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Campylobacter jejuni from no antibiotics ever (NAE) broilers: prevalence, antibiotic resistance, and virulence genes analysis

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ABSTRACT *Campylobacter jejuni* (***C. jejuni***) is a leading foodborne illness causing bacteria, and poultry is a major reservoir of this pathogen. With the recent increase in broiler production under the "no antibiotics ever" (**NAE**) system, this study aimed to assess the prevalence, antibiotic resistance, and virulence characteristics of *C. jejuni* isolated from NAE raised broilers. A total of 270 cloacal swabs were collected from the live-hang areas of 3 commercial processing plants over 9 wk. Each processing plant was visited 3 times at a 1-wk interval, and 30 samples were collected per visit. Among the total 270 cloacal swab samples, *C. jejuni* was isolated from 44 (16.3%) samples . Of these isolates, 65.9% possessed toxin-producing genes *cdtA, cdtB*, and *cdtC*, and invasion gene *ciaB*. The prevalence of antibioitc resistance genes *aph (3′)-IIIa, erm*(*B*) were 59.1%, and 50%, respectively. Nine (20.45%) *C. jejuni* isolates were identified as multidrug resistant (**MDR**), and 18 (40.9%) isolates showed resistance to at least 1 tested antibiotic. The highest resistance was observed against tetracycline (29.5%), followed by nalidixic acid (25%), whereas 22.7% of isolates were resistant to 2 clinically important antibiotics, azithromycin and ciprofloxacin. These results suggest that there ishigh prevalence level of multi-drug resistant *C. jejuni* with toxin producing virulence genes in the NAE-raised broilers sampled in this study, indicating the potential for serious human illnesses if transmitted through the food chain.

Key words: NAE raised broiler, *Campylobacter jejuni*, genotypic and phenotypic antibiotic resistance, virulence gene, food safety

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INTRODUCTION

Campylobacteriosis, primarily caused by *Campylobacter jejuni* (***C. jejuni***), is the leading global foodborne illness (Kaakoush et al., 2015; Skarp et al., 2016; Collins, 2022). Symptoms of *Campylobacter* infection to humans include acute gastroenteritis, diarrhea, vomiting, and abdominal cramps (Allos, 2001). Sometimes, *Campylobacter* infection can lead to severe extra gastrointestinal diseases such as Miller Fisher Syndrome (**MFS**), Guillain-Barre syndrome (**GBS**) (Ford et al., 2014), and reactive arthritis (Pope et al., 2007). In the United States, the incidence rate of campylobacteriosis was reported as 19.5 cases per 100,000 people, resulting in an estimated 1.5 million illnesses annually (Tack et al., 2020). The economic impact of these human infections was estimated to be around $6.9 billion annually (Scharff 2020; Tack et al., 2020). Additionally, attribution studies have identified that consumption of the contaminated chicken and turkey accounts 71.3% of nondairy human *Campylobacter* infection (IFSAC, 2021). In 2019, it was estimated that there were 458,400 antibiotic-resistant human *Campylobacter* infections, and 70 deaths occurred in the United States (CDC 2019; Francois Watkins et al., 2021). The incidence of drug-resistant *Campylobacter* outbreaks has increased by 44% from 2013 to 2019 (310,000 cases in 2013 to 448,400 cases in 2019) (CDC 2019), with drug-resistant *Campylobacter* infection doubling in the last 2 decades (NARMS 2015). Typically, fluoroquinolones (such as ciprofloxacin) and macrolides (such as azithromycin) are prescribed for the treatment of *Campylobacter* infection (Allos, 2001; Shane et al., 2017). However, the

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sensitivity of *Campylobacter* to fluoroquinolones and macrolides has decreased by approximately 30% (CDC 2019). Infection with antimicrobial resistant bacterial pathogens significantly increases the infection's severity and mortality risk (Cecchini et al., 2015; WHO, 2017; Dadgostar, 2019).

