Evaluation of a polymerase chain reaction protocol for the detection of *Salmonella* species directly from superficial samples of chicken carcasses and preenrichment broth

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ABSTRACT Chicken meat is an important vehicle of foodborne pathogens, such as *Salmonella* spp., and demands a systematic control of microbiological contamination during industrial processing. This control occurs by the adoption of quality control systems in slaughters based on the microbiological investigation on hygiene indicators and pathogens, requiring the development of fast, trustable, and precise methodologies. The objective of this study was to compare the *Salmonella* spp. conventional methodology to a protocol of PCR in chicken carcass surface samples. The PCR protocol was developed directly from the collected samples and from preenrichment broth before and after incubation. The obtained results were compared by χ^2 and McNemar tests ($P < 0.05$), and the values of concordance, sensitivity, and specificity of PCR variations were calculated considering the conventional methodology as a parameter. The obtained results indicated that although some similarities between the methodologies were observed when positive results were considered ($P > 0.05$), the PCR developed from preenrichment after incubation presented significant differences from all the other methodologies ($P < 0.05$). Wide variations were observed in the PCR performance for *Salmonella* spp. detection in chicken carcasses, which can be due to intrinsic factors inherent to the achievement of this food. Further studies are necessary to elucidate the applicability of the PCR as a tool for microbiological monitoring in quality control systems for chicken processing.

Key words: *Salmonella* spp., polymerase chain reaction, conventional method, chicken slaughtering

2010 Poultry Science 89:1524–1529
doi:10.3382/ps.2009-00368

INTRODUCTION

Salmonellosis is one of the most common foodborne diseases in the world, and poultry are the main carriers of *Salmonella* spp., which is commonly transmitted to humans through poultry products (Oliveira et al., 2003). Therefore, it is necessary to develop quality control programs in slaughterhouses, such as the Hazard Analysis and Critical Control Point, based on microbiological analyses in different stages of processing (ICMSF, 1998). Thus, there is an increasing demand for methodologies to be applied in the identification of *Salmonella* spp. employing fast tests with high performance and international acceptance (Halatsi et al., 2006).

Until now, the methodology based on the conventional microbiology is recognized worldwide as the standard method for the detection of *Salmonella* spp. in foods, which theoretically is able to detect up to one viable cell present in the analyzed sample (Busse, 1995). However, it is a laborious methodology, which demands a previous preparation of a great amount of material and culture media, and requires 5 to 7 d for a conclusive result.

More recently, alternative methods for the detection of *Salmonella* spp. in foods have been evaluated, including immunoassays (Walker et al., 2001; Alcocer and Oliveira, 2003; von Ruckert et al., 2008) and hybridization of nucleic acids and PCR (Li et al., 2000; Cortez et al., 2006; Myint et al., 2006; von Ruckert et al., 2008). The main advantages of the PCR are the capacity to detect target sequences, regardless of the antigen expression of the target cells; a higher sensitivity; and shorter time demanded to process the samples, mainly when compared with the conventional methodologies of pathogen isolation (Bennett et al., 1998; Rychlik et al., 1999).

The objective of this study was to analyze the performance of a PCR protocol for the detection of *Salmonella* spp. directly from samples of chicken carcasses

Received July 22, 2009.
Accepted March 28, 2010.
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and preenrichment broths before and after incubation to verify its feasibility to be used in quality control programs, such as Hazard Analysis and Critical Control Point.

MATERIALS AND METHODS

Sampling and Experimental Design

The present study was carried out in a slaughterhouse with the capacity of processing 4,000 poultry/d at different stages of processing (before and after evisceration and before and after passages through chiller tanks). Superficial samples of 120 chicken carcasses were collected, and each sample unit was obtained by swabs from 50 cm^2 of carcass surface, collected with the use of sterile sponges previously soaked in 10 mL of 0.1% peptone water. After the collection, the sponges were homogenized in a stomacher (400 Seward circulator, West Sussex, UK) for 1 min with 40 mL of 0.1% peptone water to obtain homogenates with a volumetric equivalent of the sampled area (1 mL = 1 cm^2).

All of the samples were submitted to the conventional methodology (**CM**) for the detection of *Salmonella* spp. A PCR protocol was used for the detection of the same pathogen from the following stages of sampling and steps of CM: directly from obtained homogenates (**PCR-S**) and also from CM preenrichment broth before (**PCR-PE**) and after (**PCR-IPE**) incubation at 37°C for 20 h.

CM for Salmonella spp.

