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Rapid detection of *Salmonella* in milk by combined immunomagnetic separation-polymerase chain reaction assay

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ABSTRACT

During the past few years, milk has presented a risk of *Salmonella* contamination; it has been implicated as the cause in several outbreaks of salmonellosis. Because conventional detection methods require 5 to 7 d for completion and involve several subcultivation stages followed by biochemical and serological tests, rapid and sensitive methods have been sought, mainly at the DNA level. Therefore, a study including milk samples was conducted to evaluate the performance of a combination of 2 techniques—immunomagnetic separation and polymerase chain reaction (PCR)—for the detection of *Salmonella*. The 16-, 14-, 12-, 10-, and 8-h nonselective pre-enrichment steps before immunomagnetic separation and the high-pure DNA preparation method before PCR were used in a combined assay. Milk samples, which were found to be *Salmonella*-negative by a reference method, were first inoculated with *Salmonella* Enteritidis. Next, the shortest pre-enrichment time that is required for detection of 1 or 10 cfu of *Salmonella*/mL by combined immunomagnetic separation-PCR assay was found by using 16-, 14-, 12-, 10-, and 8-h incubation periods. The detection limit using a 16-, 14-, or 12-h nonselective pre-enrichment was 1 to 10 cfu/mL. However, the sensitivity decreased to 10^1 and 10^2 cfu/mL, respectively, when 10- and 8-h pre-enrichments were used. This assay, in conjunction with a 12-h pre-enrichment, proved to be rapid (overall 16 h) and sensitive (1–10 cfu/mL) for the detection of *Salmonella* in milk samples and promising for routine use in the detection of *Salmonella* in milk.
Key words: immunomagnetic separation, polymerase chain reaction, *Salmonella*

INTRODUCTION

Salmonella has long been responsible for the largest number of food-poisoning outbreaks worldwide (CDC, 2003, 2007). It is estimated that approximately 1.4 million illnesses and 600 deaths are caused by nontyphoidal *Salmonella* serovars each year in the United States (Mead et al., 1999). As public awareness of the health-related impact of *Salmonella* contamination of food has increased, the development of rapid detection methods for the purpose of diagnosis, and consequently for the prevention of contamination of food, has increased (Mandrell and Wachtel, 1999).

Conventional methods for detecting *Salmonella* in food are time consuming and labor intensive, and require costly handling during analysis; therefore, rapid methods for isolation and identification of *Salmonella* in food have been developed (Garcia-del Portillo, 2000). These rapid methods often suffer from a lack of specificity or sensitivity or require expensive instruments and technically qualified personnel (Mercanoglu and Griffiths, 2005); however, PCR targeting specific genetic markers represents a major advance in terms of the speed, specificity, and sensitivity to improve food safety (Jeníková et al., 2000).

The use of PCR may result in false-negative results because of low levels of target DNA or the presence of residual food constituents in food samples (Soumet et al., 1999; Špánová et al., 2003; Löfström et al., 2004). To overcome these problems, immunomagnetic separation (**IMS**) has been suggested to eliminate the need for selective enrichment of low levels of target DNA and to remove possible inhibitory substances (Rijpens et al., 1999; Hsih and Tsen, 2001). Moreover, IMS is used for separation and concentration of the target microbial cells from food containing inhibitors or competitive microflora and can be used instead of selective enrichment in many cases (Skjerve and Olsvik, 1991; Mansfield and Forsythe, 1996).

Analyzing milk for *Salmonella* is not common but outbreaks of salmonellosis initiated by consumption of raw milk or products produced from raw milk in the United States and other countries (Cody et al., 1999; Haeghebaert et al., 2003; Mazurek et al., 2004) has proven that milk also presents a risk of *Salmonella* contamination. Therefore, in this study we present a specific, sensitive and rapid (16 h overall) detection as-

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say by evaluating IMS and PCR for the detection of *Salmonella* spp. in spiked milk samples. The combined assay was tested in conjunction with nonselective pre-enrichment in buffered peptone water (**BPW**) for 8, 10, 12, 14, and 16 h to compare its sensitivity under different conditions.

