Detection of *Salmonella enterica* in food on the next day after the sample collection using 5'-nuclease polymerase chain reaction with end-point fluorimetry

KLÁRA KRASCSENICSOVÁ - EVA KACLÍKOVÁ - DOMENICO PANGALLO - PETER SIEKEL - STEFANO GIROTTI - TOMÁŠ KUCHTA

Summary

A method for the detection of *Salmonella enterica* in food on the next day after the sample collection is presented. The method consists of a two-step enrichment, bacterial cells lysis and a closed-tube duplex 5'-nuclease PCR with an internal amplification control, which is read in an end-point mode in a 96-well fluorimeter. The 5'-nuclease PCR has an intrinsic detection limit of 10^4 CFU.ml^{-1} and facilitates monitoring of false negative results caused by the failure of amplification. The complete method for the detection of *S. enterica* in food has a detection limit of 10^0 CFU/25 g and does not produce false positive results in case of the presence of dead cells.

Keywords

Salmonella; polymerase chain reaction; rapid method

Salmonella enterica is a species of pathogenic bacteria that frequently contaminate food and represent an important cause of food-borne epidemies [1]. The pathogen is required to be absent from food in terms of a negative result of the qualitative analysis with the detection limit of 10^0 CFU/25 g [2]. An analytical method with this detection limit is available [3] but it suffers from a high time requirement of 5 to 7 days. The need for faster solutions has lead to the development of some alternative methods [4, 5]. However, for routine use in laboratories of food analysis, the alternative, faster methods for the detection of *S. enterica* should meet several criteria. These include the same detection limit as the standard method, robustness in terms of moderate requirements for a DNA-free laboratory environment, and acceptability from the economical point of view. Regarding the latter parameter, most of the recently developed alternative methods are currently hardly acceptable for a routine laboratory of food analysis since they rely on real-time PCR which requires specialized costly instrumentation.

In this article, a method for the detection of *S. enterica* in food on the next day after the sample collection by 5'-nuclease polymerase chain reaction (PCR) with end-point fluorimetry is presented. The method involves the previously developed two-step enrichment [6], 5'-nuclease PCR specific for *S. enterica* [7] and the end-point closed-tube fluorimetric detection in a 96-well fluorimeter [8], which are supplemented by a newly developed internal amplification control with its own 5'-nuclease PCR system that is detected in the duplex format.

MATERIALS AND METHODS

Bacterial strains

Salmonella enterica serovar Enteritidis CCM 4420 and *S. enterica* serovar Typhimurium CCM 4419 were obtained from the Czech Collection of Microorganisms, Brno, Czech

Klára Krascsenicsová, Eva Kaclíková, Peter Siekel, Tomáš Kuchta, Department of Microbiology and Molecular Biology, VÚP Food Research Institute, Priemyselná 4, P. O. Box 25, SK-824 75 Bratislava 26, Slovakia.
Domenico Pangallo, Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, SK-845 51 Bratislava 45, Slovakia.
Stefano Girotti, Institute of Chemical Sciences, University of Bologna, Via S. Donato 15, Bologna, Italy.

Correspondence
Tomáš Kuchta, e-mail: kuchta@vup.sk

Republic. *S. enterica* serovar Panama ALM 41 was obtained from National Institute for Public Health and the Environment, Bilthoven, the Netherlands. Bacterial strains were cultured in Nutrient Broth (Merck, Darmstadt, Germany) at 37 °C with shaking (2 Hz) overnight (16–20 h). Dead cells were prepared by boiling for 25 min and the devitalization efficiency was confirmed by plating on Nutrient Agar (Merck). Decimal dilutions of the cultures were prepared in 0.85 % NaCl. Bacterial concentration was determined by plate-count technique on plates of Nutrient Agar (Merck) incubated at 37 °C for 24 h.

Food samples

Food samples were obtained from shops in Bratislava, Slovakia. For artificial contamination, 10^0 CFU of *S. enterica* serovar Enteritidis CCM 4420 (overnight culture) was added to the plastic bag with the food sample and Buffered peptone water (BPW; Merck) prior to homogenization.

