

Article

Not peer-reviewed version

---

# Characterization of Antimicrobial Resistance in *Campylobacter* Species from Broiler Chicken Litter

---

[Tam T. Tran](#)\*, [Sylvia Checkley](#), [Niamh Caffrey](#), Chunu Mainali, [Sheryl Gow](#), [Agnes Agunos](#), Karen Liljebjelke

Posted Date: 3 June 2025

doi: 10.20944/preprints202506.0166.v1

Keywords: *Campylobacter* spp.; antibiotic resistance; poultry; conjugation; pathogen



Preprints.org is a free multidisciplinary platform providing preprint service that is dedicated to making early versions of research outputs permanently available and citable. Preprints posted at Preprints.org appear in Web of Science, Crossref, Google Scholar, Scilit, Europe PMC.

Copyright: This open access article is published under a Creative Commons CC BY 4.0 license, which permit the free download, distribution, and reuse, provided that the author and preprint are cited in any reuse.

Disclaimer/Publisher's Note: The statements, opinions, and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions, or products referred to in the content.

## Article

# Characterization of Antimicrobial Resistance in *Campylobacter* Species from Broiler Chicken Litter

Tam T. Tran <sup>1,\*</sup>, Sylvia Checkley <sup>1</sup>, Niamh Caffrey <sup>1</sup>, Chunu Mainali <sup>2</sup>, Sheryl Gow <sup>3</sup>, Agnes Agunos <sup>4</sup> and Karen Liljebjelke <sup>1</sup>

<sup>1</sup> Department of Ecosystem and Public Health, Faculty of Veterinary Medicine, University of Calgary, 3280 Hospital Dr. NW, Calgary, AB, Canada T2N 4Z6

<sup>2</sup> Alberta Agriculture and Forestry, 116 Street, Edmonton, AB, Canada T6H 4P2

<sup>3</sup> Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Dr, Saskatoon, SK, Canada S7N 5B4

<sup>4</sup> Public Health Agency of Canada, Center for Foodborne, Environmental and Zoonotic Infectious Diseases, 370 Speedvale Avenue West, Suite #201, Guelph, ON N1H 7M7

\* Correspondence: NORCE Norwegian Research Centre, Siva Innovasjonssenter, Sykehusveien 23, 9294 Tromsø, Norway; tran@norceresearch.no

**Abstract:** Campylobacteriosis in human populations is an ongoing issue in both developed and developing countries. In this study, sixty-five *Campylobacter* isolates were cultured from fecal samples collected from 17 flocks of broiler chickens in Alberta, Canada over two years (2015-2016). *Campylobacter jejuni* was the predominant species recovered during both years of sampling. There were no *Campylobacter coli* isolates found in 2015; however, approximately 33% (8/24) of isolates collected in 2016 were *Campylobacter coli*. The two most frequent antimicrobial resistance patterns in *C. jejuni* collected in 2015 were tetracycline (39%) and azithromycin/clindamycin/erythromycin/telithromycin resistance (29%). One isolate collected in 2015 has resistance pattern ciprofloxacin/nalidixic acid/tetracycline. The *tetO* gene was detected in all tetracycline resistant isolates from 2015. The *cmeB* gene was detected in all species isolates with resistance to azithromycin/clindamycin/erythromycin/ telithromycin, and from two isolates with tetracycline resistance. Alignment of the nucleotide sequences of the *cmeB* gene from *C. jejuni* isolates with different resistance patterns revealed several single nucleotide polymorphisms. A variety of multi-drug resistance patterns were observed through conjugation experiments. These data suggest that poultry production may serve as a potential reservoir for and source of transmission of multi-drug resistant *Campylobacter jejuni* and supports the need for continued surveillance.

**Keywords:** *Campylobacter* spp.; antibiotic resistance; poultry; conjugation; pathogen

## 1. Introduction

*Campylobacter* spp. are gram-negative bacteria varying in morphology from rod-, comma- or s-shape, having a single polar flagellum, bipolar flagella, or no flagellum (Kaakoush et al., 2015). Most species require microaerophilic conditions (5% O<sub>2</sub>, 10% CO<sub>2</sub>, 85% N<sub>2</sub>) for optimal growth. Some *Campylobacter* spp. can grow either in microaerophilic or anaerobic conditions (Kaakoush et al., 2015). Some species, such as *Campylobacter concisus*, *Campylobacter curvus*, *Campylobacter rectus*, *Campylobacter mucosalis*, *Campylobacter showae*, *Campylobacter gracilis*, and to a certain extent, *Campylobacter hyointestinalis*, require the presence of hydrogen or formate in culture media.

*Campylobacter* species, especially *C. jejuni* and *C. coli* are the most common cause of diarrheal illness in humans (Luber et al., 2003; Gupta et al., 2004). The number of campylobacteriosis cases reported in people in Canada was roughly ten thousand each year during 2006-2015, a rate of approximately 27 cases per 100,000 population (Public Health Agency of Canada (PHAC), 2015). In the United States there are an estimated 2.4 million cases annually (Gupta et al., 2004). *C. jejuni*

appears to cause 95% of diagnosed campylobacteriosis cases in people (Butzler, 2004). Most *Campylobacter* infections are mild and self-limiting with symptoms of acute watery or bloody diarrhea, fever, abdominal cramps and weight loss (Kaakoush et al., 2015). However, infections may become severe and prolonged especially in patients whose immune systems are compromised (Luangtongkum et al., 2010).

*Campylobacter* spp. is transmitted to human from various sources such as untreated drinking water, contaminated meat products or direct contact with live animals (Di Giannatale et al., 2019). Poultry production is recognized as a reservoir and main source of human *Campylobacter* infection, especially infection caused by *C. jejuni* (Rath et al., 2021). *C. jejuni* is a common commensal species in chicken gut microbiome (Hakeem et al., 2021). Broiler meat caused 20%–30% of human infections while 50%–80% was presumably from chicken reservoir as a whole (Di Giannatale et al., 2019). Pig production is also another *Campylobacter* reservoir and mainly associated with *C. coli* (Rath et al., 2021).

Erythromycin (macrolide) is the drug of choice for *C. jejuni* campylobacteriosis in people because of high effectiveness, low toxicity and ease of administration. Fluoroquinolones (e.g., ciprofloxacin) are the second choice for treatment because of broad spectrum activity (Altekruse et al., 1999). Alternative antibiotic choices such as chloramphenicol, clindamycin, aminoglycosides and carbapenems are effective against *Campylobacter* spp. Despite effective treatments the prevalence of resistant strains may complicate empirical treatment of campylobacteriosis (Lehtopolku et al., 2012). All antimicrobials used to treat campylobacteriosis are considered to be of very high importance (Category I) or high importance (Category II) for human medicine (Government of Canada, 2009). The use of Category I and II antimicrobials is now prohibited for poultry for preventive treatment (Anon, 2016).