Poultry provides the optimum condition for the proliferation of *C. jejuni* and serves as a reservoir host (Beery et al., 1988; Horrocks et al., 2009). Approximately 56.5% of human campylobacteriosis is connected to consuming and handling contaminated poultry (Wilson et al., 2008; EFSA, 2021). In the past, subtherapeutic levels of antibiotics (such as Bacitracin, Tetracycline, Virginiamycin) have been consistently used as feed additives in poultry to promote growth and reduce the occurrence of enteric diseases (Diarra et al., 2007). However, due to growing concerns about antibiotic resistance in both food animals and humans, the use of medically important antibiotics in food animals has been prohibited in the United States (FDA, 2012; FDA, 2013; Page et al., 2021), and all antibiotics use in food animals has been banned in the EU (ESVAC, 2018). This change in antibiotic usage policy has led to a shift in the poultry production system, with a move from the antibiotic used to reduced or no antibiotics ever (**NAE**) program (Singer et al., 2020). Approximately 50 to 60% of broilers produced in the United States are raised under the NAE system (Poultry Health, 2020). The reduction or elimination of antibiotic usage in broiler feed may have impacted the prevalence and characteristics of *Campylobacter* in the broilers. Therefore, it is crucial to assess the prevalence and characteristics of *C jejuni* in NAE-raised broilers. There is limited information available on the prevalence and characteristics of *C. jejuni* in NAE-raised broilers. The present study aims to investigate the prevalence of *C. jejuni* in NAE-raised chicken in 2 states (Mississippi and Alabama) of United States and to determine the phenotypic and genotypic antimicrobial resistance and virulence characteristics of the isolated *C jejuni* strains.

MATERIALS AND METHODS

Sample Collection

The samples were collected between July 2020 to September 2020. All procedures used were approved by the Institutional Animal Care and Use Committee (**IACUC**) of Mississippi State University with protocol numbers 19-330. A total of 270 cloacal swab samples were collected from live hang areas of 3 commercial processing plants in Mississippi and Alabama that only process NAE-raised broilers. Each processing plant was visited 3 times at 1 wk intervals andfrom each visit, 30 cloacal swab samples were collected. The swab samples were collected using CultureSwab Cary-Blair Agar Transportation System (BD BBL, Berkshire, England, UK), transported in a cooler with ice to the Mississippi State University laboratory, and processed within 4 h.

Isolation of C. Jejuni

C. jejuni isolation was performed based on the Microbiology Laboratory Guidebook (MLG) 41.04 method with some modifications as described by Moran et al. (2011). Briefly, the cloacal swab was pre-enriched using a mixture of 5 mL of 2 × Blood Free- Bolton Broth (**BF-BB**) supplemented with Oxoid Bolton Broth Selective supplements consisting of vancomycin, cefoperazone, trimethoprim, and amphotericin B (Oxoid, ThermoFisher Scientific, Waltham, MA, USA) and 5 mL of BPW, making total volume 10 mL. The broth mixture was then incubated at 42°C for 48 h under microaerobic conditions (85% nitrogen, 10% carbon dioxide, and 5% oxygen) using Mart anaerobic jars with an Anoxomat II System (Mart Microbiology B. V., Lichtenvoorde, Oost Gelre, Netherlands). The loopfull (~10 μl) enriched samples were cultured onto *Campylobacter* selective agar plates 3 times to obtain the pure single isolate. Each time a single colony was obtained from the media plate and streaked into another plate. The *Campylobacter* selective agar contained *Campylobacter* agar base (Oxoid, ThermoFisher Scientific, Waltham, MA), selective supplements (Oxoid, ThermoFisher Scientific, Waltham, MA), and 5% laked horse blood (Remel Laked Horse Blood, ThermoFisher Scientific, Waltham, MA). Cultured bacterial plates were incubated under microaerophilic conditions at 42°C for 48 h. After incubation, colony morphology was observed, and typical *C. jejuni* colonies (small to medium, grayish in coloration with an irregular or round edge and mucoid appearance) were picked for subsequent culture and storage.

Molecular Identification of C. Jejuni

A previously isolated bacterial colony was cultured in the 5 mL 1 × BF-BB supplemented with Oxoid Bolton Broth Selective supplements (Oxoid, ThermoFisher Scientific, Waltham, MA). The bacterial cell pellet was collected via centrifuge of the broth at 13,000 rpm (Eppendorf 5415 Centrifuge, Eppendorf, NY) for 3 min. The genomic DNA was extracted using QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany) following manufacturer protocol. Following the DNA extraction, the quality of the DNA was checked via agarose gel electrophoresis and quantified via NanoDrop (Thermo Scientific, Wilmington, MA). Two *C. jejuni* specific gene *hippo* [hip400F 5′ GAAGAGGGTTTGGGTGGTG, hip1134R 5′ AGCTAGCTTCGCATAATAACTTG] and *Cj-CdtC* [Cj-CdtCF 5′ TTTAGCCTTTGCAACTCCTA, Cj-CdtCR 5′ AAGGGGTAGCAGCTGTTAA] genes were amplified via PCR. PCR mixture (10 μl) containing 5 μl of 2× GoTaq Green Master Mix (Promega, Madison, WI), 0.25 μl of each forward and reverse primer (10 μM), 1 μl DNA (20 ng/μl), and 3.5 μl of sterile molecular H_2O. An Eppendorf

