Resistance to β-lactam and tetracycline in *Campylobacter* spp. isolated from broiler slaughterhouses in southern Brazil

Yuli M. Sierra-Arguello*, Rafaela B. Morgan, Gustavo Perdoncini, Leonardo M. Lima, Marcos José P. Gomes and Vladimir Pinheiro do Nascimento


The study was carried out to screen and analyze the genetic characteristics of antimicrobial resistance in *Campylobacter* spp. from poultry sources. A total of 141 strains of *Campylobacter* isolated from samples of broilers of slaughterhouses in southern Brazil was identified by phenotypic and genotypic methods. *Campylobacter* isolates were evaluated for its antimicrobial susceptibility and the presence of resistance genes. The strains were investigated for antimicrobial susceptibility against two agents (ampicillin and tetracycline) by disk diffusion method. PCR assay was used to confirm the specie and the presence of ampicillin (*bla*<sub>OXA-61</sub>), tetracycline (*tet*(O)), and the energy-dependent multi-drug efflux pump (*cme*B) genes. *Campylobacter jejuni* was the most ubiquitous; its presence was determined in 140 samples out of 141 (99.3%), whereas *Campylobacter coli* was found only in one of the contaminated samples (0.70%). The results obtained showed 65% and 35.5% of *Campylobacter* isolates resistant to β-lactams and tetracyclines, respectively. The *cme*B gene responsible for multidrug resistance was detected in 26 isolates out 141 strains (18.5%). Moreover, 36 out of 141 *Campylobacter* strains (25.6%) were found to be resistant to at least two different antimicrobia resistance markers (β-lactams and tetracyclines).

**INDEX TERMS:** *Campylobacter*, tetracycline, β-lactam, efflux pump, resistance genes, PCR.

---

**RESUMO.-** [Resistência a β-lactâmicos e tetraciclina em *Campylobacter* spp. isolados de matadouros-frigoríficos de aves no sul do Brasil.] O presente estudo foi realizado para examinar e analisar as características genéticas de resistência antimicrobiana de *Campylobacter* spp. a partir de fontes avícolas. Um total de 141 amostras de *Campylobacter* isolados em matadouros-frigoríficos de aves do estado do Rio Grande do Sul, Brasil, foi identificado por métodos fenotípicos e genotípicos. Foi analisada a susceptibilidade antimicrobiana e a presença de genes de resistência.

As cepas foram testadas para detectar sensibilidade frente a dois antimicrobianos (ampicilina e tetraciclina) pelo método de difusão em disco. A seguir, usando a reação em cadeia da polimerase foi confirmada a espécie e a presença dos genes de resistência à ampicilina (*bla*<sub>OXA-61</sub>) e tetraciclina (*tet*(O)), assim como a detecção da bomba de efluco (*cme*B). *Campylobacter jejuni* foi a espécie mais isolada, sua presença foi determinada em 140 amostras (99,3%), e *Campylobacter coli* foi encontrada em uma única amostra (0,70%). Os resultados obtidos mostraram 65% e 35,5% de *Campylobacter* isolados resistentes a β-lactâmicos e tetraciclina, respectivamente. O gene *cme*B responsável pela resistência a múltiplos antimicrobianos foi detectado em 26 amostras (18,5%). Neste contexto, 36 das 141 amostras (25,6%) foram consideradas resistentes a dois grupos diferentes de antimicrobianos (β-lactâmicos e tetraciclina).

**TERMOS DE INDEXAÇÃO:** *Campylobacter*, tetraciclina, β-lactâmicos, bomba de efluco, genes de resistência, PCR.
INTRODUCTION

