Genotyping and molecular characterization of antimicrobial resistance in thermophilic *Campylobacter* isolated from poultry breeders and their progeny in Eastern Spain

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ABSTRACT Thermophilic *Campylobacter* spp. are recognized as a major cause of acute bacterial diarrhea in humans, with broiler meat being the most common source of human infection. Antibiotic therapy is usually necessary for severe or prolonged infections, especially in immunocompromised populations such as young or elderly individuals. However, different studies have demonstrated a close association between antibiotic use in animal production and antimicrobial resistance (**AMR**) in humans. In this sense, there is social pressure to reduce antibiotic administration and find adequate alternatives to control the presence of bacterial infections in farms. However, there is a lack of information related to *Campylobacter* AMR dynamics through the entire production system from breeders to their progeny. It is unknown if resistance genes are a result of adaptation through chromosomal mutation or through horizontal gene transfer, instead of vertical transmission of DNA from the parent to their progeny. Thus, the main objectives of this study were to assess the main AMR rates present in a poultry production system, to study the relationship between *Campylobacter* AMR profiles from breeders and their progeny, and to study the presence and distribution of antibiotic resistance genes in poultry production. Regarding AMR rates, ciprofloxacin was classified as extremely high, followed by nalidixic acid and tetracyclines that were classified as very high. Moreover, this study demonstrated a relationship between the AMR patterns and genes found from *Campylobacter* strains isolated in breeders and those present in their progeny.

Key words: antimicrobial resistance, *Campylobacter* spp., resistance gene, broiler, breeder

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INTRODUCTION

Thermophilic *Campylobacter* spp. are recognized as a major cause of acute bacterial diarrhea in humans, being responsible for 400 to 500 million cases of human gastroenteritis worldwide (CDC, 2018). The primary source of human infection is poultry meat, especially raw or undercooked chicken (EFSA, 2017a; CDC, 2018). From the 25 *Campylobacter* spp. described to date, the main species implicated in human infections are *Campylobacter jejuni* and *Campylobacter coli* (Skarp et al., 2016). Several authors have studied *Campylobacter* epidemiology in broilers flocks, trying to reduce *Campylobacter* prevalence at the farm level, intending to avoid the increase of human campylobacteriosis (Allen et al., 2011; Cox et al., 2012; Agunos et al., 2014; Marin et al., 2015; Ingresa-Capaccioni et al., 2016a,b). However, there is no gold standard measure that could be successfully implemented across the poultry farming system in Europe, thus resulting in a high prevalence of bacteria at the farm and slaughterhouse level (Vidal et al., 2013; EFSA, 2018a). There is a high variation in the *Campylobacter* prevalence between countries in the European Union (**EU**)—varying from 0.6 to 13.1% in the Nordic European countries, to 74.2 to 80.0% in France, Austria, Spain, Turkey, Slovenia, or Poland (Skarp et al., 2016; EFSA, 2015).

It is essential to highlight that severe or prolonged infections can occur, especially in immunocompromised

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populations, such as young or elderly individuals. In these circumstances, antibiotic therapy of campylobacteriosis is usually necessary, using antibiotics such as azithromycin, ciprofloxacin (**CIP**), and nalidixic acid (**NA**) (Taylor et al., 2013; CDC, 2018).

Antimicrobial resistance (**AMR**) has become a major public health threat worldwide (WHO, 2014). One of the main factors contributing to the emergence of resistant bacteria has been the extensive use of antimicrobials for growth promotion and disease prevention in animal production for several years (Guo et al., 2018; Mehdi et al., 2018). Although, since 2006, the use of antibiotics in poultry is a controlled practice in EU (ESVAC, 2018), different studies have demonstrated a close association between the antibiotic administrated in animal production and AMR in humans (Marshall and Levy, 2011; EFSA, 2017b; JIACRA, 2018) by the transfer of resistance from animal products to humans (Chantziaras et al., 2013). As a result, commonly used antibiotics have become ineffective for the treatment of a wide variety of bacterial diseases (Khurana et al., 2017; EFSA, 2018b). In this sense, there is social pressure to reduce antibiotic administration and find adequate alternatives to control the presence of bacterial infections in farms (Alós, 2015; Gadde et al., 2017; Lusk, 2018). Thus, many poultry companies in Europe are making significant investments in the last step of rearing, growing farms, to grow chickens free of antibiotics (JIACRA, 2018). However, there is a lack of information related to *Campylobacter* AMR dynamics through the entire production system from breeders to their progeny. It is unknown if resistance genes are a result of adaptation through chromosomal mutation or through horizontal gene transfer, instead of vertical transmission of DNA from the parent to their progeny (Yang et al., 2019).

