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Detection of efflux pump CmeABC in enrofloxacin resistant *Campylobacter* spp. strains isolated from broiler chickens (*Gallus gallus domesticus*) in the state of Rio de Janeiro, Brazil[1]

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ABSTRACT.- Nascimento R.J., Frasão B.S., Dias T.S., Nascimento E.R., Tavares L.S.B., Almeida V.L. & Aquino M.H.C. 2019. **Detection of efflux pump CmeABC in enrofloxacin resistant *Campylobacter* spp. strains isolated from broiler chickens (*Gallus gallus domesticus*) in the state of Rio de Janeiro, Brazil.** *Pesquisa Veterinária Brasileira 39(9):728-733*. Departamento de Saúde Coletiva Veterinária e Saúde Pública, Universidade Federal Fluminense, Rua Vital Brazil Filho 64, Niterói, RJ 24230-340, Brazil. E-mail: reginajulia@gmail.com

Fowls are the main reservoirs of the highly important food-originating pathogen called *Campylobacter* spp. and broilers' meat and byproducts are the main vehicles of this microorganism. Increasing of *Campylobacter* spp. resistant strains to fluorquinolones, an antimicrobial class often employed in poultry farming and in human medicine has become a great concern to poultry breeders. In fact, several studies evaluated increasing bacterial resistance against these antimicrobial agents. The role of CmeABC efflux system has been underscored among the resistance mechanisms in *Campylobacter* spp. to fluorquinolones. This study investigated the occurrence of CmeABC efflux pump in 81 and 78 enrofloxacin resistant strains of *Campylobacter jejuni* and *C. coli* respectively, isolated from broilers collected from six abattoirs situated at São José do Vale do Rio Preto/RJ poultry center and from two commercial abattoirs situated at Metropolitan Region of Rio de Janeiro, from 2013 to 2016. The resistance to enrofloxacin was assessed by agar dilution to determine minimum inhibitory concentration (MIC). The CmeABC efflux system was investigated through the detection of genes genes *cmeA*, *cmeB* and *cmeC* by PCR. The activity of CmeABC efflux pump was investigated in 20 strains by using the efflux pump inhibitor Phenylalanine-Arginine β-Naphthylamide (PAβN). The three genes *cmeA*, *cmeB* and *cmeC* were detected in 94.3% of the strains (*C. jejuni* = 80 and *C. coli* = 70), whereas the system was absent or incomplete in 5.7% of strains (*C. jejuni* = 1 and *C. coli* = 8). MIC varied between 0.5µg/ml and 64µg/ml, and 88.7% of strains were enrofloxacin resistant and 11.3% featuring intermediate resistance. The inhibition of the efflux pump by PAβN reduced the MIC to enrofloxacin up to eight times in fifteen strains (75%). These results indicate that this system is frequent and active in *Campylobacter* spp. Resistant strains in the presence of enrofloxacin.

INDEX TERMS: Efflux pump, CmeABC, enrofloxacin, *Campylobacter* spp., broiler chickens, *Gallus gallus domesticus*, Rio de Janeiro, Brazil, resistance, chickens.

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RESUMO.- [Detecção da bomba de efluxo CmeABC em cepas de *Campylobacter* spp. resistentes à enrofloxacina isoladas de frangos de corte (*Gallus gallus domesticus*) no Estado do Rio de Janeiro, Brasil.] As aves são os principais reservatórios *de Campylobacter* spp., importante patógeno de origem alimentar e a carne de frango e produtos derivados

