Full Length Research Paper

Identification and detection of *Salmonella* strains isolated from chicken carcasses and environmental sources in Dourados, MS, Brazil

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The application of methodologies that can rapidly and precisely identify *Salmonella* in both foods and water samples is of great interest to public health. *Salmonella* sp. was isolated from chicken carcasses and water samples from aquaculture tanks using both traditional culture methods and bacteriological analyses. DNAs from these isolates were extracted and Multiplex Polymerase Chain Reactions (mPCR) were performed using primers for *invA*, *fliC* and *sefA* genes to detect the genus *Salmonella* and to differentiate the serovars Typhimurium and Enteritidis respectively. The results of these bacteriological analyses indicated that 94% of the chicken carcass samples were contaminated by 46 *Salmonella* sp. strains. mPCR analyses indicated that 43 of these strains belonged to the genus *Salmonella*. Among these isolates, 32% were genotyped as *Salmonella enteritidis* while none were identified as *Salmonella typhimurium*. Bacteriological analyses of the aquaculture tank water samples indicated that 73% were contaminated, and 11 *Salmonella* sp. strains were isolated; mPCR analyses indicated that all of them belonged to the genus *Salmonella*, but no *S. enteritidis* and *S. typhimurium* serovars were identified. Multiplex PCR was found to be a very sensitive test that allowed rapid and reliable identification of these bacteria.

Key words: *Salmonella enteritidis*, *Salmonella typhimurium*, bacteriological analyses, multiplex polymerase chain reactions, aquaculture.

INTRODUCTION

Salmonellosis has become a serious problem in many countries in recent decades. *Salmonella* is among the main causal agents of infectious diseases passed by contaminated foods and water (Malorny et al., 2003) and these illnesses have high annual costs to public health throughout the world. The economic cost of salmonellosis in the United States alone was more than $2.6 billion dollars in 2009. According to Health Ministry of Brazil data, there were 6971 reported cases of food-transmitted illnesses between 1999 and 2010, and *Salmonella* sp.

was the contaminating agent in 46% of those cases. The global increase in chicken consumption stimulated by its high protein content (similar to beef) and its accessible price has drawn the attention of producers, researchers and authorities to the necessity of controlling *Salmonella* contamination, principally during the various stages of commercial production chains. In light of that fact that chicken meat is almost exclusively sold while frozen, it will be important to assess its involvement in the spread of *Salmonella*. The capacity of *Salmonella* to survive

outside its host for relatively long periods of time adds an important dimension to the epidemiology of salmonellosis. *Salmonella* present in the natural environment represents a serious risk to animal production and public health, making constant monitoring of upmost importance (Cavada et al., 2010). In addition to its ingestion in contaminated foods, salmonellosis can also be spread by contaminated water (Kozlica et al., 2010). While *Salmonella* has only rarely been reported in aquaculture tanks, more detailed investigations and monitoring are needed due to significant global increases in fish production and consumption.

The genus *Salmonella* comprises 2579 serovars (Grimont and Weill, 2007). The serovars most frequently isolated in humans (Soumet et al., 1999) and in outbreaks of illnesses transmitted by foods (Herikstad et al., 2002) are *Salmonella enteritidis* and *Salmonella typhimurium*. Isolates of *S. enteritidis* have shown expressive increases in frequency in every part of the world since the 1980s, and more recent studies have shown *S. enteritidis* to be the most prevalent global serovar (Amini et al., 2010; Hassanein et al., 2011). Starting in the 1990s, samples of avian origin in Brazil began to show significant increases in *S. enteritidis* and *S. enteritidis* isolates in *Salmonella* samples of human origin sent to the Adolfo Lutz Institute in São Paulo increased from 1.2 to 64.9% between 1991 and 1995 (Tavechio et al., 1996). The identification of the genus *Salmonella* using conventional bacteriological analyses is laborious and time consuming and not compatible with routine processing of large numbers of samples (Mainali et al., 2011). Serovar confirmations using traditional methodologies must be performed in reference laboratories and in a country as large as Brazil only the Oswaldo Cruz Institute can perform these analyses, causing significant research delays. Consequently, the introduction of methodologies that could securely identify the principal serovars of *Salmonella* would be very important for rapid diagnoses (Silva et al., 2011).

