Low contamination of *Campylobacter* spp. on chicken carcasses in Minas Gerais state, Brazil: Molecular characterization and antimicrobial resistance

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**Abstract**

Here, we evaluated *Campylobacter* contamination on chicken carcasses and phenotypic and genotypic profiles of antimicrobial resistance of the isolated strains. A total of 95 of samples were collected from 19 slaughterhouses from Minas Gerais - Brazil, and analyzed by MPN-PCR method. *Campylobacter* was found in 16.8% of samples with microbial load ranging from 60 to 184 MPN/carcass. All isolates were resistant to at least 5 (31.2%) of the antimicrobials screened using the disk diffusion method. Thr-86-Ile mutation, *blaOxA-61* and *tet(O)* genes were found in 95%, 100% and 40% resistant isolates to ciprofloxacin, ampicillin and tetracycline, respectively. Almost all isolates (90%) showed the three genes required to synthesize the CmeABC efflux system. The use of efflux pump inhibitor (PAfIN) resulted in a significant reduction in the MICs of antimicrobials (2-128 fold), indicating the importance of efflux systems in conferring antimicrobial resistance. *Campylobacter* were detected at low concentrations in Brazilian chicken carcasses. However, high-levels of antimicrobial resistance were observed and associated with several mechanisms. This study provides a baseline survey on contamination of *Campylobacter* in Brazilian chicken carcasses and its antimicrobial resistance, giving support for actions directed at reducing this pathogen in the food chain.

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1. Introduction

*Campylobacter* spp. has been recognized as a major cause of foodborne disease worldwide. In 2011 the European Food Safety Authority (EFSA, 2013) reported that the total number of campylobacteriosis cases was 220,209 in the European Union, and it has followed a significant increasing trend in the last years. In the United States, this pathogen was the second most common infection with 14.3 cases reported per 100,000 population in FoodNet in 2012 (CDC, 2013). Although it is generally recognized that there are many contamination sources of this pathogen, chicken meat and by-products are the most important vehicles for human campylobacteriosis (Humphrey, O'brien, & Madsen, 2007). Poultry are considered natural reservoirs for this pathogen, and carcass contamination occurs more frequently via crop leakage and intestinal rupture during the evisceration stage in slaughterhouses (Smith et al., 2007). A world literature survey showed that about 58% of chicken carcasses and by-products are contaminated by *Campylobacter* spp., but great variations in the findings were observed (Suzuki & Yamamoto, 2009). Despite the importance of this pathogen, the epidemiological and risk assessment studies are recent in Brazil, and there are no legal standards yet established for its presence or counts in foods. Researches in this field become absolutely necessary in Brazil because this country is the world’s largest exporter of chicken meat since 2004 and it is the third producer, surpassed only by United States and China. The largest chicken meat producing states are Paraná, Santa Catarina, Rio Grande do Sul, São Paulo and Minas Gerais, according to the 2014 annual report of Brazilian Poultry...
Association (UBABEF, 2014). More recently, concern regarding Campylobacter contamination in foods has increased because of high levels of antimicrobial resistance, including the frequent isolation of multidrug-resistant strains (Mozina, Kurinič, Klancnik, & Mavri, 2011). Antimicrobial resistance among bacterial pathogens isolated from foods has been attributed to the indiscriminate use of antimicrobials in animal husbandry for preventive or therapeutic purposes as well as for increasing the growth rate of animals (Alfredson & Korolik, 2007). The dissemination of resistant Campylobacter to other environments via livestock and foods of animal origin may contribute to the emergence of antimicrobial resistance in humans and complicate effective clinical treatment of campylobacteriosis (Aarestrup & Engberg, 2001). Multiple mechanisms for antibiotic resistance are present in Campylobacter spp., which may be associated with mutational alteration or modification of target molecules, repression of uptake systems, activation of efflux pumps, and inactivation of the antibiotic (Iovine, 2013). Among these, the CmeABC efflux system plays a key role in resistance to a wide variety of compounds including antibiotics, bile salts, dyes and detergents, and has been broadly studied and characterized as a potential target to reduce antimicrobial resistance of Campylobacter spp. (Lin, Michel, & Zhang, 2002).

Resistance of Campylobacter isolates from chicken and by-products to antibiotics such as quinolones, macrolides, tetracycline and ampicillin as well as the molecular mechanisms of this resistance have been reported in several studies (Griggs et al., 2009; Mackiw, Korsak, Rzewuska, Tomczuk, & Różynek, 2012; Obeng et al., 2012). However, there is a lack of information regarding the molecular basis of antimicrobial resistance of Campylobacter isolates in Brazil. Most studies have focused on the prevalence of Campylobacter in food and animals and its phenotypic profiles of antimicrobial resistance (Biasi, Freitas De Macedo, Scaranello Malaquias, & Franchin, 2011; Kuana, Santos, et al., 2008; de Moura et al., 2013). Therefore, to our knowledge, there are no published data about genotypic resistance in Campylobacter isolates from chicken carcasses in Brazil. The objectives of this study were to evaluate Campylobacter contamination on chicken carcasses slaughtered in Minas Gerais State - Brazil and to determine phenotypic and genotypic profiles of antimicrobial resistance of the isolated strains.