Enrichment

A two-step enrichment procedure was used. An amount of 25 g of the food sample was homogenized in 225 ml of BPW (Merck) using Stomacher 400 homogenizer (Seward, Basingstoke, England) and incubated at 37 °C for 18–20 h. A volume of 0.1 ml of the pre-enriched sample was added to 10 ml of the Rappaport-Vassiliadis medium with soya (Merck) and incubated at 41.5 °C for 5 h statically in tubes [6].

Preparation of DNA

DNA was released from the cultures using the cell lysis by boiling. A volume of 1 ml of the bacterial suspension was centrifuged at 10 000 *g* for 5 min, the sediment was resuspended in 0.85 % NaCl and centrifuged again at 10 000 *g* for 5 min. The washed sediment was resuspended in 100 μl of 1x buffer supplied with HotStarTaq DNA polymerase (Qiagen, Hilden, Germany) and incubated at 95 °C for 20 min [9].

Internal amplification control

The plasmid pDP2 to be used as an internal amplification control (IAC) was prepared by cloning a fragment of the gene *phtAc* from *Mycobacterium vanbaalenii* (AY365117) to the plasmid pDrive cloning vector (Qiagen). The sequence of the cloned gene fragment was confirmed by sequencing and primers as well as a probe for 5'-nuclease PCR targeting the sequence were designed using the computer programme Primer Express (Applied Biosystems, Foster City, California, USA). The uniqueness of the designed primers and of the probe was confirmed by BLASTn software on a GenBank database (National Center for Biotechnology Information, Bethesda, Maryland, USA).

Polymerase chain reaction with end-point fluorimetry

Each reaction sample (volume 65 μl) contained 300 nmol.l^{-1} of the primer Srt2F (5'-ata aat ccg gcg gcc tga tg-3'), 300 nmol.l^{-1} of the primer Srt2R (5'-tgg tat cga cgc ctt tat ctg aga-3'), 200 nmol.l^{-1} of the probe Srt2P labelled with 6-carboxyfluorescein and quenched with Black Hole Quencher 1 (FAM-tta cac cgg agt gga tta aac ggc tgg g-BHQ1 [7]; oligonucleotides synthesized by Qiagen Operon, Köln, Germany), approx. 10^3 copies of the plasmid pDP2, 300 nmol.l^{-1} of the primer phtAc11F (tta taa agg cta agc gcg agg tat g), 300 nmol.l^{-1} of the primer phtAc131R (gcg gaa gga acc tcc gtc tt), 200 nmol.l^{-1} of the probe phtAc42P labelled with Yakima Yellow and quenched with Black Hole Quencher 1 (YY-cta cgc gaa ctg tgt cgt tgc ggc c-BHQ1; oligonucleotides synthesized by Qiagen Operon), 400 μmol.l^{-1} of each dNTP (Applied Biosystems), 4.5 mmol.l^{-1} $MgCl_2$, 2 U HotStarTaq DNA polymerase (Qiagen), 6.5 μl of 10x concentrated PCR buffer supplied with the polymerase and 2.5 μl of the DNA preparation. Reactions were performed in TopYield 8-strips (Nunc, Roskilde, Denmark) in a GeneAmp 9700 thermal cycler (Applied Biosystems) using a programme consisting of the initial denaturation at 95 °C for 15 min and 35 cycles (denaturation at 95 °C for 15 s and annealing with polymerization at 60 °C for 60 s). After PCR, amplification was measured by fluorimetry directly in the microtubes in a Genios 96-well reader (Tecan, Grödig bei Salzburg, Austria) equipped with an excitation filter with a pass maximum of 492 nm (bandwidth, 10 nm) and an emission filter with a pass maximum of 520 nm (bandwidth, 10 nm) for channel 1 and an excitation filter with a pass maximum of 515 nm (bandwidth, 10 nm) and an emission filter with a pass maximum of 546 nm (bandwidth, 10 nm) for channel 2. Ten flashes per measurement, integration time 40 μs, manual gain of 75 for channel 1 and 100 for channel 2, and measurement from the bottom orientation were used. To define the positivity threshold, three no-template control samples were always analysed and read in the fluorimeter along with the samples, mean value and the standard deviation (SD) were calculated and the positivity threshold for individual channels was set to (mean + 3 SD).

