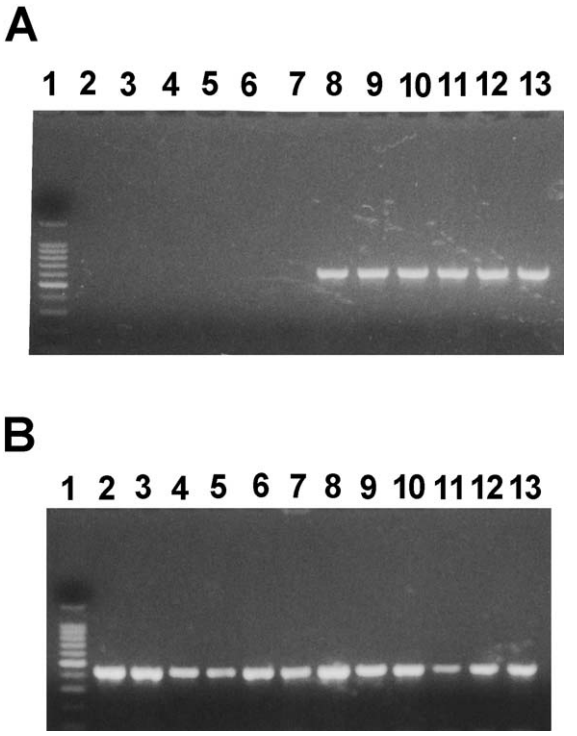

Fig. 3. Agarose gel electrophoresis of PCR products obtained from *Listeria* spp. Lane 1, 100-bp DNA ladder used as a size standard. Lane 2, *L. ivanovii*; lane 3, *L. innocua*; lane 4, *L. welshimeri*; lane 5, *L. seeligeri*; lane 6, *L. grayi*; lane 7, *L. murrayi*; lane 8, *L. monocytogenes* ATCC 19114; lane 9, *L. monocytogenes* ATCC 19115; lane 10, *L. monocytogenes* ATCC 19117; lane 11, *L. monocytogenes* ATCC 19118; lane 12, *L. monocytogenes* HPB410 and lane 13, *L. monocytogenes* ATCC 35152. (A) PCR was performed with DG69/DG74 primers to amplify 636-bp *hlyA* gene segment. (B) Conditions similar to (A) except for primers. Primer set DG75/DG76 was used to amplify the 421-bp 16S rRNA gene segment.

3. Results

3.1. Detection of L. monocytogenes in milk

The sequences of the primers used in this work are listed in Table 2. According to the published sequence of the listeriolysin O (*hlyA*) gene (Domann and Chakraborty, 1989), eight primers were designed. Primer sets DG71/72 (Furrer et al., 1991) and

DG73/74 (Golsteyn Thomas et al., 1991) have been published previously and amplify 234 and 560 bp, respectively. To find the appropriate primer set for the PCR, the band intensity of PCR products using five different primer sets was compared. The band intensity was quite varied among primer sets. DG69/74 and DG73/74 produced more DNA copies than the other primer sets, and thicker bands were observed (Fig. 2). Since the primer set DG69/74 was as efficient as primer set DG73/74 and expected to be more useful for the cPCR (after cleavage with *Eco*RI, proper size of DNA band for cPCR is produced; this will be described in a later section), following experiments

