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POLYMERASE CHAIN REACTION (PCR) FOR THE DETECTION OF *Salmonella* IN ARTIFICIALLY INOCULATED CHICKEN MEAT

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SUMMARY

The aim of this study was to develop a polymerase chain reaction (PCR) protocol for the detection of *Salmonella* in artificially contaminated chicken meat. Tests were performed with different dilutions of *Salmonella* Typhimurium or *Salmonella* Enteritidis cells (10^{-7}, 10^{-8} or 10^{-9} CFU/mL) inoculated in chicken meat samples, in order to establish the limits of detection, incubation times (0, 6, 8 and 24 hours of pre-enrichment in PBW 1%) and three DNA extraction protocols (phenol-chloroform, thermal treatment and thermal treatment and Sephaglass). The assay was able to detect until 10^{-9} CFU/mL of initial dilution of *Salmonella* cells inoculated in chicken meat, which allows detection of *Salmonella* within 48 hours, including 24 hours of pre-enrichment and using the phenol-chloroform DNA extraction protocol. As the results are obtained in a shorter time period than that of microbiological culture, this procedure will be useful in the methodology for detection of *Salmonella* in chicken.

KEYWORDS: *Salmonella*; PCR; Chicken meat.

INTRODUCTION

Salmonella infections may be severe, especially in very young, older or immunodepressed people, with a possible infecting dose for healthy persons of 10^5 to 10^7 CFU [7]. In the State of Rio Grande do Sul, Brazil, during the period of 1994 to 1995, the number of cases of foodborne outbreaks clinically compatible with salmonellosis increased, many of them associated with the consumption of poultry products or products including poultry as an ingredient. During the same period, a survey of the contamination indices for *Salmonella* in chicken carcasses, performed at the Centre for Diagnostics and Research in Avian Pathology (CDPA), showed that 17.5% of the 1300 carcasses examined were positive for *Salmonella*[3].

Polymerase chain reaction (PCR) is a molecular biology technique which has taken up an increasingly significant space in the field of laboratory diagnostics, allowing the detection of various pathogens, such as *Listeria monocytogenes*, *Campylobacter* sp., *Yersinia enterocolitica*, *Vibrio cholerae*, *Shigella flexneri*, *Escherichia coli* and *Salmonella*, in different kinds of food, such as meat and milk. PCR can reduce the time required to detect and identify the agent[1,4]. Thus, the purpose of this study was to establish a PCR protocol in order to detect *Salmonella* in experimentally infected chicken meat samples.

MATERIAL AND METHODS

Bacterial strains and inoculation of food products: Aliquots of 10 µL of *Salmonella* Typhimurium (ATCC 14028), *Salmonella* Enteritidis (ATCC 1980) and 18 other bacteria (Table 1) maintained in DTS (PI9500076-3 INPI) were transferred to BHI broth (Brain Heart Infusion, Merck) and incubated for 18 h at 37 °C. The *Salmonella* samples were diluted in buffered peptone water 0.1% (PBW, Merck) until 10^{-9}, and then counts of *S.* Enteritidis (10^{-7}:120 CFU) and *S.* Typhimurium (10^{-7}:200 CFU) were determined in plate count agar (PCA, Merck). A pool of the other 18 bacteria was obtained by mixing 1mL of each bacterium in a sterile tube. The diluted 10^{-2} pool contained between 10^{-5} to 10^{-6} CFU/g.

Sample preparation: Thirty-two samples of chicken meat were used. From each sample, 25 grams were weighted in sterile bags (Seward Medical Stomacher® 400 sterile bags) and inoculated with 1 mL of a dilution (10^{-7},10^{-8} or 10^{-9}) of *S.* Enteritidis or *S.* Typhimurium and 1 mL of the other 18 bacteria pool diluted 10^{-2}. In two of the samples, only the pool was inoculated. Finally, 225 mL of PBW 1% were added and homogenized in a Stomacher (Laboratory Blender Stomacher 400, Seward Medical, England) for 30 seconds. Aliquots intended to be used in the PCR were collected immediately after homogenization, and after 6, 8 and 24 hours of pre-enrichment in PBW 1% at 37 °C, in which case they were frozen until PCR assays were performed.

