Food Control 51 (2015) 15–22

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

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

Humberto Moreira Hungaro [a, *], Regina Célia Santos Mendonça [a], Vinícius Ornelas Rosa [a], Andréa Cátia Leal Badaró [b], Maria Aparecida Scatamburlo Moreira [c], José Benício Paes Chaves [a]

[a] Department of Food Technology, Federal University of Viçosa (UFV), Av. PH Rolfs s/n, Campus Universitário, Viçosa, 36570-900, Minas Gerais, Brazil
[b] Department of Food Technology, Technological Federal University of Paraná (UTFPR), Av. Santa Bárbara s/n, Francisco Beltrão, 85601-970, Paraná, Brazil
[c] Department of Veterinary Medicine, Federal University of Viçosa (UFV), Av. PH Rolfs s/n, Campus Universitário, Viçosa, 36570-900, Minas Gerais, Brazil

ARTICLE INFO

Article history:
Received 9 June 2014
Received in revised form
1 October 2014
Accepted 1 November 2014
Available online 8 November 2014

Keywords:
Foodborne pathogen
Poultry
Multidrug resistance
MPN
CmeABC
PAβN

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 *gyrA* mutation, bla_{OxA-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 (PAβN) 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.

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

* Corresponding author. Department of Pharmaceutical Sciences, José Lourenço Kelmer, s/n – Bairro São Pedro, Campus Universitário, Juiz de Fora, 36036-900, Minas Gerais, Brazil. Tel.: +55 32 2102 3804; fax: +55 32 2102 3893.
E-mail address: humbertomh@gmail.com (H.M. Hungaro).

http://dx.doi.org/10.1016/j.foodcont.2014.11.001
0956-7135/

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 (Možina, Kurinčič, Klančnik, & 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; Maćkiw, Korsak, Rzewuska, Tomczuk, & Rożynek, 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 determinate 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 UI l^{-1} 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% H_2, 5% O_2, 10% CO_2 and 83% N_2) 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 hippuricase gene (*hipO*) specific for *Campylobacter jejuni*, and siderophore transport gene (*ceuE*) specific for *Campylobacter coli*. Amplifications were carried out in 25 μl reactions containing 2.5 μl of 10 × PCR buffer; 0.2 mmol l^{-1} of deoxynucleoside 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
Primers and annealing temperature used for PCR in this study.

Target gene	Primer sequence (5′–3′)	Annealing temperature	Reference
16S rRNA	ATCTAATGGCTTAACCATTAAAC GGACGGTAACTAGTTTAGTAT	59 °C	Denis et al. (1999)
hipO	GAAGAGGGTTTGGGTGGTG AGCTAGCTTCGCATAATAACTTG	66 °C	Linton, Lawson, Owen, and Stanley (1997)
ceuE	TGATTTTATTATTTGTAGCAGCG AATTGAAAATTGCTCCAACTATG	59 °C	Denis et al. (1999)
tet(O)	GCGTTTTGTTTATGTGCG ATGGACAACCCGACAGAAG	54 °C	Pratt and Korolik (2005)
bla_{OxA-61}	AGAGTATAATACAAGCG TAGTGAGTTGTCAAGCC	54 °C	Obeng et al. (2012)
gyrA	TTTTTAGCAAAGATTCTGAT CAAAGCATCATAAACTGCAA	48 °C	Zirnstein et al. (1999)
cmeA	TTTGGATCCTTGATGGCTAAGGC AACTTTC CTCCAATTTCTTAAGCTTCGCTACCAA	50 °C	Lin et al. (2002)
cmeB Cj[a]	GGTACAGATCCTGATCAAGCC AGGAATAAGTGTTGCACGGAAATT	50 °C	Lin et al. (2002)
cmeB Cc[b]	TCCTAGCAGCACAATATG AGCTTCGATAGCTGCATC	54 °C	Obeng et al. (2012)
cmeC	GCTTGGATCCTTATCTTGGGAAAAA TTTTTAAAGCTTTAAGGTAATTTTCTT	50 °C	Lin et al. (2002)

[a] Cj – *C. jejuni*.
[b] Cc – *C. coli*.

