




Evaluation of a rapid detection method of *Salmonella* in comparison with the culture method and microbiological quality in fish from the Brazilian Amazon

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Abstract

Microbiological safety of fish is a concern of consumers, industries and regulatory agencies worldwide. Among the pathogenic microorganisms, *Salmonella* spp. is one of the main agents of foodborne diseases and should be absent in animal products. Rapid and accurate identification of pathogens in the supply chain is important for both quality assurance and tracking infectious agents within the chain. In this context, this study aimed to evaluate the equivalence of two rapid detection tests, as alternative methods to the conventional *Salmonella* detection method, as well as to verify the microbiological quality parameters of two commercially important fish species in the Amazon biome. The plate count of aerobic bacteria ranged from 7.76×10^4 to 8.71×10^7 CFU.g⁻¹ for mesophiles and 1.70×10^6 to 4.27×10^8 CFU.g⁻¹ for psychrotrophic whereas the maximum for this group of microorganisms in fresh fish is 10^6 CFU.g⁻¹. Regarding the *Staphylococcus* count, the two species presented variations between 1.35×10^4 to 1.51×10^5 CFU.g⁻¹. This represents unsatisfactory conditions of handling, storage and conservation of fish species. The immunoenzymatic and molecular methods have been shown to be reliable, fast and effective in the detection of *Salmonella* and for its high index of agreement with the conventional detection method. We also emphasize the convenience of multiplex PCR application due to the high sensitivity, specificity, speed and accuracy of *Salmonella* detection.

Keywords: *Brachyplatystoma filamentosum*; *Ilisha amazonica*; mPCR; Tecra *Salmonella*.

Practical Application: Identify the presence of salmonella through fast methods and molecular identification techniques.

1 Introduction

Microbiological safety of fish is a concern of consumers, industries and regulatory agencies around the world nowadays. The rapid and accurate identification of pathogens in the supply chain is important both for quality assurance and for tracking infectious agents within the chain (Välilä et al., 2015; Sebastião, et al., 2015; Buncic, et al., 2019). According to data from regulatory agencies and health inspection agencies worldwide *Salmonella* stands out among the pathogens, as the main bacterial agent, responsible for transmit diseases associated with food consumption nowadays (Centers for Disease Control and Prevention, 2016; European Food Safety Authority, 2018). In Europe, authorities point to *Salmonella* as the second most important agent in foodborne disease transmission, with over 91,857 cases of salmonellosis (European Food Safety Authority, 2018; Trimoulinard et al., 2017). In the United States, the incidence of *Salmonella* represents 15.89% of the cases associated with diseases transmitted by food consumption (Centers for Disease Control and Prevention, 2016). In Brazil, *Salmonella* is responsible for more than 30% of foodborne diseases, according to the Brazilian Ministry of Health (Brasil, 2017).

Although *Salmonella* is not a biologically natural pathogen of fish, it can be introduced to the fish chain through improper handling and hygiene in processing or contact with contaminated water by discharging sewage effluent into fishing basins. *Salmonella* are facultative intracellular parasites that invade the mucous membrane with the human and animal intestinal tract being the main reservoir of this pathogen (Álvarez-Ordóñez et al., 2012; Ertas Onmaz et al., 2015; Nhung et al., 2018). The conventional method used for the detection of *Salmonella* in food is based on pre-enrichment in buffered peptone water (BPW) and enrichment in selective medium, followed by differential isolation and serological confirmation (Suo & Wang, 2014; Lee, et al., 2015; Li, et al., 2017). The main limitation of this method is that these tests usually require a minimum of 5 to 6 days. However, infection control increasingly depends on faster and more accurate tests for the diagnosis of this pathogen, especially for monitoring the production of animal products, food manufacturing and end products (Andrade et al., 2010; Frickmann, et al., 2013; Gokduman et al., 2016; Lobato & O'Sullivan, 2018). In addition, there is a strong industrial demand to comply with legislation and