The preenrichment was performed in an aliquot of 25 mL of the homogenate in 225 mL of 1% buffered peptone water, with incubation at 37°C for 20 h. Next, 1 mL was transferred to selenite cystine broth, followed by incubation at 37°C for 24 h and 0.1 mL in Rappaport-Vassiliadis broth, incubated at 42.5°C for 24 h. The obtained cultures were streaked in brilliant green phenol red lactose sucrose agar and xylose lysine deoxycholate agar and incubated at 37°C for 24 h. Once each one of the typical colonies were identified (brilliant green phenol red lactose sucrose agar: colorless or pinkish colonies; xylose lysine deoxycholate agar: opaque colonies with or without the production of H_2S), they were submitted to the preliminary biochemical identification in triple sugar iron agar and lysine iron and incubated at 37°C for 24 h. The final confirmation was carried out by serological tests of agglutination with somatic and flagellar polyvalent antisera (Probac do Brasil, São Paulo, Brazil). The samples with characteristic biochemical reactions and with positive reactions in both serological tests were considered positive (Ministério da Agricultura, Pecuária e Abastecimento, 2003).

All of the cultures presenting positive results in the serological tests were also submitted to confirmation of the genus by PCR (Galán et al., 1992; Oliveira et al., 2003) and serotyping by Fundação Oswaldo Cruz (Rio de Janeiro, Brazil). All of the analyses were carried out simultaneously with *Salmonella* Enteritidis ATCC 13076 as positive control. Only Oxoid culture media (Oxoid Ltd., Basingstoke, UK) were used in all experiments.

Alternative Methodology: PCR

DNA Extraction. The DNA present in the samples analyzed and in the culture used as control was extracted by the phenol-chloroform technique (Oliveira et al., 2003). The DNA extraction presented the following stages: 1 mL of the sample homogenates (PCR-S), the preenrichment broth sample before (PCR-PE) or after incubation (PCR-IPE), or the suspect *Salmonella* spp. cultures obtained by the CM (after being sown in 1% peptone water and incubated at 37°C for 24 h) were centrifuged at 894 × *g* for 4 min at room temperature. The obtained sediment was resuspended in 444 μL of a buffer solution (10 mmol/L of Tris-HCl, pH 8, 1 mmol/L of EDTA); 30 μL of lysozyme (50 mg/mL) was added and incubated at 4°C for 30 min; 25 μL of sodium dodecyl sulfate at 10% and 1.25 μL of proteinase K (20 mg/mL) were added and incubated at 55°C for 30 min; 500 μL of phenol-chloroform (1:1) was added and centrifuged for 4 min at 8,050 × *g* in 2 replications; and then a final washing was carried out only with chloroform. The obtained material was centrifuged at 8,050 × *g* for 4 min at room temperature; 35 μL of sodium acetate (3 mol/L) and 350 μL of cooled isopropanol were added and the mixture was incubated at −20°C for 30 min; the DNA was centrifuged at 10,956 × *g* for 10 min at 4°C; and the sediment was washed with 1 mL of refrigerated ethanol at 80% and resuspended in 20 μL of sterile ultrapure Milli-Q water for immediate use or stored at −20°C.

DNA Amplification. The mixture for the PCR reaction was prepared in sterile ultrapure Milli-Q water, 10 m*M* Tris-HCl (pH 9.0 at 25°C), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.3 m*M* of each one of the deoxynucleoside triphosphates, 0.3 *M* of each oligonucleotide, and 1 U/μL of Taq DNA polymerase. Aliquots of 21 μL of the reaction mixture were transferred to 0.2-mL tubes (Oliveira et al., 2002). After the DNA addition (4 μL), the mixture was transferred to a thermocycler (PTC-100, MJC Inc., Marietta, GA). The oligonucleotides were designed to detect and amplify a DNA fragment of 284 bp of the gene *inv*A of *Salmonella* spp. (Table 1). The thermocycler was programmed for an initial denaturing at 95°C for 1 min, followed by 35 cycles of denaturing temperature at 95°C for 30 s, annealing temperature at 60°C for 30 s and extension temperature at 72°C for 30 s, followed by a final extension temperature at 72°C for 7 min. The products of amplification were separated by 1% agarose gel electrophoresis, stained with ethidium bromide, and visualized in a transilluminator.