Master cycler (Eppendorf, Westbury, NY) thermocycler was used under the following conditions: 2 min at 95°C; and 35 cycles of 30 s denaturation at 95°C, 30 s annealing at 60°C, and 60 s for the extension at 72°C and final extension of 5 min at 72°C (Linton et al., 2000; On and Jordan, 2003; Asakura et al., 2008). The amplified PCR product was visualized via gel electrophoresis using 2% agarose gel, and the gel image was visualized using a Kodak Gel Logic 200 Imaging System (Eastman Kodak Co., Rochester, NY).

Detection of Virulence Gene

The prevalence of thirteen virulence genes responsible for adhesion (*cadF, jlpA, pebA, porA, and pldA*), invasion (*ciaB*), toxin production (*cdtA, cdtB,* and *cdtC*), flagella gene (*flaAB, flgB,* and *flhB*) and type secretion system IV (*virB9*) were tested. Information about the primer is listed in Table 1. The virulence gene was detected following the procedure and primer described by Poudel et al. (2022).

Motility Assay

The *C. jejuni* motility assay was conducted in 0.4% semi-solid Muller-Hinton Agar Plates following the procedure of Pascoe et al. (2019) with slight modifications. Briefly, the *C. jejuni* colonies were picked from the subcultured agar plates and suspended in Muller-Hinton Broth (**MHB**) (Oxoid, ThermoFisher Scientific, Waltham, MA). The MHB was standardized with 0.5 McFarland Standard solution using spectrophotometry (Thermo Scientific Sensitire Nephelometer, ThermoFisher Scientific, Waltham, MA). The standardized 1 mL solution was then transferred to 5 mL of MHB, and 2 μl diluted MHB was pipetted into the center of 60 mm semi-solid Muller-Hinton Agar plates in duplicates. The cultured agar plates were incubated at 42 °C for 24 h under microaerophilic conditions. Following the incubation, the halo diameter of the bacterial motility was measured using a vernier caliper. Based on the diameter of the spread, bacteria were categorized into 3 categories nonmotile, motile, and hypermotile (Pascoe et al., 2019). Bacteria that did not spread across the agar plate were categorized as nonmotile. If a bacterial spread diameter was >0.5 cm, then categorized as motile, whereas a diameter >1.5 cm was categorized as hypermotile.

Detection of Antibiotics Resistance Genes

Prevalence of 6 antibiotics resistance genes (**ARG**) was tested *tet(O), aph(2′)-Ig, aph(3″)-IIIa,* bla_{OXA-61}, $bla_{OXA-184}$, and *ermB*. The tested ARGs are responsible for the resistance to tetracycline [*tet(O)*], aminoglycosides [*aph(2′)-Ig, aph(3″)-IIIa*], β-lactam (bla_{OXA-61}, $bla_{OXA-184}$), and erythromycin (*ermB*). The PCR was conducted following the primer and thermocycler conditions as previously described by Poudel et al. (2022) (Table 2).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (**AST**) was conducted following the broth micro dilution method using the Sensitire *Campylobacter* CAMPY2 plate (ThermoFisher Scientific, Waltham, MA) following manufacturer protocol. Briefly, the previously isolated and identified *C. jejuni* were subcultured in *Campylobacter* agar base for 48 h at 42 °C under microaerophilic conditions. The colonies were picked up from the agar plate and suspended in cation adjusted MHB (Thermo Scientific, Waltham, MA). The MHB was standardized with 0.5 McFarland Standard solution using spectrophotometry (Thermo Scientific Sensitire Nephelometer, CatLog: V301). A 100 μl of standardized MHB solution was then transferred to 11 mL MHB with lysed horse blood (Thermo Scientific, Waltham, MA). Subsequently, 50 μl broth solution was dispensed into CAMPY2 microtiter plates (Trek Diagnostics System, Thermo Scientific, Waltham, MA), which contains known concentrations ranging between 0.12 μg/mL to 128 μg/mL of 8 antibiotics (azithromycin, ciprofloxacin, clindamycin, erythromycin, florfenicol, gentamicin, tetracycline, and nalidixic acid). The microtiter plates were incubated at 37°C for 48 h under microaerophilic conditions. After incubation, the micro titer plate was visualized using the Vision System (Thermo Scientific, Waltham, MA), and the results were read manually. *C. jejuni* ATCC 33560 strain was used as the positive control.

