

## Thermophilic *Campylobacter* Survey in Chilled and Frozen Poultry Meat at Retail in Concórdia, Santa Catarina\*

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### ABSTRACT

**Background:** Human campylobacteriosis has become a major foodborne disease, although little information is available about the role it plays in the scenario of foodborne diseases in Brazil. Since thermophilic *Campylobacter* (*C.*) species are often found in the intestinal tract of broiler chickens, consumption of contaminated poultry meat has been considered a risk factor for *Campylobacter* human infection. *Campylobacter* has been described as extremely susceptible to a variety of environmental stresses; hence the difficulty to establish cultures of the microorganism in the laboratory. In addition, it has been shown to decline in refrigerated and frozen foods. Currently there is a need for data on *Campylobacter* contamination level in Brazilian poultry meat. This work describes a survey performed in chilled and frozen poultry meat obtained from different retailers in Concórdia, Santa Catarina, Brazil.

**Materials, Methods & Results:** This study analyzed 24 samples of fresh (chilled or frozen) poultry meat portions (thighs and drumsticks) produced by three different Brazilian broiler chicken processors, which were purchased from three different retailers in Concórdia, Santa Catarina. *Campylobacter* isolation was performed according to ISO 10272-1:2006. Individual samples (25g) were enriched in 225 mL of Bolton Broth in microaerobic atmosphere at 37°C for 4h to 6h, then at 41.5°C for 44h (+/- 4h). Aliquots were streaked in Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) and Campy-Cefex Agar at 41.5°C in a microaerobic atmosphere for 44h (+/- 4h). Suspected colonies were subcultured in Blood Agar no 2 plates for confirmation by morphology, Gram staining, tests for catalase, oxidase and hippurate and indoxyl acetate hydrolysis test. Enriched Bolton Broth aliquots were also analyzed by Polymerase Chain Reaction (PCR) to amplify a 287 bp sequence of the 16S rRNA gene from thermophilic *Campylobacter*. Although *C. jejuni* positive control was isolated and confirmed by the morphological and biochemical tests described, all samples analyzed were negative for the presence of thermophilic *Campylobacter*. However, either mCCDA or Campy-Cefex Agar showed an abundant growth of non-*Campylobacter* cells. Moreover, all samples analyzed were negative in PCR analysis, although the *C. jejuni* reference strain used as PCR positive control showed the expected DNA fragment amplified.

**Discussion:** Higher levels of *Campylobacter* contamination in poultry meat have been found by other studies, which used different isolation protocols in comparison to the present work. This study shows that *Campylobacter* isolation procedure according to ISO 10272-1:2006 allowed the growth of contaminants in both selective media used. It might be result of enrichment for 48 h or proliferation of ESBL producing *Escherichia coli*, able to hydrolyze cefoperazone in the selective medium used, which could underestimate the presence of *Campylobacter* in samples. In this sense, the PCR assay was essential to corroborate the negative result in *Campylobacter* isolation. Because poultry meat analyzed was negative for the presence of thermophilic *Campylobacter*, it was not possible to assess any difference between chilled or frozen storage on the bacteria survival. The present study might reflect low rates of *Campylobacter* contamination in poultry meat available at retail in Concórdia, Santa Catarina, Brazil, although it cannot replace good hygienic practices as well as consumer education.

**Keywords:** *Campylobacter*, poultry meat, retail, PCR, ISO 10272-1:2006.

## INTRODUCTION

Thermophilic *Campylobacter* (*C.*) species (*C. jejuni*, *C. coli* and *C. lari*) are commensals of gut in a variety of food-producing animals, including poultry. Despite the absence of clinical symptoms in poultry, high *Campylobacter* counts are recovered from the avian cecum, which might contaminate poultry carcass during processing, representing a public health risk [12]. Currently campylobacteriosis is a major human foodborne disease worldwide [6,12], which is predominantly believed to be associated to consumption of improperly handled or undercooked contaminated poultry meat [5,15].

*Campylobacter* is highly susceptible to environmental stresses, like variations in temperature, humidity, osmolarity, presence of sunlight and atmospheric oxygen [12]. Hence the difficulty to establish cultures of the microorganism in the laboratory. Due to its fastidious growth requirements and absence of growth below 30°C, *Campylobacter* does not multiply in foods held at room temperature [3]. However, it can survive and remain infective during the refrigerated or frozen storage of poultry meat, the latter representing a positive effect by decreasing viable *Campylobacter* cells [3,5,17].