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the rapid release of food products to the market. Recent advances in technologies for the detection and identification of contaminated foodborne pathogens have provided faster, more sensitive and specific alternatives to conventional methods (Paula, et al. 2002; Kawasaki, et al., 2009; Koo, et al., 2016; Suo, et al., 2017). These assays are generally referred as “fast” or alternative methods, terms commonly used to describe a variety of assays including miniaturized biochemical kits, immunoassays, DNA/RNA-based assays, and combinations with cultural methods (Lofstrom et al., 2010; Almeida et al., 2013; Kim, et al., 2017). Molecular testing uses a specific sequence of bacterial nucleic acids as a target for pathogen detection and this is the category of alternative methods that most improved in recent years (Valderrama et al., 2016; Hu, et al., 2018; Bundidamorn, et al., 2018). The PCR assay, Polymerase Chain Reaction, is the molecular test that has also been widely used to detect *Salmonella* in food. It is a highly sensitive technique based on enzymatic amplification of specific segments of DNA in vitro, which enables to obtain thousands of copies from a single nucleic acid sequence, within two or three hours (Shabarinath et al., 2007; Singh et al., 2019).

Another rapid assay that has been extensively studied in the analysis for pathogen detection is *multiplex* PCR. This technique involves more than one pair of oligonucleotides and allows, among other applications, to perform a single reaction to detect various types of microorganism, their serotypes or different genes of the same microorganism (Malorny et al., 2009; Wang, et al., 2015; Chin et al., 2017).

In this context, the objective of this study was to evaluate the equivalence of two rapid detection tests as alternative methods to the conventional method of bacteriological detection of *Salmonella* in two fish species with commercial importance and large occurrence in the Amazon biome and associated with this verify the parameters of microbiological qualities of the two species of fish.

2 Materials and methods

2.1 Samples and procedure for isolation and identification

The fish samples included in this study were *Brachyplatystoma filamentosum* and *Ilisha amazonica* species. These two species are of large occurrence in the Amazon biome and commercially important for their high consumption. The total of 10 collections of fish samples were performed in the period from January to June 2018, at the main North fish landing port in Brazil (Ver-o-Peso market, 01°27'21”S and 48°30'14”W). Four batches of fish (without evisceration) of each species, which constituted separate samples, were placed in sterile polyethylene bags, stored under refrigeration (5 °C) and transported to the laboratory of the Federal University of Pará-UFPa, where the analyzes were initiated immediately upon arrival.

2.2 Sample preparation

For the preparation of the material to be submitted to microbiological analysis and isolation of *Salmonella* from fish samples, the protocol described in the Compendium of Methods for Microbiological Examination of Foods (American

Public Health Association, 2001) was followed. Briefly, 25 g of the samples were homogenized with 225 mL buffered peptone water (Oxoid CM509) using a *stomacher* mixer (Logen Scientific, LS1901n, ALPAX) at 2000 rpm for 2 min. From the initial suspension, decimal dilutions were prepared by transferring 1 mL from the previous dilution to 9 mL of buffered peptone water and so on until reaching the desired dilution. From the decimal dilutions obtained, analyzes of total and thermotolerant coliforms, *Staphylococcus aureus* and total and psychrotrophic aerobic bacteria count were performed as follows.

2.3 Microbiological analysis

The presumptive coliforms test was performed using the CC Petrifilm™ commercial kit (3M Company, St. Paul, MN, USA), where 1 mL of the dilution, obtained according to item 2.2, was inoculated into the kit plates, according to manufacturer's instructions. The plates were incubated at 35 °C for 48 h, and the result was expressed in MPN of total coliforms per gram of sample. For Thermotolerant Coliforms, 1 mL aliquots of three dilutions of the sample obtained according to item 2.2 were inoculated into a series of 3 tubes containing 10 mL of Tryptose Lauryl Sulfate Broth - LST (ACUMEDIA 7142) and incubated at 35 °C. After 48 h, 1 mL aliquots of each positive tube of LST were transferred to tubes containing 10 mL of *Escherichia coli* broth - EC (Acumedia 7206) and incubated at 45 °C for 24 h in a water bath, and the result was expressed in MPN of thermotolerant coliforms per gram of sample. For quantification of *Staphylococcus aureus*, the commercial Petrifilm™ Staph express Cout Plate kit (3M Company, St. Paul, MN, USA) was used, where 1 mL of the sample dilution obtained according to item 2.2 was inoculated into kit plates, and the plates were incubated at 36 °C for 24 h according to the manufacturer's instructions. After the plates incubation period, typical *Staphylococcus* colonies were counted and the result expressed in CFU.g⁻¹. In the mesophilic and psychrotrophic aerobic bacterial count assays, the “*pour plate*” technique with standard agar (PCA) was performed. The plates were incubated at 35 °C for 48 h (mesophilic) and 7 °C for 10 days (psychrotrophic). The result of the count was expressed in CFU.g⁻¹.