NaCl wt v^{-1}) from colonies grown on Müller-Hinton agar containing 5% (v v^{-1}) sheep blood (M–H blood) at 42 °C for 24 h under microaerobic conditions, and adjusted to an absorbance of 0.13 at 625 nm equivalent to 10^8 CFU ml^{-1}. Sterile cotton-tipped swabs were used to transfer the inoculum onto M–H blood plates to produce a confluent lawn of bacterial growth. After the inoculum the plates were dried, the antibiotic disks were distributed over the inoculated plates and these were incubated at 42 °C for 48 h under microaerobic conditions. The standard disks (Laborclin, Brazil) containing the following antibiotics were used in this assay: ciprofloxacin (5 μg); nalidixic acid (30 μg); chloramphenicol (30 μg); tetracycline (30 μg); gentamicin (10 μg); imipenem (10 μg); ampicillin (10 μg); amoxicillin-clavulanic acid (10 μg); cephalothin (30 μg); cefoxitin (30 μg); cefuroxime (30 μg); cefotaxime (30 μg); ceftazidime (30 μg); cefepime (30 μg); trimethoprim-sulfamethoxazole (23.75/1.25 μg); and erythromycin (15 μg). The inhibition zone diameter obtained for these antibiotics was interpreted according to the breakpoints of the Clinical and Laboratory Standards Institute for Enterobacteriaceae (CLSI, 2012), except for erythromycin, which followed recommendations from The European Committee on Antimicrobial Susceptibility Testing – EUCAST (http://www.eucast.org). The multiple antibiotic resistance (MAR) index was calculated using the formula: a/b, where 'a' represents the number of antibiotics to which a particular isolates was resistant and 'b' the total number of antibiotics tested.

2.5. Detection of antibiotic resistance genes

All the *Campylobacter* spp. were tested for the presence of $tet_{(O)}$, bla_{OxA-61}, *gyrA* (Thr-86-Ile mutation), *cmeA*, *cmeB*, and *cmeC* genes, representing resistance to tetracycline, ampicillin and quinolones, and CmeABC efflux system components, respectively. Each amplification reaction consisted of 2.5 μl of 10 × PCR buffer; 0.2 mmol l^{-1} of deoxynucleoside 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 in a total reaction volume of 25 μl. Simplex PCR assays for each gene were performed using the following protocol: initial denaturation step of 96 °C for 1 min, followed by 30 cycles of 94 °C for 30 s, specific annealing temperature for 45 s, and 72 °C for 1 min, and a final extension step at 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 10^5 CFU ml^{-1}. Two-fold serial dilutions of antibiotics and bile salts were performed in the 96-well plates for concentrations from 0.125 to 512 μg ml^{-1} and 0.05–25 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 (living cells – red; dead cells – blue) (Salvat, Antonnacci, 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 cephalosporins (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

Table 2
Campylobacter spp. enumeration in chicken carcass samples using MPN technique.

Slaughterhouses	Sample code	Positive tubes (Dilution)	MPN carcass^{-1} [a]
		D-10^{-1}-10^{-2}	
A	A52013	0-1-0	60
B	B12013	1-0-0	72
C	C42013	1-0-0	72
D	D22013	2-0-0	184
	D32013	1-0-0	72
	D42013	1-1-0	148
E	E22013	1-0-0	72
	E32013	2-0-0	184
	E52013	1-0-0	72
F	F12013	2-0-0	184
	F22013	1-0-0	72
	F32013	1-0-0	72
G	G12013	1-0-0	72
	G52013	1-0-0	72
H	H42013	1-0-0	72
I	I52013	0-1-0	72

[a] Remaining samples showed *Campylobacter* spp. < 60 MPN carcass^{-1}.

selected three antibiotics (ciprofloxacin, tetracycline and ampicillin) and bile salts for an investigation of the MICs and molecular mechanisms contributing to antimicrobial resistance. Eight isolated strains (40%) showed multi-drug resistance to ampicillin, tetracycline and ciprofloxacin, and only one was sensitive to the three antibiotics evaluated using the broth microdilution method (Table 4). The MIC values of bile salts were highly variable, with resistance rates between 0.390 and >25 mg ml^{-1}.

The results of phenotypic and genetic analysis of ciprofloxacin susceptibility were sufficiently compatible, which was not confirmed for tetracycline and ampicillin. All ciprofloxacin-resistant isolates had a point mutation Thr-86-Ile in *gyr*A gene of DNA gyrase, except for one. In contrast, ten isolates were resistant to tetracycline, but only four showed the $tet_{(O)}$ gene responsible for the synthesis of a ribosomal protection protein that confers resistance to the inhibitory effect of tetracycline on protein synthesis.

Table 3
Antibiotic resistance profile and multiple antibiotic resistance (MAR) index of *Campylobacter* spp. isolates from chicken carcasses.