MATERIALS AND METHODS

Inoculation of Milk Samples

Salmonella enterica ssp. *enterica* serovar Enteritidis (*Salmonella* Enteritidis; ATCC 13076) used in this study was inoculated into tryptic soy (**TS**) broth (Merck, Darmstadt, Germany) and incubated at 37°C. This *Salmonella* Enteritidis culture was diluted with a lambda buffer [5.8 g of NaCl, 2.0 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of gelatin 0.01%, 50 mL of 1 *M* Tris-HCl (pH 7.5) in 1 L of distilled water] to achieve final cell concentrations increasing in 1-log-cycle increments between 10^0 and 10^8 cfu/mL; spread plate counts were performed in triplicate for each sample using TS agar (Merck) with overnight incubation at 37°C. One-milliliter aliquots of the suitable dilutions (10^1, 10^2, and 10^3 cfu/mL; the average spread plate counts of these dilutions were 12, 123, and 1,089 cfu, respectively) were subsequently taken as inocula for 9-mL milk samples in which no *Salmonella* contamination had been determined by using the ISO 6579 reference method (ISO, 2002). Each of these inoculated samples was mixed in 90 mL of BPW (Merck) that was used for the nonselective pre-enrichment of bacterial cells in milk samples. After mixing, the mixed sample was incubated for 8, 10, 12, 14, and 16 h at 37°C to find the shortest nonselective pre-enrichment time needed for the detection of approximately 1 to 10 cfu of the target bacteria/mL of milk using the combined IMS-PCR assay. An uninoculated milk sample served as negative control. After 8, 10, 12, 14, and 16 h of pre-enrichment of all samples, a 1-mL portion of each sample was subjected to the IMS technique. Each experiment was repeated 5 times.