2.4 Salmonella spp. detection

In *Salmonella* detection were applied three protocols, which are described briefly as follows:

Traditional method

By the traditional method, the procedures were performed according to the methodology described in ISO 6579 (American Public Health Association, 2001), where aliquots of the sample prepared according to item 2.2 were incubated at 37 °C for 24 h. From the BPW pre-enriched culture, 0.1 mL was transferred to tubes containing 10 mL of Rappaport-Vassiliadis Soya (RVS) Broth. RVS broth was incubated at 42 °C for 24 h. From the selective enrichment broth, streaks were performed on plates with *Salmonella Shigella* (SS) Agar and Xylose-Lysine-Deoxycholate (XLD) Agar, and incubated at 35 °C for 24 h. Plates showing typical colony growth were collected for biochemical and serological

identification to confirm the presence of *Salmonella*, according to the methodology described in the Compendium of Methods for Microbiological Examination of Foods (American Public Health Association, 2001).

Fast detection immunoenzymatic method

In this type of rapid detection assay, the investigated pathogens, if present in the test product, are captured by specific antibodies adsorbed on the surface of a solid matrix. In this study, the immunoenzymatic assay was performed using the Tecra *Salmonella* kit (3M Company, St. Paul, MN, USA), which briefly, 1 mL of pre-enriched mixture in RVS broth was transferred to tubes containing 10 mL of M-broth and incubated at 35 °C for 8 h. Subsequently, a 1 mL aliquot of M-broth was heated in a water bath (~ 100 °C) for 15 min. After cooling, 200 µL aliquots of M-broth were placed in kit's individual wells following the manufacturer's procedures protocol for detection of salmonella in the product. Samples showing positive detection for *Salmonella* were submitted to biochemical and serological identification to confirm the presence of *Salmonella* according to the protocol described in the Compendium of Methods for Microbiological Examination of Foods (American Public Health Association, 2001).

Rapid detection mPCR molecular method

The multiplex Polymerase Chain Reaction (mPCR) assay is one of the most widely used molecular tests for *Salmonella* detection and its fundamental feature is the enzymatic amplification of specific segments of DNA in vitro, which enables to obtain thousands of copies from a single sequence of nucleic acid in a short time. In preparing samples for the mPCR assay, DNA extraction was performed using the protocol described by Darwish et al. (2009), with some modifications. A 2 mL aliquot of the sample prepared according to item 2.2 and reactivated in Brain Heart Infusion (BHI) broth at 37 °C for 18 h was transferred to *Eppendorf* and centrifuged at $13,200.0 \times g$ for 5 min. The supernatant was discarded and the pellet formed resuspended in 600 µL of Lysis buffer T1 (Kasvi, Brazil) and 10 µL of proteinase K (20 mg/mL) were then added to the suspension. After incubation in a 56 °C water bath for 6 h the samples were centrifuged at $10,000 \times g$ for 10 min. To optimize the separation and precipitation of protein was added 700 µL of phenol-chloroform (1:1), cooled to 5 °C for 5 min, and a further centrifugation was performed at $14,000 \times g$ for 20 min. An aliquot of 400 µL of the supernatant was transferred to a new flask containing 600 µL of isopropanol, then centrifuged at $14,000 \times g$ for 1 min. The supernatant was completely discarded and 600 µL of 70% ethanol was added and centrifuged at $14,000 \times g$ for 1 min. The supernatant was discarded again and the flasks were oven dried at 37° C for 30 min. DNA samples were hydrated with 100 µL TE buffer solution (10 mM Tris-HCl, 1 mM EDTA) and stored at -18° C until use in mPCR analysis. For the target gene, the following *primers* were used: prime Forward: 5' TATCGCCACGTTTCGGGCAA 3' and Reverse: 5' TCGCACCGTCAAAGGAACC 3' amplifying 275bp sequences, *prime* Forward: 5' CGGTGTTGCCAGGTTGGTAAT 3' and Reverse: 5' ACTGGTAAAGATGGCT 3', which amplify 620bp

