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Detection of Salmonella spp., Salmonella Enteritidis, Salmonella Typhi and Salmonella Typhimurium in cream cakes by polymerase chain reaction (PCR)

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Summary

The aims of this study were: (i) to determine the incidence of Salmonella spp. by conventional culture methods in cream cakes, (ii) to detect invA gene by PCR for the confirmation of the isolates, (iii) to analyze isolates for S. Enteritidis, S. Typhi, and S. Typhimurium by PCR based on Sdf I, ViaB, Spy gene sequences, respectively, and (iv) to identify isolates in comparison to the reference strain by DNA sequence analysis.

A total of 81 unpackaged cream cake samples were obtained from different patisseries in Hatay, Turkey. Salmonella spp. was detected in 13 (16%) out of 81 cream cake samples by the conventional culture method. A total of 45 isolates from the 13 positive samples were confirmed as Salmonella spp. by PCR. Homology among the reference strain and isolates and homology within the isolates was found to be 98.97-100%.

Cream cake samples analyzed in this study were found to be contaminated with Salmonella, thus posing a potential health hazard for the consumer. To protect public health, food safety management systems such as HACCP, GMP and GHP could be carried out in cream cake production.

Keywords: Salmonella, cream cake, invA gene, sequence analysis

Salmonellosis is a major foodborne disease posing a public health risk. A global survey carried out in 1995 showed that *S.* Enteritidis, *S.* Typhimurium, and *S.* Typhi were the most frequently isolated serotypes in humans. *S.* Typhi is a human specific serotype and causes typhoid fever. *S.* Enteritidis and *S.* Typhimurium are the major causes of gastroenteritis and are found in both humans and animals (3, 5, 7, 10, 16).

The cream cake is an ideal medium for the growth of many pathogenic microorganisms, as it has a rich nutrient content, high moisture, and an appropriate pH and water activity (a_w) (4, 18, 20). Eggs and milk provide a potential source of *Salmonella* spp. in cream cakes. Egg contamination by *Salmonella* spp. can be caused by penetration of the shell by organisms as eggs pass through the cloaca or by transovarial transmission (8).

Bakery ingredients can usually be contaminated with *Salmonella* through inadequate pasteurisation or by cross-contamination. *Salmonella* can be destroyed through pasteurisation, but the use of raw and polluted eggs in the icing increases the risk of contamination (3, 19). Because bakery products may contain *Salmonella* spp., to detect the incidence of *Salmonella* and its serotypes Enteritidis, Typhi, and Typhimurium in cream cakes is very important for public health. Recently, PCR-based methods have been used effectively for the detection and identification of the *Salmonella* serovars.

The aims of this study were: (i) to determine the incidence of *Salmonella* spp. in cream cakes by conventional culture methods, (ii) to detect *invA* gene by PCR for the confirmation of the isolates, (iii) to analyse isolates for *S.* Enteritidis, *S.* Typhi and *S.* Typhimurium by PCR based on *Sdf I*, *ViaB*, and *Spy* gene sequences, respectively, and (iv) to identify isolates in comparison to the reference strain by DNA sequence analysis.

Tab. 1. Target genes, the size of amplified products, and primers used in this study

Bacteria	Target gene	Primer sequence (5'-3')	Size (bp)	Reference
Salmonella spp.	invA	Forward Primer: GTGAAATTATCGCCACGTTCGGGCAA Reverse Primer: TCATCGCACCGTCAAAGGAACC	284	(17)
S. Enteritidis	Sdf I	Forward Primer: TGTGTTTTATCTGATGCAAGAGG Reverse Primer: TGAACTACGTTCGTTCTTCTGG	304	(1)
S. Typhi	ViaB	Forward Primer: CACGCACCATCATTTCACCG Reverse Primer: AACAGGCTGTAGCGATTTAGG	738	(10)
S. Typhimurium	Spy	Forward Primer: TTGTTCACTTTTTACCCCTGAA Reverse Primer: CCCTGACAGCCGTTAGATATT	401	(13)

Material and methods

Sample collection. A total of 81 unpackaged cream cake samples were obtained from different patisseries in Hatay, Turkey. The samples were transported to the laboratory at +4°C and analyzed on the same day.

