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APPLICATION OF THE END POINT POLYMERASE CHAIN REACTION METHOD TO THE DETECTION OF CERTAIN MILK-BORNE BACTERIA

Nassib, T. A.[1], W. M. El-Sharoud[1], E. A. Essawy [2] and Aliaa A. Darwish[2]

[1] Dairy Department, Faculty of Agriculture, Mansoura University

[2] Dairy Department, Food Technology Research Institute, Agricultural Research Center, Giza, Egypt

ABSTRACT

The polymerase chain reaction (PCR) is a modern method that could differentiate microorganisms from each other by detecting certain DNA sequences uniquely associated with each species. This study aimed to develop a relevant end point PCR assay to detect *Salmonella* ser. Typhimurium in milk. To pursue this objective, the conditions of both DNA extraction and PCR reactions were developed in terms of the boiling time for DNA extraction and primer concentration for the PCR assay. Results showed that all of the three examined boiling times of 10, 15 and 20 min were equally effective for carrying out efficient DNA extraction. PCR reaction mixture involving a primer concentration of 1300 nM was the most efficient concentration, compared to 650 nM, and 325 nM for detecting *Salmonella* ser. Typhimurium. The sensitivity of the developed PCR method to detect different viable numbers of *Salmonella* Typhimurium in tryptone soya broth (TSB) and reconstituted skim milk (RSM) was examined. The PCR assay was able to detect 10^9, 10^8, 10^7, and 10^6 cfu mL^{-1} of *Salmonella* in TSB. Whereas, it could detect 10^9, and 10^8 cfu mL^{-1} of *Salmonella* in RSM. These results suggested that the developed PCR method had higher sensitivity to detect *Salmonella* in TSB, compared to RSM. This was attributed to some milk components that could be inhibitory to PCR reactions.

Keywords: *Salmonella* ser. Typhimurium, PCR assay, boiling time, primer concentration.

INTRODUCTION

Despite the advancement in food safety approaches, food-borne *Salmonella* continues as a leading causative agent of gastroenteritis worldwide (D'Aoust, 2000; Pui *et al*., 2011 a & b; Scallan *et al*., 2011). *Salmonella* is usually transmitted via foods from animal sources including milk, dairy products, poultry and egg. Developing highly sensitive, rapid methods for detecting *Salmonella* is an effective measure to ensure the eradication of this pathogen from foodstuffs (Baia *et al*., 2013). Traditional culture-based methods are highly reliable for detecting *Salmonella* in food products, but they require 3-4 days for their completion (Mercanoglu and Griffiths, 2005).

The polymerase chain reaction (PCR) method is a sensitive molecular-based protocol that has proved to speed up the detection process of food-borne pathogens (Bruce, 1994; Hill, 1996 and Wang *et al.,* 1997). It relies on the amplification and detection of DNA sequences that are unique to each bacterial species. In its end point format, PCR involves DNA extraction from

cells, followed by amplification of a certain DNA sequence, if present, and gel electrophoresis detection of the amplified product. However, the application of PCR to the detection of bacterial pathogens requires optimization steps aimed at adapting the protocol to the examined microorganism and food products. The present study was thus designed to optimize the boiling time for DNA extraction from *Salmonella* cells, and primer concentration used for DNA amplification. The study also examined the efficiency of the developed PCR protocol for the detection of *Salmonella* in a synthetic culture medium and reconstituted skim milk.

MATERIALS AND METHODS

Cultures and growth conditions

Salmonella ser. Typhimurium was used throughout the whole study. *S*. Typhimurium was maintained on plates of tryptone soya agar (TSA) (Oxoid, Basingstoke, UK) at 4°C. Prior to each use, a single colony of *S*. Typhimurium was inoculated into tryptone soya broth (TSB) (Oxoid), followed by incubation at 37°C for 24 h.

