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Prevalence and antimicrobial resistance of *Campylobacter jejun*i and *Campylobacter coli* in slaughtered broiler chickens in Paraguay

Prevalencia y resistencia antimicrobiana de *Campylobacter jejuni* y *Campylobacter coli* en pollos parrilleros de una planta frigorífica en Paraguay

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ABSTRACT

Campylobacteriosis is one of the main zoonotic diseases transmitted through food, particularly chicken meat and its derivatives, which are relevant sources of transmission to humans. This study aimed to ascertain both prevalence and antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* in Cobb 500TM broiler chickens from a slaughterhouse in Paraguay. From 300 cloacal swab samples collected randomly, microbiological analysis were performed followed by bacterial confirmation through molecular polymerase chain reaction (PCR). *Campylobacter spp.* prevalence was 63.6 %, with 97.3 % corresponding to *C. jejuni* and 2.7 % to *C. coli*. Bacterial susceptibility to ciprofloxacin and erythromycin was determined using the E-test®. Resistance to ciprofloxacin was observed in 85 % of *C. jejuni* and in 100 % of *C. coli* isolates. Erythromycin resistance was identified in only one *C. jejuni* issolate (0.5 %). This research highlights the significance of *C. jejuni* prevalence and resistance to ciprofloxacin. These findings underscore the public health risk associated with chicken meat consumption, possibly containing antibiotic-resistant *Campylobacter* species. Hence, the necessity of implementing health control measures, concerning antibiotic treatments in poultry production can be emphasized.

Keywords: Poultry, antimicrobials, *Campylobacter spp*, food pathogens, PCR, public health.

RESUMEN

La campylobacteriosis es una de las principales enfermedades zoonóticas transmitidas por los alimentos, en particular la carne de pollos parrilleros y sus derivados, representando fuentes importantes de transmisión a los humanos. Esta investigación tuvo como objetivo verificar la prevalencia y resistencia antimicrobiana de *Campylobacter jejuni* y *Campylobacter coli* en pollos parrilleros Cobb 500™ de una planta frigorífica en Paraguay. A partir de 300 muestras de hisopado cloacal colectadas aleatoriamente, fueron realizados análisis microbiológico seguido de confirmación por reacción en cadena de la polimerasa (PCR). La prevalencia *Campylobacter spp.* fue de 63.6 %, con 97.3 % correspondiente a *C. jejuni* y 2.7 % a *C. coli*. La susceptibilidad antimicrobiana a la ciprofloxacina y eritromicina fue determinada por E-test®. La resistencia a la ciprofloxacina fue de 85 % en aislados de *C. jejuni* y en 100 % de *C. coli*. La resistencia para la eritromicina fue encontrada en un aislado de *C. jejuni* (0.5 %). Este trabajo alerta valores relevantes de resistencia a la ciprofloxacina y de alta prevalencia de *C. jejuni*. Estos resultados informan el riesgo que representa, para la salud pública, el consumo de carne de pollos parrilleros contaminada con especies de *Campylobacter* resistentes a antibióticos. Por lo tanto, puede ser enfatizada la necesidad de implementar medidas de control sobre la utilización de antibióticos en la producción avícola.

Palabras clave: avicultura, *Campylobacter spp*, patógenos de alimentos, PCR, salud pública.

INTRODUCTION

Poultry industry represents one of the primary sectors in the Paraguayan economy, with an exponential growth in the number of poultry farms dedicated to broiler chickens production (Rojas *et al*., 2010). This is directly related to the increase of local poultry meat consumption, which is even higher than that of beef and pork (USAID, 2010). However, technological advances have intensified exploitation systems, favoring proliferation and dissemination of crucial pathogens for public health, with birds as important reservoirs of zoonotic microorganisms. In poultry production, antimicrobial agents are massively used, not only as growth promoters but also for diseases prevention and control (Zendehbad *et al*., 2015).