Statistical Analysis

The MIC breaking point from Central for Disease Control and Prevention (https://www.cdc.gov/narms/antibiotics-tested.html) was utilized to interpret phenotypic antibiotic susceptibility test results; the breaking point value is listed in Table 3. For the concordance analysis and Cohen's kappa test (κ) value between the genotypic and phenotypic antibiotic resistance was only calculated for antibiotic tetracycline and *tet(O)* using methods described by Mackinnon, (2000). Even though 8 other antibiotics were tested we were unable to do concordance analysis due to the presence of the ≥1 cells having value zero resulting error, during calculation, which might be due to the lower positive sample. Correlation between the flagellar genes (*flgB, flhB, flaAB*) and phenotypic motility (combined hypermotile and motile) of *C. jejuni* was assessed using logistic regression utilizing SAS 9.4 (SAS, NC) (SAS Institute, 2013). The level of significance was determined at $P \leq 0.05$. The descriptive calculation and graph were created using GraphPad Prism 9.1.2 (GraphPad Software, San Diego, CA).

RESULTS

C. jejuni Prevalence, Virulence, and Motility

The overall prevalence of *C. jejuni* was found to be 16.3% (44/270) (Table 4). There was variation in prevalence of *C. jejuni* between the sampling point and

Table 1. List of primers used and thermocycler setting[1] used for the amplification of the virulence genes.

Set	Target genes	Accession No.	Primer name	Sequence (5′−3′)	Length (nt)	Tm (°C)	Amplicon Size(bp)	References
A[2]	*ciaB*	NP_282066	*ciaB.F335*	GGTCTAACTTCATCAACCCTTTGC	24	62.9	658	Poudel et al. (2022)
			ciaB.R992	CTCATGCGGTGGCATTAGAATG	22	62.7		
	cadF	NP_282616	*cadF.F20*	GCATCCACTCTTCTATTATCCGC	23	62.8	543	Poudel et al. (2022)
			cadF.R562	ATTCCGTCTTAGTGATTCTTTGGC	24	61.2		
	cdtA	NP_281292	*cdtA.F3*	ATCGTACCTCTCCTTGGCG	19	62.3	440	Poudel et al. (2022)
			cdtAR442	CGGAGCAGCTTTAACGGTTTG	21	62.6		
	cdtC	NP_281290	*cdtC.F260*	GCTCCAAAGGTTCCATCTTCTAAG	24	62.9	263	Poudel et al. (2022)
			cdtC.R522	GCAACTCCTACTGGAGATTTGAAAG	25	62.9		
	cdtB	NP_281291	*cdtB.F152*	GCTTGAGTTGCGCTAGTTGG	20	62.4	180	Poudel et al. (2022)
			cdtB.R331	TGGAGGAACAGATGTAGGAGC	21	62.6		
B[2]	*virB9*	YP_980061	*virB9.F429*	AAGAACACGCTTTGCAATGGC	21	60.6	535	Poudel et al. (2022)
			virB9.R964	CGATGATCCTAGTCTCTACTGGAC	24	64.6		
	pebA	NP_282073	*pebA.F40*	GCTCTAGGTGCTTGTGTTGC	20	62.4	436	Poudel et al. (2022)
			pebA.R476	GTAGTTGCAGCTTGAGCCAC	20	62.4		
	porA	NP_282406	*porA.F740*	TCAACTGGACACTTGAAGGTGC	22	62.7	342	Poudel et al. (2022)
			porA.R1082	CCACCATATACGAAGTCAGCACC	23	64.8		
	flhB	NP_281526	*flhB.F531*	GGTTGCACAGCTTACTTGGC	20	62.4	257	Poudel et al. (2022)
			flhB.R788	ACATCCGCACCTGCAACATC	20	62.4		
	jlpA	NP_282133	*jlpA.F998*	GCACACAGGGAATCGACAGC	20	64.5	119	Poudel et al. (2022)
			jlpA.R1116	AAATGACGCTCCGCCCATTAAC	22	62.7		
C[3]	*flaAB*	NP_282485 (*flaA*) NP_282484 (*flaB*)	*flaA.R1094*	CAGTTGGAACAGGACTTGGAG	21	62.6	∼1500	Poudel et al. (2022)
			flaB.R253	GCTCATCCATAGCCTTATCAGCAG	24	64.6		
	pldA	Part of NC_002163	*pldA.F422*	GCCTATACTCAAACTTCTTGGTGG	24	60.6	499	Poudel et al. (2022)
			pldA.R940	AGTCTATAAGGCTTTCTCCATAGCC	25	62.9		
	flgB	NP_281712	*flgB.F25*	GAACTGGTCACTGGTGCTTTAGC	23	64.6	224	Poudel et al. (2022)

