Journal of Food Safety and Food Quality 70, Heft 4 (2019), Seiten 91–124

Arch Lebensmittelhyg 70,
94–98 (2019)
DOI 10.2376/0003-925X-70-94

ISSN 0003-925X

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Occurrence and antimicrobial resistance profile of *Salmonella* isolated from native fish slaughtered and commercialised in Brazil

Vorkommen und Antibiotikaresistenzprofil von Salmonellen aus in Brasilien geschlachteten und vermarkteten einheimischen Fischen

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Summary

Brazil is one of the largest freshwater fish producers worldwide, producing and supplying thousands of tons to the entire population. Fish-carrying *Salmonella* has been implicated in foodborne disease worldwide. In this context, this study aimed to investigate the occurrence of *Salmonella,* identify the serotypes present in fish samples slaughtered and commercialized in Brazil, and evaluate the antimicrobial resistance profiles of the isolated strains. Fifty-two samples of commercialized native fish were evaluated by classical microbiological culture and by a multiplex PCR. *Salmonella* was isolated and detected in three (5.76%) of the 52 analyzed fish samples. We identified the presence of two uncommon serovars in fish samples: *S.* Abony and *S.* Schwarzengrund. This is a novel worldwide report on the occurrence of *S.* Abony in freshwater fish. All strains demonstrated a single resistance to sulphamethoxazole + trimethoprim. This study is crucial for *Salmonella* surveillance in the entire country and can provide data to formulate control measures for the effective treatment and prevention of foodborne and zoonotic pathogens.

Keywords: *Salmonella* Abony, *Salmonella* Schwarzengrund, sulphamethoxazole, trimethoprim, foodborne disease

Zusammenfassung

Brasilien ist einer der größten Süßwasserfischproduzenten der Welt und produziert und liefert Tausende von Tonnen an die gesamte Bevölkerung. Mit Salmonellen belastete Fische waren weltweit an lebensmittelbedingten Erkrankungen beteiligt. Das Ziel dieser Studie war es, das Auftreten von Salmonellen in Brasilien geschlachteten und vermarkteten Fischproben zu untersuchen, die Serotypen von zu identifizieren und die antimikrobiellen Resistenzprofile der isolierten Stämme zu bewerten. 52 kommerziell gefangene, einheimische Fischproben wurden bakteriologisch-kulturell und mit Multiplex-PCR untersucht. Salmonellen wurden in drei (5,76%) der 52 Fischproben nachgewiesen. Es wurden zwei seltene Serovare in den Fischproben nachgewiesen: *S.* Abony und *S.* Schwarzengrund. Dies ist ein neuer, weltweiter Bericht über das Vorkommen von *S.* Abony in Süßwasserfischen. Alle Stämme zeigten ausschließlich eine Resistenz gegenüber Sulfamethoxazol + Trimethoprim. Diese Studie ist für die Salmonellenüberwachung im gesamten Land von Bedeutung und kann Daten liefern, um Kontrollmaßnahmen für die wirksame Behandlung und Prävention von Lebensmittel- und Zoonoseerregern zu entwickeln.

Schlüsselwörter: *Salmonella* Abony, *Salmonella* Schwarzengrund, Sulfamethoxazol, Trimethoprim, Lebensmittelvergiftung

Introduction

Infectious diseases transmitted between animals and humans can be acquired by direct contact with animals, by environmental exposure, or by eating contaminated food (European Food Safety et al., 2015). *Salmonella enterica* is one of the main microorganisms carried by products of animal origin, which are responsible for Foodborne Diseases (FBD). Also, this microorganism was classified as the seventh major cause of diarrheal diseases in the world (Havelaar et al., 2015). *Salmonella* is a gram-negative bacillus belonging to the *Enterobacteriaceae* family, classified according to the Kauffmann-White scheme in two species, *bongori* and *enterica* (Issenhuth-Jeanjean et al., 2014). The latter is divided into six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica*. The *enterica* subspecies presents approximately 2,600 serotypes, of which 99% can cause infections in both animals and humans (Issenhuth-Jeanjean et al., 2014).