2. Materials and methods

2.1. Sample collection

A total of 95 chicken carcasses were collected from 19 slaughterhouses localized in Minas Gerais state, Brazil, during January and February 2012. This is the fifth largest producer and exporting Brazilian state of chicken meat, surpassing 930,000 and 185,000 tons of carcasses produced and exported in 2013, respectively (UBABEF, 2014). All selected establishments were representative of slaughterhouses located in Minas Gerais state, Brazil, regarding slaughtering frequency, numbers of slaughtered poultry and geographical distribution into state. They were previously selected by simple random sampling, representing 56% of the total number of federally inspected slaughterhouses in this Brazilian state. Five samples were randomly selected from each slaughterhouse after the chilling stage. Each carcass was aseptically packed in a sterile plastic bag and transported to the laboratory under cooled conditions. Upon arrival at the laboratory, the carcass samples were weighed and subject to Campylobacter enumeration.

2.2. Enumeration of Campylobacter spp

Previously, each carcass was aseptically transferred into a new sterile plastic bag, to which was added 200 ml of 0.1% buffered peptone water – BPW (Himedia, Mumbai, India), and vigorously shaken by hand for 1 min. This chicken carcass rinse was serially diluted (10-fold) using BPW and analyzed by the Most Probable Number Method (MPN) as described by Scherer, Bartelt, Sommerfeld, and Hildebrandt (2006a) with some modifications. Briefly, aliquots of 1 ml from each dilution (direct homogenate, 10−1 and 10−2) were brought into three MPN-tubes containing 9 ml Preston Enrichment Broth Base (Himedia, Mumbai, India) containing 0.4% charcoal and supplemented with antibiotics (20 mg l−1 of trimethoprim lactate, 15 mg l−1 of cephalothin, 20 mg l−1 of vancomycin, 2,500 U l−1 of polymyxin B and 2 mg l−1 of amphotericin B – Sigma–Aldrich, Saint Louis, USA) and 5% reductive solution FBP (0.5% of ferrous sulfate, sodium metabisulfite, and sodium pyruvate – Vetec, Rio de Janeiro, Brazil). The MPN-tubes were incubated under microaerobic conditions (approximately 2% H2, 5% O2, 10% CO2 and 83% N2) at 42 °C for 24 h using an anaerobic chamber (Forma 1025 Thermo Scientific, USA). Then, 10 µl from each tube were streaked onto Campylobacter Agar Base (Himedia, Mumbai, India) supplemented as previously described for Preston broth. After an incubation time of 48 h at 42 °C under microaerobic conditions, plates were examined for presence of presumptive colonies. At least five colonies per plate were selected, Gram stained and examined by phase-contrast microscopy for typical Campylobacter morphology. One colony per tube-NMP showing typical cellular morphology was isolated and confirmed using polymerase chain reaction (PCR). After molecular confirmation of these colonies, the number of positive tubes at each dilution was determined and the Campylobacter spp. counts present in the rinse fluid and, consequently, in the chicken carcass samples were computed from the statistical MPN table of de Man (1983) (95% confidence interval).

2.3. Confirmation and species identification

The isolates were confirmed and identified using genus- and species-specific PCR assays. DNA extraction was carried out using a boiled-cell method. Fresh cultures of isolates were suspended in 500 µl of sterile ultra-pure water and boiled at 95 °C for 10 min. After centrifugation at 13,000 g for 2 min, the supernatants were collected and stored at −20 °C until use as template DNA in the PCR reactions. Primer sequences for the 16S rRNA gene were used to confirm isolates as belonging to the Campylobacter genus. The species were identified using primer sequences to detect hipo case gene (hipO) specific for Campylobacter jejuni, and siderophore transport gene (ceuE) specific for Campylobacter coli. Amplification were carried out in 25 µl reactions containing 2.5 µl of 10 × PCR buffer; 0.2 mmol l−1 of deoxyribonucleoside triphosphate mix (ATP, GTP, TTP and CTP); 0.4 µmol l−1 of each primer; 2.5 U Taq PCR polymerase (Promega, Madison, USA) and 2 µl of DNA preparation. PCR reaction mixtures were heated at 96 °C for 2 min as an initial denaturation step followed by 30 cycles of denaturation at 95 °C for 30 s, annealing for 45 s and extension 72 °C for 60 s, and a final extension at 72 °C for 5 min. The sequence of primers and annealing temperatures are listed in Table 1. The amplified products were electrophoresed in 1.5% agarose gel containing ethidium bromide (0.5 µg ml−1) at 90 V for 40 min and visualized using a UV gel documentation system (Quantum ST4 1100/26MX – Vilber Lourmat). A 100-bp DNA ladder (New England Biolabs, MA, USA) was used as a standard for molecular size determinations.