Table 1. Oligonucleotides used in the PCR for the detection of *Salmonella* spp. in superficial samples of chicken carcasses and confirmation of *Salmonella* spp. isolates achieved by the conventional methodology[1]

Oligonucleotide	Target gene	Size	Sequence (5′ → 3′)	Amplification product (bp)
139 (forward)	*inv*A	26	5′-GTGAAATTATCGCCACGTTCGGGCAA-3′	284
141 (reverse)	*inv*A	22	5′-TCATCGCACCGTCAAAGGAACC-3′	284

[1]Source: Galan et al. (1992).

All of the used reagents were from Phoneutria (Phoneutria Biotecnologia e Serviços Ltda., Belo Horizonte, Minas Gerais, Brazil), Promega Co. (Madison, WI), and Integrated DNA Technologies Inc. (Coralville, IA). All of the molecular analyses were carried out using a culture of *Salmonella* Enteritidis ATCC 13076 as positive control.

PCR Detection Limit. A PCR product of the positive control (*Salmonella* Enteritidis ATCC 13076) was previously cloned using the Vector pGEM T Easy kit (Promega Co.), purified by the use of the Wizard Plus SV Minipreps DNA Purification System kit (Promega Co.), and quantified by spectrophotometry (optical density at 260 nm). The kits were used according to the manufacturer's instructions. The obtained *Salmonella* recombinant plasmid was used as a DNA model for detection tests, diluted for the final concentrations of 10^{10} to 10^{1} copies of plasmid/μL, and used as a standard for sample quantification. The genome mass calculation was carried out by the ABI Prism 7500 User's Manual software system (Applied Biosystems, Foster City, CA).

Analysis of the Results

The frequencies of *Salmonella* spp. obtained by CM and PCR variations were compared by the χ^2 test, and the significant differences were identified by the Marascuilo procedure ($P < 0.05$) using Statistica 7.0 software (StatSoft Inc., Tulsa, OK) and XLStat 2009.1.02 (Addinsoft, New York, NY). The McNemar test was used to verify the occurrence of significant differences between the obtained results (positive and negative) by all of the evaluated methodologies ($P < 0.05$). The performance rates (sensitivity, specificity, and agreement) of the PCR variations were calculated considering the CM as reference according to the following mathematical equations (Doohoo et al., 2003; Mäde et al., 2004):

$$\text{Sensitivity} = \frac{\text{Number of positive results by conventional and tested methodologies}}{\text{Number of positive results by conventional methodology}} \times 100$$

$$\text{Specificity} = \frac{\text{Number of negative results by conventional and tested methodologies}}{\text{Number of negative results by conventional methodology}} \times 100$$

$$\text{Agreement} = \frac{\text{Number of positive and negative results by conventional and tested methodologies}}{\text{Number of positive and negative results by conventional methodology}} \times 100.$$

RESULTS AND DISCUSSION

The frequencies of positive results for *Salmonella* spp. in superficial samples of chicken carcasses obtained by CM and by PCR variations are presented in Table 2. The positive PCR reactions are shown in Figure 1. All of the *Salmonella* spp. cultures isolated by the CM were confirmed by the PCR and were serologically identified as *Salmonella* Enteritidis.

By the analysis of the results in Table 2, it is observed that the CM did not differ statistically from

Table 2. Frequencies of positive results for *Salmonella* spp. in superficial samples of chicken carcasses achieved by the conventional methodology of isolation (CM) and a PCR protocol directly from the samples (PCR-S) and the preenrichment broth before (PCR-PE) and after (PCR-IPE) incubation at 37°C for 20 h

Number of samples	CM	PCR			χ^2
		PCR-S	PCR-PE	PCR-IPE	
Analyzed	119	120	120	120	
Positive results	6^{ab}	0^{a}	2^{a}	16^{b}	26.61; df = 3; $P < 0.0001$

[a,b]Different superscripts indicate a significant difference by the Marascuilo procedure ($P > 0.05$).

