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Detection of *Salmonella* spp. in Hamburgers: a Comparison Between Modified Standard and Salmosyst Methods

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Abstract

Salmonella spp. was analyzed in beef and chicken and in beef hamburgers using two different microbiological methods with slight modifications in the biochemical test stage. Of the 80 hamburger samples analyzed, 22 (27.5%) were positive for *Salmonella* spp., 10 (12.5%) beef and 12 (15%) chicken and beef hamburgers. Of the 22 positive samples, 11 (50.0%) were detected by the standard method alone, 5 (22.73%) by the Salmosyst method, and 6 (27.27%) were detected using both methods. Sensitivity and specificity of methods was investigated. The standard method was the most effective to identify real positive results, while Salmosyst method afforded to characterize real negative results. As for accuracy, both methods were acceptable to analyze *Salmonella* spp. in hamburgers. The importance of streaking *Salmonella* spp. colonies obtained using the TSI and LIA agars, with atypical characteristics 1 and 2 was also revealed, since these two sets of characteristics may be used to isolate atypical *Salmonella* spp. serotypes, which ferments lactose and *Salmonella* Parathyphi, which does not decarboxylate lysine, respectively. Secondary screening was efficient as regards the differentiation between *Proteus* spp. and *Salmonella* spp. colonies.

Key words: *Salmonella*; Hamburger; Standard Method; Salmosyst Method.

Introduction

Food poisoning outbreaks pose a challenge to the food industry and health authorities, and in this sense the genus *Salmonella* stands as one of the most important and common foodborne pathogenic bacteria (Siqueira et al., 2003). These microorganisms invade the intestinal tract mucosa, and are transmitted to humans through contaminated water, meat, eggs and poultry products via the fecal-oral pathway. More specifically, food poisoning caused by *Salmonella* spp. is termed salmonellosis, a disease with considerable social impact and that is held responsible for an array of different kinds of economic losses worldwide (Giombelli and Silva, 2002).

Salmonellosis may be caused by any of the almost 2,500 *Salmonella* serotypes known. It is the most ancient and common form of food poisoning, with total mortality rates that may reach 0.2% (Gooding and Choudary, 1999; Gutiérrez-Cogco et al., 2000; Oliveira et al., 2003). Additionally, it is the most prevalent disease transmitted to humans from animals (D'Aoust, 1991; Ferretti et al., 2001; Kwang et al., 1996; Riyaz-Ul-Hassan et al., 2004). It has been reported that the disease starts when food or water contaminated with sufficiently high bacteria counts are ingested, resulting in the colonization of the small intestine (Van Pouke, 1990). The main clinical signs of salmonellosis include acute gastroenteritis, bacteremia with or without extraintestinal infection, and fever, though asymptomatic carriers are also reported.

Salmonella spp. is detected mainly in animal food products, among which hamburgers, prepared with beef alone or as a combination of beef and chicken. The

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hamburger is an extensively industrialized meat product consumed largely because of ease of preparation and excellent sensory characteristics. The main raw material is minced beef, though fat and other ingredients may be included. Intensive handling and complex preservation issues in preparation promote pathogen growth and transmission in hamburgers. High probabilities of some kind of contamination have been observed throughout the hamburger production process. This contamination may be caused by a variety of agents, such as specific organic compounds used in pastures, management techniques, transportation of animals, inappropriate slaughter procedures (contamination in carcasses), apart from problems in handling, storing and even in preparation and consumption.

In this sense, microbiological assessment of hygiene and sanitation procedures in hamburger production becomes necessary to warrant safe and contamination-free consumption. Techniques developed to evaluate bacterial contamination in the food production chain, more specifically of animal origin, aims mostly at reducing risks of foodborne diseases and testing the quality of these products. The detection and characterization of *Salmonella* spp. in foods and water is very important in the control and prevention of food poisoning outbreaks. However, concerning health inspection routines, which require fast and reliable results so as to issue approval certificates for commercialization and consumption, standard methodologies to evaluate *Salmonella* spp. in foods based on classical culture media are time-consuming and impractical. As a rule, standard microbiological techniques require between 5 and 7 days of laboratory work, especially when a large number of samples has to be processed, as observed in the food industry. These methods are also prone to producing false negative results, mainly due to the interference of other microorganisms in food samples that are direct competitors with *Salmonella* spp. bacterial cells.