são os principais veículos desse microrganismo. O aumento de cepas de *Campylobacter* spp. resistentes às fluorquinolonas, uma classe antimicrobiana frequentemente empregada na avicultura e na medicina humana, tornou-se uma grande preocupação para os produtores de aves e vários estudos avaliaram o aumento da resistência bacteriana a esses antimicrobianos. O papel do sistema de efluxo CmeABC tem sido enfatizado entre os mecanismos de resistência em *Campylobacter* spp. à fluorquinolonas. O presente estudo investigou a ocorrência da bomba de efluxo CmeABC em 81 cepas de *Campylobacter jejuni* e 78 cepas de *Campylobacter coli* resistentes à enrofloxacina, isoladas de frangos de corte coletados em seis abatedouros situados no polo avícola de São José do Rio Preto/RJ e de dois abatedouros comerciais situados na Região Metropolitana do Rio de Janeiro, de 2013 a 2016. A resistência à enrofloxacina foi avaliada pelo método de diluição em ágar para determinar a concentração inibitória mínima (CIM). O sistema de efluxo CmeABC foi investigado através da detecção dos genes *cmeA, cmeB e cmeC* por PCR. A atividade da bomba de efluxo CmeABC foi investigada em 20 cepas utilizando o inibidor da bomba de efluxo Phenylalanine-Arginine β-Naftilamida (PAβN). Os três genes *cmeA, cmeB e cmeC* foram detectados em 94,3% das cepas (C. jejuni = 80 e C. coli = 70), enquanto o sistema estava ausente ou incompleto em 5,7% das cepas (*C. jejuni* = 1 e *C coli* = 8). A CIM variou entre 0,5µg/ml e 64µg/ml e 88,7% das cepas foram resistentes à enrofloxacina, enquanto 11,3% apresentaram resistência intermediária. A inibição da bomba de efluxo pelo PAβN reduziu a CIM da enrofloxacina até oito vezes em quinze cepas (75%). Estes resultados indicam que este sistema é frequente e ativo em cepas resistentes de *Campylobacter* spp. na presença de enrofloxacina.

TERMOS DE INDEXAÇÃO: Bomba de efluxo, CmeABC, cepas, *Campylobacter* spp., enrofloxacina, frangos de corte, *Gallus gallus domesticus*, Rio de Janeiro, Brasil, resistência.

INTRODUCTION

The food-borne zoonotic pathogen *Campylobacter* spp. is one of the main gastroenteritis agents worldwide, especially in developing countries (Zhou et al. 2016). In the EU, 246.307 cases of campylobacteriosis were reported in 2016, featuring the most relevant cause of gastroenteritis since 2005 (EFSA & ECDC 2017). In the USA, *Campylobacter* was the most registered cause of bacterial gastroenteritis, with an estimate of 1.3 million cases a year (CDC 2017). Most campylobacteriosis cases are associated to the ingestion of raw and undercooked chicken meat or to cross-contaminated meat. Its prevalence in broilers' carcasses ranges from 0.29% to 96.7% (Aquino et al. 2002, Garin et al. 2012, Wang et al. 2013).

In developing countries, such as Brazil, information on outbreaks is often incomplete and diarrheal diseases are endemic. Although Brazil is the largest exporter of chicken meat in the world, the presence of *Campylobacter* spp. is not investigated in most cases of human bacterial gastroenteritis. This is probably due to the particularities of its isolation and characterization methodology, which is different from that applied to detect enteropathogenic bacteria, such as *Escherichia coli*, *Salmonella* and *Shigella* (Panzenhagen et al. 2016a).

Resistance to fluoroquinolones in *Campylobacter* spp. strains has been reported in several countries, with different results in the treatment of infections (Helms et al. 2005). The first reported resistance case in human treatment for *C. jejuni* occurred when enrofloxacin was used in the treatment of broilers in Holland in the winter of 1987 (Endtz et al. 1991).

Mutation in the Quinolones Resistance-Determining Region (QRDR) of gene *gyrA*, which codifies for subunit A of the DNA *gyrase* enzyme, substitutes Tre-86-Ile and is the main resistance mechanism to fluoroquinolones (Wieczorek & Osek 2013). However, several studies show that mutation in QRDR may not be the sole mechanism in the resistance to these drugs and reveal that efflux pump codified by *cmeA*, *cmeB* and *cmeC* genes is an important factor in *Campylobacter* spp. resistance to several antimicrobial agents (Poole 2005, Iovine 2013, Wieczorek & Osek 2013), alone or coupled to other mechanisms.

