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FULL PAPER

Multiplex touchdown Polymerase Chain Reaction for rapid detection of *Salmonella enterica* subsp. *enterica* serovars Enteritidis and Typhimurium in food

[1,2]Sandrasaigaran, P., [3]Kuan, C.H., [1]Son, R., [2]Gobal, D., [4]Abidin, U.F.U.Z., [1]Rukayadi, Y. and [1,5,*]Hasan, H.

[1]*Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400, UPM Serdang, Selangor, Malaysia*
[2]*Department of Biotechnology, School of Biotechnology, Manipal International University, 71800 Nilai, Negeri Sembilan, Malaysia*
[3]*Department of Food Science and Nutrition, Faculty of Applied Sciences, UCSI University, 56000 Cheras, Kuala Lumpur, Malaysia*
[4]*Department of Food Service and Management, Faculty of Food Science and Technology, University Putra Malaysia, 43400, UPM Serdang, Selangor, Malaysia*
[5]*Halal Products Research Institute, Universiti Putra Malaysia, 43400, UPM Serdang, Selangor, Malaysia*

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Abstract

The *Salmonella* outbreak is one of the leading foodborne diseases in the world with increasing cases being reported annually. However, the current methods for *Salmonella* detection in foods are outdated, laborious and time-consuming. This necessitated developing a technique that is rapid for *Salmonella* detection in foods. Thus, the current study aimed to develop a multiplex touchdown PCR (m-TdPCR) protocol for rapid and simultaneous detection of *Salmonella enterica* subsp. *enterica* serovars Enteritidis and Typhimurium in foods. A two-phase m-TdPCR protocol was developed and optimized with primer pairs targeting the *Salmonella enterica* subsp. *enterica* (ST11/ST15-0.15 µM), serovars Enteritidis (*sdfI* gene-1.2 µM), Typhimurium (*fliC* gene-1.5 µM) and an internal amplification control (16S rRNA-0.08 µM). It was found that the m-TdPCR protocol is highly sensitive detecting up to 1 ng of *Salmonella* DNA and its specificity was verified using the *in-silico* method. Furthermore, the developed m-TdPCR shows no non-specific PCR amplicons and is able to detect both *S. enterica* ser. Enteritidis and *S. enterica* ser. Typhimurium in real-time when tested against the artificially contaminated food samples at up to 10^{-3} dilutions. Therefore, the validated m-TdPCR protocol in this study can be used as a tool for rapid detection of *S. enterica* ser. Enteritidis and *S. enterica* ser. Typhimurium in food samples and this may significantly reduce any related foodborne incidences in future.

1. Introduction

The *Salmonella enterica* subsp. *enterica* is a clinically relevant bacteria that have caused a significant threat to public health and animal welfare in recent years. This subspecies is generally divided into typhoidal and nontyphoidal salmonellae, 99% of which can cause disease in humans and animals (Issenhuth-Jeanjean *et al*., 2014). The typhoid fever causing *Salmonella enterica* serovar Typhi and *S. enterica* ser. Paratyphi are largely restricted to the human host, non-zoonotic and are commonly witnessed in many underdeveloped countries due to poor sanitation practices (Havelaar *et al*., 2015). Contrarily, the non-typhoidal salmonellae (NTS) are zoonotic, foodborne, and found to be widespread in many industrialized countries, primarily due to the consumption of poultry and food products contaminated by these bacteria (Eng *et al*., 2015; Bangera *et al*., 2019). The two most prevalent NTS serotypes reported worldwide were *S. enterica* ser. Enteritidis (65%) and *S. enterica* ser. Typhimurium (12%) (Eng *et al*., 2015). In general, NTS contributes to a low mortality rate in humans, but due to its high morbidity and environmental widespread distributions, the prevalence of these bacteria in the global food chain has substantially increased the clinical and economic