Fish do not represent a *Salmonella* reservoir, and the presence of this microorganism in this matrix might be *indica*tive of fecal contamination in the aquatic environment. Also, the detection of *Salmonella* in processed products thus suggests cross-contamination during fish processing stages, such as evisceration, cut into big steaks, ribs and fish fillets. Fish-carrying *Salmonella* has been implicated in foodborne disease (FDB) cases by 1% in the European Union according to the World Health Organization. From 1996 to 2014, fish were ranked as the main FBD-causing microorganism vehicle from imported food in the United States of America (Gould et al., 2017). In Brazil so far, there are very few studies regarding the occurrence of *Salmonella* originated from fish. For this reason, health authorities were not capable to publish any official national report. This study may be essential to provide data to help Brazilian authorities to determine the real occurrence of FBD caused by *Salmonella* from fish. In addition, investigating the antimicrobial resistance profile of *Salmonella* strains isolated from fish is necessary since infection by strains carrying resistance to the antimicrobials of choice for salmonellosis treatment causes severe impacts on public health.

The state of Mato Grosso, Brazil is the third largest national freshwater fish producer, producing approximately 49 thousand tons in 2016. The abundance of fresh water and favorable climate result in higher fish production in this region and justify our interest in evaluating *Salmonella* occurrence in this food matrix. Moreover, the perception of the frequency and identification of *Salmonella* serovars in fish from these regions are unrevealed and will fill a gap in this understanding. In this context, we aimed to reveal the frequency of *Salmonella* serotypes isolated from Brazilian native fish specimens commercialized in the States of Mato Grosso and characterize their antimicrobial susceptibility profiles.

Materials and methods

Sample collection

The 52 samples of native fish, eviscerated or not, fresh, chilled and frozen were randoly colectted from processing facilities and retail trade in municipalities of the state of Mato Grosso – Brazil. Forty samples of Tambaqui *(Colossoma macropomum),* two of Pirarucu *(Arapaima gigas),* three of Pintado *(Pseudoplastystoma corruscans),* one of the hybrid Tambacu – Tamabaqui *(Colossoma macropomum)* X Pacu-caranha *(Piaractus mesopotamicus),* and 6 of the hybrid Tambatinga – Tamabaqui *(Colossoma macropomum)* + Pirapitinga *(Piaractus brachypomus)* were analyzed for the presence or absence of *Salmonella*.

Isolation and identification of *Salmonella* species

Samples were collected and stored aseptically in thermal boxes with ice and transported to the Federal University of Mato Grosso for microbiological and molecular analyses. The isolation method was based on the protocol recommended by the International Standardization Organization ISO-6579 (ISO, 2017). Briefly, 25 grams of each sample were inoculated in Buffered Peptone Water (Himedia®, Mumbai, India), incubated at 37°C for 24h, enriched in Rappaport-Vassiliadis Broth (Oxoid®, United Kingdom), incubated at 42°C for 24h and then in Muller Kauffmann Novobiocin Tetrathionate Broth (Himedia®, Mumbai, India) at 37°C for 24h, with subsequent plating on Xylose Lysine Deoxycholate Agar (Himedia®, Mumbai, India) and Rambach Agar (Merck, Darmstadt, Germany), incubated at 37°C for 24h. Typical colonies were selected, purified on Nutrient Agar and subsequently inoculated on API 20E (BioMérieux®, Lyon, France). Strains showing a typical *Salmonella* reaction were subjected to serum-agglutination by the anti-salmonela polyvalent O serum.