Microbiology method: Following 24 hours of preenrichment in PBW 1%, 0.1 mL was transferred to 9.9 mL of Rappaport-Vassiliadis (Merck) broth and 1 mL to tetrathionate broth (Merck) for selective enrichment at 42 °C and 37 °C for 24 h, respectively. One loopful of broth cultures were streaked onto brilliant green agar (Merck) and

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Table 1
Non- *Salmonella* strains examined in this study

Strains	Source
Escherichia coli	ATCC 25922
Enterobacter cloacae	LARA
Enterobacter aerogenes	ATCC 13048
Proteus mirabilis	LARA
Proteus vulgaris	ATCC 13315
Pseudomonas fluorescens	LARA 0612
Schewanella putrefaciens	LARA 2122
Bacillus cereus	LARA
Bacilus subtilis	LARA
Bacillus alvei	LARA
Bacillus laterosporus	LARA
Klebsiella pneumoniae	ATCC 13883
Citrobacter freundii	LARA 0676
Staphylococcus aureus	ATCC 25923
Staphylococcus epidermidis	ATCC 12228
Crysiomonas luteola	LARA 6491
Aeromonas sobria	LARA 4578
Aeromonas hydrofila	LARA 5621

ATCC: American Type Culture Collection; LARA: Regional Laboratory of Animal Health.

Rambach agar (Merck). The plates were incubated at 37 °C for 24 h. Presumptively positive colonies were inoculated into TSI, LI, SIM, urea and nutrient agar (Merck). After incubation at 37 °C for 18-24 h, confirmations were done serologically, using *Salmonella* polyvalent O and H agglutinating sera (Difco).

DNA extraction using phenol-chloroform, adapted from WILSON *et al.*[8]: A 1 mL aliquot of each sample was centrifuged (Centrifuge 5415C, Germany) at 5000 rpm for 4 min and the supernatant was discarded. The pellet was suspended in 1 mL of 10 mM Tris - 1 mM EDTA (TE pH 8), vortexed for 10 s (Pachane tube shaker, Brazil) and the resulting mixture was centrifuged twice, as described above. The supernatant was discarded and the pellet was suspended in 350 µL of TE and vortexed for 10 s. Thirty microlitre of lysozyme (50 mg/mL, Pharmacia Biotech,) was added to the suspension, and the mixture was vortexed for 10 s and placed on ice for 30 min to lyse the cells. After lysis, 40 µL of 10% sodium dodecyl sulphate (SDS, VETEC, Brazil) solution in distilled H_2O was added and mixed for 1 min or until it reached an homogeneous, milky suspension. Forty microlitre of proteinase K solution (20 mg/mL, GibcoBRL) was then added; the suspension was mixed by inversion, and the resulting mixture was incubated at 37 °C for one hour in a water bath (Precision, USA). DNA was extracted by adding 800 µL of phenol pH 7 (Merck) to a microcentrifuge tube containing the above-described mixture. The tube was shaked vigorously until a white, milky emulsion was formed and then centrifuged at 13000 rpm for 1 min. After centrifugation, the aqueous phase was transferred to a clean microcentrifuge tube containing 150 µL of TE buffer. A 700 µL volume of a 1:1 mixture of phenol (pH 7) and chloroform-isoamyl alcohol (25:1 vol/vol) was added. The tube was again shaked vigorously until a white emulsion was formed and then centrifuged at 13000 rpm for 1 min. Six hundred microlitre of the aqueous phase were transferred to a clean tube. Approximately 800 µL of chloroform-isoamyl alcohol (25:1) were added, and the tube was inverted several times before centrifugation as before. Following centrifugation, 325 µL of the aqueous phase were transferred to a tube containing 75 µL of 3M sodium acetate pH 7.2 (ACS). The mixture was mixed briefly with a micropipette. Approximately 1 mL of ethanol was added and the tube was inverted five or six times prior to being placed on ice for 10 min. The precipitated DNA was pelleted by centrifugation for 15 min at 13000 rpm; the supernatant was decanted and discarded. The open tube of pelleted DNA was inverted on absorbent paper for 30 min. The pellet was suspended in 50 µL of TE and stored at –20 °C.

DNA extraction with thermal treatment: A 1 mL aliquot of each sample was centrifuged at 5000 rpm for 4 min and the supernatant was discarded. The pellet was suspended in 1 mL of TE, vortexed for 10 s and the resulting mixture was centrifuged twice, as described above. The supernatant was discarded; the pellet was suspended in 100 µL of TE and vortexed for 10 s. The sample was placed on a thermal block (Multi-Blok Heater, Baxter, EUA) at 95 °C for 10 min. The mixture was centrifuged for 20s at 5000 rpm and the supernatant was stored at –20 °C.