Campylobacter is recognized as the leading causes of bacterial foodborne diarrheal disease throughout the worldwide (Park 2002, Silva et al. 2011). Campylobacteriosis is estimated to cause about 1.3 million infections, 13,000 hospitalizations and 120 deaths each year in the United States (CDC 2013). It is also the most commonly reported antecedent infection in the development of Guillain Barré syndrome (GBS) and Miller Fisher syndrome (MFS) (Godschalk et al. 2004, Hardy et al. 2011, Van den Berg et al. 2014). A risk factor for human disease is the consumption of contaminated poultry products (Conlan et al. 2007, Ellsrøm et al. 2014). Transmission to man usually results in sporadic infection, and is often associated with improper handling or cooking of food (Moore et al. 2005). Campylobacter jejuni (C. jejuni) and Campylobacter coli (C. coli) are considered to be zoonotic pathogens, antimicrobial resistance among isolates in the animal reservoir has serious implications for the treatment in humans (Moore et al. 2006, EFSA 2011). The majority of cases of clinical Campylobacter spp. enteritis are generally mild or self-limiting disease not to require of antimicrobial chemotherapy (Moore et al. 2005). However, antimicrobial therapy may be used in a subset of patients with severe and prolonged systemic complications or to control infection (Avrane et al. 2003, Janssen et al. 2008). Currently, macrolides and fluoroquinolones are the antimicrobial agents of choice when therapeutic intervention is warranted (Engberg et al. 2001, Moore et al. 2005). Bacterial populations can respond to the threat of an antimicrobial agent by evolving some type of resistance mechanism(s) (Rowe-Magnus et al. 2002, Luangtongkum et al. 2009). These resistant bacteria may be transferred to humans either through the food supply or by direct contact with animals (Khachatourians 1998, Angulo et al. 2004). Ampicillin and tetracycline have activity against Campylobacter, but in general, are not recommended for the treatment of Campylobacter infections (Blaser 1995, Dasti et al. 2007). However, the increment of resistant strains to commonly used antimicrobials in clinical practices makes it necessary to consider alternatives therapies as well as the search of easy and reliable methods to study antimicrobial susceptibility (Luangtongkum et al. 2009). The aim of the present study was to determine the occurrence of Campylobacter spp. strains carrying resistance genes (tetracycline and β-lactam) and the energy-dependent multi-drug efflux pump through phenotypic and molecular analyses in poultry sources from slaughterhouses in Rio Grande do Sul state, Brazil.

MATERIALS AND METHODS

Sample collection. Since January 2012 to December 2013, a total of 141 isolates from: carcasses through slaughter line (n=115); water collected from the chiller tank (n=18); and from swabs (cloacal and boxes of transport) (n=8) were obtained from broiler slaughterhouses of Rio Grande do Sul state, Brazil.

Antimicrobial susceptibility screening. Isolation was performed in accordance with the International Standards Organization guidelines (ISO 10272-1:2006). Campylobacter isolates were analyzed for antimicrobial resistance using the agar disk diffusion method. The suspension was adjusted to match the 0.5 McFarland turbidity standards as recommended by the Clinical and Laboratory Standards Institute (CLSI 2010). Isolated cultures were analyzed for antimicrobial resistance using the disk diffusion assay on Mueller-Hinton agar plates (CM0337 Oxoid®, containing 5% sheep blood) incubated under microaerophilic conditions using a gas tank with a mixture (10% CO₂, 5% H₂, 5% O₂ and 85% N₂) for 48 hr at 41.5°C. Sheep blood agar plates were inoculated, and disks (Oxoid®) including tetracycline (30 µg), and ampicillin (10 µg) were added. Plates were incubated as described above. In view of lack of interpretative CLSI criteria for Campylobacter strains, the criteria used for the Enterobacteriaceae family were employed as breakpoints for Campylobacter resistance (CLSI 2012). C. jejuni ATCC33560 strain was used as control throughout the testing period.

DNA extraction. Genomic DNA was extracted using an adapted protocol described by Borosoi et al. (2009). Stored Campylobacter isolates were cultured on 5% sheep blood agar plates and incubated at 41.5°C for 48 hrs in microaerophilic conditions (10% CO₂, 5% O₂ and 85% N₂). One milliliter of bacterial culture was centrifuged at 12,000 r.p.m. for 2 min (5415C Microcentrifuge, Eppendorf, Hamburg, Germany) and the supernatant was discarded. The pellet was suspended in 800 µL of sterile distilled water and the resulting mixture was centrifuged at 12,000 r.p.m. for 2 min. The pellet was again suspended in 200 µL of sterile distilled water. The sample was placed on a thermal block (Multi-Block Heater, Baxter, USA) at 95°C for 10 min. The mixture was centrifuged as described above and the supernatants were transferred into fresh Eppendorf tubes to serve as a DNA template for subsequent processing.

Multiplex-PCR assay. The isolates were confirmed by Multiplex-PCR based detection of 16S rRNA, cveE and mapA genes (Denis et al. 1999).