The aim of this study was to characterize antimicrobial resistance phenotypes and genotypes *Campylobacter* strains isolated from commercial broiler chicken production in Alberta during the period of 2015-2016. The mobility of AMR phenotypes was also observed using conjugation assays.

## 2. Materials and Methods

### 2.1. Bacterial isolation and speciation

Four pooled fecal samples from one randomly selected barn per farm were collected in 2015 and 2016, as part of the Canadian Integrated Program for Antimicrobial Resistance Surveillance program - CIPARS (Public Health Agency of Canada, 2015). In total, 68 fecal samples were collected from 17 flocks of broiler chickens in Alberta, Canada (Table S1). Pooled fecal samples were sent on ice in coolers to Alberta Agriculture and Forestry for bacterial isolation and speciation.

*Campylobacter* was isolated using the standard CIPARS methodology, which is as follows: 25 g portion of each composite fecal sample was mixed with 225 mL of buffered peptone water (BPW) and incubated at  $35 \pm 1^\circ\text{C}$  for 24 hours. The BPW mixture was serially diluted with Bolton broth (BB) in the ratios of 1:100 and 1:1000, then incubated in a microaerophilic atmosphere at  $37^\circ\text{C}$  for 4 hours. After that, they were incubated at  $42^\circ\text{C}$  for 20 to 24 hours. The BB tube contents were next streaked on modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) plates, followed by microaerophilic incubation at  $42^\circ\text{C}$  for 72 hours. Finally, presumptive *Campylobacter* colonies were identified using biochemical tests (Gram stain, catalase test, oxidase test) and multiplex PCR for speciation.

The multiplex PCR speciation of *C. jejuni* and *C. coli* was performed as previously described (Persson & Olsen, 2005). Three pairs of oligo primers were added into the PCR mix (Table 1).

Table 1. Primers used in the study.

Gene target (size)	Primers	Reference
16s universal ribosomal RNA sequence (1062 bp)	16s-F: 5'-GGAGGCAGCAGTAGGGAATA-3' 16s-R: 5'-TGACGGGCGGTGAGTACAAG-3'	(Persson & Olsen, 2005)
Aspartokinase to detect <i>C. coli</i> (500 bp)	CC18-F: 5'-GGTATGATTTCTACAAGCGAG-3' CC519-R: 5'-ATAAAAGACTATCGTCGCGTG-3'	(Persson & Olsen, 2005)
Hippuricase to detect <i>C. jejuni</i>	hipO-F: 5'-GACTTCGTGCAGATATGGATGCTT-3' hipO-R: 5'-GCTATAACTATCCGAAGAAGCCATCA-3'	(Persson & Olsen, 2005)
<i>tetA</i> gene (888 bp)	tetA_F: 5'-GTGAAACCCAACATACCCC-3' tetA_R: 5'-GAAGGCAAGCAGGATGTAG-3'	(Abdi-Hachesoo et al., 2014)
<i>teO</i> gene (559 bp)	tetO_F: 5'-GGCGTTTTGTTTATGTGCG-3' tetO_R: 5'-ATGGACAACCCGACAGAAGC-3'	(Bacon et al., 2000)
<i>cmeB</i> gene (819 bp)	cmeB_F: 5'-GGTACAGATCCTGATCAAGCC-3' cmeB_R: 5'-AGGAATAAGTGTGCACGGAAATT-3'	(Lin et al., 2002)

The bacterial isolates were then shipped to University of Calgary for further characterization. Mueller-Hinton agar/broth (MHA/MHB) (Becton Dickinson - BD, Franklin Lakes, NJ, USA) was used to recover these isolates. *Campylobacter* was cultured under microaerophilic condition at 37°C for 3 days or at 42°C for 2 days. Microaerophilic conditions were achieved by placing activated sachets in a BD BBL GasPak™ jar (BD GasPak™ EZ Campy Container System, BD, Franklin Lakes, NJ, USA) with inoculated growth medium.

2.2. Antimicrobial susceptibility assays

The minimal inhibitory concentration (MIC) values for *Campylobacter* were determined by broth microdilution assay as part of the CIPARS program and was performed by Public Health Agency Canada (PHAC). The detailed procedure was previously described in a previous report by PHAC (Public Health Agency of Canada, 2015). Briefly, the CAMPY plates designed by NARMS and containing 9 dehydrated antimicrobials were used. After incubation period, plates were read using the Sensititre Vizion System. The MIC values obtained were compared with those of CLSI standards.

The MIC values for transconjugants were also determined using a broth microdilution method. Sensititre™ *Campylobacter* MIC plates (Trek Diagnostic Systems, Thermo Fisher Scientific Inc., Mississauga, ON, Canada). Strains were streaked on Mueller-Hinton agar plates and incubated in microaerophilic conditions at 37°C for 72 hrs or 42°C for 48 hrs. Several colonies were selected and inoculated into 5 ml Sensititre™ cation-adjusted Mueller-Hinton broth with TES buffer - CAMHBT (Trek Diagnostic Systems, Thermo Fisher Scientific Inc., Mississauga, ON, Canada) and adjusted to a 0.5 McFarland Standard using a Sensititre™ nephelometer. The inoculated CAMHBT was then mixed well, and subsequently 100 µl was transferred into Sensititre cation adjusted Mueller-Hinton broth with TES buffer and lysed horse blood - CAMHBT+ LHB (Trek Diagnostic Systems, Thermo Fisher Scientific Inc., Mississauga, ON, Canada). The inoculated CAMHBT + LHB was mixed, and 100 µl was inoculated into each well on MIC plate using Sensititre™ Auto-Inoculator. The microtiter plate was incubated in microaerophilic conditions at 37°C for 48 hours before reading results using the Sensititre™ Manual Viewer. For interpretation of manually read results we followed MIC Interpretive guidelines as provided by the CLSI (Clinical and Laboratory Standards Institute (CLSI), 2017).