*Corresponding author.
Email: *mhanan@upm.edu.my*

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burden. Studies have shown that both *S. enterica* ser. Enteritidis and *S. enterica* ser. Typhimurium was found not only in raw carcasses but also in many cooked and pre-cooked foods (Anukampa *et al*., 2017; Siala *et al*., 2017). Consequently, the food poisoning incidents documented due to the NTS were seen to be on the rise and an estimated 93.8 million cases occur globally each year, 80.3 million cases of which were linked to the foodborne spread resulting in 155 000 deaths annually (Majowicz *et al*., 2010). The infection caused by NTS primarily elicits gastroenteritis and bacteraemia; besides, it may also cause other extra-intestinal complications such as pneumonia, meningitis, urinary tract infections and endocarditis (Arii *et al*., 2002). As such, the median cost for treatments (2006 – 2008) was estimated at US$ 468 for gastroenteritis, US$ 1465 for septicaemia and US$ 1918 for infections at other sites (Chen *et al*., 2012).

The medical cost for treating salmonellosis is ballooning and could implicate severe financial distress to both industrialised and developing countries. However, the current practice for *Salmonella* detection in foods is rather outdated, laborious, and time-consuming. Today, the gold standards in *Salmonella* identification in many laboratories are the conventional culture method using selective media, followed by biochemical characterization and molecular serotyping. These methods typically take more than five days to confirm the serovar, and at the critical outbreak, the infection would have deeply spread in the community and sustained further damage by the time the results were made available (Lee *et al*., 2015). Furthermore, the detection and identification of *Salmonella* in foods using the culture methods demand highly trained biomedical personnel to avoid reporting false negative or false positive (Paniel and Noguer, 2019). Conversely, many of the commercially available *Salmonella* detection kits such as serological assays and miniaturized biochemical assays are mostly automated and rapid but at the same time, they are too costly (Lee *et al*., 2015). Thus, this necessitated the need to develop a rapid, sensitive, and cost-effective method to detect and identify the NTS. Such criteria were fairly fulfilled by the polymerase chain reaction (PCR) techniques in pathogen detection and identification, particularly in the food samples (Lee *et al*., 2015). However, the method may still require further optimization, especially in the simultaneous detection of several bacteria and their targets in food samples.

Foods sold in the outlets and premises can be easily contaminated by various foodborne pathogens (Latchumaya *et al*., 2021). Since the PCR assay is highly sensitive, it can produce misleading non-specific amplification for the non-targeted bacteria even when the DNA is presented at a trace amount (Garibyan, and Avashia, 2013). On top of that, the development of multiplex PCR for simultaneous detection of several targets can lead to low amplification efficiency, low reproducibility, and succumb to self-inhibition due to the interference among the multiple primer pairs used in the PCR master mix (Xu and Shang, 2016). To prevent this, hot-start PCR and touchdown PCR can be used to improve the target specificity and avoid any non-specific PCR amplifications. Thus, this study aimed to develop a multiplex-touchdown PCR (m-TdPCR) which would be rapid and capable of simultaneously detecting *S. enterica* serovars Enteritidis and Typhimurium in food samples. The development of this protocol will be cost-effective and will add simplicity besides reducing the time for NTS detection in food samples. This may substantially prevent any significant impact of foodborne NTS outbreaks in future.

2. Materials and methods

2.1 Bacterial strains and DNA isolation

Salmonella enterica subsp. *enterica* serovar Typhimurium (ATCC® 14028™) and *Salmonella enterica* subsp. *enterica* serovar Enteritidis (ATCC® 13076™) were obtained from the Department of Food Science Laboratory, Universiti Putra Malaysia. These strains were individually inoculated overnight in nutrient broth (Chem Soln, India) at 37.0±1.0°C for DNA isolation using the cell boiling method. The DNA isolation was conducted according to the method defined by Pui *et al*. (2011).