In spite of these apparent disadvantages, these techniques remain as the most used, and are the reference methodology officially recommended in the Brazilian legislation on the subject. Concerning the specific regulation on hamburger origin and quality, Ordinance Nº. 20 (Brasil, 2000) established that the current legislation is to be enforced concerning microbiological contamination criteria. Microbiological contamination thresholds and standards for hamburgers defined in Resolution Nº. 12, issued by the Board of the National Sanitation Authority (ANVISA) (Brasil, 2001) establish that indicative or representative food samples must be free of *Salmonella* spp. bacterial cells.

In this scenario, the main goal of present study was to investigate the presence of *Salmonella* spp. in beef hamburgers and combined beef and chicken meat hamburgers sold in the city of Niterói, state of Rio de Janeiro, RJ, Brazil, using modified versions of the standard microbiological methodology and the Salmosyst method.

Investigation of Salmonella spp. using the standard methodology

The standard *Salmonella* spp. detection method for foods was developed to investigate the presence of the pathogen, even in extremely unfavorable conditions, as in foods hosting a competing microbiota that overwhelms *Salmonella* spp. population and in items in which the pathogen's cells are injured or stressed by conservation strategies like heat, freezing and/or drying. Variations in choice of culture media and sample preparation routines are observed, though essentially four steps are applicable in the analysis of any kind of food: pre-enrichment in non-selective broth; enrichment in selective broth; differential selective plating (isolation and selection); confirmation (biochemical identification and seroagglutination test) (Brasil, 2003).

In general, these stages include pre-enrichment, which promotes the regeneration and the multiplication of injured *Salmonella* spp. cells, the selective enrichment to increase the ratio of *Salmonella* spp. cells to competing microorganisms, and selective/differential plating to allow the identification and isolation of *Salmonella* spp. colonies by suppressing the growth of the competing microbiota. Biochemical and serological confirmation is required whenever typical *Salmonella* spp. colonies grow in selective/differential media. In theory, the detection threshold of these methods is one *Salmonella* spp. cell in a 25 g food sample, though detection may be hampered by the presence of competing microorganisms during enrichment (Beckers et al., 1987; Blackburn, 1993; Fricker, 1987).

PRE-ENRICHMENT IN NON-SELECTIVE BROTH

In this step, a 25 mL sample is inoculated at 36℃ for 16 to 20 h in 225 mL Buffered Saline Peptone Solution (BSPS) or Buffered Peptone Water (BPW). This procedure minimizes the effects of the industrial processing of foods, which causes stress to *Salmonella* spp. cells without biologically inactivating them. Also, BSPS or BPW help maintain pH, preventing acidification of the medium by other bacteria present, a condition that hampers the recovery of *Salmonella* spp. (Brasil, 2003).

Isolation of *Salmonella* spp. is influenced mainly by the presence of competing microorganisms. Nevertheless, the competing microbiota is not representative of the same microbiota actually occurring in foods. By adding the reference material to a mixture of BPW and the food being analyzed, before incubation, the competing microorganisms naturally present in the sample may be used (Beckers et al., 1985). These investigation procedures usually include the pre-enrichment of a food sample in a non-selective medium to facilitate and allow the repair of stressed or injured *Salmonella* spp. cells, commonly detected in raw as well as in processed foods. These cells may then multiply. Growth should afford sufficiently high counts of these microorganisms, in an attempt to warrant survival in a

highly selective medium used in the next step (D'Aoust et al., 1992; Van Schothorst and Renaud, 1985).

ENRICHMENT IN SELECTIVE BROTH

This step is carried out at 41℃ for 24 to 30 h, in culture media containing substances that prevent the growth of most competing microorganisms. The selective media used are the Rappaport-Vassiliadis Broth (RV) medium and the Selenite-Cystine Broth (SC), though Tetrathionate Broth (TT) is occasionally employed. In the RV Broth, malachite green and magnesium chloride, in an appropriate temperature, act as selective agents on the competing microbiota, while soy peptone stimulates *Salmonella* spp. growth. As for the SC Broth, sodium selenite inhibits coliforms and enterococci, while TT Broth selectiveness is indicated by tetrathionate and brilliant green (Brasil, 2003).

