Journal of Food: Microbiology, Safety & Hygiene

Oliveira et al., J Food Microbiol Saf Hyg 2018, 3:1
DOI: 10.4172/2476-2059.1000130

Research Article Open Access

Validating the Efficiency of a Simplex PCR and Quantitative SYBR Green qPCR for the Identification of *Salmonella spp*. DNA

Andrey Carlos do Sacramento de Oliveira[1*], Matheus C Rosa[2], Jéssica L Borchardt[2], Yasmine A Menegon[2], Milena Martins Andrade Fernandes[1], Gabrielle Virgínia Ferreira Cardoso[1], Andréia Silva da Silva[1], Roberta S Sousa[1], Josyane Brasil da Silva[1], Fábio FL Leite[2], Talita Bandeira Roos[1] and Carina Martins de Moraes[1]

[1] Food Hygiene and Quality Laboratory and Microbiology Laboratory, School of Veterinary Medicine and Graduate Program in Animal Health in the Amazon, Federal University of Pará (Universidade Federal do Pará-UFPA), Castanhal, Brazil

[2]Technological Development Center (Centro de Desenvolvimento Tecnológico – ®CDTec), Federal University of Pelotas (Universidade Federal de Pelotas-UFPel), Pelotas, Brazil

[*]Corresponding author: Andrey Carlos do Sacramento de Oliveira, Food Hygiene and Quality Laboratory and Microbiology Laboratory, School of Veterinary Medicine and Graduate Program in Animal Health in the Amazon, Federal University of Pará (Universidade Federal do Pará-UFPA), Castanhal, Pará, Brazil, E-mail: andrey.oliveira@zootecnista.com.br

Received date: December 18, 2017**; Accepted date:** January 16, 2018; **Published date:** January 23, 2018

Copyright: ©2018 Oliveira ACS, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Abstract

This study aimed to perform a comparative validation of the efficiency of quantitative SYBR Green qPCR and simplex PCR identification of *Salmonella spp*. DNA. For this, the samples of DNA from the *Salmonella typhimurium* were diluted up to 10^{-5} in duplicate. The results showed that the same primers were effective for both simplex PCR and qPCR. It was possible to detect the bovine species up to a dilution of 10^{-1} using simplex PCR. For all dilutions, it was possible to obtain qPCR amplification with a minimum Ct value of 15.13 for the 10^{-1} dilution for *Salmonella spp*. Next, the SYBR Green qPCR amplicons were separated using agarose gel electrophoresis for confirmation of the amplified fragment size. The superiority of qPCR over multiplex PCR was validated in terms of sensitivity, even with the use of SYBR Green dye, suggesting the possible use for quality control in foodstuffs.

Keywords: *S. typhimurium*; Food pathogens; Food quality; Public health; Bacterial detection; Food contamination

Introduction

Despite current knowledge of microbial pathogenicity, modern methods of food production and rigorous industrial hygiene, causative agents of food-borne diseases are still commonplace. These food-borne illnesses can range from mild aggravations to life-threatening situations [1].

One of the main pathogenic microorganisms is *Salmonella spp.* due to frequent associated with human diseases. According to Dawoud et al. [2], *Salmonella spp.* continues to be one of the more major food-borne pathogens in the world from a public health standpoint due to the biology of *Salmonella* and your ubiquity in nature, related to its ability to inhabit diverse ecosystems, being part of the normal microbiota of the human body, animals and plants.

According to the European legislation on microbiological criteria for foodstuffs, the established standard is the analysis of multiple test portions from the same food item, of which none must be positive for a certain test portion size for *Salmonella spp*. [3]. The European legislation on microbiological criteria for foodstuffs provides a standard for the rest of the world.

Therefore, mechanisms of identification of these pathogens like *Salmonella spp.* is becoming increasingly necessary. Among the several biomolecular techniques for the detection of *Salmonella spp.*, one of the most frequently used is polymerase chain reaction (PCR). Simplex PCR has been used both for the identification of different species and for the bacterial genus *Salmonella spp.*, although it is only a qualitative analysis. There is also real-time quantitative PCR (qPCR), which shows high sensitivity and reproducibility in the amplification of specific fragments and has the ability to quantify the presence of DNA of *Salmonella spp.* in foodstuffs.

Simplex PCR and qPCR are of note owing to their particularities. PCR simplex has proven its effectiveness for decades because it allows the qualitative identification of different species of *Salmonella* in different foods. By contrast, qPCR, in addition to enabling the determination of *Salmonella*, makes it possible to quantify with precision the genetic material identified.