2.2 PCR primers and in-silico primers specificity

A total of four primer pairs (Vivantis, Malaysia) developed from previous studies were used to optimize the PCR amplification of targeted sequences as shown in Table 1; ST11/ ST15 primers (429 bp), *sdfI* gene (304 bp), *fliC* gene (620 bp), bacterial 16S rRNA universal primers (180 bp) (Soumet *et al*., 1999; Alvarez *et al*., 2004; Kang *et al*., 2017). Besides, these primer pairs were also verified for bacterial specificity using the *in-silico* PCR amplification program (insilico.ehu.es) against the complete genome (including their plasmids) of *Salmonella* genus (n = 45) and other common and related Enterobacteriaceae families such as *Escherichia* spp. (n = 65), *Yersinia* spp. (n = 19) and *Shigella* spp. (n = 10). Up to two mismatches between the template DNA and primers were allowed.

2.3 Multiplex-touchdown PCR development

Both singleplex and multiplex PCR (mPCR) were optimized with the 2× Taq master mix (Vivantis, Malaysia) prior to developing the m-TdPCR protocol.

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Table 1. PCR primers ST11/ ST15, *sdfI*, *fliC*, and Universal total bacteria 16S rRNA optimized in the multiplex-touchdown PCR reaction.

Primers		Sequence (5' - 3')	Amplicon/ target size (bp)	Reference
Random sequence of *Salmonella* sp.	Forward (ST11)	GCCAACCATTGCTAAATTGGCGCA	429	Soumet *et al.* (1999)
	Reverse (ST15)	GGTAGAAATTCCCAGCGGGTACTGG		
sdfI for *S. enterica* ser. Enteritidis	Forward (ENTF)	TGTGTTTTATCTGATGCAAGAGG	304	Alvarez *et al.* (2004)
	Reverse (ENTR)	TGAACTACGTTCGTTCTTCTGG		
fliC gene for *S. enterica* ser. Typhimurium	Forward (Fli15)	CGGTGTTGCCCAGGTTGGTAAT	620	Soumet *et al.* (1999)
	Reverse (Typ04)	ACTGGTAAAGATGGCT		
Universal total bacterial 16S rRNA	U16srt-F	ACTCCTACGGGAGGCAGCAGT	180	Kang *et al.* (2017)
	U16srt-R	TATTACCGCGGCTGCTGGC		

For the singleplex PCR, the master mix was prepared by adding the PCR mixture with 0.5 µM primers (four primers for each sample, in separate PCR tubes) and 1 ng of *S. enterica* ser. Enteritidis or *S. enterica* ser. Typhimurium DNA template giving the final reaction mixture of 25 µL/reaction tube. The annealing temperature for each primer pair was varied; ST11/ST15 (59°C), *sdfI* (56°C), *fliC* (56°C) and the universal bacterial 16S rRNA (60°C). For the mPCR, all four primers concentrations were optimized: ST11/ST15 (0.15 µM), *sdfI* (1.2 µM), *fliC* (1.5 µM) and the universal bacterial 16S rRNA (0.08 µM). Besides that, the $MgCl_2$ (Vivantis, Malaysia) and Taq polymerase (Vivantis, Malaysia) was also optimized to the final concentration of 3.5 mM and 2.0 U, respectively. Additionally, 5% DMSO (Duchefa, Netherlands) and nuclease-free H_2O were added to prepare a final mixture of 25 µL/reaction tube. The annealing temperature was optimized at 58°C. The DNA concentration was measured by Nanodrop one^{TM} (Thermo Fisher, USA) and added to the master mix with at least 1 ng concentration per reaction mix. Two-phase m-TdPCR protocol (Table 2) for the rapid detection of *S. enterica* ser. Enteritidis and *S. enterica* ser. Typhimurium was adopted from a previous study (Korbie and Mattick, 2008) where the annealing temperature was further optimized as depicted in Table 2. *Escherichia coli* DH5α strain was used as the control. The PCR mix was amplified in a thermocycler (Bio-Rad, USA), and the amplicons were analyzed in the gel documentation system (UVP, UK) after the gel electrophoresis [1.5%] step.