End point polymerase chain reaction (PCR) analysis

End point PCR analysis of *S*. Typhimurium involved three stages of a) DNA extraction, b) PCR amplification, and c) agarose gel electrophoresis. These stages were conducted as follows:

DNA extraction

The boiling in water method was used to extract DNA from *S*. Typhimurium cultures in TSB. Briefly, 1 mL of each *Salmonella* culture was centrifuged at 14,000 rpm for 10 min using a bench centrifuge (Hettich, Aulendorf, Germany). Cell pellet was re-suspended in 100 µl sterilized distilled water and incubated at 100°C in dry heating block (Cleaver Scientific Ltd, Swift Valley Rugby, UK) for 10 min, 15 min, and 20 min. Samples were cooled for 2 min at room temperature and centrifuged at 14,000 rpm for 10 min. DNA containing supernatant was collected and preserved at -20°C until use.

PCR amplification

DNA extracted from *Salmonella* was subjected to PCR amplification targeting the invasive gene (*invA*) (Rahn *et al*. 1992). This involved using the *invA-1* (5′-GTG AAATTA TCG CCA CGT TCG GGC AA-3′) and *invA-2* (5′-TCATCG CAC CGT CAA AGG AAC C-3′) primers. PCR was conducted using a reaction mixture of a total volume of 15 µL consisting of 7.5 µl master mix (OneTaq Quick-Load 2X, New England Biolabs, UK), 2 µl of each of *invA-1* (forward primer), and *invA-2* (reverse primer) to give different concentrations of 1300 nM, 650 nM, and 325 nM, 3 µl DNA template extracted from *Salmonella*, and 0.5 µl sterilized distilled water. PCR reactions were conducted employing the conditions described by Rahn *et al*. (1992) and Mercanoglu and Griffiths (2005) with some modifications as follows. PCR reaction mixture was initially heated at 50 °C for 2 min, followed by 95 °C for 10 min and 45 cycles of 95 °C for 30 s, 65 °C for 1 min, and 72 °C for 2 min.

PCR reactions were conducted using the Primus 25 Advanced® PCR thermal cycler (PEQLAB Biotechnology GmbH, Erlangen, Germany).

Agarose gel electrophoresis

PCR amplified DNA product (amplicon) of different samples was examined using agarose gel electrophoresis as follows. Samples of 10 µl of each amplicon was electrophoresed on 2% (w/v) agarose gel in TBE buffer (1x) for 40 min at 110 V. Separated bands were visualized and photographed using the BioDocAnalyzer gel documentation system (Biometra, Goettinge, Germany).

RESULTS AND DISCUSSION

Optimizing the boiling time for DNA extraction for PCR detection of *Salmonella* ser. Typhimurium

The effect of different boiling times used for DNA extraction on the efficiency of PCR was assessed. DNA was extracted from a 24 h culture of *S*. ser. Typhimurim using different boiling times of 10 min, 15 min, and 20 min, and subjected to PCR analysis targeting the *invA* gene. PCR amplicons were then analysed using agarose gel electrophoresis. Figure 1 shows the results of the gel electrophoresis. It could be seen that all treatments resulted in resolving a DNA band of the size 284 bp. This was the expected size of the DNA amplicon resulting from PCR amplification of the targeted fragment of the *invA* gene (Rahn *et al*. 1992). This indicated that all of the three boiling times of 10 min, 15 min, and 20 min were equally efficient in DNA extraction. One of these three boiling times, 15 min, was thus used in the next experiments.