The inhibitor of efflux pump Phenylalanine-Arginine β-Naphthylamide (PAβN) is efficient against several Gram-negative bacteria and compromises nutrient uptake and excretion of toxic compounds through its inhibition. Several authors also underscore that the inhibition of the CmeABC efflux system by PAβN increases the susceptibility of *C. jejuni* to different antibiotics, including macrolides and fluoroquinolones, which are important drugs for the treatment of human campylobacteriosis (Mamelli et al. 2003, Chollet et al. 2004, Hasdemir et al. 2004, Saenz et al. 2004).

This study investigated the presence of the CmeABC efflux system and its activity in enrofloxacin resistant *C. jejuni* and *C. coli* strains isolated from broilers in the state of Rio de Janeiro, Brazil.

MATERIALS AND METHODS

Sample collection and species identification. Strains were obtained from samples of broilers' intestines, retrieved immediately after evisceration collected from six abattoirs situated at São José do Vale do Rio Preto poultry center with state inspection, and from two commercial abattoirs of State of Rio de Janeiro, from 2013 to 2016. In each collect, 10 broilers' intestines were collected and taken to the laboratory, for processing at the same day. Swabs with caecum material were diluted in 2mL of sterilized distilled water, 0.30ml was filtered by cellulose acetate membrane (Sartorius) (0.65µm), and spreaded on Columbia agar plates, supplemented with activated coal (0.4%) and CAMPYLOFAR® (CEFAR) (Aquino et al. 2002). Plates were incubated at 37°C for 48 hours in microaerophilic conditions. Suspect colonies were confirmed by PCR technique (Harmon et al. 1997) and *Campylobacter jejuni* ATCC 33560 and *Campylobacter coli* ATCC 33559 strains were employed as positive control.

Detection of antibiotic resistance genes. Eighty-one *Campylobacter jejuni* strains and 78 *C. coli* strains, classified as resistant to enrofloxacina by the agar dilution assay (CLSI 2013),were investigated to detect the CmeABC efflux system. Genes *cmeA*, *cmeB* and *cmeC* were detected by PCR, following Lin et al. (2002) and Obeng et al. (2012) as described: 25uL with 2µl of sample DNA; 2.5µl of 10X PCR Buffer; 0.2mmol l^{-1} of deoxyribonucleotide phosphates (dATP, dCTP, dGTP and dTTP); 0.4µmol l^{-1} of each starter (Table 1); 2.5U of Taq polymerase (Ludwig, Alvorada, Brasil) were employed and Thermal Cycler (ThermoElectron Corporation - Px2 ThermalCycler) was used to run the PCR. Initial denaturalization was undertaken at 96°C for 1 min, followed by 30 cycles with denaturalization for 30 seconds at 94°C; annealing temperature at 54°C for 45 seconds; at 72°C for 1 minute; final extension at 72°C for 5 min. Table 1 provides sequences of primers. For the visualization

Table 1. Primers used in the PCR to detect the CmeABC efflux system in *Campylobacter* strains

Target gene	Primer sequence (5′-3′)		Amplicon size (bp)	Reference
	Forward	Reverse		
cmeA	TTTGGATCCTTGATGGCTAAGGCAACTTTC	CTCCAATTTCTTAAGCTTCGCTACCAA	771	(Lin et al. 2002)
cmeB C. jejuni	GGTACAGATCCTGATCAAGCC	AGGAATAAGTGTTGCACGGAAATT	820	(Lin et al. 2002)
cmeB C. coli	TCCTAGCAGCACAATATG	AGCTTCGATAGCTGCATC	241	(Obeng et al. 2012)
cmeC	GCTTGGATCCTTATCTTGGGAAAAA	TTTTTAAAGCTTTAAGGTAATTTTCTT	624	(Lin et al. 2002)

of the PCR product, 5µL of amplicon was performed in a horizontal electrophoresis tank 'Electrophoresis Cell (BioAmérica) at Power Pac 300 (Bio-Rad) in agar gel 1.5%, buffer TBE (1.0M Tris, 0.01M boric acid, 0.01M EDTA, pH 8.0) (Ludwig, Alvorada, Brasil), and 1µlGelRed with 1µl loading buffer. Image was visualized and photographed in ultraviolet transilluminator (Nova Instruments).