sequences and *prime* Forward: 5' AGATTGGGCACTACACGTGT '3 and Reverse: 5' TGTACTCCACCAGGTAATTG 3', which amplify 535bp sequences. These *primers* were previously configured based on studies described in the literature (Wang et al., 1997; Aabo et al., 1993; Soumet et al., 1999). *Primers* were synthesized by Ludwig Biotec® and prepared according to the manufacturer's instructions and diluted in sterile ultrapure water and TE 8.0 pH buffer to 100 pmol µL⁻¹. Amplifications were performed using a thermal cycler (Applied Biosystems VERITI 96) programmed for initial denaturation at 94 °C for 30 s, followed by 35 cycles with denaturation for 30 s, annealing for 1 min at 55 °C and extension for 30 s at 72 °C, followed by a final extension at 72 °C for 7 min. Finally, the samples were kept at 4 °C. The PCR products were separated by 1.5% agarose gel electrophoresis, stained with Safer dye (KASVI) and visualized by transillumination (Gel Documentation System, Gel Doc™ XR+, Bio-Rad) with the aid of Image Lab Software Version 5.2.1.

DNA sequencing

The sequencing was conducted as described by Malorny et al. (2009). Briefly, the sequencing was performed using the AB 3500 Genetic Analyzer automated sequencer armed with 50 cm capillaries and POP-7 polymer (Applied Biosystems). DNA templates, previously selected, were labeled using 2.5 pmol. The resulting sequences after editing with Chromas Lite® and Bioedit software were then submitted to Genbank (National Center for Biotechnology Information, 2016) where they could be compared by similarity to the other sequences already deposited, using BLAST online software. This analysis procedure provided the gene fragment identification as belonging to a particular genus or species, considering the similarity percentage above 98%.

2.5 Statistical analysis

Analyses were performed in triplicate (n = 3). Tests of the significance of the results were performed using an one-way analysis of variance (ANOVA) using the Microsoft Office Excel for Windows 7.0.

3 Results and discussion

3.1 Microbiological quality

The microbiological evaluation results of *in natura* *Brachyplatystoma filamentosum* and *Ilisha amazonica* species, related to hygiene indicator microorganisms or processing, fecal contamination, handling and enteric waterborne pathogens are shown in Table 1. The aerobic bacteria plate count, used as indicator of hygiene and temperature control during processing for *Brachyplatystoma filamentosum* species, showed variations between 8×10^4 to 1.1×10^6 CFU.g⁻¹ for mesophiles and 7.4×10^7 to 1.3×10^7 CFU.g⁻¹ for psychrotrophic. It was verified by one-way ANOVA, that the statistical analysis globally indicated, for the lots of *Brachyplatystoma filamentosum* samples throughout the six months of collection, the existence of significant fact ($p < 0.05$), linked to the count of mesophilic and psychrotrophic aerobic bacteria. It is also observed (Table 1) that in the group of mesophilic bacteria, from the total collections performed

Table 1. Microbiological quality of *Brachyplatystoma filamentosum* and *Ilisha amazonica* species.