RESULTS AND DISCUSSION

Intrinsic detection limit

Analytical parameters of the 5'-nuclease PCR with primers Srt2F, Srt2R and the probe Srt2P had been previously determined [7], however, its detection limit might have been impaired by the additional use of the internal control in the duplex mode. For this reason, the intrinsic detection limit of the duplex 5'-nuclease PCR with end-point fluorimetry was determined using decimal dilutions of the *S. enterica* serovar Enteritidis CCM 4420 culture, when fluorescence values greater than the positivity threshold in channel 1 indicated the positive result (Fig. 1). The determined value of 10^4 CFU.ml^{-1} is comparable to that of real-time PCR [5,7] and is compatible with the two-step enrichment [6]. IAC was detectable in all samples, as indicated by fluorescence values greater than the positivity threshold in channel 2, which means that IAC was functional in monitoring the success of the amplification and thus revealing potential false negative results (data not shown). The IAC signal was not detected in channel 1 and hence the use of IAC did not lead to false positive results. Similar results were obtained with two other strains, namely, *S. enterica* serovar Typhimurium CCM 4419 and *S. enterica* serovar Panama ALM 41 (data not shown).

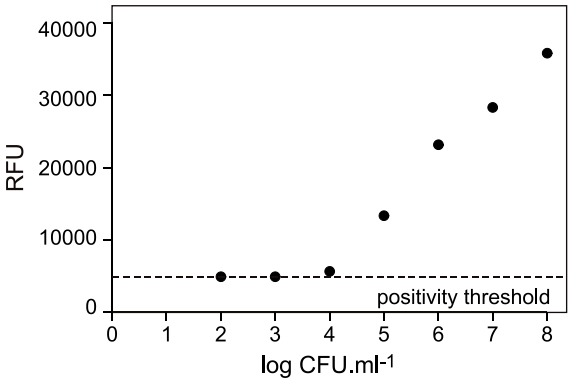

Fig. 1. Results of the duplex 5'-nuclease PCR with decimal dilutions of *S. enterica* serovar Enteritidis CCM 4420 culture and with the internal amplification control; values of mean fluorescence of three replicates of the *Salmonella*-specific PCR in channel 1 (FAM) are presented; standard deviation values were too low to be depicted at the given scale.

Practical performance of the method with artificially contaminated food samples

The complete method for the detection of *S. enterica* in food consisting of the two-step enrichment, bacterial cell lysis and the duplex 5'-nuclease PCR with end-point fluorimetry was evaluated with food samples artificially contaminated with live *S. enterica* serovar Enteritidis CCM 4420 cells at a level of 10^0 CFU/25 g and with respective dead cells at a level of 10^8 CFU/25 g. Experiments were carried out with various food matrices to cover cases when different mixed bacterial populations interfere with the enrichment and thus impair the detection limit. The results confirmed the detection limit of 10^0 CFU/25 g (Tab. 1) which is required by the legislation [2]. Artificially contaminated food samples are considered not to be fully equivalent to the naturally contaminated ones and the detection limit determined with artificially contaminated food samples may be an underestimation in certain cases. However, since proper pre-enrichment was used in the presented method, our results obtained with artificially contaminated food samples can be extrapolated to the naturally contaminated ones in terms of ISO 16140 [10]. The results also demonstrate that the method did not

Tab. 1. Results of the complete method for the detection of *S. enterica* in artificially contaminated food samples; results of three replicates are presented.