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Campylobacteriosis is recognized as one of the most important gastric diseases in the world, characterized by diarrhoea, abdominal pain and fever in humans (Hungaro *et al*., 2015; Silva *et al*., 2016; Borck *et al*., 2016; Seliwiorstow *et al*., 2016). This disease is produced by thermophilic bacteria of the genus *Campylobacter*, among which *Campylobacter jejuni* and *Campylobacter coli* are the most associated species with frequent humans infection (Prachantasena *et al*., 2016). In broilers, scientific evidence have shown that campylobacteriosis prevalence is very high, reaching over 70 % (Saleha, 2002; Hue *et al*., 2011; Saiyudthong *et al*., 2015). However, some countries such as Italy, China, Czech Republic and Brazil reported lower prevalence values, between 32.7 and 63.1 % (Bardon *et al*., 2009; Chen *et al*., 2010; Giacomelli *et al*., 2012; Zendehbad *et al*., 2015; Wang *et al*., 2016). In this context, contaminated chicken meat represents the main transmission source for Campylobacteriosis to humans (Robyn *et al*., 2015; Abu-Madi *et al*., 2016).

Besides this, antibiotics are used in poultry for campylobacteriosis treatment, including macrolides and fluoroquinolones, such as erythromycin and ciprofloxacin, respectively (Payot *et al*., 2006). However, literature has demonstrated antimicrobial resistance to cyprofloxacin, tetracycline, nalidixic acid in some *Campylobacter* strains, fact that implies real sanitary risks to human health (Jamali *et al*., 2015; Wieczorek *et al*., 2015). Furthermore, antibiotic resistance in humans has been also observed. The literature argue that antibiotic resistance behaviour is linked with high campylobacteriosis prevalence in poultry farms, making the human population vulnerable due to the increased chicken meat consumption (Mäesaar *et al*., 2016). Thus, the objective of this study was to verify the prevalence of *C. jejuni* and *C. coli* in broilers from the Central Department of Paraguay and to evaluate their antimicrobial susceptibility to ciprofloxacin and erythromycin.

MATERIAL AND METHODS

Fecal samples collection and management

Cloacal swabs samples in chicken broilers was carried out with non-invasive procedures, without entering the birds' body cavity, at the slaughter installations. All the prevailing local, national and international regulations and conventions, as well as normal scientific ethical practices, were respected.

A total of 300 samples of cloacal swabs from Cobb 500™ broiler chickens of approximately 38 to 41 days of life were randomly collected. The samples were obtained from six different slaughter lots of a cold storage chamber located in Paraguay. Feces were collected before slaughter using sterile cotton swabs and transported in Cary-Blair medium (Oxoid, Dardilly, France).

Campylobacter spp. isolation and identification

Cloacal swabs samples were analyzed at the Laboratorio de Diagnóstico de los Animales Domésticos, Facultad de Ciencias Veterinarias, Universidad Nacional de Asunción. Bacterial culture consisted in direct sowing the swabs samples in *Campylobacter* Agar Preston enrichment medium (Oxoid, Basingstoke, UK), and then incubated in microaerophilic atmosphere with commercial CampyGen™ kit (Oxoid, Basingstoke, UK) at 42 °C during 48 h. Suspected colonies were collected on base blood agar (Oxoid, Basingstoke, UK) and incubated at 42 °C for 24 h. After incubation, petri dishes were examined for identification of *Campylobacter* spp (Silva *et al*., 2016). Identification at species level was performed by catalase test, indoxyl acetate hydrolysis, hippurate hydrolysis, and susceptibility to nalidixic acid and cephalothin (Ingresa-Capaccioni *et al*., 2015) and then confirmed by multiplex PCR (WHO, 2007).

DNA extraction for *Campylobacter spp.* identification and PCR conditions

Genomic DNA was extracted using a modified protocol of bacterial cell lysis, according to Giacomelli *et al*. (2012). The reaction mixture conformed the final volume of 25 µL, containing 2.5 µL Buffer (50 mM Tris-HCl 10X), 1 µL Cl_2Mg (50 mM), 2 µL of each dNTP (2.5 mM), 2.5 µL of each primer (10 µM), 0.25 µL of Taq polymerase (5U / µL Invitrogen™, USA), 4.25 µL of molecular quality water and 5 µL template DNA (100 ng/µL). The oligonucleotide sequence of each primer was previously described by Vandamme *et al*. (1997), for *Campylobacter* spp. identification, as shown in Table 1.

Bacterial DNA was amplified in a C1000™ thermocycler (BIO-RAD, Singapore) with the following amplification conditions: Initial denaturation of 94 °C during for minutes, eight cycles of one minute at 94 °C, with a decreacing gradient of 2 °C every two cycles, beginning with 64 °C and 72 °C; followed by 30 cycles at 94 °C, 54 °C and 72 °C for 1 min and a final extension stage at 72°C for 10 min (WHO, 2007).

