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Study of Thermophilic *Campylobacter* Contamination of a Broiler Batch at Slaughter

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ABSTRACT

***Background*:** Broilers are a reservoir of *Campylobacter* (*C.*), an important causal agent of gastroenteritis mostly associated to handling and consumption of broiler meat. The majority of broiler flocks are colonized by thermophilic *Campylobacter* at the slaughter age, and carcasses might be contaminated throughout the processing line. Since surveillance is crucial to evaluate and improve approaches to reduce *Campylobacter* spread during broiler processing, a cross-sectional study was carried out to detect the level of *Campylobacter* contamination in a broiler at slaughter.

***Materials, Methods & Results*:** Cloacal swabs, caeca and whole carcasses were taken from a broiler flock slaughtered in Southern Brazil. Samples were individually inoculated in Bolton Broth (BB) and incubated at 41.5°C in a microaerobic atmosphere for 44 h, when the enriched culture was inoculated onto modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) and Campy-Cefex Agar (CCA) plates. All plates were incubated at 41.5°C in the microaerobic atmosphere for 44 h. Aliquots of each enriched BB were collected and submitted to polymerase chain reaction (PCR), while the genetic relatedness of isolates was analyzed by pulsed-field gel electrophoresis (PFGE). A total of 3 (9.4%) cloacal swabs were positive for *C. jejuni*. No *Campylobacter* was isolated from any of the caecal contents or broiler carcasses analyzed. In addition, negative mCCDA and CCA plates showed an abundant growth of contaminant cells. The PCR assay detected all thermophilic *Campylobacter* reference strains tested and also the *Arcobacter* species. No amplified product was obtained from the non-related bacterial species analyzed. It was possible to identify 29 (90.6%) cloacal swabs, 32 (97.0%) caecal contents and 31 (100%) broiler carcasses *Campylobacter*-positive by PCR analysis. PFGE typing of the *C. jejuni* isolated resulted in two clearly distinguished genotypes which were grouped into different clusters.

***Discussion*:** The detection of *C. jejuni* in only few cloacal swabs sampled contrasts with higher frequencies of *Campylobacter* previously described in broilers. However, the enrichment culture of fecal samples might be compromised by the many competing non-target bacteria present, which may have prevented the detection of *Campylobacter*-positive samples. In addition, the BB and selective media containing cefoperazone might have allowed the growth of cefoperazone-resistant contaminant cells from fecal and carcasses samples, which masked *Campylobacter* cells onto mCCDA and CCA. To improve the detection of *Campylobacter* in broiler samples, alternative antimicrobial supplements or reduction of the time of enrichment has already been suggested. PCR showed a higher number of positive samples, which might reflect the increased ability of the PCR assay to detect either injured cells in conventional enrichment culture or *Campylobacter* that were masked by the proliferation of competing cells onto selective media used. The PCR assay was able to detect all the reference strains of thermophilic *Campylobacter*, but also the related *Arcobacter* species. However, the temperature of incubation of the enriched cultures associated to the selective pressure of the antimicrobials present in the BB restricts the growth of *Arcobacter* and the false-positive results observed using PCR. The subtypes of the *C. jejuni* strains isolated showed that the target broiler flock was simultaneously colonized by more than one *C. jejuni* strain which might be the result of introduction of *Campylobacter* from different sources at farm. PCR analysis showed high *Campylobacter* contamination level of the target flock at slaughter, pointing to the need for additional studies to investigate *Campylobacter* sources at broiler processing.

Keywords: *Campylobacter*, broiler, broiler carcass, enrichment culture, PCR, PFGE.

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INTRODUCTION

Broilers are considered a reservoir of thermophilic *Campylobacter* (*C*.) species which are thought to be an important cause of bacterial gastroenteritis mostly associated to handling and consumption of contaminated raw or undercooked broiler meat worldwide [7]. At slaughter, over 90% of the flocks might be colonized by thermophilic *Campylobacter* [8,11,31], which is found in high numbers in the intestinal content [12,26,30].