In this context, the main objectives of this study were to assess the main AMR rates present in a poultry production system, to study the relationship between *Campylobacter* AMR profiles from breeders and their progeny, and to study the presence and distribution of antibiotic resistance genes in poultry production.

MATERIALS AND METHODS

Campylobacter Isolates Origin

Sixty-five *Campylobacter* isolates were obtained from January 2012 to August 2013, in a longitudinal and vertical study of the whole poultry production cycle (breeders and their progeny) in the Valencia region (Eastern Spain), carried out by the Center of Poultry Quality and Animal Feeding of the Valencian Community (CECAV) and CEU Cardenal Herrera University (Ingresa-Capaccioni et al., 2016a,b). The farms involved in the study belonged to 2 companies that handle the majority of poultry slaughtered in Spain. One flock from each farm was studied. To participate in the study, farms had to be commercial broiler farms with chickens reared on the floor. All the animals were kept indoor under controlled conditions with a density of 3 to 5 birds/m^2 and 33 kg/m^2 for breeders and broilers, respectively. Facilities were provided with programmable electrical lights, automated electric heating, and forced ventilation (Cobb, 2008; BOE, 2010).

Different types of samples were used for *Campylobacter* isolation. Strains were isolated from 4 batches of breeders (PS1, PS2, PS3, and PS4), and 12 batches of their progeny (3 per breeder batch: A, B, and C). To do so, breeder birds were monitored from the time just before housing the 1-day-old chicks in the houses (rearing), then throughout the laying period (0–60 wk), and throughout their progeny phase (broiler fattening, 1–42 D) until slaughter. With respect to sample collection, from each flock of breeders, ceca samples from 10 birds upon their arrival at the farm were taken and cloacal swabs were collected during the rearing period (1, 8, 12, 16, and 20 wk) and during the laying period (26, 31, 48, and 60 wk). Regarding broiler flocks, each flock was sampled just before placing 1-day-old chicks (d1). Then, cloacal samples were collected at weekly intervals during the fattening period (7, 14, 21, 28, 35, and 42 D) (Ingresa-Capaccioni et al., 2016a,b).

Campylobacter Isolation

Campylobacter isolation and identification were performed according to ISO 10272-1:2006. For the ceca content, pools of 10 animals per flock were created by removing and homogenizing 0.02 g per animal. From all samples, 10 µL aliquots of each pool suspension were cultured directly onto modified charcoal cefoperazone deoxycholate agar (Oxoid, Dardilly, France) and Preston agar (CM0689, Oxoid). Agar plates were incubated at 41.5 ± 1°C in a micro-aerobic atmosphere (84% N_2, 10% CO_2, 6% O_2) for 44 ± 4 h to detect the presence of suspected colonies consistent to *Campylobacter* spp. For cloacal swabs, they were directly plated onto modified charcoal cefoperazone deoxycholate agar and Preston agar and incubated as previously described.

After incubation, 5 *Campylobacter*-like colonies were plated onto Columbia Blood Agar (AES Laboratories, Bruz Cedex, France) for further characterization. Colony morphology and motility were evaluated under dark field microscopy. Confirmation of suspicious colonies was established by oxidase and catalase tests and plating at different temperatures and atmospheres (41.5°C under microaerophilic conditions and 25°C under aerobic conditions) in Columbia Blood Agar (AES Laboratories) (Ingresa-Capaccioni et al., 2016a,b). Finally, characterization of the bacterial species was done by the hippurate hydrolysis test. All isolates were stored at −80°C until use.

The selected isolates were unfrozen and plated onto Columbia Blood Agar (Oxoid Ltd., England, UK). Plates were incubated at 41.5 ± 1°C for 44 ± 4 h under a micro-aerobic atmosphere (84% N_2, 10% CO_2, and 6% O_2) (CampyGen, Oxoid Ltd.). Identification was confirmed by observing the mobility in a dark field microscope.

Campylobacter *Molecular Identification*

DNA extraction A loop of *Campylobacter* pure overnight culture of each strain was used to perform the DNA extraction. All DNA extractions were carried out by using a commercial kit (GenElute Bacterial Genomic DNA Kit, Sigma-Aldrich, St. Louis, MO), according to the manufacturer recommendations. DNA solutions were kept at −20°C until the PCR was carried out.

Multiplex PCR A multiplex PCR was performed to analyze if the isolate belonged to the *Campylobacter* genus, and to differentiate between thermophilic species and other *Campylobacter* spp. Primers (Table 1) were directed to the following target genes: a part of the gene aspartokinase (asp), specific of *C. coli* (Linton et al., 1997); the hipuricase gene (hipO), specific of *C. jejuni*; and a universal 16S rDNA sequence common to all *Campylobacter* spp. (Persson et al., 2005).

