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Conventional Microbiology, Salmosyst Method and Polymerase Chain Reaction: A Comparison in the Detection of *Salmonella* spp. in Raw Hamburgers

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Abstract

The fast and reliable detection of *Salmonella* spp. in foods is increasingly important. Although conventional microbiological analyses are the reference and the most widely used methodologies for that end, the polymerase chain reaction (PCR) technique is today regarded as a sensitive and specific protocol to detect the bacteria. The present study compares the conventional microbiology method, the Salmosyst method and PCR to detect the presence of *Salmonella* spp. in raw beef and chicken and beef mix hamburgers marketed in the city of Niterói, RJ, Brazil. Of the 80 hamburger samples analyzed, 32 (40.0%) were contaminated with *Salmonella* spp., of which 14 (17.5%) were identified only by conventional microbiology, 10 (12.5) only by PCR, and 8 (10.0%) by both methods. Conventional microbiology and PCR produced similar true positive rates, though conventional microbiology was more efficient to detect true negatives. Both beef and chicken and beef mix hamburgers pose health risks. Both methodologies were efficient, proving the usefulness in microbiological monitoring and controlling in the food industry.

Key words: Hamburger; Conventional Microbiology; Salmosyst; PCR; *Salmonella*

Introduction

The detection and identification of bacteria of the genus *Salmonella* in food play an essential role in the prevention of food poisonings outbreaks. However, between 5 and 7 days are required when conventional methods and culture media are used for that end. Additionally, the direct competition between *Salmonella* spp. bacterial cells and other microorganisms may also lead to false positive results.

In this sense, speeding up and improving reliability in the detection of these microorganisms in food are increasingly important. New methodologies, among which biomolecular methods, are continuously developed and perfected in the attempt to make microbiological analyses faster and less labor intensive.

A previous study discovered that, as a rule, the reliable detection of *Salmonella* spp. is influenced by how the isolation of the bacteria in foods is conducted (Tortora *et al.* 2012). Yet, these isolation procedures have a few disadvantages, like the need for specific, selective and differential culture media, apart from the long time spans to arrive at a diagnosis and/or identify the pathogen. Also, the small *Salmonella* spp. counts in food samples may render the detection of the pathogen more difficult.

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Conventional microbiological analyses are still the most commonly used in the detection of microorganisms, and are the reference method appointed by Brazilian legislation. Nevertheless, these analyses are time consuming, since a conclusive diagnosis may take up to seven days to be reached, especially when large numbers of samples are investigated. In this sense, in spite of being standardized and reliable, conventional microbiology is not very practical, mainly in routine food inspection procedures, which approve foods for human consumption based on consistent and fast results.

Currently, tests based on chromophore and/or fluorophore groups (chromogenic and/or fluorogenic media) associated with different types of cell substrates are among the main alternatives in the detection and identification of *Salmonella* bacteria. In this scenario, tests that use the polymerase chain reaction (PCR) are considered today the most promising to detect *Salmonella* spp. genetic material in food samples, which may be viable and therefore culturable. The technique is sensitive and specific to detect several microorganisms, and is used in the analysis of animal products. PCR detects a conserved region of a microorganism's genome, allowing the *in vitro* replication of sequences defined by the DNA of the microorganism studied, that is, only the region amplified by a specific primer (Mullis and Faloona 1987; Mullis 1990).

In general, when compared to conventional microbiology methods, PCR is more specific, fast and reliable. However, the technique's main limitation is that it does not differentiate viable from non-viable genetic material, leading to false positive results. In other words, the PCR technique may indicate the presence of the pathogen, even when there is no food poisoning hazard.

This study compares the conventional microbiology method, the Salmosyst method and PCR to detect the presence of *Salmonella* spp. in raw beef and chicken and beef mix hamburgers marketed in the city of Niterói, state of Rio de Janeiro, Brazil.

Materials and Methods

This study was conducted in the Laboratory of Microbiological Control of Animal Product and Laboratory of Molecular Epidemiology, Veterinary School, Universidade Federal Fluminense. In total, 80 samples were analyzed, 40 beef hamburgers and 40 hamburgers made with beef and chicken meat. Hamburgers were purchased in local supermarkets and butcher's shops in the city of Niterói, RJ, Brazil, in the original packaging. Hamburgers weighed between 56 g and 120 g. Four hamburgers were considered a sample unit. As of purchase, hamburgers were kept in the original packaging, placed in individual low-density polyethylene bags with hermetic lock, transferred to a portable thermal container and transported to the laboratory for analyses.

Microbiological standards. *Salmonella* spp. prevalence in hamburgers samples was compared with the standards defined in Resolution No. 12, issued by the Board of the National Sanitation Authority (ANVISA), Ministry of Health (Brasil 2001), which establishes sanitary microbiological standards and interpretation criteria in the microbiological analyses of foods destined for human consumption.