[1]PCR thermocycler condition was initial denaturation at 95 °C for 3 min followed by 35 cycles of 95° C for 30 s, 60° C for 30 s, 72° C for 60 s, and a final extension step of 72° C for 5 min.
[2]Virulence genes run as a pentaplex PCR.
[3]Virulence gene run individually for PCR.

Table 2. List of primers used and thermocycler setting[1] used for the amplification of the antimicrobial resistance genes.

Set	Target genes	Accession No.	Orientation	Sequence (5′−3′)	Length (nt)	Tm (°C)	Amplicon Size (bp)	Reference
A[2]	*aph (3′)-IIIa*	NG_047420	Forward	TGCACTTTGAACGGCATGATG	21	56.5	432	Poudel et al. (2022)
			Reverse	TGTCATACCACTTGTCCGCC	20	57.3		
	aph (2″)-Ig	NG_047407	Forward	GATTTACCTGCCTTGATTCCGG	22	56.0	523	Poudel et al. (2022)
			Reverse	TTCGCCGAAATCTTTCCCA	19	54.6		
	$bla_{\text{OXA-184}}$	NG_049485	Forward	GCTCTCAAGTGCCTGCTTTT	20	56.0	317	Poudel et al. (2022)
			Reverse	AAATCCAACAATCCAAGCCAAA	22	53.6		
	bla_{OXA-61}	NG_049801	Forward	CTTTCTCTCCCGCTTCCACT	20	56.8	203	Poudel et al. (2022)
			Reverse	ACCAATTCTTCTTGCCACTTCTTT	24	55.3		
	tet(O)	NG_048260	Forward	AATATTCAGAGAAAAGGCGGCG	22	55.7	686	Poudel et al. (2022)
			Reverse	GCAGCCATAAAGAACCCCCT	20	57.6		
B[3]	*ermB*	KC575115	Forward	GGGCATTTAACGACGAAACTGG	22	62.7	421	Cheng et al. (2020)

[1]PCR thermocycler condition was initial denaturation at 95 °C for 3 min followed by 35 cycles of 95° C for 30 s, 55° C for 30 s, 72° C for 60 s, and a final extension step of 72° C for 5 min.
[2]Antibiotics resistance genes run as a pentaplex PCR.
[3]Antibiotics resistance gene run individually for PCR.

Table 3. Cut-off values used to determine the antibiotic resistance of *Campylobacter* along with the information about the concentration of the disc used.

CLSI[1] Class	Antimicrobial agents	Antimicrobial concentration range (μg/mL)	MIC[2] interpretive criteria (μg/mL)		
			Susceptible	Resistance	Reference source
Macrolides	AZI	0.015-64	≤0.25	≥0.5	NARMS[3]
	ERY	0.03-64	≤4	≥8	NARMS
Lincosamides	CLI	0.03-16	≤0.5	≥1	NARMS
Aminoglycosides	GEN	0.25-32	≤2	≥4	NARMS
Quinolones	CIP	0.015-64	≤0.5	≥1	NARMS
	NAL	4-64	≤16	≥32	NARMS
Tetracyclines	TET	0.06-64	≤1	≥2	NARMS
Phenicol	FLO	0.03-64	≤4	≥8	NARMS

Abbreviations: AZI, azithromycin; CIP, ciprofloxacin; CM, clindamycin; ERY, erythromycin; FLO, florfenicol; GEN, gentamycin; NAL, nalidixic acid; TET, tetracycline.
[1]Clinical and Laboratory Standards Institute.
[2]Minimum Inhibatory Concentration.
[3]National Antimicrobial Resistance Monitoring System.

Table 4. Isolation statistics of chicken *C. jejuni* strains.