2.4 Bacterial artificial contamination and multiplex-touchdown PCR bacterial detection in food sample

Bacterial cultures (*S. enterica* ser. Typhimurium and *S. enterica* ser. Enteritidis) were prepared at 10^8 CFU/ mL concentration and artificially contaminated with a protocol modified by Rohde *et al.* (2015). The contamination of food sample protocol was adapted from a previous study (Rohde *et al.*, 2015). This study used cooked and autoclaved chicken nuggets (25 g) to represent food samples and it was inoculated with 10^8 CFU/mL *S. enterica* ser. Typhimurium and *S. enterica* ser. Enteritidis culture. The inoculum was incubated at room temperature for 4 hrs and then added to 225 mL of Buffered Peptone Water (BPW) (Chemsoln, India) for homogenization. Serial dilution (10^{-2} and 10^{-3}) were prepared in triplicates, and these tubes were then subjected to DNA isolation using the cell boiling method. The resulting DNA was used as a template in the m-TdPCR amplification.

3. Results and discussion

In this study, four primers pairs were utilized to develop an m-TdPCR protocol for simultaneous and rapid detection of *Salmonella enterica* subspecies *enterica* (subsp. I), *S. enterica* ser. Enteritidis and *S. enterica* ser. Typhimurium from food samples. The universal total bacterial 16S rRNA primers were used as

Table 2. Two phase m-TdPCR protocol for a rapid detection of *Salmonella enterica* subsp., *S. enterica* ser. Enteritidis and *S. enterica* ser. Typhimurium in food.

PCR Stages		Temperature and Time	
Phase 1	Initial denaturation	94°C for 5 mins.	
	Denaturation	94°C for 45 s	
	Annealing	68°C (-1°C per cycle) for 1 min	(10 cycles)
	Elongation	72°C for 1 min	
Phase 2	Denaturation	94°C for 45 s	
	Annealing	53°C (Ta-5°C), 1 min	(20 cycles)
	Elongation	72°C for 1 min	
	Final Elongation	72°C for 7 mins	
Hold		12°C	

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the internal amplification control (IAC) in this protocol. The incorporation of the IAC is important to avoid false-negative results in the enumeration of bacteria from the food samples using the PCR method (Copin *et al*., 2012).

Table 3 shows the *in-silico* PCR outcome against ST11/ ST15 primer pair, three out of forty-five *Salmonella* strains [*S. bongori* (n = 2) and *S. enterica* subsp. *arizonae* (n = 1)] did not show any PCR band. No PCR band was also observed when the same primer pair was tested against the *Escherichia* spp. (n = 65), *Yersinia* spp. (n = 19), and *Shigella* spp. (n = 10). The failure of the ST11/ST15 primers to detect *S. bongori* and *S. enterica* subsp. *arizonae* is much anticipated as these oligo primers has been shown to lack specificity for *Salmonella enterica* subspecies II, III, IV, V and VI earlier (Aabo *et al*., 1993). The primers to amplify *sdfI* was only visible for the *S. enterica* ser. Enteritidis (n = 1) and was not present in any other *Salmonella* strains, *Escherichia* spp., *Yersinia* spp. and *Shigella* spp. A similar outcome was also observed for the primers targeted *fliC* with the band only visible for the *S. enterica* ser. Typhimurium (n = 13), but no cross-reactivity was noticed with closely related bacterial clades. This theoretically warrants that the primers ST11/ ST15, primers for *sdfI* and the primers for *fliC* are specific to the *S. enterica* subspecies *enterica* (subsp. I), *S. enterica* ser. Enteritidis and *S. enterica* ser. Typhimurium, respectively. The *in-silico* PCR and the primers validation methods are highly specific, and it is a validated technique. A study conducted by Goay *et al*. (2016), looked into the *S. enterica* ser. Typhi gene amplification compared the study's outcome using the *in -silico* and *in vitro* PCR methods. When a whole genome of bacteria was made available (n = 5), the predicted *in-silico* PCR showed 100% specificity, and a similar outcome was obtained using *in vitro* PCR method. However, this outcome was achieved if only the whole bacterial genome were available for the *in-silico* test (Goay *et al*., 2016). Thus, the amplification specificity testing using the *in-silico* PCR technique is equally valid compared to the *in vitro* PCR technique. On top of that, the *in-silico* PCR technique is also cheap, labour effortless and safe as it prevents any risk of contracting harmful pathogens (van Weezep *et al*., 2019). Assessing the cross-reactivity among the primers used in this study against other important Enterobacteriaceae is vital. Bacteria such as *E. coli*, *Yersinia* spp. and *Shigella* spp. are closely related to the *Salmonella* genus, and they not only possess similar clinical symptoms but are also found to have high similarity in their genome and their G + C content (Forsdyke, 1996). As such, careful scrutinization for the cross-reactivity within the primer pairs in the developed m-TdPCR protocol is necessary for simultaneous and rapid detection of *S. enterica*., *S. enterica* ser. Enteritidis and *S. enterica* ser. Typhimurium to prevent any false-negative or false-positive outcomes.