Strain	Antibiotic resistant profile[a]	MAR index
A52013	CipNalCphCfoCrxCtxCazSut	0.50
B12013	CipNalTetAmpCfphCfoCrxCtxCazSut	0.63
C42013	CipNalTetCphCfoCrxCtxCazSut	0.56
D22013a	CipNalTetAmpAmcCphCfoCrxCtxCazSut	0.69
D22013b	CipNalTetAmpAmcCphCfoCrxCtxCazSut	0.69
D32013	CipNalAmpCphCfoCrxCtxCazSut	0.56
D42013a	CipNalCphCfoCrxCtxCazSut	0.50
D42013b	CipNalTetCphCfoCrxCtxCazSut	0.56
E22013	CipNalCphCfoCrxCtxCazSut	0.50
E32013a	CipNalCphCfoCrxCazSut	0.44
E32013b	CipNalCphCfoCrxCtxCazSut	0.50
E52013	CipNalTetCphCfoCrxCtxCazSut	0.56
F12013a	CipNalTetAmpCphCfoCrxCtxCazSut	0.63
F12013b	CipNalTetAmpCphCfoCrxCtxCazSut	0.63
F22013	CipNalTetAmpCphCfoCrxCtxCazSut	0.63
F32013	CipNalTetAmpCphCfoCrxCtxCazSut	0.63
G12013	CipNalCphCfoCrxSut	0.38
G52013	CipNalCphCfoCazSut	0.38
H42013	AmpCphCfoCrxSut	0.31
I52013	CipNalCphCfoCrxCazSut	0.44

[a] Ciprofloxacin (Cip) 5 μg; Nalidixic acid (Nal) 30 μg; Chloramphenicol (Clo) 30 μg; Tetracycline (Tet) 30 μg; Gentamicin (Gen) 10 μg; Imipenem (Imp) 10 μg; Ampicillin (Amp) 10 μg; Amoxicillin-clavulanic acid (Amc) 10 μg; Cephalothin (Cfl) 30 μg; Cefoxitin (Cfo) 30 μg; Cefuroxime (Crx) 30 μg; Cefotaxime (Ctx) 30 μg; Ceftazidime (Caz) 30 μg; Cefepime (Cpm) 30 μg; Trimethoprim-sulfamethoxazole (Sut) 23.75/1.25 μg; Erythromycin (15 μg).

Eight ampicillin-resistant isolates had a β-lactamase gene (bla_{OxA-61}), and this fact was also observed in ten sensitive isolates. Moreover, we also investigated the presence of the three genes that encode the CmeABC efflux pump in *Campylobacter* spp. These three genes, named *cmeA*, *cmeB* and *cmeC*, are arranged in an operon, and encode for a periplasmic fusion protein, an inner membrane drug transporter and an outer membrane protein, respectively. The *cmeB* gene was found in all isolates, whereas *cmeA* was absent in two isolates, one of which also showed no *cmeC*.

We used phenylalanine-arginine β-naphthylamide (PAβN) to inhibit the CmeABC efflux system from *Campylobacter* isolates in order to evaluate its effect on transport and resistance to antimicrobials. 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, 50 and 25% of the isolates had transport of bile salts, ciprofloxacin, ampicillin and tetracycline affected by PAβN, respectively. In combination with PAβN, 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 *cmeA* and *cmeC* genes in two isolates did not influence the resistance 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 *Campylobacter* 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 prevalence rates of *Campylobacter* may be attributed to poultry breeding methods, hygienic practices during slaughter, sampling strategies and 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 expected 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, Bartelt, 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 carcasses may be of concern to food safety authorities as because

Table 4
Minimum inhibitory concentrations (MICs) of antimicrobials in the absence and presence of the efflux pump inhibitor (EPI) against *Campylobacter* isolates and research on resistance genes.