Busse (1995) reports that these selective enrichment media in general are used to suppress the accompanying microbiota as well as Gram-positive bacteria. The selective media for *Salmonella* spp. are totally efficient, among which the most commonly used are the selenite broth, tetrathionate broth with brilliant green, bile green or malachite green combined with high amounts of magnesium chloride. Patil and Parhad (1986), apart from confirming that enrichment media play an important role in the isolation of *Salmonella* spp., since usually the populations of this microorganisms are smaller compared to other bacteria, also stress the fact that enrichment media were initially developed to isolate *Salmonella* spp. in feces, and were later used in the analyses of other materials, like food and water.

However, Busse (1995) warns that the isolation of *Salmonella* spp. in foods is somewhat prone to failure. The main risk lies in faulty sampling procedures, since bacterial cells initially present in a sample may be lost during enrichment. If *Salmonella* spp. are the prevailing microorganisms in the enrichment broth, detection will always be successful. Nevertheless, if populations of competing microorganisms overwhelm *Salmonella* spp. counts, then detection may not succeed.

DIFFERENTIAL SELECTIVE PLATING (ISOLATION AND SELECTION)

In this step, *Salmonella* spp. colonies are isolated using at least two of the following sold selective media: (i) Brilliant-Green Phenol-Red Lactose Sucrose Agar (BPLS); (ii) Rambach Agar (RA); (iii) Differential Salmonella Agar (DSA); (iv) Hektoen Enteric Agar (HE); (v) *Salmonella-Shigella* Agar (SS); Xylose Lysine Desoxycholate Agar (XLD); Xylose-Lysine Tergitol 4 Agar (XLT4); Mannitol Lysine Crystal Violet Brilliant Green Agar (MLCB). In BPLS, novobiocin is added to inhibit mainly *Proteus* spp., while bovine bile, a stain derived from triphenyl methane (brilliant green) inhibits Gram-positive bacteria. In RA, differentiation between *Salmonella* spp. and other microorganisms is due to the presence of propylene glycol and a chromogen that indicates the hydrolysis of beta-galactosidase. In MLCB, magnesium ions promote the growth of *Salmonella* spp., while brilliant green inhibits the accompanying microbiota (Brasil, 2003).

The choice of a selective plating medium is object of special attention. Appropriate plating media are essential for the efficient isolation of all bacteria, which should grow on solid media. Efficient plating media should exhibit a variety of characteristics, like supporting the growth of a wide array of specific microorganisms, while inhibiting the growth of other bacteria, as far as possible. Apart from this, these media should afford to distinguish the organisms being investigated from those that may also establish colonies in the same media. Additionally, efficient differentiation not only increases the rates of success in isolation, but also reduces the number of colonies that have to be characterized using biochemical and physiological methods to confirm the presence of the microorganism studied (Fricker, 1987).

BIOCHEMICAL IDENTIFICATION

Suspected *Salmonella* spp. colonies are screened in media that provide indications of the biochemical characteristics of the microorganisms. The media used are: (i) Triple Sugar Iron Agar (TSI); (ii) Lysine Iron Agar (LIA); (iii) Kliger Iron Agar (KIA) (Siqueira, 1995).

COMPLEMENTARY BIOCHEMICAL TESTS

These tests provide evidence of the physiological and metabolic properties of suspected colonies, among which: (i) presence of cytochrome oxidase; (ii) urease production; (iii) glucose fermentation; (iv) saccharose and lactose detection in TSI; (v) beta-galactosidase detection; (vi) lysine decarboxylation; (vii) sulfide production (H_2S); (viii) motility; and (ix) indol production. The following culture media are used: (i) Urea Broth; (ii) SIM medium; (iii) oxidase test; (iv) Phenylalanine Agar; (v) VM-VP Broth; and (vi) Citrate Agar (Brasil, 2003).

SEROAGGLUTINATION TEST (SEROLOGY)

This test is based on the antigen-antibody reaction, which caused antigen agglutination by antiserum for polyvalent *Salmonella* spp. (Brasil, 2003).

Chromogenic media to detect Salmonella spp.

According to Reis and Camargo (2008), chromogenic media are used to differentiate and isolate *Salmonella* spp. from other genera and also other numerous species. It has been published that standard methods to detect *Salmonella* spp. suffer from poor specificity and therefore leads to a considerable number of false positive results (which include the detection of *Citrobacter* spp. and *Proteus* spp.) among very few positive results for *Salmonella* spp. (Manafi, 2000). Since these labor-intensive techniques may often produce false results in routine laboratory analysis, new chromogenic and fluorogenic methods that lead to faster

and more reliable *Salmonella* spp. detection become necessary.