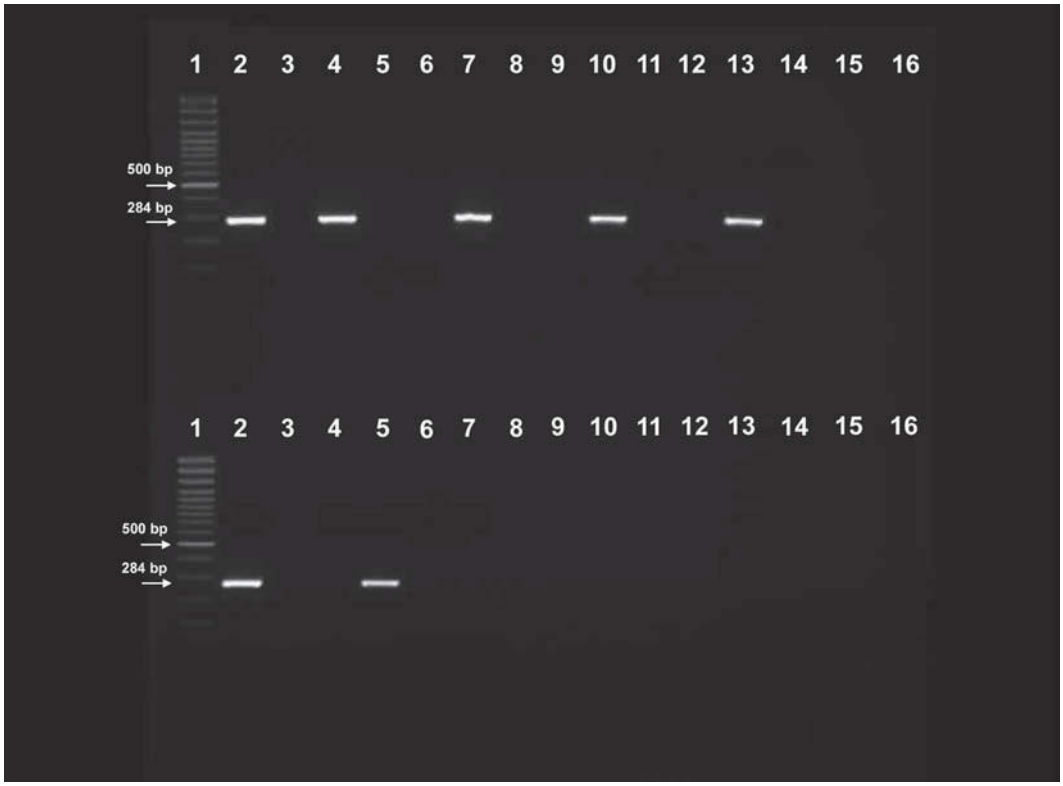

Figure 1. Agarose gel analysis of amplified DNA obtained from 1-mL artificially contaminated milk samples. Samples were collected after 8 h of pre-incubation and immunomagnetic separation. Lane 1 (top and bottom) = 100-bp molecular weight ladder; lane 2 (top and bottom) = positive control; lanes 4, 5, and 6 (top) = first replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lanes 7, 8, and 9 (top) = second replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lanes 10, 11, and 12 (top) = third replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lanes 13 and 14 (top) and 4 (bottom) = fourth replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lanes 5, 6, and 7 (bottom) = fifth replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lane 16 (top and bottom) = negative control; lanes 3 and 15 were empty.

Magnetic Separation

Anti-*Salmonella* beads (20 μL) were incubated with 1 mL of each pre-enriched sample at room temperature for 20 min with continuous rotation at maximum speed on Dynal MX3 sample mixer (Dynal, Oslo, Norway), so that the target bacteria from the pre-enriched samples were specifically bound onto anti-*Salmonella* Dynabeads (Dynal). These complexes of bacteria and beads were then placed in a magnetic particle concentrator for 3 min to separate the complexes from the sample. Washing buffer [1 mL of 0.15 *M* NaCl, 0.01 *M* sodium phosphate buffer (pH 7.4), 0.05% Tween 20] was added to wash the complexes. The separation steps were repeated 3 times to remove food debris and other microorganisms. The beads were then resuspended in 200 μL of the lambda buffer.

DNA Isolation Procedure

All suspensions were treated with a high-pure PCR template preparation (HPPTP) kit (Roche, Mannheim, Germany), and the DNA isolation procedure was performed as in the study of Mercanoglu and Griffiths (2005). According to this procedure, 5 μL of a 10 mg/mL lysozyme solution (Roche) was added to each 200-μL bead-bacteria suspension and the suspension was then incubated at 37°C for 15 min. Next, 200 μL of binding buffer and 40 μL of proteinase K were added and the suspension was incubated at 70°C for 10 min. The samples were then mixed with 100 μL of isopropanol and each suspension was applied to the combined filter-collection tube. After centrifugation at 8,000 × *g* for 1 min (Hettich Mikro 200, Tuttlingen, Germany), the filter was washed once with 500 μL of inhibitor removal buffer and twice with 500 μL of wash buffer from the HPPTP kit. The final DNA extract was eluted in 200 μL of prewarmed (70°C) elution buffer and centrifuged at 8,000 × *g* for 1 min. The resulting template DNA was subjected to PCR.

PCR Conditions and DNA Analysis

The purified DNA (1.5 μL) was subjected to PCR in 50 μL of PCR mixture comprising 0.5 μ*M* of each

Figure 2. Agarose gel analysis of amplified DNA obtained from 1-mL artificially contaminated milk samples. Samples were collected after 10 h of pre-incubation and immunomagnetic separation. Lane 1 (top and bottom) = 100-bp molecular weight ladder; lane 2 (top and bottom) = positive control; lanes 4, 5, and 6 (top) = first replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lanes 7, 8, and 9 (top) = second replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lanes 10, 11, and 12 (top) = third replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lanes 13 and 14 (top) and 4 (bottom) = fourth replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lanes 5, 6, and 7 (bottom) = fifth replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lane 16 (top and bottom) = negative control; lanes 3 and 15 were empty.