Strain	MIC of antibiotics (mg l^{-1})								Resistance genes					
	Cip		Tet		Amp		Bile salts		*gyrA*	*tet(O)*	*bla$_{OxA}$*	*cmeA*	*cmeB*	*cmeC*
	−P	+P	−P	+P	−P	+P	−P	+P						
A52013	4^R	**0.5**	$<0.5^S$	<0.5	4^S	**2**	6250	**390.6**	+	−	+	+	+	+
B12013	8^R	8	128^R	**64**	32^R	**8**	25,000	**390.6**	+	+	+	+	+	+
C42013	8^R	**4**	256^R	256	2^S	**1**	3125	**390.6**	+	−	−	−	+	+
D22013a	8^R	**4**	64^R	**32**	128^R	**64**	12,500	**390.6**	+	+	+	+	+	+
D22013b	32^R	**16**	256^R	256	512^R	**256**	25,000	**781.3**	−	+	+	−	+	−
D32013	8^R	8	$<0.12^S$	<0.12	2^S	2	3125	**195.3**	+	−	+	+	+	+
D42013a	32^R	**16**	$<0.12^S$	<0.12	8^S	8	12,500	**1562.5**	+	−	+	+	+	+
D42013b	8^R	**4**	64^R	**16**	128^R	**64**	3125	**390.6**	+	+	+	+	+	+
E22013	4^R	4	$<0.12^S$	<0.12	1^S	1	1562.5	**781.25**	+	−	−	+	+	+
E32013a	8^R	**4**	$<0.12^S$	<0.12	8^S	**4**	>25,000	>25,000	+	−	+	+	+	+
E32013b	16^R	**8**	$<0.12^S$	<0.12	8^S	**4**	>25,000	>25,000	+	−	+	+	+	+
E52013	8^R	8	64^R	**32**	8^S	**2**	12,500	**390.6**	+	−	+	+	+	+
F12013a	4^R	4	128^R	128	128^R	128	390.6	**97.65**	+	−	+	+	+	+
F12013b	8^R	**4**	512^R	**256**	256^R	128	25,000	**781.3**	+	−	+	+	+	+
F22013	8^R	8	128^R	128	64^R	64	12,500	**195.3**	+	−	+	+	+	+
F32013	8^R	8	64^R	64	64^R	64	12,500	**390.6**	+	−	+	+	+	+
G12013	8^R	**4**	$<0.12^S$	<0.12	4^S	4	1562.5	**781.3**	+	−	+	+	+	+
G52013	8^R	**4**	$<0.12^S$	<0.12	4^S	4	1562.5	**781.3**	+	−	+	+	+	+
H42013	0.5^S	0.5	0.5^S	0.5	8^S	8	3125	**1562.5**	−	−	+	+	+	+
I52013	4^R	4	$<0.12^S$	<0.12	4^S	**2**	25,000	**195.3**	+	−	+	+	+	+

−P: absence of phenylalanine-arginine β-naphthylamide (PAβN); +P: presence of phenylalanine-arginine β-naphthylamide (PAβN); ^{R} − resistant; ^{S} − 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.

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 $carcass^{-1}$ (Chrystal, Hargraves, Boa, & Ironside, 2008; Figueroa, Troncoso, López, Rivas, & Toro, 2009; Johannessen, Johnsen, Økland, Cudjoe, & Hofshagen, 2007; Kuana, 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, Cagney, 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 cephalothin, 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 β-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 β-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, & Laisney, 1995; Jacob, Mdegela, & Nonga, 2011).

Resistance to a large number of β-lactam antimicrobial agents is becoming more widespread in foodborne bacteria, including *Campylobacter* (Li, Mehrotra, Ghimire, & Adewoye, 2007). Besides cephalosporins, we evaluated the antimicrobial resistance of *Campylobacter* to other β-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 amoxicillin-clavulanic acid. In previous studies, these antimicrobial groups 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 efficacy of β-lactams for the treatment of infections of this nature (Hakanen et al., 2003).

Campylobacter isolated in the present study were also susceptible to gentamicin, erythromycin and chloramphenicol. Results similar to ours were reported by other authors, who observed that *Campylobacter* isolated from chicken were highly sensitive to these three antibiotics (EFSA, 2012; NARMS, 2011; Zhao et al., 2010). In contrast, higher resistances to these three antimicrobials were observed by de Moura et al. (2013) and Nobile, Costantino, Bianco,

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; Maćkiw 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; Maćkiw et al., 2012). These concerns have become more severe because fluoroquinolones and tetracycline, along with macrolides, are used for treating human infections (Maćkiw 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 their use 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 (Maćkiw et al., 2012; Wieczorek, Kania, & Osek, 2013; Zirnstein, 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 (Maćkiw 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 $bla_{OxA\text{-}61}$ gene, which is associated with β-lactamase production (Alfredson & Korolik, 2005). Griggs et al. (2009) observed that 91% of the ampicillin-resistant isolates carried the $bla_{OxA\text{-}61}$ 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-susceptible *Campylobacter* isolates from chicken were able to encode the $bla_{OxA\text{-}61}$ gene. Such findings indicate that the $bla_{OxA\text{-}61}$ gene in these *Campylobacter* isolates may has 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 Možina (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, & Pagés, 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 to either 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 $bla_{OxA\text{-}61}$ 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 Kurinčič, Klančnik, and Smole Možina (2012a). With regards to tetracycline, Kurinčič 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 Možina (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

(Klančnik, Gröblacher, Kovač, Bucar, & Možina, 2012; Kurinčič, Klančnik, & Smole Možina, 2012b).

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.

Acknowledgments

This investigation was supported by CNPQ and FAPEMIG, Brazil, and Humberto Moreira Hungaro was supported by a fellowship from CAPES. We would like to thank "Coleção de *Campylobacter* (CCAMP)", Instituto Oswaldo Cruz, Rio de Janeiro, Brazil for donation of the reference strains.

References

Aarestrup, F. M., & Engberg, J. (2001). Antimicrobial resistance of thermophilic Campylobacter. *Veterinary Research, 32*, 311–321.