Polymerase Chain Reaction (PCR) is a simple, rapid, very specific, reliable, and relatively inexpensive technique (Rampersad et al., 2008) that has been widely used in detecting *Salmonella* sp. and in differentiating serovars of greatest importance to public health. Most of the virulence factors in *Salmonella* are bar-coded in genes grouped in various genetically stable *Salmonella* Pathogenity Islands (SPI) in the bacterial chromosome. SPI1 is present in all of the phylogenetic lines of the genus *Salmonella* but absent in other bacteria (such as *E. coli*). SPI1 contains genes (among them *invA*) that code for proteins necessary for pathogen entry into the epithelial cells lining the host's intestines (Galán, 1996). The *sefA* gene (*fimbrial antigen* of *S. enteritidis*), specific for the detection of the Enteritidis serovar, was identified in all of the strains of *S. enteritidis* isolated from birds, pigs, humans and cattle, but in no other serovar tested (Murugkar et al., 2003). The *fliC* gene (a flagellar gene of

Salmonella) is responsible for the expression of a flagellar protein specific to *S. typhimurium* (Soumet et al., 1999).

The development of tests that would allow efficient and rapid confirmations of water or food contamination by *Salmonella* would be very important to health officials and the food industry in Mato Grosso do Sul State, Brazil. The present study analyzed frozen chicken carcasses and water samples from aquaculture tanks in Dourados-MS for *Salmonella* using both standard bacteriological analyses and mPCR as an alternative method to test for the presence of the genus *Salmonella* and to specifically identify Enteritidis and Typhimurium serovars.

MATERIALS AND METHODS

Obtaining samples

Analyses were performed on 36 samples of chicken carcasses available at local market in Dourados, Mato Grosso do Sul state (MS), Brazil. We selected chickens randomly that had been obtained from abattoirs in MS state. The material was transported under refrigeration to the microbiology laboratory at UFGD for processing and analyses. Water analyses were performed using 15 samples taken from aquaculture tanks. The water samples were stored in sterile glass flasks and transported under refrigeration to the microbiology laboratory for subsequent analyses.

Microbiological analyses of the food and water samples

According to the methodology recommended by APHA (2001), 25 g of different parts of the chicken carcasses were homogenized in 225 ml of sterile buffered peptone (Oxoid). The pond water samples were processed by homogenizing 10 ml of water with 90 ml of sterile buffered peptone (Oxoid). The samples (water or chicken) were incubated for 24 h at 37°C (pre-enrichment), and 1 ml of this pre-enrichment liquid was transferred to 10 ml of Selenite Cystine broth (SC - Acumedia) and 0.1 to 10 ml Rappaport Vassiliadis broth (RV - Isofar) (selective enrichment). The broths were incubated at 37°C for 24 h (SC) or at 42°C for 48 h (RV). A sterilized platinum loop was used to transfer samples from each of the two selective enrichment broths to petri dishes containing Hektoen Enteric agar (Merck) that were incubated at 37°C for 24 h. The colonies with characteristics of *Salmonella* sp. (1 to 2 mm in diameter, with darkened centers and a colorless halo) were submitted a biochemical tests using the Triple Sugar Iron agar (TSI - Merck) and Motility Indole Lysine Medium (MIL – Merck). The isolates with biochemical profiles typical of *Salmonella* were submitted to serum agglutination reactions on slides using polyvalent *Salmonella* serum (Probac) to confirm the diagnosis.

Results were verified by conventional serotyping methods using Probac on basis of the bacterial cell wall antigenic components according to the Kauffman-White scheme. Subsequent confirmations of the genus and detection of the Enteritidis and Typhimurium serovars of these isolates were performed using multiple PCR.

Positive and negative control strains

The positive (*S. enteritidis* and *S. typhimurium*) and negative (*E. coli*, *Proteus mirabilis* and *Citrobacter* sp.) control strains were obtained from the Food Microbiology Laboratory of the Department

Table 1. Microorganisms, target genes, sequences and sizes of the amplified fragments.

	Bacteria	Target gene	Primer sequence 5' - 3'	Fragment size (bp)	Reference
Genus	*Salmonella* sp.	*invA*	F – GTGAAATTATCGCCACGTTCGG R – TCATCGCACCGTCAAAGG	284	Rahn et al. (1992)
S. enterica serovar	Typhimurium	*fliC*	F – CGGTGTTGCCCAGGTTGGTAAT R - ACTGGTAAAGATGGCT	620	Soumet et al. (1997)
	Enteritidis	*sefA*	F – GCCGTACACGAGCTTATAGA R - ACCTACAGGGGCACAATAAC	250	Soumet et al. (1997)

of Clinical and Biomedical Analyses at the State University of Maringá (UEM) at Maringá/PR.