2.4. Antibiotic susceptibility screening

The Campylobacter isolates were analyzed for antimicrobial resistance using the agar disk diffusion method (CLSI, 2010). The bacterial suspension was prepared in sterile saline solution (0.85%
Table 1

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence (5’→3’)</th>
<th>Annealing temperature</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>tet(O)</td>
<td>TGATTTTTTATTGAGGCCCAGG</td>
<td>59°C</td>
<td>Denis et al. (1999)</td>
</tr>
<tr>
<td>tet(D)</td>
<td>GCATTTTATTGATTTGAGCG</td>
<td>59°C</td>
<td>Denis et al. (1999)</td>
</tr>
<tr>
<td>blaox-61</td>
<td>AGATCGATCTGCAATCTTGGTG</td>
<td>59°C</td>
<td>Pratt and Korolik (2005)</td>
</tr>
<tr>
<td>gyrA</td>
<td>TTGTTTGAGCAGAGATCTGAT</td>
<td>48°C</td>
<td>Zirnstein et al. (1999)</td>
</tr>
<tr>
<td>cmeA</td>
<td>TTTTGAGCTGCAGTGCAGGAGGC</td>
<td>50°C</td>
<td>Lin et al. (2002)</td>
</tr>
<tr>
<td>cmeB Cj</td>
<td>GGATCAGATGCTGACAAGCC</td>
<td>50°C</td>
<td>Lin et al. (2002)</td>
</tr>
<tr>
<td>cmeB Cg</td>
<td>AGAATTGATGCTGACAGAATT</td>
<td>54°C</td>
<td>Obeng et al. (2012)</td>
</tr>
<tr>
<td>cmeC</td>
<td>GGTGATGCTTCATTTGCTGGGAAA</td>
<td>50°C</td>
<td>Lin et al. (2002)</td>
</tr>
</tbody>
</table>

References:

C Obeng et al. (2012)
C Pratt and Korolik (2005)
C Denis et al. (1999)
C Linton, Lawson, Owen, and Stanley (1997)
C Lin et al. (2002)
C Godoy, 2001)
C Denis et al. (1999)
C H.M. Hungaro et al. / Food Control 51 (2015) 15–22
C for 24 h under microaerobic conditions.
C for 48 h under microaerobic conditions.
C for 5 min. The sequence of primers and annealing temperature for 45 s, and 72°C for 5 min. The sequence of primers and annealing temperatures are listed in Table 1. The various amplicons were electrophoresed and visualized as previously described.

2.6. Effect of efflux pump inhibitor on antimicrobial resistance

To investigate the contribution of efflux systems to resistance to ampicillin, tetracycline, ciprofloxacin (Sigma–Aldrich, Saint Louis, USA) and bile salts (50% sodium cholate and 50% sodium deoxycholate) (Becton Dickinson, Sparks, USA), the minimum inhibitory concentrations (MIC) of these antimicrobials were determined in all isolates using broth microdilution method (CLSI, 2010) in the presence and absence of phenylalanine—arginine β-naphthylamide (PAβN) (Sigma–Aldrich, Saint Louis, USA), a well-known efflux pump inhibitor. The resistance assays were carried out in the 96-well microtiter plates containing a final volume of 100 μl per well of Mueller Hinton broth (HiMedia, Mumbai, India), with addition of testing agents and final inoculum of 10⁵ CFU ml⁻¹. Two-fold serial dilutions of antibiotics and bile salts were performed in the 96-well plates for concentrations of 0.125 to 512 μg ml⁻¹ and 0.05–16 mg ml⁻¹, respectively. The PAβN was used to a final concentration of 5 μg ml⁻¹ defined by a preliminary microdilution assay, and that had no inhibitory effects on bacterial growth for any of the isolates. All of the MIC measurements were carried out in triplicate, and positive and negative control wells were included. The microtiter plates were incubated at 42°C for 48 h under microaerobic conditions. Antibacterial activity was detected using a colorimetric method by adding 30 μl of resazurin staining (0.01%) aqueous solution in each well at the end of the incubation period (at least 4 h before). The MICs were defined as the lowest concentration of an antimicrobial where no metabolic activity is seen after the incubation period, and they were determined by visual observation on the basis of change in resazurin staining (live cells – red; dead cells – blue) (Salvat, Antonacci, Fortunato, Suarez, & Godoy, 2001).