| Brachyplatystoma filamentosu | Samples | | | | | | | | | |
|---|------------------------------|------------------------------|------------------------------|------------------------------|-------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Mesophiles (UFC.g ⁻¹) | 1.32 x 10 ⁷ ± 2.5 | 7.94 x 10 ⁶ ± 5.1 | 4.47 x 10 ⁵ ± 3.1 | 7.76 x 10 ⁴ ± 1.2 | 1.10 x 10 ⁶ ± 1.5 | 5.37 x 10 ⁵ ± 6.0 | 2.34 x 10 ⁵ ± 3.2 | 5.8 x 10 ⁵ ± 3.1 | 4.36 x 10 ⁵ ± 6.3 | 1.1 x 10 ⁶ ± 2.1 |
| Psychrotrophic (UFC.g ⁻¹) | 1.95 x 10 ⁷ ± 1.4 | 6.17 x 10 ⁷ ± 1.1 | 1.0 x 10 ⁷ ± 1.1 | 2.13 x 10 ⁶ ± 3.4 | 4.07 x 10 ⁶ ± 16.6 | 4.89 x 10 ⁶ ± 3.4 | 5.88 x 10 ⁷ ± 2.8 | 1.2 x 10 ⁷ ± 15.1 | 1.62 x 10 ⁷ ± 3.3 | 4.17 x 10 ⁶ ± 1.5 |
| Coliforms at 35 °C (NMP.g ⁻¹) | 4.15 ± 0.54 | 4.47 ± 0.6 | 5.40 ± 0.11 | 5.41 ± 0.13 | 3.21 ± 0.24 | 3.25 ± 0.15 | 4.13 ± 1.39 | 5.40 ± 0.10 | 4.05 ± 0.67 | 4.89 ± 0.13 |
| Coliforms at 45 °C (NMP.g ⁻¹) | 4.48 ± 0.12 | 2.50 ± 0.19 | 4.38 ± 0.10 | 4.47 ± 0.21 | 4.67 ± 0.11 | 3.56 ± 0.31 | 3.84 ± 0.90 | 2.80 ± 0.24 | 2.36 ± 0.68 | 3.08 ± 0.12 |
| Staphylococcus aureus (UFC.g ⁻¹) | 2.24 x 10 ⁴ ± 1.0 | 2.51 x 10 ⁴ ± 1.3 | 3.16 x 10 ⁴ ± 1.3 | 2.08 x 10 ⁴ ± 1.3 | 2.51 x 10 ⁴ ± 1.2 | 2.45 x 10 ⁴ ± 2.6 | 9.12 x 10 ⁴ ± 2.6 | 1.35 x 10 ⁴ ± 6.0 | 2.51 x 10 ⁴ ± 1.3 | 2.19 x 10 ⁴ ± 1.3 |
| Salmonella (C.M.) | - | - | - | - | - | - | - | - | - | - |
| Salmonella (I.M) | - | - | - | - | - | - | - | - | - | - |
| <i>Ilisha amazônica</i> | | | | | | | | | | |
| Mesophiles (UFC.g ⁻¹) | 8.71 x 10 ⁷ ± 4.5 | 3.15 x 10 ⁵ ± 3.9 | 3.71 x 10 ⁵ ± 1.3 | 2.81 x 10 ⁵ ± 1.3 | 5.01 x 10 ⁶ ± 9.8 | 1.51 x 10 ⁶ ± 1.1 | 1.2 x 10 ⁵ ± 3.8 | 4.0 x 10 ⁵ ± 4.1 | 2.51 x 10 ⁸ ± 1.1 | 6.45 x 10 ⁴ ± 1.7 |
| Psychrotrophic (UFC.g ⁻¹) | 4.26 x 10 ⁸ ± 4.4 | 5.62 x 10 ⁷ ± 1.3 | 8.12 x 10 ⁶ ± 1.7 | 1.0 x 10 ⁷ ± 1.3 | 2.69 x 10 ⁷ ± 1.2 | 3.98 x 10 ⁷ ± 3.4 | 6.31 x 10 ⁶ ± 1.1 | 8.91 x 10 ⁶ ± 1.2 | 2.45 x 10 ⁷ ± 1.4 | 1.69 x 10 ⁶ ± 2.1 |
| Coliforms at 35 °C (NMP.g ⁻¹) | 2.65 ± 0.33 | 5.24 ± 0.12 | 5.62 ± 0.13 | 4.05 ± 0.28 | 5.10 ± 0.11 | 4.22 ± 0.54 | 3.94 ± 0.66 | 5.40 ± 0.15 | 5.00 ± 0.20 | 4.81 ± 0.11 |
| Coliforms at 45 °C (NMP.g ⁻¹) | 4.81 ± 0.56 | 2.56 ± 0.19 | 4.48 ± 0.31 | 4.32 ± 0.16 | 4.48 ± 0.21 | 3.23 ± 0.30 | 4.51 ± 0.30 | 2.16 ± 0.28 | 2.67 ± 0.04 | 3.63 ± 0.61 |
| Staphylococcus aureus (UFC.g ⁻¹) | 2.51 x 10 ⁴ ± 1.3 | 8.31 x 10 ⁴ ± 1.3 | 2.39 x 10 ⁴ ± 1.2 | 2.51 x 10 ⁴ ± 1.7 | 2.63 x 10 ⁴ ± 1.2 | 4.6 x 10 ⁴ ± 2.4 | 3.63 x 10 ⁴ ± 2.0 | 1.65 x 10 ⁴ ± 1.8 | 5.24 x 10 ⁴ ± 1.7 | 1.51 x 10 ⁵ ± 1.5 |
| Salmonella (C.M.) | - | - | - | - | - | - | - | - | - | - |
| Salmonella (I.M) | - | - | - | - | - | - | - | - | - | - |

