

***Aeromonas hydrophila* and *Campylobacter jejuni* isolated in fresh tuna (*Thunnus* spp.) sold in São Paulo, Brazil**

Detecção de *Aeromonas hydrophila* e *Campylobacter jejuni* em atum (*Thunnus* spp.) fresco comercializado em São Paulo, Brasil

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Abstract

Aeromonas hydrophila and *Campylobacter jejuni* are bacteria of emerging importance in public health. However, little has been published about fish contaminated by these pathogens. The present study aimed to verify the presence of *Aeromonas hydrophila* and *Campylobacter jejuni* in fresh tuna samples (*Thunnus* spp.) caught off the coast of Santa Catarina State and distributed in the wholesale market of São Paulo/SP. A total of 85 tuna fillet samples were collected and examined by PCR and bacteriological analyses. *Aeromonas* spp. was detected in 11/85 (13%) samples, with 10/11 (90.9 %) being confirmed as *Aeromonas hydrophila* by PCR. *Campylobacter* spp. was found in 10/85 (11.7%) samples, 10/10 (100%) identified as *Campylobacter jejuni* by PCR and conventional biochemical analyses. Both pathogens were found in 2/85 (2.3%) samples. This is the first report on the contamination of fresh tuna by *Campylobacter jejuni* and *Aeromonas hydrophila* in Brazil. In addition to show that tuna can be a vehicle for transmission of pathogens when consumed raw, it emphasizes the importance of further studies to support the control these pathogens in fish.

Keywords: Fresh tuna. *Aeromonas hydrophila*. *Campylobacter jejuni*. São Paulo.

Resumo

Aeromonas hydrophila e *Campylobacter jejuni* são bactérias de importância emergente em saúde pública, porém com escassos trabalhos publicados na área de pescado. O presente estudo investigou a presença de *Aeromonas hydrophila* e *Campylobacter jejuni* em amostras de atum (*Thunnus* spp.) fresco, capturados no litoral de Santa Catarina e distribuídos no comércio atacadista de São Paulo, SP. Foram colhidas 85 amostras de filé de atum e processadas por análises bacteriológicas e PCR. Do total, 11/85 (13%) amostras foram positivas para *Aeromonas* spp., sendo 10/11 (90,9%) confirmadas como *Aeromonas hydrophila* pela PCR. Para *Campylobacter* spp., 10/85 (11,7%) foram positivas, sendo 10/10 (100%) identificadas como *Campylobacter jejuni* pelas provas bioquímicas tradicionais e PCR ressaltando-se que duas (2/85 - 2,3%) amostras de atum albergavam ambos os patógenos. Trata-se do primeiro relato no Brasil de contaminação de atum fresco por *Campylobacter jejuni* e *Aeromonas hydrophila*, indicando que este alimento ingerido *in natura* pode ser um veículo de transmissão de agentes patogênicos, ressaltando-se a importância de estudos adicionais que deem suporte ao controle desses microrganismos em pescado consumido cru.

Palavras-chave: Atum fresco. *Aeromonas hydrophila*. *Campylobacter jejuni*. São Paulo.

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Received: 26/03/2015

Approved: 26/02/2016

Introduction

The genus *Aeromonas* is associated with aquatic environments and since 1894 some species are known to be pathogenic to fish (MARTINELLI et al., 2011). In humans, *Aeromonas* spp. can cause intestinal and extra-intestinal infections, such as cellulitis and

septicemia. It can also lead to wound, urinary tract, hepatobiliary tract and soft tissue infections, and occasionally meningitis and peritonitis. In immunocompromised children, this pathogen can cause more serious complications, such as hemolytic-uremic syndrome (HUS) and necrotizing fasciitis, though further studies are needed to establish such associations (FOOD AND DRUG ADMINISTRATION, FDA, 2009; KHAJANCHI et al., 2010).

Since 1970 campylobacteriosis is considered one of the major emerging foodborne diseases, with recognized importance in public health. It is associated with the consumption of contaminated unpasteurized milk, raw or undercooked meat and specially poultry, contaminated water and vegetables or direct contact with carrier animals (KUMAR et al., 2001; CALIL et al., 2008). The main symptoms in humans include diarrhea (watery or bloody), abdominal pain, fever, headache, nausea and vomiting. Complications such as appendicitis, pancreatitis, edema of the colon and arthritis may occur. *C. jejuni* is associated with the Guillain-Barré syndrome, an autoimmune disease involving the peripheral nerves of the muscular system (GERMANO; GERMANO, 2001; CALIL et al., 2008).