Processing Plant	Location	Sampling Time	No of swab	No. of PCR positive (%)	No. of PCR positive (%)
Plant 1	Mississippi	2020 July	30	4 (13.3%)	14 (15.5%)
	Mississippi	2020 July	30	7 (23.3%)	
	Mississippi	2020 August	30	3 (10.0%)	
Plant 2	Alabama	2020 August	30	1 (3.3%)	7 (7.7%)
	Alabama	2020 August	30	4 (13.3%)	
	Alabama	2020 August	30	2 (6.7%)	
Plant 3	Mississippi	2020 September	30	2 (6.7%)	23 (25.5%)
	Mississippi	2020 September	30	0 (0%)	
	Mississippi	2020 September	30	21 (70.0%)	
			270	44 (16.3%)	

between the processing plant. Among the 3 processing plant visit, plant 3 had higher prevalence compared to plant 1 and 2. Of the 44 *C. jejuni* positive samples, the adhesion gene *pldA* and flagellar gene *flgB* were present in 81.8% of the isolates, followed by another flagellar gene *flaAB* in 79.55% of the isolates. The *Campylobacter* adherence factor gene *cadF*, toxin gene cluster *cdtA, cdtB,* and *cdtC*, and invasion gene *ciaB* were present in 65.9% of the isolates. However, the genes *porA* and type IV secretion gene *virB9* were absent in all isolates (Figure 1).

The swarming motility of the isolated *C. jejuni* was determined in the semisolid agar. Out of the 44 *C. jejuni* isolates, 61.36% (27/44) were hypermotile, 15.9% (7/44) were motile, and 22.7% (10/44) were nonmotile (Figure 2). The phenotypic motility (combined hypermotile and motile) of *C. jejuni* was highly correlated with all 3 tested flagellar genes (*flgB, flhB, flaAB)*, with respective *P*-values of $P < 0.001$, 0.0059, and <0.001.

C. Jejuni Antibiotics Resistance and Antibiotics Susceptibility

Six antibiotic resistance genes (*aph(3′)-IIIa, aph(2″)-Ig, bla*$_{\text{OXA-61}}$, *bla*$_{\text{OXA-184}}$, *tet(O),* and *ermB*) associated with resistance against 4 antibiotic classes (aminoglycoside, β-lactamase, tetracycline, and macrolide) were tested. The most prevalent resistance gene was *aph(3′)-IIIa* conferring resistance to aminoglycoside detected in 26 (59.9%) isolates. The erythromycin resistance gene *ermB* was detected in 22 (50%) isolates. The tetracycline resistance gene *tet(O)* was present in 21 (47.7%) isolates. The β-lactamase resistance gene *bla*$_{\text{OXA-184}}$ was detected in 4 (9.09%) isolates, whereas *bla*$_{\text{OXA-61}}$ was absent in all isolates (Figure 3).

The 44 *C. jejuni* isolates were tested for susceptibility to 8 antibiotics from 6 different antimicrobial classes, and the results are summarized in Figure 4. The highest resistance was observed against tetracycline, with 13