However, qPCR has specific features that are a challenge at the time of standardization of a reaction, among them the generation of primers amplifying a fragment between 50 and 150 base pairs (bp). Another important feature is the choice of dye used for the quantification of DNA, one of the most popular and most economically accessible is SYBR Green, which appears to be an intercalating dye that may generate doubts about the specificity of qPCR [4]. Many researchers around the world despite having standardized simplex PCR reactions decide to decide to synthesize new primers, for the same target species, with smaller fragments for qPCR and avoid using intercalating dyes [5].

Therefore, the objective of this work was to perform a comparative validation of the efficiency of using simplex PCR and quantitative SYBR Green qPCR for the identification of *Salmonella spp.*, using the same set of primers and the same thermal profile for both techniques.

Material and Methods

For the accomplishment of the present research, initially strain of *Salmonella tiphymurium* (ATCC 14028) was used as positive control. Next, the DNA of *Salmonella tiphymurium* was extracted using the

protocol described by Darwish et al. [6], but with modifications as suggested by Silva et al. [7]. The samples were separated using 0.8% agarose gel (Inlab™) electrophoresis (Bio-Rad®), run in Tris-borate-ethylenediaminetetraacetic acid (EDTA) (TBE) buffer, which contains Tris-Base (Promega®), boric acid (Alphatec®) and 0.5% EDTA (Ludwig®), and stained with 6X GelRed™ (Biotium) (1 µL of the dye/5 µL of the sample). The analysis of the electrophoresis results was performed using a UV transilluminator (Gel Documentation System, Gel Doc™, Bio-Rad®). DNA quantification and purity determinations were performed using a BioTek® Gen5™ spectrophotometer at 230 nm, 260 nm and 280 nm according to the Beer-Lambert law [8,9]. In parallel, the samples of DNA from the *Salmonella typhimurium* were diluted up to 10^{-5} in duplicate.

The simplex PCR and qPCR reactions were performed with primers amplifying specific sequences for *Salmonella spp.* (primer reverse 5' - ACTGGTAAAGATGGCT- 3' and primer forward 5' - CGGTGTTGCCCAGGTTGGTAAT- 3'), described by Soumet et al. [10]. These primers generate sequences of 429 bp and were prepared according to the manufacturer's instructions (Ludwig Biotec®), and were eluted in TE buffer (pH 8.0) to a concentration of 100 pmol/µL.

The proposed simplex PCR methodology was applied according to methods previously described by Darwish et al. [6], with some modifications as suggested by Oliveira et al. [7]. The PCR solution contained 50 mM $MgCl_2$ (Ludwing Biotec®), 10X buffer, 10 mM dNTP mix (Ludwing Biotec®), approximately 232 ng of template DNA, 1U Taq DNA Polymerase (Ludwing Biotec®), 10 pmol of each primer (Ludwing Biotec®) and sterilized ultrapure water to a final volume of 25 µL per reaction. The thermocycler (Applied Biosystems VERITI® 96) was programmed for 40 cycles, with denaturing, annealing and extension temperatures and times of 94°C/30 s, 55°C/1 min and 72°C/30 s, respectively. In addition, an initial denaturing step was performed at 94°C for 2 min, and a final extension step was performed at 72°C for 10 min. The amplicons were separated using 1.5% agarose gel (Inlab™) electrophoresis (Bio-Rad®) run in TBE buffer and stained with 6X GelRed™ (Biotium) (1 µL of the dye/5 µL of the sample). The analysis of the electrophoresis results was performed using a UV transilluminator (Gel Documentation System, Gel Doc™, Bio-Rad®).

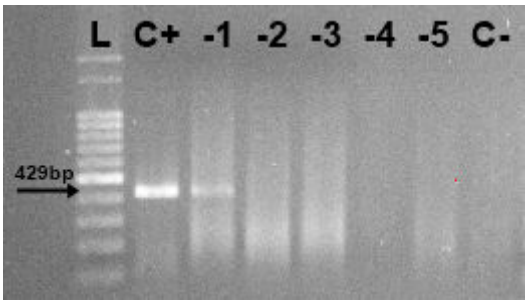

Figure 1: 1.5% agarose gel showing the presence of *Salmonella* (429 bp) DNA fragments obtained using simplex PCR performed with serial dilutions of *Salmonella spp.* DNA. L: 1 kb molecular marker; C+: positive control; -1: 10^{-1} *Salmonella typhimurium* DNA dilution; -2: 10^{-2} *Salmonella typhimurium* DNA dilution; -3: 10^{-3} *Salmonella typhimurium* DNA dilution; -4: 10^{-4} *Salmonella typhimurium* DNA dilution; -5: 10^{-5} *Salmonella typhimurium* DNA dilution and C-: negative control.