2.3. Genomic DNA extraction

Genomic DNA extraction was performed using a previously published method with modifications (Meade et al., 1982). Briefly, overnight culture was harvested, re-suspended in 500 µl TES (10 mM Tris, 25 mM EDTA, pH 8.0) and lysed using lysis solution (20 µl of 25% SDS, 50 µl of 5 mg/ml of predigested Pronase E and 50 µl of 5 M NaCl) at 68°C for 30 minutes. Proteins were precipitated by adding 260 µl of 7.5 M ammonium acetate to the lysate kept on ice for 20 minutes. Precipitated protein was separated from the lysate by centrifuging at 13,000 rpm for 15 minutes. DNA was extracted from the lysate supernatant by adding chloroform of the same volume and subsequent



centrifugation at 13,000 rpm for 15 mins. After centrifugation, the top layer was transferred to a new tube containing 780 µl of isopropanol and the mixture was incubated on ice for 30 mins to precipitate DNA. DNA was pelleted, washed with 500 µl of 70% ethanol and pelleted again at 13,000 rpm for 1 min. After the supernatant was discarded, DNA was dissolved in 50 µl of TE buffer.

#### 2.4. PCR assay to detect AMR gene

A PCR assay was performed on genomic DNA prep to determine the presence of *tetA*, *tetO* and *cmeB* genes with previously published primers (Table 1).

Two *tetO* PCR products from isolates 96.3 and 13.3 were sent for Sanger sequencing (<http://www.ucalgary.ca/dnalab/sequencing>) to confirm their sequences. Four *cmeB* PCR products from isolates 13.3, 14.3, 85.3 and 86.3 were also sent for Sanger sequencing (<http://www.ucalgary.ca/dnalab/sequencing>) to confirm their sequences. Multiple sequence alignment was done using online tool (<http://multalin.toulouse.inra.fr/multalin/>).

#### Conjugation Assay

All *C. jejuni* isolates displaying various resistance patterns selected for conjugation assays were from the 2015 sampling. The *C. jejuni* isolate number 13.3, with MDR phenotype: azithromycin/clindamycin/erythromycin/telithromycin (AzClErTl) was used for mating assays with four randomly picked *C. jejuni* isolates (6.3, 33.3, 85.3, 96.3) that displayed resistance to tetracycline (Te). The donor and recipient strains were mated in the ratio of 1:1 on MHA plates (Tran et al., 2021). After 3-day incubation at 37°C, conjugation spots were transferred to selective media: MHA plates supplemented with erythromycin (5 µg/ml) and tetracycline (5 µg/ml), to select for transconjugants. Conjugation spots were also spotted individually onto MHA plates and then transferred to selective media (MHA plates supplemented with erythromycin (5 µg/ml) and tetracycline (5 µg/ml)), as negative controls.

In a second conjugation experiment, the *C. jejuni* isolate number 96.3 which had a ciprofloxacin/nalidixic acid/tetracycline resistance phenotype (CiNaTe) was used as the donor in a mating assay with three isolates (No. 13.3, 113.3, 117.3) which had the AzClErTl resistance phenotype. The conjugation protocol was similar to the protocol mentioned above, except for the use of different selective media. In this conjugation experiment, the selective medium was MHA supplemented with erythromycin (5 µg/ml) and nalidixic acid (15 µg/ml).

A third conjugation experiment was performed similarly as described in the second conjugation using the same donors and recipients, except for selective media. In the third conjugation, selective media were MHA supplemented with erythromycin (5 µg/ml), tetracycline (5 µg/ml) and nalidixic acid (15 µg/ml).

#### Statistical analyses

All analysis was completed in Stata 15 (StataCorp. 2017. *Stata Statistical Software: Release 15*. College Station, TX: StataCorp LLC).

Fisher's exact test was used to assess resistance to different antimicrobials between 2015 and 2016.

### 3. Results

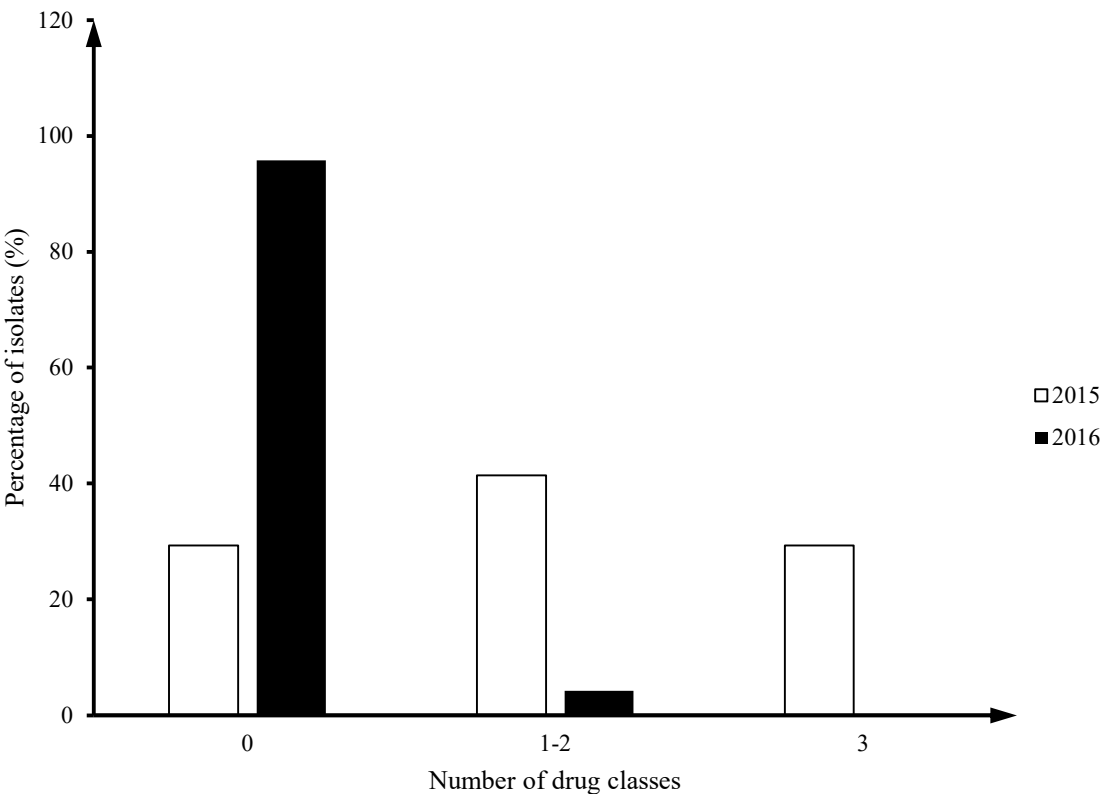
#### 3.1. Flock characteristics

Sixty-five *Campylobacter* isolates were isolated from fecal samples in 17 flocks over two years. Just eight isolates were *C. coli*, all of which were detected in 2016. Resistance to one or more drugs was detected in 9/17 flocks. Two flocks had isolates that were either susceptible or intermediate to all drugs, and isolates from the remaining six flocks were susceptible to all drugs tested. Chicks originated from three different hatcheries; however, 80% were from the same hatchery. Two flocks

reported no use of antibiotics, the remaining 15 flocks were conventionally raised (i.e. reported using antibiotics). Management practices, including methods used to clean barn and water lines, and antimicrobials used in flocks are summarized (Table S1). The most commonly used antibiotics were bacitracin (n = 14), salinomycin (n = 9) and monensin (n = 5).