PCR reaction was performed in a total reaction volume of 25 μL containing 10x NH_4 reaction buffer, 1.5 mmol $MgCl_2$, 0.5 mmol of each of the deoxyribonucleotide triphosphates (**dNTP**), 0.05 U Taq polymerase, and 2.5 μL of DNA. The concentrations of primers were 0.4 μmol of the primers CC18F and CC519R, 0.2 μmol of the primers hipO-F and hipO-R, and 0.05 μmol of the primers 16S-F and 16S-R (Persson and Olsen, 2005). Reference strains *C. coli* DSM-4689 and *C. jejuni* DSM-4688 were used as positive controls. The PCR products were analyzed by electrophoresis in a 1.2% agarose gel (Pronadisa, Spain) in 1% TAE buffer with RedSafe (iNtRON Biotechnology, Kirkland, WA). Gels were run for 1 h at 100 V and visualized by a Vilber Lourmat transilluminator (09 200272) with UV light. The size of the products was confirmed by comparison with the molecular marker GeneRuler 100-bp DNA Ladder.

Antimicrobial Susceptibility

Determination of AMR was carried out by a standard disc diffusion assay (Antimicrobial Susceptibility Test Disc, Oxoid Ltd.) in Müeller-Hinton Agar medium (Müeller-Hinton Broth, Scharlau, Barcelona, Spain) enriched with 5% defibrinated horse blood (Oxoid Ltd.).

An inoculum of each isolate was diluted in a 0.9% saline solution (Scharlau) and adjusted to a concentration of 2.0 on the McFarland scale. Incubation conditions were 41.5 ± 1°C during 44 ± 4 h, specific for thermophilic *Campylobacter*, under a micro-aerobic atmosphere (84% N_2, 10% CO_2, and 6% O_2) (CampyGen, Oxoid Ltd.). *Campylobacter* isolates were tested against 8 antimicrobials belonging to 5 families of antimicrobials commonly used for the treatment of campylobacteriosis in both humans and veterinary significance (Mensa et al., 2014; EFSA/ECDC, 2017)—2 fluoroquinolones: CIP (5 μg) and NA (30 μg); 2 β-lactamases: amoxicillin-clavulanic acid (**AMC**, 3 μg) and ampicillin (**AMP**, 10 μg); 2 aminoglycosides: gentamicin (**CN**, 10 μg) and streptomycin (**S**, 10 μg); one macrolide: erythromycin (**E**, 15 μg); and one tetracycline (**TE**, 30 μg) (Oxoid Antimicrobial Susceptibility Testing Disc Dispenser, Antimicrobial Susceptibility Test Disc, Oxoid Ltd.). The measurement and interpretation of the results were carried out following the guidelines of the European Committee on Antimicrobial Susceptibility Testing (ECDC, 2016). Isolates were considered multidrug resistant (**MDR**) when the isolate was resistant to at least 2 antimicrobial classes (ECDC, 2016). Resistance level were classified based on the values indicated by European Food Safety Authority and ECDC (2018a,b): sporadic < 0.1%; very low 0.1 to 1.0%; low > 1.0 to 10.0%; moderate > 10.0 to 20.0%; elevated > 20.0 to 50.0%; very high 50.0 to 70.0%; and extremely high > 70.0%.

Detection of AMR Genes

Resistance genes against β-lactams, TE, E, and quinolones were detected using 4 different PCRs (Table 2). The β-lactamase genes bla_{TEM}, bla_{SHV}, and bla_{CMY-2} and the primary resistance genes for TE tetA, tetB, and tetC were tested by multiplex PCRs using the primers and conditions described by Kozak et al. (2009) and Lanz et al. (2003). A single gene (ermB) was analyzed to determine the resistance against E, according to Chen et al. (2007). For the analysis of resistance to quinolones, a single gene (qnrS) was evaluated according to Marti and Balcázar (2013). *Escherichia coli* PCR positive amplicons for each gene belonging to previous studies were used as positive controls (Fenollar et al., 2019).

All PCRs were performed by using 25 μL mixtures of 1x NH_4 reaction buffer (BIOTAQ DNA Polymerase, Bioline, London, UK), dNTP (dNTP Mix 100 mM, Bioline) at 0.5 mM each, $MgCl_2$ (BIOTAQ DNA Polymerase), Taq DNA polymerase (BIOTAQ DNA Polymerase) at 1.25 U, and 2.5 μL of DNA sample.