Since high numbers of thermophilic *Campylobacter* might be present in the intestinal tract but also in feathers and skin of broilers, it can be found throughout the slaughter line, whose contamination of broiler carcasses may occur after visceral breakage at evisceration [1,12,26,30], during scalding [1,26], defeathering [1,2,27] or cross-contamination in the processing line [1]. *Campylobacter* has been identified in broiler carcasses sampled after chilling in levels up to 6.7 log10 CFU per carcass [1,17,26].

Despite the high levels of *Campylobacter* in the intestinal contents, the overall proportion of positive broiler carcasses is relatively low following the processing of contaminated flocks [15,30], which might be attributed to the technology used at the slaughterhouse associated to control measures based on good hygienic practices [26]. Nevertheless, surveillance is crucial to evaluate and improve approaches to reduce *Campylobacter* contamination during broiler processing [7]. Hence, the purpose of this study was to assess the thermophilic *Campylobacter* contamination in a commercial broiler batch at the slaughter line.

MATERIALS AND METHODS

Broiler samples

Sampling took place in May 2010 in a broiler slaughterhouse in Southern Brazil which operates in accordance to Federal inspection. A total of 32 cloacal swabs, 33 caeca and 31 carcasses were taken from the first 1,000 broilers to be slaughtered in the working day under the slaughterhouse schedule. The number of samples was estimated based on an expected prevalence of 25%, with a precision of 15% and a confidence of 95%. Cloacal swabs were collected in the hanging-on area and were individually placed in screw-cap tubes with Cary-Blair transport medium. Intact broiler caecum were collected during evisceration and were individually placed in plastic bags, while whole carcasses were sampled from the processing line immediately after chill tank cooling and individually placed in plastic bags. All samples were transported to the laboratory in insulated boxes with ice packs and processed within 7 h of sampling.

Thermophilic Campylobacter isolation procedure

At the laboratory, cloacal swabs were individually inoculated in 15 mL of Bolton Broth[1] (BB) plus supplements (0.02 g/L cefoperazone, 0.02 g/L vancomycin, 0.02 g/L trimethoprim lactate and 0.01 g/L amphotericin B)[2] with no lysed defibrinated horse blood. Caecal contents were aseptically collected and inoculated in BB in a 1:10 ratio. Neck and breast skin fragments were collected from each broiler carcass and individually inoculated in BB in a 1:10 ratio. Samples were incubated at 41.5°C in a microaerobic atmosphere (5% O_2, 10% CO_2 with the balance N_2)[3] for 44 h (±4 h), when a sterile loop was used to inoculated the enriched culture onto duplicate modified Charcoal Cefoperazone Deoxycholate Agar[1] (mCCDA) plates plus supplements (0.032 g/L cefoperazone and 0.01 g/L amphotericin B)[2] and Campy-Cefex Agar (CCA) [6] plates plus 5% lysed ovine blood and supplements (0.033 g/L cefoperazone and 0.2 g/L cycloheximide)[2], as the second isolation medium. All plates were incubated at 41.5°C in the microaerobic atmosphere for 44 h (±4 h). Typical or suspect bacterial colonies were subcultured onto Blood Agar no. 2[1](BA2) plates for confirmation, which were incubated in the microaerobic atmosphere at 41.5°C for 24 h to 48 h. Gram negative colonies exhibiting curved or spiral rods were presumptively identified as *Campylobacter* and were tested for catalase, oxidase, hippurate hydrolysis and hydrolysis of indoxyl acetate [13]. A strain of thermophilic *Campylobacter* (FIOCUZ 419) was inoculated in BB and used as positive control.