The qPCR reactions were run in a real-time PCR thermocycler (LightCycler®96 System, Roche Life Science) using the same primers described above for simplex PCR. The qPCR reactions were run in 96-well semi-skirted polyethylene plates (Corning®), using 1 µL (232 ng) of DNA, 6.25 µL of SYBR Green PCR Master Mix (Applied Biosystems™), 10 pmol of each primer, and 3.75 µL of DNAse-free water (Sigma Aldrich) for a total volume of 12 µL, and all the samples were analyzed in duplicate. The thermal cycle profile was the same model used for simplex PCR described here. Next, the SYBR Green qPCR amplicons were separated using agarose gel electrophoresis, under the same conditions described above for simplex PCR, for confirmation of the amplified fragment size.

In the qPCR, the samples were considered positive when the threshold cycle (Ct) value was less than 35 and when amplification was detected for both replicates. The amplification efficiency was calculated using the slope value obtained in the qPCR for each DNA dilution. The melting curve was analyzed to evaluate the specificity of the primers. Based on the initial quantification, titer values were defined for each dilution and its respective concentrations in ng/µL. These values were used to obtain the standard curve that included the Ct values on the Y-axis and the logarithm of the DNA concentration on the X-axis. The linear regression formula generated was the basis for the subsequent calculations to determine unknown samples.

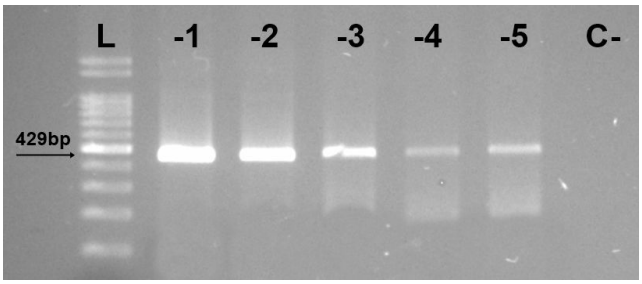

Figure 2: 1.5% agarose gel showing the presence of *Salmonella* (429 bp) DNA fragments obtained using the qPCR amplicons performed with different dilutions of DNA. L: 1 kb molecular marker; -1: 10^{-1} *Salmonella typhimurium* DNA dilution; -2: 10^{-2} *Salmonella typhimurium* DNA dilution; -3: 10^{-3} *Salmonella typhimurium* DNA dilution; -4: 10^{-4} *Salmonella typhimurium* DNA dilution; -5: 10^{-5} *Salmonella typhimurium* DNA dilution and C-: negative control.

Results

The 0.8% agarose gel electrophoresis profile and the results obtained through the Beer-Lambert law [7,8] showed that DNA extraction resulted in an extracted material with a quality and concentration sufficient for simplex PCR and qPCR. The purity and yield of the total DNA extracted was confirmed by measuring the absorbance at 230 nm, 260 nm and 280 nm, which indicated values that met DNA purity parameters and with a mean concentration of 232 ng/µL.

The set of primers for the detection of *Salmonella spp.* presented good accuracy and precision in both simplex PCR and qPCR. Figure 1 shows the results obtained using simplex PCR performed with serial dilutions of *Salmonella spp.* DNA.

The Figure 2 presents the results obtained using the qPCR amplicons performed with different dilutions of DNA.

Table 1 presents the Ct value and amplification efficiency obtained using qPCR performed with different dilutions of DNA from the same species.

Species	Sample	Ct[a] (evaluation)	Efficiency (slope)[b]
Salmonella typhimurium	*Salmonella* control	12.05 (+)	89 (-3.63)
	Negative control	-	-
	100	12.05 (+)	89 (-3.61)
	10^{-1}	15.13 (+)	89 (-3.59)
	10^{-2}	18.86 (+)	90 (-3.58)
	10^{-3}	20.90 (+)	111 (-3.08)
	10^{-4}	21.45 (+)	113 (-3.04)
	10^{-5}	21.56 (+)	104 (-3.22)

Table 1: Real-time PCR results (Ct and efficiency) for the *Salmonella typhimurium* controls and dilutions; [a]Cut-off used: positive result (+): Ct <35; negative result (-): Ct ≥ 35; [b]Percent efficiency of qPCR amplifications: E=(10(-1/slope)-1) × 100%.

The melting curves obtained presented high specificity for the target fragment, with approximately 80°C (Figure 3).