3. Results

A total number of 95 chicken carcasses from 19 slaughterhouses were analyzed for the presence of Campylobacter spp. by the Most Probable Number Method (MPN) associated with PCR confirmation. Sixteen samples from nine slaughterhouses were contaminated with microbial load ranging from 60 to 184 MPN carcass⁻¹ (Table 2). The overall contamination of chicken carcasses with Campylobacter spp. was 16.8%, while the variation in positive samples between the slaughterhouses was 20–60%. C. jejuni was the species most frequently isolated, representing 93.8% of the positive samples, whereas C. coli was present in only one sample. A total of 20 Campylobacter isolates made up of 18 C. jejuni and 2 C. coli isolates were obtained during sampling of typical colonies grown on Campylobacter agar, corresponding to one isolate per positive MPN-tube. An initial screening was performed to identify the antimicrobial susceptibility of 20 Campylobacter strains against 16 antimicrobial agents by using the disk diffusion test. All isolates were resistant to at least 5 (31.2%) of the antimicrobial agents tested (Table 3). The highest resistance rates were noted for cefalosporins (75–100%), ciprofloxacin (95%), nalidixic acid (95%) and trimethoprim-sulfamethoxazole (100%). A similar level of resistance was found for tetracycline (50%) and ampicillin (45%), whereas a lower frequency of resistance was observed toward amoxicillin-clavulanic acid (10%). On the other hand, no resistance to chloramphenicol, gentamicin, imipenem, cefepime or erythromycin was observed in any of the isolates. From these data, we
Eight ampicillin-resistant isolates had a β-lactamase gene (blaOxa-
61), and this fact was also observed in ten sensitive isolates. More-
over, we also investigated the presence of the three genes that
encode the CmeABC efflux pump in Campylobacter spp. These three
genes, named cmrA, cmrB and cmrC, are arranged in an operon, and
encode for a periplasmic fusion protein, an inner membrane drug
transporter and an outer membrane protein, respectively. The cmrB
gene was found in all isolates, whereas cmrA was absent in two
isolates, one of which also showed no cmrC.

We used phenylalanine-arginine β-naphthylamide (PﬁJN) to
inhibit the CmeABC efflux system from Campylobacter isolates in
order to evaluate its effect on transport and resistance to anti-
microbials. A correlation between this efflux system and resistance
to bile salts was observed for almost all isolates, whereas with other
antibiotics it was not so evident. Approximately 90, 55, 25% and 25%
of the isolates had transport of bile salts, ciprofloxacin, ampicillin
tetracycline affected by PﬁJN, respectively. In combination with
PﬁJN, the MIC of ciprofloxacin, tetracycline and ampicillin decreased
from 2 to 8-fold for at least one of the isolates evaluated, whereas
for bile salts the decrease ranged from 2 to 128-fold (Table 4). In addition, our results revealed that the absence of

cmrA and cmrC genes in two isolates did not influence the resis-
tance or transport of antimicrobials.

4. Discussion

Our results revealed low prevalence (16.8%) and low numbers
(60–184 MPN carcass−1) of Campylobacter spp. in chicken carcasses
obtained from slaughterhouses from Minas Gerais state, Brazil.
However, high resistance levels were observed in isolates for most
of the antibiotics, which proved to be caused by different molecular
mechanisms. Campylobacter contamination rates in this type of
food vary widely among countries, including Brazil. In a study of 13
European Union member states performed in 2011, the chicken
carcass contamination rate was 31.3% on average, but ranged from
3.2% to 84.6% (EFSA, 2013). Likewise, a literature survey of studies
performed in Brazil also showed that the occurrence of Campylo-
bacter spp. in chickens and by-products is widespread and ranges from
6.7 to 100% (Franchin, Ogliari, & Batista, 2007; Gonçalves, Yamanaka, Almeida, Chano, & Ribeiro, 2013; de Moura et al., 2013; Oliveira & Oliveira, 2013). These variations in the preva-
ce rates of Campylobacter may be attributed to poultry breeding
methods, hygienic practices during slaughter, sampling strategies
differences in analytical methods (Daskalov & Maramski, 2012).

Although many studies have conﬁrmed the prevalence of
Campylobacter in chicken carcasses, a quantitative risk assessment
of human campylobacteriosis depends on quantitative data, which
has been highlighted as a major hindrance in carrying out such an
assessment in developing countries (FAO/WHO, 2002). The choice
of most appropriate quantification method depends on the ex-
pected level of Campylobacter in the sample examined and any
competitive bacterial flora that may be present (Son, Englen, Berrang, Fedorka-Cray, & Harrison, 2007). In this study, we chose to use the Most Probable Number Method (MPN) associated with
identiﬁcation of Campylobacter by using a species-speciﬁc PCR
technique, which proved to be more suitable for enumeration of
this pathogen in Brazilian chicken carcasses. Our results showed
that the concentration of Campylobacter spp. in chicken carcasses
was low and similar to results reported by Tang et al. (2010), Wong
et al. (2007) and Scherer, Bartel, Sommerfeld, and Hildebrandt
(2006b), who found the contamination of most samples ranging
from <100 MPN g−1, <0.3 to 10 MPN g−1, and <0.3 MPN g−1,
respectively. However, even a low contamination of chicken car-
casses may be of concern to food safety authorities as because