PCR products were analyzed by electrophoresis at 100 V for 1 h through 1.2% (w/v) agarose gels in TBE buffer, pH 8.3, with RedSafe (iNtRON biotechnology) at 5%, to visualize the DNA under UV light. A 100-bp DNA ladder was used as a molecular weight marker.

All the assays were performed in duplicate.

Statistical Analysis

The isolated strains were categorized as sensitive, intermediary, or resistant, based on the Clinical and Laboratory Standards Institute interpretative criteria (CLSI, 2015). A GLM was used to compare the resistance between *Campylobacter* spp. Also, AMR and MDR rates between breeders and their progeny were analyzed. Thus, binomial data for each sample were assigned a value of 1 if the isolate was resistant or 0 if it was sensitive or intermediate. A *P*-value of less than 0.05 was considered to indicate a statistically significant difference. All statistical analyses were carried out using SPSS 16.0 software (SPSS Inc., Chicago, IL).

Table 1. Primers, sequences, and product size in the PCRs used for *Campylobacter* identification.

Gene	Primer name	Primer sequence (5′-3′)	Product	Reference	*Campylobacter* ID
asp	CC18 CC519	F-GGTATGATTTCTACAAAGCGAG R-ATAAAAGACTATCGTCGCGTG	500 bp	Linton et al. (1997)	*Campylobacter coli*
hipO	hipO	F-GACTTCGTGCAGATATGGATGCTT R-GCTATAACTATCCGAAGAAGCCATCA	344 bp	Persson et al. (2005)	*Campylobacter jejuni*
16S	Campy	F-GGAGGCAGCAGTAGGGAATA R-TGACGGGCGGTGAGTACAAG	1,062 bp	Persson et al. (2005)	*Campylobacter* spp.

Abbreviations: bp, base pairs; ID, identification.

RESULTS

Confirmation of Genus and Species by PCR

Five of the 65 strains analyzed showed inconclusive or non-repeatable PCR results, and were discarded from the analysis. Six isolates out of the resting 60 strains were not *C. jejuni* or *C. coli*, according to the PCR results, and were classified as *Campylobacter* spp. Overall, 66.7% (n = 40 of 60) were identified as *C. jejuni* and 23.3% (n = 14 of 60) as *C. coli*.

Besides, the PCR technique demonstrated that 7 isolates previously identified by phenotypic tests were not correctly described: 5 hippurate positive isolates were identified as *C. coli* by PCR, while 2 hippurate negative isolates were found to belong to *C. jejuni* species.

Prevalence of AMR

The percentages of AMR among the analyzed *Campylobacter* isolates are summarized in Table 3. All isolates were resistant to at least one out of the 8 antibiotics tested (n = 60 of 60). Furthermore, statistically significant differences in AMR rates were detected according to the species (*C. jejuni* or *C. coli*), except for CN and TE (P-value > 0.05) (Table 4). For *C. jejuni*, the results showed an extremely high resistance level to CIP (95%, n = 38 of 40) and NA (92%, n = 37 of 40). Moreover, no resistance against CN, E, and S was found. For *C. coli* isolates, the AMR rates obtained were significantly lower than for *C. jejuni* (P-value < 0.05), with the highest levels recorded for CIP (71%, n = 10 of 14), followed by NA (64%, n = 9 of 14), and moderate levels for AMC (14%, n = 2 of 14) and AMP (14%, n = 2 of 14). It is essential to highlight the fact that *C. jejuni* and *C. coli* showed a very high level of resistance to TE (60%, n = 24 of 40; 57%, n = 8 of 14, respectively). Finally, regarding the unknown *Campylobacter* spp. isolates, 100% (n = 6 of 6) were resistant to CN, AMP, E, CIP, NA, and TE. Moreover, 83% (n = 5 of 6) AMR was observed for S. No resistance was observed against AMC.

No significant differences were found between AMR rates from breeders and their progeny (P-value > 0.05), except for the flock PS2, where significant differences were found in resistances to CN and S (P-value < 0.05) (Figure 1).

Overall, a total of 80% (n = 48 of 60) *Campylobacter* isolates was resistant to 2 or more antimicrobials. MDR rates were much higher for *C. jejuni* than *C. coli* (85%, n = 34 of 40 vs. 43%, n = 6 of 14, respectively) ($P < 0.05$) (Table 4).

Antibiotic Resistance Patterns

The resistance patterns per isolate are summarized in Figure 2. For *C. jejuni*, 5 different patterns were observed: QNL; QNL-TE; βLAC-TE; β-LAC-QNL; and β-LAC-QNL-TE. For *C. coli*, 8 different patterns were observed: TE; QNL; QNL-TE; β-LAC-QLN; AMG-QNL-TE; AMG-MCL-QNL-TE; AMG-β-LAC-QNL-TE; and AMG-β-LAC-MCL-QNL-TE.