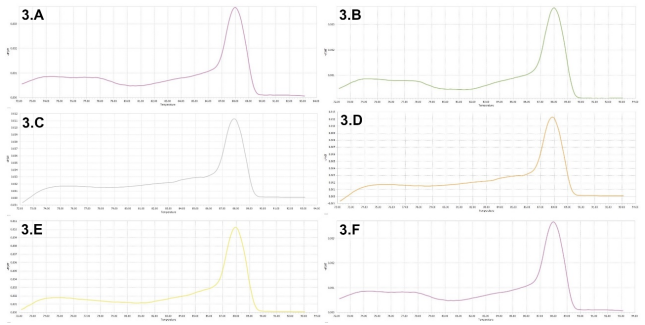

Figure 3: Melting curves obtained after qPCR amplification with SYBR Green dye, where 3.A: positive control; 3.B: 10^{-1} *Salmonella typhimurium* DNA dilution; 3.C: 10^{-2} *Salmonella typhimurium* DNA dilution; 3.D: 10^{-3} *Salmonella typhimurium* DNA dilution; 3.E: 10^{-4} *Salmonella typhimurium* DNA dilution and 3.F: 10^{-5} *Salmonella typhimurium* DNA dilution.

Discussion

Salmonellosis represents a severe economic and public health concern due to wide spread by different foods. To this end, an early diagnosis and a rapid laboratory confirmation are crucial for public health measures, to contain problems related to the outbreaks. Many researchers highlight simplex PCR as a simple technique for species-specific identification. These researchers detected Salmonella spp. using a rapid and specific PCR, with methodologies and results similar to those obtained in this study. However, while the technique is extremely efficient, it does not provide quantitative data, which is often relevant when it comes to the sensitivity of the technique, which increases the importance of techniques such as SYBR Green qPCR used in this study

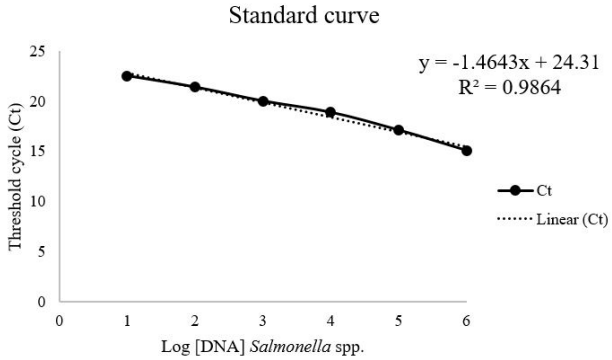

Figure 4: Standard curves showing the logarithm of the DNA concentration *vs.* the threshold cycle (Ct) for *Salmonella spp.*

Figure 4 shows the standard curves with specific formulas relative to the logarithmic scale of the concentration for each duplicate of the *Salmonella spp.* DNA dilutions; all showed a linear regression coefficient (R^2) ≥ 0.97 (Figure 4).

In addition, the amplification of the *Salmonella* DNA control at a concentration of 232 ng confirmed the precision of the data presented by Soumet et al. [10], who developed a multiplex PCR here for the detection of all the serotypes of *Salmonella* and for the identification of *Salmonella typhimurium* and *enteritidis*. In addition to the amplification of the controls, in our study it was possible, by simplex PCR, to detect *Salmonella spp.* DNA up to a 10^{-1} dilution (23.2 ng), as shown in Figure 1. These results aid in the determination of the sensitivity of the technique and allow the comparative validation with qPCR results.

In spite of the advantages of simplex PCR, related to the determination species-specific and the high speed when compared with standard methods for detection of these pathogens, this technique presents some limitations, since it allows only a qualitative analysis of the results and requires an additional step for interpretation of the results. When comparing the different existing methods, SYBR Green qPCR produces results within a relatively short period of time and with high sensitivity when compared to simplex PCR and is the simplest and least expensive of qPCR methods. This fact was confirmed in our study because although the extraction of DNA was equal for both techniques there were differences in the following phases.

Some factors must be considered in the development of simplex PCR and qPCR, including primer design, annealing temperature and choice of dye, which represent fundamental factors for high efficiency and specific amplification [11]. In this study, we were able to adjust the protocols so that the same primer set was used in both the simplex PCR and SYBR Green qPCR applications. In addition, these adjustments allowed an ideal annealing temperature of 55°C for both techniques. One of the innovative features of the SYBR Green qPCR approach proposed here is that the set of primers amplifies fragments of 429 bp (Figures 1 and 2). This exceeds the fragment size, considered ideal, for qPCR development, which, according to most researchers, cannot be higher than 150 bp [12].