3.2. Distribution frequency and minimum inhibition concentration (MIC) for *Campylobacter* spp. isolates from 2015 and 2016

All 2015 isolates (100%, 41/41) were *C. jejuni*, while in 2016 there were 66.7% *C. jejuni* (16/24) and 33.3% *C. coli* (8/24). Twenty nine percent of *Campylobacter jejuni* isolates from 2015 (12/41) had multiclass drug resistance (MDR;  $\geq 3$  drug classes), but no MDR isolates were identified in 2016 (Figure 1).



**Figure 1.** Comparison of distribution frequency by the number of drug classes between *Campylobacter* in year 2015 and 2016.

The distribution of MICs around the resistance breakpoint for each antimicrobial showed that some isolates in 2015 had MICs larger than the maximum value of the tested range (Table 2): TEL (12/12), NAL (1/41), ERY (12/41), AZM (12/41). All 2016 isolates had MIC of all antimicrobials falling within the tested range (Table 2).

**Table 2.** Minimum inhibitory concentration’s distributions for resistance to each drug in *Campylobacter* isolates from Alberta poultry farms in year 2015 (n=41) and in year 2016 (n=24).

Year	Antimicrobial	Distribution of isolates (count) across dilution range													
		<0.015	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	>64
2015	Gentamicin							1	40						
	Telithromycin							21	8				12		
	Clindamycin				6	22	1					5	7		
	Nalidixic acid										27	13			1
	Ciprofloxacin					40						1			

	Erythromycin					18	11										12
	Azithromycin	1		14	9	4	1										12
	Florphenicol							3	38								
	Tetracycline				2	5	17						6	2			9
2016	Gentamicin							6	18								
	Telithromycin							9	7	1	3	4					
	Clindamycin				2	13	2	7									
	Nalidixic acid										7	7	9	1			
	Ciprofloxacin				9	15											
	Erythromycin					1	12	3	2	6							
	Azithromycin			13	3	3	5										
	Florfenicol								20	4							
	Tetracycline					14	1				1	7			1		

**Note:** Clear areas indicate the range tested for each antimicrobial. Numbers in shaded areas indicate isolates having MIC outside tested range. Double vertical bars represent resistance breakpoints. Single vertical bars represent susceptibility breakpoints.

The association between resistance to individual drugs and year of isolation was examined in *C. jejuni* using Fisher’s exact test. A significant difference in the number of resistant isolates from 2015 (17/41, 41% ) to 2016 (1/24, 4%) was detected for tetracycline ( $P = 0.011$ ), and the pattern of telithromycin, erythromycin, clindamycin and azithromycin resistance ( $P = 0.013$ ).

3.3. Resistance patterns of *Campylobacter* isolates from 2015 and 2016

Two main resistance patterns found in *Campylobacter* isolates from 2015 were azithromycin/clindamycin/erythromycin/ telithromycin resistance (AzClErTl<sup>R</sup> - pattern 1, drug classes: macrolide-lincosamide-ketolide) and tetracycline resistance (Te<sup>R</sup> – pattern 2, drug classes: tetracycline). In 2015, 39% (16/41) exhibited Te<sup>R</sup> pattern, and 29% (12/41) exhibited the AzClErTl<sup>R</sup> pattern. There was only one isolate 96.3 which displayed resistance to ciprofloxacin/nalidixic acid/tetracycline resistance (CiNaTe<sup>R</sup>, drug classes: quinolone-tetracycline).

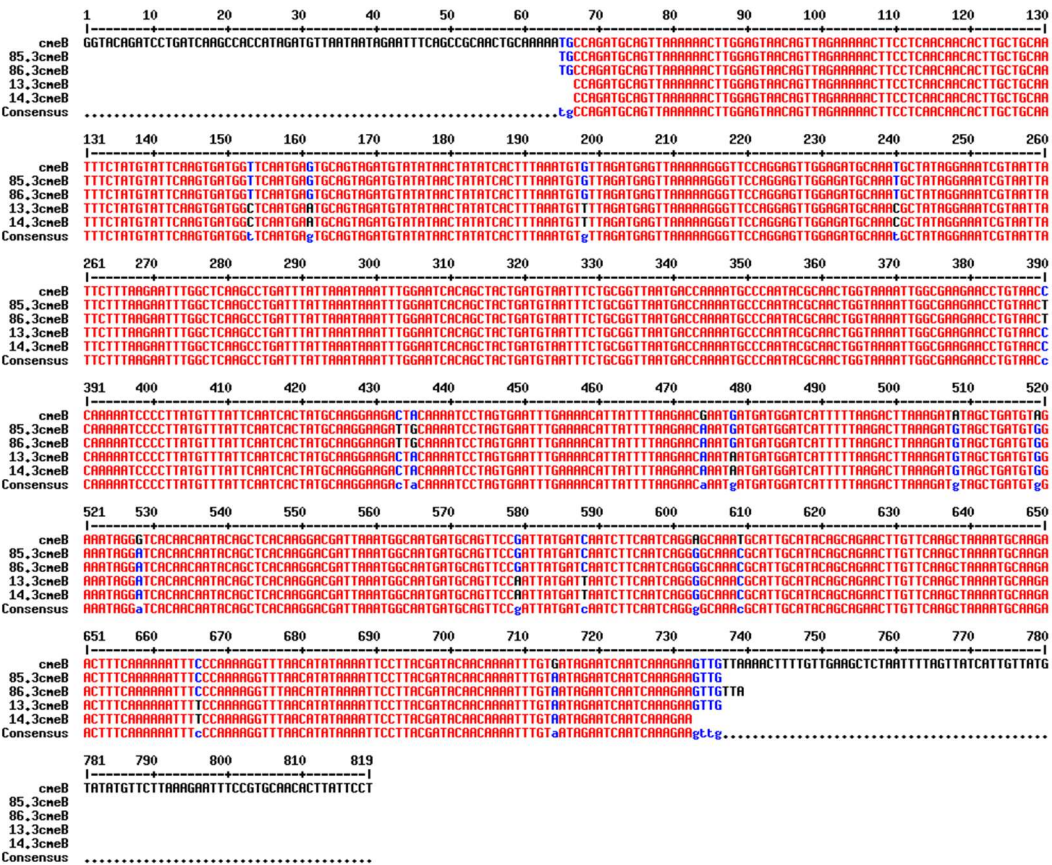
*C. jejuni* isolates with the resistance pattern AzClErTl<sup>R</sup> were identified in three flocks in 2015. All three flocks obtained their chicks from the same hatchery, and all three farms used salinomycin for treatment of coccidiosis. These farms had the same floor space area (8,000 ft<sup>2</sup>) and stocking density (0.54 ft<sup>2</sup> per bird), used Ross 308 birds, and were all multi-age facilities (Public Health Agency of Canada, 2015). Sanitation on these three farms was done using a hot water wash between productions periods, with chlorine in a pressurized form used as the disinfectant of choice. These three farms did not disinfect their water lines between flocks but did use chlorine to treat the water lines during the production cycle (Public Health Agency of Canada, 2015).