Food sample	Artificial contamination level	RFU ± SD	
		Channel 1 (*S. enterica*)	Channel 2 (IAC)
Ice cream	10^0 CFU live	29 299 ± 121	15 718 ± 104
	10^8 CFU dead	5166 ± 6	11 877 ± 47
	positivity threshold	5500	11 197
Brawn	10^0 CFU live	9 330 ± 130	21 953 ± 204
	10^8 CFU dead	5023 ± 11	12196 ± 79
	positivity threshold	5500	11 197
Cheese	10^0 CFU live	16 435 ± 129	32 389 ± 131
	10^8 CFU dead	5360 ± 28	12 637 ± 87
	positivity threshold	5500	11 197

detect dead cells in a density of up to 10^8 CFU/25 g (Tab. 1) which means that it has a potential to avoid false positive results caused by the presence of dead cells. This is an important feature which is absent in other currently available methods [4, 5].

CONCLUSIONS

The presented closed-tube 5'-nuclease PCR with an internal amplification control has a detection limit of 10^4 CFU.ml^{-1} and facilitates monitoring of false negative results caused by the failure of amplification. When coupled to the two-step enrichment and bacterial cell lysis, the complete method for the detection of *S. enterica* in food has a detection limit of 10^0 CFU/25 g, does not produce false positive results due to dead cells and produces results on the next day after the sample collection.

Acknowledgements

This research was done in frames of the Slovakian State Programme of Research and Development, project "Food Quality and Safety", and in frames of the bilateral project of the scientific cooperation between Slovakia and Italy "Rapid detection of *Salmonella* spp. and *Listeria monocytogenes* in food by endpoint 5'-nuclease PCR and PCR-ELISA".

REFERENCES

1. Tietjen, M. - Fung, D. Y. C.: Salmonellae and food safety. Critical Reviews in Microbiology, *21*, 1995, pp. 53-83.
2. Potravinový kódex SR. Druhá časť. 4. hlava. Mikrobiologické požiadavky na potraviny, kozmetické prostriedky a obaly na ich balenie [online]. Published on 29.12.2004 [cited on 28.1.2005]. <http://www.svssr.sk/sk/legislativa/kodex/2_04.asp>
3. EN ISO 6579:2002 / Cor. 1:2004 Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp. Brussels : European Committee for Standardization, 2004. 29 pp.
4. Uyttendaele, M. - Vanwildemeersch, K. - Debevere, J.: Evaluation of real-time PCR vs automated ELISA and a conventional culture method using a semi-solid medium for detection of *Salmonella*. Letters in Applied Microbiology, *37*, 2003, pp. 386-391.
5. Malorny, B. - Paccassoni, E. - Fach, P. - Bunge, C. - Martin, A. - Helmuth, R.: Diagnostic real-time PCR for detection of *Salmonella* in food. Applied and Environmental Microbiology, *70*, 2004, pp. 7046-7052.
6. Kuchta, T. - Kaclíková, E. - Krascsenicsová, K.: Štúdium selektívneho rastu kultúr salmonel v potravinových zmesných kultúrach použitím kvantitatívnej polymerázovej reťazovej reakcie. [Final report.] Bratislava : Výskumný ústav potravinársky, 2005. 25 pp.
7. Piknová, Ľ. - Kaclíková, E. - Pangallo, D. - Polek, B. - Kuchta, T.: Quantification of *Salmonella* by 5'-nuclease real-time polymerase chain reaction targeted to *fimC* gene. Current Microbiology, *50*, 2005, pp. 38-42.
8. Kaclíková, E. - Krascsenicsová, K. - Pangallo, D. - Kuchta, T.: Detection and quantification of *Citrobacter freundii* and *C. braakii* by 5'-nuclease polymerase chain reaction. Current Microbiology, *51*, 2005, pp. 229-232.
9. Abolmaaty, A. - El-Shemy, M. G. - Khallaf, M. F. - Levin, R. E.: Effect of lysing methods and their variables on the yield of *Escherichia coli* O157:H7 DNA and its PCR amplification. Journal of Microbiological Methods, *34*, 1998, pp. 133-141.
10. ISO 16140. Microbiology of food and animal feeding stuffs - Protocol for the validation of alternative methods. Geneva : International Organization for Standardization, 2003, 74 pp.

Received 16 December 2005; accepted 21 February 2006.