Genomic DNA extraction

Two non-phenolic genomic DNA extraction protocols were performed; both applied to cloacal swabs, caecal contents and skin fragments individually enriched in BB for 44 h (±4 h). In the protocol A [3], a 0.1 mL aliquot of each enriched culture was added to 40 µL of a suspension of diatomaceous silica[2] and 0.9 mL of lysis buffer (5 M guanidinum thiocyanate, 0.1 M Tris-HCl pH 6.4, 0.2 M EDTA pH 8.0,

2.6%[v/v] Triton X-100), mixed thoroughly and incubated at room temperature for 10 min. Suspension was centrifuged at 12,000 X g for 15 s and the pellet obtained was washed twice in the washing buffer (5 M guanidinum thiocyanate, 0.1 M Tris-HCl pH 6.4), following by washes in 70% ethanol and acetone. DNA was resuspended in Tris-EDTA (TE; 10mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -20°C until required.

According to protocol B [24], a 0.4 mL aliquot of each BB enriched 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 phosphate--buffered saline (PBS; 0.01 M phosphate buffer pH 7.4; 0.85% NaCl), lysed with 5 M guanidinum thiocyanate, 100 mM EDTA and 5% (v/v) sarcosine at room temperature for 10 min. Lysed suspensions were mixed with 0.25 mL of 2.5 M ammonium acetate and kept in ice for 10 min. Further, chloroform and isoamyl alcohol (24:1) (v/v) were added, and the phases were mixed thoroughly, centrifuged at 12,000 X g at 4°C for 10 min. Next, 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, resuspended in TE and stored at -20°C until required. Concentration of genomic DNA obtained by both protocols was determined by absorbance readings at 260 nm and 280 nm.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) assay was performed to detect a 287 base pairs (bp) sequence of the 16S rRNA gene from *C. jejuni*, *C. coli* and *C. lari* [16]. The relative PCR sensitivity was determined by 10-fold serial dilutions of a thermophilic *Campylobacter* strain culture (FIOCRUZ 419) previously enriched in BB and incubated at 41.5°C in the microaerobic atmosphere for 44 h (±4 h), whose genomic DNA was extracted using both non-phenolic protocols described above. Aliquots of 100µL of each dilution from 10^{-1} up to 10^{-6} were plated out for counting the CFU. Two replicates of each serial dilution were performed to determine the minimum number of CFUs detectable by PCR assay. *C. jejuni* subsp. *jejuni* (ATCC 33560), *C. jejuni* subsp. *doylei* (ATCC 49349), *C. coli* (ATCC 33559), *C. lari* (ATCC 35221), *C. upsaliensis* (ATCC 43953), *Arcobacter* (*A*.) *butzleri* (ATCC 49616), *A. skirrowii* (ATCC 51132), *Escherichia coli* (ATCC 25922) and *Salmonella Enteritidis* (ATCC 11826) strains were used for the PCR specificity determination.

PCR reactions consisted of 1X Go*Taq* Flexi buffer[4], 2.0 mM $MgCl_2$[4], 200 mM of each dNTP[4], 12 pMol of each forward (5'-CTGCTTAACACAAGTTGAGTAGG-3') and reverse (5'-TTCCTTAGGTACCGTCAGAA-3') primers[5] [16], 1 U of Go*Taq* DNA polymerase[4], 2 µL of genomic DNA as template and water in 25 µL. PCR reactions were performed using genomic DNA obtained by protocol A and protocol B, respectively. PCR amplification was carried out in a Mastercycler Personal DNA Thermal Cycler[6] with 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. Amplified products were electrophoresed in a 1.5% (v/v) agarose gel in Tris-Borate buffer (TBE; 0.045 M Tris-borate, 0.001 M EDTA) which was stained with ethidium bromide (EtBr; 0.5 µg/mL). DNA from a strain of thermophilic *Campylobacter* (FIOCRUZ 419) was the positive control, while negative control consisted either of all reagents except the template DNA or a reaction containing DNA of *C. fetus* subsp. *fetus*. Randomly selected PCR products from positive samples were purified using the GFX PCR Purification kit[7], marked by BigBye Terminator 3.1 Cycle Sequencing[8] and sequenced on an ABI 3130 Genetic Analyzer[8]. Data was collected with Data Collection 1.0.1[8]. DNA sequences were compared to the published sequences for thermophilic Campylobacter with BLAST and aligned by Clustal W.

Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed as previously described [28]. Briefly, cell suspensions were prepared by removing *Campylobacter* colonies from BA2 plates with a cotton swab, suspended in PBS and adjusted to1.8 X 10^9 CFU. DNA in agarose plugs was prepared with the adjusted cell suspension, lysed with 50 mM Tris, 50 mM EDTA pH 8.0, 1% sarcosine (v/v), and 0.1 mg of proteinase K/mL; and incubated in restriction enzyme mixture containing 40 U of *Sma*I[9] at room temperature for 2 h. DNA fragments were electrophoresed with an initial switch time of 6.75 s and a final switch time of 38.35 s in a CHEF Mapper system[10] on a 1% agarose gel PFGE certified[10] with 0.5X TBE as running buffer for 18 h at 6 V/cm and angle of 120°. DNA of *Salmonella* Typhimurium LT2 strain digested with *Xba*I[9] was used as size standard. Agarose gel was stained by EtBr (1 µg/ml). DNA macrorestriction patterns were analyzed by BioNumerics[11] software. Similarity was calculated by

the Dice coefficient and a dendrogram was generated by cluster analysis using the unweighted pair group method with arithmetic averages (UPGMA). *C. fetus* subsp. *fetus* and also thermophilic *Campylobacter* field strains (FIOCRUZ 419, 1006 and 1012) were used as controls.

RESULTS

Thermophilic Campylobacter isolation

A total of three (9.4%) cloacal swabs were positive for thermophilic *Campylobacter*. Two cloacal swabs were found to be *Campylobacter*-positive in both mCCDA and CCA, while another one was positive only in mCCDA. Cells from these colonies were Gram negative stained, exhibited curved shape and were positive upon oxidase, catalase, hippurate hydrolysis and hydrolysis of indoxyl acetate tests, which characterized *C. jejuni*. No thermophilic *Campylobacter* was isolated from any of the caecal contents or broiler carcasses analyzed. In addition, negative enriched cultures plated onto mCCDA and CCA showed an abundant growth of contaminant cells. Positive control (thermophilic *Campylobacter* FIOCRUZ 419) was recovered from the enriched culture streaked both onto mCCDA and CCA.

Polymerase Chain Reaction

Absorbance readings showed similar average recovery of genomic DNA from BB enriched cultures using both protocol A (438.6 ng/μL) and protocol B (422.4 ng/μL). However, because PCR assay produced detectable amplification with mean concentrations of 7.1 X 10^1 CFU/mL (protocol A) and 7.1 X 10^3 CFU/mL (protocol B), PCR results obtained with DNA extracted using protocol A were considered. The PCR assay detected all thermophilic *Campylobacter* reference strains analyzed and also the related species *A. butzleri* and *A. skirrowii*. No amplified product was obtained from non-related bacterial species analyzed.

According to the amplification of a 287 bp product in length (Figure 1), it was possible to identify 29 (90.6%) cloacal swabs, 32 (97.0%) caecal contents and 31 (100%) broiler carcasses *Campylobacter*-positive by PCR analysis. Positive PCR control yielded the expected product whereas any amplification was visualized in the negative control. DNA sequences obtained from the amplified products showed 99% nucleotide identity to the thermophilic *Campylobacter* 16S rRNA sequences published in GenBank.