Akiba, M., Lin, J., Barton, Y.-W., & Zhang, Q. (2006). Interaction of CmeABC and CmeDEF in conferring antimicrobial resistance and maintaining cell viability in *Campylobacter jejuni*. *Journal of Antimicrobial Chemotherapy, 57*, 52–60.

Alfredson, D. A., & Korolik, V. (2005). Isolation and expression of a novel molecular class D β-lactamase, OXA-61, from *Campylobacter jejuni*. *Antimicrobial Agents and Chemotherapy, 49*, 2515–2518.

Alfredson, D. A., & Korolik, V. (2007). Antibiotic resistance and resistance mechanisms in *Campylobacter jejuni* and *Campylobacter coli*. *FEMS Microbiology Letters, 277*, 123–132.

Biasi, R. S., Freitas De Macedo, R. E., Scaranello Malaquias, M. A., & Franchin, P. R. (2011). Prevalence, strain identification and antimicrobial resistance of *Campylobacter* spp. isolated from slaughtered pig carcasses in Brazil. *Food Control, 22*, 702–707.

Black, R. E., Levine, M. M., Clements, M. L., Hughes, T. P., & Blaser, M. J. (1988). Experimental *Campylobacter jejuni* infection in humans. *The Journal of Infectious Diseases, 157*, 472–479.

Bolla, J., De, E., & Dorez, A. (2000). Purification, characterization and sequence analysis of Omp50, a new porin isolated from *Campylobacter jejuni*. *Biochemical Journal, 352*, 637–643.

Bolla, J.-M., Loret, E., Zalewski, M., & Pagés, J.-M. (1995). Conformational analysis of the *Campylobacter jejuni* porin. *Journal of Bacteriology, 177*, 4266–4271.

CDC. (2013). *Center for diseases control and Prevention – CDC. Estimates of foodborne Illness in the United States*. Available at http://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html Accessed 20.01.13.

Chrystal, N. D., Hargraves, S. J., Boa, A. C., & Ironside, C. J. (2008). Counts of *Campylobacter* spp. and prevalence of *Salmonella* associated with New Zealand broiler carcasses. *Journal of Food Protection, 71*, 2526–2532.

CLSI. (2010). *Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria; approved guideline* (2nd ed.). Wayne, PA: Clinical and Laboratory Standards Institute (M45–A2).

CLSI. (2012). *Performance standards for antimicrobial susceptibility testing, twenty-second informational supplement*. Wayne, PA: Clinical and Laboratory Standards Institute (M100–S22).

Corcoran, D., Quinn, T., Cotter, L., & Fanning, S. (2005). Relative contribution of target gene mutation and efflux to varying quinolone resistance in Irish *Campylobacter* isolates. *FEMS Microbiology Letters, 253*, 39–46.

Corry, J. E., Post, D., Colin, P., & Laisney, M. (1995). Culture media for the isolation of *Campylobacters*. *International Journal of Food Microbiology, 26*, 43–76.

Daskalov, H., & Maramski, A. (2012). Prevalence and factors affecting the presence of *Campylobacter* spp. in broiler carcasses in Bulgaria. *Turkish Journal of Veterinary and Animal Sciences, 36*, 539–545.

Dasti, J. I., Groß, U., Pohl, S., Lugert, R., Weig, M., & Schmidt-Ott, R. (2007). Role of the plasmid-encoded *tet (O)* gene in tetracycline-resistant clinical isolates of *Campylobacter jejuni* and *Campylobacter coli*. *Journal of Medical Microbiology, 56*, 833–837.

Denis, M., Soumet, C., Rivoal, K., Ermel, G., Blivet, D., Salvat, G., et al. (1999). Development of am-PCR assay for simultaneous identification of *Campylobacter jejuni* and *C. coli*. *Letters in Applied Microbiology, 29*, 406–410.

Dzieciol, M., Wagner, M., & Hein, I. (2011). CmeR-dependent gene *Cj0561c* is induced more effectively by bile salts than the CmeABC efflux pump in both human and poultry *Campylobacter jejuni* strains. *Research in Microbiology, 162*, 991–998.

EFSA. (2012). The European Union Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2010. *EFSA Journal, 10*, 2598.

EFSA. (2013). Trends and sources of zoonoses and zoonotic agents and food-borne outbreaks in 2011. *EFSA Journal, 11*, 3129.

FAO/WHO [Food and Agriculture Organization of the United Nations/World Health Organization]. (2002). *Risk assessment of Campylobacter spp. in broiler chickens and Vibrio spp. in seafood*. Report of a Joint FAO/WHO Expert consultation. Available at http://www.who.int/foodsafety/publications/micro/aug2002.pdf Accessed 20.01.13.