These methods may be used directly after primary growth, abolishing the need for long procedures of isolation and identification. By using synthetic substrates it becomes possible to conduct several reactions simultaneously in one same dish. Chromophore and/or fluorophore groups are associated to different types of protein, glycoside, and alcohol substrates, among others which, compared to standard media, are responsible for the greater detection accuracy exhibited by chomogenic and fluorogenic media (Bascombs, 1987; Manafi et al., 1991; Manafi et al., 1996; Tsoraeva and Marques, 2005).

Acknowledging the limitations in the use of phenotypic characteristics currently used to detect *Salmonella* spp., Rambach (1990) developed a medium to presumptively detect these microorganisms, differentiating *Salmonella* spp. from *Proteus* spp. The method also allows the identification of colonies of bacteria of the Enterobacteriacea family. Additionally, *Salmonella* spp. are distinctively stained, facilitating the recognition of colonies of other microorganisms.

Rambach (1990) reports a new phenotypic characteristic: the production of acid from ethylene glycol. This parameter may be used instead of H_2S production to identify *Salmonella* spp. in selective media. Organisms that use lactose were then identified based on a chromogenic indicator of beta-galactosidase, discriminating *Salmonella* spp. from members of the Enterobacteriacea family that also use this enzyme. Desoxycholate was included as an inhibitor of Gram-positive bacteria, though not every compound was used at the concentrations that usually do not inhibit pathogenic microorganisms and coliforms.

A chromogenic medium relies on the ability of *Salmonella* spp. to produce acid from propylene glycol, which differentiates these species from other enteric bacteria. Apart from this, the presence of the chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) affords to detect the production of beta-galactosidase D produced by another enterobacterium. *Salmonella* spp. cultures are positive only in the first reaction (acid production from propylene glycol). These colonies are brilliant red in color, while *Escherichia coli* and other coliforms are positive only for beta-galactosidase D, and form blue colonies. *Proteus* spp. are negative for both reactions and therefore its colonies are transparent. *Citrobacter* spp. form violet colonies due to the combination of colors (red and blue), since the two reactions are observed (Garrick and Smith, 1994; Pignato et al., 1995). Manafi (1996; 2000) also stated that chromogenic substrates of the enzyme are the compounds that act as substrate to specific enzymes and change color due to the action of this same enzyme. Generally speaking, four groups of chromogenic compounds are described, based on the chemical reaction they participate in: indol compounds (soluble in water and thermostable), and the most commonly used compounds, like 5-bromo-4-chloro-indolyl (colorless), 5-bromo-6-chloro-3-indolyl (magenta) and 6-chloro-3 indolyl (salmon pink), which do not diffuse in agar plates.

The ability to detect the presence of a specific and exclusive enzyme using appropriate substrates, more specifically the chromogenic and fluorogenic enzyme substrates, has led to the development of a large number of methods to identify microorganisms, even in media used for preliminary isolation. The addition of these substrates to a selective medium may abolish the need for subculture, affording to use biochemical tests to establish the identity of determined microorganisms (Manafi, 2000).

Salmonella spp. detection using the Salmosyst method

The Salmosyst method is a double enrichment method consisting of a pre-enrichment stage in non-selective Salmosyst Broth Base (SYBB) containing only nutrients, electrolytes and buffers that regulate pH. In the enrichment stage, selective agents are added as Salmosyst selective supplement tablets, which dissolve gradually during 30 min, producing a selective gradient in the medium and allowing a progressive adaptation of *Salmonella* spp. cells to the medium, and increasing survival rates of these cells (Fierens and Huyghebaert, 1996).

After the enrichment stage, a selective and differential chromogenic agar medium is used to distinguish *Salmonella* spp. from other enteric bacteria. This medium is prepared mainly with propylene glycol, sodium desoxycholate, neutral red (as a pH indicator) and a chromogenic substrate for beta-galactosidase. The differentiation between bacteria is based on the production of acid from propylene glycol by *Salmonella* spp. and/or beta-galactosidase activity (Fierens and Huyghebaert, 1996; Rambach, 1990). The pink or red colonies obtained in the chromogenic agar medium are then identified using biochemical and serological methods (Pignato et al., 1995).

