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Detection of *Salmonella* in Milk by Polymerase Chain Reaction

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ABSTRACT – The polymerase chain reaction was used to selectively detect sequences within the fimbrial antigen of *Salmonella enteritidis*. Sterile milk was artificially inoculated with known amount of *S. enteritidis* and then DNA was extracted with guanidine thiocyanate/phenol/chloroform, followed by PCR. A detection limit of as few as 100 colony forming unit (cfu) per 0.5 ml milk was obtained with this method. For the whole procedure, it took only 5 h. A semi-quantitative polymerase chain reaction assay which allows an estimation of colony forming unit of *S. enteritidis* was developed. Known amount of standard plasmid pGem-4Z-Sef B(-) containing cloned *S. enteritidis* fimbrial antigen gene was co-amplified with *Salmonella* genomic DNA isolated from artificially inoculated milk. The same set of primers were used for the amplification and the products were cleaved with *Bam* HI. The concentration of the target DNA could be estimated by comparing the intensity of the two bands after electrophoresis. The PCR-based protocol described in this paper provides a rapid, simple, and sensitive method for detecting *S. enteritidis* in milk.

Key words □ Fimbrial antigen, Genomic DNA.

Salmonella species are the important etiologic agents of food poisoning salmonellosis. Although many different conventional culture media and enrichment protocols have been proposed for isolating *Salmonella* species from food samples,[1,3] these organisms are still difficult to detect, or enumerate from the natural food systems. In addition, conventional plate counting methods are tedious and usually underestimate the numbers.[5]

The develpoment of polymerase chain reaction (PCR) technology has a potential to solve these problems. PCR is a rapid *in vitro* procedure for enzymatic amplification which increases the number of copies of the target sequence.[6] This allows to increase sensitivity of detection of a DNA sequence present in trace amounts in food samples.[2,4,8,9] However, there is no report to quantitate the *Salmonella* in food by PCR. In this study, several template preparation methods for the detection of fimbrial antigen by a PCR procedure were tested. Semi-quantitative PCR procedure using cloned and genetically modified plasmid DNA was also tried.

Materials and Methods

Bacterial strains. *Salmonella enteritidis* ATCC 13076 (obtained from Korean NIH) were grown overnight at 37°C in Tetrathionate broth base (Difco) or LB. The cultures were serially diluted in 0.85% NaCl and their colony numbers determined by plating on LB plates or BG sulfa agar (Difco).

Reagents. Restriction enzymes were purchased from Promega. The *Taq* DNA polymerase and reagents for the PCR were obtained from Takara. Sequenase was purchased from Amersham Pharmacia biotech. Klenow enzyme was bought from New England Biolab. All chemicals unless otherwise noted were purchased from Sigma Chemical Co.

Primer design. The DNA primers DG50 (5' ATTTTTGG AATTCTTGTAAT 3')/ DG 51 (5' AAATAAGCTTAG TTTTGATA 3') were designed to create *Eco* RI and *Hind* III for cloning fimbrial antigen gene into the pGem 4Z vector. DG58 (5' CAGCATCTGCAGTAGC AGTT 3')/ DG 59 (5' TGAACGTAGAAGGTCGCAGT 3') amplifies a 472 base pair sequences. DG44 (5' CGT AAATCAGCATCTGCAGTAGCA 3') was used to confirm the removal of the *Bam* HI site by sequencing. These primers were prepared from GenoTech (Daejon, Korea).