Figueroa, G., Troncoso, M., López, C., Rivas, P., & Toro, M. (2009). Occurrence and enumeration of *Campylobacter* spp. during the processing of Chilean broilers. *BMC Microbiology, 9*, 94.

Franchin, P., Ogliari, P., & Batista, C. (2007). Frequency of thermophilic *Campylobacter* in broiler chickens during industrial processing in a Southern Brazil slaughterhouse. *British Poultry Science, 48*, 127–132.

Garin, B., Gouali, M., Wouafo, M., Perchec, A.-M., Thu, P. M., Ravaonindrina, N., et al. (2012). Prevalence, quantification and antimicrobial resistance of *Campylobacter* spp. on chicken neck-skins at points of slaughter in 5 major cities located on 4 continents. *International Journal of Food Microbiology, 157*, 102–107.

Gibreel, A., Wetsch, N. M., & Taylor, D. E. (2007). Contribution of the CmeABC efflux pump to macrolide and tetracycline resistance in *Campylobacter jejuni*. *Antimicrobial Agents and Chemotherapy, 51*, 3212–3216.

Gonçalves, K., Yamanaka, E. H. U., Almeida, A. P. I., Chano, L., & Ribeiro, A. B. (2013). Pesquisa de *Campylobacter* spp. em carnes de frango comercializadas na cidade de Campo Mourão-PR Study about the *Campylobacter* spp. present in chicken meat sold in the city of Campo Mourão-PR. *Alimentos e Nutrição Araraquara, 23*, 211–216.

Griggs, D. J., Peake, L., Johnson, M. M., Ghori, S., Mott, A., & Piddock, L. J. V. (2009). β-Lactamase-Mediated β-lactam resistance in *Campylobacter* species: prevalence of *Cj0299* (bla_{OxA-61}) and evidence for a novel β-lactamase in *C. jejuni*. *Antimicrobial Agents and Chemotherapy, 53*, 3357–3364.

Gritchina, A. V., Mishchuk, V. I., & Pozhalostina, L. V. (2005). Antibiotic susceptibility of *Campylobacter* isolated in the lipetsk region. *Antibiotiki i Khimioterapiya, 50*, 51–54.

Hakanen, A. J., Lehtopolku, M., Siitonen, A., Huovinen, P., & Kotilainen, P. (2003). Multidrug resistance in *Campylobacter jejuni* strains collected from Finnish patients during 1995–2000. *Journal of Antimicrobial Chemotherapy, 52*, 1035–1039.

Humphrey, T., O'brien, S., & Madsen, M. (2007). *Campylobacters* as zoonotic pathogens: a food production perspective. *International Journal of Food Microbiology, 117*, 237–257.

Iovine, N. M. (2013). Resistance mechanisms in *Campylobacter jejuni*. *Virulence, 4*, 230–240.

Iovine, N. M., & Blaser, M. J. (2004). Antibiotics in animal feed and spread of resistant *Campylobacter* from poultry to humans. *Emerging Infectious Diseases, 10*, 1158.

Jacob, P., Mdegela, R. H., & Nonga, H. E. (2011). Comparison of Cape Town and Skirrow's *Campylobacter* isolation protocols in humans and broilers in Morogoro, Tanzania. *Tropical Animal Health and Production, 43*, 1007–1013.

Jeon, B., Wang, Y., Hao, H., Barton, Y.-W., & Zhang, Q. (2011). Contribution of CmeG to antibiotic and oxidative stress resistance in *Campylobacter jejuni*. *Journal of Antimicrobial Chemotherapy, 66*, 79–85.

Johannessen, G., Johnsen, G., Økland, M., Cudjoe, K., & Hofshagen, M. (2007). Enumeration of thermotolerant *Campylobacter* spp. from poultry carcasses at the end of the slaughter-line. *Letters in Applied Microbiology, 44*, 92–97.

Klančnik, A., Gröblacher, B., Kovač, J., Bucar, F., & Možina, S. S. (2012). Anti-*Campylobacter* and resistance-modifying activity of *Alpinia katsumadai* seed extracts. *Journal of Applied Microbiology, 113*, 1249–1262.

Kuana, S. L., Dos Santos, L. R., Rodrigues, L. B., Salle, C. T. P., Moraes, H. L. D. S., & Do Nascimento, V. P. (2008). Ocorrência de *Campylobacter* sp em lotes de frangos de corte e nas carcaças correspondentes. *Ciência Animal Brasileira, 9*, 480–486.

Kuana, S. L., Santos, L. R. D., Rodrigues, L. B., Borsoi, A., Moraes, H. L. D. S., Salle, C. T. P., et al. (2008). Antimicrobial resistance in *Campylobacter* spp isolated from broiler flocks. *Brazilian Journal of Microbiology, 39*, 738–740.