Genotypic antimicrobial resistance. The confirmed C. jejuni isolates were screened for the presence of three genes: tetracycline (tetO), β-lactam (blaOXA-1), and the energy-dependent multi-drug efflux pump (cmeB). Primers, PCR conditions and lengths of products generated in this study are listed (Table 1). The PCR conditions were adapted (Pratt & Korolik 2005, Obeng et al. 2012). All PCR amplifications were performed in a mixture (25 µL) consisting of 5 µL of 10X PCR Buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 0.25 µL (5U/µL) of Taq thermostable DNA polymerase (Invitrogen®), 2 µmol 1¹ of MgCl₂ (25 mM), 2 µL dNTPs (dATP, dCTP, dGTP and dTTP, each at 2.5 mM), 2 µL extracted template DNA and 0.5 µL (10 pmol ¹) of each primer. Sterile Mill-Q water was added q.s 25 µL. All amplification reactions are performed in thermal cycler (Peltier Thermal Cycler Biocycler-MJ96+/MJ96G). The cycles were performed as described in Table 1. For visualization of PCR products, 10 µL aliquots were subjected to electrophoresis in a 2% agarose gel (Invitrogen®) in Tris-Acetate EDTA (TAE) buffer. DNA bands were stained with ethidium bromide and photographed (Fig. 1). The size of the PCR amplicons was compared to the 100 bp DNA ladder (Invitrogen®).

Statistical analysis. Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) v18 (IBM). Discrete variables were expressed as percentages, and proportions were compared using the Chi-square test with the significance level defined at P value <0.05.

RESULTS AND DISCUSSION

All the isolates were confirmed by Multiplex-PCR based detection of 16S rRNA, cveE and mapA genes. The most ubiquitous of the thermotolerant Campylobacter spp. was
C. jejuni. It was found in 140 of the contaminated samples (99.3%), whereas C. coli was identified in the remaining sample (0.7%). The PCR-amplified products of Campylobacter species and three resistance associated genes in agarose gel are summarized in Figure 1. The most prevalent species in this study was C. jejuni detected in 99.3% of positive findings, similar to what has been observed by other researchers (Garin et al. 2012, Hungaro et al. 2015).

An initial screening was performed to identify the antimicrobial susceptibility of 141 Campylobacter strains against ampicillin and tetracycline by using the disk diffusion test. Resistance of Campylobacter isolates among this method to ampicillin and tetracycline was detected in 65% and 35.5%, respectively (Fig. 2). The resistance we found to β-lactam among both our Campylobacter spp. isolates was at the lower range of that reported for amoxicillin (87.5%) in the prior Brazilian study (De Moura et al. 2013). Expression of a penicillinase-type of β-lactamase in Campylobacter confers resistance to amoxicillin, ampicillin and ticarcillin (Iovine 2013). This enzyme provided resistance to penicillins but not to cefotaxime and imipenem (Alfredson & Korolik 2005). The level of resistance to Campylobacter spp. to tetracycline by phenotypic method was similar to that reported in other studies in Brazil (Kuana et al. 2008). However, in contrast high presence of resistance (93.75%) was found by De Moura et al. (2013).

Ampicillin is not recommended for the treatment of Campylobacter infections. However, the increasing resistance to fluoroquinolones, tetracycline and erythromycin of C. coli and C. jejuni strains, might compromise the effectiveness of treatment (Aarestrup & Engberg 2001, Engberg et al. 2001, Gibreel & Taylor 2006, Alfredson & Korolik 2007, Silva et al. 2011). Recently, it was proposed that an oral β-lactam may provide an alternative therapy, although epidemiological studies have shown β-lactam resistance to be commonly observed in Campylobacter spp. (Griggs et al. 2009, Gormley et al. 2010). β-lactam antimicrobials, which inhibit bacterial cell wall biosynthesis, have been the most commercially available antimicrobials in the market because of their high specificity and potent killing effect (Zeng et al. 2014). Several major β-lactam resistance mechanisms have been characterized, including the production of β-lactamases (Lachance et al. 1991, Griggs et al. 2009). A majority of C. jejuni and C. coli isolates are able to produce β-lactamases, which inactivate the β-lactam molecule by hydrolysis the structural lactam ring (Tajada et al. 1996, Wieczorek & Osek 2013). In Campylobacter, ampicillin resistance and the associated β-lactamase production are chromosomally encoded (Alfredson & Korolik 2005). A single nucleotide mutation (G→T transversion) upstream of bla_{oxa-61} was identified in the ampicillin-resistant derivative of C. jejuni NCTC 11168 (Zeng et al. 2014). The results of phenotypic and genetic analyses of antimicrobial susceptibility were fully concordant. We showed that ampicillin resistance by disk diffusion in campylobacters isolated was 87.5% for Campylobacter genus identification and 589 for C. jejuni species identification. We showed that ampicillin resistance by disk diffusion in campylobacters isolated was 87.5% for Campylobacter genus identification and 589 for C. jejuni species identification.