Therefore, PCR products were separated by electrophoresis in 2 % agarose gel at 100 V and 400 mA for 40 min, in Tris-Acetate-EDTA (TAE 1X), and stained with ethidium bromide to be visualized on Digidoc-It® Imaging System (UVP, Canada). The reference strains used were *C. jejuni* ATCC 29428 and *C. coli* ATCC 33559.

Antimicrobial susceptibility testing

The minimal inhibitory concentration (MIC) to ciprofloxacin and erythromycin were assessed by E-test® (AB Biodisk, Sweden) using Mueller-Hinton agar, under microaerophilic

Table 1. Primers used in the polymerase chain reaction (PCR) to identify *Campylobacter jejuni* and *Campylobacter coli* in fecal samples from slaughtered broiler chickens in Paraguay.
Tabla 1. Iniciadores utilizados en la reacción en cadena de la polimerasa (PCR) para identificar *Campylobacter jejuni* y *Campylobacter coli* en muestras fecales de pollos parrilleros en Paraguay.

Target species	Target gene[a]	Primer sequence (5′ → 3′)	Product size (bp)
Campylobacter jejuni	Random	F: CA TCT TCC CTA GTC AAG CCT R: AAG ATA TGG CTC TAG CAA GAC	773
Campylobacter coli	Random	F: AG GCA AGG GAG CCT TTA ATC R: TAT CCC TAT CTA CAA ATT CGC	364

[a]Assay performed as a multiplex reaction for detection of both C. jejuni and C. coli. F=Forward; R= Reverse

atmosphere at 37 °C during 48 h (Albert, 2013), at the Laboratorio Central de Salud Pública, Ministerio de Salud Pública y Bienestar Social. The cut-off points were interpreted according to CLSI M45-2 document (CLSI, 2012), where MIC of ≤ 8 is considered as sensitive, 16 as intermediate and ≥ 32 as resistant strain for Erythromycin; and for Ciprofloxacin, when ≤ 1 is considered as sensitive, 2 as intermediate and ≥ 4 as resistant strain. *C. jejuni* ATCC 29428 strains were included in the experimental assay for quality control.

Statistical analyses

To investigate *C. jejuni* and *C. coli* prevalence in different batches of Cobb 500™ broiler chickens, non-parametric ANOVA with sensitive and resistant samples was used within each batch. The samples in each batch, ractor considered as fixed effect, were used to asses differences between possitive (1) and negative (0) porcentages for *Campylobacter spp.* trough a generalized linear model (GLM) with binomial distribution. Each sample was considered as random effect. Statistical analyses were executed using R (R Core Team, 2016) with lme4 package (Bates *et al*., 2015) considering 5 % of significance level ($p < 0.05$).

RESULTS AND DISCUSSION

Table 2 shows the absolute frequencies of *Campylobacter spp.* found in the current study. From the 300 samples, 191 (63.6 %) were positive for *Campylobacter spp*., 186 (97.3 %) of which corresponded to *C. jejuni* and 5 (2.7 %) to *C. coli*. Furthermore, Figure 1 evidence the electrophoresis gel from *C. jejuni* and *C. coli*, as result from PCR identification.

Particulary, the differences between *C. jejuni* and *C. coli* prevalence values, could be related to the fact that poultry chicken are hosts of *C. jejuni* and that they serve as a reservoir for this pathogen (Sahin *et al*., 2002; Lee and Newell, 2006) and, there is evidence for the season of the year influencing *C. jejuni* and *C. coli* sprouts, resulting in largest numbers of *C. jejuni* cases in spring (Wieczorek *et al*., 2020), coinciding with the results of the current work, considering that the samples were obtained from October to December (spring), therefore, the largest cases of *C. coli* are present in autumn.

Prevalence studies were developed in countries with relevant meat produciton from poultry farming. In commercial broiler chickens production from Brazil, Malaysia, and the United Kingdom, high *Campylobacter* spp. prevalence were found, ranging from 58 to 95 % in fecal samples (Saleha, 2002; Yew *et al*., 2010; Colles *et al*., 2011; Silva *et al*., 2016). In the same context, Italy and Spain commercial broiler chicken produciton showed prevalence values between 65 to 61.9 %, respectively (Giacomelli *et al*., 2012; Ingresa-Capaccioni *et al*., 2015), which are similar to those found in this research. Other studies showed lower values found in samples of cecal content (37.1 and 35.9 %), meat (18.9 %) and broiler carcasses (16.8 %) (Chen *et al*., 2010; Wang *et al*., 2016; Mäesaar *et al*., 2016). These differences can be attributed to the type of sample, bacterial isolation procedures, DNA extraction methods, primers sequences used for PCR, management practices or broiler chickens age sampled.