Out of 16 *C. jejuni* isolates collected in 2016, there was only one isolate which had resistance to Tetracycline (Te<sup>R</sup>). The rest 15 *C. jejuni* and 8 *C. coli* isolates in 2016 were pan-sensitive.

3.4. Detection of AMR genes

All Te<sup>R</sup> isolates from the 2015 batch (n=17) harbored *tetO* gene as determined by PCR assay and sequence analysis. The *tetO* nucleotide sequence had 99% agreement with the sequence encoding TetM/TetW/TetO/TetS family tetracycline resistance protein published in the GenBank database (Accession No. CP023546.1). The *tetA* gene sequence was not detected in any of our Te resistant isolates. For the PCR assays, *C. jejuni* isolate 13.3 with the AzClErTl<sup>R</sup> pattern was included as a negative control as no *tetO* gene was amplified from this tetracycline sensitive isolate.

The *cmeB* gene was detected in all *C. jejuni* strains collected in 2015 which had the AzClErTl<sup>R</sup> pattern (n=12). In addition, out of five *C. jejuni* isolates which had other resistance patterns (Te<sup>R</sup> and CiNaTe<sup>R</sup>), the *cmeB* gene was detected in two Te<sup>R</sup> *C. jejuni* isolates. Alignment of the *cmeB* nucleotide sequence obtained by PCR revealed that isolates with the same resistance pattern (AzClErTl<sup>R</sup> or Te<sup>R</sup>) shared the same nucleotide sequence and single-nucleotide polymorphisms of the *cmeB* gene (Figure 2). The *cmeB* gene was not detected in a *C. jejuni* isolate with the CiNaTe<sup>R</sup> pattern nor in two *C. jejuni* isolates with Te<sup>R</sup> pattern.



**Figure 2.** Multiple sequence alignment of *cmeB* gene amplification from four isolates against *cmeB* gene from Genbank database (Access No. AB894099.1) using online tool (<http://multalin.toulouse.inra.fr/multalin/>). Two of them (85.4 and 86.4) were *C.jejuni* isolates with Te resistance pattern, and the other two (13.3 and 14.3) were *C.jejuni* isolates with AzCIERTl resistance pattern.

3.5. *Campylobacter jejuni* antimicrobial resistance phenotypes transferred via in vitro conjugation

Three out of four *C. jejuni* isolates from 2015 with either Te<sup>R</sup> or CiNaTe<sup>R</sup> were able to produce transconjugants when mated with the *C. jejuni* isolate from 2015 with AzCIERTl<sup>R</sup> pattern (Table 3). The AMR pattern (AzCIERTlTe<sup>R</sup>) of transconjugants 13.6, 13.85 and 13.96 was then confirmed by antimicrobial susceptibility assay (Table 3). All the transconjugants were characterized as having a combined AMR pattern of Te from the donor and AzCIERTl from the recipient strain. Although all of the transconjugants had phenotypic resistance to clindamycin, their MICs (MIC = 8 µg/ml) were half of the parental isolate's original MIC value (MIC = 16 µg/ml). The isolate (96.3) which had the CiNaTe<sup>R</sup> pattern was in contact with the isolates of AzCIERTl<sup>R</sup> pattern producing transconjugants of the combined resistance pattern (AzCIERTl<sup>R</sup> + Te<sup>R</sup>). All the transconjugants had the same MICs for nalidixic acid as the parental isolate 13.3. These MICs (8 and 16 µg/ml) were higher than MICs in two of the likely donor isolates 6.3 and 85.3 (MIC ≤ 4 µg/ml), but lower than the MIC (MIC > 64 µg/ml) of likely recipient isolate 96.3.

**Table 3.** Minimum inhibition concentration (MIC) and AMR patterns of isolates used in conjugation assays and the resulting transconjugants.

Strains	MIC (µg/ml)										Interpretation								AMR patterns
	AZM	CIP	CLI	ERY	FLR	GEN	NAL	TEL	TET	AZM	CIP	CLI	ERY	FLR	GEN	NAL	TEL	TET	
<i>C. jejuni</i>																			
ATCC 33250 <sup>a</sup>	0.03	0.06	0.25	<0.25	1	0.5	<=4	<0.5	<0.12	S	S	S	S	S	S	S	S	S	Susceptible
6.3	0.12	0.12	0.12	0.5	1	1	<=4	1	>64	S	S	S	S	S	S	S	S	R	Te
85.3	0.12	0.12	0.12	0.5	1	0.5	<=4	0.5	>64	S	S	S	S	S	S	S	S	R	Te
96.3	0.06	8	0.12	0.25	1	1	>64	0.5	>64	S	R	S	S	S	S	R	S	R	CiNaTe



13.3	>64	0.12	16	>64	1	1	8	>8	0.25	R	S	R	R	S	S	S	R	S	AzClErTl
113.3	>64	0.12	8	>64	1	1	8	>8	0.25	R	S	R	R	S	S	S	R	S	AzClErTl
117.3	>64	0.12	16	>64	1	1	8	>8	0.25	R	S	R	R	S	S	S	R	S	AzClErTl
13.6 <sup>b</sup>	>64	0.12	8	>64	1	0.25	8	>8	>64	R	S	R	R	S	S	S	R	R	AzClErTlTe
13.85 <sup>b</sup>	>64	0.12	8	>64	1	0.25	8	8	>64	R	S	R	R	S	S	S	R	R	AzClErTlTe
13.96 <sup>b</sup>	>64	0.12	8	>64	2	0.25	8	>8	>64	R	S	R	R	S	S	S	R	R	AzClErTlTe
96.13 <sup>c</sup>	>64	16	16	>64	1	0.5	>64	>8	0.12	R	R	R	R	S	S	R	R	S	CiNaAzClErTl
96.113 <sup>c</sup>	>64	16	16	>64	2	0.5	>64	>8	0.25	R	R	R	R	S	S	R	R	S	CiNaAzClErTl
96.117 <sup>c</sup>	>64	8	16	>64	1	0.25	>64	>8	0.12	R	R	R	R	S	S	R	R	S	CiNaAzClErTl