Multiplex-PCR

The strains biochemically identified as *Salmonella* were inoculated in 10 mL of BHI Broth (Brain Heart Infusion) and incubated at 35°C for 24h. A 1.5 mL aliquot was then centrifuged at 14,000 x g for 5 minutes; the pellet was dissolved in 500 µL Mili-Q water, and heated at 100°C for 10 minutes on a heating plate (BioGPRO, Brazil), then cooled at 4°C for 10 minutes. The lysate was then centrifuged at 14,000 x g for 5 minutes, and 200 µL of the supernatant was removed, maintained in a freezer and subsequently subjected to multiplex PCR. The reaction was performed in a total volume of 25 µL containing 1U Taq Polymerase (Invitrogen®), 1x Taq buffer (5 mM KCl Tris-HCl, pH 8.5) 1.5 mM MgCl 2, 0.1 mM dNTP's (Promega®), 0.9 MM primer Inv-A (Fratamico, 2003) and 0.4 µM of IE-1 (Wang and Yeh, 2002) and Flic-C (Paião et al., 2013) primers (Invitrogen®). The conditions were based on the study performed by Paião et al. (2013). The m-PCR assay was performed with an initial denaturation step for 5 minutes at 95°C, followed by 30 one-minute cycles at 95°C, 1 minute at 58°C, and 30 seconds at 75°C, with a final extension step at 72°C for 7 minutes. The PCR products were analyzed by electrophoresis on 1.5% agarose gels, with TBE buffer (45 mmol L^{-1} Tris pH 8.3, 45 mmol L^{-1} borate, and 2 mmol L^{-1} EDTA) as the running buffer. The gels were then stained with Gel Red (Invitrogen®) and photo-documented (Mini-Bis-Pro DNT, Bio-Imaging Systems®).

Antimicrobial Susceptibility Test

Antimicrobial susceptibility tests were performed with the disk-diffusion test according to Clinical and Laboratory Standards Institute (CLSI, 2017). *Salmonella* isolates were inoculated into Mueller-Hinton broth (Himedia, Pennsylvania, US) and incubated at 37°C overnight (16–18 h). The suspensions were adjusted to the turbidity of 0.5 McFarland scale (about 108 cfu/ml) and streaked onto Mueller-Hinton agar (Himedia). Antimicrobial disks (Cecon®, Brazil) were dispensed onto the surface of the inoculated agar plates and the plates were incubated at 37°C for 16 to 20h. The following 15 antimicrobials were

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tested: ampicillin (10µg), aztreonam (30µg), cephalothin (30µg), cefoxitin (30µg), ceftiofur (30µg), chloramphenicol (30µg), florfenicol (30µg), streptomycin (300µg), gentamicin (10µg), nalidixic acid (30µg), ciprofloxacin (5µg), enrofloxacin (5µg), tetracycline (300µg), sulfamethoxazole-trimethoprim (25µg) and nitrofurantoin (300µg). The results were recorded as susceptible, intermediate or resistant by measuring the inhibition zone diameter according to the interpretative standards of CLSI in *Enterobacteriaceae* section M02-07 (2017). The reference strain Escherichia coli ATCC 25922 was used as the quality control organism and included with each batch of isolates tested. Data from all quality-control E. coli were within appropriate CLSI quality ranges (CLSI, 2017).

Salmonella serotyping

Salmonella serotype identification was carried out at the National Reference Laboratory Diagnosis of Enteric Bacteria, Oswaldo Cruz Institute, Oswaldo Cruz Foundation (FIOCRUZ), by detection of somatic and flagellar antigens, using polyvalent and monovalent antisera, with or without flagellar phase induction (Voss-Rech et al., 2015). Serotypes were assigned according to the Kauffmann-White scheme (Grimont and François-Xavier, 2007).

Results

Out of the 52 fish samples analyzed, 5.76% (3/52) were contaminated with *Salmonella*. They comprised one sample of frozen eviscerated fish, one of rib toothpick and another one of frozen fillet of Pintado (Table 1). The isolates showed the presence of Inv-A amplicon which confirmed they belong to the genus *Salmonella* spp. Additionally, we use Multiplex-PCR for fast detection and screening of serovars *Salmonella* Typhimurium (ST) and Enteritidis (SE). However, the technique did not amplify the genes FliC e IE-1 which means there were no ST and SE among the isolates. Subsequently, serotyping identified two strains as *S.* Abony and *S.* Schwarzengrund but was not able to serotype the third strain isolated from the frozen fillet of Pintado (Table 1). Antimicrobial resistance was tested against 15 agents. The three strains isolated presented the same unique profile of antimicrobial resistance against sulfamethoxazole-trimethoprim (SUT).