In São Paulo, Brazil, there has been a growing consumption of raw fish, such as tuna and salmon, mainly used for sushi and sashimi. Moreover, few reports on tuna contaminated by *Aeromonas hydrophila* and *Campylobacter* spp. are found in the literature. Thus, the aim of this study was to verify the presence of these bacteria in fresh tuna (*Thunnus* spp.) sold in São Paulo/ SP, according to microbiological and molecular analyses.

Materials and Methods

A total of 85 samples of fresh tuna (*Thunnus* spp.), caught off the coast of Santa Catarina State and acquired at the wholesale fish market in the metropolitan region of São Paulo/SP, were analyzed

from August 2011 to February 2012. Samples were identified by numbers (01-85).

The samples were stored in sterile plastic bags (Nasco Whirl-Pak, Radnor, USA), transported to the laboratory in cool boxes with ice packs (temperature 2-8°C) and processed within 24h. Aliquots of 25 g of tuna were macerated in 100 mL of sterile physiological saline (0.9%), using a Stomacher 80 (Lab System, Port Saint Lucie, USA) for 4 min. Supernatants of homogenates were used for the isolation and biochemical identification of *Campylobacter* spp., according to OFFICE INTERNATIONAL DES EPIZOOTIES (OIE) (2008) and Carvalho et al. (2010). A total of 2 mL of the supernatant was passed through a cellulose ester membrane filter (pores size 0.65 µm – Millipore Inc., Darmstadt, Germany.) using a plastic support (Swinex – Millipore Inc., Darmstadt, Germany) and sterile syringe to inoculate 100 µL of filtrate on to Brucella agar medium (Difco, Sparks, USA) with 5% defibrinated sheep blood (ABS – Biotério Boa Vista, Valinhos, Brasil). Also, 100 µL of the supernatant were inoculated on to ABS medium supplemented with an antibiotic (Inlab, São Paulo, Brazil) mixture: polymyxin B (1,000 IU/L), cycloheximide (20 mg/L), novobiocin (5 mg/L), and bacitracin (15,000 IU/L) (ABS-ATB). The plates were incubated for 48-72 h at 37°C in a microaerophilic chamber (5%CO₂, 10%O₂). After the incubation period, Gram staining and oxidase test were carried out. Colonies suspected of belonging to the genus *Campylobacter* (gray or colorless, flat, nonhemolytic, irregular and spreading colonies) were subjected to biochemical screening tests: H₂S production, TSI fermentation or oxidation, oxidase, catalase, hydrolysis of hippurate and susceptibility to nalidixic acid and to cephalothin. (HOLT et al., 1994).

Supernatants from the homogenates were also subjected to bacteriological procedure for the isolation and biochemical identification (oxidase, TSI fermentation, lysine decarboxylase, indole, citrate, urease and gelatinase) of *Aeromonas* spp., according

to Abbott et al. (2003); Songer and Post (2005) and Hirsch et al. (2006). A total of 100 µL of the supernatant was inoculated on to Tryptic Soy Agar (TSA- Difco, Sparks, USA) medium plus ampicillin (10 mg/L) and 5% defibrinated sheep blood. A 100 µL sample was also inoculated on to MacConkey Agar medium (Difco, Sparks, USA) and ABS-ATB. The plates were incubated for 48-72 h at 37°C in a microaerophilic chamber (5%CO₂, 10%O₂). Suspected colonies (beta-hemolytic) were subjected to genus-specific biochemical tests: oxidase, TSI fermentation, lysine decarboxylase, indole, citrate, urease and gelatinase (ABBOT et al., 2003).

The isolated suspected *Aeromonas* spp. and *Campylobacter* spp. samples had their DNA extracted using the commercial kit illustra bacteria genomicPrep Mini Spin (GE Healthcare, Buckinghamshire, UK), according to manufacturer specifications. *Aeromonas hydrophila* ATCC 7966 and *Campylobacter jejuni* ATCC 33291 were used as controls.

In order to confirm the detection of *Aeromonas hydrophila* carried out by biochemical tests, the conserved **16S rDNA** gene region, which corresponds to a fragment of 685 bp, was screened by PCR (CHU; LU, 2005). The oligonucleotide primers used in DNA amplification were **16S rDNA1** 5'-GAA AGG TTG ATG CCT AAT ACG TA-3' and **16S DNA2** 5'-CGT GCT GGC AAC AAA GGA CAG-3'.