**Note:** Grey shading separates transconjugants from isolates used in conjugation experiments. S: Susceptible, R: Resistant (in bold). AZM/Az = Azithromycin, CIP/Ci = Ciprofloxacin, CLI/Cl = Clindamycin, ERY/Er = Erythromycin, FLR = Florfenicol, GEN = Gentamicin, NAL/Na = Nalidixic acid, TEL/Tl = Telithromycin, TET/Te = Tetracycline. <sup>a</sup> Negative control strain was used in antimicrobial susceptibility tests. <sup>b</sup> Transconjugants 13.6, 13.85 and 13.96 were obtained via conjugation between the isolate 13.3 with isolates 6.3, 85.3 and 96.3 in the first conjugation assay, respectively. <sup>c</sup> Transconjugants 96.13, 96.113 and 96.117 were obtained via conjugation between the isolate 96.3 with isolates 13.3, 113.3 and 117.3 in the second conjugation assay, respectively.

In the second conjugation assay, three isolates (13.3, 113.3 and 117.3) with AzClErTl<sup>R</sup> pattern were mated with the isolate 96.3 with CiNaTe<sup>R</sup> pattern (Table 3). All transconjugants displayed the combined patterns CiNa<sup>R</sup> + AzClErTl<sup>R</sup> but they were all susceptible to Te; and two of them had an increased MIC (16 µg/ml) for ciprofloxacin as compared to the parental strain (8 µg/ml).

In the third conjugation, the presence of tetracycline in the selective media helped maintain Te resistance in the transconjugants. Presumable *C. jejuni* donors (13.3, 113.3 and 117.3) with AzClErTl<sup>R</sup> pattern was mated with the presumable *C. jejuni* recipient 96.3 with CiNaTe<sup>R</sup> pattern and selected on medium containing tetracycline. The transconjugants had a combined multi-drug resistance pattern of CiNaTeAzClErTl (Fluoroquinolones – CiNa, Tetracycline – Te, Macrolides – AzEr, Lincosamide – Cl, Ketolide – Tl).

4. Discussion

*Campylobacter jejuni* was the only *Campylobacter* species identified in samples from broiler production in Alberta in 2015, while both *C. jejuni* (66.7%) and *C. coli* (33.3%) were identified in samples collected in 2016. Overall, *C. jejuni* was the predominant *Campylobacter* species isolated, which is similar to a previous study (Luber et al., 2003). This is a clinically grave concern in humans when considering the fact that the majority (95 to 98%) of human cases of *Campylobacter* gastroenteritis were caused by *C. jejuni*, followed by *C. coli* cases (2 to 5 %) (Taylor & Courvalin, 1988; Luber et al., 2003). Thirty years ago very few *Campylobacter* strains (< 1%) in Canada and the United Kingdom were resistant to erythromycin (Taylor & Courvalin, 1988). In contrast, our study showed almost 30% of *C. jejuni* isolates from poultry in 2015 having phenotypic resistance to erythromycin. In addition, while other studies otherwise reported that *C. coli* was more likely to be associated with macrolide resistance, macrolide resistance phenotype was only found in *C. jejuni* isolates in our study (Bolinger & Kathariou, 2017).

Tetracycline-resistant isolates were found to make up 41% of 2015 *Campylobacter* isolates. The *tetO* gene was identified in all our 2015 *C. jejuni* isolates (n = 17) with phenotypic resistance to tetracycline. This result is similar to previous studies where the *tetO* gene was identified as the most common tetracycline resistance gene in all *C. jejuni* and *C. coli* isolates with resistance to tetracycline (Dasti et al., 2007; Abdi-Hachesoo et al., 2014; Pratt & Korolik, 2005). Transmissible plasmids carrying the *tetO* gene have been found not only in *C. jejuni* and *C. coli* but also in other bacteria such *Enterococcus faecalis* and *Streptococcus* spp. (Zilhao et al., 1988). These *tetO*-carrying conjugative plasmids were also associated with genes encoding for different aminoglycoside inactivating enzymes, transposase- like genes, and multiple other genes (Nirdnoy et al., 2005; Marasini et al., 2020). The *tetO* gene can also be located on the bacterial chromosome, especially in *C. coli* (Dasti et al., 2007; Pratt & Korolik, 2005). The phenotypes of our transconjugants imply that the *tetO* gene in some of our isolates is plasmid-encoded and transmissible. The isolate that was unable to transfer the

tetracycline resistance phenotype to the recipient might carry a chromosomally encoded *tetO* gene, or the gene may be located on a separate mobile element which was not transferred.

The *cmeB* gene encodes for an inner membrane efflux transporter and is a part of three-gene operon (designated *cmeABC*) that contributes to multidrug resistance in *C. jejuni* (Lin et al., 2002). It was shown that mutation of the *cmeB* gene resulted in decreasing MICs to a wide range of antimicrobial agents (i.e. ciprofloxacin, nalidixic acid, erythromycin, tetracycline), heavy metals and bile salts between 2 and 4,000-fold. In these *cmeB* mutant strains resistance to ciprofloxacin was decreased 8-fold, resistance to nalidixic acid decreased 2-fold, to erythromycin decreased 4-fold, and tetracycline resistance decreased 8-fold, (Lin et al., 2002). In our study, the *cmeB* gene (819 bp) was selected for screening in a subset of *Campylobacter* isolates, especially the ones with AzCIerTl phenotype because the gene was likely to confer resistance to antibiotics of different classes. All isolates with AzCIerTl resistance pattern carried the *cmeB* gene. The *cmeB* gene was also present in two *C. jejuni* isolates with Te resistance pattern. However, they did not share the same sequence identity of *cmeB* genes with *C. jejuni* isolates of AzCIerTl resistance pattern. A significant increase of *cmeB* mutations in *C. jejuni* strains carrying *cmeB* gene compared to those in the *CmeB* null mutant strains at 10X and 32X the concentration (Yan et al., 2006). However, there were no further investigation of these mutations in their study.

In this study, only one *C. jejuni* isolate was resistant to ciprofloxacin, nalidixic acid and tetracycline. The *cmeB* gene was not detected in the *C. jejuni* isolate with the CiNaTe phenotype although this gene was shown to be associated with the resistance to ciprofloxacin, nalidixic acid and tetracycline in several previous studies (Lin et al., 2002; Ge et al., 2005; Yan et al., 2006). We postulate that some mutation in the *gyrA* gene most likely accounted for the resistance to fluoroquinolones (ciprofloxacin and nalidixic acid) in this isolate because these drugs target the DNA gyrase encoded by *gyrA* gene (Aksomaitiene et al., 2018; Hakanen et al., 2002).