DNA extraction using thermal treatment and Sephaglass, adapted from ROSSETI *et al.*[5]**:** One mL of the sample was centrifuged for 10 min at 5000 rpm and the supernatant was discarded. The pellet was suspended in 200 µL of TE, vortexed for 10 s, and the resulting mixture was recentrifuged twice as described above. The supernatant was discarded; the pellet was suspended in 50 µL of TE and vortexed for 10 s. The sample was placed on a thermal block at 95 °C for 10 min. The mixture was centrifuged for 20 s at 5000 rpm and the supernatant was transferred to a microtube with 5 µL of Sephaglass (Sephaglass TM BandPrep Kit, Pharmacia Biotech). It was then mixed gently for 5 min and centrifuged for 1 min at 5000 rpm. After that, the supernatant was discarded. Approximately 200 µL of ethanol 70% was added and the resulting mixture gently shaked; the tube was centrifuged for 20 s at 5000 rpm and the supernatant discarded. This last step was repeated twice. The open tube of pelleted DNA was inverted on absorbent paper for 30 min. The pellet was suspended in 30 µL of TE, vortexed for 10 s and centrifuged for 2 min at 14000 rpm. The supernatant was stored at -20 °C.

PCR, adapted of RAHN *et al.*[4]**:** Reaction mixtures (25 µL) for each sample contained 2.5 µL reaction buffer 10X (15 mM $MgCl_2$ 500 mM KCL, 100 mM Tris-HCL pH 8.3, (Cenbiot[Enzimas]), 1.2 µL of BSA (8 µg/ml), 5 mM of each deoxyribonucleotide, 5 ρmol of each oligonucleotide (149:5'GTGAAATTATCGCCACGTTCGGGCAA3', 151:5'TCATCGCACACGTCAAAGGAACC3'), 1U of *taq* DNA polymerase (Cenbiot[Enzimas]) and 20 ng purified DNA. The cycling programme was set in a Perkin Elmer Gen Amp PCR System 2400 thermocycler (Perkin Elmer Cetus, Norwalk, CT, USA). Samples were denatured at 94 °C for 5 min. Thirty-five cycles of amplification were run for 30 s at 94 °C, 30 s at 54 °C and 30 s at 72 °C, with the final extension continuing for 7 min. Twenty-five microlitre aliquots of the reaction mixtures were electrophoresed through 2.0% agarose gel and fragments were revealed by staining with ethidium bromide.

RESULTS AND DISCUSSION

In the specificity assay, all samples of *Salmonella* DNA tested were amplified, generating a characteristic 284 bp product. These results are

similar to those of RAHN *et al*.[4], that studying 630 isolates of *Salmonella* and 142 of other bacteria, amplified all the samples of *Salmonella*, except *S*. Litchfield and *S*. Senftenberg. In the authors' opinion, the apparent absence of gene *invA* in these two serovars suggests that these organisms are not invasive, or that they have alternative pathways to penetrate cells, with, an as yet, unknown pathogenic potential. One of the critical points of the technique used is the choice of the sequence to be amplified, which must be common to most of the serovars and do not present homology with other organisms. In the present study, when using primers 149 and 151, which are derived from gene *invA* and code for proteins related to cell invasion, a non-specific amplification of DNA of a sample of *Escherichia coli* was observed, which could be safely differentiated from *Salmonella,* since the former presents a band of around 300 bp (Fig. 1) and, by so, it does not allow any misinterpretation of the results.

When assays were performed using artificially contaminated chicken meat samples after 24 hours of pre-enrichment and using the DNA extraction protocol with phenol-chloroform, until 10^{-9} CFU/mL of initial dilution of *Salmonella* cells inoculated in chicken meat could be detected

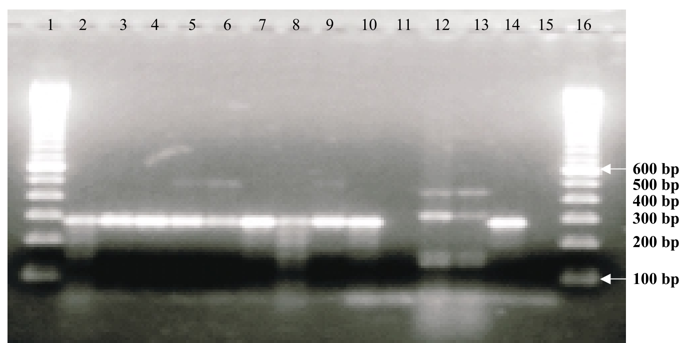

Fig. 1 - Agarose gel electrophoresis of the PCR products obtained when testing the artificially contaminated chicken meat samples after 24 hours of pre-enrichment and using the DNA extraction protocol with phenol-chloroform and controls.

1- DNA ladder 100 bp; **2-** *S*. Enteritidis 10^{-7} control; **3-** *S*. Enteritidis 10^{-8} control; **4-** *S*. Enteritidis 10^{-9} control; **5-** chicken meat contaminated with *S*. Enteritidis 10^{-7}; **6-** chicken meat contaminated with *S*. Enteritidis 10^{-8}; **7-** chicken meat contaminated with *S*. Enteritidis 10^{-9}; **8-** chicken meat contaminated with *S*. Typhimurium 10^{-7}; **9-** chicken meat contaminated with *S*. Typhimurium 10^{-8}; **10-** chicken meat contaminated with *S*. Typhimurium 10^{-9}; **11-** chicken meat contaminated with pool diluted 10^{-2}; **12** and **13-** *E. coli* ATCC 25922; **14-** Positive control (20 ng *Salmonella* Typhimurium purified DNA); **15-** Negative control; **16-** DNA ladder 100 bp.