Conventional culture method. Cream cake samples were analyzed for *Salmonella* spp. according to the ISO 6579:2002 reference method. From each sample, 25 g was homogenized with 225 mL of buffered peptone water (Oxoid, Basingstoke, Hampshire, England) for preenrichment. After incubation at 37°C for 24 h, 0.1 mL was transferred to 10 mL of selective Rappaport-Vassiliadis broth (Oxoid) and incubated for 24 h at 42°C. A loopful of broth culture was streaked on xylose-lysine-desoxycholate-agar (XLD agar) (Oxoid), Brilliance Salmonella agar (Oxoid), and incubated at 37°C for 24 to 48 h.

Presumptive *Salmonella* colonies were identified on the basis of Gram stain and standard biochemical tests (oxidation/fermentation of glucose, lysine decarboxylation, urease test) and confirmed with *Salmonella* latex test (Oxoid). Each isolate of *Salmonella* spp. was stored at –20°C in cryovials until PCR analysis.

Bacterial strains. Reference strains of *S.* Typhimurium ATCC 14028, *S.* Enteritidis and *S.* Typhi were used as positive controls in this study. The *S.* Enteritidis strain was provided by the Department of Microbiology, Faculty of Veterinary Medicine, University of Erciyes, Kayseri, Turkey. The *S.* Typhi strain was obtained from the Department of National Type Culture Collection Laboratories, Public Health Institutes of Turkey.

PCR method. A fragment of 284 base pairs (bp) of the *invA* gene was used for genus identification. This gene encodes an important function that allows *Salmonella* to enter intestinal epithelial cells. A 304 bp *Sdf I* gene, 401 bp *Spy* gene, and 738 bp *ViaB* gene fragments were selected, respectively, for *S.* Enteritidis, *S.* Typhimurium and *S.* Typhi. The primers used in this study are shown in Tab. 1.

From the overnight broth culture of each isolate, 1 mL was transferred to Eppendorf tubes and centrifuged for 2 min at 6,000 × g. Then supernatant was completely removed. DNA extraction was performed with Bacterial DNA Extraction kit (Vivantis, Malaysia) following the manufacturer's instructions. Cell suspensions were treated with lysozyme and proteinase K. After this treatment, these cell lysates were stored at –20°C.

The PCR assay was carried out in a 50 µl reaction solution containing 0.5 µM of each primer, 20 units mL^{-1} Phusion DNA polymerase, 0.2 mM each of dNTP, 1X Phusion HF Buffer, and 10 µL template DNA using Phusion High Fidelity PCR Master Mix (Biolabs, New England).

PCR amplification conditions suggested by Phusion High Fidelity PCR Master Mix were applied by modifying the following: initial denaturation of 98°C for 30 s, followed by 30 cycles, each consisting of 98°C for 7 s, 60°C for 20 s, 72°C for 20 s, and a final extension cycle of 7 min at 72°C (Boeco, Hamburg, Germany).

In this study, the primers' annealing temperature for *Salmonella* spp., *S.* Enteritidis, *S.* Typhi and *S.* Typhimurium was applied at 60°C. *Salmonella* spp., *S.* Enteritidis, and *S.* Typhi were detected in this primer annealing temperature, but *S.* Typhimurium was not detected. When the primer annealing temperature was modified as 20 seconds at 55°C, *S.* Typhimurium was detected.

Amplification products were detected by agarose gel (1.5%) electrophoresis performed at 120 V for 40 min (Cleaver, CS-300V, England) and visualised under UV transillumination (UVP, Upland, USA).

DNA sequence analysis. DNA sequence analyses of the isolates were performed. The *invA* amplification products were purified using Agencourt Ampure purification kit (Beckman Coulter, Beverly, USA). The sequence reaction was carried out using a Dye Terminator Cycle Sequencing Quick Start kit (Beckman Coulter).

Sequence PCR products were purified using a Dye-Terminator removal kit (Agencourt CleanSEQ; Beckman Coulter). DNA sequences of the purified products were identified using Beckman Coulter 8000 equipment. The isolates were identified through comparison to the DNA reference strain (*S.* Typhimurium partial 16S rRNA gene, strain ATCC 14028, GenBank: FM207099.1) with data stored in the GenBank using the Basic Local Alignment Search Tool (BLAST) program.