primer [based on the sequence of *invA* gene, 139: (5′-GTG AAA TTA TCG CCA CGT TCG GGC AA-3′) and 141: (5′-TCA TCG CAC CGT CAA AGG AAC C-3′) (MWG Biotech, Ebersberg, Germany) originally designed by Rahn et al., 1992], 200 μ*M* of each dNTP, 2 m*M* $MgCl_2$ (with 1× PCR buffer, containing 10 m*M* Tris, 50 m*M* KCl, pH 8.3), 0.025 U/μL FastStart Taq DNA polymerase (Roche), and ultrapure water. The mixture was subjected to 35 cycles in a Primus 96 thermocycler (THE-MWG). The amplification reactions included an initial denaturation step at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 52°C for 45 s, and extension at 72°C for 60 s. A final elongation of 72°C for 7 min was also applied. The PCR products were detected in 1.5% agarose gel (that was stained with 1 mg/mL ethidium bromide solution), with a 100-bp GeneRuler DNA ladder plus (ready-to-use, Fermentas, Vilnius, Lithuania), in Tris-borate EDTA buffer; bands were visualized by using the InGenius gel visualization and analysis system (Syngene, Cambridge, UK). The purified DNA from *Salmonella* Enteritidis was used as a positive control and the DNA from the uninoculated, *Salmonella*-free milk sample served as a negative control. A positive result (i.e., the *Salmonella*-specific band) was indicated by a fluorescent band at 284 bp. Additional confirmation was established by utilizing the ABI Prism 310 DNA sequencing system (Applied Biosystems, Foster City, CA).

RESULTS AND DISCUSSION

In this study, a rapid combined assay based on *Salmonella*-specific magnetic beads and PCR technique targeting the *invA* gene was optimized for the detection of *Salmonella* in milk samples. The assay consisted of a nonselective pre-enrichment stage of the milk samples in BPW carried out over 8, 10, 12, 14, and 16 h, followed by magnetic separation and isolation and purification of the DNA. Finally, the DNA was analyzed by PCR for the presence of *Salmonella* Enteritidis. The expected molecular weight for the PCR products amplified from *Salmonella* with primers 139 and 141 was 284 bp. Non-*Salmonella* bacteria,