At species level, this research verified that *C. jejuni* prevalence was higher than *C. coli*. The same relation has been widely described (Bardon *et al*., 2009; Hungaro *et al*., 2015;

Table 2. Frequency (n) and percentage (%) of *Campylobacter jejuni* and *Campylobacter coli* prevalence found by polymerase chain reaction (PCR), from fecal samples of slaughtered broiler chickens in Paraguay (N = 300).
Tabla 2. Frecuencia (n) y porcentaje (%) de la prevalencia de *Campylobacter jejuni* y *Campylobacter coli*, determinada mediante la reacción en cadena de la polimerasa (PCR), de muestras fecales de pollos parrilleros en Paraguay (N= 300).

	Samples			*Campylobacter*	
Batch	**N**	**Positive**	**Negative**	***jejuni***	***coli***
A	50	42 (84.0)	8 (16.0)	42 (100.0)	/
B	50	40 (80.0)	10 (20.0)	40 (100.0)	/
C	50	15 (30.0)	35 (70.0)	15 (100.0)	/
D	50	26 (52.0)	24 (48.0)	25 (96.2)	1 (3.8)
E	50	40 (80.0)	10 (40.0)	39 (97.5)	1 (2.5)
F	50	28 (56.0)	22 (44.0)	25 (89.2)	3 (10.8)
Total	300	191 (63.6)	119 (36.4)	186 (97.3)	5 (2.7)

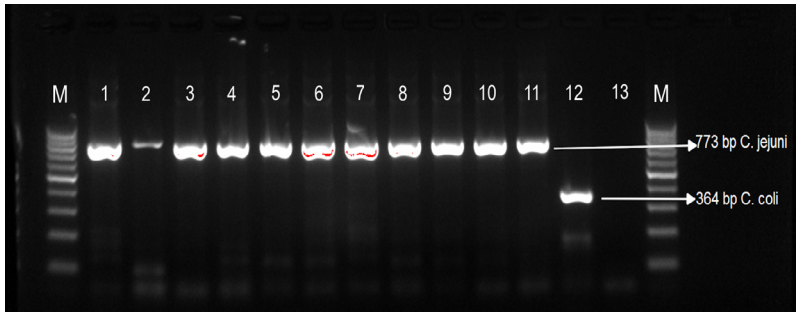

Figure 1. PCR agarose electrophoresis gel of bacterial DNA samples from fecal samples, obtained in slaughtered broiler chickens in Paraguay. *Lane M: 100 bp molecular weight marker. Lanes 1 to 10: Campylobacter jejuni positive samples. Lane 11: Campylobacter jejuni positive control. Lane 12: Campylobacter coli positive control. Lane 13: Negative control.*
Figura 1. Gel de electroforesis en agarosa de PCR a partir de DNA bacteriano de muestras fecales, obtenidas de pollos parrilleros en Paraguay. *Carril M: Marcador de tamaño molecular de 100 bp. Carriles 1 a 10: Muestras positivas a Campylobacter jejuni. Carril 11: Control positivo de Campylobacter jejuni. Carril 12: Control positivo de Campylobacter coli. Carril 13: Control negativo.*

Saiyudthong *et al*., 2015; Mäesaar *et al*., 2016). In contrast, Wang *et al*. (2016) found percentages of *C. coli* positive samples in broiler chickens, slightly higher when compared to *C. jejuni* in the same samples; as well as in commercial pigs with 98.7 % for *C. coli* and 1.2 % for *C. jejuni*. In studies developed in human faecal samples, *C. jejuni* prevalence was higher than *C. coli* (Thakur *et al*., 2010; Rivera *et al*., 2011; Tamborini *et al*., 2012; Quetz *et al*., 2016).

The present work found a high antimicrobial resistance to ciprofloxacin (85 %) while resistance to erythromycin was very low (0.5 %) ($p < 0.05$). (Table 3).