Results and discussion

Salmonella spp. was detected in 13 (16%) out of 81 analyzed samples. A total of 45 isolates from the 13 positive samples were investigated in this study. The *invA* gene was detected in all 45 (100%) isolates by PCR (Fig. 1). The PCR of positive controls for

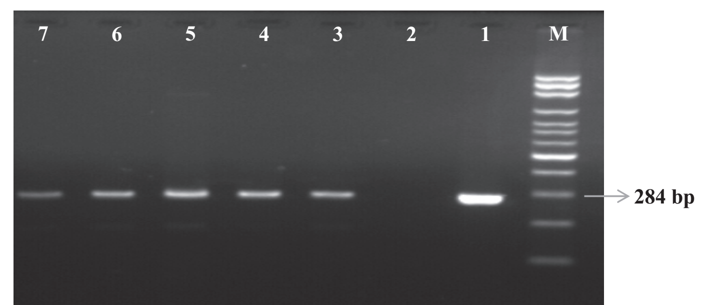

Fig. 1. Electrophoresis image of *invA* positive *Salmonella* spp. isolates

Explanations: M: 100 bp DNA marker (Axygen), 1: Positive control (*S.* Typhimurium ATCC 14028), 2: Negative control (Nuclease free water; Promega, Madison, USA), 3-7: *invA* positive *Salmonella* spp. isolates

Salmonella genus and its serotypes *S.* Enteritidis, *S.* Typhi, and *S.* Typhimurium is shown in Fig. 2.

No isolates were detected as *S.* Enteritidis, *S.* Typhi, or *S.* Typhimurium when PCR was applied on the isolates identified as *Salmonella* positive (the average annealing temperature of primers was arranged to 60°C for PCR assay). When the annealing temperature was applied at 55°C only for *S.* Typhimurium, all of the isolates were defined as *S.* Typhimurium (Fig. 3).

After PCR, DNA sequence analyses of the isolates were performed and the homology among the reference strain and isolates and homology within the isolates was 98.97-100%. Nucleotide sequence data of the isolates were entered into the BLAST program. All of the isolates were defined as *Salmonella* enterica subsp. enterica serovar Typhimurium in the GenBank.

According to studies performed in different parts of Turkey, Elmali et al. (4) isolated *Salmonella* spp. from 6 of the 75 cream pastries (8%) by conventional culture method; three from fruit cream, one from chocolate cream, and two from butter cream pastries. This level of contamination is lower than the results of the current study.

Unlike this study, Gumus et al. (6) and Siriken et al. (18) did not find *Salmonella* in any of the cream cake samples. It was reported that cream cake samples could be a potential risk for *Bacillus cereus* (18).

A total of 555 egg and egg products (250 chicken eggs, 180 quail eggs, 100 mayonnaises, and 25 icings) collected from the Ankara region were examined by Öktem et al. (14) and *Salmonella* was isolated in 15 samples (6%) of chicken eggs. However, *Salmonella* was not isolated from quail eggs, mayonnaise, or icing samples.

The authors think that poor microbiological quality of the ingredients (e.g. raw and polluted eggs) in cake mixes, cross-contamination, food handlers and also storage at improper holding temperatures prior to serving could contribute to contamination of cream cakes with *Salmonella*.

In Wales, between the years 2003-2005, 3391 ready-to-eat foods, including dairy cream cakes, were examined for microbiological quality. *Salmonella* and *L. monocytogenes* were not detected in dairy cream cakes, but they were found unsatisfactory for *E. coli*, *S. aureus*, and *B. cereus* (12). On the other hand, Kotzekidou (9) detected *Salmonella* spp. at a higher percentage (28.6%) in frozen pastries.

Piknova et al. (15) used pre-PCR enrichment protocol in order to achieve the same sensitivity of the method to *Salmonella* serovars as in ISO 6579. At the evaluation of the PCR-based method on food samples (including cream cakes, ice cream, mayonnaise, egg mélange, minced and separated meat, ham, and salami) identical results as with the reference method were obtained. *Salmonella* was not detected in cream cakes by PCR technique parallel to ISO 6579. It was reported that the PCR-based method may be used as a faster alternative for the detection of *Salmonella* in food.

