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Detection of fluoroquinolone resistance by mutation in *gyr*A gene of *Campylobacter* spp. isolates from broiler and laying (*Gallus gallus domesticus*) hens, from Rio de Janeiro State, Brazil

Detecção de resistência às fluoroquinolonas através da mutação no gene *gyr*A em *Campylobacter* spp. isolados de frangos de corte e galinhas (*Gallus gallus domesticus*) poedeiras, no Estado do Rio de Janeiro, Brasil

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ABSTRACT

*Poultry are considered to be the main reservoir of **Campylobacter** spp. bacteria, an important pathogen for humans. Many studies have reported a rapid selection of fluoroquinolone-resistant strains following the widespread use of these antimicrobials in poultry production and human medicine. The main mechanism of fluoroquinolone resistance in **Campylobacter** is a mutation in the Quinolone Resistance Determinant Region (QRDR) in the gyrA gene, which codes for the subunit of the enzyme DNA gyrase, the target for fluoroquinolone. The aim of this study was to investigate the mutation in QRDR in the gyrA gene of **Campylobacter** strains previously isolated from broiler carcasses and feces of laying hens. Thirty-eight strains of **C. jejuni** and 19 **C. coli** strains (n=57), previously characterized as resistant to ciprofloxacin and enrofloxacin by the disk diffusion method and minimum inhibitory concentration (MIC), were selected. For detection of the mutation, a fragment of 454pb QRDR in the gyrA gene was used for direct sequencing. All strains presented the QRDR mutation in the gyrA gene at codon 86 (Thr-86-Ile), which confers resistance to fluoroquinolones. Other known silent mutations were observed. This genotypic characterization of fluoroquinolone resistance in **Campylobacter** strains has confirmed the prior phenotypic detection of the resistance. The Thr-86-Ile mutation was observed in all samples confirming that this is the predominant mutation in enrofloxacin and ciprofloxacin resistant strains of **C. jejuni** and **C. coli**.*

Key words: *enrofloxacin, ciprofloxacin, poultry, **C. jejuni**, **C. coli**.*

RESUMO

*As aves são consideradas o principal reservatório de **Campylobacter** spp., um importante patógeno para humanos e muitos estudos têm relatado uma rápida seleção de cepas resistentes às fluoroquinolonas após o uso destes antimicrobianos na produção avícola e na medicina humana. O principal mecanismo de resistência às fluoroquinolonas em **Campylobacter** consiste na mutação na Região Determinantes de Resistência às Quinolonas (RDRQ) do gene gyrA, que codifica para a subunidade A da enzima DNA girase, alvo das fluoroquinolonas. O objetivo deste estudo foi investigar a mutação na RDRQ do gene gyrA em cepas de **Campylobacter** previamente isolados de carcaças de frangos de corte e fezes de galinhas poedeiras. Foram selecionadas 38 cepas de **C. jejuni** e 19 cepas de **C. coli** (n=57), previamente caracterizadas como resistentes à ciprofloxacina e enrofloxacina, pelo método da difusão em disco e pela determinação da concentração inibitória mínima. Para detecção da mutação, foi utilizado sequenciamento direto de um fragmento de 454pb da RDRQ do gene gyrA gerado por PCR. Todas as cepas apresentaram a mutação na RDRQ do gene gyrA no códon 86 (Tre-86-Ile), que confere resistência às fluoroquinolonas e outras mutações silenciosas foram observadas. A caracterização genotípica da resistência às fluoroquinolonas em **Campylobacter** confirmou a prévia detecção fenotípica dessa resistência e a mutação Tre-86-Ile foi observada na totalidade das amostras, comprovando ser esta a mutação predominante em cepas de **C. jejuni** e **C. coli** resistentes à enrofloxacina e ciprofloxacina.*

Palavras-chave: *enrofloxacina, ciprofloxacina, aves, **C. jejuni**, **C. coli**.*

INTRODUCTION

Campylobacter jejuni and, less often, ***C. coli*** have emerged in recent decades as the leading cause of food-borne gastroenteritis in developed countries (WANG et al., 2013). Poultry are considered the main source of transmission to humans (BOLTON et al., 2014)

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due to the frequent presence of this microorganism in the gastrointestinal tract of these animals, in particular in the caecum, which provides a favorable environment for its development (MEREDITH et al., 2013).