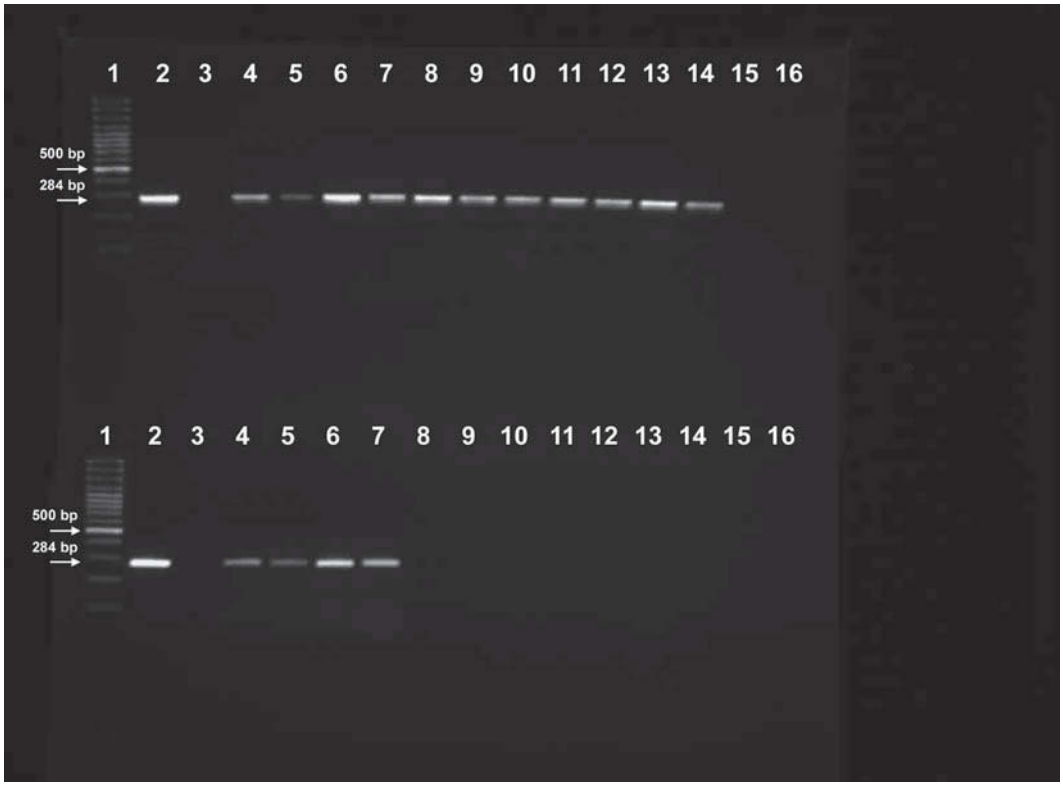

Figure 3. Agarose gel analysis of amplified DNA obtained from 1-mL artificially contaminated milk samples. Samples were collected after 12 h of pre-incubation and immunomagnetic separation. Lane 1 (top and bottom) = 100-bp molecular weight ladder; lane 2 (top and bottom) = positive control; lanes 4, 5, and 6 (top) = first replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lanes 7, 8, and 9 (top) = second replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lanes 10, 11, and 12 (top) = third replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lanes 13 and 14 (top) and 4 (bottom) = fourth replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lanes 5, 6, and 7 (bottom) = fifth replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lane 16 (top and bottom) = negative control; lanes 3 and 15 were empty.

which may bind nonspecifically to the beads or show cross-reactivity with the antibody, were not detected in the PCR when this *Salmonella*-specific primer pair was used; all uninoculated milk control samples tested negative. In addition, DNA sequencing was applied to all positive samples for additional confirmation; DNA analysis of sequenced amplicons showed 97% homology to the *inv*A gene sequence in GenBank (accession no. AL627276.1; data not shown).

Figures 1, 2, 3, 4, and 5 present agarose gel analysis of amplified DNA obtained from 1-mL artificially contaminated milk samples, which were collected after 8, 10, 12, 14, and 16 h, respectively, of preincubation and magnetic separation. Because of these repeated experiments, it was found that the combined IMS-PCR assay was sensitive and capable of identifying *Salmonella* at approximately 1 to 10 cfu in 1-mL milk samples after at least a 12-h pre-enrichment step. However, the minimum number of *Salmonella* detectable by the combined IMS-PCR assay increased to 10 and 100 cfu after 10- and 8-h pre-enrichment steps, respectively. These results are presented in Table 1. Using the combined assay, an overall 16-h analysis time (combined IMS-PCR assay in conjunction with a 12-h nonselective pre-enrichment) was evaluated as sufficient for detection of approximately 1 to 10 cfu of *Salmonella*/mL of milk.

Oliveira et al. (2002) demonstrated the specificity of the 139 and 141 primers to the genus *Salmonella* with 135 *Salmonella* strains and 13 other genera; their results mostly agreed with the work of Rahn et al. (1992). In addition, Mercanoglu and Griffiths (2005) checked the specificity of these primers to several *Salmonella* strains and strains belonging to other common foodborne bacterial genera and reported no cross-reactivity with strains other than *Salmonella*.

The specificity level of magnetic separation that was used both for concentration of the *Salmonella* density to a detectable level by PCR and for reduction of PCR inhibitors by the inclusion of washing steps was also demonstrated in our previous study (Mercanoglu and Aytac, 2002). Špánová et al. (2003) concluded that a higher number of false-negative results was obtained when using PCR without magnetic separation compared with PCR with magnetic separation. Stevens and