Construction of the plasmid encoding *Salmonella enteritidis* fimbrial antigen gene

All DNA manipulations were performed according to standard procedures.[7] Restriction enzymes and

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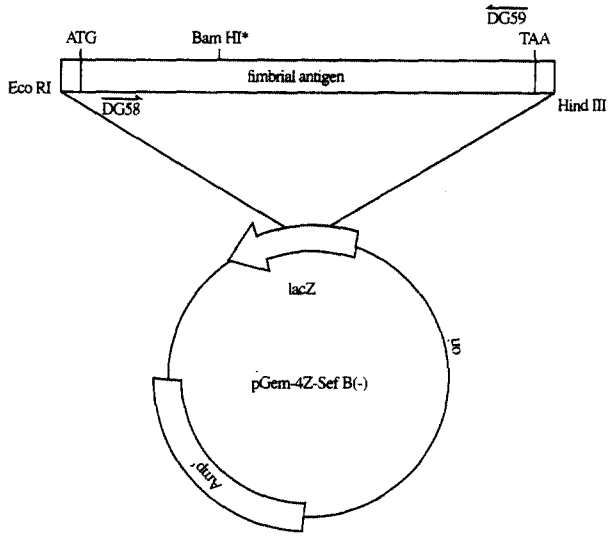

Fig. 1. Construction of pGem-4Z-Sef B(-) encoding *Salmonella enteritidis* fimbrial antigen. }
A DNA segment which includes a whole fimbrial antigen and few extra sequences was amplified by PCR and was ligated into the pGem-4Z vector to get pGem-4Z-Sef. The pGem-4Z-Sef was cleaved with *Bam* HI, gap-filled with dNTPs, religated to remove *Bam* HI site. The final clone was designated pGem-4Z-Sef B(-). PCR with primers DG58/DG59 amplifies *S. enteritidis* specific 472 base sequences.

DNA modification enzymes were used according to the manufacturer's instructions. A DNA fragment of the *S. enteritidis* fimbrial antigen (whole fimbrial antigen plus few extra sequences) was amplified by PCR (Perkin-Elmer 2400, Foster, CA) with synthetic DNA primers DG50 and DG51. For the PCR reactions, the *S. enteritidis* genomic DNA was used as the template. For the preparation of template DNA, 1 ml of a *S. enteritidis* culture was centrifuged at 15000 rpm for 5 min and the pellet was washed three times with distilled water. The washed pellet was suspended with 100 μl of 0.125% sosium dodecylsulfate/0.05 M NaOH and was boiled for 10 min. After centrifuge, 1 μl of the supernatant was used for the PCR. The amplified DNA fragment was cleaved with *Eco* RI/*Hind* III, cloned into the pGem-4Z plasmid and resulted in the clone pGem-4Z Sef. The pGem-4Z Sef DNA was cleaved with *Bam* HI, gap-filled with dNTP using Klenow, religated and was introduced into *E. coli* DH5α. The colonies were screened for the absence of *Bam* HI site by restriction digestion. After sequencing, the final clone was designated pGem-4Z-Sef B(-) (Fig. 1). The DNA was sequenced by using Sequenase version 2.0 sequencing kit (Amershampharmacia biotech) with primer DG44.

DNA purification from milk.

Salmonella enteritidis genomic DNA to be used as PCR templates were extracted from milk by three different methods. 0.5 ml milk, which was artificially inoculated with serially diluted *S. enteritidis*, was used for the template preparation. (i)Phenol-chloroform method. The artificially inoculated milk was extracted once with 0.5 ml of phenol/chloroform (1:1) and the aqueous phase was precipitated with ethanol and sodium acetate. The pellet was washed once with 70% ethanol, dried and used for the PCR. (ii) Guanidine thiocyanate-phenol-chloroform method. The artificially inoculated milk was extracted 0.25 ml solution D (4 M guanidine 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. (iii) SDS-NaOH method. To the artificially inoculated milk 100 μl of 0.125% sosium dodecylsulfate/0.05 M NaOH was added and was boiled for 10 min. After centrifuge, 1 μl of the supernatant was used for the template.

PCR.

Each 50 μl of PCR reaction 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 (Takara), 100 pmol of each primer and varying amounts of template DNA. Samples were denatured at 94°C for 45 sec, and subjected to 10-35 amplification cycles in a thermocycler. Each PCR cycle consisted of a 45 sec annealing step (55°C), a 45 sec extension step (72°C), and a 45 sec denaturation step (94°C). Finally, products were extended for 7 min at 72°C at the completion of amplification cycles.