For the analysis of SYBR Green qPCR results, the cut-off Ct value was defined as 35 and all the DNA dilutions were considered strongly positive because they yielded Ct <35 (Table 1). According to Pfaffl [13], PCR efficiency must be 100 ± 20%, which corresponds to slope values ranging from -3.6 to -3.1. In our study, we obtained an efficiency between 89% and 113%, all within the acceptance parameters for an efficient qPCR (Table 1).

Next, the SYBR Green qPCR amplicons were removed from the plate and separated using agarose gel electrophoresis for confirmation of the amplified fragment of 429 bp (Figure 2). In addition, the visualization of a single peak in the melting curve for all the dilutions indicated a single specific fragment, the absence of primer dimers, and the lack of nonspecific products, which is similar to the results previously obtained for simplex PCR (Figure 3). These results for the melting curve and electrophoresis also validate the qPCR protocol proposed here, in particular the use of SYBR Green, which is a nonspecific intercalating dye capable of detecting any double-stranded DNA present in the sample [8].

This is a peculiarity of our research, since it makes possible the use of a more financially accessible dye without compromising the efficiency and specificity of qPCR, in addition, it optimizes the use of primers by the possibility of making a single set that is functional in different PCR variations and for the detection of this bacterial genus in different products of animal origin.

An analysis of the standard curve allows the conclusion that qPCR was highly sensitive for the detection of *Salmonella* at all dilutions used (Figure 4). Even the less concentrated sample was detected at Ct=21.56 whereas in the simplex PCR no dilution below 10^{-1} was detected neither in the plateau phase in cycle 40. The linear regression formula generated will be the basis for the subsequent calculations to determine unknown samples.

Thus, the association and correlation of the results presented in this study show that the use of primers that are initially designed to be used in simplex PCR and the use of SYBR Green did not affect the efficiency and specificity of the qPCR approach (Table 1).

Conclusion

We conclude that simplex PCR and SYBR Green qPCR validation was possible using the same set of primers and the same thermal profile for both techniques for the detection of *Salmonella spp.* DNA. The superiority of qPCR compared to simplex PCR was validated in terms of sensitivity, even when using SYBR Green dye, which suggests its potential use for the authentication of different foodstuffs.

References

1. Fleckenstein JM, Bartels SR, Drevets PD, Bronze MS, Drevets DA (2010) Infectious Agents of Food and Water Borne Illness. Am J Med Sci 340: 238-246.
2. Dawoud TM, Shi Z, Kwon YM, Ricke SC (2017) Overview of Salmonellosis and Food-borne, *Salmonella*: Historical and Current Perspectives. Producing Safe Eggs 7: 133-138.
3. EC, European Community (2005) Commission Regulation, 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Eur Lex.
4. Prada-Arismendy J, Castellanos JE (2011) Real time PCR. Application in dengue studies. Colomb Med 42: 243-258.
5. Liu Y, Cai X, Zhang X, Gao Q, Yang X, et al. (2006) Real time PCR using TaqMan and SYBR Green for detection of *Enterobacter sakazakii* in infant formula. J Microbiol Methods 65: 21 – 31.
6. Darwish SF, Allam, H A, AminAS (2009) Evaluation of PCR assay for detection of cow's milk in water buffalo's milk. World Appl Sci J 7: 461-467.
7. Oliveira ACSO, Ferreira BCA, Cardoso GVF, Silva CL, Silva AS, et al. (2015) Avaliação da técnica PCR multiplex para detecção de fraude por adição de carne bubalina em carne moída bovina. Rev Inst Adolfo Lutz 74: 371-379.
8. Lambert JH (1760) Photometria, sive de mensure et gradibus luminis, colorum et umbrae. German: German Edition, 2.
9. Beer A (1852) Bestimmung der Absorption des rothen Lichts in farbien Flüssigkeiten. Annalen der Physik 152: 78–82.
10. Soumet C, Ermel G, Rose N, Rose V, Drouin P, et al. (1999) Evaluation of a multiplex PCR assay for simultaneous identification of *Salmonella sp., Salmonella enteritidis* and *Salmonella typhimurium* from environmental swabs of poultry houses. Lett Appl Microbiol 28: 113-7.
11. Ye J, Feng J, Zhang Y, Jiang, X, Dai Z, et al. (2016) Identification of four squid species by quantitative real-time polymerase chain reaction. Mol Cell Probes 30: 22-29.
12. Arvidsson S, Kwasniewski M, Riaño-Pachón DM, Mueller-Roeber B (2008) QuantPrime – a flexible tool for reliable high-throughput primer design for quantitative PCR. BMC Bioinformatics 9: 465.
13. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: 45