Kurinčič, M., Klančnik, A., & Smole Možina, S. (2012a). Effects of efflux pump inhibitors on erythromycin, ciprofloxacin, and tetracycline resistance in *Campylobacter* spp. isolates. *Microbial Drug Resistance, 18*, 492–501.

Kurinčič, M., Klančnik, A., & Smole Možina, S. (2012b). Epigallocatechin gallate as a modulator of *Campylobacter* resistance to macrolide antibiotics. *International Journal of Antimicrobial Agents, 40*, 467–471.

Li, X.-Z., Mehrotra, M., Ghimire, S., & Adewoye, L. (2007). β-lactam resistance and β-lactamases in bacteria of animal origin. *Veterinary Microbiology, 121*, 197–214.

Lin, J., & Martinez, A. L. (2006). Effect of efflux pump inhibitors on bile resistance and in vivo colonization of *Campylobacter jejuni*. *Journal of Antimicrobial Chemotherapy, 58*, 966–972.

Lin, J., Michel, L. O., & Zhang, Q. (2002). CmeABC functions as a multidrug efflux system in *Campylobacter jejuni*. *Antimicrobial Agents and Chemotherapy, 46*, 2124–2131.

Lin, J., Sahin, O., Michel, L. O., & Zhang, Q. (2003). Critical role of multidrug efflux pump CmeABC in bile resistance and in vivo colonization of *Campylobacter jejuni*. *Infection and Immunity, 71*, 4250–4259.

Linton, D., Lawson, A., Owen, R., & Stanley, J. (1997). PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. *Journal of Clinical Microbiology, 35*, 2568–2572.

Lomovskaya, O., & Bostian, K. A. (2006). Practical applications and feasibility of efflux pump inhibitors in the clinic–a vision for applied use. *Biochemical Pharmacology, 71*, 910–918.

Lomovskaya, O., & Watkins, W. (2001). Inhibition of efflux pumps as a novel approach to combat drug resistance in bacteria. *Journal of Molecular Microbiology and Biotechnology, 3*, 225–236.

Luo, N., Sahin, O., Lin, J., Michel, L. O., & Zhang, Q. (2003). In vivo selection of *Campylobacter* isolates with high levels of fluoroquinolone resistance associated with *gyrA* mutations and the function of the CmeABC efflux pump. *Antimicrobial Agents and Chemotherapy, 47*, 390–394.

Lynch, Ó. A., Cagney, C., Mcdowell, D. A., & Duffy, G. (2011). Occurrence of fastidious *Campylobacter* spp. in fresh meat and poultry using an adapted cultural protocol. *International Journal of Food Microbiology, 150*, 171–177.

Maćkiw, E., Korsak, D., Rzewuska, K., Tomczuk, K., & Rożynek, E. (2012). Antibiotic resistance in *Campylobacter jejuni* and *Campylobacter coli* isolated from food in Poland. *Food Control, 23*, 297–301.

Man, J. C. (1983). MPN tables, corrected. *European Journal of Applied Microbiology and Biotechnology, 17*, 301–305.

Martinez, A. L., & Lin, J. (2006). Effect of an efflux pump inhibitor on the function of the multidrug efflux pump CmeABC and antimicrobial resistance in *Campylobacter. Foodborne Pathogens and Disease, 3*, 393–402.

Mavri, A., & Smole Možina, S. (2013). Resistance to bile salts and sodium deoxycholate in macrolide-and fluoroquinolone-susceptible and resistant *Campylobacter jejuni* and *Campylobacter coli* strains. *Microbial Drug Resistance, 19*, 168–174.

de Moura, H. M., Silva, P. R., Da Silva, P. H. C., Souza, N. R., Racanicci, A. M. C., & Santana, A. P. (2013). Antimicrobial resistance of *Campylobacter jejuni* isolated from chicken carcasses in the federal district, Brazil. *Journal of Food Protection, 76*, 691–693.

Možina, S. S., Kurinčič, M., Klančnik, A., & Mavri, A. (2011). *Campylobacter* and its multi-resistance in the food chain. *Trends in Food Science & Technology, 22*, 91–98.

NARMS. (2011). *NARMS retail meat annual report, 2011.* Available at http://www.fda.gov/downloads/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/UCM334834.pdf Accessed 20.01.13.

Nobile, C. G., Costantino, R., Bianco, A., Pileggi, C., & Pavia, M. (2013). Prevalence and pattern of antibiotic resistance of *Campylobacter* spp. in poultry meat in Southern Italy. *Food Control, 32*, 715–718.

