Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola

ISSN 1516-635X Jan - Mar 2019 / v.21 / n.1 / 001-006

http://dx.doi.org/10.1590/1806-9061-2018-0776

Original Articles

Duplex Real-Time PCR Using Sybr Green I for Quantification and Differential Diagnosis between Salmonella Enteritidis and Salmonella Typhimurium

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■Keywords

Identification, melting curve, non-typhoidal salmonellosis, standard curve.

Submitted: 11/March/2018
Approved: 15/May/2018

ABSTRACT

The incidence of foodborne diseases caused by the genus *Salmonella* spp. in industrialized countries is often high in epidemiological surveys. Obtaining a rapid diagnostic test for identification of bacteria is crucial in order to rapidly implement control measures to contain bacterial spread, to reduce losses in animal production and to avoid risks from food-borne infections to human health. The aim of this study was to standardize duplex real-time PCR using SYBr Green I for differential and quantitative diagnosis of *S*. Typhimurium and *S*. Enteritidis. According to the experiment, the melting temperature of 85°C was observed for a 206bp amplified product when *S*. Enteritidis DNA was added to the reaction. *S*. Typhimurium DNA showed that the melting temperature of 79°C when observed for a 62bp amplified product. The standard curve showed the high sensitivity of the proposed test, since it was possible to obtain eight quantification points, starting at 10^8 CFU/mL and ending at 10^1 CFU/mL. As a result of the present study, a real-time PCR duplex reaction with high sensitivity, specificity and based on the fluorescence of SYBr Green I was standardized. In addition, this methodology aligns low cost to the faster diagnostic result, in relation to other molecular tests, making it attractive for application in routine laboratory analyzes.

INTRODUCTION

The incidence of foodborne diseases caused by the genus *Salmonella* spp. in industrialized countries is often high in epidemiological surveys (Ma *et al.*, 2014). At least 1.3 billion cases of human salmonellosis are reported annually worldwide, with 93.8 million cases of acute gastroenteritis, 155000 deaths and approximately 85% of which are estimated to be foodborne (FAO/WHO, 2016). These outbreaks cause significant damage to the health of infected animals and humans and economic losses (Chen *et al.*, 2010).

A report published in Brazil, by the Secretary of Health Surveillance (SVS), showed that the incidence of foodborne diseases in this country was reduced by 35% in comparison to the last report published in 2014, but in 58.8% of these outbreaks, the isolation and identification of the pathogens involved was not performed. However, when the agent was identified, 14.3% of these isolates came from bacteria of the genus *Salmonella* spp., being the most frequently isolated pathogen among the analyzed samples (SVS, 2015).

Products of poultry origin are often associated with food born pathogen outbreaks from *Salmonella* spp., because they are affected by both host-specific and non-specific serovars (O'Regan *et al.*, 2008). The importance of their control at all scales of the poultry production is due to the fact that they are capable of infecting commercial birds. Consequently, there will be the contamination of poultry products which

are subsequently destined for human consumption (Löfström *et al.*, 2010).

A rapid diagnostic test for identification of bacteria is crucial in order to rapidly implement control measures to contain bacterial spread, to reduce losses in animal production and to avoid risks from food-borne infections to human health (Chen *et al.*, 2010; Cheraghchi *et al.*, 2014). With this purpose, the real-time PCR (qPCR) has been outstanding for the diagnosis of pathogens, since they obtain results faster than the microbiological diagnosis and with reduction of the stages used in the reaction, associated with higher sensitivity, when compared to conventional PCR (Park *et al.*, 2011; Ma et al. 2014). The aim of this study was to standardize duplex qPCR using SYBr Green for differential and quantitative diagnosis between *S.* Enteritidisand *S.* Typhimurium.

MATERIAL AND METHODS

Bacterial strains for validation of the qPCR assay

Bacterial strains are used for standardization of the multiplex quantitative PCR in real time, including the sequenced strain *S.* Enteritidis P125109 (accession number: AM933172.1) (Thomson *et al.*, 2008) and *S.* Typhimurium F98 (Barrow *et al.*, 1990). Although the primers had already undergone validation in published research (Malorny *et al.*, 2007; Park *et al.*, 2013), 21 *Salmonella* spp. and six non-*Salmonella* spp. strains were used as negative controls for validation of the primers and the multiplex reaction specificity (Table 1). All bacteria were stored at -80°C in the Laboratory of Avian Pathology at the Agricultural and Veterinary Sciences School (FCAV-Unesp/Jaboticabal/SP).