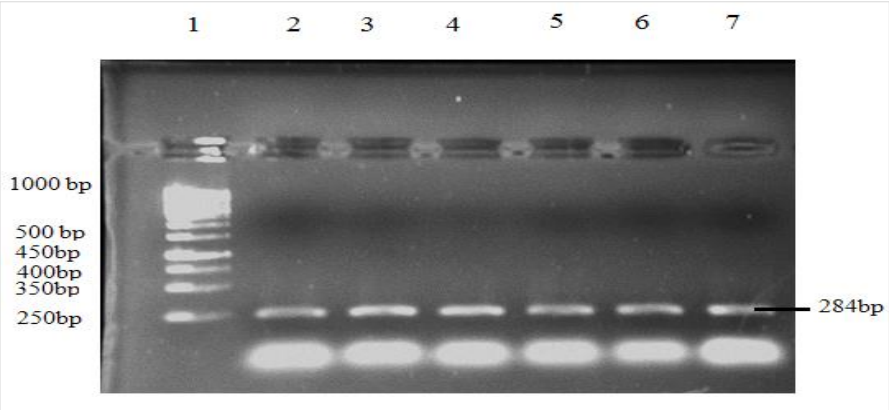

Figure 1: Effect of different boiling times used for DNA extraction on the efficiency of end point PCR detection of *S.* Typhimurium. Lane 1: DNA ladder (250 bp – 1000 bp), lanes 2 & 3: PCR amplicons using 10 min boiling, lane 4 & 5: PCR amplicons using 15 min, and lane 6 & 7: PCR amplicons using 20 min.

Optimization of primer concentration in the PCR reaction for the detection of *Salmonella* ser. Typhimurium

The concentration of the forward and reverse primers (*invA*-1 and *invA*-2, respectivley) used for PCR detection targeting the *invA* gene in *Salmonella* ser. Typhimurium was optimized. DNA was extracted from a 24 h culture of *Salmonella* ser. Typhimurium by boiling for 15 min. Extracted DNA was then subjected to end point PCR analysis using different concentrations of 1300 nM, 650 nM, and 325 nM of each of the *invA*-1 and *invA*-2 primers. Figure 2 shows the results of the gel electrophoresis of PCR amplification using different primer concentrations. It could be seen that only the PCR reaction mixture involving a primer concentration of 1300 nM produced a 284 bp DNA band. This represented the expected size of the DNA amplicon resulting from PCR amplification of the targeted fragment of the *invA* gene (Rahn *et al*. 1992). Whereas, the 650 nM, and 325 nM primer concentrations did not give any positive results. It could thus be concluded that the primer concentration of 1300 nM was the most efficient concentration compared to 650 nM, and 325 nM for the end point PCR detection of *Salmonella* ser. Typhimurim.

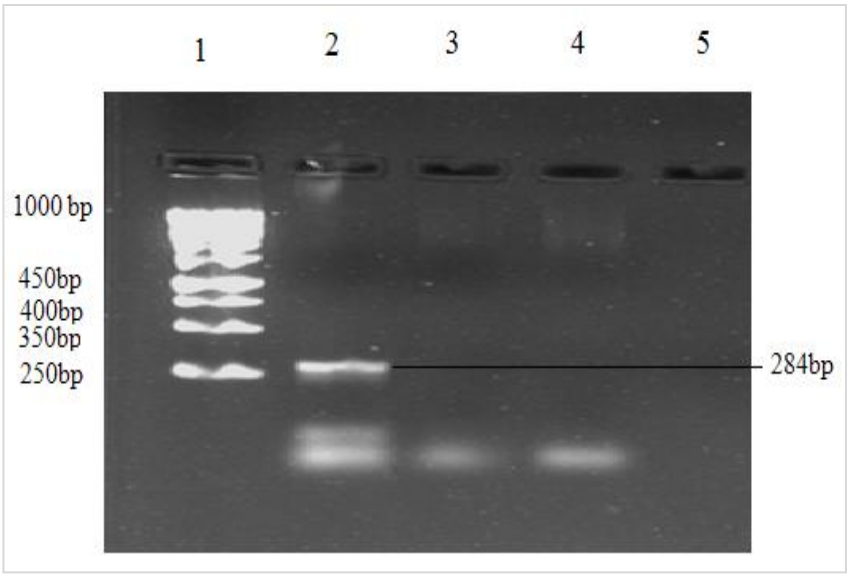

Figure 2: Effect of different primer concentrations on the efficiency of end point PCR detection of *S* Typhimurium. Lane 1: DNA ladder (250 bp – 1000 bp), lane 2: PCR amplicon using 1300 nM of each primer, lane 3: PCR amplicon using 650 nM of each primer, and lane 4: PCR amplicon using 325 nM of each primer.