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**Table 2**

<table>
<thead>
<tr>
<th>Slaughterhouses</th>
<th>Sample code</th>
<th>Positive tubes (Dilution)</th>
<th>MPN carcass−1 (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>D-10¹, 10⁻²</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>AS2013</td>
<td>9-1-0</td>
<td>60</td>
</tr>
<tr>
<td>B</td>
<td>B12013</td>
<td>1-0-0</td>
<td>72</td>
</tr>
<tr>
<td>C</td>
<td>C42013</td>
<td>1-0-0</td>
<td>72</td>
</tr>
<tr>
<td>D</td>
<td>D22013</td>
<td>2-0-0</td>
<td>184</td>
</tr>
<tr>
<td>E</td>
<td>E22013</td>
<td>1-0-0</td>
<td>148</td>
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<tr>
<td>F</td>
<td>F12013</td>
<td>2-0-0</td>
<td>184</td>
</tr>
<tr>
<td>G</td>
<td>G12013</td>
<td>1-0-0</td>
<td>72</td>
</tr>
<tr>
<td>H</td>
<td>H42013</td>
<td>1-0-0</td>
<td>72</td>
</tr>
<tr>
<td>I</td>
<td>I52013</td>
<td>0-1-0</td>
<td>72</td>
</tr>
</tbody>
</table>

* Remaining samples showed Campylobacter spp. < 60 MPN carcass⁻¹.

**Table 3**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Antibiotic resistant profile*</th>
<th>MAR index</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS2013</td>
<td>CipNaCphTcrCxtCazSut</td>
<td>0.50</td>
</tr>
<tr>
<td>B12013</td>
<td>CipNaTetAmpCphCtcCxtCazSut</td>
<td>0.63</td>
</tr>
<tr>
<td>C42013</td>
<td>CipNaTetAmpCphCtcCxtCazSut</td>
<td>0.56</td>
</tr>
<tr>
<td>D22013a</td>
<td>CipNaTetAmpAmcCphCtcCxtCazSut</td>
<td>0.69</td>
</tr>
<tr>
<td>D22013b</td>
<td>CipNaTetAmpAmcCphCtcCxtCazSut</td>
<td>0.69</td>
</tr>
<tr>
<td>D22013c</td>
<td>CipNaTetAmpAmcCphCtcCxtCazSut</td>
<td>0.56</td>
</tr>
<tr>
<td>E22013</td>
<td>CipNaCphTcrCxtCazSut</td>
<td>0.50</td>
</tr>
<tr>
<td>E23013a</td>
<td>CipNaCphTcrCxtCazSut</td>
<td>0.44</td>
</tr>
<tr>
<td>E23013b</td>
<td>CipNaCphTcrCxtCazSut</td>
<td>0.50</td>
</tr>
<tr>
<td>E23013c</td>
<td>CipNaCphTcrCxtCazSut</td>
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<td>E32013</td>
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<td>F12013</td>
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<td>0.56</td>
</tr>
<tr>
<td>F12013b</td>
<td>CipNaCphTcrCxtCazSut</td>
<td>0.63</td>
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<tr>
<td>G2013</td>
<td>CipNaTetCxtSut</td>
<td>0.38</td>
</tr>
<tr>
<td>H42013</td>
<td>CipNaCphCtcCxtSut</td>
<td>0.31</td>
</tr>
<tr>
<td>I52013</td>
<td>CipNaCphTcrCxtCazSut</td>
<td>0.44</td>
</tr>
</tbody>
</table>

* Ciprofloxacin (Cip) 5 μg; Nadifloxin (Nal) 30 μg; Chloramphenicol (Clo) 30 μg; Tetracycline (Tet) 30 μg; Gentamycin (Gen) 10 μg; Imipenem (Imp) 10 μg; Ampicillin (Amp) 10 μg; Amoxicillin-clavulanic acid (Amc) 10 μg; Cephalothin (Cf) 30 μg; Cefoxitin (Clo) 30 μg; Cefuroxime (Cfx) 30 μg; Cefotaxime (Ctx) 30 μg; Cefazidime (Caz) 30 μg; Cefepime (Cpm) 30 μg; Trimethoprim-sulfamethoxazole (Sut) 23.75/1.25 μg; Erythromycin (15 μg).

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Campylobacter strains are highly infectious, with the infective dose being as low as 800 cells (Black, Levine, Clements, Hughes, and Blaser, 1988). A number of authors have quantified Campylobacter on chicken carcasses using enumeration methods other than MPN, and they observed that counts ranged from <1.0 to 8.0 log_{10} CFU carcass^{-1} (Chrystl, Hargraves, Boa, & Ironside, 2008; Figueroa, Troncoso, López, Rivas, & Toro, 2009; Johannessen, Johnsen, Økland, Cudjoe, & Hofshagen, 2007; Kuan, dos Santos, et al., 2008; Stern et al., 2007). However, it is difficult to compare results across studies, owing to the use of different materials and enumeration methods, which consequently impedes the risk assessment studies (Scherer et al., 2006a).