DNA genomic extraction

Bacterial isolates from the chicken carcasses and the pond water that had been identified as the genus *Salmonella* using the serum agglutination reaction, as well as positive and negative control strains that had been held on nutrient agar, were reactivated in buffered peptone water at 37°C for 24 h. The samples were diluted in sterile saline solution (85%) in the proportions of 9:1 (9 ml of the saline solution and 1 ml of the buffered peptone water containing the bacteria). The samples diluted in saline solution were submitted to DNA extraction using chloroform, as adapted from Wilson et al. (1992). 300 µL aliquots of each solution were placed in 2 mL microtubes and 2.5 µL of proteinase K (Bioline) (20 mg/ml) and 500 µL of 20% sodium dodecyl sulfate-Vetec (SDS) were added and the mixture homogenized in a vortex, and incubated at 60°C in a water bath for 2 h. After incubation, 800 µL of chloroform were added and the solutions were agitated until completely homogenized, 350 µL of protein precipitation solution were added and the mixture homogenized in a vortex. The samples were centrifuged at 18.000 x g for 10 min, the liquid phase was removed and transferred to another microtube and 1 ml of 100% ethanol was added. The material was centrifuged again at 13.000 x g for 5 min, the super-natant discarded, and 1 ml of 70% ethanol was added. The mixture was centrifuged again for 2 min and the supernatant again dis-carded. The microtube was inverted to dry the sediment and 100 µL of TE (Tris-EDTA) pH 8.7 with 0.1 µL of RNase (Fermentas) (10 mg/ml) was added.

The isolated DNA was re-suspended in TE and incubated at 37°C for 1 h and stored in a freezer at –20°C.

Quantitative and qualitative analyses of DNA

The quantity and purity of the DNA extracted from each sample was determined by measuring its optical density in a spectrophotometer (NanoDrop® ND-1000 UV-Vis). Additionally, 10 µL of DNA, with added 2 µL of Blue Orange Loading Dye buffer (Fermentas) was submitted to electrophoresis in 2% agarose gel colored with ethidium bromide. The DNA was visualized in the gel and photo-graphically registered under UV light.

PCR and primers

The DNA samples were submitted to multiplex polymerase chain reactions (mPCR) adapted from Santos et al. (2001). Three pairs of primers were used: one primer sequence to detect the genus *Salmonella* (gene *invA*) and the others to identify the two serovars (Fli15-Typ04, selected from the *fliC* gene and specific for *S. typhimurium*; and S1 to S4, selected from the *sefA* gene associated with virulence and specific for *S. enteritidis*). The oligonucleotide primer sequences and their expected product sizes were listed in Table 1. All the primer sequences were synthesized by Integrated DNA Technologies (IDT), USA.

DNA amplification and detection

The PCR amplification was performed in 25 µL of reaction mixture containing 12.5 µL of PCR Master Mix (Fermentas), 1.5 µL (10 pM/µL) of each of the primers and 10 to 50 ng of genomic DNA. The polymerase chain reactions were run in a thermal cycler (Biorad) and consisted of initial denaturation at 94°C for 5 min before initiating the 35 cycles, each of which consisted of holding at 94°C for 30 s, at 55°C for 30 s for primer annealing and extension, at 72°C for 30 s catalyzed by DNA polymerase activity, with a final extension for 7 min at 72°C. All of the reactions were performed using a negative control in which the DNA was substituted by an equal volume of ultra-pure water. The reproducible natures of the tests were confirmed by running each isolate twice. We also used DNA from control strains of *S. enteritidis*, *S. thyphimurium*, *E. coli*, *Proteus mirabilis* and *Citrobacter* sp. The multiplex PCR products were added to the loading buffer (Fermentas) and submitted to electrophoresis in 2% agarose gel. The gel was prepared using TBE buffer colored with ethidium bromide (10 mg/ml). The products amplified sizes were identified using 50 base pair DNA ladder (Promega).

The amplified products were visualized under UV Transillumi-nator using Gel Documentation System and photographed using a photo documentation system (UVP).

Tests of the genomic *Salmonella* sp. DNA sensitivity

DNA samples of Salmonella containing 21 ng/µL were diluted with DNA-free water at $1:10^0$, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} and subjected to same PCR amplification. 2 µL of each sample were added to the PCR reagents, using only the primers for the *invA* gene, following the protocol described earlier.