Isolation and identification of *Salmonella* spp.

a. Conventional microbiology

The conventional microbiology method used was based on Ordinance No. 62, issued by the Ministry of Agriculture, Livestock and Supplies (MAPA), Office for the Protection of Agriculture and Livestock (SDA), which publishes the official analytical methods used in the microbiological analyses of animal products and water (Brasil 2003).

Initially, 25 g of each sample were placed in a homogenization bag and weighed on a digital precision scale. Then, 225 mL of buffered peptone saline 1% (PBS) were added to bags. Next, samples were homogenized in a stomacher for 60 s at normal speed. Bags were sealed and left at room temperature for 1 h. After that, homogenized samples were incubated in bags in a stove at 35ºC for 24 h. For enrichment, samples were inoculated in Selenite-Cystin broth (SC) and Tetrathionate broth (TT) and incubated at 35ºC for 24 h. For selective plating, aliquots of SC and TT enrichments were transferred to individual petri dishes containing solid Hektoen Enteric Agar (HE), *Salmonella-Shigella* Agar (SS), Xylose Lysine Desoxycholate Agar (XLD) and Differential Salmonella Agar (DSA). All culture media were incubated at 35ºC for 18-24 h. Selective plating was carried out in duplicate for each pre-enrichment broth. Typical *Salmonella* spp. colonies were identified based on classical biochemistry methods and serology.

b. Salmosyst method

Twenty-five grams of the sample were weighed as described above. Then, 225 mL of Salmosyst Broth Base (SYBB) for pre-enrichment was added and the mixture was homogenized in a stomacher for 60 s at normal speed. The bag was sealed and left at room temperature for 1 h, and incubated at 35ºC for 6 h. Selective Salmosyst enrichment was carried out transferring 10 mL of the SYBB to test tubes containing 1 mL Salmosyst Selective Supplement, to become pre-enrichment Selective Supplement broth. Incubation at 35ºC for 18 h ensued, after which this broth was streaked on petri dishes containing DSA. Dishes were incubated at 35ºC for 18 h. Pink or red colonies were identified based on classical biochemistry and serology (Pignato *et al*. 1995).

c. Polymerase chain reaction

Aliquots (0.7 mL) of the pre-enrichment broth (PBS) used in the conventional microbiology was transferred to sterile polypropylene microtubes 6 h and 24 h after the beginning of incubation for DNA extraction and amplification. Similarly, 0.7-mL aliquots of SYBB was transferred to different sterile polypropylene microtubes 6 h

after the beginning of incubation. Aliquots of identical volume of the Selective Supplement Broth were transferred to identical microtubes 24 h after the beginning of incubation. Then, 0.7 mL of glycerol was added to every tube. Contents were stored at -20ºC, upon DNA extraction.

The PCR protocol adopted was based on Flôres *et al.* (2001) and Santos *et al.* (2001a; 2001b), in which DNA extraction is carried out by thermal treatment. The PCR reaction was conducted using a pair of primers that amplifies 284 bp, containing the oligonucleotide sequence derived from the *invA* gene: primer 1, *invA* 159: 5' GTG AAA TTA TCG CCA CGT TCG GGC AA 3', and primer 2, invA 141: 5' TCA TCG CAC ACG TCA AAG GAC C 3'.

DNA extraction. DNA was extracted using 300 µL aliquots from the microtubes' contents using micropipettes with tips. Aliquots were transferred to new microtubes. After, 300 µL tris-EDTA buffer was added. Contents were homogenized in an automatic shaker for 30 s. Immediately after, microtubes were centrifuged for 30 min at 13,500 rpm and 10ºC. The supernatant was discarded and 100 µL of tris-EDTA buffer was added to the remaining 50 µL of the sediment. Contents were then homogenized once again for 10 s in an automatic shaker. After, microtubes were kept on a thermal block at 94ºC for 10 min and then immediately centrifuged for 1 min at 13,500 rpm and 10ºC. The supernatant obtained was transferred to new microtubes and kept in a freezer at -20ºC for 10 min, prior to DNA amplification.

DNA amplification. PCR reactions were carried out in conic 200-µL polypropylene microtubes containing 4.4 µL ultrapure water, 5.0 µL 10X buffer, 5.0 µL $MgCl_2$ 50 mM, 5.0 µL dNTP mix (25 µM each nucleotide), 1.0 µL (100 ρmol) primer 1, 1.0 µL (100 ρmol) primer 2, 1.2 µL bovine serum albumin (BSA) 400 µg/mL, 0.4 µL *Taq* DNA polymerase (2.0 U) and 2.0 µL of purified sample DNA, to a final volume of 25.0 µL. *Salmonella* Enteriditis DNA (2.0 µL) was used as positive control, and ultrapure water (2 µL) was used as negative control. Amplification conditions were as follows: one 5-min cycle at 94ºC (denaturation), 35 cycles of 30 s at 94ºC (denaturation), 30 s at 54ºC (annealing) and 30 s 72ºC (extension), and a final 7-min extension cycle at 72ºC. after amplification, the total reaction volume was electrophoresed on agarose gels 1.5% in tris/borate/EDTA (TBE) 0.5X stained with 5.5 mg/mL ethidium bromide. Amplified fragments were visualized in a UV transluminator and photodocumented.