Regarding antimicrobial patterns from the breeders and each progeny, results showed that for breeder flock PS1, the antimicrobial patterns observed were QNL-TE (50%, n = 2 of 4) and βLAC-TE (25%, n = 1 of 4). Strains isolated from the progeny presented a combination of

Table 2. Primers, sequences, and product size in the PCRs used for detection of resistance genes.

PCR	Gene	Primer sequence (5′-3′)	Product	Reference
1	bla_{TEM}	F-TTAACTGGCGAACTACTTAC R- GTCTATTTCGTTCATCCATA	247 pb	Kozak et al. (2009)
	bla_{SHV}	F-AGGATTGACTGCCTTTTTG R-ATTTGCTGATTTCGCTCG	393 pb	Colom et al. (2003)
	bla_{CMY-2}	F- GACAGCCTCTTTCTCCACA R-TGGACACGAAGGCTACGTA	1,000 pb	Kozak et al. (2009)
2	tetA	F- GGCGGTCTTCTTCATCATGC R- GGCAGGCAGAGCAAGTAGA	502 pb	Lanz et al. (2003)
	tetB	F- CGCCCAGTGCTGTTGTTGTC R- GCGTTGAGAAGCTGAGGTG	173 pb	Goswami et al. (2008)
	tetC	F- GCTGTAGGCATAGGCTTGGT R- CCGGAAGCGAGAAGAATCA	888 pb	Lanz et al. (2003)
3	ermB-2	F-GATACC GTTTACGAAATTGG R-GAATCGAGACTTGAGTGTGC	364 pb	Chen et al. (2007)
4	qnrS	F- GACGTGCTAACTTGCGTGAT R-TGGCATTGTTGGAAACTTG	120 pb	Marti and Balcázar (2013)

Table 3. Percentage of antimicrobial resistance against *Campylobacter* spp. strains.

	Resistance		Intermediate		Susceptibility	
Antibiotics	No. of isolates	Ratio (%)	No. of isolates	Ratio (%)	No. of isolates	Ratio (%)
CN	8	13	0	0	52	87
S	9	15	0	0	51	85
AMC	19	32	30	50	11	18
AMP	37	62	8	13	15	25
E	8	13	1	2	51	85
CIP	57	95	0	0	3	5
NA	56	93	3	5	1	2
TE	39	65	0	0	21	35

Abbreviations: AMC, amoxicillin-clavulanic acid; AMP, ampicillin; CIP, ciprofloxacin; CN, gentamicin; E, erythromycin; NA, nalidixic acid; S, streptomycin; TE, tetracycline.

both patterns (βLAC-QNL-TE) in 57% of cases (n = 4 of 11). In breeder flock PS2, the pattern observed was AMG-βLAC-MCL-QNL-TE (4 of 4), and 30% (3 of 10) of the strains isolated from its progeny presented the same antimicrobial pattern. In breeder flock PS3, 60% (3 of 5) of the isolated strains had a βLAC-QNL-TE pattern, and its progeny shared the same pattern in 45% of cases (5 of 11). Finally, in breeder flock PS4, 2 patterns were found: QNL-TE (60%, 3 of 5) and βLAC-QNL-TE (40%, 2 of 5). Its progeny presented the βLAC-QNL-TE antimicrobial pattern in 29% of cases (4 of 14), while none of the isolates showed the QNL-TE pattern.

Detection of AMR Genes

The results obtained for the PCR detection of AMR genes are shown in Table 5. Overall, at least one of the antibiotic resistance genes studied was detected in 38.3% (23 of 60) of isolates. The more frequently detected gene was qnrS (69.6%, 16 of 23), related to the resistance to quinolones, followed by the bla_{TEM} gene (34.8%, 8 of 23) and tetC gene (13.0%, 3 of 23). It is remarkable that in some isolates, while the PCR result showed the presence of different AMR genes, no AMR pattern was observed, or it was intermediate.

By sample origin, all the bla_{TEM} positives isolates belonged to one of the breeding flocks (PS2) and one of the broiler groups of their offspring (PS2-A). The gene bla_{CMY-2} was only observed in one strain isolated from breeding flock PS4. The 3 tetC positive strains belonged to a batch of broilers (PS3-C). Meanwhile, none were detected from the breeding flock PS3. The ermB positive strain also belonged to a unique batch of broilers (PS2-A). On the other hand, the qnrS positive isolates were distributed among the 3 batches (PS2, PS3, and PS1) of both breeders and their progeny (Table 5).