In the singleplex PCR amplification, the *Salmonella* bacteria were amplified against all four primer pairs (Figure 1a). For the ST11/ ST15 and the Universal primers 16S rRNA, both *S. enterica* ser. Enteritidis and *S. enterica* ser. Typhimurium amplicons were observed. Alternatively, for the primers targeting the *sdfI* and *fliC*, the amplicons were only noticed for the *S. enterica* ser. Enteritidis and *S. enterica* ser. Typhimurium, respectively, with no cross-reactivity. Conversely, the control *E. coli* DH5α amplification occurred only for the universal bacterial 16S rRNA primers. Besides that, when a multiplex PCR protocol was developed with the same four primer pairs, all amplicons with respective size bands were observed in a single PCR reaction (Figure 1b). The minimal DNA concentration required was 1 ng in this protocol. This was optimized in the sensitivity experiment where mPCR amplification with 0.1 ng of DNA concentration failed to obtain results for all four amplicons. A study conducted by Wang *et al*. (2019) to detect six pathogenic bacteria in poultry using mPCR showed that the developed protocol's sensitivity

Table 3. PCR primers and its specificity towards *Salmonella* enterica subsp. enterica, *Escherichia* spp., *Yersinia* spp. and *Shigella* spp.

Primers	Closely related Enterobacteriaceae			
	Salmonella enterica subsp. *enterica* (n = 45).	*Escherichia coli* (n = 63), *Escherichia blattae* (n = 1) and *Escherichia fergusonii* (n = 1)	*Yersinia* spp. (n = 19)	*Shigella* spp. (n = 10)
ST11/ ST15	No PCR bands were noticed in all two *S. bongori* and one *S. arizonae*. The remaining 42 *Salmonella* enterica subsp. *enterica* strains were observed PCR bands.	No bands	No bands	No bands
sdfI	PCR band was observed only in one *S. enterica ser.* Enteritidis strain.	No bands	No bands	No bands
fliC	PCR band was observed in 13 *S. enterica ser.* Typhimurium strains	No bands	No bands	No bands

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(detection limit) was mostly up to 0.5 – 1 ng (Wang *et al*., 2019). Moreover, a typical PCR may require a 3×10^5 DNA template molecule which is reasonable to start with 1 ng of bacterial genomic DNA (Møller, 2016).