Statistical analysis. Results of microbiological analyses (conventional microbiology and Salmosyst method) and PCR were compared by the chi-square test at 95% (α=0.05) and 99% (α=0.01) significance level (df=5). Concordance in the detection of *Salmonella* spp. in hamburgers using the different methods was assessed using the McNemar test. All statistical analyses were carried out in the *BioStat*™ 3.0 software, version 2003 (Ayres *et al*. 2003).

Results

All samples analyzed were previously inspected by federal sanitation authorities. Of the 80 samples analyzed, 32 (40%) were contaminated with *Salmonella* spp., and were thus inappropriate for human consumption, as established in Resolution No. 12, issued by the Board of the National Sanitation Authority (ANVISA), Ministry of Health (Brasil 2001). Of these, 14 (17.5%) were identified as positive for the microorganism only by conventional microbiology and the Salmosyst methods, while 10 (12.5%) were positive in the PCR protocol and eight (10%) were positive using both analyses (Figure 1).

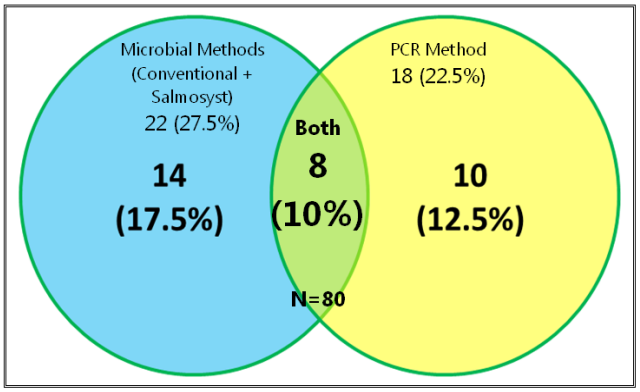

Figure 1. Number of samples positive for *Salmonella* spp. using the conventional microbiology methods, Salmosyst method and PCR.

More specifically, when conventional microbiology and the Salmosyst method are compared, *Salmonella* spp. was detected in 17 (21.25%) and 11 (13.75%) samples, respectively, while PCR identified 18 (22.5%) samples contaminated with the pathogen (Table 1). Only tree (7.5%) of the samples were shown to contain the microorganism using the three methods.

No statistically significant difference was observed in the detection of *Salmonella* spp. using the three methods in terms of the type of hamburger analyzed (beef and a beef and chicken mix) using the chi-square test (χ^2=3.209, *p*=0.6679, below significance values α=0.01, 15.09, and α=0.05, 11.07). In other words, the detection of *Salmonella* spp. was not influenced by the method used or type of hamburger.

As for the PCR analysis, eight (10%) samples were positive for *Salmonella* spp. using the classical pre-enrichment broth (PBS) for a 24-h incubation period, eight (10%) using the Selective Salmosyst Supplement enrichment broth for a 24-h incubation period, and two (2.5%) using both broths (Table 2).

No statistically significant difference was observed between detection of *Salmonella* spp. using conventional microbiology, PCR using PBS culture, PCR using Selective Salmosyst Supplement enrichment broth (χ^2=3.333, p=0.3430, below significance values α=0.01, 15.09, and α=0.05, 7.81). The detection of *Salmonella* spp. was not influenced by the method used.

When PCR results are individually compared with conventional microbiology and the Salmosyst method, the McNemar test showed no statistically significant difference in the power to detect *Salmonella* spp. (1.7413, p=0.1904 and 0.0, p=1.0, respectively, below the significance levels α=0.01, 6.64, and α=0.05, 3.84).

Concerning sensitivity, microbiological methods were poorly sensitive to detect *Salmonella* spp. in raw hamburgers (36.36%0, which may increase the risk of false negative results. However, these methods were highly specific (82.76%), indicating the capacity to characterize negative results more efficiently. In turn, PCR presented low sensitivity (44.44%) and average specificity (77.42%). Precision for both methods was 70%.

Table 1. Positive *Salmonella* spp. samples using the conventional microbiology, Salmosyst method and PCR for beef and chicken and beef hamburgers.

Hamburger type	Conventional microbiology		Salmosyst		PCR	
	Salmonella (+)	*Salmonella* (–)	*Salmonella* (+)	*Salmonella* (–)	*Salmonella* (+)	*Salmonella* (–)
Beef	9	31	3	37	8	32
Chicken and beef	8	32	8	32	10	30
TOTAL	**17**	**63**	**11**	**69**	**18**	**62**

Table 2. Positive *Salmonella* spp. samples using the microbiological methods (conventional and Salmosyst) and PCR.