C.M. (Conventional Method); I.M. (Immunoenzymatic Method); Positive result (+); Negative result (-).

in this study for *Brachyplatystoma filamentosum* species, 40% exceeded the maximum limit of 10⁶ CFU.g⁻¹ established by the International Commission on Microbiological Specifications for Foods standards (International Commission on Microbiological Specifications for Foods, 2002). However, regarding the group of psychrotrophic bacteria, 80% of the samples collected for *Brachyplatystoma filamentosum* species exceeded the limit of 10⁶ CFU.g⁻¹. The high count of these bacteria in fish represents unsatisfactory storage and conservation conditions of the product. Thus, the proper handling and storage of *Brachyplatystoma filamentosum* becomes essential, as psychrotrophic bacteria are endowed with proteolytic and lipolytic characteristics that may continue their multiplication process, which may affect the durability of fish even stored in favorable temperatures. Regarding the *Ilisha amazonica* species, the count of mesophilic and psychrotrophic aerobic bacteria showed variations between 1.0x10⁶ to 7.8x10⁵ CFU.g⁻¹ and 1.10 x 10⁷ to 8.9 x 10⁶ CFU.g⁻¹ respectively. Statistical analysis also showed that the aerobic bacteria counts showed significant variations (p < 0.05) over the six months of collection. It was also verified (Table 1) that the species *Ilisha amazonica* presented the same nonconformities as *Brachyplatystoma filamentosum*, since 60% of the samples collected exceeded the limit of 10⁶ CFU.g⁻¹ for the group of psychrotrophic bacteria. The results related to the total and thermotolerant coliforms group of *Brachyplatystoma filamentosum* showed variations between 1.7 x 10³ to 8.5 x 10⁴ MPN.g⁻¹ and 1.2 x 10³ to 3.0 x 10⁴ MPN.g⁻¹ respectively. While *Ilisha amazonica* presented total coliforms between 1.0x10⁵ to 8.0 x 10⁴ MPN.g⁻¹

and thermotolerant coliform variations between 1.0 x 10⁵ to 1.3 x 10⁴ MPN.g⁻¹. Total and thermotolerant coliform counts in fresh fish can be considered as indicator of quality, because the presence of this group is commonly associated with pathogenic bacteria, being considered as risk to consumer health. The International Commission on Microbiological Specifications for Foods (2002) establishes for this group of microorganisms the maximum limit of 10³ MPN.g⁻¹ in fresh fish. In the present study, the bacteriological results (Table 1) related to the group of total and thermotolerant coliforms, for the two fish species evaluated, evidence inadequate hygiene and sanitation practices, according to the standards required for processing this food. Statistical analysis also indicates that, globally, for both *Brachyplatystoma filamentosum* and *Ilisha amazonica* species the bacteriological counts related to the total and thermotolerant coliforms group showed significant variations (p < 0.05) over the collection period. Regarding the *Staphylococcus* count, *Brachyplatystoma filamentosum* showed variations between >1.0 x 10¹ to 2.5 x 10⁴ CFU.g⁻¹, while the *Ilisha amazonica* species ranged from >1.0 x 10¹ to 5.6 x 10⁴ CFU.g⁻¹. The International Commission on Microbiological Specifications for Foods (2002) establishes for this group of microorganisms the maximum limit of 10³ CFU.g⁻¹ in fresh fish. In the present study 40% of the samples collected related to the two fish species evaluated exceeded the maximum limit established. Although *Staphylococcus* is not part of neither evaluated fish species microbiota, the presence of this group of microorganisms in the samples is likely to result from cross-contamination, as these bacteria are common to human