Material and Methods

Study area and sample collection

This study was conducted in the Laboratory of Microbiological Control of Animal Products, Department of Food Technology, Veterinary Medicine School, Universidade Federal Fluminense, Rio de Janeiro, RJ, Brazil. Eighty samples of hamburgers prepared with beef alone (n=40) or as a combination of beef and chicken (n=40) were randomly purchased as routinely commercialized in double or single polypropylene bags by local supermarkets or butchers' shops in the city of Niterói, RJ, Brazil, between May and December, 2011. Four hamburgers were considered a whole sample. All hamburgers were certified by the federal inspection authority of Brazil (SIF). Hamburgers weighed between 56 g and 120 g. As of purchase, hamburgers were kept in the original package, which were placed in individual low-

density polyethylene bags with hermetic lock, transferred to a portable thermal container and transported to the laboratory for analyses.

***Salmonella spp* analysis**

The microbiological methods used to isolate and identify *Salmonella* spp. were those defined in 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).

The results of *Salmonella* spp. analyses obtained were compared to the national standard, based on Resolution Nº. 12, issued by the Board of the National Sanitation Authority (ANVISA), Ministry of Health (Brasil, 2001). These standards define sanitary and microbiological thresholds for foods, and determine the criteria to be adopted in the interpretation of results of microbiological analyses of foods for human consumption (Brasil, 2001).

Standard microbiology

In the laboratory, a sample was defined as a 25 g aliquot was retrieved from a hamburger sample. For pre-enrichment, samples were placed in a plastic bag and weighed in a digital precision scale. Then, 225 mL of BSPS 1% were added and the mixture was homogenized in a stomacher for 60 s at normal speed. The bag containing the sample was hermetically closed and left at room temperature for 1 h. After, bags were placed in a stove, where samples were incubated at 36ºC for 16 to 20 h.

For enrichment, samples were inoculated with SC and TT broths. Aliquots of 1 mL were individually transferred to tubes containing 10 mL of each broth, and were inoculated at 41ºC for 24 to 30 h.

Selective plating was carried out using the selective enrichment broths. Selective media were streaked on dry Petri dishes using a bacteriological loop so as to produce isolated CFU. Two dishes were used for each culture medium (one culture from SC and one from TT), which were: HE, SS, XLD and DSA. All dishes were incubated in a stove at 36ºC for 18 to 24 h.

During screening, here called initial screening, up to typical 3 CFU were chosen from each Petri dish used in selective plating. These CFU were streaked onto two different tubes, one containing TSI and the other containing LIA. Tubes were incubated in a stove at 36ºC for 24 to 30 h. After incubation, TSA and LIA tubes were inspected for typical *Salmonella* spp. characteristics, with or without H_2S production. The expected phenotypical characteristics observed were: acid bottom (yellow) and alkaline slant (red) in TSI, and alkaline bottom and slant (purple) in LIA. The first set of atypical characteristics in TSI/LIA were: TSI with acid bottom and slant (yellow), and LIA with acid bottom (yellow) and alkaline slant (purple). In turn, the second set of atypical characteristics in TSI/LIA were: TSI with acid bottom (yellow) and alkaline slant (red), and LIA with acid bottom (yellow) and alkaline slant (purple). TSI tubes presenting acid bottom and slant (yellow) and LIA tubes with acid bottom (yellow) and alkaline slant (purple) were excluded.

This initial screening of typical and atypical *Salmonella* spp. characteristics was used in the secondary screening (complementary biochemical tests). Using a bacteriological loop, colonies were streaked in tubes containing culture media that revealed physiological and metabolic properties of suspected *Salmonella* spp. colonies by assessing urease production (Urea Broth) and phenylalanine deamination (Phenylalanine Agar). This stage was important in order to differentiate *Proteus* spp. cells, which produce positive urease results (i.e., hydrolyze urea) and deaminate phenylalanine, from *Salmonella* spp. cells.

In the urease production assay, colonies were massively seeded in test tubes containing 3.0 mL Urea Broth and were then incubated at 36ºC for 24 to 30 h. Colonies that retained their initial color indicated that urea hydrolysis did not occur. Change in color to pink showed the alkalinization of the medium due to the action of urease over urea, since *Salmonella* spp. do not produce urease. In the phenylalanine deamination test, the slant surface of the Phenylalanine Agar was streaked and incubated at 36ºC for 18 to 24 h. Then, 2 to 3 drops of ferrous chloride 10% were added. Phenylanine deamination was indicated by a green color on the slant, since *Salmonella* spp. do not deaminate phenylalanine.