In spite of its relevance as foodborne agent, there is little information about the human disease or *Campylobacter* contamination level in retail poultry meat in Brazil. Therefore, thermophilic *Campylobacter* surveillance in poultry products is essential to assess its potential as human health risk. The aim of this study was to analyze the presence of thermophilic *Campylobacter* in retail chilled and frozen poultry meat in Concórdia, Santa Catarina, Brazil.

## MATERIALS AND METHODS

### Sample collection

A total of 12 chilled and 12 frozen poultry meat portions (thighs and drumsticks) packed in tray packs with unadulterated overwrap were individually analyzed. All poultry meat sampled was produced by three different Brazilian broiler chicken processors which operate under the official inspection (SIF), and were purchased from three different retailers located in Concórdia, Santa Catarina (Southern Brazil). Three chilled and 3 frozen packs were obtained per week between February and March 2010, and were transported to the laboratory in chilled containers. Chilled samples

were analyzed within 2 h of purchase, while frozen samples were analyzed after thawed at 4°C for 18 h.

### Isolation of Thermophilic *Campylobacter*

Thermophilic *Campylobacter* isolation was performed according to Microbiology of food and animal feeding stuffs – Horizontal method for detection of *Campylobacter* spp. (ISO 10272-1:2006) [9]. For analysis, slices were cut aseptically from the thighs and drumsticks. The initial suspension was prepared introducing 25 g of slices in 250 mL of the enrichment medium Bolton Broth<sup>1</sup> plus supplements<sup>2</sup> (0.02 g/L cefoperazone, 0.02 g/L vancomycin, 0.02 g/L trimethoprim lactate and 0.01 g/L amphotericin B) without the addition of lysed defibrinated horse blood, which was incubated in a microaerobic atmosphere (5% O<sub>2</sub>, 10% CO<sub>2</sub> and 85% N<sub>2</sub>) at 37°C for 4h to 6h, then at 41.5°C for 44h (+/- 4h). The enriched culture was inoculated with a sterile loop onto duplicate Modified Charcoal Cefoperazone Deoxycholate Agar<sup>1</sup> (mCCDA) plates plus supplements<sup>2</sup> (0.032 g/L cefoperazone and 0.01 g/L amphotericin B) and Campy-Cefex Agar [6] plates, as the second isolation medium, plus 5% lysed ovine blood and supplements<sup>2</sup> (0.033 g/L cefoperazone and 0.2 g/L cycloheximide), which were incubated at 41.5°C in a microaerobic atmosphere for 44h (+/- 4h). Suspected colonies were taken from each selective medium and streaked onto Blood Agar no 2<sup>1</sup> plates for confirmation. The plates were incubated in a microaerobic atmosphere at 41.5°C for 24h to 48h for examination of morphology, Gram staining, tests for catalase and oxidase, hippurate hydrolysis and hydrolysis identification of indoxyl acetate. A *Campylobacter jejuni* strain (FIOCUZ 419) was inoculated in Bolton Broth and used as positive control.

### Genomic DNA extraction

A 0.5 mL aliquot of each enriched Bolton Broth culture was harvested at the end of incubation period and centrifuged at 10,000 X g at 4°C for 5 min. Cells were resuspended in PBS pH 7.4, when the genomic DNA was obtained as previously described [16]. Briefly, resuspended cells were lysed with 5 M guanidinium thiocyanate<sup>3</sup>, 100mM EDTA<sup>2</sup> and 5% (v/v) sarkosyl<sup>2</sup> at room temperature for 10 min. Lysed suspensions were mixture with 0.25 mL of 2.5 M ammonium acetate and held on ice for 10 min. Further, it was added chloroform and isoamyl alcohol (24:1) (v/v) and the phases were mixed thoroughly,

centrifuged at 12,000 X g at 4°C for 10 min, when the upper phase was transferred to a 1.5 mL tube. The DNA was precipitated by 0.54 v of cold isopropanol, centrifuged at 6,500 X g for 20 s, washed in 70% ethanol and resuspended in Tris-EDTA (10mMTris-HCl, 1 mM EDTA, pH 8.0). Aliquots of sterilized Bolton Broth and the positive control (*Campylobacter jejuni* enriched in Bolton Broth) were used as DNA extraction procedure control, respectively.