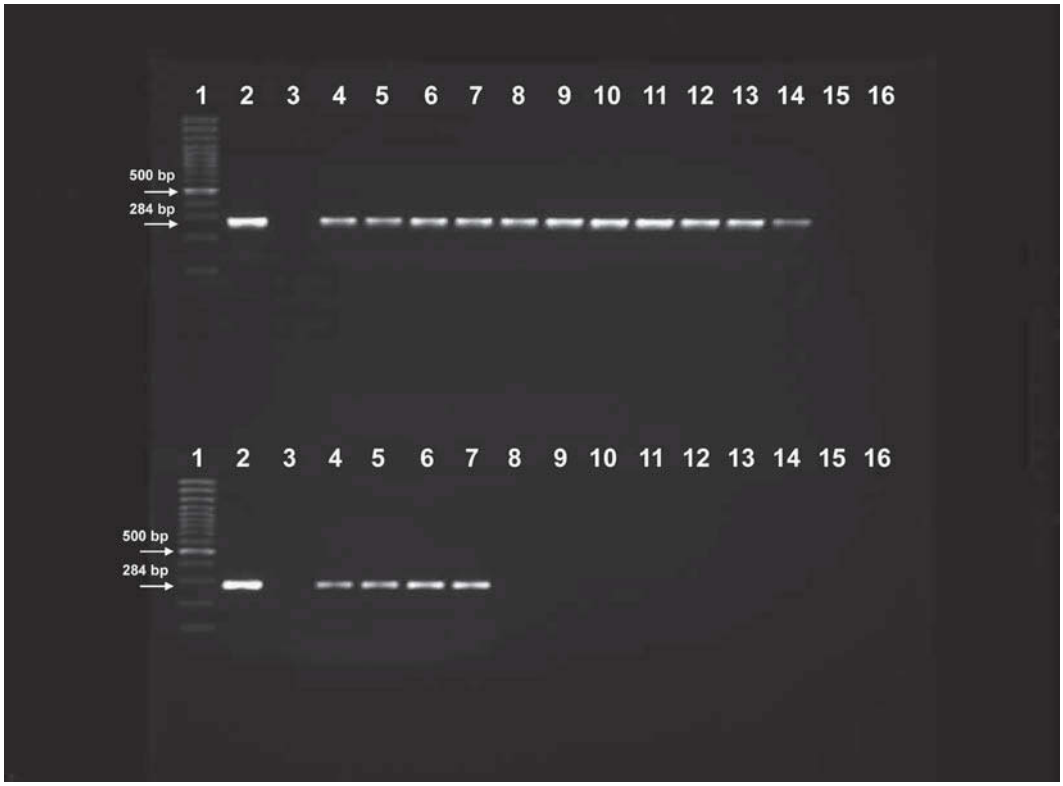

Figure 4. Agarose gel analysis of amplified DNA obtained from 1-mL artificially contaminated milk samples. Samples were collected after 14 h of pre-incubation and immunomagnetic separation. Lane 1 (top and bottom) = 100-bp molecular weight ladder; lane 2 (top and bottom) = positive control; lanes 4, 5, and 6 (top) = first replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lanes 7, 8, and 9 (top) = second replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lanes 10, 11, and 12 (top) = third replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lanes 13 and 14 (top) and 4 (bottom) = fourth replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lanes 5, 6, and 7 (bottom) = fifth replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lane 16 (top and bottom) = negative control; lanes 3 and 15 were empty.

Table 1. Frequency of detection of *Salmonella* in artificially contaminated milk samples[1] by combined immunomagnetic separation-PCR assay with pre-enrichment of 8, 10, 12, 14, or 16 h

Inoculation level, cfu/mL of milk	Positive samples after pre-enrichment[2]				
	8 h	10 h	12 h	14 h	16 h
10^2	5/5	5/5	5/5	5/5	5/5
10^1	0/5	5/5	5/5	5/5	5/5
10^0 (= 1)	0/5	0/5	5/5	5/5	5/5

[1]No amplification was observed in the uninoculated 1-mL milk sample.

[2]Frequency of detection is the number of *Salmonella* positive samples/number of samples tested.

Jaykus (2004) also focused on a dairy matrix-associated PCR inhibition when PCR was used as the only technique for determination of *Salmonella*. Therefore, in this study, magnetic beads were used in conjunction with PCR and the shortest overall analysis time was optimized for the determination of approximately 1 to 10 cfu of *Salmonella*/mL of milk.

Although Widjojoatmodjo et al. (1991) were able to detect 100 cfu of *Salmonella* in unenriched food samples that underwent magnetic separation and PCR, a pre-enrichment step is highly recommended in detection of *Salmonella* in foods because the detection level of most PCR assays for *Salmonella* in unenriched food samples is generally high and the infective dose is low (Suslow et al., 2002). Rijpens et al. (1999) showed that a minimum pre-enrichment time of 16 h is required to obtain a PCR-detectable amount of *Salmonella*. They demonstrated that 5.9 cfu of *Salmonella* from 25 g of egg, ice cream, and cheese samples can be isolated by PCR after a 16-h pre-enrichment in BPW, followed by magnetic separation and a 4-h selective enrichment in Rappaport-Vassiliadis broth. Thus, in this study, pre-enrichment steps of 8, 10, 12, 14, and 16 h in BPW were studied to find the shortest nonselective pre-enrichment time needed for the detection of levels of target bacteria of approximately 1 to 10 cfu/mL of milk by the combined IMS-PCR assay.