RESULTS

Thirty-four isolates were identified from the chicken car-casses analyzed as having the biochemical profile of

Table 2. Contamination samples and *Salmonella* isolates identified by biochemical tests and multiplex PCR.

| Samples analyzed		Chicken carcasses	Pond water
Nº of samples collected		36	15
Numbers of contaminated samples detected by biochemical tests		34	11
Numbers of contaminated samples detected by PCR		32	11
Total number of *Salmonella* isolates confirmed by biochemical tests		46	11
Numbers of *Salmonella* isolates confirmed by mPCR	*Salmonella* sp.	43	11
	S. Enteritidis	14	0
	S. Typhimurium	0	0
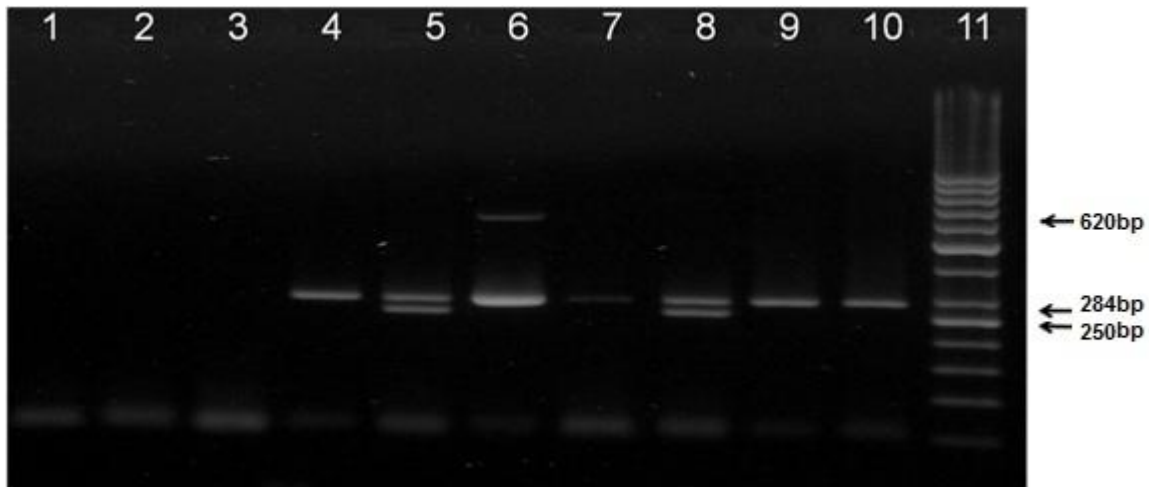
Figure 1. Agarose gel electrophoresis of the amplified products of multiplex PCR to determine the strains of the genus *Salmonella* (gene *invA*, 284 pb) in isolates of chicken carcasses and fish pond water and to identify Enteritidis (gene *sefA*, 250 pb) and Typhimurium (gene *fliC*, 620 pb) serotypes. Lane 1: negative control; lane 2: *Proteus mirabilis*; lane 3: *Citrobacter* sp.; lanes 4, 5 and 6: positive controls for *Salmonella* sp., *Salmonella enteritidis*, and *Salmonella typhimurium* respectively; lanes 7 and 8: chicken carcass samples; lanes 9 and 10: aquaculture water samples; lane 11: 50 pb molecular weight marker.

Salmonella sp. in Selenite Cystine broth, and 12 isolates in the Rappaport Vassiliadis broth, totaling 46 isolates demonstrated agglutination in sero-agglutination tests, confirming the diagnosis of the genus *Salmonella*. A contamination rate of 94% of the chicken carcasses was observed in this analysis (Table 2). The 46 *Salmonella* sp. isolates confirmed by serum agglutination were submitted to multiplex PCR (mPCR). The amplifications of the mPCR products were visualized in agarose gel and a 284 base pair fragment (gene *invA*) was identified in the positive samples for the genus *Salmonella*; a 250 base pair fragment (gene *sefA*) for the samples belonging to the Enteritidis serovar and a 620 base pair fragment (gene *fliC*) for the samples belonging to the Typhimurium serovar (Figure 1). It was confirmed that 43 of the 46 isolates belonged to the genus *Salmonella*, and among these, 14 were identified as *S. enteritidis*. The Typhimurium serovar was not detected among these studied samples. A contamination rate of 89% of the chicken carcasses

was confirmed by mPCR. Of the 15 analyses of water from aquaculture tanks, 11 (73%) tested positive for *Salmonella* sp. using bacteriological analysis and were confirmed by mPCR, although the Enteritidis and Typhimurium serovars were not detected in any of these samples.