The most prevalent species in this study was C. jejuni detected in 93.8% of positive findings, similar to what has been observed in other studies researching prevalence of this pathogen in chicken and by-products (Garin et al., 2012; Tang et al., 2010; Zhao et al., 2010). The higher rate of detection of C. jejuni in several studies may be a reflection of the methods used, rather than the actual incidence in food (Lynch, Caygner, Mcdowell, & Duffy, 2011). Besides C. jejuni and C. coli, other Campylobacter species were not detected by our study. Nonetheless, Campylobacter lari, Campylobacter upsaliensis, Campylobacter concisus and Campylobacter fetus have also been isolated from chicken and by-products in other studies, even if at a lower frequency (Garin et al., 2012; Lynch et al., 2011).

Our study also represents one of the few reports on antibiotic susceptibility of Campylobacter spp. in chicken carcasses from Minas Gerais, Brazil. We used disk diffusion test to perform an initial screening in order to investigate antimicrobial resistance of 20 Campylobacter isolates against 16 antimicrobials. All Campylobacter spp. evaluated in this study were resistant to at least five antimicrobials, thus characterized as multidrug-resistant isolates. The percentage of antibiotics to which a particular isolate was resistant ranged from 31% to 69%. We observed a generalized resistance to first- and second-generation cephalosporin, namely cephalexin, cefoxitin and cefuroxime, whereas some isolates were susceptible to third-generation cephalosporin, cefotaxime and ceftazidime. Surprisingly, all isolates were susceptible to cefepime, a fourth-generation cephalosporin. Similar results were observed by Gritchina, Mishchuk, and Pozhalostina (2005), who demonstrated high resistance of Campylobacter to the first-generation cephalosporins, and general susceptibility to cefepime. The cephalosporins belong to a diverse class of compounds named ß-lactam antibiotics and individual differences among these molecules, such as the amount and variety of side chains, may explain the variation in antimicrobial resistance (Iovine, 2013). In addition, high resistance of Campylobacter to ß-lactams may be a consequence of selective pressure caused by isolation and quantification methods, whose culture media are supplemented with antibiotics of this class to inhibit enteric flora contaminants (Corry, Post, Colin, & Laisyne, 1995; Jacob, Mdegela, & Nonga, 2011).

Resistance to a large number of ß-lactam antimicrobial agents is becoming more widespread in foodborne bacteria, including Campylobacter (Li, Mehrrota, Ghimire, & Adewoye, 2007). Besides cephalosporins, we evaluated the antimicrobial resistance of Campylobacter to other ß-lactams, including ampicillin, amoxicillin-clavulanic acid and imipenem. We observed a high level of resistance to ampicillin, which was similar to that reported in other studies (Griggs et al., 2009; Obeng et al., 2012). In contrast, all isolates were susceptible to imipenem, and most of them also to aztreonam and ceftazidime. In previous studies, these antimicrobial agents have also shown excellent in vitro activities against Campylobacter spp. (Griggs et al., 2009; Hakanen, Lehtopolku, Siitonen, Huovinen, & Kotilainen, 2003). Our results suggest that these agents might be candidates for clinical trials in enteritis caused by multi-drug resistant Campylobacter. Nevertheless, there are few data on the clinical efficiency of ß-lactams for the treatment of infections of this nature (Hakanen et al., 2003).

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC of antibiotics (mg l^{-1})</th>
<th>Resistance genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cip (Tet)</td>
<td>Amp</td>
</tr>
<tr>
<td>A52013</td>
<td>4^R</td>
<td>0.5</td>
</tr>
<tr>
<td>B12013</td>
<td>8^R</td>
<td>128</td>
</tr>
<tr>
<td>C42013</td>
<td>8^R</td>
<td>256</td>
</tr>
<tr>
<td>D22013a</td>
<td>8^R</td>
<td>64^R</td>
</tr>
<tr>
<td>D22013b</td>
<td>32^R</td>
<td>16</td>
</tr>
<tr>
<td>D32013</td>
<td>8^R</td>
<td>&lt;0.12^R</td>
</tr>
<tr>
<td>D42013a</td>
<td>32^R</td>
<td>16</td>
</tr>
<tr>
<td>D42013b</td>
<td>8^R</td>
<td>64^R</td>
</tr>
<tr>
<td>E22013</td>
<td>4^R</td>
<td>&lt;0.12^R</td>
</tr>
<tr>
<td>E32013a</td>
<td>8^R</td>
<td>&lt;0.12^R</td>
</tr>
<tr>
<td>E32013b</td>
<td>16^R</td>
<td>&lt;0.12^R</td>
</tr>
<tr>
<td>E52013</td>
<td>8^R</td>
<td>64^R</td>
</tr>
<tr>
<td>F12013a</td>
<td>4^R</td>
<td>128</td>
</tr>
<tr>
<td>F12013b</td>
<td>8^R</td>
<td>512^R</td>
</tr>
<tr>
<td>F22013</td>
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<td>128</td>
</tr>
<tr>
<td>F32013</td>
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<td>64^R</td>
</tr>
<tr>
<td>G12013</td>
<td>8^R</td>
<td>&lt;0.12^R</td>
</tr>
<tr>
<td>G52013</td>
<td>8^R</td>
<td>&lt;0.12^R</td>
</tr>
<tr>
<td>H42013</td>
<td>0.5^R</td>
<td>0.5</td>
</tr>
<tr>
<td>I2S013</td>
<td>4^R</td>
<td>&lt;0.12^R</td>
</tr>
</tbody>
</table>