Discussion

Salmonella presence in fish could be explained due to the contamination of the aquatic environment by sewage, animal or human waste; or due to the handling process from harvest areas to the fish market resulting in deterioration of the quality of fish; or may also occur by the cross-contamination during the processing and preparation of the fish fillet. As a limitation of our study, we did not have access to these fish aquatic environments and processing stages once the samples we collected samples from the retails. Therefore, the accurate information about the primary source of *Salmonella* cannot be clarified in this study. Besides, the small number of samples analyzed in this study might be a concerning issue to determine the real occurrence of *Salmonella* in this specimen. Nevertheless, the detection of samples contaminated with *Salmonella* reported here revealed a population health hazard by the consumption of contaminated fish with potential microbial pathogens.

Some authors commonly agreed that the presence of *Salmonella* in eviscerated fish, steaks, fish ribs and salted cod in the supermarkets of cities in the central, northeast and southeast regions of Brazil *indica*tes a cross-contamination during the fish processing (Almeida et al., 2004; Duarte et al., 2010; Mayrla et al., 2017). One of our previous studies has provided strong evidence that *Salmonella* contamination in the fish environment and processing industry in Mato Grosso State might have originated from the poultry production waste. In a paper published at Poultry Science (Cunha-Neto et al., 2018), we demonstrated that the occurrence of *Salmonella* spp. in chicken carcasses in Mato Grosso State was 3.7% (31/850) and the serovars Abony and Schwarzengrund comprised 25.8% and 9.7% of the strains isolated, respectively. The Mato Grosso State has one of the highest poultry production in Brazil, so it is much more evident that poultry production was the primary source of fish contamination than conversely. Also corroborating with our hypothesis, there is a recently gradual reduction of the most common *Salmonella* serotypes in the poultry industry such as Enteritidis (Voss-Rech et al., 2015). This reduction is a consequence of the *Salmonella* control program of immunization with inactivated vaccines implemented by the Brazilian biosecurity authorities. Hence, when some serovars decrease, others less frequently such as Schwarzengrund and Abony find a free niche to grow with less competition resulting in a faster and better replication rate. It may be possible that higher level of these serovars originated from poultry production resulted in transmission towards the fish production.

TABLE 1: *Distribution and antibiotic resistance profile of Salmonella serotypes by fish specimens and post-processing presentation of the sample from the retail trade in Mato Grosso, Brazil.*

Name	Sample type	Number of Samples	Number of Isolates (%)	PCR (invA) detection	Serovars	AMR profile
Tambatinga[1]	Frozen eviscerated fish	4	0	negative	-	-
Tambatinga[1]	Fish with viscera and refrigerated	2	0	negative	-	-
Tambacu[2]	Fish with viscera and refrigerated	1	0	negative	-	-
Tambaqui *(Colossoma macropomum)*	Frozen eviscerated fish	23	1 (4.35%)	positive	Schwarzengrund	SUT
Tambaqui *(Colossoma macropomum)*	Freshly gutted fish	1	0	negative	-	-
Tambaqui *(Colossoma macropomum)*	Frozen toothpick rib	16	1 (6.25%)	positive	Abony	SUT
Pintado *(Pseudoplastystoma corruscans)*	Frozen fish fillet	3	1 (33.3%)	positive	*Salmonella* spp.	SUT
Pirarucu *(Arapaima gigas)*	Frozen fish fillet	2	0	negative	-	-
Total		**52**	**3 (5.76%)**			

[1] Tamabaqui *(Colossoma macropomum)* x Pirapitinga *(Piaractus brachypomus);* [2] Tamabaqui *(Colossoma macropomum)* x Pacu-caranha *(Piaractus mesopotamicus);* SUT (trimethoprim+sulfamethoxazole)