Based on the negative results of the urease and deamination tests, typical of *Salmonella* spp. cells, the corresponding colonies grown in TSI tubes were streaked onto slanted tubes containing nutrient agar and incubated at 36ºC for 18 to 24 h. After, the growth obtained was resuspended in approximately 2.0 mL saline 0.85%. Next, one drop of saline 2% and then one drop of polyvalent anti-*Salmonella* serum were laid on two glass slides, straight from the container. One drop of the test solution was added to each slide. Reactions were observed by spinning slides gently over a black background for 1 to 2 min. Positive results for *Salmonella* spp. were defined by the presence of agglutination only in the slides containing bacterial growth and antiserum. Negative results were defined as the absence of any agglutination in both slides, and non-specific reaction was observed as agglutination in both slides.

Apart from this, at each stage of the microbiological analysis (standard and Salmosyst methods), aliquots of broths and colonies in plates and tubes were transferred to glass slides using a microbiological loop for staining smear according to Gram's method (morphostaining characteristics) to detect Gram-negative rods under an optical microscope equipped with an immersion objective lens.

***Salmonella spp.* detection using the Salmosyst metho**

A 25 g aliquot was retrieved from a hamburger sample and placed in a plastic bag. This sample was and weighed in a digital precision scale. 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 hermetically closed and left to stand at room temperature for 1 h. Then, samples were incubated in a stove at 35ºC for 6 h. Selective enrichment was carried out transferring 10 mL of SYBB to test tubes containing 10 mL of the Selective Supplement. This selective medium was incubated at 35ºC for 18 h. After incubation, it was streaked on DSA and incubated again at 35ºC for 18 h. Pink and red colonies were submitted to biochemical and serological investigation, as described in standard methodologies.

Statistical analyses

A transversal observational analytical evaluation of data was carried out. Data were expressed and analyzed as absolute and percent frequencies. Additionally, the chi-square test (χ^2), the McNemar test and diagnostic tests were executed using the statistical software *BioStat*™ 3.0, version 2003.

The statistical analysis of significance of the differences observed in results of microbiological analysis (standard microbiology and Salmosyst method) was carried out using the chi-square (χ^2) test at 95% significance ($\alpha = 0.05$) and 99% ($\alpha = 0.01$). Results of *Salmonella* spp. in hamburgers by standard methods were compared with the results by the Salmosyst method using the McNemar test. Sensitivity, specificity and accuracy of results of standard and Salmosyst methods were evaluated using diagnostic tests.

Results

Of the 80 samples of hamburgers analyzed (40 pure beef and 40 beef and chicken hamburgers), which were all inspected by the federal inspection authority of Brazil (SIF), 22 (27.5%) were contaminated by *Salmonella* spp. and were thus considered inappropriate for human consumption (according to Resolution Nº. 12, issued by the Board of the National Sanitation Authority (ANVISA) (Brasil, 2001).

Of the 22 *Salmonella* spp. positive samples (**Tab. 1**), 10 (12.5%) were pure beef and 12 (15%) were beef and chicken hamburgers.

Table 1. *Salmonella* spp. detected in beef and chicken and beef hamburger samples.

Type of hamburger	*Salmonella* spp.		**TOTAL**
	Positive	Negative	
Beef	10	30	**40**
Chicken and beef	12	28	**40**
TOTAL	**22 (27.5%)**	**58 72.5%)**	**80 (100%)**

The value obtained in the chi-square (χ^2) test, used to assess the relationship between type of hamburger and *Salmonella* spp. prevalence using both methods, was 2.71, with p=0.4386 (df=3). Since this value was lower than values considering significance levels α=0.01 (11.34) and α=0.05 (7.81), showing that the differences were non-significant. In other words, the differences observed were not related to the analytical methods employed to analyze either hamburger type (pure beef and chicken and beef hamburger).

However, considering the parameter type of meat used in hamburger preparation, it was observed that among the 40 beef hamburger samples, 10 (12.5%) were positive for *Salmonella* spp., and of the 40 chicken and beef hamburgers, 12 (30.0%) were contaminated with *Salmonella* spp.