skin, nasal cavity and oral cavity, being used as indicators of hygienic character deficiencies in the food obtaining process and in the operations of manipulation. Statistical analysis also indicates that globally, for both *Brachyplatystoma filamentosum* and *Ilisha amazonica* species, bacteriological counts related to *Staphylococcus* showed no significant variations ($p > 0.05$) over the collection period.

3.2 *Salmonella* spp. detection

The results in Table 1 show the incidence of *Salmonella* in various samples for both *Brachyplatystoma filamentosum* and *Ilisha amazonica* species detected by conventional method and confirmed by the immunoenzymatic method performed with Tecra commercial kit. Considering the positive confirmed results and negative results together, it was found that of the 40 samples analyzed by the conventional method over the 10 collections, 30% of the samples of *Brachyplatystoma filamentosum* presented *Salmonella* contamination. For *Ilisha amazonica*, the contamination was 50% of the collected samples. The total number of *Salmonella* positive samples was 8. In this study, the agreement rate between the conventional *Salmonella* detection method and the detection by the immunoenzymatic method performed with the commercial Tecra kit was 92.5% for samples of specie *Brachyplatystoma filamentosum* and 95.0% for samples of specie *Ilisha amazonica* (Table 1). Taking this result as a reference point, we emphasize the convenience of applying this method for *Salmonella* detection due to its simplicity of execution and rapid availability of the screening result, so the immunoenzymatic method applicability performed by the Tecra commercial kit may satisfy the need for rapid diagnosis of the industry and regulatory agencies with excellent accuracy.

For the genetic identification by molecular method of serotypes isolated from *Brachyplatystoma filamentosum* and *Ilisha amazonica* species using the conventional *Salmonella*

detection method, the multiplex PCR method was applied. This method makes possible to confirm the genus and to detect the species simultaneously by combining several specific primers for target genes, as two or more DNA fragments are amplified in the same reaction. Figure 1 shows the result of multiplex PCR agarose gel where it is possible to observe two bands corresponding to fragments 239, 527 and 578 bp, confirming positive contamination of fish by *Salmonella* and pointing out the existence of 2 different serotypes. After the subsequent alignment of the previously selected primers and BLAST evaluation with the GenBank genomic database, the result achieved in this test was satisfactory to obtain a multiplex PCR capable of detecting *Salmonella* spp. and differentiate between *Salmonella typhimurium* (Figure 1A) and *Salmonella enteritidis* (Figure 1B) subspecies. The amplification of the target genes occurred as expected: pure culture *Salmonella* samples showed bands corresponding to the 239 bp fragment with 98% of identification and coverage for the CP018219.1 gene available on the GenBanck data platform, and *Salmonella typhimurium* presented the bands corresponding to the 578 bp fragment with 98% of identification and 99% of coverage for the CP024619.1 gene available on the GenBanck data platform. *Salmonella enteritidis* exhibited bands corresponding to the 527 bp fragment showing 98% of identification and coverage for the CP032851.1 gene available on the GenBanck data platform. In the *Brachyplatystoma filamentosum* species the serotype identified was only *Salmonella Enteritidis* whereas in the species *Ilisha amazonica* both *Salmonella Enteritidis* and *Salmonella typhimurium* serotypes were detected. Taking this result as a reference point, we emphasize the convenience of applying multiplex PCR developed in this study, demonstrated by its high sensitivity, specificity and accuracy, besides showing strong agreement with the reference method for the detection of *Salmonella*. It is important to highlight that *Salmonella Enteritidis* and *Salmonella typhimurium* are common serotypes