Results

DNA extraction methods

To find suitable DNA extraction methods, *S.*

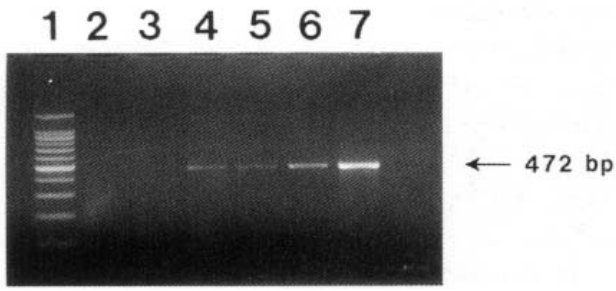

Fig. 2. Agarose gel electrophoresis of 472 base products from *S. enteritidis* detection in terms of cfu.
Lane 1 shows 100 bp DNA ladder as a size standard. Approximately sized PCR products were detected from the amplification of DNA from 1, 10, 10^2, 10^3, 10^4, 10^5 cfu of *S. enteritidis* per 0.5 ml milk (lanes 2 - 7).

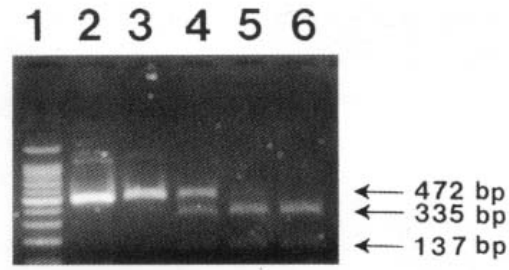

Fig. 3. Quantitation of *Salmonella enteritidis* DNA from milk.
The DNA bands were electrophoretically resolved in 2% agarose gel and stained with ethidium bromide. Upper band (472 bp) is derived from pGem-4Z-Sef B (-) standard DNA and lower bands (335 bp, 137 bp) are from target sequence after cleavage with *Bam* HI. 100 bp DNA ladder was used as size marker. lane 2: bands for 10^5 copies of standard DNA and 10^3 cfu *S. enteritidis* DNA, lane 3: bands for 10^4 copies of standard DNA and 10^3 cfu *S. enteritidis* DNA, lane 4: bands for 10^3 copies of standard DNA and 10^3 cfu S. enteritidis DNA, lane 5: bands for 10^2 copies of standard DNA and 10^3 cfu *S. enteritidis* DNA, lane 6: bands for 10 copies of standard DNA and 10^3 cfu *S. enteritidis* DNA.

enteritidis DNAs to be used as PCR templates were extracted from artificially inoculated milk by three methods (see Material & Methods). Boiling the inoculated milk in SDS/NaOH solution were ineffective. A method based on the lysis of bacteria using guanidine thiocyanate/phenol/chloroform took less than 2 h and yielded DNAs suitable for amplification by the PCR. The DNAs prepared by this method yielded about 50-100 fold as much PCR product as did DNA extracted with phenol-chloroform (data not shown). Therefore, template DNAs for the subsequent experiments were extracted exclusively by guanidine thiocyanate/phenol/chloroform method.

Detection of Salmonella from milk

Detection limits and sensitivities were examined with artificially inoculated milk samples. For this purpose, the sterile milk samples were inoculated with 1-10^5 cfu *S. enteritidis* per 0.5 ml. DNAs were extracted and were subjected to PCR amplification. The sensitivity limit of simple detection was 100 cfu per 0.5 ml milk (Fig. 2). Experiments were carried out 3 times and good reproducibility was observed. Increasing the cycle number or another round of PCR didn't raise the detection limit.