In the first conjugation assay, the transconjugants with a combined resistance pattern (AzCIerTl + Te) can be made by mating isolates of AzCIerTl and Te phenotypes. Based on the MICs of transconjugants, isolates with Te phenotypes were likely to be the donors which transferred Te phenotype into the recipient isolate 13.3 with AzCIerTl phenotype. In the second conjugation assay, isolate 96.3 with CiNaTe phenotype was used as the recipient and mated it with isolates exhibiting AzCIerTl resistance pattern to determine the transferability of AzCIerTl phenotype. Interestingly, the transconjugants displayed resistance not only to erythromycin, which was used as a selection marker, but also to azithromycin, clindamycin, and telithromycin. More surprisingly, all transconjugants maintained resistance to CiNa but lost resistance to Te after conjugation. It is well-known that the strains normally suffer a fitness cost to maintain plasmids; therefore, they can easily lose plasmids in antibiotic-free environment (Millan & Maclean, 2017). In the last conjugation experiment, we wanted to see if the transconjugants still maintain resistance to Te when we added tetracycline into the media in addition to nalidixic acid and erythromycin. The results suggested that they were able to maintain resistance to all antimicrobials provided that selection pressure was present in the media.

In conclusion, the study isolated and speciated *Campylobacter* isolates from broiler chickens in Alberta. In both years, *C. jejuni* was the predominant species isolated, and *C. coli* was only isolated in 2016. Twenty nine percent of *C. jejuni* isolates from 2015 (12/41) had multiclass drug resistance (MDR) ( $\geq 3$  drug classes), but no MDR isolates were identified in 2016. Several single nucleotide polymorphisms were found in the *cmeB* gene of isolates of different resistance patterns. We also showed the potential for resistance pattern transfer during conjugation. The demonstration of transmission of multi-drug resistance via conjugation between strains supports the importance of continued antimicrobial resistance surveillance in food borne pathogens.

**Supplementary Materials:** The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Table S1. Flock level characteristics including antibiotics given in feed and methods used to disinfect barns.

**Author Contributions:** SC, KL, SG, CM conceptualized the research idea and obtained research funding. AAF (PI :SC). SG and AA developed the CIPARS AMU-AMR farm surveillance framework, farm surveillance tools (questionnaire) and protocols, and validated the recovery and AMR datasets. Bacterial isolation and initial antimicrobial susceptibility testing were performed by RC. TT and KL were responsible for experimental design. TT conducted research and laboratory analysis. NC conducted statistical analysis. TT and NC designed and drafted the manuscript. All authors contributed to manuscript development. The authors read and approved the final manuscript.

**Funding:** This study was funded by Alberta Agriculture and Forestry (AAF) [grant number 2015R025R] with significant in a kind support from PHAC and the AAF, Agri-Food Laboratories.

**Data Availability Statement:** Data available upon request.

**Acknowledgments:** We would like to thank the poultry veterinarians and producers who voluntarily participated in the CIPARS farm surveillance program and enabled data and sample collection. We are grateful to the Chicken Farmers of Canada and the Alberta Chicken Producers for their valuable input to the framework development and technical discussions. TT also wishes to thank Odd-Gunnar Wikmark and the Norwegian Research Council (MARMIB - project number 315812) for their support in finishing this work.

**Conflicts of Interest:** Competing interests: The authors declare there are no competing interests.

## Reference

- Abdi-Hachesoo, B., Khoshbakht, R., Sharifiyazdi, H., Tabatabaei, M., Hosseinzadeh, S. & Asasi, K. 2014. Tetracycline resistance genes in *Campylobacter jejuni* and *C. coli* isolated from poultry carcasses. *Jundishapur Journal of Microbiology*, 7(9): 7–11.
- Aksomaitiene, J., Ramonaite, S., Olsen, J.E. & Malakauskas, M. 2018. Prevalence of Genetic Determinants and Phenotypic Resistance to Ciprofloxacin in *Campylobacter jejuni* from Lithuania Bacterial Strains and Culture Conditions. *Frontiers in Microbiology*, 9: 1–6.
- Altekruse, S.F., Stern, N.J., Fields, P.I. & Swerdlow, D.L. 1999. *Campylobacter jejuni* - An emerging foodborne pathogen. *Emerging Infectious Diseases*, 5(1): 28–35.
- Anon. 2016. *Chicken farmers of Canada*. <http://www.chickenfarmers.ca/wp-content/uploads/2017/03/2016-Annual-Report-ENG-web.pdf>.
- Bacon, D.J., Alm, R., Burr, D.H., Hu, L., Kopecko, D.J., Ewing, C.P., Trust, T.J. & Guerry, P. 2000. Involvement of a plasmid in virulence of *Campylobacter jejuni* 81-176. *Infection and Immunity*, 68(8): 4384–4390.
- Bolinger, H. & Kathariou, S. 2017. The Current State of Macrolide Resistance in *Campylobacter* spp.: Trends and Impacts of Resistance Mechanisms. *Applied and Environmental Microbiology*, 83(12): 1–9.
- Butzler, J. 2004. *Campylobacter*, from obscurity to celebrity. *Clinical Microbiology and Infection*, 10(10): 868–876. <http://dx.doi.org/10.1111/j.1469-0691.2004.00983.x>.
- Clinical and Laboratory Standards Institute (CLSI). 2017. *Performance Standards for Antimicrobial Susceptibility*. M45-A2.
- Dasti, J.I., Groß, U., Pohl, S., Lugert, R., Weig, M. & Schmidt-Ott, R. 2007. Role of the plasmid-encoded *tet(O)* gene in tetracycline-resistant clinical isolates of *Campylobacter jejuni* and *Campylobacter coli*. *Journal of Medical Microbiology*, 56(6): 833–837.
- Di Giannatale, E., Calistri, P., Di Donato, G., Decastelli, L., Goffredo, E., Adriano, D., Mancini, M. E., Galleggiante, A., Neri, D., Antoci, S., Marfoglia, C., Marotta, F., Nuvoloni, R., & Migliorati, G. 2019. Thermotolerant *Campylobacter* spp. in chicken and bovine meat in Italy: Prevalence, level of contamination and molecular characterization of isolates. *PloS one*, 14(12), e0225957. <https://doi.org/10.1371/journal.pone.0225957>
- Ge, B., Mcdermott, P.F., White, D.G., Meng, J. & Hemother, A.N.A.G.C. 2005. Role of Efflux Pumps and Topoisomerase Mutations in Fluoroquinolone Resistance in *Campylobacter jejuni* and *Campylobacter coli*. , 49(8): 3347–3354.

