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Development of a multiplex real-time PCR assay with an internal amplification control for the detection of *Campylobacter* spp. and *Salmonella* spp. in chicken meat

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ABSTRACT

Conventional methods for the detection of *Campylobacter* and *Salmonella* based on culturing are time consuming and laborious. The aim of this study was to develop a multiplex real-time PCR assay with an internal amplification control for the simultaneous detection of *Campylobacter* spp. and *Salmonella* spp. in chicken meat. Boiling was used for DNA extraction, followed by nucleic acid purification with phenol-chloroform. Assay specificity was 100%, and the detection limit was 10^3 CFU of *Campylobacter* spp. and 10^6 CFU of *Salmonella* spp. per milliliter of spiked chicken meat rinse without an enrichment step. After 24 h of the selective enrichment of *Campylobacter* spp. and the non-selective enrichment of *Salmonella* spp., the assay sensitivity was 1 CFU of each of these pathogens per milliliter of rinse. To our knowledge, the present study is the first multiplex real-time PCR assay developed for the simultaneous detection of these pathogens with the inclusion of an internal amplification control to monitor PCR inhibitors. The developed assay is a relatively inexpensive and efficient means to detect *Campylobacter* spp. and *Salmonella* spp. in chicken meat after enrichment, and can be a useful alternative in food processing to prevent the distribution of contaminated food.

1. Introduction

Campylobacter spp. and *Salmonella* spp. are the most common causes of bacterial foodborne disease in several countries, and poultry products are the main vehicles of transmission to humans (WHO, 2009). Brazil is the third largest producer of chicken meat (behind the United States and China) and is the largest exporter of this product (followed by the United States and the European Union). In 2013, Brazil produced 12.30 million tons of chicken, and 31.6% of this was exported. In Brazil, the *per capita* consumption of chicken increased from 29.91 kg in 2000 to 41.8 kg in 2013 (UBA, 2014).

Conventional methods for the detection of *Salmonella* and *Campylobacter,* based on culturing, are time consuming and laborious (Candrian, 1995; Omiccioli, Amagliani, Brandi, & Magnani, 2009). A rapid and specific detection method for these pathogens is essential due to the perishable nature of raw chicken meat. Multiplex PCR allow simultaneous amplification of more than one target sequence in a single PCR reaction. A multiplex PCR assay has the potential to produce considerable savings of time, reagent costs and effort, without compromising the results (Omiccioli et al., 2009; Perry et al., 2007). The elimination of post amplification steps increases the reliability and reproducibility of the assay and decreases the time of analyses (Lund, Nordentoft, Pedersen, & Madsen, 2004).

Real-time PCR is based on fluorescence measurements during the PCR run. Hydrolysis probes use a short oligonucleotide of 20–30 bases coupled on the 5′ end with a fluorescent reporter dye and on the 3′ end with a quencher dye. The quencher dye absorbs the fluorescence from the reporter, preventing the light signal from reaching the detector. During amplification, the 5′ nuclease activity of the DNA polymerase hydrolyzes the probe bound to the target amplification product. The released reporter dye is no longer quenched and then can be detected (Malorny, Huehn, Dieckmann, Krämer, & Helmuth, 2009).

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A problem in real-time PCR is DNA amplification failure due to the presence of inhibitory substances in the samples that can produce false negative results. The European Standardization Committee, in collaboration with International Standard Organization, has proposed a general guideline for PCR testing that requires the presence of an internal amplification control (IAC) in each PCR reaction. Thus, an IAC must be included when a PCR assay is to be validated through a multicenter collaborative trial (Hoorfar, Malorny, Wagner, Abdulmawjood, & Fach, 2003; Lund & Madsen, 2006).

The rapid detection of *Campylobacter* spp. and *Salmonella* spp. in food can identify sources of contamination and help food supply monitors to take appropriate measures to prevent the distribution of contaminated food. The aim of this study was to develop a multiplex real-time PCR assay for the simultaneous detection of *Campylobacter* spp. and *Salmonella* spp. in chicken meat using a hydrolysis probe assay and an IAC.

2. Material and methods

2.1. Bacterial strains and growth conditions

Salmonella serovars, *Campylobacter* strains and other bacterial strains used in this study are listed in Table 1. *Campylobacter* strains were grown in Bolton Broth (CM 0983, Oxoid, Basingstoke, Hampshire, United Kingdom) at 42 °C for 48 h (h) under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2) created with the Microaerobac system (Probac, São Paulo, SP, Brazil). *Salmonella* and other bacterial strains were grown in brain heart infusion broth (BHI, Acumedia, Lansing, Michigan, USA) at 37 °C for 24 h.