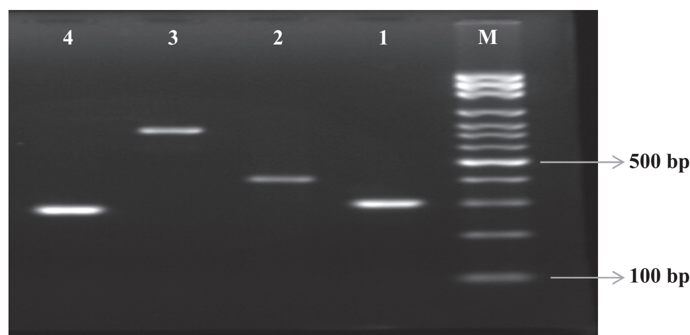

Fig. 2. PCR of positive controls for *Salmonella* genus and its serotypes Enteritidis, Typhi and Typhimurium
Explanations: M: 100 bp DNA marker (Axygen), 1: 304 bp fragment of serotype Enteritidis, 2: 401 bp fragment of serotype Typhimurium, 3: 738 bp fragment of serotype Typhi, 4: Fragment of 284 bp of *Salmonella* spp.

Fig. 3. PCR for identification of *Salmonella* Typhimurium
Explanations: M: 100 bp DNA marker (Axygen), 1: Positive control (*Salmonella* Typhimurium ATCC 14028), 9: Negative control (Nuclease free water; Promega, Madison, USA), 2-8: Positive isolates for the serovar Typhimurium

De Freitas et al. (5) adapted multiplex PCR (m-PCR) for the detection of *Salmonella* spp., as well as serotypes *S.* Enteritidis, *S.* Typhi, and *S.* Typhimurium in poultry meat. Primer sequences and the annealing temperature of primers for *S.* Typhimurium were selected in the same way as in this study. Results indicate that the m-PCR was able to detect bacteria in a short period of time.

A collaborative study involving four European laboratories was conducted to investigate the diagnostic accuracy of a *Salmonella* specific PCR-based method. Similar to our study, a 284 bp sequence of the *invA* gene was used for specific amplification of *Salmonella* DNA. The interlaboratory diagnostic accuracy was found to be 97.5% and showed the applicability of the PCR technique as an alternative to conventional culture method (11).

In Japan, Akiba et al. (2) developed m-PCR assays for identifying the seven major serovars of *Salmonella*, i.e. *S.* Typhimurium, *S.* Choleraesuis, *S.* Infantis, *S.* Hadar, *S.* Enteritidis, *S.* Dublin, and *S.* Gallinarum. As in the current study, the *Salmonella*-specific *invA*

gene was used to confirm the isolates. The annealing temperature of the primers was established at 60°C for 30 s and 68°C for 30 s. However, it was not clearly explained which annealing temperature was selected for amplification of each one of the serotypes. In this study, the annealing temperature was applied at 55°C only for *S.* Typhimurium as different from the annealing temperature selected for *S.* Typhi and *S.* Enteritidis. Although Akiba et al. (2) observed false-positive results they indicated that these assays were sufficiently specific for identifying the seven *Salmonella* serovars.

In another study, Pui et al. (16) optimized m-PCR for the detection of *Salmonella* spp., *S.* Typhi, and *S.* Typhimurium. Primer pairs selected for m-PCR were different from the primers used in the present study. Their results showed that the annealing temperature most appropriate for all three *Salmonella* groups (*Salmonella* spp., *S.* Typhi, and *S.* Typhimurium) was 53°C.

A PCR assay in the multiplex format was also developed by Kumar et al. (10) for the specific detection of *S.* Typhi from water and food samples. Primers for *invA*, *prt*, *fliC-d*, and *viaB* genes were used and 60°C was applied as the annealing temperature. All cultures of *Salmonella* were identified by the PCR assay with no nonspecific amplification in other cultures. They indicate that the assay can be useful for identifying *S.* Typhi in environmental samples.

As a result, cream cake samples analyzed in this study were found to be contaminated with *Salmonella*; therefore, taking hygienic measures is necessary for both food safety and food quality.

Conventional culture methods used for the detection of *Salmonella* spp. are time consuming and not able to provide information on serotypes. By PCR-based techniques, *Salmonella* specific primers are used, and thus the DNA sequences of other species could not be amplified. Consenquently, molecular methods should be very effective and useful for rapid detection of the *Salmonella* serovars in foods.

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