The isolates of *Salmonella* sp. from the chicken carcasses and/or aquaculture tank water samples demonstrated average genomic DNA contents of 5.39 μg/μL. The ratio of their absorbances at 260/280 yielded an average of 1.84, indicating an acceptable degree of purity. The limits of detection by PCR using oligonucleotides primers to amplify the *Salmonella invA* gene are shown in Figure 2. When the genomic DNA of *Salmonella* sp. was tested using dilutions with ultra-pure water, a specific band at 284 bp could be seen at concentrations of 42 fg/μL. These results demonstrated the sensitivity of the analyses used in the present work, permitting the detection of this pathogen even in samples with reduced DNA concentrations.
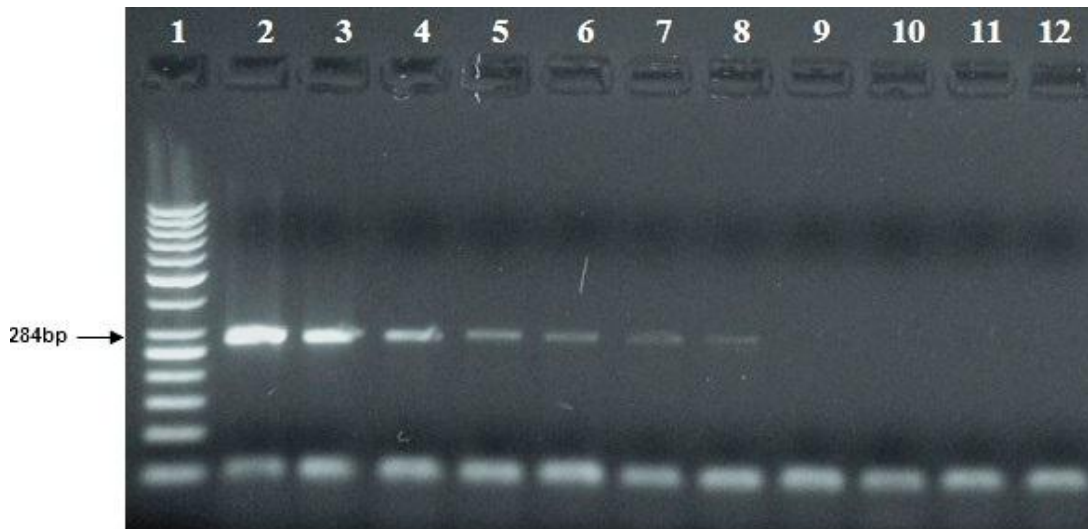
Figure 2. Agarose gel electrophoresis results showing the sensitivity of the PCR assay for the detection of genomic DNA of *Salmonella* sp. using *invA* gene (284 pb). Lane 1: 50 pb molecular weight marker; lane 12: negative control; lanes 2 to 11: 42 ng, 4.2 ng, 0.42 ng, 42 pg, 4.2 pg, 420 fg, 42 fg, 4.2 fg, 0.42 fg and 42 ag of genomic DNA diluted in ultra-pure water respectively.

DISCUSSION

Recent research has indicated wide variations in the rates of chicken carcass contaminations by *Salmonella* sp. in Brazil and in other countries, but most studies have reported indices well below the rates encountered here. Contamination rates of 16% were detected in Iran (Salehi et al., 2005). Antunes et al. (2003) detected 60% contamination by *Salmonella* sp. in Portugal, with prevalence of the Enteritidis serovar. Uyttendaele et al. (1998) reported contamination rates of 19, 24, 22 and 37% in chicken carcasses in Belgium in the year 1993 to 1996, respectively. Malkawi et al. (2004) reported contamination rates of 30% in Jordan, with 49% of the isolates being identified as *S. typhimurium* and 47% as the Enteritidis serovar – data which goes against an apparent global tendency of the presence of *S. enteritidis*, as well as the data observed in the present work – indicating that the prevalences of different serovars in different regions of the world can vary greatly. Santos et al. (2000) analyzed 150 frozen chicken carcasses in São Paulo State and detected *Salmonella* sp. in 48 (32%), of which 60% were of the Enteritidis serovar. Duarte et al. (2009) encountered contamination rates of 9.6% in Pernambuco State, of which 25% were identified as *S. enteritidis*. Boni et al. (2011) detected *Salmonella* in 257 samples from various processing steps in abattoirs located in the central region of Mato Grosso do Sul State and found a contamination rate of 11% for *Salmonella*. The differences in these contamination percentages are probably related to numerous factors, including the origin of the chicken lots, the hygiene-sanitary conditions in the abattoirs, and cross-contamination that occurred during plucking, washing, cooling and wrapping.