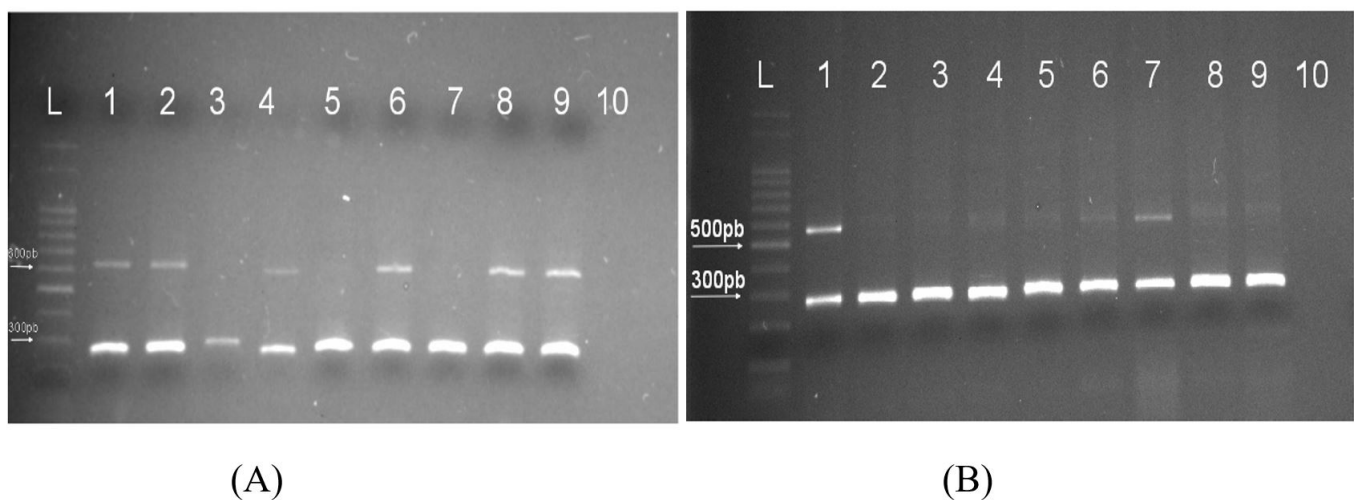


Figure 1. (A) 1.5% agarose gel electrophoresis demonstrating the presence of DNA fragment genus *Salmonella* spp and serovar typhimurium. Where, L: molecular weight marker (100bp), 1: positive control of *Salmonella* spp., And typhimurium, 2 to 9: strains isolated from fish samples, 10: negative control; (B) 1.5% agarose gel electrophoresis demonstrating the presence of *Salmonella* spp DNA fragment and serovar enteritidis. Where, L: molecular weight marker (100bp), 1: positive control of *Salmonella* spp., And enteritidis, 2 to 9: strains isolated from fish samples, 10: negative control.

in contaminated estuaries and coastal waters, and can be found in fish and shellfish from tropical waters having multiresistant strains for disinfectants. In addition, they have a reasonable ability to form biofilm providing them a certain factor of permanence in the environment, which is crucial in the contamination of equipment and accessories used in fish processing.

4 Conclusion

The results obtained from the microbiological evaluation of *Brachyplatystoma filamentosum* and *Ilisha amazonica in natura* species show that some hygienic conditions along the fishing chain were not adequate due to the high count values of microorganisms not belonging to the normal flora of these two fish species. Therefore, it is recommended to apply improved hygienic practices in fish handling and processing until it arrives at the selling point in order to provide a product with quality and health safety. The immunoenzymatic and molecular methods have been shown to be reliable, fast and effective in the detection of *Salmonella* and its high level of agreement with the conventional method of detection of this pathogen. The study also reveals that the multiplex PCR protocol applied was able to detect *Salmonella Enteritidis* and *Salmonella typhimurium* serotypes simultaneously and could serve as an alternative in the quality control laboratory routine of fish industry and regulatory agencies in the diagnosis of foodborne pathogenic bacteria such as *Salmonella*.

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