Bacterial DNA extraction

Each isolate was cultured in lysogeny broth (LB) at 37 °C, shaking at 100 rpm for a period of 18 hours, including the bacteria used as negative controls. Before DNA extraction, all *S.* Enteritidis and *S.* Typhimurium cultures were quantified by plating serial dilutions in Brilliant Green agar (Oxoid CM0263) of the culture with subsequent incubation at 37°C for 24 hours. After incubation, the bacterial numbers of each culture were evaluated and the colony forming units per mL (CFU/mL) were transformed to Log_{10}. DNA extraction of all bacterial cultures was performed using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer instructions. The purity

Table 1 – Bacterial strains used to duplex real-time qPCR validation for differential diagnosis between *S.* Enteritidis and *S.* Typhimurium.

Strain	Code	Strain	Code
Salmonella spp.		Non - *Salmonella* spp.	
S. Agona	CR104/10	*Escherichia coli*	Field strains
S. Albany	CR189/07	*Citrobacter freundii*	Field strains
S. Enteritidis	P125088	*Listeria monocitogenes*	FC00266
S. Enteritidis	SE 147/139-2	*Pseudomonas* sp.	Field strains
S. Enteritidis	SE 16742	*Shigella sonnei*	ATCC23931
S. Enteritidis	SE 174	*Yersinia enterocolitica*	ATCC9610
S. Gallinarum	SG 287/91		
S. Hadar	CR111/10		
S. Heidelberg	CR239/08		
S. Infantis	CR142/10		
S. Newport	CR044/10		
S. Paratyphi B	CR423/06		
S. Pullorum	SP FCAV198		
S. Saintpaul	CR325/08		
S. Schwarzengrund	CR121/10		
S. Senftenberg	CR119/10		
S. Typhimurium	STM 1116		
S. Typhimurium	STM 723		
S. Typhimurium	STM F98		
S. Typhimurium	STM 986		
S. Typhimurium	STM 985/11		

Bacterial isolates were obtained from: National Agricultural and Livestock Laboratories – LANAGRO (CR/AV); Field strains isolated and maintained at School of Agricultural and Veterinary Sciences, São Paulo State University, FCAV/UNESP, Jaboticabal Campus, SP, Brazil.

and concentration of bacterial DNA was analyzed by spectrophotometry (Nanodrop 1000 ThermoFisher Scientific, USA).

Primers used for differential diagnosis between *S*. Enteritidis and *S*. Typhimurium

Two pairs of primers were used and the sequences and details are in Table 2. The primer spSE were based on Malorny *et al.* (2007) to detect the *S.* Enteritidis and the primer spSTM were based on Park *et al.* (2013) to detect *S.* Typhimurium. Primers were validated *in silico* using the primer BLAST tool and with 21 *Salmonella* spp. and six non - *Salmonella* spp. strains. For the standardization of this reaction, the primers were submitted to a temperature and concentration gradient evaluation, to obtain the best annealing temperature for both primers and the best reaction inclusion concentration for each primer pair (not shown).

Cloning of the target DNA amplicon

The target genome regions used for the specific amplification and diagnostic of each biovar, were cloned into plasmids to use as positive controls and to obtain the quantitative standard curve in the qPCR, according to the adapted protocol described by Sambrook & Russel (2001). Briefly, the amplicons were obtained with conventional PCR which had as reaction mix 10X of Buffer, 2 µM of dNTP, 25 µM of $MgCl_2$, 4 µM of each primer, 2 µL de DNA, 5 U/mL de Taq DNA polimerase and ultrapure water to make the final volume 20 µL (Sigma-Aldrich, St. Louis, Missouri, USA), with cycling protocol starting with one denaturation cycle at 94 °C for 3 minutes, followed by 30 cycles at 94°C for 40 seconds, 63°C for 60 seconds and 72°C for 60 seconds and one final extension cycle at 72°C for 7 minutes.

Table 2 – Specifications of the primers used in the study.