The carcasses are also susceptible to additional contamination during transport and commercialization (Corry et al., 2002). In addition to chickens, fishes are considered healthy alternative foods with low caloric value, and fish consumption by humans has greatly increased, principally in coastal cities and those near major rivers (Almeida et al., 2002). Fresh water fish production in Dourados-MS grew by 567% between 1999 and 2002, reaching fully 62% of the total production of Mato Grosso do Sul state by 2002, although, inadequate hygiene practices, lack of worker training, inadequate nutritional programs, and poorly planned installations have compromised the microbiological quality of the water. These conditions can result the contamination of fish by a wide variety of microorganisms and making it an important vector of pathogenic agents, including *Salmonella*, with numerous outbreaks among human populations (Liuson, 2003). PCR of the *invA* gene represents a relatively low-cost but specific test that has the advantage of generating results within just a few hours after isolating the bacteria in selective media. Another advantage of PCR of the *invA* gene is that it is capable of specifically identifying *S. enterica* among other *Enterobacteriaceae* (Nucera et al., 2006). The *invA* gene of *Salmonella* contains unique sequences of this genus and has been shown to be an adequate target for PCR for diagnostic purposes (Rhan et al., 1992). This gene is recognized internationally as a standard for detecting the genus *Salmonella*, and its amplification has been used by many workers to detect contamination (Malorny et al., 2003; Jeyasekaran et al., 2011) in chicken carcasses (Salehi et al., 2005; Hassanein et al., 2011) and environmental samples (Moganedi et al., 2007). Of the 46 *Salmonella* isolates from chicken carcasses tested in the present work, 43 were confirmed

using mPCR.

Jamshidi et al. (2009) working in Iran was able to identify all of the isolates examined as being representatives of *Salmonella* genes by way of replication of the *invA* gene (using the same primer employed in this research). The lack of confirmation of three isolates in the present work by PCR may indicate that some serovars cannot be detected using this gene. This observation corroborates the conclusions of Rahn et al. (1992) that some Salmonella serovars such as *Salmonella litchfield* and *Salmonella senftenberg* could not be detected by PCR. The *fliC* gene is responsible for the expression of a protein known as flagellin in *Salmonella* sp. the *sefA* gene codes for the fimbrial protein SEF14 that has unique specificity to *S. enteritidis* and its amplification can be used to identify this serovar (Thorns et al., 1996).

Researchers have used the specific sequences of these genes to detect *S. typhimurium* and *S. enteritidis*. Rahn et al. (1992) reported a detection limit of 27 pg/µL, Stone et al. (1994) reported 30 pg/µL, Upadhyay et al. (2010) noted 10 pg/µL, Santos et al. (2001) reported 63 fg/µL similar that we observed in this research, 42 fg/µL. The confirmation of the presence of *Salmonella* using mPCR yielded results similar to traditional bacteriological methods – but reduced the time necessary for those analyses (Jofre et al., 2005). The simultaneous application of more than one DNA region of interest in a single PCR reduces the work effort, time, costs and risks of cross-contamination. As such, various authors (Freitas et al., 2010; Silva et al., 2011) have standardized mPCR to detect the presence of *Salmonella* and its serovars that are important in food safety and public health.

The contamination levels of chicken carcasses detected in the present work were high, indicating the necessity of better hygiene and sanitary conditions in the various stages of chicken production in Mato Grosso do Sul State, Brazil. There is likewise a need to establish more efficient water quality controls in aquaculture tanks located in the region around Grande Dourados-MS, Brazil. Although, our results are still preliminary, they strongly indicate that mPCR assays are an effective alternative to traditional methods of identification and differentiation of the most relevant *Salmonella* types. They could be used to rapidly and reliably confirm the presence of the genus *Salmonella*, including the Enteritidis and Typhimurium serovars (two of the most relevant agents in terms of human contamination by *Salmonella*). The use of mPCR assays would make it possible to establish parameters for studies requiring large numbers of samples - principally in studies directed toward the rapidly growing sector of aquaculture in the region around Dourados, MS.

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