The value calculated in the chi-square (χ^2) test to evaluate the relationship between hamburger type and *Salmonella* spp. prevalence, 0.251 (df=1; p=0.6165), was lower than values considering significance levels α=0.01 (6.64) and α=0.05 (3.84), showing that there is no significant statistical difference between frequencies of the pathogens observed and expected.

Considering the methods used, in the analysis of 80 hamburger samples by the standard method, 17 (21.25%) were positive for *Salmonella* spp., 9 (22.5%) of which were beef hamburgers and 8 (20.0%) were beef and chicken hamburgers. Considering the Salmosyst method, 11 (13.75%) were shown to be contaminated with the bacteria, 3 (7.5%) beef and 8 (20.0%) beef and chicken hamburgers (**Tab. 2**).

Table 2. Number of hamburger samples positive for *Salmonella* spp. in each microbiological method used.

Type of hamburger	Method	
	Standard	Salmosyst
Beef	9 (52.94%)*	3 (27.27%)
Chicken and beef	8 (47.06%)	8 (72.73%)
TOTAL	**17 (100%)**	**11 (100%)**

* Percentages calculated based on total numbers of samples used in each method.

The value of the chi-square (χ^2) test carried out to evaluate the association between method and *Salmonella* spp. prevalence was 1.558 (df=1; p=0.2119), lower than the values considering significance levels $\alpha = 0.01$ (6.64) and α=0.05 (3.84), showing the absence of statistical significance in the differences between frequencies of the pathogen measured and expected.

It is important to observe that, of the 22 hamburger samples positive for *Salmonella* spp., 11 (50.0%) were detected only using the standard methods, 5 (22.73%) were

detected using the Salmosyst method, and 6 (27.27%) were detected using both methods (**Tab. 3**).

Table 3. Number of hamburger samples positive for *Salmonella* spp. in each microbiological method used, and number of samples positive for the pathogen in both methods.

Type of hamburger	Method			TOTAL
	Standard	Salmosyst	Both	
Beef	7	1	2	**10 (45.45%)**
Chicken and beef	4	4	4	**12 (54.55%)**
TOTAL	**11 (50.0%)**	**5 (22.73%)**	**6 (27.27%)**	**22 (100%)**

The values calculated in the McNemar test was 1.5625 (for the two kinds of hamburgers together), 3.125 (for beef hamburgers), and 0.125 (for chicken and beef). All values were below the values considering significance levels α=0.01 (6.64) and α=0.05 (3.84), indicating that there is no statistically significant difference between *Salmonella* spp. prevalences using standard methodology and the Salmosyst method.

In the diagnostic tests, the results obtained show that the standard method to detect *Salmonella* spp. in hamburgers had intermediate sensitivity (54.55%) and high specificity (84.06%) and accuracy (80.0%) values. In turn, the values observed using the Salmosyst method were low for sensitivity (35.29%), indicating the likelihood of false negative results, and high for specificity (92.06%), pointing to a considerable reliability to detect false negative results. As in the standard method, accuracy was 80.0%.

In this sense, the conventional method was the most effective to identify hamburger samples that were really positive for *Salmonella* spp., while the Salmosyst method afforded to detect false negative results more successfully. As regard accuracy, both methods led to acceptable results for the detection of *Salmonella* spp. in hamburgers.

Discussion

In a similar study carried out to detect *Salmonella* spp. in turkey hamburgers sold in the city of Niterói, Brazil, Almeida Filho et al. (2006) analyzed 30 samples, of which 15 (30%) were contaminated. Similarly, Parra et al. (2002) observed that, among 27 hamburger samples collected in the city of Maracaibo, Venezuela, of which 18 were beef hamburgers and 9 were chicken burgers, 9 (33.33%) beef hamburgers were contaminated with the pathogen.

In turn, in a study by Leal et al. (2008) that analyzed 60 hamburger samples, of which 30 were home-made and 30 were industrialized products, *Salmonella* spp. was isolated in one (1.66%) home-made hamburger.

In a study that analyzed 81 hamburger samples stored at different temperatures (-15℃, 5℃ and 15℃) and for distinct periods of time (0 h, 48 h and 96 h), only one sample (1.23%) stored at 15℃ for 96 h presented contamination by *Salmonella* spp., showing that the result may be ascribed to a combined effect of storage temperature and time that allowed the microorganism to thrive, if present in a sample, concomitantly with a decrease in initial microbiota, since *Salmonella* spp. is a weak competitor.