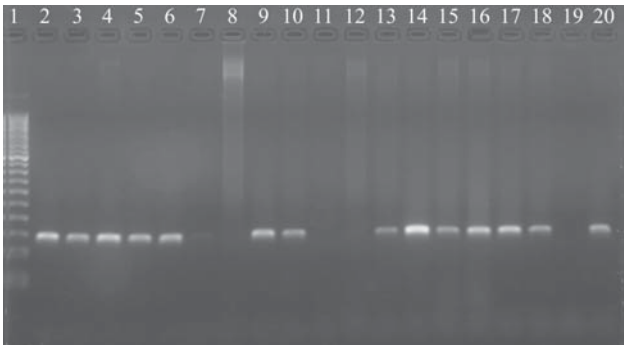

Figure 1. PCR detection of a 287 bp product from thermophilic *Campylobacter* in cloacal swabs, caecal contents and broiler carcasses. 1: 100-bp DNA ladder; 2-7, 9-10, 13-18: positive samples; 8,11,12: negative samples; 19: negative control (PCR reaction containing no genomic DNA); 20: positive control (thermophilic *Campylobacter* strain FIOCRUZ 419).

Pulsed-Field Gel Electrophoresis

PFGE typing of the three *C. jejuni* isolated from cloacal swabs resulted in two genotypes that were clearly distinguished from the non-related *Campylobacter* strains included as DNA macrorestriction controls. The genetic diversity of both PFGE profiles from *C. jejuni* allowed them to be grouped into different clusters (Figure 2).

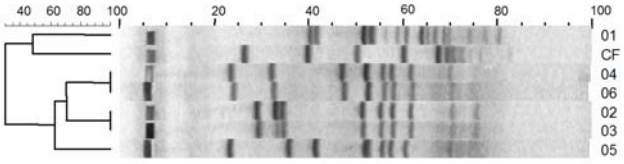

Figure 2. Dendrogram derived from PFGE profiles of *Campylobacter jejuni* isolated from cloacal swabs. 01, 02, 03: FIOCRUZ 419, 1006 and 1012 (thermophilic *Campylobacter* field strains used as control, respectively); 04, 05, 06: *Campylobacter jejuni* strains isolated from cloacal swabs; CF: *Campylobacter fetus* subsp. *fetus* (control). Similarity was calculated by Dice coefficient and cluster analysis was generated by UPGMA.

DISCUSSION

Broiler meat is established as a significant source of thermophilic *Campylobacter*, and detection of the bacteria in the various stages of the broiler processing can help to evaluate control measures based on good hygienic practices implemented by the industry. For this reason, conventional enrichment culture is widely used to detect thermophlic *Campylobacter* in broiler samples at the processing line [11,12,26]. *C. jejuni*, which causes the majority of human cases of *Campylobacter*-associated gastroenteritis [7], was detected in 9.4% of the cloacal swabs taken from the broiler flock slaughtered. This finding contrasts which other studies that showed much higher frequencies of *Campylobacter* in broilers at the pre-harvest age. *Campylobacter* spp. was isolated from 77.2% of caecal contents from French broiler flocks [12] or 80.4% of

cloacal swabs from Brazilian flocks [17]. On the other hand, a low *Campylobacter* percentage (4.9%) has already been detected in broiler samples collected in processing lines in Brazil [5]. It must be considered that direct comparison between results obtained in different studies might be difficult, because of the strategy of sampling, transport conditions and the wide variety of culturing methodologies used.

The large number of viable *Campylobacter* cells in the intestinal content of broilers [12,26,30] allows detecting the bacteria by direct plating onto selective media [23]. On the other hand, the enrichment culture of fecal samples can be severely compromised by the many competing non-target bacteria present in the sample [23,29], which might have prevented the detection of cloacal and caecal contents *Campylobacter*-positive samples in this study. It is important to note that recovery of *Campylobacter* from samples might be influenced by the sensitivity of the selective media [10,25]. Therefore, CCA was used as a second isolation media, which failed to detect *C. jejuni* from one cloacal swab positive by mCCDA. Although both CCA and mCCDA have already showed similar efficiency to detect *Campylobacter* from samples [6,25], direct culture onto mCCDA has been evaluated as a most sensitive method for detection of *Campylobacter* in caecal contents [29].