Primer	Sequence (5' - 3')	Product Size (pb)	Concentration (µM)
pSE	F - ATATCGTCGTTGCTGCTTCC R - CATTGTTCCACCGTCACTTTG	206	0.05
pSTM	F - GCGCACCTCAACATCTTTC R - CGGTCAAATAACCCACGTTCA	62	0.025
SalI	5'GTCGAC3' 3'CAGCTG5'	---	---
PstI	5'CTGCAG3' 3'GACGTC5'	---	---
BamHI	5'GGATCC3' 3'CCTAGG5'	---	---
HindIII	5'AAGCTT3' 3'TTCGAA5'	---	---

pSE: *Salmonella* Enteritidis specific primers; pSTM: *Salmonella* Typhimurium specific primers.
F: Primer forward; R: Primer reverse. SalI, PstI, BamHI and HindIII: restriction enzymes.

The primer used for *S*. Enteritidis, were designed with SalI and PstI (Thermo Scientific, Waltham, Massachusetts, EUA); and for *S*. Typhimurium were designed with BamHI and HindIII (Thermo Scientific, Waltham, Massachusetts, EUA) subsequently both amplicons were cloned in plasmid (pMAL™-p4X; NEB #E8000). Competent cells of *E. coli* DH5α™ using thermal transformation were used. After cloning, the accurate copy number of the target amplicons was calculated (NEBioCalculator™ v 1.6.0) and the DNA of each clone was diluted, corresponding to 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 CFU of *S*. Enteritidis or *S*. Typhimurium, to obtain the quantitative standard curve.

Conditions of the duplex real-time qPCR

The duplex qPCR was performed using the following optimized reaction mix of 12.5 µL Luminoct® SYBr® Green qPCR Readymix (Sigma-Aldrich, St. Louis, Missouri, USA), 0.05 µM of the primer pSE, 0.025 µM of primers pSTM, 2.5 µL of serially diluted DNA or eluted DNA after the extraction from the tested samples, and ultrapure water (Sigma-Aldrich, St. Louis, Missouri, USA) to make the final volume 25 µL. The qPCR in real time was performed in the C1000 Touch™ Thermal Cycler (Bio-Rad, Hercules, California, USA) and Low-Profile 0.2 ml 8-Tube Strips (Bio-Rad, Hercules, California, USA), with cycling protocol starting with one denaturation cycle at 94 °C for 2 minutes, followed by 40 cycles at 94°C for 15 seconds and 63°C for 21 seconds. After amplification, the melting curve was performed to obtain the specific melting temperature (*Tm*) of each amplicon for the differential diagnosis between *S*. Enteritidis and *S*. Typhimurium, in which 5°C per 5 seconds were used for the temperature and time range.