The human campylobacteriosis in most of the cases is sporadic and in many cases are not diagnosed or reported and it is estimated that each year more than 2.4 million people are infected (CDC, 2013). Usually ***Campylobacter*** infections are self-limiting, but in some cases, as in persistent and severe infections or pregnant women, children and elderly and immunosuppressed the use of antimicrobials is necessary (WIECZOREK & OSEK, 2013). In some cases, after infection, in long term, there may be serious consequences as Miller Fisher syndrome, irritable bowel syndrome and Guillain-Barre Syndrome, one of the most common sequela in ***Campylobacter*** spp. infections, characterized by a demyelinating polyneuropathy (WHO, 2012).

Transmission of ***Campylobacter*** to humans most often occurs by direct contact with feces or by cross-contamination in the food chain, being the chicken meat the food most responsible (MILLER et al., 2010). Studies in Brazil have revealed the presence of resistance to fluoroquinolones in up to 100% of chicken-hosted ***Campylobacter*** strains (BADARÓ, 2013; MOURA, 2010). In the 1990s, when the use of enrofloxacin in animal production was introduced, the resistance to fluoroquinolones, especially ciprofloxacin, began to increase among ***Campylobacter*** strains isolated from humans in Asia and in Europe (ENDTZ et al., 1991). The same occurred in the United Kingdom and the United States after the approval of its use in Veterinary Medicine (NACHAMKIN et al., 2002). The acquisition of resistance affects the treatment of patients, increasing the duration of gastrointestinal symptoms in patients infected with resistant strains to fluoroquinolones (SMITH et al., 1999; ENGBERG et al., 2004).

Fluoroquinolones act by inhibiting bacterial DNA replication by its action on the enzymes DNA gyrase and topoisomerase IV (ALTE et al., 2012). The main mechanism of resistance to fluoroquinolones in ***Campylobacter*** is a mutation in the quinolone resistance determinant region (QRDR) in the *gyr*A gene, which codes for the 'A' subunit of the enzyme DNA gyrase, conferring a decreased sensitivity to these antibiotics. Most of the highly resistant ***C. jejuni*** strains have the Thr-86-Ile mutation, resulting in the replacement of the amino acid threonine by isoleucine (WILSON et al., 2000). Other substitutions were reported, such as Asp-90-Asn, Ala-70-Thr, Asp-85-Tyr, Pro-104-Ser (IOVINE, 2013; QIN et al., 2011; WIECZOREK & OSEK, 2013), but a role in the resistance to fluoroquinolones has not been established for these mutations. Other mutations seen as changes in the *gyr*B gene have not been documented in ***Campylobacter*** and few studies show mutations in the *par*C gene associated with resistance to fluoroquinolones (PIDDOCK et al., 2003).

The aim of this study was to determine the presence of mutation in the Quinolone Resistance Determinant Region in the *gyr*A gene in fluoroquinolone-resistant strains of ***Campylobacter jejuni*** and ***C. coli*** isolated from broilers and laying hens in the state of Rio de Janeiro, Brazil.

MATERIAL AND METHODS

A total of 57 ***Campylobacter*** strains resistant to fluoroquinolones isolated from chicken carcasses (n=49) belonging to 6 different flocks and laying hens (n=8) from three farms situated at Rio de Janeiro State were studied. The previous detection of resistance to fluoroquinolones was obtained by the disk diffusion method (NCCLS, 2003) and by determining the minimum inhibitory concentration (MIC) to enrofloxacin and ciprofloxacin (NCCLS, 2003). The strains showing a high value of MIC for enrofloxacin ($\geq$8-64μg mL^{-1}) and ciprofloxacin ($\geq$16-128μg mL^{-1}) were selected. Frozen strains at -20°C in peptone broth (Himedia/India) with 25% glycerol (Sigma-Aldrich/USA) were thawed and spread on Agar Columbia (Himedia/India) supplemented with 0.4% activated charcoal (Vetec Fine Chemicals/Brazil) and incubated at 37°C for 48 hours in a microaerophilic atmosphere. Multiplex PCR assay using primers C1288_R C412_F for 16S rRNA gene (816 bp) specific to the genus Campylobacter (LINTON et al., 1996) and the primers C1 and C4 for oxidoreductase gene (160 bp) specific for ***C. jejuni*** (WINTERS & SLAVIK, 1995) was carried for confirmation of the identification. Primers Col1 and Col2 for the *ceu*E gene (894 bp) (GONZALEZ et al., 1997) were used to confirm the species ***C. coli***. ***C. jejuni*** ATCC® 33560 and ***C. coli*** ATCC® 33559, kindly provided by the National Institute of Quality Control in Health from Oswaldo Cruz Foundation (INCQS/FIOCRUZ), were included as controls. For PCR, DNA was extracted by using a QIAGEN commercial extraction kit (QIAGEN Companies - Uniscience Brazil) according to the manual instructions. For multiplex PCR, an Invitrogen kit (Life Technologies/USA) was used. The final volume was 50μL, containing 5μL DNA, 1X PCR Buffer (500mM KCl, 100mM Tris-HCl [pH 9.0]); 1μL (200μM each) dATP, dCTP, dGTP e dTTP; 20pmol from each primer; 2.5U Taq