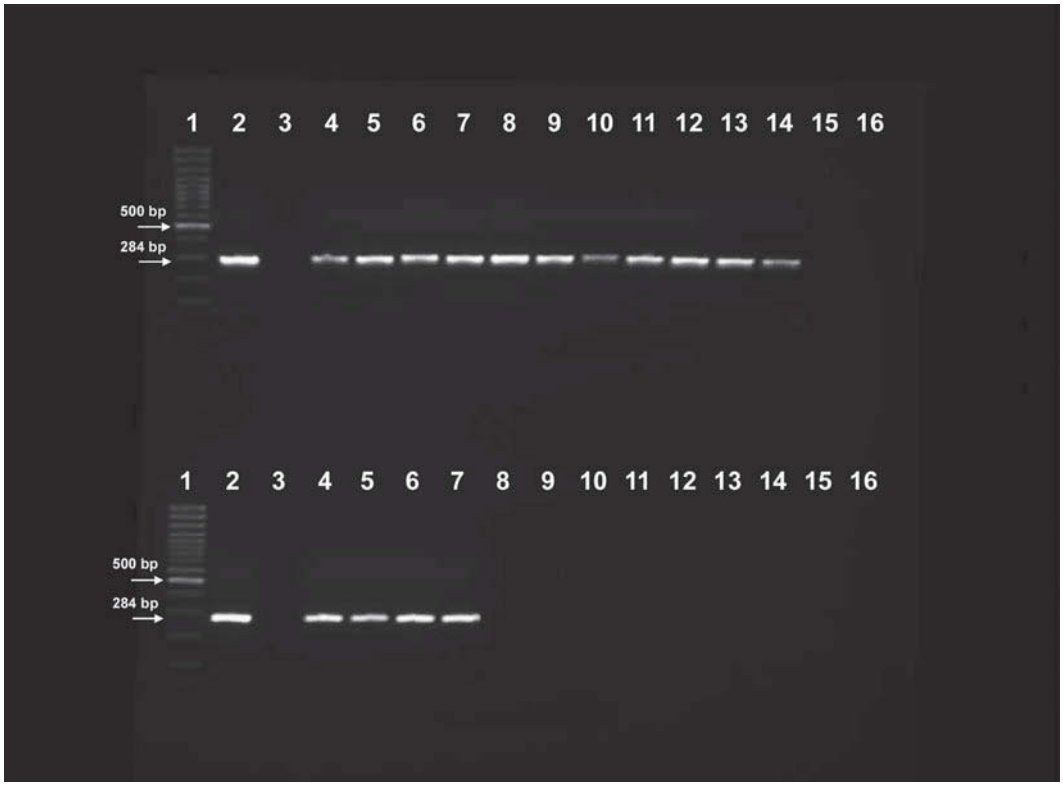

Figure 5. Agarose gel analysis of amplified DNA obtained from 1-mL artificially contaminated milk samples. Samples were collected after 16 h of pre-incubation and immunomagnetic separation. Lane 1 (top and bottom) = 100-bp molecular weight ladder; lane 2 (top and bottom) = positive control; lanes 4, 5, and 6 (top) = first replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lanes 7, 8, and 9 (top) = second replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lanes 10, 11, and 12 (top) = third replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lanes 13 and 14 (top) and 4 (bottom) = fourth replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lanes 5, 6, and 7 (bottom) = fifth replicate with 10^2, 10^1, and 1 cfu/mL of milk, respectively; lane 16 (top and bottom) = negative control; lanes 3 and 15 were empty.

CONCLUSIONS

Some components in milk may inhibit PCR or impair the sensitivity of PCR, and these factors may be removed by using the IMS technique before PCR; this study demonstrates that by using the combined IMS-PCR assay, it is possible to detect approximately 1 to 10 cfu of *Salmonella* in 1-mL milk samples in an overall 16-h method (12 h for pre-enrichment and 4 h for IMS, DNA isolation, PCR, and gel electrophoresis). Therefore, this study may aid the dairy industry and public health authorities in identifying and recalling *Salmonella*-contaminated milk as quickly as possible. Because the IMS technique saves 1 d over conventional methods by concentrating *Salmonella* and reduces potential PCR inhibitors through the washing steps, this rapid, sensitive, and specific combined assay could be applied to other food and clinical samples. In conclusion, this combined assay is suitable as a specific, sensitive, and rapid screening method for the detection of *Salmonella* spp. in milk compared with conventional detection methods.

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