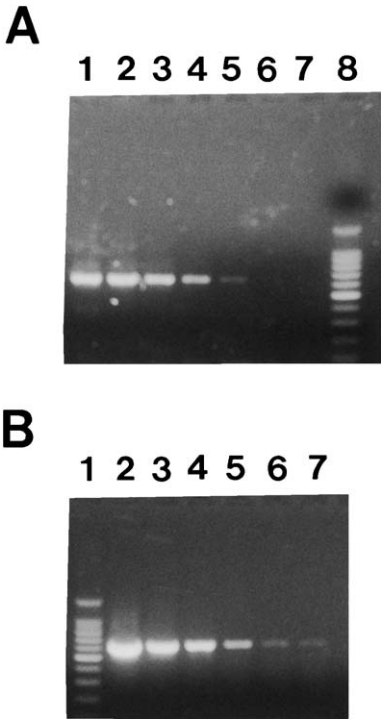

Fig. 4. (A) Detection limit of *L. monocytogenes* in milk. 636-bp PCR products were detected from 10^7, 10^6, 10^5, 10^4 and 10^3 cfu of *L. monocytogenes* (ATCC 19113) per 0.5 ml of milk (lanes 1–5), but no band was detected from 10^2 and 10 cfu of *L. monocytogenes* per 0.5 ml of milk (lanes 6 and 7). Lane 8 shows 100-bp DNA ladder as a size standard. (B) Detection limit after growing in TSBY. Artificially, inoculated milk was diluted 20-fold with TSBY and incubated at 25 °C for 15 h at 150 rpm. 0.5 ml of cultured broth was taken, and DNA was extracted and then PCR was performed. The inoculum size was 10^5 (lane 2), 10^4 (lane 3), 10^3 (lane 4), 10^2 (lane 5), 10 (lane 6) and 1 cfu (lane 7). Lane 1 shows 100-bp DNA ladder as a size standard.

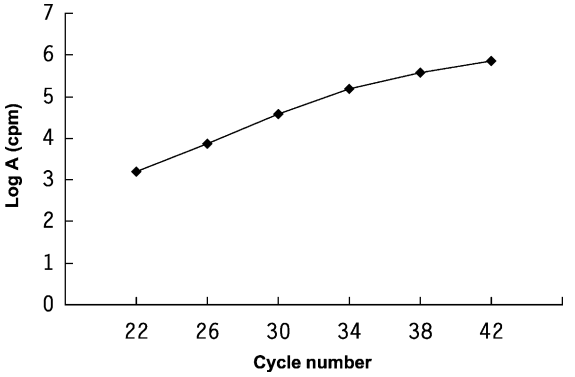

Fig. 5. Kinetics of amplification of the *hlyA* gene specific sequences. After 22 amplification cycles, and after each of additional cycles, a small portion of the reaction was removed and the products resolved on an 8% polyacrylamide gel electrophoresis. After staining with ethidium bromide, bands were excised from the gel, and the amount of incorporated radioactivity was determined by scintillation counting. The log of the amount of PCR product (cpm) was graphed as a function of cycle number. Each data point represents the average of two assays.

were performed using DG69/74. Gel electrophoresis after PCR using DG69/74 yielded one specific 636 bp band from all six *L. monocytogenes* tested, but no band appeared from the other six *Listeria* spp. (Fig. 3A). Under the same conditions, a control experiment produced 421-bp fragments of 16S ribosomal RNA gene with primers DG75/76 from all the tested *Listeria* spp. (Fig. 3B).

To measure the sensitivity, sterile milk samples were artificially inoculated with $10–10^5$ colony-forming unit (cfu) *L. monocytogenes* per 0.5 ml milk. DNAs were directly extracted and were subjected to PCR amplification. The limit of detection was 10^3 cfu/0.5 ml milk (Fig. 4A). Experiments were carried out three times and good reproducibility was observed. The cycle extension up to 50 cycles or another 30 cycles of PCR did not increase the sensitivity. After 20-fold dilution with TSBY and incubation at 25 °C for 15 h, improved sensitivity could be achieved and even a single bacterium could be detected by PCR (Fig. 4B).

3.2. cPCR for the direct enumeration of L. monocytogenes in milk

The amount of PCR product is proportional to the starting amount of the template DNA if PCR amplification proceeds exponentially. To find conditions for