Semi-Quantitative PCR

For the quantification, standard plasmid pGem-4Z-Sef B(-) containing fimbrial antigen was co-amplified with genomic DNA from artificially inoculated *Salmonella* (Fig. 3). To establish conditions for exponential amplification of *S. enteritidis* specific sequences, kinetic analysis of Sef specific amplification using the pGem-4Z-Sef B(-) was performed. DG58 primer was end labeled with ^{32}P by using T4 polynucleotide kinase. PCR products were analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide. Bands corresponding to each specific products were excised from the gels and the amount of incorporated radioactivity was determined by scintillation counting. At cycle numbers 30, the slopes of the reactions using 1000 or 100 copies template were still in the exponential range (data not shown).

For the quantitation of *S. enteritis* DNA, PCR was carried out in the presence of a known copy numbers of competetor template molecules at various concentrations that recognize the same primers in the exponential range. Since *Bam* HI site was removed in the pGem-4Z-Sef B(-), 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 amplified DNA with *Bam* HI.(Fig. 3) Using this method, artificially inoculated milk samples were tested. From the competitive PCR, the

cfu and DNA copies number were almost equal.

Discussion

A rapid PCR-based method for the detection of *Salmonella* in milk was developed in this study. Specific primers for the fimbrial antigen of *S. enteritidis* were used for the PCR. For the DNA-template preparation, 3 protocols of cell lysis were compared. The protocol developed in this study has detection limit of 100 cfu per 0.5 ml milk (Fig. 2). For the whole procedure, it takes only 5 h to identify *S. enteritidis* in milk, while conventional method requires culturing followed by a series of presumative and confirmation tests that takes 5 to 7 days to complete.[1,3] Detection limit wasn't changed by increasing the cycle number or performing another round of PCR. It is possible that detection limit was defined by the limit of DNA extraction. Therefore, it would be necessary to enrich the milk in case it contains *Salmonella* less than 200 cfu per ml.

For the quantitation of *S. enteritidis*, PCR was carried out in the exponential range in the presence of competing template. Since the competing template pGem-4Z-Sef B(-) doesn't have *Bam* HI site, initial concentration of the target DNA could be estimated by comparing the intensity of the two bands after digesting with *Bam* HI (Fig. 3, 335, 472 base pairs each). The difference in the melting temperature as well as the efficiency of polymerization might be negligible because the amplified fragments are almost equal (only 4 bases difference) and the same primers are used for the amplification. Practically, calculated DNA copy number of reference DNA and colony forming units were almost equal in this procedure (Fig. 3). However, it shohld be mentioned that with this method it is only possible to estimate the DNA copy numcer and cannot count the exact one.

In conclusion, the PCR-based protocol described in this paper provides a rapid, simple, and sensitive method for detecting *S. enteritis* in milk. An estimation of DNA copy number is possible by competitive PCR using cloned reference competitor DNA and *S. enteritidis* genomic DNA from milk as template. The result was well correlated with plating method.

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국문요약

우유에 포함된 *Salmonella enteritidis*를 효과적으로 분리하는 방법을 찾고 이를 이용하여 우유속의 *S. enteritidis*의 량을 추정하는 방법을 개발하였다. 일정량의 *S. enteritidis*를 접종한 우유로부터 guanidine thiocyanate/phenol/chloroform을 이용하여 DNA를 추출한 후 중합효소반응으로 *S. enteritidis* 섬모항원 유전자를 선택적으로 검출함으로써 우유 1 ml당 200 colony forming unit까지 검출이 가능하였고 전체 과정의 수행에 단지 5시간 정도 걸렸다. *S. enteritidis* 섬모항원 유전자를 cloning한 pGem-4Z-Sef B(-) DNA와 인위적으로 접종된 우유로부터 추출한 *Salmonella* DNA를 함께 중합효소반응으로 증폭한 후 제한효소로 잘라 전기영동을 행하여 band의 강도를 비교함으로써 *Salmonella* DNA copy수를 추정하는 것이 가능하였다.

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