Sensitivity of the end point PCR method to detect various viable numbers of *Salmonella* in a synthetic culture medium and reconstituted skim milk

The sensitivity of the optimized end point PCR method to detect different viable numbers of *Salmonella* Typhimurium in tryptone soya broth (TSB) and reconstituted skim milk (RSM) was examined. A 24 h culture of *Salmonella* was serially diluted in TSB and RSM to provide viable numbers ranging from 10^9 to 10 cfu mL^{-1}. Figure 3 shows the results of the gel electrophoresis of PCR amplification of different concentrations of *Salmonella* in TSB. It could be seen that a DNA band of the size of the targeted sequence of the *invA* gene, i.e. 284 bp, could be detected in PCR amplicons resulted from the analysis of 10^9, 10^8, 10^7, and 10^6 cfu mL^{-1} of *Salmonella* in TSB. No positive results could be observed with cell numbers lower than 10^6 cfu mL^{-1} of *Salmonella* (figure 3). This suggested that the minimum detection level of *Salmonella* in TSB by the end point PCR method was 10^6 cfu mL^{-1}.

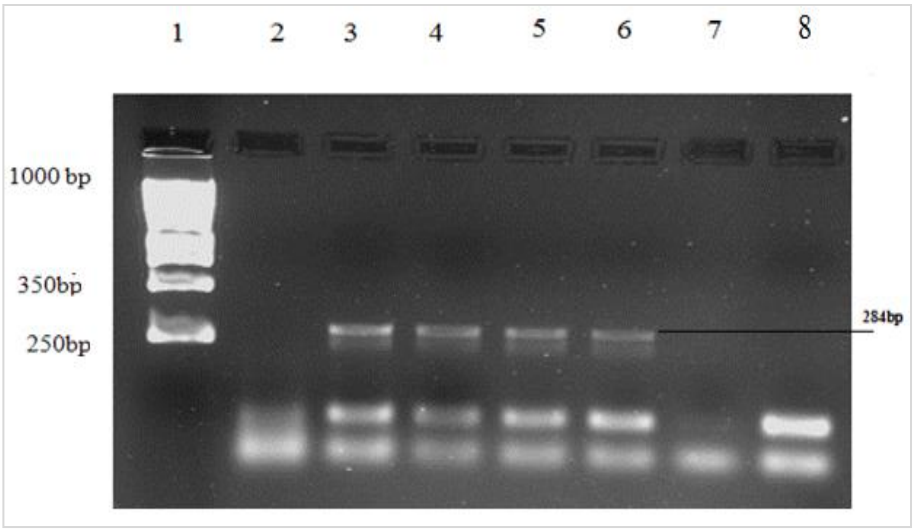

Figure 3: PCR Detection of different viable numbers of *S.* Typhimurium in TSB. Lane 1: DNA ladder (250 bp – 1000 bp), lanes 3, 4, 5, and 6: PCR amplicons of 10^9 cfu mL^{-1}, 10^8 cfu mL^{-1} 10^7 cfu mL^{-1}, and 10^6 cfu mL^{-1} of *Salmonella* in TSB, respectively, Lanes 7 & 8: PCR amplicons of 10^5 cfu mL^{-1}, 10^4 cfu mL^{-1} of *Salmonella* in TSB, respectively.

The use of the same end point PCR method to detect *Salmonella* in reconstituted skim milk (RSM) generated a higher detection level. It could be noted in Figure 4 that only two positive DNA bands of the size 284 bp were detected in PCR amplicons resulted from the analysis of 10^9 and 10^8 cfu mL^{-1} of *Salmonella* in RSM. No positive results could be reported with viable numbers lower than 10^8 cfu mL^{-1}. Attempts to improve the sensitivity of the

assay using 10 mL of RSM cultures containing *Salmonella* rather than 1 mL for DNA extraction were unsuccessful. Figure 5 shows the results of the analysis of 10 mL samples of RSM containing different viable numbers of *Salmonella.* It could be seen that no improvement could be observed with the higher sample volume of 10 mL since positive DNA bands were only observed with the analysis of 10^9 and 10^8 cfu mL^{-1}. This indicated that the sensitivity of the end point PCR method to detect *Salmonella* in RSM was lower compared to TSB. This could be attributed to the presence of macromolecules like proteins and fat in milk, which might interfere with the PCR assay (Oliveira and Lencastre, 2002). However, Bickley *et al*. (1996) reported that PCR inhibition in milk samples is mainly dependant on the concentration of calcium, whereas the fat content seems to have only minor influence on the amplification efficiency. Powell *et al*. (1994) have also identified plasmin as a natural PCR inhibitor in milk that could degrade the *Taq* polymerase.