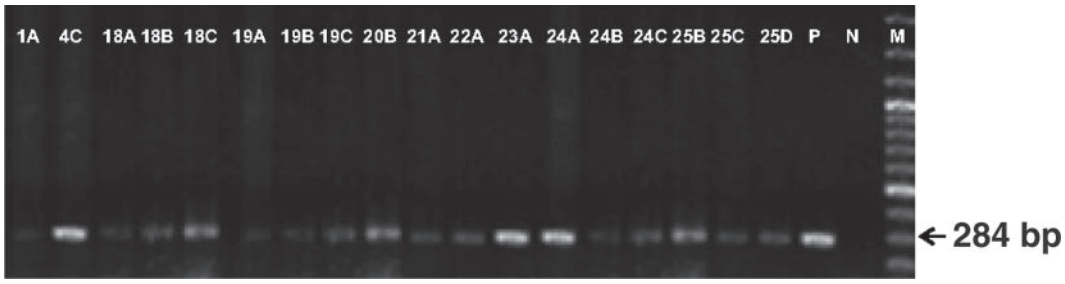

Figure 1. Gel electrophoresis with amplification products obtained by the PCR from preenrichment broths sown with superficial samples of chicken carcasses before (1A and 22A) and after (4C, 18A, 18B, 18C, 19A, 19B, 19C, 20B, 21A, 23A, 24A, 24B, 24C, 25B, 25C, 25D) incubation at 37°C for 20 h. M = Promega molecular marker of 100 bp (Promega Co., Madison, WI); P = *Salmonella* Enteritidis ATCC 13076 as positive control with a segment of 284 bp; N = Milli-Q water (Millipore, Billerica, MA) as negative control.

PCR-S, PCR-PE, and PCR-IPE. As to the PCR procedures, there was a statistical difference between PCR-S and PCR-IPE and between PCR-PE and PCR-IPE. There was no statistical difference between PCR-S and PCR-PE. Considering only the positive results, it can be observed that similarities occurred between the CM and the PCR variations. However, considering the same positive results, significant differences were observed between the PCR variations.

However, considering both the positive and negative results, significant differences were identified (Table 3). The CM presented a significant difference only in relation to the PCR-S and PCR-IPE. Considering the PCR variations, significant differences were observed between PCR-IPE and PCR-S and between PCR-IPE and PCR-PE (Table 3). These results indicate that the similarity and the equivalence between the methodologies should not be evaluated considering only the positive results for *Salmonella* spp.: other parameters must also be considered in these evaluations, such as sensitivity, specificity, and agreement (Doohoo et al., 2003). Considering the CM as reference, the values of sensitivity, specificity, and agreement were calculated and can be observed in Table 4.

Several techniques of fast diagnosis have been developed in recent years, and PCR is one of the most promising. Several authors report its high sensitivity and specificity (Soumet et al., 1997; Uyttendaele et al., 2003; Shabarinath et al., 2007), but few studies are carried out on naturally contaminated samples (Oliveira et al., 2003). The PCR is usually employed in the analysis of pathogens in foods based on enriched samples, after the stages of preenrichment from CM, when, theoretically, the target microorganism achieves a high concentration, allowing the proper detection (Dickinson et al., 1995; Myint et al., 2006). The PCR-IPE was evaluated considering these observations, but when compared with the CM, it presented a sensitivity of 33.3% (Table 4) and absence of equivalence (Table 3), although it presented similar frequencies of positive results (Table 2).

Table 3. Levels of significance (*P*-value) achieved by the McNemar test to verify the significant differences between the methodologies for the detection of *Salmonella* spp. [conventional methodology (CM), PCR directly from the samples (PCR-S) and from enrichment broths before (PCR-PE) and after (PCR-IPE) incubation at 37°C for 20 h] in superficial samples of chicken carcasses

Methodology	*P*-value[1]
CM × PCR-S	0.031
CM × PCR-PE	0.289
CM × PCR-IPE	0.031
PCR-S × PCR-PE	0.500
PCR-S × PCR-IPE	<0.0001
PCR-PE × PCR-IPE	0.001

[1]Values below 0.05 indicate significant differences between the methodologies compared.

The performance of alternative methodologies is usually evaluated with artificially contaminated samples, and using as reference of comparison the CM. However, some aspects must be considered on these evaluations. Candrian (1995) stated that autochthonous microorganisms adhere more tightly to food particles than the inoculated microorganisms, which interferes with their recovery in the in the enrichment media. The autochthonous microbiota of naturally contaminated food samples may interfere significantly in the performance of the methodologies (Gouws et al., 1998), mainly in the CM (Nero et al., 2009), once it is able to generate unfavorable conditions for the survival and detection of the target pathogen, such as the pH reduction and the toxicity of the culture media due to the production of metabolites, besides the chemical residues present in food (D'Aoust et al., 1992; Busse, 1995). Despite these limitations, the CM is recognized by many countries, including Brazil, as the official method for the diagnosis of *Salmonella* spp. and other foodborne pathogens (Ministério da Agricultura, Pecuária e Abastecimento, 2003; Cortez et al., 2006; FDA, 2009).