Antibiotic susceptibility test. Resistance to enrofloxacin was investigated by agar dilution method to determine Minimum Inhibitory Concentration (MIC) (CLSI 2013). Suspension of inoculated *Campylobacter* was adjusted to turbidity, equivalent at McFarland 0.5 standard. Mueller Hinton agar plates were supplemented with sheep blood (7%) and enrofloxacin (64µg/ml, 32µg/ml, 16µg/ml, 8µg/ml, 4µg/ml, 2µg/ml, 1µg/ml, 0.5µg/ml, 0.25µg/ml, and 0.125µg/ml) to determine MIC. The plates were incubated at 37°C for 48 hours under microaerophilic conditions. The strains were classified according to the MIC value (µg/ml) as resistant (≥2µg/ml), intermediate (1µg/ml-0.5µg/ml) or susceptible (≤0.25µg/ml).

Effect of efflux pump inhibitor on antimicrobial resistance. The twenty most recently isolated strains harboring the three genes (by *cmeA*, *cmeB* and *cmeC*) had the activity of CmeABC efflux investigated, by efflux pump inhibitor Phenylalanine-Arginine β-Naphthylamide (PAβN) (MP Biomedicals, Santa Ana, California, USA). To determine the efflux pump activity, MIC was determined by agar dilution method with and without the inhibitor PaβN according to Hungaro et al. (2015). Mueller Hinton agar plates were supplemented with sheep blood (7%), inhibitor PAβN (5µg ml^{-1}) and enrofloxacin (64µg/ml, 32µg/ml, 16µg/ml, 8µg/ml, 4µg/ml, 2µg/ml, 1µg/ml, 0.5µg/ml, 0.25µg/ml, 0.125µg/ml). The plates were incubated at 37°C for 48 hours under microaerophilic conditions and changes in the MIC value were investigated.

RESULTS AND DISCUSSION

The three genes, *cmeA*, *cmeB* and *cmeC*, were detected in 94.3% (150) of 159 strains. Nine strains (5.7%) failed to have the full efflux system as described below: in one *Campylobacter coli* strain, the three genes were absent and in five *C. coli* strains the *cmeB* was not detected; the genes *cmeB* and *cmeC* were not detected in one *C. coli* strain and *cmeA* was not detected in one *Campylobacter jejuni* and in one *C. coli* strain.

In this study, the efflux pump system was observed in most *C. jejuni* e *C. coli* strains isolated from broilers. These results are in agreement with those of others authors from Brazil and other countries (Hungaro et al. 2015, Lin et al. 2002, Van Deun et al. 2007, Cantero et al. 2018) which frequently observed the presence of this efflux system in *Campylobacter* strains. Of the nine strains in which the full efflux system was not detected, one was isolated from a commercial abattoir and eight were from the poultry center located São José do Vale do Rio Preto.

Two efflux systems involved in the resistance mechanism of *Campylobacter* have been well characterized, namely, *Campylobacter* multidrug efflux CmeABC and CmeDEF, belonging to the proton motive force-dependent group, and Resistance-Nodulation-Division family (RND) (Jeon et al. 2011). The family RND is usually registered in Gram-negative bacteria and its efflux system works by a tripartite system that includes a periplasmatic membrane fusion protein, an inner membrane drug transporter and an outer membrane protein, which are codified, respectively, by genes *cmeA, cmeB* and *cmeC* in *Campylobacter* (Lin et al. 2005, Poole 2005). The three proteins are codified by a three-gene operon, (CmeABC), working together to expel toxic substrates from the interior of the bacterial cell (Lin et al. 2002).

Efflux systems in *Campylobacter* participate in the uptake of essential nutrients and ions, in the excretion of bacterial metabolism products and toxic substances. They also participate in the communication processes between cells and the environment. They have also been studied as a potential target to reduce resistance to antibiotics in *Campylobacter* spp. and as an alternative strategy to limit contaminations and prevent infections (Možina et al. 2011, Nikaido & Jean-Marie 2012).