#### Polymerase Chain Reaction

The PCR procedure was based on a 287 bp sequence of the 16S rRNA gene from *C. jejuni*, *C. coli* and *C. lari* [10]. PCR mixture contained the 1X GoTaq Flexi buffer<sup>4</sup>, 2.0 mM MgCl<sub>2</sub><sup>4</sup>, 200 mM of each dNTP<sup>4</sup>, 12 pMol of each forward (5'-CT-GCTTAACACAAGTTGAGTAGG-3') and reverse (5'-TTCCTTAGGTACCGTCAGAA-3') primers<sup>5</sup>, 1 U of GoTaqDNA polymerase<sup>4</sup>, 2 µL of genomic DNA as template and ultra-pure water in a total volume of 25 µL. The reaction mixture was amplified under the following conditions: an initial denaturing step of 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 58°C for 15 s and 72°C for 30 s. All PCR assays were performed in a Mastercycler Personal DNA thermal cycler<sup>6</sup>. Amplified fragments were electrophoresed in a 1.5% (wt/vol) agarose gel in Tris-borate buffer (0.045 M Tris-borate, 0.001M EDTA). Agarose gel was stained with ethidium bromide (0.5 µg/mL) and

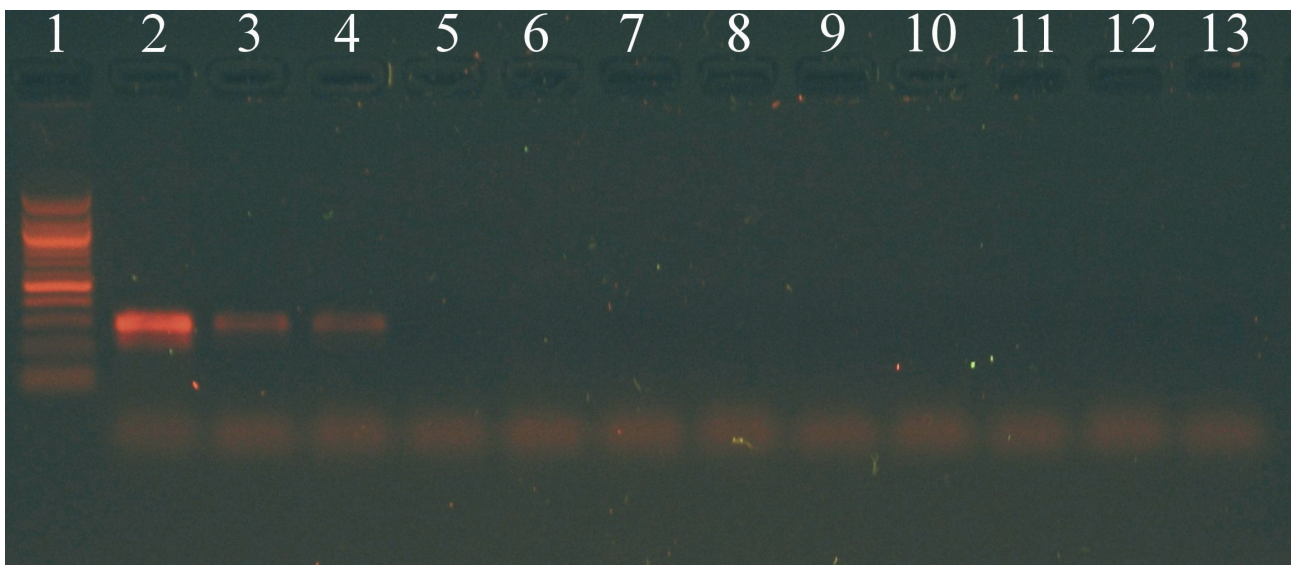
recorded by digital image capture. *C. jejuni* (FIOCRUZ 419) was used as positive control, while negative controls consisted of all PCR components, except the DNA template, and also *C. fetus* subsp. *fetus*, respectively. All PCR reactions were made in duplicate.

#### RESULTS

All samples analyzed were negative for the presence of thermophilic *Campylobacter* by the current isolation procedure. *Campylobacter* was identified only in the positive control plates, where it was grown pure. All plates, either mCCDA or Campy-Cefex Agar, showed an abundant growth of contaminant cells that were phenotypically incompatible with *Campylobacter*. Although PCR assays were all negative, PCR positive control showed the 287 bp DNA fragment amplified (Figure 1). Neither reaction containing DNA extracted from sterilized Bolton Broth aliquots nor PCR negative controls were amplified by PCR. Since poultry meat analyzed were negative for the presence of thermophilic *Campylobacter*, it was not possible to access any difference between chilled or frozen storage on the bacteria survival.