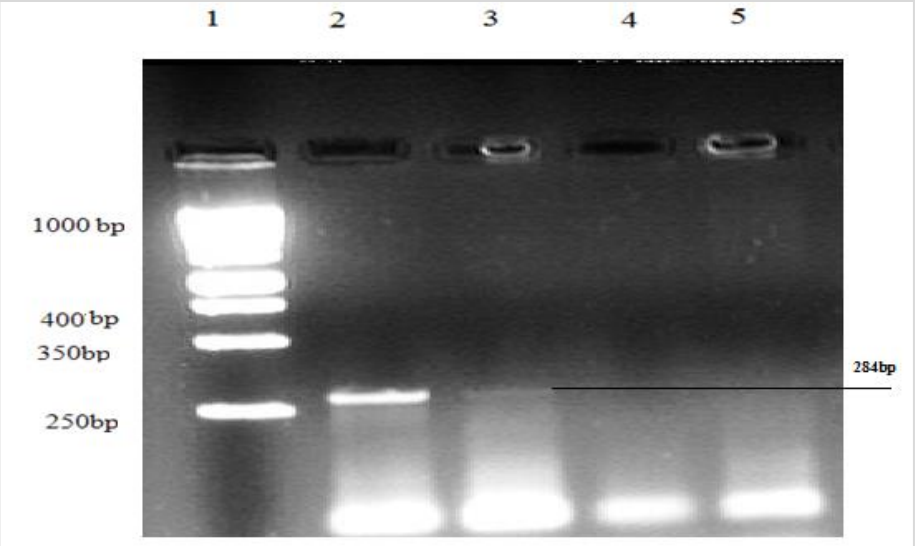

Figure 4: PCR Detection of different viable numbers of *S.* Typhimurium in reconstituted skim milk, using 1 mL samples. Lane 1: DNA ladder (250 bp – 1000 bp), lanes 2 & 3: PCR amplicons of 10^9 cfu mL^{-1}, and 10^8 cfu mL^{-1} of *Salmonella* in RSM, respectively, lanes 4 & 5: PCR amplicons of 10^7 cfu mL^{-1}, and 10^6 cfu mL^{-1} of *Salmonella* in RSM, respectively.