Obeng, A., Rickard, H., Sexton, M., Pang, Y., Peng, H., & Barton, M. (2012). Antimicrobial susceptibilities and resistance genes in *Campylobacter* strains isolated from poultry and pigs in Australia. *Journal of Applied Microbiology, 113*, 294–307.

Oliveira, A. D. L., & Oliveira, R. B. P. D. (2013). Enumeração de *Campylobacter* spp. e presença de *Campylobacter jejuni* em carcaças de frango no Estado de Minas Gerais. *Ciência Rural, 43*, 480–484.

Pratt, A., & Korolik, V. (2005). Tetracycline resistance of Australian *Campylobacter jejuni* and *Campylobacter coli* isolates. *Journal of Antimicrobial Chemotherapy, 55*, 452–460.

Salvat, A., Antonnacci, L., Fortunato, R. H., Suarez, E. Y., & Godoy, H. M. (2001). Screening of some plants from Northern Argentina for their antimicrobial activity. *Letters in Applied Microbiology, 32*, 293–297.

Scherer, K., Bartelt, E., Sommerfeld, C., & Hildebrandt, G. (2006a). Comparison of different sampling techniques and enumeration methods for the isolation and quantification of *Campylobacter* spp. in raw retail chicken legs. *International Journal of Food Microbiology, 108*, 115–119.

Scherer, K., Bartelt, E., Sommerfeld, C., & Hildebrandt, G. (2006b). Quantification of *Campylobacter* on the surface and in the muscle of chicken legs at retail. *Journal of Food Protection, 69*, 757–761.

Smith, D., Northcutt, J., Cason, J., Hinton, A., Buhr, R., & Ingram, K. (2007). Effect of external or internal fecal contamination on numbers of bacteria on prechilled broiler carcasses. *Poultry Science, 86*, 1241–1244.

Son, I., Englen, M. D., Berrang, M. E., Fedorka-Cray, P. J., & Harrison, M. A. (2007). Prevalence of *Arcobacter* and *Campylobacter* on broiler carcasses during processing. *International Journal of Food Microbiology, 113*, 16–22.

Stern, N., Georgsson, F., Lowman, R., Bisaillon, J.-R., Reiersen, J., Callicott, K., et al. (2007). Frequency and enumeration of *Campylobacter* species from processed broiler carcasses by weep and rinse samples. *Poultry Science, 86*, 394–399.

Suzuki, H., & Yamamoto, S. (2009). *Campylobacter* contamination in retail poultry meats and by-products in the world: a literature survey. *Journal of Veterinary Medical Science, 71*, 255–261.

Tang, J. Y. H., Ghazali, F. M., Saleha, A. A., Nakaguchi, Y., Nishibuchi, M., & Radu, S. (2010). MPN-PCR enumeration of *Campylobacter* spp. in raw chicken meats and by-products. *Frontiers of Agriculture in China, 4*, 501–506.

UBABEF. (2014). *Annual report.* http://www.ubabef.com.br/files/publicacoes/8ca705e70f0cb110ae 3aed67d29c8842.pdf Accessed 28.08.14.

Wassenaar, T., Fernández-Astorga, A., Alonso, R., Marteinsson, V., Magnusson, S., Kristoffersen, A., et al. (2009). Comparison of *Campylobacter* fla-SVR genotypes isolated from humans and poultry in three European regions. *Letters in Applied Microbiology, 49*, 388–395.

Wieczorek, K., Kania, I., & Osek, J. (2013). Prevalence and antimicrobial resistance of *Campylobacter* spp. Isolated from poultry carcasses in Poland. *Journal of Food Protection, 76*, 1451–1455.

Wilson, I. (2003). Antibiotic resistance of *Campylobacter* in raw retail chickens and imported chicken portions. *Epidemiology and Infection, 131*, 1181–1186.

Wong, T. L., Hollis, L., Cornelius, A., Nicol, C., Cook, R., & Hudson, J. A. (2007). Prevalence, numbers, and subtypes of *Campylobacter jejuni* and *Campylobacter coli* in uncooked retail meat samples. *Journal of Food Protection, 70*, 566–573.

Zhao, S., Young, S., Tong, E., Abbott, J., Womack, N., Friedman, S., et al. (2010). Antimicrobial resistance of *Campylobacter* isolates from retail meat in the United States between 2002 and 2007. *Applied and Environmental Microbiology, 76*, 7949–7956.

Zirnstein, G., Li, Y., Swaminathan, B., & Angulo, F. (1999). Ciprofloxacin resistance in *Campylobacter jejuni* isolates: detection of *gyrA* resistance mutations by mismatch amplification mutation assay PCR and DNA sequence analysis. *Journal of Clinical Microbiology, 37*, 3276–3280.