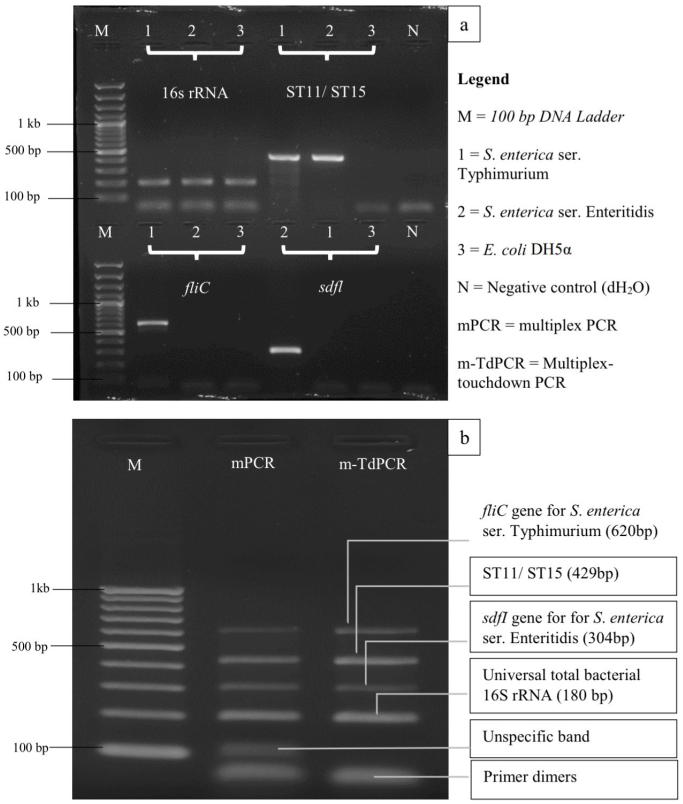

Figure 1. Detection of *Salmonella enterica* subsp., *S. enterica* ser. Enteritidis and *S. enterica* ser. Typhimurium using PCR. An unspecific band (~100 bp) was noticed in the mPCR but not in the Td-mPCR protocol.

However, the protocol developed in mPCR presented a non-specific amplicon at approximately 100 bp even after the annealing temperature and other PCR reagents in the master mix were optimized. The non-specific amplicon in the mPCR is expected as impurities or inhibitors in the PCR reaction mixture due to the cell boiling method during the DNA extraction in this study. Nevertheless, the cell-boiling DNA extraction method is more cost-effective and less laborious compared to the phenol/chloroform or DNA extraction kits (Oliveira *et al*., 2014). *Salmonella* genus generally has high G + C regions in their genome, contributing to many inter and intra hairpin loops. This can generate many complex secondary structure formations, which may inhibit target amplification in the PCR reactions (Musso *et al*., 2006). Higher G + C rich regions could also resist template denaturation and lead to improper annealing conditions, thus preventing primers annealing at the desired site (Hubé *et al*., 2005). This may subsequently add to non-specific target amplification. This issue was addressed when the two-phase m-TdPCR was introduced where the non-specific band was not observed, therefore increasing the accuracy and specificity of the target. This could be due to the wide annealing temperature range employed in the m-TdPCR that enhances the specificity of the amplicon formation (Korbie and Mattick, 2008).

Besides that, all three dilution tubes (10^{-1}, 10^{-2} and 10^{-3}) in the artificially contaminated food experiment were subjected to the m-TdPCR amplification, respective amplicon bands for both *S. enterica* ser. Enteritidis (ST11/ ST15, *sdfI* gene and universal total bacteria 16S rRNA) and *S. enterica* ser. Typhimurium (ST11/ ST15, *fliC* gene and universal total bacteria 16S rRNA) were evident (Figure 2). This has demonstrated that the m-TdPCR protocol can detect and identify the presence of *S. enterica* ser. Enteritidis and *S. enterica* ser. Typhimurium in the food samples with no unspecific amplicons observed. Several studies in the past had successfully developed the m-TdPCR protocol for various pathogen detections in food products. For instance, a study conducted by Moezi *et al*. (2019) showed that the m-TdPCR has better sensitivity and specificity for *E. coli* O157:H7, *L. monocytogenes*, *Staphylococcus aureus*, and *S. enterica* ser. Enteritidis detection in the raw milk samples. The same study also had shown that the m-TdPCR protocol had successfully