-P: absence of phenylalanine-arginine ß-naphthylamide (PA[NI]); +P: presence of phenylalanine-arginine ß-naphthylamide (PA[N]). - resistant; ß sensitive; (+) presence of genes; - absence of genes. Breakpoint - ciprofloxacin ≥4 mg l^{-1} (Cip); tetracycline ≥16 mg l^{-1} (Tet); and ampicillin ≥32 mg l^{-1} (Amp) (CLSI, 2012 – Enterobacteriaceae). Values highlighted in bold indicate difference in the MIC in presence of the EPI.
Pileggi, and Pavia (2013) from Campylobacter isolated from chicken carcasses in Brazil and Italy, respectively.

Alarmingly, we observed high resistance to quinolones (ciprofloxacin and nalidixic acid) and tetracycline. High levels of Campylobacter resistant to these antibiotics have also been reported by other authors (EFSA, 2012; Mackiw et al., 2012). The rates of resistance to quinolones and tetracycline vary worldwide, and in the last years the high prevalence of Campylobacter spp. resistant to these drug classes has been increasingly frequent and worrying (Alfredson & Korolik, 2007; EFSA, 2012; Mackiw et al., 2012). These concerns have become more severe because fluoroquinolones and tetracycline, along with macrolides, are used for treating human infections (Mackiw et al., 2012; Obeng et al., 2012).

Overall, we observed both similarities and differences amongst our results for antimicrobial resistance and those reported by other authors. These variations in resistance profiles of Campylobacter spp. might be expected, and occur due to the variations in the use of antibiotics among countries, the origin and type of the samples, the geographical distribution of resistant species and the methodology applied to evaluate susceptibility (Wassenaar et al., 2009; Wilson, 2003; Zhao et al., 2010). The occurrence of high resistance rates to some of the antibiotics investigated in this study could be explained by these usage as growth promoters in animal feed and veterinary medicine (Iovine & Blaser, 2004), and reflects the extent to which these antimicrobial agents are used in Brazil.

In order to investigate the molecular basis of antimicrobial resistance of Campylobacter isolates, we evaluated the presence of resistance and efflux system genes as well as the minimum inhibitory concentrations in the absence and presence of the efflux pump inhibitor for three antibiotics selected and bile salts. Bile salts were included in this last assay because their resistance in Campylobacter has proved to be mediated by CmeABC efflux system (Lin, Sahin, Michel, & Zhang, 2003). The high resistance to ciprofloxacin observed in this study was strongly correlated with the Thr-86-Ile modification of GyRA. This mutation is the main mechanism of resistance to fluoroquinolones in Campylobacter, and has been frequently reported in other studies (Mackiw et al., 2012; Wieczorek, Kania, & Osek, 2013; Zirmstein, Li, Swaminathan, & Angulo, 1999). In our study only one isolate was resistant to ciprofloxacin and did not show the Thr-86-Ile mutation, and therefore its resistance may be associated with other mechanisms, such as other mutations in the gyrA, and multidrug efflux systems (Lin et al., 2002; Luo, Sahin, Lin, Michel, & Zhang, 2003). On the other hand, a relatively low percentage (40%) of the tetracycline-resistant isolates showed the tet(O) gene, which encodes a protein that protects the ribosome from the inhibitory effect of this antibiotic (Dasti et al., 2007). Low correlation between tetracycline resistance and tet(O) gene was also observed by Obeng et al. (2012). Whilst, in other studies high levels of phenotypic resistance to tetracycline were attributed almost solely to presence of this gene (Mackiw et al., 2012; Wieczorek et al., 2013). Beyond the tet(O) gene, nonspecific efflux systems such as CmeABC multidrug efflux pump may decrease susceptibility to tetracycline (Gibreel, Wetsch, & Taylor, 2007; Iovine, 2013).

Concerning the genetic basis of ampicillin resistance, all resistant isolates showed the blaOxA-61 gene, which is associated with β-lactamase production (Alfredson & Korolik, 2005; Griggs et al., 2005) observed that 91% of the ampicillin-resistant isolates carried the blaOxA-61 gene. However, it is worth noting that in our study many susceptible isolates also showed this ampicillin resistance gene. These results were in agreement with a previous observation by Obeng et al. (2012). They reported that 10 of the ampicillin-resistant Campylobacter isolates from chicken were able to encode the blaOxA-61 gene. Such findings indicate that the blaOxA-61 gene in these Campylobacter isolates may have been inactivated or is not being expressed. Beyond synthesis of β-lactamases, β-lactam resistance may also be conferred by reduced uptake due to alterations in outer membrane porins and efflux systems (Iovine, 2013).