- Government of Canada. 2009. Categorization of Antimicrobial Drugs Based on Importance in Human Medicine. <https://www.canada.ca/en/health-canada/services/drugs-health-products/veterinary-drugs/antimicrobial-resistance/categorization-antimicrobial-drugs-based-importance-human-medicine.html>.
- Gupta, A., Nelson, J.M., Barrett, T.J., Tauxe, R. V., Rossiter, S.P., Friedman, C.R., Joyce, K.W., Smith, K.E., Jones, T.F., Hawkins, M.A., Shiferaw, B., Beebe, J.L., Vugia, D.J., Rabatsky-Ehr, T., Benson, J.A., Root, T.P. & Angulo, F.J. 2004. Antimicrobial resistance among *Campylobacter* strains, United States, 1997-2001. *Emerging Infectious Diseases*, 10(6): 1102–1109.
- Hakanen, A., Jalava, J., Kotilainen, P., Jousimies-somer, H., Siitonen, A. & Huovinen, P. 2002. *gyrA* Polymorphism in *Campylobacter jejuni*: Detection of *gyrA* Mutations in 162 *C. jejuni* Isolates by Single-Strand Conformation Polymorphism and DNA Sequencing. *Antimicrobial agents and chemotherapy*, 46(8): 2644–2647.
- Hakeem, M. J., & Lu, X. 2021. Survival and Control of *Campylobacter* in Poultry Production Environment. *Frontiers in cellular and infection microbiology*, 10, 615049. <https://doi.org/10.3389/fcimb.2020.615049>
- Kaakoush, N.O., Castaño-Rodríguez, N., Mitchell, H.M. & Man, S.M. 2015. Global epidemiology of campylobacter infection. *Clinical Microbiology Reviews*, 28(3): 687–720.
- Lehtopolku, M., Kotilainen, P., Puukka, P., Nakari, U.M., Siitonen, A., Eerola, E., Huovinen, P. & Hakanen, A.J. 2012. Inaccuracy of the disk diffusion method compared with the agar dilution method for susceptibility testing of *Campylobacter* spp. *Journal of Clinical Microbiology*, 50(1): 52–56.
- Lin, J., Michel, L.O. & Zhang, Q. 2002. CmeABC Functions as a Multidrug Efflux System in *Campylobacter jejuni*. *Society*, 46(7): 2124–2131.
- Luangtongkum, T., Jeon, B., Han, J., Plummer, P., Logue, C.M. & Zhang, Q. 2010. Antibiotic resistance in *Campylobacter*: emergence, transmission and persistence. *Future Microbiology*, 4(2): 189–200.
- Luber, P., Wagner, J., Hahn, H. & Bartelt, E. 2003. Antimicrobial Resistance in *Campylobacter jejuni* and *Campylobacter coli* Strains Isolated in 1991 and 2001-2002 from Poultry and Humans in Berlin , Germany. *Antimicrobial Agents and Chemotherapy*, 47(12): 3825–3830.
- Marasini, D., Karki, A.B., Bryant, J.M., Sheaff, R.J. & Fakhr, M.K. 2020. Molecular characterization of megaplasms encoding the type VI secretion system in *Campylobacter jejuni* isolated from chicken livers and gizzards. *Scientific Reports*, 10: 12514. <https://doi.org/10.1038/s41598-020-69155-z>.
- Meade, H.M., Long, S.R., Ruvkun, G.B., Brown, S.E. & Ausubel, M. 1982. Physical and Genetic Characterization of Symbiotic and Auxotrophic Mutants of *Rhizobium meliloti* Induced by Transposon Tn5 Mutagenesis. *J. Bacteriol*, 149(1): 114–122.
- Millan, A.S.A.N. & Maclean, R.C. 2017. Fitness Costs of Plasmids : a Limit to Plasmid Transmission. *Microbiol Spectrum*, 5(5): 1–12.
- Nirdnoy, W., Mason, C.J. & Guerry, P. 2005. Mosaic structure of a multiple-drug-resistant, conjugative plasmid from *Campylobacter jejuni*. *Antimicrobial Agents and Chemotherapy*, 49(6): 2454–2459.
- Persson, S. & Olsen, K.E.P. 2005. Multiplex PCR for identification of *Campylobacter coli* and *Campylobacter jejuni* from pure cultures and directly on stool samples. *Journal of Medical Microbiology*, 54: 1043–1047.
- Pratt, A. & Korolik, V. 2005. Tetracycline resistance of Australian *Campylobacter jejuni* and *Campylobacter coli* isolates. *Journal of Antimicrobial Chemotherapy*, 55(4): 452–460.
- Public Health Agency of Canada. 2015. *Canadian Integrated Program for Antimicrobial (CIPARS) Annual Report 2015*. [http://publications.gc.ca/collections/collection\\_2017/aspc-phac/HP2-4-2015-eng.pdf](http://publications.gc.ca/collections/collection_2017/aspc-phac/HP2-4-2015-eng.pdf).
- Public Health Agency of Canada (PHAC). 2015. Canadian Notifiable Disease Section. <http://diseases.canada.ca/notifiable/charts?c=pl>.
- Rath, A., Rautenschlein, S., Rzeznitzek, J., Breves, G., Hewicker-Trautwein, M., Waldmann, K. H., & von Altrock, A. 2021. Impact of *Campylobacter* spp. on the Integrity of the Porcine Gut. *Animals : an open access journal from MDPI*, 11(9), 2742. <https://doi.org/10.3390/ani11092742>
- Taylor, D.E. & Courvalin, P. 1988. Mechanisms of antibiotic resistance in *Campylobacter*. *Antimicrob. Agents Chemother.*, 32(8): 1107–1112.
- Tran, T., Checkley, S., Caffrey, N., Mainali, C. & Gow, S. 2021. Genetic Characterization of AmpC and Extended-Spectrum Beta- Lactamase Phenotypes in *Escherichia coli* and *Salmonella* From Alberta Broiler Chickens. *Front. Cell. Infect. Microbiol.*, 11(March): 1–12.



- Yan, M., Sahin, O., Lin, J. & Zhang, Q. 2006. Role of the CmeABC efflux pump in the emergence of fluoroquinolone-resistant *Campylobacter* under selection pressure. *Journal of Antimicrobial Chemotherapy*, 58(October): 1154–1159.
- Zilhao, R., Papadopoulou, B. & Courvalin, P. 1988. Occurrence of the *Campylobacter* resistance gene *tetO* in *Enterococcus* and *Streptococcus* spp. *Antimicrobial Agents and Chemotherapy*, 32(12): 1793–1796.

**Disclaimer/Publisher's Note:** The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.