In Table 2, it can be observed that it was not possible to isolate *Salmonella* spp. by PCR-S. The PCR from the nonenriched samples, namely, directly from food, would be a practicable and fast alternative. This methodology is efficient for pure nucleic acid solutions, and its sensitivity may be reduced when the food contains salts, lipids, proteins, and other microorganisms (Lantz et al., 2000), which may have affected the performance

Table 4. Positive results for *Salmonella* spp. in superficial samples of chicken carcasses by a PCR protocol directly from the samples (PCR-S) and the preenrichment broth before (PCR-PE) and after (PCR-IPE) incubation at 37°C for 20 h, considering the conventional methodology (CM) as a reference for the calculation of the performance rates

	Alternative methodology		
Number of samples	PCR-S	PCR-PE	PCR-IPE
Tested	119	119	119
Positive by the CM	6	6	6
Positive by the methodology tested	0	2	16
Positive exclusively by the CM	6	6	4
Positive exclusively by the methodology tested	0	2	14
Positive simultaneously by the CM and tested methodologies	0	0	2
Sensitivity (%)	0.0	0.0	33.3
Specificity (%)	100.0	98.2	87.6
Agreement (%)	95.0	93.3	83.1

of PCR-S (Table 2). Similar results were observed by Candrian (1995) and Myint et al. (2006), who were not successful in isolating *Salmonella* spp. directly from food. Dickinson et al. (1995) carried out the PCR directly from food samples and were successful in the isolation of *Yersinia enterocolitica* and *Listeria monocytogenes*, but they stressed the importance of obtaining DNA free from residues for adequate reactions in the PCR technique. Agarwal et al. (2002) were also able to detect *Salmonella* spp. by PCR directly from artificially contaminated foods.

A more sensitive PCR, such as the nested PCR, would be more efficient for *Salmonella* spp. detection in samples without the preenrichment stage. Rychlik et al. (1999) performed nested PCR in feces naturally contaminated and concluded that, even without the preenrichment, the detection limit of the PCR technique was 10^5 cfu/g. Waage et al. (1999), on the other hand, did not isolate *Salmonella* spp. directly from naturally contaminated food, even with the nested PCR.

The detection limit of the PCR protocol was 3.3×10^7 cfu/mL. Considering this limit, the PCR protocol requires a very high concentration of the target microorganism ($\geq 3.3 \times 10^7$ cfu/mL), which is usually obtained only after sample enrichment. This relative high value of detection limit may explain why 14 samples presented positive results by PCR-IPE and, simultaneously, negative by CM and PCR-S (Table 4), indicating its better performance on *Salmonella* spp. detection in chicken carcasses. Similar results were reported by Oliveira et al. (2002), who found a great variation in the detection limit among several *Salmonella* spp. serotypes. Kim et al. (2007) found a detection limit of 10^5 cfu/mL using the same target gene (*inv*A) but with different oligonucleotides.

The selection of the sequences that flank the DNA segments intended to be amplified by the PCR is a critical point of the technique. The PCR specificity is a result from the precision with which the originators hybridize with the target DNA. For the detection of *Salmonella* spp., the chosen region must be common to most strains and codify for proteins that are relevant for the microorganism pathogenicity without presenting homology with other microorganisms (Bäumler et al., 1997). The *inv*A gene, used in this study, codifies proteins related to the invasion and is present in most strains of *Salmonella* spp. That is why it is chosen for most studies (Rahn et al., 1992; Waage et al., 1999). However, Shabarinath et al. (2007) demonstrated a low efficiency of this gene when compared with the *hns* gene. Ginocchio et al. (1997) stated that the gene *inv*A belongs to a pathogenicity island, being instable for some *Salmonella* spp. serotypes. The PCR aiming the detection of genes belonging to pathogenicity islands may provide false-negative results (Saroj et al., 2008).

The obtained results indicated that the evaluated PCR protocols presented distinct performances on detection of *Salmonella* spp. from surface samples of chicken carcasses when compared with CM. Despite these differences, the PCR of preenrichment broth after incubation at 37°C for 20 h presented the best performance. However, interference factors must be considered in this evaluation, leading to further studies to evaluate the applicability of the PCR variations as tools for the monitoring of *Salmonella* spp. in chicken slaughter.

ACKNOWLEDGMENTS

This work was supported by Coordenação de Aperfeiçoamento de Pessoal do Nível Superior (CAPES, B. G. Matias, master scholarship) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG, publication fees, and L. A. Nero, CVZ-PPM-00093-09).

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