DISCUSSION

The present study assessed the AMR prevalence in poultry *Campylobacter* isolates and their AMR genes dynamics, from breeders to their progeny. To the best of our knowledge, this is the first field study in scientific literature that evaluated the relationship between AMR patterns and resistance genes in *Campylobacter* strains isolated from breeders and their progeny.

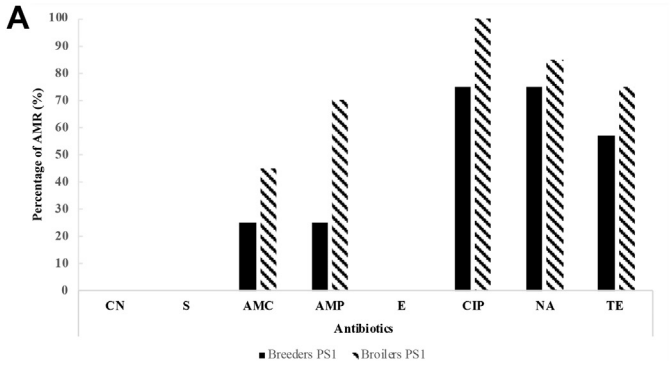
At present, there is social pressure on the poultry production system that demands the prohibition of antibiotic administration during the growing period. It has been demonstrated that stringent programs for AMR control result in lower AMR rates in poultry production (Miflin et al., 2007; Zhang et al., 2019). Thus, the poultry sector is making a significant effort to eliminate antibiotic administration as much as possible from growing farms. Despite the origin of the bacteria, in this study, 100% of the *Campylobacter* isolates analyzed were resistant at least to one antibiotic. Besides, 80% were MDR isolates. In 2016, the European Food Safety Authority reported that 73.1% of *Campylobacter* spp. isolated from broilers in the EU were resistant to at least one antibiotic. However, there were significant differences in AMR rates between the EU Member States, being notably lower in Nordic countries and higher in southern countries, especially Spain (EFSA, 2018b).

Regarding the AMR rates obtained in this study, it is essential to highlight that CIP and NA resistance levels were classified as extremely high for *C. jejuni* and very high for *C. coli*. These results are relevant, as they are

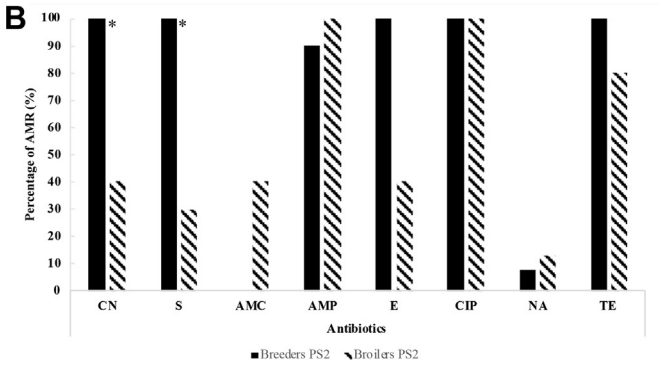
Table 4. Percentage of antimicrobial resistance among *Campylobacter jejuni* and *Campylobacter coli* isolated strains.

	Percentage of resistance								
MO	MDR	CN	S	AMC	AMP	E	CIP	NA	TE
C. jejuni	85	0	0	42	73	0	95	92	60
C. coli	43	7	28	14	14	14	71	64	57
P-value	<0.05	>0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	>0.05

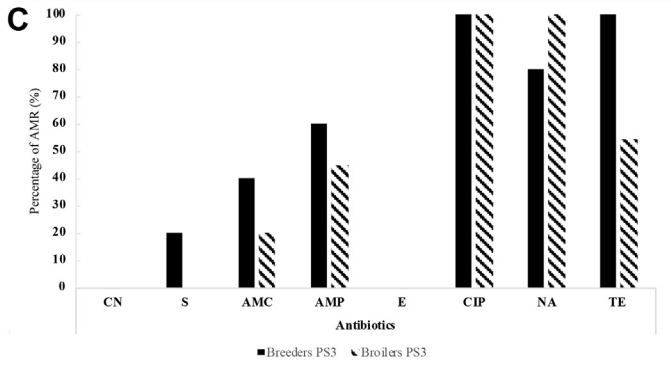
Abbreviations: AMC, amoxicillin-clavulanic acid; AMP, ampicillin; CIP, ciprofloxacin; CN, gentamicin; E, erythromycin; MDR, multidrug resistance (2 or more antibiotic-resistant); MO, microorganism; NA, nalidixic acid; S, streptomycin; TE, tetracycline.