#### DISCUSSION

Thermophilic *Campylobacter* has been isolated from between 71.3% [7] and 99.0% [11] of broiler chicken carcasses collected in Brazilian slaughterhouses. In spite of this high prevalence, little



**Figure 1.** PCR detection of a 287 bp sequence from 16S rRNA gene in thermophilic *Campylobacter*. 1= 100-bp ladder; 2-4= Positive control (*C. jejuni* strain and *C. jejuni* enriched in Bolton Broth, respectively); 5-7= negative reaction (chilled samples); 8-10= negative reaction (frozen samples), 11-13= negative control (sterilized Bolton Broth, *Campylobacter fetus* and PCR reaction containing no DNA template, respectively).



information about *Campylobacter* in retailed poultry meat is available in Brazil. *Campylobacter* was isolated from 32.6% (17/52) [4] or 75% (12/16) [8] of different chilled poultry meat portions sampled in Brazil, whereas the pathogen was isolated from 59.6% (37/62) of fresh poultry carcasses [1]. However, these results from retailed samples were obtained using different isolation procedures, as compared to the present work, whose samples analyzed were all negative for *Campylobacter*.

In fact, *Campylobacter* is difficult to culture in the laboratory because of its sensitivity to environmental stress [12]. Moreover, low counts of the pathogen may be found in the greater presence of competitor cells; additionally, it may be sublethally injured by food processing conditions, which affect the isolation method performance [2,18]. *Campylobacter* detection by ISO 10272-1:2006 [9] is a validated procedure for food samples which has been used worldwide [17,18]. However, enrichment for 48 h has allowed the growth of contaminants [18]. For this reason, sub-culture in Bolton Broth after 24 h could be more efficient than within 48 h [13], but it was not performed in this work. Furthermore, the contaminants that overgrew on mCCDA or Campy-Cefex Agar plates might be result of proliferation of extended spectrum  $\beta$ -lactamase (ESBL) producing *Escherichia coli*, able to hydrolyze cefoperazone present in Bolton Broth, mCCDA or Campy-Cefex Agar used in the present work [14]. Consequently, it has been suggested that the ISO 10272-1:2006 procedure might underestimate the prevalence of *Campylobacter* in poultry meat [13,14], which was not considered in our results, since negative isolation was confirmed by PCR analysis.

PCR may provide faster results than *Campylobacter* conventional culture. In order to improve the capacity to detect viable *Campylobacter* in poultry meat analyzed, PCR detection was preceded by an enrichment period. Although it might be a time-consuming procedure, enrichment in Bolton Broth allows primary multiplication of low *Campylobacter* counts in a greater presence of competitor cells [2,15]. Since the presence of blood in enrichment broth can inhibit the PCR reaction [10,18], blood-free Bolton Broth was used in this work. As previously described, the addition of blood to Bolton Broth did not improve

*Campylobacter* isolation [10,15], while PCR detection can be more easily applied to blood-free enrichment broth [18].

On the other hand, *Campylobacter* in poultry meat has exhibited different declining patterns during chilled and frozen storage [5,17]. For instance, maximum reduction in viable *Campylobacter* counts on artificially contaminated poultry skin refrigerated at 4°C for 7 days was 0.63 log<sub>10</sub> CFU/g, while a reduction of 3.39 log<sub>10</sub> CFU/g was obtained after 2 weeks at -20°C [3]. The presence of *Campylobacter* in chilled and frozen poultry meat samples was investigated, since it was expected that *Campylobacter* rates were lower in frozen samples. However, because samples were negative at microbiology and molecular analysis, it was not possible associate chilled or frozen storage to *Campylobacter* level in poultry meat sampled.

## CONCLUSIONS

Retailed poultry meat samples were negative at conventional culture and PCR analysis, which might reflect low rates of thermophilic *Campylobacter* contamination in poultry thighs and drumsticks available at retail in Concórdia, Santa Catarina. However, in order to prevent *Campylobacter* contamination and foodborne disease, continued good hygienic practices at poultry meat processing and handling as well as consumer education should be considered.

## SOURCES AND MANUFACTURES

<sup>1</sup>Oxoid, Basingstoke, UK.

<sup>2</sup>Sigma, St. Louis, USA.

<sup>3</sup>Acros Organics, Belgium.

<sup>4</sup>Promega, Wisconsin, USA.

<sup>5</sup>Integrated DNA Technologies, Iowa, USA.

<sup>6</sup>Eppendorf, Hamburg, Germany.

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