Resistance-nodulation-division (RND) efflux systems contribute towards *Campylobacter*'s intrinsic resistance for a wide range of structurally non-related antimicrobial agents. They may acquire resistance to fluoroquinolones by super-expression of efflux proteins or by synergic interaction with resistance mechanisms to non-efflux fluoroquinolones, such as mutations in gene *gyr*A (Lin et al. 2002, Luo et al. 2003). Luangtongkum et al. (2009) showed that CmeABC system works synergically with *gyr*A in the mediation of resistance to fluoroquinolones, whereas strains resistant to fluoroquinolones without Tre-86-Ile mutation have already been reported (Hungaro et al. 2015).

Minimum Inhibitory Concentration (MIC) in this study ranged from 0.5 to 64µg/mL, and 88.7% of the strains were resistant, whereas 11.3% showed intermediate resistance. The highest levels of resistance were observed in abattoir 3 (Table 2), a commercial abattoir, located at metropolitan region of Rio de Janeiro In Brazil, enrofloxacin is frequently employed in poultry breeding, and there is no effective control by regulatory agencies of its use in small producers, as may be verified in the report of the State Program for the Control of Veterinary Medicine Wastes in Animal-derived Food (PAMvet) in the state of Paraná, Brazil (Machinski Junior et al. 2005). In fact, Panzenhagen et al. (2016b) registered enrofloxacin wastes in 72.2% of broilers' samples collected in the state of Rio de Janeiro, analyzed by Enzyme-Linked Immunosorbent Assay (ELISA), albeit at rates lower than the Maximum Waste Limits. In Spain, Cantero et al. (2018) reported that

Table 2. Strains, origin and determination of MIC with and without PAβN inhibitor in 20 *Campylobacter* spp. strains

Campylobacter strains	Source	*CmeA*	*CmeB*	*CmeC*	MIC (μg/mL)	MIC with PAβN (μg/mL)
C. coli	Abattoir 1	+	+	+	4(R)	1(I)
C. coli	Abattoir 1	+	+	+	2(R)	1(I)
C. coli	Abattoir 2	+	+	+	2(R)	1(I)
C. coli	Abattoir 2	+	+	+	2(R)	1(I)
C. coli	Abattoir 2	+	+	+	1(I)	1(I)
C. coli	Abattoir 2	+	+	+	2(R)	1(I)
C. coli	Abattoir 2	+	+	+	1(I)	1(I)
C. coli	Abattoir 2	+	+	+	2(R)	1(I)
C. coli	Abattoir 3	+	+	+	64(R)	8(R)
C. coli	Abattoir 3	+	+	+	8(R)	4(R)
C. coli	Abattoir 3	+	+	+	8(R)	8(R)
C. coli	Abattoir 3	+	+	+	8(R)	1(I)
C. coli	Abattoir 3	+	+	+	8(R)	8(R)
C. coli	Abattoir 3	+	+	+	8(R)	4(R)
C. coli	Abattoir 3	+	+	+	8(R)	4(R)
C. coli	Abattoir 3	+	+	+	16(R)	2(R)
C. jejuni	Abattoir 3	+	+	+	4(R)	2(R)
C. jejuni	Abattoir 3	+	+	+	4(R)	2(R)
C. jejuni	Abattoir 3	+	+	+	4(R)	4(R)
C. jejuni	Abattoir 3	+	+	+	16(R)	8(R)

Abattoir 1 = located in São José do Vale do Rio Preto, Abattoir 2 and 3 = located in Metropolitan Region of Rio de Janeiro, R = resistant strain, I = intermediate resistance.

isolated strains of broilers were predominantly resistant to quinolones, perhaps due to the frequent use of enrofloxacin in poultry breeding. The 2016 EU´s report similarly described resistance to antimicrobial agents in zoonotic bacteria and in indicating bacteria from humans, animals and food. In fact, *Campylobacter* strains isolated from humans and broilers showed high to extremely high resistance to ciprofloxacin (EFSA & ECDC 2018).High minimum inhibitory concentration for enrofloxacin in broiler-derived strains in this study may compromise the efficaciousness of fluoroquinolones in the treatment of human infections caused by poultry-derived strains.