Great variations in bile salts resistance were observed among Campylobacter isolates, which was consistent with the findings from previous studies by Dzieciol, Wagner, and Hein (2011) and Mavri and Smole Mozina (2013). The CmeABC efflux system plays a key role in mediating bile salts resistance and is essential for Campylobacter growth in bile-containing media such as the animal intestinal tract (Lin et al., 2003). The observed difference in bile salts resistance in our study could not be explained by failures in the CmeABC efflux system, since most of the isolates showed the three genes (cmeA, cmeB and cmeC) required for its synthesis, and even the absence of some of them did not influence the levels of resistance. Therefore, other mechanisms of resistance to bile salts such as the presence of porins (Omp50 and PorA) and other efflux systems (CmeDEF and CmeG) are probably involved in these results (Akiba, Lin, Barton, & Zhang, 2006; Bolla, De, & Dorez, 2000; Bolla, Loret, Zalewski, & Pagès, 1995; Jeon, Wang, Hao, Barton, & Zhang, 2011).

The efflux inhibitor (PAβN) slightly decreased the MIC of ciprofloxacin, tetracycline and ampicillin, but did not restore the susceptibility of them in the Campylobacter isolates. The effects of PAβN were mostly of 2–4 fold MIC reduction for these antibiotics. The activity of PAβN on quinolone resistance in C. jejuni and C. coli is controversial. Our results showing the effect of PAβN on ampicillin resistance were similar to those reported by Martinez and Lin (2006), who observed that the MIC of β-lactams (cefotaxime and ampicillin) decreased 2-fold using this EPI. In another study performed by Griggs et al. (2009) the presence of PAβN had no effect on the MICs of β-lactams, including ampicillin, for the Campylobacter isolates or their respective blaOxA-61 mutants. Similarly, Corcoran, Quinn, Cotter, and Fanning (2005) have demonstrated that PAβN did not produce a significant decrease on the resistance to quinolones in Campylobacter. However, significant decreases in the MIC of fluoroquinolones and restoration of susceptibility in Campylobacter isolates were reported by Kunicic, Klanicnik, and Smole Mozina (2012a). With regards to tetracycline, Kunicic et al. (2012a) demonstrated that PAβN caused MIC reductions of at least 2-fold–64-fold, and this EPI also restored susceptibility to some tetracycline-resistant isolates. In contrast, lower effects of PAβN on tetracycline MIC (1.3–2.6-fold decrease) were observed by Gibreel et al. (2007). Although our data, as well as those from other studies, demonstrate a lack of efficacy of PAβN in reversing fluoroquinolone and tetracycline resistance, cmeB mutants of Campylobacter have shown a significant decrease in their resistance to these antibiotics classes (Lin et al., 2002; Luo et al., 2003). This fact suggests that the CmeABC system acts synergistically with other mechanisms to contribute to antimicrobial resistance.

On the other hand, PAβN had a strong effect on bile salts resistance in almost all isolates in our study, causing MIC reductions of up to 128-fold. Other studies performed by Lin and Martinez (2006) and Mavri and Smole Mozina (2013) also demonstrated that the MICs of bile salts were dramatically decreased in the presence of PAβN, around 16–512 fold and 2–64 fold, respectively. The different effect of PAβN on susceptibility of antimicrobials indicates that the inhibition occurs via binding site specific on the cmeABC system and in a competitive manner, as suggested in previous studies (Lomovskaya & Bostian, 2006). It has been proposed that inhibition of multidrug efflux systems by EPIs is a novel approach to enhance drug accumulation inside the bacterial cell, thereby increasing bacterial susceptibility to antimicrobials (Lomovskaya & Watkins, 2001). In recent years, some studies have been developed in order to find out new compounds which exhibit such properties.
5. Conclusion

This work provides a baseline study on Campylobacter contamination and its antimicrobial resistance and molecular basis in chicken carcasses in Minas Gerais state, Brazil. Despite the low prevalence and the low concentration of Campylobacter in chicken carcass samples, the identification of high levels of antimicrobial resistance and multidrug-resistant isolates make this issue even more serious. Our results point to the need for more frequent monitoring of the prevalence and antimicrobial resistance of Campylobacter in order to provide support for actions directed at reducing this pathogen in the food chain. In addition, we also suggest that efflux pump inhibitors may be an important tool to reduce the antimicrobial resistance and colonization of Campylobacter in animals raised for food purposes.

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References


(Klanik, Gröblacher, Kovač, Bučar, & Moszina, 2012; Kurinić, Klancnik, & Smole Moszina, 2012b).