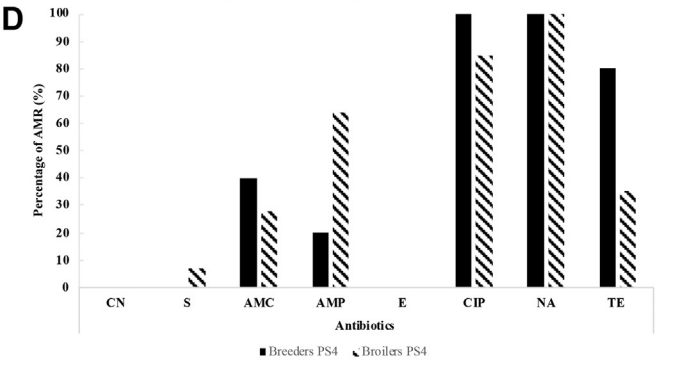
Flock PS1: Percentage of antimicrobial resistance between breeders and each progeny. No statistical differences were observed (*p-value* > 0.05).



Flock PS2: Percentage of antimicrobial resistance between breeders and each progeny. *Statistical differences were observed in CN and S antimicrobial resistance (*p-value* < 0.05).



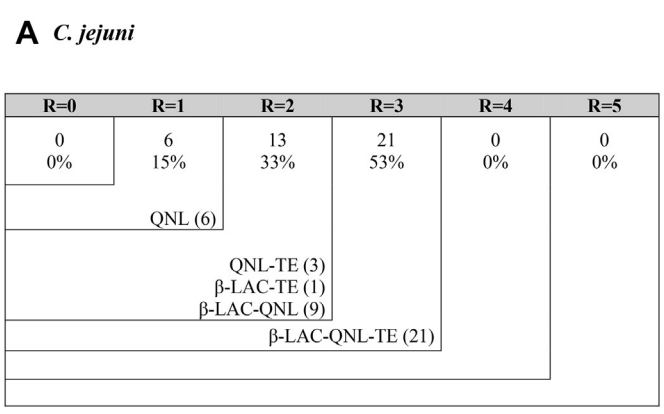
Flock PS3: Percentage of antimicrobial resistance between breeders and each progeny. No statistical differences were observed (*p-value* > 0.05).



Flock PS4: Percentage of antimicrobial resistance between breeders and each progeny. No statistical differences were observed (*p-value* > 0.05).

Figure 1. Percentage of antimicrobial resistance per batch of breeders and each progeny. (A) Flock PS1: no statistical differences were observed (*P*-value > 0.05). (B) Flock PS2: *statistical differences were observed in CN and S antimicrobial resistance (*P*-value < 0.05). (C) Flock PS3: no statistical differences were observed (*P*-value > 0.05). (D) Flock PS4: no statistical differences were observed (*P*-value > 0.05). Abbreviations: AMC, amoxicillin-clavulanic acid; AMP, ampicillin; CIP, ciprofloxacin; CN, gentamicin; E, erythromycin; NA, nalidixic acid; S, streptomycin; TE, tetracycline.

the first-choice drugs in human clinical treatments, in concordance with the Spanish trend of an increase in quinolone resistance (EFSA, 2019). Hence, many hypotheses explain how bacteria could acquire AMR. Throughout the years, *Campylobacter* has developed several types of antibiotic resistances, including quinolones, due to their low cost and widespread availability for livestock use (Di Giannatale et al., 2014; Du et al., 2018). Moreover, EFSA (2018b), for the 24 EU Member States also reported very high levels for CIP, NA, and TE. This fact can be explained by the use of *Campylobacter* in animal production for several years, to treat infectious diseases and as a growth promotor, and also in human medicine (Lee et al., 2017) probably to a greater extent. This is concerning because once quinolone-resistant *Campylobacter* becomes dominant in poultry, it can sustain in the farm environment even in the absence of antibiotic selection pressure (Panzenhagen



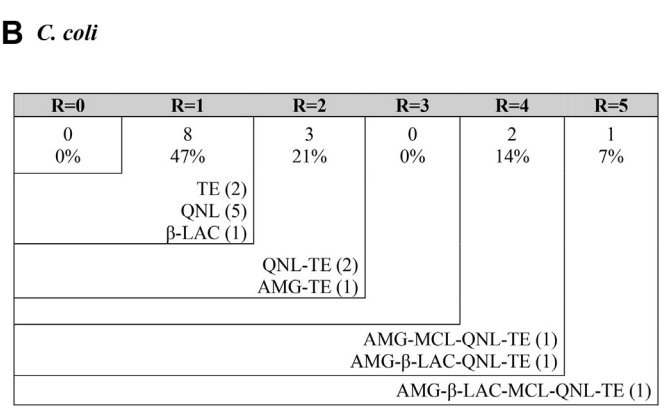


Figure 2. Patterns of resistance for *Campylobacter jejuni* (A) and *Campylobacter coli* (B) isolates. Number within parentheses denotes the number of isolates with the indicated pattern. Abbreviations: AMG, aminoglycosides; β-LAC, β-lactamases; MCL, macrolides; QNL, quinolones; R, number of antibiotic resistances; TE, tetracycline.