MIC varied from 8 to 32μg/mL in strains without *cmeA* or *cmeC,* whereas resistance ranged from 0.5 to 32μg/mL in 7 strains in which the gene *cmeB* was not detected. These findings suggest the participation of another resistance mechanism besides the efflux pump. The lack of genes *cmeA* and *cmeC* in a study by Hungaro et al. (2015) failed to affect MIC, whereas Pumbwe & Piddock (2002) reported that the absence of *cmeB* made possible the recovery of levels of sensitiveness. The gene *cmeB* codifies the internal membrane which transports drugs and it is fundamental for the functioning of the CmeABC efflux system. Our study investigated the activity of efflux pump with inhibitor PAβN in *Campylobacter* spp. strains with complete efflux system and revealed a decrease in MIC of enrofloxacin from two to eight times in 15 (75%) strains. Despite the MIC decreasing, eight strains remained resistant and seven strains showed intermediate resistance (Table 2). No change in the MIC of 5 (25%) strains with the complete efflux system was observed, suggesting that the efflux system was inactive.

Effective participation of the CmeABC efflux system in the *Campylobacter* fluoroquinolone resistance has not always been reported. Mavri & Možina (2012) reported MIC reductions for ciprofloxacin with PAβN inhibitor in only 50% of strains derived from broilers, pigs, humans and surface water. Corcoran et al. (2005) demonstrated that PAβN inhibitor did not cause significant decrease in resistance to fluoroquinolones in *Campylobacter* spp. isolates from broilers and humans. On the other hand, Kurinčič et al. (2012) reported MIC decrease in fluoroquinolones and the reestablishment of susceptibility with PAβN inhibitor in *Campylobacter* isolates from food, animals, water and humans. These results indicate that the efflux mechanism is involved in the resistance to enrofloxacin in *Campylobacter*, but not as a single mechanism. In fact, in our study, several resistant strains did not have the full CmeABC efflux system or the activity of the efflux pump was not reduced by the use of PAβN inhibitor.

Several studies have been undertaken recently on natural compounds which may inhibit the efflux system of bacteria (Chérigo et al. 2009, Fadli et al.2011, Ramalhete et al. 2011, Roy et al. 2012, Možina et al.2018). Due to the crucial role of the CmeABC system in the adaptation of *Campylobacter* spp. in the intestine tract, it has been suggested that this would be a proper target for the control of infections by *Campylobacter* spp. The inhibition of efflux systems in *C. jejuni* may be a new approach to decrease resistance to antibiotics and prevent infection by *Campylobacter* spp. in human beings and animal reservoirs. The employment of *CmeABC* inhibitors as food additives in poultry breeding systems to prevent or decrease intestine colonization and reduce the dissemination of *Campylobacter* resistant strains may be considered an advance in the use of inhibitors. In fact, many cases of human campylobacteriosis are attributed to the intake of raw or undercooked chicken meat (Hungaro et al. 2015, Možina et al. 2018). Further, several regulating mechanisms that change CmeABC expression have been described (Lin et al. 2005) and information on them and knowledge on the structure, functions and regulation of other efflux pumps in *Campylobacter* may identify new targets for the therapeutic intervention of *Campylobacter* (Možina et al. 2018).

CONCLUSIONS

Our findings suggest that CmeABC efflux system is an important mechanism in the *Campylobacter* resistance to enrofloxacin .However, the lack of a full efflux system in resistant strains and the MIC maintenance in the presence of the inhibitor PAβN indicates the participation of other mechanisms or a synergic activity in the resistance of enrofloxacin.

The use of efflux pump inhibitors may offer perspectives to reduce antimicrobial resistance and the colonization of *Campylobacter* spp. in broilers.

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