Results	Microbiology Methods		PCR	
	Conventional microbiology	Salmosyst	Pre-enrichment broth used in conventional microbiology (24 h)	Pre-enrichment broth used with Salmosyst selective supplement (24 h)
Salmonella spp. positive	17 (21.25%)	11 (13.75%)	10 (12.5%)	10 (12.5%)
Salmonella spp. negative	63 (78.75%)	69 (86.25%)	70 (87.5%)	70 (87.5%)
TOTAL	**80**	**80**	**80**	**80**

Discussion

Microbiological methods and PCR performed similarly concerning the identification of true positive *Salmonella* spp. in raw hamburgers. However, the microbiological methods were more efficient at identifying true negatives. As regards precision, both methods yielded acceptable results to detect *Salmonella* spp.

As observed in the present study, the importance of pre-enrichment has been underlined in the detection of *Salmonella* spp. In a previous study, of 30 chicken carcasses analyzed, five (16.7%) were positive for the bacteria (Rissato *et al*. 2011). The authors also observed that the sensitivity of the PCR technique after pre-enrichment and a 24-h incubation period was 3.3 % (1/30), and increased to 16.7% (5/30) in samples incubated for 48 h. In another study, Mynt *et al.* (2003) observed that when pre-enrichment was not used, the PCR technique did not detect *Salmonella* spp. Nevertheless, sensitivity increased to 79% when pre-enrichment was used. This increase in PCR sensitivity with pre-enrichment was also reported in other papers (Croci *et al*. 2004; Santos *et al*. 2001b).

Additionally, the advantages of pre-enrichment in the detection of microorganisms by PCR were elegantly

discussed in a study that reported that pre-enrichment broths are usually inexpensive, are not labor-intensive, dilute substances that may inhibit the PCR and increase the number of viable bacterial cells (Stone *et al*. 1995). Also, for Cheung and Kam (2012) pre-enrichment allows multiplying the bacterial cells in the sample, improving detection of *Salmonella* spp. This step reduces the chances of amplifying sequences of dead cells present in the sample.

In a study that compared the conventional microbiological isolation procedure with PCR in the detection of *Salmonella* spp. in experimentally challenged calves, Silva *et al.* (2011) concluded that the conventional method was more effective than PCR to detect *Salmonella* Typhimurium, as revealed by the McNemar test. Similarly, Oliveira *et al.* (2003) state that the PCR carried out with non-selective pre-enrichment was the least sensitive to generically detect *Salmonella*, compared to the conventional method.

In turn, Rall *et al*. (2009) investigated the presence of *Salmonella* spp. in 50 samples of chicken and 75 samples of fresh sausages, and observed that 27 (54%) of the chicken samples were positive in the PCR and only four (8%) were positive for the microorganisms in the conventional methodology. In sausage samples, 42 (56%) and seven (9.3%) were positive for *Salmonella* spp. using the PCR and conventional methods, respectively. The authors also observed that PCR was more sensitive than conventional microbiology.

Here, the percentage prevalence of *Salmonella* spp., assessed using both microbiological methods and the PCR technique, differed from the findings reported in previous studies. In a paper reporting the presence of *Salmonella* spp. in 300 chicken meat samples, Dickel *et al.* (2005) observed 170 (56.6%) and 225 (75%) positive samples using the conventional methodology and PCR, respectively. In a study that investigated the presence of *Salmonella* spp. in 98 samples of a variety of foods, Kawasaki *et al*. (2001) reported that 27 (37.76%), 42 (42.86%) and 34 (34.69%) were positive for the bacteria using conventional microbiology, PCR, and both methods, respectively. Analyzing 268 pork samples, Castagna *et al*. (2005) reported the presence of *Salmonella* spp. in 54 samples (20.15%) using the PCR, 42 (15.67%) using the conventional methodology, and 39 (14.55%) using both methods. However, none of these studies analyzed raw beef or beef and chicken hamburgers. These differences reported for the efficiency of the PCR protocol and conventional methods do detect *Salmonella* spp. in foods point to the importance of further studies to shed more light on how far a given type of sample may influence the sensitivity and specificity of these techniques.

Conclusion

Both beef and beef and chicken hamburgers pose a health hazard, indicating the need for improvement in inspection routines and sanitary control of these products to reduce the contamination by *Salmonella* spp. as well as other pathogenic microorganisms. The results obtained in the present study suggest that more than one detection method may make the detection of *Salmonella* spp. in hamburgers more reliable. Also, the three analysis approaches used were efficient, and are useful in the microbiological monitoring and control of contamination sources in the food industry.

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