Table 5. Resistance genes detected in *Campylobacter* spp. from breeder flocks and their progeny.

Antimicrobial group	Gene	Positive samples (n = 60)	Breeding flock	Broiler
β-LAC	bla_{TEM}	8	PS2 (4)	PS2-A (4)
	bla_{CMY-2}	1	PS4 (1)	–
TE	tetC	3	–	PS3-C (3)
E	ermB	1	–	PS2-A (1)
QNL	qnrS	16	PS2 (1), PS3 (1), PS1 (1)	PS2-A (2), PS3-A (1), PS3-B (2), PS3-C (3), PS4-A (2), PS4_B (1), PS4-C (1), PS1-A (1)

Abbreviations: β-LAC, β-lactamases; E, erythromycin; QNL, quinolones; TE, tetracycline.
Numbers within parentheses denote the number of isolates that present the indicated gene.

et al., 2015). On the other hand, high resistance to TE was found in both species, according to previous studies (Di Giannatale et al., 2014; Varga et al., 2019). This fact can be explained by its use to treat bacterial diseases in poultry, which signifies the importance of antimicrobial control in flocks (Varga et al., 2019).

Our study demonstrated a relationship between the AMR patterns found from *Campylobacter* strains isolated in breeders and those isolated from their progeny. Our results emphasize the importance to control the administration of antibiotics not only during growing but also in breeders, because AMR seems to be transferred due to the co-selection and to mobile genetic elements (Tooke et al., 2019), even to not commonly used antibiotics. In addition, the PCR test improvises on the phenotypic test in AMR studies as was observed in this study; therefore, antibiotic surveillance ought to include AMR genes search in order to give a global perspective about antibiotic resistance in the poultry farming system.

This study demonstrated a relationship between AMR genes in breeders and those present in their progeny. Several studies have investigated the role of vertical transmission in resistant genes spreading (Projahn et al., 2017a, 2017b; Okorafor et al., 2019). Authors have studied how MDR bacteria and resistance genes in poultry could be transferred (Borjesson et al., 2016; Daehre et al., 2017; Osman et al., 2018). Moreno et al. (2019) reported resistance percentages in bacteria from 1-day-old chicks to be higher compared to hens, with a progressive decrease in frequency and variability during growth. This decrease could indicate the vertical transmission from parent flocks. In the same line, Jiménez-Belenguer et al. (2016) demonstrated the existence of a high percentage of resistant *E. coli* strains in 1-day-old chickens, not exposed previously to any antibiotic.

However, some patterns that are relevant for horizontal transfer on AMR dissemination were not found in breeders and their progeny. Agyare et al. (2018) demonstrated by genomic analysis that some bacteria could acquire resistances by incorporating different genetic elements through horizontal gene transfer. The presence of the ermB gene in *Campylobacter* spp. has been reported as this gene is present on mobile genetic elements and is usually responsible for a very high level of macrolide resistance (EFSA, 2019). Furthermore, Moreno et al. (2019) reported that the most frequently detected genes from 1-day-old chicks and hens were tetA and bla_{TEM}, which could indicate a co-selection in their transmission. It may also be the case of the high prevalence of qnr genes detected in our study. Some authors (Robicsek et al., 2006) have reported an association between extended-spectrum β-lactamase and qnr genes, so isolates with extended-spectrum β-lactamase phenotype are possible carriers of quinolone-resistant genes. Thus, further field studies are needed to assess the role of horizontal and vertical transmission on AMR dissemination.

In conclusion, despite the origin of the bacteria, all strains from this study were resistant to at least one antibiotic, and 80% were MDR. *C. jejuni* showed higher MDR rates than *C. coli*; however, both were especially resistant to CIP, NA, and TE. Moreover, this study demonstrated a relationship between AMR patterns and genes found from *Campylobacter* strains isolated in breeders, and those isolated from their progeny. However, some patterns, which are relevant for horizontal transfer on AMR dissemination, were not observed in breeders and their progeny. Thus, our results emphasize the importance of controlling antibiotic administration not only during the growing period but also in breeders.

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