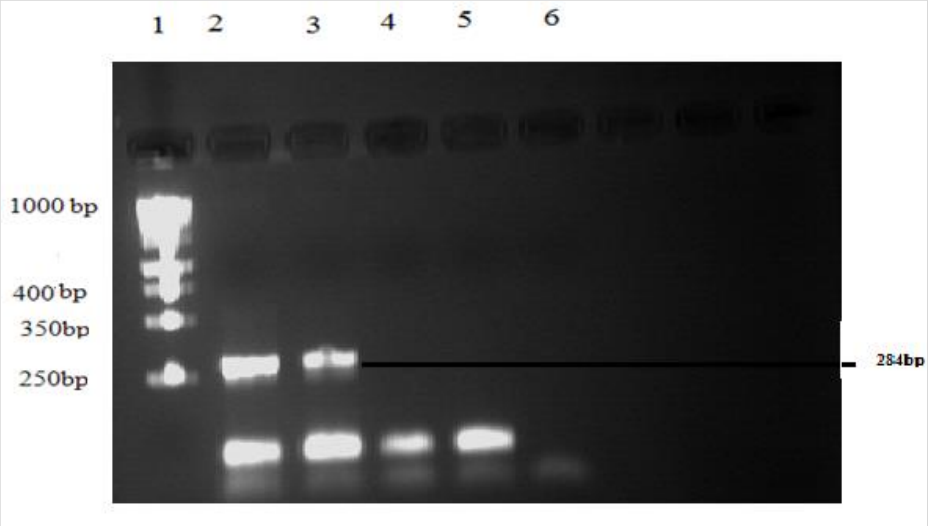

Figure 5: PCR Detection of different viable numbers of *S.* Typhimurium in reconstituted skim milk, using 10 mL samples. Lane 1: DNA ladder (250 bp – 1000 bp), lanes 2 & 3: PCR amplicons of 10^9 cfu mL^{-1}, and 10^8 cfu mL^{-1} of *Salmonella* in RSM, respectively, lanes 4, 5 & 6: PCR amplicons of 10^7 cfu mL^{-1}, 10^6 cfu mL^{-1}, and 10^5 cfu mL^{-1} of *Salmonella* in RSM, respectively.

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إستخدام طريقة تفاعل البلمرة المتسلسل في الكشف عن أنواع معينه من البكتريا المرتبطة باللبن

طه عبدالحليم نصيب[١] ، وليد محمود الشارود[١] , ايهاب عبدالباقي عيسوي[٢]و علياء علي درويش[٢]

[١]قسم الألبان, كلية الزراعة, جامعة المنصورة, المنصوره, مصر.

[٢] قسم الالبان, معهد بحوث تكنولوجيا الاغذيه, مركز البحوث الزراعيه, جيزه, مصر.

تفاعل السلسلة المتبلمر (PCR) هو طريقة حديثة يمكن إستخدامها في التمييز بين الميكروبات وذلك بالكشف عن تتابعات معينة من DNA تكون مميزة لكل نوع ميكروبي. وقد إستهدفت هذه الدراسة تطوير طريقة PCR مناسبة يمكن إستخدامها في الكشف عن ميكروب *Salmonella* ser. Typhimurium في اللبن. حيث أهتمت الدراسة بتحديد أفضل الظروف اللازمة لإستخلاص DNA وإجراء تفاعلات PCR وذلك فيما يتعلق بوقت الغليان اللازم لإستخلاص DNA من الخلايا، وتركيز البرايمر المستخدم في تفاعلات PCR. وقد أظهرت النتائج أن كل أوقات الغليان التي تم إختبارها وهي ١٠, ١٥, ٢٠ دقيقه كانت ذات كفاءة متساوية للقيام بعملية إستخلاص DNA فعالة. كما وُجد أن إستخدام مخلوط لتفاعل PCR ذو تركيز ١٣٠٠ نانومولر من البرايمر كان الأكثر كفاءة وذلك بالمقارنة بمخلوط التفاعل المحتوي على تركيز ٦٥٠ أو ٣٢٥ نانومولر. تم إستخدام طريقة PCR المطورة في الكشف عن *Salmonella* ser. Typhimurium في بيئة TSB و اللبن الفرز المسترجع. وقد إستطاعت الطريقة الكشف عن الميكروب بأعداد حية تصل إلي $١٠^{٩}$، $١٠^{٨}$، $١٠^{٧}$، $١٠^{٦}$ وحدة تكوين مستعمرة في المللي من بيئة TSB، ولكنها أعطت نتائج سلبية مع الأعداد الأقل من $١٠^{٦}$ وحدة تكوين مستعمرة في المللي. كما أمكن تقدير الميكروب بأعداد $١٠^{٩}$، $١٠^{٨}$ وحدة تكوين مستعمرة في المللي من اللبن الفرز المسترجع مع الحصول علي نتائج سلبية مع الأعداد الأقل من $١٠^{٨}$ وحدة تكوين مستعمرة في المللي. أوضحت هذه النتائج أن طريقة PCR المطورة كانت ذات حساسية أعلي للكشف عن *Salmonella* ser. Typhimurium في بيئة TSB وذلك بالمقارنة باللبن الفرز المسترجع، وقد أعزي ذلك إلي امكانية تثبيط تفاعل PCR بواسطة بعض المركبات اللبنية.