Other studies conducted to analyze *Salmonella* spp. in hamburgers did not reveal the presence of the pathogen in this food. Tavares and Serafini (2003) carried out a microbiological investigation on the quality of 100 hamburgers sold in fast food trailers in the city of Goiânia, Brazil, detecting no *Salmonella* spp. The same result was reported by Bezerra et al. (2010) in a study that analyzed 105 hamburger samples sold in the streets of Cuiabá, Brazil, using the standard microbiological method.

Narváez et al. (2005) reported no *Salmonella* spp. in 56 packaged hamburgers ready to be sold sampled in a small-scale hamburger processing plant in the city of Maracaibo, Venezuela. However, the microorganism was observed in samples collected during the molding stage (66.0%), followed by the mincing and mixing stage (22.0%) and the stage when the meat is diced (11.0%).

Disagreeing with these results, Pignato et al. (1995) used the standard and the Salmosyst methods to analyze 519 food samples, of which 85 were poultry, 246 were red meat (beef, pork and horse) as well as 188 eggs. *Salmonella* spp. was detected in 39 (7.5%) and 47 (9.0%) samples using the standard and the Salmosyst methods, respectively. The sensitivity for the Salmosyst method was (97.9%), while for the standard method sensitivity was 81.2%. The authors believe that the Salmosyst method, in the analysis of foods, presents several advantages. First, only two media are required, one liquid medium (used in pre-enrichment as well as in selective enrichment), and a solid medium (used in selective isolation), compared to the five media required in the standard method. Apart from this, the total time for analysis is shortened, from four to six days, down to 48 h. In actual fact, the pre-enrichment in Salmosyst broth and selective enrichment take only 6 and 18 h, respectively, while an additional period of 24 h is needed to perform incubation of dishes in solid medium, for selective isolation.

Corroborating the results observed herein, Fierens and Huyghebaert (1996) reported 27 *Salmonella* spp. positive samples in 217 foods of animal and plant origin. The standard method detected the pathogen in 17 (81.0%) and the Salmosyst indicated the presence of *Salmonella* spp. in only 8 (38.1%) of the samples analyzed. The authors also state that, in the Salmosyst method, in which enrichment in a liquid medium is shortened, s analysis times were reduced by 24 h, compared to the standard method. However, the low recovery of *Salmonella* spp. may be due to several factors, like a short pre-enrichment stage (of 6 to 8 h),

enrichment in a low selectivity medium, or incubation at 37ºC.

For Balckburn (1993), shortening incubation times in pre-enrichment and enrichment to 6 to 8 h has led to results considered reasonably reliable, compared to the standard methods in which times are more conservatively observed. However, in some cases higher numbers of false negative results may be observed.

Conclusion

Of the hamburger samples analyzed, 22 (27.5%) were contaminated with *Salmonella* spp., and were thus considered inappropriate for human consumption and under inadequate sanitation conditions, as defined in Resolution Nº. 12, issued by the Board of the National Sanitation Authority (ANVISA), Ministry of Health (Brasil, 2001). Of the 22 positive samples, the pathogen was detected in 11 (50.0%) using the standard method, in 5 (22.73%) using the Salmosyst method, and in 5 (27.27%) in both methods concomitantly.

The standard method was the most effective to identify real positive results, while Salmosyst method afforded to characterize real negative results. As for accuracy, both methods were acceptable to analyze *Salmonella* spp. in hamburgers. However, no statistically significant differences were observed between results obtained using these methods.

DSA played an important role in the selective plating, due to the ease of identification of typical *Salmonella* spp. colonies that would then be streaked on TSI and LIA tubes (initial screening).

The importance of streaking *Salmonella* spp. colonies obtained using the TSI and LIA agars, with atypical characteristics 1 and 2 was also revealed, since these two sets of characteristics may be used to isolate atypical *Salmonella* spp. serotypes, which ferments lactose and *Salmonella* Parathyphi, which does not decarboxylate lysine, respectively.

Secondary screening was efficient as regards the differentiation between *Proteus* spp. and *Salmonella* spp. colonies, since it indicates the characteristic *Salmonella* spp. colonies that should undergo the seroagglutination test, that is, colonies negative for the urease test and the phenylalanine deamination test.

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