(Table 2). The microbiology method used presented the same detection limit, but supplied results after 96 hours of analysis. In this particular aspect, these results are not in accordance with those from MAHON *et al*.[2], who demonstrated, experimentally, a greater sensitivity of the PCR to detect *Salmonella* in chicken skin samples, when compared with that of the microbiological culture. RAHN *et al*.[4], abolishing the pre-enrichment phase, were not able to amplify *Salmonella* DNA directly from chicken litter and carcasses. Their results are in accordance with those from the current experiment, where negative results were obtained when meat samples were processed immediately after homogenization with peptone water, or at the sixth and eight hour of the incubation period (Table 2). These results agree with those from STONE *et al*.[6], who indicated that the inclusion of a pre-enrichment step presents advantages over the direct extraction of DNA, since the enrichment broth are relatively cheap, require little manipulation, dilute substances which could inhibit PCR and increase the number of bacterial cells. On the other hand, substances found in meat samples, such as blood and fat, could also interfere in the PCR results.

Thus, the capacity to isolate the target bacteria, as well as the influence of matrix components, could be limiting factors for PCR reliability. These problems could be overcome with procedures such as dilutions of the sample, previous isolation of the pathogens being studied, and DNA extraction protocols, as well as corrections in PCR reagent concentrations. In this experiment, when the protocols for extraction with thermal treatment and Sephaglass were used, negative results were observed at all incubation periods tested (Table 2). Differently, the protocol for phenol-chloroform extraction supplied positive results after 24 hours of sample's pre-enrichment. With this protocol, the reduction of analysis time from approximately 96 hours, with microbiology method, to 48 hours with PCR, appears to be the main advantage of the technique, since the detection limits of both methods (PCR and conventional microbiology) were the same (Table 2). The specificity of PCR is another indication of its good applicability in diagnostic routines. However, there are reports indicating the occurrence of false negative results when naturally contaminated chicken meat samples are analyzed by the PCR method. Thus, the next stage of these experiments should include the comparison between the PCR protocol developed in this study and microbiological analysis for the detection of *Salmonella* in naturally infected chicken meat samples.

Table 2

Results of detection of *Salmonella* in chicken samples artificially contaminated with 10^{-7}, 10^{-8} or 10^{-9} dilutions of *S*. Enteritidis or *S*. Typhimurium and pool of 18 other bacteria by PCR following DNA extractions procedure after four incubation times in PBW 1%

DNA extraction procedure and PCR	Incubation times in PBW 1%			
	Zero	6 hours	8 hours	24 hours
	10^{-7}, 10^{-8} or 10^{-9} UFC/mL	10^{-7}, 10^{-8} or 10^{-9} UFC/mL	10^{-7}, 10^{-8} or 10^{-9} UFC/mL	10^{-7}, 10^{-8} or 10^{-9} UFC/mL
Phenol-chloroform	negative	negative	negative	positive
Thermal treatment	negative	negative	negative	negative
Thermal treatment and Sephaglass	negative	negative	negative	negative

RESUMO

Reação em cadeia pela polimerase (PCR) para detecção de *Salmonella* em carne de frango artificialmente contaminada

O objetivo deste trabalho foi adequar um protocolo de reação em cadeia pela polimerase (PCR) para detecção de *Salmonella* em carne de frango artificialmente contaminada. Foram realizados ensaios com amostras de carne de frango inoculadas com diluições de *Salmonella* Typhimurium ou *Salmonella* Enteritidis (10^{-7}, 10^{-8} ou 10^{-9} UFC/mL), procurando-se determinar o limite de detecção da técnica, intervalos de incubação das amostras (0, 6, 8 ou 24 horas de pré-enriquecimento em água peptonada 1%) e três protocolos de extração de DNA (fenol-clorofórmio, tratamento térmico ou tratamento térmico e Sephaglass). Foi possível amplificar DNA de S*almonella* nas amostras de carne de frango inoculadas inicialmente com diluições de até 10^{-9} UFC/mL, utilizando-se protocolo de extração de DNA por fenol-clorofórmio e após um período de 24 horas de pré-enriquecimento, totalizando 48 horas de análise. Como os resultados são obtidos mais rapidamente que a cultura, este procedimento será útil na metodologia para detecção de *Salmonella* em carne de frango.

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