DNA polymerase and 2mM $MgCl_2$. For conventional PCR, 1.5mM $MgCl_2$ and 25pmol from each primer was used. The amplification reaction was performed in a thermocycler (Eppendorf® AG 22331, Hamburg). With multiplex PCR, the initial denaturation was at 94°C for 3 minutes and 25 cycles were performed with denaturation at 94°C for 1 minute, primer annealing at 50°C for 1 minute, extension at 72°C for 1 minute, and final extension at 72°C for 5 minutes. For conventional PCR, 30 cycles were performed with denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds and extension at 72°C for 1 minute. For the visualization of the PCR product, 12µL of amplicon was used for electrophoresis in agarose gel 1.5% with TBE buffer (Invitrogen - Life Technologies/USA) and ethidium bromide ($3mg.ml^{-1}$) and compared with a molecular weight of 100bp (Invitrogen - Life Technologies/USA).

For sequencing, PCR was performed with specific primers for amplification of QRDR in the *gyr*A gene, *Cjgyr*A QRDR F and *Cjgyr*A QRDR R (PARKHILL et al., 2000; PRICE et al., 2005). The Invitrogen kit (Life Technologies/USA) was used for the reaction, with 1X PCR Buffer (500mM KCl, 100mM Tris-HCl [pH 9.0]); 5µL (1mM each) dATP, dCTP, dGTP e dTTP; 0.5µL from each primer, 1.0U Taq DNA polymerase and 2mM $MgCl_2$. The amplification reaction was performed in a thermocycler (Thermo Electron Corporation – Px2 Thermal Cycler), with an initial denaturation at 94°C for 5 minutes, 35 cycles of denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds, extension at 72°C for 1 minute, and final extension at 72°C for 10 minutes. The amplification product was purified with the Commercial Purification Kit GE®, following the manual instructions.

The dosage of the purified DNA was performed as recommended in the 'Low DNA Mass Ladder' (Invitrogen - Life Technologies/USA) protocol, and 4µL of the purified amplicon was used for electrophoresis under the conditions already described. An automated sequencer (ABI PRISM-3100® GeneticAnalyze) was used, with capillaries of 50cm and polymer POP6 (Applied Biosystems - Life Technologies/USA). The sequences obtained in the chromatograms were processed using the BioEdit Sequence Alignment Editor software (Hall, 1999) and Molecular Evolutionary Genetics Analysis Version 6.0, MEGA6 (TAMURA et al. 2013). The sequence of two strains, ***C. jejuni*** (L04566.1) and ***C. coli*** (U63413.1) collected from GenBank, were used as standard sensitive strains to compare with the field isolates.

RESULTS

Within the 57 selected strains, 38 (66.67%) were characterized as ***C. jejuni*** and 19 (33.33%) as ***C. coli***. Within the strains isolated from chicken carcasses, 19 were confirmed as ***C. coli*** species and 30 as ***C. jejuni***, while all isolates from laying hens were confirmed as ***C. jejuni***.

All of the investigated strains, except for ***C. jejuni*** ATCC 33560 and ***C. coli*** ATCC 33559, had a mutation at codon 86 (Thr-86-Ile) in the QRDR fragment of the *gyr*A gene. In all isolates from laying hens, the silent mutations His-81-His, Ser-119-Ser and Ala-120-Ala were observed. All ***C. jejuni*** strains from chicken carcasses had the silent mutation Gly-74-Gly and one strain had two more silent mutations, Asp-75-Asp and Ser-79-Ser. All the ***C. coli*** strains from chicken carcasses had the silent mutations Phe-99-Phe and Ala-122-Ala (Table 1).