### Table 1. List of primers and PCR conditions used in this study

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primers</th>
<th>Sequence (5'→3')</th>
<th>PCR conditions</th>
<th>Product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>MD16S^R</td>
<td>ATCTAATGGGCTAACATTAAC</td>
<td>95°C/10 min, 35 cycles: 16S</td>
<td>100 bp marker</td>
<td></td>
</tr>
<tr>
<td>mapA</td>
<td>MDmapA^R</td>
<td>GCGTTTTGTTTATGTGCG</td>
<td>94°C/5 min, 30 cycles:</td>
<td>372 bp</td>
<td></td>
</tr>
<tr>
<td>ceuE</td>
<td>col3F</td>
<td>TAGTATTGATTTGAGTGCTTG</td>
<td>72°C/1 min, and 72°C/10 min.</td>
<td>462 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ceuE-R</td>
<td>AGCTTCGATAGCTGCATC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tetO</td>
<td>tetO^F</td>
<td>TTTTCAATTGTTTCTG</td>
<td>94°C/5 min, 30 cycles:</td>
<td>559 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tetO^R</td>
<td>TTCTAGCAGCACAATATG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bla_{oxa-61}</td>
<td>F</td>
<td>AGAGTATGAAACCCGACAGAAG</td>
<td>94°C/30 s, 372 bp</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>AGCTTCGATAGCTGCATC</td>
<td>54°C/30 s, 857 bp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cmeB</td>
<td>cmeB-F</td>
<td>TGCATGAGCACAATATG</td>
<td>72°C/1 min, and 72°C/7 min.</td>
<td>241 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cmeB-R</td>
<td>AGCTTCGATAGCTGCATC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Fig.1.** Agarose gel electrophoresis of PCR products from C. jejuni. Lanes: M, 100 bp marker; 1-2, bla_{oxa-61} (372 bp); 3-4, cmeB (241bp); 5, tetO (559 bp); 7, mapA (589 bp); 8, ceuE (462 bp); 9-10, Reaction control.

**Fig.2.** The occurrence of Campylobacter spp. strains carrying resistance genes (tetracycline and β-lactam) and the energy-dependent multi-drug efflux pump.

This study found a high level of resistance to β-lactam gene in 65% (91/141) of isolates. Our results are similar to those described by Obeng et al. (2012), who observed resistance of 59-65.4% for C. jejuni. In Brazilian studies, Hungaro et al. (2015) was found 100% resistant isolates to ampicillin.

An increase in tetracycline resistance in C. jejuni and C. coli strains has been observed in recent years (Luangtongkum et al. 2008). The tetO gene was detected in 35.5% (50/141) of isolates. Previous reports have indicated that tetO is present in 40% of C. jejuni isolates from chicken carcasses (Hungaro et al. 2015), and of C. jejuni from chickens 19.2-40.7% (Obeng et al. 2012). Tetracyclines have been suggested as an alternative choice in the treatment of clinical campylobacteriosis, but in practice are rarely used (Moore et al. 2006). The tet(O) gene in Campylobacter encodes the Tet(O) protein that protects the ribosome from the inhibitory effect of tetracycline (Connell et al. 2003). Its primary antimicrobial effect takes place by direct steric hindrance by binding to the A site in the 30S subunit, thus hindering the movement of transfer RNA and inhibits peptide elongation (Wierczezok & Osek 2013). Tet(O) is coded by a gene located on plasmids of different sizes and in some strains chromosomally (Lee et al. 1994, Gibreel et al. 2004, Dasti et al. 2007). However, studies provide evidence that tetracycline–resistance Campylobacter have been recovered from organic and other production systems in which no antimicrobial have been used, which indicates that prior or current use may not be a defining attribution to resistance (Piddock et al. 2000, Luangtongkum et al. 2008, Cox et al. 2009).

In Campylobacter is described a multidrug efflux system (CmeABC), belonging to the Resistance Nodulation Division (RND) family of transporters that conferring intrinsic resistance to various antimicrobials and toxic compounds (Pumbwe & Piddock 2002, Lin et al. 2002, Guo et al. 2010). This pump is widely distributed in Campylobacter, including C. coli, and is constitutively expressed (Payot et al. 2002). Lin et al. (2003) showed that this efflux pump is essential for the growth and survival of C. jejuni in chicken intestinal extracts, and their report indicates that, through mediating resistance to bile salts in the intestinal tract, CmeABC allows C. jejuni to colonize chickens successfully. It is also possible that, in some species, efflux pumps that export antimicrobial agents also export virulence determinants, such as adhesins, toxins or other proteins that are important for the colonization and infection of human and animal cells (Piddock 2006). The role of efflux pumps in biofilm formation, persistence, resistance to antimicrobials and biocides was largely hypothetical; until some recent studies have discovered that expression of efflux pumps contribute to antimicrobial resistance by biofilms (Zhang & Mah 2008, Andersen et al. 2015). The higher expression of efflux pumps offers physiological advantage in the form of expelling toxic wastes from the densely populated biofilms and in the process efflux antimicrobial compounds, which is manifested as enhanced antimicrobial resistances of biofilms (Andersen et al. 2015). In addition, adaptation to the environment, such as quorum sensing and biofilm formation can also contribute to bacterial persistence (Martins et al. 2013). In this study, twenty six isolates (18.5%) presents the gene that encode a multidrug efflux pump. The CmeABC efflux pump may also contribute to β-lactam resistance (Iovine 2013). Gibreel et al. (2007) reported that inactivation of the cmeB gene in the resistant isolates examined led to a 16 to 128-fold decrease in tetracycline minimum inhibitory concentration (MIC), resulting in the complete restoration of tetracycline susceptibility. Studies also suggested that when both CmeABC and tetO are functional, the impact on tetracycline resistance is synergistic (Lin et al. 2002, Pumbwe & Piddock 2002).