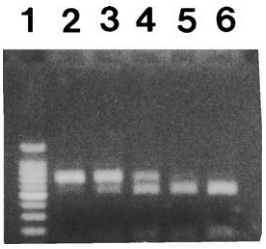

Fig. 6. An agarose gel electrophoresis after cPCR. Upper band (636 bp) is derived from pGem-4Z LMRI(−) standard DNA and lower bands (488 bp plus 4-base overhang, 144 bp plus 4 bp overhang) are from artificially inoculated *L. monocytogenes* (ATCC 19113) DNA after cleavage with *Eco*RI. A 100-bp DNA ladder was used as a size marker. Lane 2, bands for 10^6 copies of standard DNA and 10^4 cfu *L. monocytogenes* DNA; lane 3, bands for 10^5 copies of standard DNA and 10^4 cfu *L. monocytogenes* DNA; lane 4, bands for 10^4 copies of standard DNA and 10^4 cfu *L. monocytogenes* DNA; lane 5, bands for 10^3 copies of standard DNA and 10^4 cfu *L. monocytogenes* DNA; and lane 6, bands for 10^2 copies of standard DNA and 10^4 cfu *L. monocytogenes* DNA.

exponential amplification of *hlyA* specific sequences, kinetic analysis using pGem4Z-LMRI(−) was performed. At cycle number 30, the slopes of the reactions using 10^5 copies template were still in the exponential range (Fig. 5).

For the quantification of *L. monocytogenes* DNA, DNA from artificially inoculated milk was coamplified in the presence of known copy numbers of a competitor DNA pGem-4ZLMRI(−) that binds the same primers (DG69/74) for 30 cycles. The initial concentration of the target DNA could be estimated by comparing the intensity of the two bands of the amplified fragments after digesting the coamplified DNA with *Eco*RI (Fig. 6). Calculated DNA copy number of standard DNA and colony-forming unit from conventional plate counting were almost equal in this procedure.

4. Discussion

In this study, we described a cPCR method for the rapid enumeration of *L. monocytogenes* in milk as a model system. Segment of listeriolysin O (*hlyA*) gene was cloned into the pGem-4Z vector, and *Eco*RI site was knocked-out for the distinction from natural sequences. The efficiency of amplification of two different kinds of DNA should be almost equal because the same primers (DG69/74) were used for the amplification. The difference between *hlyA* gene of the bacterial chromosome and modified *hlyA* gene of the pGem-4Z LMRI(−) is just 4 bp, which was derived from gap-fill. Since the relative amounts of the cPCR products generated from amplification reflect the relative initial levels of the two different kinds of DNA, the colony-forming unit of *L. monocytogenes* could be easily estimated by comparing the intensity of the two bands after electrophoresis.

A recent paper described a quantitative detection of *L. monocytogenes* based on 5′ -nuclease PCR analysis (Nogva et al., 2000). Even though the procedure provides automated, direct detection and quantification of *L. monocytogenes*, it requires an expensive special machine. Depending on the purpose, sometimes detection and rough estimation of colony-forming unit alone is adequate for routine control. The procedure described in this study does not provide accurate numbers of the contaminated *L. monocytogenes*. However, it will be useful for rapid enumeration under conditions when expensive equipment such as ABI Prism Sequence Detection system (PE Biosystems, Foster City, CA) is not available.

Since the procedure described in this paper is basically PCR-based detection and quantification system, it counts dead cells as viable ones and can yield exaggerated result. To overcome this problem, experiments are ongoing to develop RT-cPCR to determine viable cells because RT-PCR amplification of mRNA has been successfully applied for the specific detection of viable *L. monocytogenes* in food (Klein and Juneja, 1997).

Although the band intensity produced from DG73/74 was comparable with DG69/74, DG69/74 set was selected for cPCR because the DG69/74 set yields bands with appropriate distance after *Eco*RI cleavage. Actually, DG73/74 set produces 560, 488 and 144 bp bands while DG69/74 set produces 636, 488 and 144-bp bands after *Eco*RI cleavage. At present, it is not clear why DG69/DG74 set and/or DG73/DG74 set produce more DNA copies than other primer sets. One possible explanation might be a T_m value difference (Table 2). Still, T_m value difference alone could not explain why these two primer sets are better than the others.

In summary, we have cloned and modified *hlyA* gene and have demonstrated that cPCR using modified *hlyA* DNA segment can be utilized for the rapid

enumeration of *L. monocytogenes* contaminated in milk. The results of this study provide the basis for cPCR as a rapid, inexpensive and convenient method for the *L. monocytogenes* assay in a practical field such as HACCP.

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