In spite of the highly contaminated broilers, a quantitative reduction of *Campylobacter* on carcasses from positive flocks has been observed throughout the slaughter line [1,26,30]. Otherwise, *Campylobacter* cells might be injured by some further processing procedures of carcasses, such as freezing [4,27,30]. However, because *Campylobacter* has already been detected in nearly all carcasses from low colonized broiler flocks [1], it was expected to find *Campylobacter*-positive carcasses from the target *C. jejuni*-colonized broiler batch. Currently, BB enriched culture is recommended to detect these low numbers of *Campylobacter* in food products [13]. Nevertheless, the growth of cefoperazone-resistant non-*Campylobacter* cells has been described in BB enriched cultures of broiler meat plated onto selective media containing cefoperazone [9,14,22]. In line with these thoughts, the diffuse growth of the non-*Campylobacter* cells identified in samples plated onto mCCDA and CCA after enrichment in BB might be overlooked the low number of *Campylobacter* in the carcasses sampled, leading to false-negative results. A combination of other enrichment broth or selective media [10,14], addition of alternative antimicrobial supplement than cefoperazone [22], or reduction of the time of enrichment [10,20] have already been suggested to improve the detection of *Campylobacter* in broiler meat.

Because of the difficulty to culture *Campylobacter*, the enriched cultures were also analyzed by PCR. In contrast to the low recovery of *Campylobacter* by the conventional enrichment culture, PCR assay showed a higher number of thermophilic *Campylobacter*-positive samples. This might reflect the increased ability of the PCR assay to detect injured cells that otherwise would be undetectable by conventional enrichment culture or the detection of *Campylobacter* which were masked by competing microflora onto mCCDA and CCA plates. As previously described, recovery of *Campylobacter* onto mCCDA was negatively affected by overgrowth of other competing bacteria after enrichment; however, *Campylobacter* detection from these enriched cultures by PCR was not affected [18]. Overall, the enrichment step prior to the PCR assay ensured this higher level of *Campylobacter* detection by increasing the number of the target cells and reducing the chance of detection of dead cells [16]. PCR reaction detected all reference strains of thermophilic *Campylobacter* tested, including *C. upsaliensis*. Although the PCR assay was able to detect the close-related *Arcobacter* species tested, cultures enriched in BB at a similar temperature (42°C) prevent the growth of *Arcobacter* spp. [6,21]. After that, the incubation temperature of 41.5°C of the enriched cultures associated to the selective pressure of the antimicrobials present in the BB seen to restrict the growth of *Arcobacter* species and the further false-positive results on PCR analysis of the samples analyzed.

On the other hand, the genetic characterization of strains helps the identification of potential sources of contamination of broilers and determination of routes of transmission to more accurately limit the spread of *Campylobacter* infections in flocks [28]. Although only a few number of *C. jejuni* strains was subtyped by PFGE, the two non-related *SmaI* PFGE patterns showed that the target broiler flock was simultaneously colonized by more than one *C. jejuni* strain. According to previous studies, broiler flocks might be colonized by different subtypes of *Campylobacter* at the slaughter

age [11,19]. Although any definitive conclusion must be drawn from our finding, the identification of different genotypes of *C. jejuni* might be result of the introduction of *Campylobacter* from different sources at farm [11].

CONCLUSIONS

Conventional enrichment culture allowed detecting *C. jejuni* in 9.4% of the cloacal swabs analyzed, whereas the genotypes of the isolates showed that the target broiler flock was colonized by two distinct strains, which might be related to introduction of *Campylobacter* from different sources at farm. The highest number of *Campylobacter*-positive samples detected by PCR analysis may reflect the increased ability of the PCR assay to detect either injured thermophilic *Campylobacter* cells that otherwise were undetectable during enrichment culturing, or *Campylobacter* that were masked by the proliferation of contaminant cells onto selective media used. Finally, PCR analysis showed high *Campylobacter* contamination level of the target flock at slaughter, pointing to the need for additional studies to investigate *Campylobacter* sources at broiler processing.

SOURCES AND MANUFACTURES

[1]Oxoid, Basingstoke, UK.
[2]Sigma, St. Louis, USA.
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[6]Eppendorf, Hamburg, Germany.
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