The obtained data revealed that 7.1% Campylobacter strains possess three resistance determinant (n=10), and 43 isolates (30.5%) presents two resistance-associated genes. A percentage 35.5% of isolates presents one markers (n=50). No resistance markers were found in 38 isolates (27%). Moreover, 36 out of the 141 Campylobacter strains (25.6%) were found to be resistant to at least two different antimicrobial resistance groups (blaOXA-61 and tetO). The distribution of resistance markers among Campylobacter spp. tested according to the source of isolation are summarized in Table 2. This study has found a high genetic diversity of Campylobacter spp. isolated from broiler slaughterhouses. This observation suggests that such genotypes may be particularly adapted to survive cleaning and disinfection stress in poultry slaughterhouses and may contaminate the carcasses during processing (Peyrat et al. 2008). Additionally, our results showed that Campylobacter may easily contaminate poultry carcasses at slaughter process.

Antimicrobial resistance in both medicine and agriculture is documented by the World Health Organization (WHO), along with other various national authorities as a major emerging problem of public health importance, as well as consequences for animal treatment (Moore et al. 2006, Silva et al. 2011). Bacteria have developed multiple ways of becoming resistant to antimicrobials; in most cases bacteria are exposed to these substances, but have found a way to evade or resist the antimicrobial agent (Lin et al. 2003, Griggs et al. 2009, Wierczezok & Osek 2013). Further research in understanding the antimicrobial resistance mechanisms will facilitate the selection of antimicrobials for

<table>
<thead>
<tr>
<th>Sources</th>
<th>Percentage (number) of strains</th>
<th>tetO</th>
<th>cmeB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cloacal swab (n=5)</td>
<td>60 (3/5)</td>
<td>60 (3/5)</td>
<td>40 (2/5)</td>
</tr>
<tr>
<td>Swab of broiler transportation cage (n=3)</td>
<td>66.6 (2/3)</td>
<td>0</td>
<td>33.3 (1/3)</td>
</tr>
<tr>
<td>Broiler carcasses through slaughter process (n=115)</td>
<td>25 (1/4)</td>
<td>50 (2/4)</td>
<td>0</td>
</tr>
<tr>
<td>Deplaning (n=10)</td>
<td>80 (8/10)</td>
<td>30 (3/10)</td>
<td>40 (4/10)</td>
</tr>
<tr>
<td>Evisceration (n=5)</td>
<td>60 (3/5)</td>
<td>40 (2/5)</td>
<td>60 (3/5)</td>
</tr>
<tr>
<td>Spray-washing (n=7)</td>
<td>57.1 (4/7)</td>
<td>57.1 (4/7)</td>
<td>28.5 (2/7)</td>
</tr>
<tr>
<td>Cooling (n=89)</td>
<td>66.3 (59/89)</td>
<td>30.33 (27/89)</td>
<td>11.2 (10/89)</td>
</tr>
<tr>
<td>Chiller tank processing water (n=18)</td>
<td>61.11 (11/18)</td>
<td>50 (9/18)</td>
<td>22.2 (4/18)</td>
</tr>
<tr>
<td>Total</td>
<td>65 (91/141)</td>
<td>35.5 (50/141)</td>
<td>18.5 (26/141)</td>
</tr>
</tbody>
</table>
clinical treatment and the formulation of diagnostic media for various *Campylobacter* spp. To our knowledge, this is the first study to determine the frequency of the bla *OXA-61*, tetO and cmeB genes in campylobacters from poultry origin of Rio Grande do Sul state, Brazil. Our results also emphasize the need for a surveillance and monitoring system and risk analyzes for the prevalence and resistances of *Campylobacter* in poultry and other food animals.

### REFERENCES