Standard curve for absolute bacterial quantification

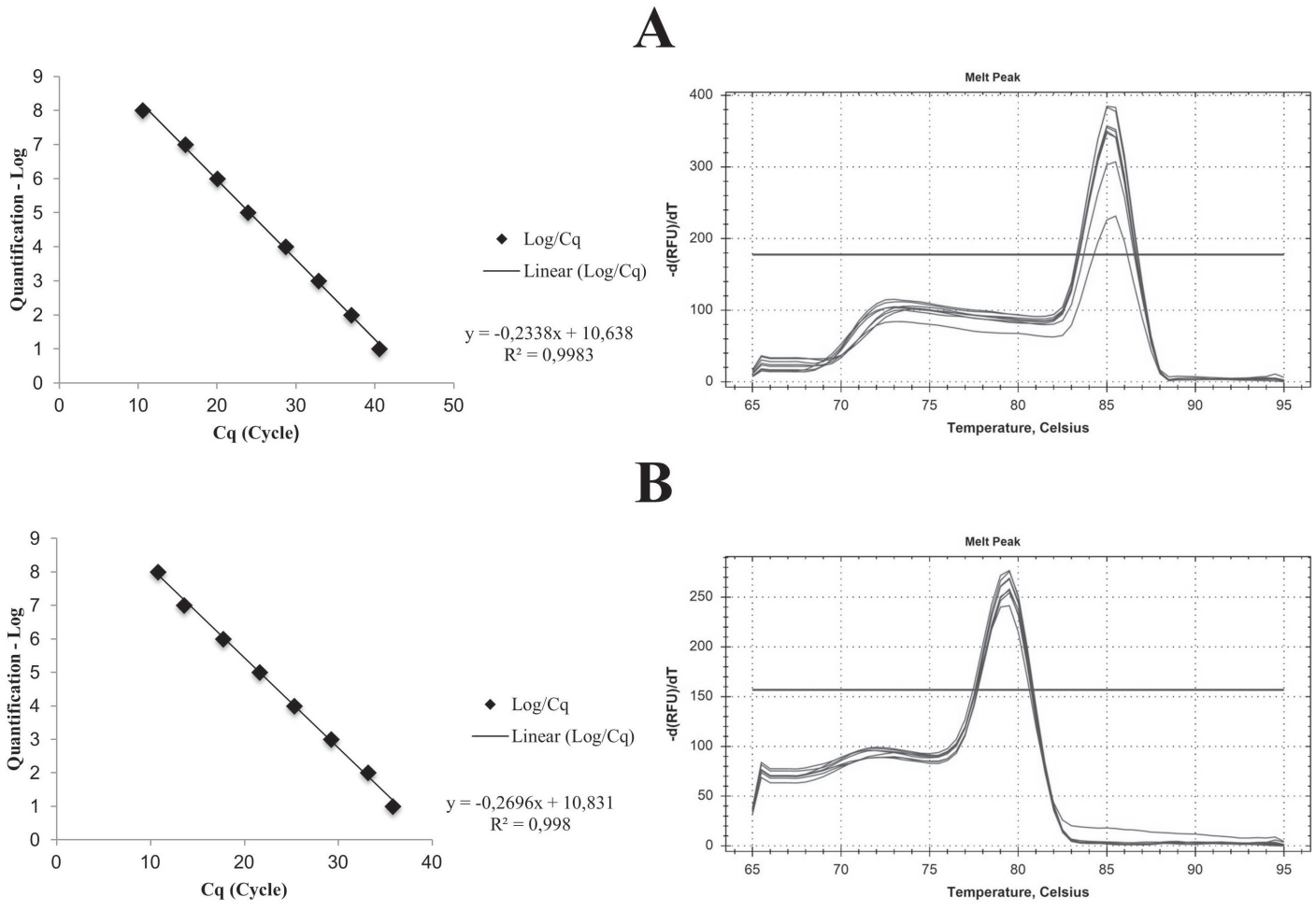
Serially diluted plasmid DNA containing the cloned DNA from *S*. Enteritidis and *S*. Typhimurium, in triplicate, were used to prepare the quantitative standard curve. The reactions followed the optimized qPCR conditions for the multiplex reactions. The CFX Manager™ 3.1 Software (Bio-Rad, Hercules, California, USA) was used to calculate the regression coefficients, standard deviation and standard curves efficiency ratio. The linear standard curve was used to quantify the number of gene copies that corresponded to the number of CFU of each serovar.

RESULTS AND DISCUSSION

The effects on public health caused by bacteria of the genus *Salmonella* spp. are known to be harmful to animal productions, due to their high occurrence in reports of food outbreaks and in industrialized or non-industrialized countries, with higher incidence of *S*. Enteritidis and *S*. Typhimurium isolates (Aktas *et al.*, 2007; Arshad *et al.*, 2008; Freitas *et al.*, 2010; Matheson *et al.*, 2010; Favier *et al.*, 2013; CDC, 2014; SVS, 2015; EU, 2016). Due to this, qualitative and quantitative studies with these bacteria are of great value, by properly identifying the pathogen that is occurring, the place from which they are derived and the bacterial amount that is necessary to cause damage to the animals and humans health (Ellingson *et al.*, 2004).

With the standardization of the reaction, the results obtained in the present study (Figure 1) demonstrate the possibility to differentiate and quantify two serovars, *S*. Enteritidis and *S*. Typhimurium, in a single reaction and using the fluorophore SYBr Green I. Because it does not require the use of probes and more than one reaction to obtain the differential diagnosis, this protocol becomes less expensive and faster, in order to enable the implementation of adequate control measures to eradicate these important pathogens from the public health view.