A similar procedure was adopted to confirm the detection of *Campylobacter* spp., by screening the *hip* gene encoding the enzyme hippuricase, which corresponds to a 735 bp fragment, specific for *Campylobacter jejuni*, according to Linton et al. (1997). The oligonucleotide primers used in DNA amplification were **HIP400F** 5'-GAA GAG GGT TTG GGT GGT G-3' and **HIP1134R** 5'-AGC TAG CTT CGC ATA ATA ACT TG-3'.

The analyses of the amplified products were carried out by electrophoresis in 2% agarose gel. The gel was stained with ethidium bromide (0.5 mg/mL), photographed under UV light (300-320 nm) with a

DC/120 Kodak Digital Zoom camera and analyzed with 1D Image Analysis software (Kodak Digital Science).

Results

A total of 11/85 (13%) tuna samples tested for *Aeromonas* spp. by biochemical analyses (oxidase positive, lysine decarboxylase negative, indole positive, citrate negative, urease negative and gelatinase positive, showing TSI fermentation with gas production) were positive, with 10/11 (90.9%) confirmed by PCR as *Aeromonas hydrophila* (Table 1).

A total of 10/85 (11.7%) tuna samples were positive by biochemical analyses for *Campylobacter* spp., with 10/10 (100%) identified as *Campylobacter jejuni* by biochemical analyses and PCR (Table 1). In addition, to morpho-staining characteristics observed after the Gram staining for *Campylobacter jejuni*, further biochemical tests showed neither H₂S production nor TSI fermentation and positive oxidase and catalase, positive hydrolysis of hippurate, sensitivity to nalidixic acid and resistance to cephalothin.

Aeromonas hydrophila and *Campylobacter jejuni* were simultaneously found in 2/85 (2.3%) tuna samples (Table 1).

A total of 9/11 (81.8%) *Aeromonas* spp. strains were isolated in the selective medium for *Campylobacter* spp. (Brucella blood agar plus antibiotic mixture – ABS-ATB), whereas 1/ 11 (9.1%) was isolated in the medium specific for *Aeromonas* spp. (TSA, blood and ampicillin). A single strain (1/11; 9.1%) was positive in both selective media.

It was not possible to identify *Aeromonas* spp. in the MacConkey agar medium, due to intense contamination by other bacterial genera.

All *Campylobacter* spp. strains (10/10; 100%) were isolated in Brucella blood agar medium (ABS), after filtration through a cellulose ester membrane (pores size 0.65 µm) (Table 1).

Table 1 – Classification of *Aeromonas* spp. and *Campylobacter* spp. strains isolated in fresh tuna (*Thunnus* spp) sold in São Paulo, Brazil, according to the medium of the isolation and conventional PCR identification. Samples collected from August 2011 to February 2012

Sample	<i>Aeromonas</i> spp.	Medium		PCR	<i>Campylobacter</i> spp.	Medium		PCR
	isolate	TSA-AMP	ABS-ATB	<i>A. hydrophila</i>	isolate	ABS-FLT.	ABS-ATB	<i>C. jejuni</i>
1	+	+	-	+	-	-	-	-
3	-	-	-	-	+	+	-	+
4	-	-	-	-	+	+	-	+
27	+	-	+	+	-	-	-	-
31	-	-	-	-	+	+	-	+
32	-	-	-	-	+	+	-	+
42	-	-	-	-	+	+	-	+
43	+	-	+	+	-	-	-	-
51	+	+	+	+	-	-	-	-
54	+	-	+	+	+	+	-	+
56	+	-	+	+	-	-	-	-
58	-	-	-	-	+	+	-	+
59	+	-	+	+	-	-	-	-
60	+	-	+	-	-	-	-	-
66	+	-	+	+	-	-	-	-
71	-	-	-	-	+	+	-	+
75	+	-	+	+	-	-	-	-
80	-	-	-	-	+	+	-	+
82	+	-	+	+	+	+	-	+

TSA- AMP: Tryptic Soy Agar Blood plus ampicillin; ABS- ATB: Brucella blood agar plus antibiotics; ATB: polymyxin B (1,000 IU/L), cycloheximide (20 mg/L), novobiocin (5 mg/L), and bacitracin (15,000 IU/L); ABS- FLT: Filtration 0.65 μm pore size and inoculation in Brucella blood agar. Positive (+) and negative (-)

Discussion

Thunnus spp. inhabits the deep waters of the high seas. Tuna samples analyzed in this study were captured off the coast of Santa Catarina State and sent to the wholesale market of São Paulo/SP. The samples were subject to contamination originating at various stages of the distribution cycle, which can range from sea pollution, fishing conditions, shipping and handling during filleting.