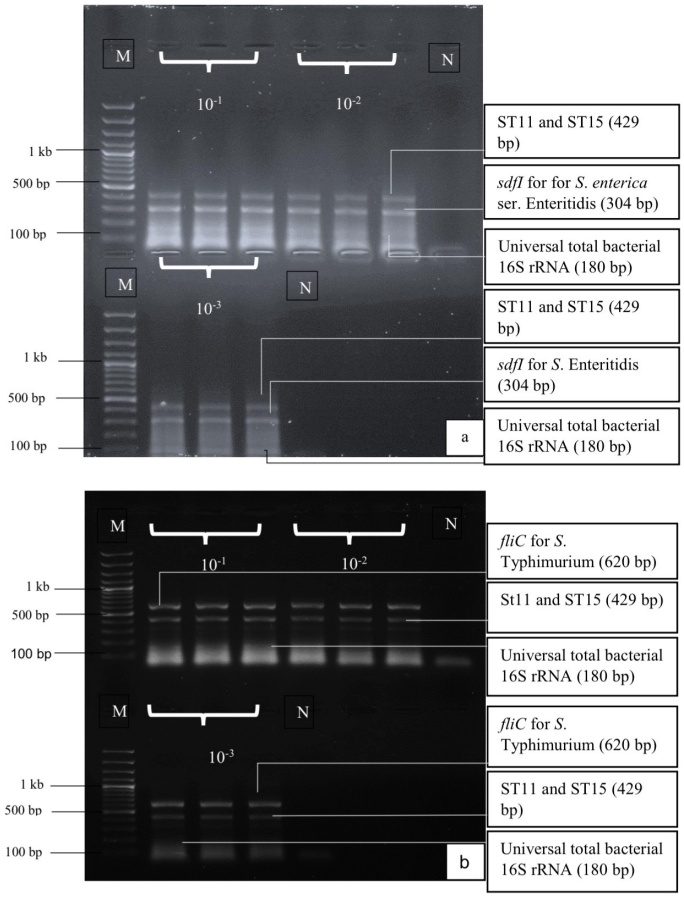

Figure 2. Gel electrophoresis analysis of the m-TdPCR for artificially contaminated food experiment. Artificially contaminated home cooked nuggets with *S. enterica* ser. Enteritidis (a) and *S. enterica* ser. Typhimurium (b) able to be detected in all triplet dilution tubes (10^{-1}, 10^{-2} and 10^{-3}) during the m-TdPCR amplification. The figure shown is a representation of PCR results in triplicates. M: 100 bp DNA Ladder (Thermo Scientific, US); N: Negative Control (dH_2O as template).

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eliminated any non-specific bands that appeared in the mPCR besides delivering comparatively similar pathogen detection outcomes to the conventional culture method (Moezi *et al*., 2019). As the pathogen detection using the conventional method may take up to 2 – 5 days, the currently developed m-TdPCR protocol was shown rapid (detection within 24 hrs), sensitive and specific in detecting *S. enterica* ser. Enteritidis and *S. enterica* ser. Typhimurium in the food samples.

4. Conclusion

The developed multiplex touchdown PCR protocol was shown to have high sensitivity (1 ng of DNA template added to 25 µl PCR mix) in detecting *S. enterica* ser. Enteritidis and *S. enterica* ser. Typhimurium. Besides, the *in-silico* results had proved that the PCR primers used in this protocol were highly specific as no cross-reactivity was observed within the *Salmonella* genus and other closely related bacterial clades. On top of that, when the food samples were artificially contaminated with *S. enterica* ser. Enteritidis and *S. enterica* ser. Typhimurium, the m-TdPCR was able to detect the bacteria up to 10^{-3} dilutions. Additionally, the inclusion of the internal amplification control (IAC) in the protocol can rule out any possible false-negative result using this m-TdPCR protocol. Thus, the developed m-TdPCR protocol is suitable for detecting NTS such as *S. enterica* ser. Enteritidis and *S. enterica* ser. Typhimurium in the food samples as it is rapid, sensitive, and specific.

Conflict of interest

The authors declare no conflict of interest.

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