This methodology for the simultaneous detection of pathogens is frequently reported in the literature with the use of hydrolysis probes. However, the manufacture of this type of fluorophore has a higher



cost than the fluorophore SYBr Green I. In the absence of probes, the specificity of the reaction and the differentiation of pathogens with the use of DNA intercalators is determined by the melting temperature of the amplified product (Nam *et al.*, 2005). The differentiation of *S*. Enteritidis and *S*. Typhimurium serovars by the duplex reaction qPCR in the present work was possible due to the difference between the size generated for each amplified product and melting temperature (*Tm*) of the specific primers. According to the experiment, a *Tm* of 85°C was observed for a 206bp amplified product when *S*. Enteritidis DNA was added to the reaction. When *S*. Typhimurium DNA was added a *Tm* of 79°C was observed for a 62bp amplified product. In contrast, when carrying out the validation of the reaction with the negative control samples, no *Tm* was observed, or it was different from the positive controls obtained for the duplex reaction.

Akiba *et al.* (2011) performed a multiplex conventional PCR reaction for the differentiation of the *S*. Enteritidis and *S*. Typhimurium serovars, however, it required electrophoresis as an additional step and required 7 pairs of primers for its differentiation. In addition, due to the difficulty in interpreting the results, through the large number of primers used in the reaction, the occurrence of false negative reactions were reported. Similarly, Freitas *et al.* (2010) standardized a multiplex reaction to differentiate these serovars by conventional PCR and although they reported the need for only 2 primers for their differentiation by viewing bands, a post-PCR step was also required, which requires more time and increases the risk of contamination or loss of the analyzed samples.

With a standard curve for quantification of samples for the duplex reaction qPCR showed the high sensitivity of the proposed test, since it was possible to obtain eight quantification points, starting at 10^8 CFU/mL and ending at 10^1 CFU/mL. This fact demonstrates the potential of detection that qPCR possesses, obtaining the diagnostic result from the DNA of 10^1 CFU/mL, which could not be done only by conventional microbiology, which according to Malorny *et al.* (2008), the limit of bacterial detection by the microbiological technique is 10^2 CFU/mL, using the most probable number (MPN) with reference.

In an epidemiological study in Michigan (USA), Arshad *et al.* (2008) reported that cases of *Salmonella* spp. are underreported, probably due to low bacterial load in faecal samples and the presence of mild symptoms in the analyzed patients. However, they did not quantify the samples from the reported fact, reinforcing the importance of having a standard curve for quantification of this type of clinical sample and for epidemiological surveys, in order to adequately delineate the spread, course and predispositions of the disease.

Similar to the present study, Park & Ricke (2010) performed a reaction for *S*. Enteritidis and *S*. Typhimurium serovars differentiation and quantification of the samples. However, they performed the differentiation in conventional PCR and with specific primers for each serovar. The quantification of the samples was done by qPCR and with a primer specific only for the genus *Salmonella* spp. This fact contrasts with the present study, which also differentiated and quantified the serovars and the diagnostic result was obtained in only one type of reaction, without the need for subsequent steps and allowing its quantification and differentiation in only one reaction.

Hein *et al.* (2006) to standardize a reaction based on the *inv*A gene for the detection of *Salmonella* spp., performed a quantification curve with *S*. Typhimurium DNA in order to evaluate the limit of detection of the reaction. At the end of the standardization, they reported a limit of detection of 10^2 CFU/mL for their reaction, a fact contrary to the present experiment, which allows the identification of samples with bacterial load referring to 10^1 CFU/ml. This fact shows the high sensitivity obtained with the reaction of the present study.

As a result of the present study, a duplex qPCR with high sensitivity, specificity and based on the fluorescence of SYBr Green I was standardized for differential and quantitative diagnosis between *S*. Enteritidis and *S*. Typhimurium. In addition, this methodology aligns low cost to the speed in obtaining the diagnostic result, making it attractive for application in routine laboratory analyzes.

ACKNOWLEDGEMENTS

The authors thank the Sao Paulo Research Foundation (FAPESP) and Coordination of Improvement of Higher Level Personnel (CAPES) for the financial support.M.S.R. was funded by doctorate scholarship (Grant n. 2014/05496-7) and R.A.C.P.F. was funded by post-doctoral scholarship (Grant n. 2012/24017-7).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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