As shown in Table 1, 11/85 (13%) tuna samples were positive for *Aeromonas* spp., with 10/11 (90.9%) were identified as *A. hydrophila*. Similar studies were carried out with fish sold in São Paulo State. Nespolo et al. (2012) found *Aeromonas* spp. in 13/31 (41.95%) samples of salmon from markets in São Paulo State, but no proliferation by *Aeromonas hydrophila* was detected. Silva et al. (2010) investigated the presence

of pathogens in fish sold at street markets in the municipality of São Paulo. Fish contamination by *Aeromonas* spp. was found, with 50% of contaminated samples being identified as *A. hydrophila*. According to the authors, these results emphasize that contamination can occur by the bacteria lodged in the skin, gills and intestine of fish.

Similar results were reported in other countries. In Switzerland, Gobat and Jemmi (1993) isolated *Aeromonas* spp., with *A. hydrophila* being identified in 10.9% of salmon samples. Sharma and Kumar (2011) detected *Aeromonas* spp. in 18/137 (13.13%) fish fillets in India, 62.5 % being identified as *A. hydrophila*.

According to Stratev et al. (2012), the preponderance of *Aeromonas hydrophila* in fish

contaminated by *Aeromonas* spp. is reported in the international literature, as observed in this study (90.9%).

Only a few studies could be found in the literature about the isolation and identification of *Campylobacter* spp. isolates in fish, especially fresh tuna. These studies describe the species *C. jejuni* and *C. lari* associated with the consumption of seafood, especially oysters and mussels. Whyte et al. (2004) found *C. jejuni* in 3/117 (2.5%) oysters from Ireland. Endtz et al. (1997) discovered that 27% of oysters (11/41) and 69% of mussels (41/59) were positive for *C. lari* in the waters of an estuary in the Netherlands.

In the present study, 10/85 (11.7%) of tuna samples were positive for *C. jejuni*. This information indicates that tuna, frequently used in the preparation of sushi and sashimi, could harbor *Campylobacter* spp., suggesting that fish also should be investigated for the presence of *Campylobacter* spp., like seafood, such as oysters and mussels.

Table 1 shows a higher incidence of *Aeromonas* spp. isolates (9/11 - 81.8%) in the selective medium for *Campylobacter* spp. (Brucella blood agar plus antibiotics, ABS-ATB) than in the selective medium recommended for the isolation of *Aeromonas* spp. (1/11 - 9.1%) (TSA, blood and ampicillin). Only one strain (1/11 9.1%) was positive in both selective media. This can be explained by the intense contamination in samples where the presence of only one antibiotic (ampicillin) in TSA medium was not enough to stop the contamination. However, contamination was significantly reduced in ABS-ATB medium, with three antibiotics (polymyxin B, novobiocin and bacitracin) and an antifungal agent (cycloheximide), thus favoring the isolation of *Aeromonas* spp.

Table 1 also shows that 10/10 (100%) of the *Campylobacter* spp. strains were isolated in ABS

medium after filtration through a cellulose ester membrane filter (pores size 0.65 μ M), confirming the superiority of the filtration technique in relation to ABS-ATB selective medium when there is intense contamination of the sample by other bacterial genera.

Conventional PCR was a more advantageous method in terms of specificity, speed and low cost when compared to laborious and extensive biochemical analyses, indicated for the identification of *Aeromonas hydrophila* (CHU; LU, 2005). According to Abbott et al. (2003), 63 phenotypic tests (biochemical properties) are required for the differentiation of the 14 *Aeromonas* genomospecies.

In the present study, 2/85 (2.3%) tuna samples (tuna 54 and tuna 82) contained both genera, *Aeromonas hydrophila* and *Campylobacter jejuni*, posing a high risk of causing gastroenteritis, especially if fish are stored at inadequate temperatures and are eaten raw, considering that *Campylobacter* spp. and *Aeromonas* spp. remain viable under refrigeration, but are sensitive to freezing (SCARCELLI et al., 2005; NESPOLO et al., 2012). The increasing raw fish consumption in Brazil (sushi and sashimi) is leading to a growing concern with the sanitary quality of the fish and its various preparations. Fish consumed raw can be a vehicle for transmission of pathogens, such as *Aeromonas hydrophila* and *Campylobacter jejuni*, requiring measures of hygiene and sanitary control throughout its processing.

This is the first report in Brazil showing the presence of *Campylobacter jejuni* and *Aeromonas hydrophila* in fresh tuna, emphasizing the importance of further studies to support the control these pathogens in fish.

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