2.2. DNA extraction and purification

Aliquots of 1 mL of bacterial suspensions were centrifuged at 16.000×*g* for 10 min (min). The pellet was washed twice with 1 mL buffered peptone water (Acumedia, Lansing, Michigan, USA), centrifuged at 16.000×*g* for 10 min and resuspended in a 300 μL of solution of Triton X-100 (Nuclear) 1%. Suspensions were heated at 100 °C for 10 min, cooled in ice, and centrifuged at 16.000×*g* for 5 min.

For DNA purification, 300 μL of a solution of phenol, chloroform and isoamyl alcohol (25:24:1) (v/v) was added to the supernatant. The mixture was shaken by inversion, centrifuged at 13.000×*g* for 10 min and the aqueous phase transferred to a new tube, to which 30 μL of sodium acetate (3 M) and 270 μL of absolute ethanol were added. Tubes were kept for 12 min at −80 °C, and centrifuged at 13.000×*g* for 10 min. The pellet was washed with 70% ethanol, and centrifuged again as described above. DNA was dried at 37 °C and dissolved in 50 μL of sterile nuclease free water.

The extracted DNA concentration (ng/μL) and purity was determined by measuring the absorbance at 260 and 280 nm (GeneQuant™ Amersham Biosciences, USA). The DNA samples were diluted in sterile nuclease free water to a stock concentration of 20 ng/μL stored at −20 °C and used in the optimization of the multiplex real-time PCR assay.

2.3. Primers and fluorogenic probes

Primers, sequences and the size of the amplified DNA sequences are listed in Table 2. The internal amplification control, TaqMan® Exogenous Internal Positive Control (Applied Biosystems, Foster City, USA) was used according to manufacturer's recommendation.

Specific probes for *Campylobacter, Salmonella* and IAC were labeled at the 5'-end with Cy5, 6-FAM and VIC fluorophores, respectively. Fluorophores were selected taking into account their maximum emission wavelengths (Cy5, 651; 6-FAM, 494; VIC, 550 nm), and the filters provided in the ABI 7500 Fast Real-time PCR System (Applied Biosystems) were used to reduce spectral overlapping. All probes were also labeled at the 3′-end with non-fluorescent quenchers (dark quencher).

2.4. Development of multiplex real-time PCR assay

Multiplex real-time PCRs were carried out in an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Inc.). A 100 ng concentration of DNA from *Salmonella* and *Campylobacter* was used to optimize the concentrations of primers, probes, and $MgCl_2$. The following concentrations were assayed for simultaneous

Table 1
Salmonella serovars, *Campylobacter* and other bacterial strains used in the development and evaluation of the multiplex real-time PCR assay.

Serovar[a]	Origin	Strains[a]	Origin
Salmonella. Anatum	LACEN[d]	*Bacillus cereus*	UEL[c]
S. Agona	UEL[c]	*Campylobacter coli* CCAMP 1003	FIOCRUZ[b]
S. Bredeney	LACEN[d]	*C. coli* CCAMP 1008	FIOCRUZ[b]
S. Dublin	LACEN[d]	*C. coli* CCAMP 595	FIOCRUZ[b]
S. Derby	LACEN[d]	*Campylobacter jejuni* ATCC 33291	FIOCRUZ[b]
S. Enteritidis ATCC 13076	UEL[c]	*C. jejuni* CCAMP 971	FIOCRUZ[b]
S. Infantis	UEL[c]	*C. jejuni* CCAMP 594	FIOCRUZ[b]
S. Johannesburg	LACEN[d]	*C. jejuni* CCAMP 1014	FIOCRUZ[b]
S. Kentucky	IB[e]	*Campylobacter lari*	LACEN[d]
S. London	LACEN[d]	*Citrobacter freundii*	UEL[c]
S. Montevideo	UEL[c]	*Enterobacter aerogenes*	UEL[c]
S. Muenchen	LACEN[d]	*E. cloacae*	UEL[c]
S. Newport	UEL[c]	*Escherichia coli*	UEL[c]
S. Panama	LACEN[d]	*Klebsiella pneumoniae*	UEL[c]
S. Senftenberg	LACEN[d]	*Morganella morganii*	UEL[c]
S. Typhi	UEL[c]	*Proteus mirabilis*	UEL[c]
S. Typhimurium ATCC 14028	UEL[c]	*Shigella sonnei*	UEL[c]
		Staphylococcus aureus	UEL[c]
		S. saprophyticus	UEL[c]

[a] One isolate of each strain was tested.
[b] Strain obtained from the Oswaldo Cruz Foundation Collection (FIOCRUZ), Rio de Janeiro, Brazil.
[c] Strain belongs to the Food Microbiology Laboratory Collection, Londrina State University, Londrina, Paraná, Brazil.
[d] Central Laboratory of Paraná State (LACEN), Paraná, Brazil.
[e] São Paulo Biological Institute (IB), São Paulo, Brazil.