DISCUSSION

In this study, all fluoroquinolone-resistant strains had the mutation that replaces the amino acid threonine by isoleucine. The same was observed by DUARTE et al. (2014) in Portugal, who found the mutation in all ciprofloxacin-resistant strains in other tests. Ruiz et al. (1998), demonstrated that strains with MIC for ciprofloxacin $\geq$16µg mL^{-1} had the mutation Thr-86-Ile, with one exception due to the occurrence of Thr-86-Lys mutation, while all strains with MIC of ciprofloxacin $\leq$0.25µg mL^{-1} had no mutation in codon Thr-86. In this study, all of the investigated strains had high-level resistance, substantiated by the values obtained at CIM, ranging from $\geq$8µg mL^{-1} to $\leq$64µg mL^{-1} for enrofloxacin; and from $\geq$16µg mL^{-1} to $\leq$128µg mL^{-1} for ciprofloxacin, confirming that this change is always related with high MICs to fluoroquinolones (SAID et al., 2010).

Beyond the Thr-86-Ile mutation, no other mutations that confer resistance to fluoroquinolones were detected in the strains investigated. All ***C. jejuni*** strains had a nucleotide 257 change from ACA to ATA and all ***C. coli*** strains had a change from ACT to ATT as described previously (SAID et al., 2010). Ciprofloxacin is the main metabolite of enrofloxacin and the antimicrobial activity of the latter has been linked in part to the action of this metabolite (IDOWU et al., 2010). Although previous research addressed only the mutations found in ciprofloxacin-resistant strains, in this study the strains were also resistant to enrofloxacin, suggesting the same resistance acquisition mechanism.

Table 1 - Nucleotide and amino acid changes due to silent and missense mutation in the quinolone resistance-determining region of *gyr*A gene of DNAgyrase of 57 strains of ***Campylobacter jejuni*** and ***C. coli*** isolated from poultry.

Type of Mutation	Nucleotide change	Amino acid change	---------- N. of isolates from species ----------	
			C. jejuni (38 strains)	***C. coli*** (19 strains)
Silent Mutation	CAC → CAT	His-81 → His		
	AGT → AGC	Ser-119 → Ser	8	---
	GCC → GCT	Ala-120 → Ala		
	GGT → GGG	Gly-74 → Gly	29	---
	GGT → GGG	Gly-74 → Gly		
	GCT → GAT	Asp-75 → Asp	1	---
	CGT → AGT	Ser-79 → Ser		
	TTT → TTC	Phe-99 → Phe	---	19
	GCG → GCA	Ala-122 → Ala		
Missense mutation	ACA → ATA	Thr-86 → Ile	38	---
	ACT → ATT	Thr-86 → Ile	---	19

Silent mutations are often described in fluoroquinolone sensitive and resistant strains and many combinations of transitions and mutations may exist. BECKMANN et al. (2004) reported the same silent mutations detected in this research, His-81-His and Ser-119-Ser, in quinolone sensitive strains. The silent mutation Ala-120-Ala, found in this study, was also previously described by WILSON et al. (2000) and HAKANEN et al. (2002).

The increasing level of resistance to fluoroquinolones currently observed in ***Campylobacter*** spp. strains isolated from poultry reveals the impact of the use of these antibiotics in poultry production (ENDTZ et al., 1991; SMITH et al., 1999; ENGBERG et al., 2004; HUMPHREY et al., 2005). Resistance levels vary according to the country, variable factors including the permission to use this drug in poultry production (CHEN et al., 2010). In Brazil, the use of this drug has been allowed for therapeutic purposes in poultry production. However, in Norway and Australia, where the use of these antibiotics in aviculture is not allowed, fluoroquinolone resistant ***Campylobacter*** were not detected in studies in poultry slaughterhouses (NORSTRÖM et al., 2007; OBENG et al., 2012).

CONCLUSION

The genotypic characterization of resistance to fluoroquinolones in ***Campylobacter*** confirmed the previous phenotypic detection of this resistance. Thr-86-Ile mutation was observed in all the resistant strains, proving this to be the predominant mutation in ***C. jejuni*** and ***C. coli*** resistant to ciprofloxacin and enrofloxacin. More extensive studies of genotyping and the level of resistance to these antibiotics in ***Campylobacter*** spp. isolated from humans and poultry must be performed in order to investigate the role of these resistant strains, originated from poultry production, in human infections.

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