Salmonella Schwarzengrund is cited among the top 20 serotypes responsible for FBD cases on the Asian continent (Hendriksen et al., 2011). Also, this serotype was related to cases of FBD in humans involving pet food in the United States and Canada (Behravesh et al., 2010; Li et al., 2012). However, the presence of S. Schwarzengrund in fish samples is sporadic around the world. Few isolations were reported in Africa and also in rations for fish produced and imported by Norway (Lunestad et al., 2007; Traoré et al., 2015). A 9 year (1990–98) study of the presence of *Salmonella* in imported and domestic seafood products carried out by the US FDA *indica*ted that *Salmonella* Schwarzengrund is rarely detected in seafood (Heinitz et al., 2000). In our acknowledge, the presence of S. Schwarzengrund in fish have not been reported yet in Brazil. This evidence also corroborates with the hypothesis discussed above which the transmission of this serovar is likely coming from poultry toward fish production.

We detected no reports about the occurrence of the *S.* Abony in fish worldwide. However, this serotype has been described occurring in surface waters of lakes and rivers in northern Greece (Arvanitidou et al., 1997), and in mud, water and shrimp ponds of crustacean in southwest Asia (Reilly and Twiddy, 1992). Our study seems to be the first to report the isolation of *S.* Abony from frozen fish worldwide. Currently, we have the interest in understanding whether if this Abony serovar origin is much more related to the hypothesis of transmission from poultry production or if the Brazilian water of lakes, ponds and rivers harbor *Salmonella* as reported in northern Greece and southwest. Shortly, an extensive study of *Salmonella* in aquaculture in the various States of Brazil will be performed by our research group to provide solid answers to confirm our hypothesis. Also, the Whole Genome Sequencing of the *S.* Abony strain is ongoing to compare with the genome of others exemplars isolated from poultry origin.

The isolates of *Salmonella* spp., *S.* abony and *S.* Schwarzengrund were tested against seventeen antimicrobials. The antimicrobials tested were carefully selected to include the most commonly used veterinary drugs in the Brazilian animal production and the first-choice drugs for the treatment of human enteric infections. The isolates showed a single resistance against trimethoprim + sulfamethoxazole – SUT (Table 1). The resistance against this antimicrobial seems to be very common in *Salmonella* isolated from fish worldwide. Similar studies also detected resistance against trimethoprim + sulfamethoxazole in isolates from fish farmed in tanks in Rio de Janeiro, Brazil, from fish found in the retail trade in China, and fish caught in Nigeria, Africa, and fish purchased from the Turkish trade (Ertas Onmaz et al., 2015; Raufu et al., 2014; Ribeiro et al., 2010; Yang et al., 2015). Brazil is a country of continental dimensions, with producers of chicken and swine in all its regions. The possible explanation for the common SUT resistance among *Salmonella* isolates should be attributed to the extended use of trimethoprim + sulfamethoxazole to treat infections by enterobacteria and as a growth promoter in the Brazilian animal production. The indiscriminate use of SUT during the swine and chicken production in Mato Grosso State might have selected resistant strains which were transmitted to the fish production in the region and resulted in the isolation of *Salmonella* strains with this resistance profile.

Conclusion

This study provides interesting data that are critical for assessing and controlling the risk associated with the presence of *Salmonella* in fish. The consumption of fish infected with *Salmonella* can instigate a public health problem, so we suggest that fish should be appropriately processed before consumption. Finally, this study is crucial for *Salmonella* surveillance in the entire country and can provide data to formulate control measures for the effective treatment and prevention of foodborne and zoonotic pathogens.

Acknowledgments

The authors would like to thank the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (process no. E-26/201.185/2014, FAPERJ, Brazil) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (process no. 311422/2016-0, CNPq, Brazil), for financial support.

Conflict of interest

The authors declare there is no conflict of interest.

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