In broiler chickens, there is scientific evidence showing resistance to ciprofloxacin, with values ranging from 60.2 to 99.5 % in *C. jejuni* and 44.4 % to 100 % in *C. coli*; and to erythromycin, with values ranging from 1 % to 98.3 % in *C. jejuni* and 33.3 to 100 % in *C. coli* (Bardon *et al*., 2009; Chen *et al*., 2010; Yew *et al*., 2010; Zendehbad *et al*., 2015; Wang *et al*., 2016; Mäesaar *et al*., 2016). The main cause of the high *C. jejuni* ciprofloxacin resistance in the present study could be due to genetic and environmental effects. On one hand, *C. jejuni* does not have one of the main action target sites of Topoisomerase IV, due to a punctual gene mutation, allowing it to show high resistance to ciprofloxacin; and also, it could be related to the indiscriminate use of antimicrobials in poultry industry (Orrego *et al*., 2014). Furtermore, one of the batches sampled in the present research, batch A, showed *C. jejuni* isolates with similar percentage between sensitive and resistant strains ($p > 0,05$), provably due to the slaughter of animals with less antibiotical use as growing promoter when compared to the other batches, with more than 90 % of the samples resistant to ciprofloxacin ($p < 0,05$), fact that could be related to the massive use of this antibiotic, aiming to treat and prevent of diseases, as well as growth promoters in broiler chickens commercial production (McDermott *et al*., 2002; Gouvêa *et al*., 2015)

In *C. jejuni* isolates of human origin, resistance to ciprofloxacin have been found to be between 68 % and 65 % (Tamborini *et al*., 2012; Mäesaar *et al*., 2016). Increased resistance to erythromycin, ciprofloxacin and tetracyclines has also been found in isolates from patients with diarrhoea, limiting their use for health treatment in humans (Albert, 2013). This is important for public health, since these antibiotics are commonly used for humans campylobacteriosis treatment (Wieczorek *et al*., 2015). In the present study, a single *C. jejuni* isolate was found to be resistant to both antibiotics. Several authors have described antibiotic multiresistance of *C. coli* when compared to *C. jejuni*, probably due to the intrinsic capacity of the microorganism to develop resistance to antibiotics (Zhao *et al*., 2010; Chen *et al*., 2010), wich was not observed in the present work, possibly due to the standarized poultry management in broiler chicken farms from Paraguay, sharing similar animal and antibiotic handling and production paterns.

CONCLUSIONS

This research demonstrated the presence of thermotolerant *Campylobacter* species, mainly *C. jejuni and C. coli*, in cloacal swabs from broiler chickens. The isolates studied showed high *C. jejuni* resistance to ciprofloxacin. This work provides unprecedented information on the prevalence and antimicrobial resistance of *Campylobacter* species from a slaughtered broiler chickens of Paraguay.

CONFLICTS OF INTEREST

The authors declare they have no conflicts of interest with regard to the work presented in this report.

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Table 3. Frequency (n) and percentage (%) of *Campylobacter jejuni* and *Campylobacter coli* antimicrobial susceptibility, obtained from fecal samples of slaughtered broiler chickens in Paraguay (N = 191).
Tabla 3. Frecuencia (n) y porcentaje (%) de susceptibilidad antimicrobiana de *Campylobacter jejuni* y *Campylobacter coli*, obtenida de muestras fecales de pollos parrilleros en Paraguay (N= 191).

	Campylobacter jejuni				***Campylobacter coli***			
	Ciprofloxacin		**Erythromycin**		**Ciprofloxacin**		**Erythromycin**	
Batch	**Sensitive**	**Resistant**	**Sensitive**	**Resistant**	**Sensitive**	**Resistant**	**Sensitive**	**Resistant**
A	21 (50.0)[a]	21 (50.0)[a]	42 (100)	/	/	/	/	/
B	2 (5.0)[b]	38 (95.0)[a]	40 (100)	/	/	/	/	/
C	/	15 (100.0)	15 (100)	/	/	/	/	/
D	1 (4.0)[b]	24 (96.0)[a]	25 (100)	/	/	1 (100)	1 (100)	/
E	2 (5.0)[b]	37 (95.0)[a]	39 (100)	/	/	1 (100)	1 (100)	/
F	2 (8.0)[b]	23 (92.0)[a]	24 (96.0)[a]	1 (4.0)[b]	/	3 (100)	3 (100)	/
Total	28 (15.0)[b]	158 (85.0)[a]	185 (99.5)[a]	1 (0.5)[b]	/	5 (100)	5 (100)	/

[a,b] Rows with different superscripts letters are significantly different ($p<0.05$)

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