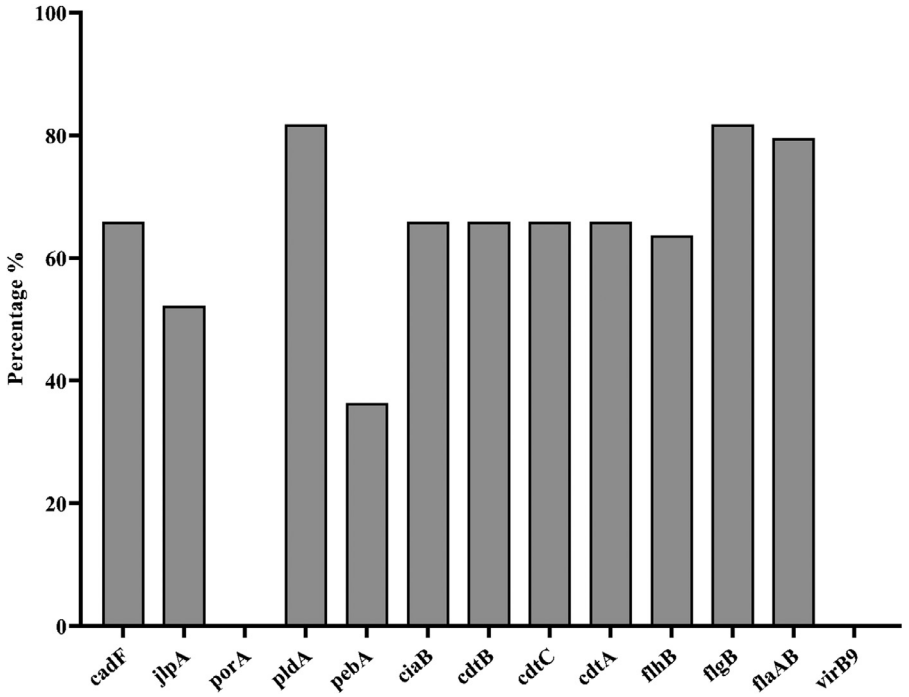

Figure 1. Percentage of virulence genes in *C. jejuni* isolates from NAE-raised broilers (n = 44). The bar plots display the presence of virulence genes in *C. jejuni* isolates obtained from broilers. These results provide insights into the genetic profiles of *C. jejuni* strains and their potential virulence factors in broiler.

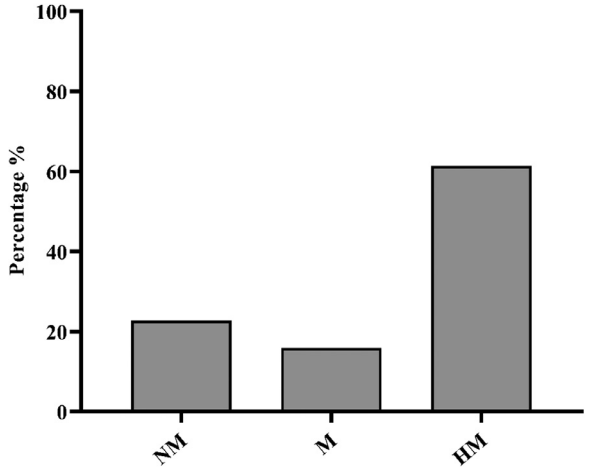

Figure 2. Motility profiles of *C. jejuni* isolates from NAE-raised broiler cloacal swabs (n = 44). The bar plot shows the distribution of motility profiles among *C. jejuni* isolates obtained from broiler cloacal swabs. The categories include nonmotile (NM), motile (M), and hypermotile (HM) isolates.

(29.5%) isolates showing resistance, followed by nalidixic acid with 11 (25%) isolates, azithromycin and ciprofloxacin with 10 (22.7%) isolates each, clindamycin with 8 (18.1%) isolates, erythromycin and gentamicin with 6 (13.6%) isolates each. The lowest resistance was observed against florfenicol, with 2 (4.5%) isolates (Figure 4). Among the tested antibiotics, 9 (20.45%) *C. jejuni* isolates exhibited multidrug resistance (**MDR**), and 26 (59.09%) isolates were susceptible to all tested antibiotics. One isolate (2.27%) showed resistance to all 6-antimicrobial classes, followed by 3 (6.81%) isolates resistant to 5 antimicrobial classes (Table 5).

The concordance analysis of the *tet(O)* and tetracycline antibiotics shows moderate agreement between the antibiotic's resistance and presence of the gene in the genome, as we observed the Cohen's kappa test (κ) value 0.68 with the specificity 87% and 48% sensitivity.

DISCUSSION

In the present study, we aimed to evaluate the prevalence, virulence genes, ARGs, and phenotypic antimicrobial susceptibility (**AST**) of *C. jejuni* isolates obtained from NAE-raised broilers. Our study revealed a prevalence of 16.3% for *C. jejuni* in NAE-raised broilers, which aligns with our previous investigation reporting a 15.7% prevalence of *C. jejuni* in environmental samples including broiler feces, cloacal swab, and litter samples from NAE broiler farms (Poudel et al., 2022). Current *C. jejuni* prevalence in NAE-raised birds was similar to (15 % -19.7 %) the previous studies, which studied the prevalence of *C. jejuni* in conventionally raised birds (Rosenquist et al., 2013; Gaucher et al., 2015; Golden and Mishra, 2020). Additionally, a meta-analysis predicts the prevalence of *Campylobacter spp* in conventionally raised birds to be 15.8% (Golden and Mishra, 2020). These findings indicate that the prevalence of *C. jejuni* has not increased in NAE-raised birds compared to conventionally raised birds. Although it is generally expected that the removal of antibiotics from the bird's diet could potentially increase enteric pathogen

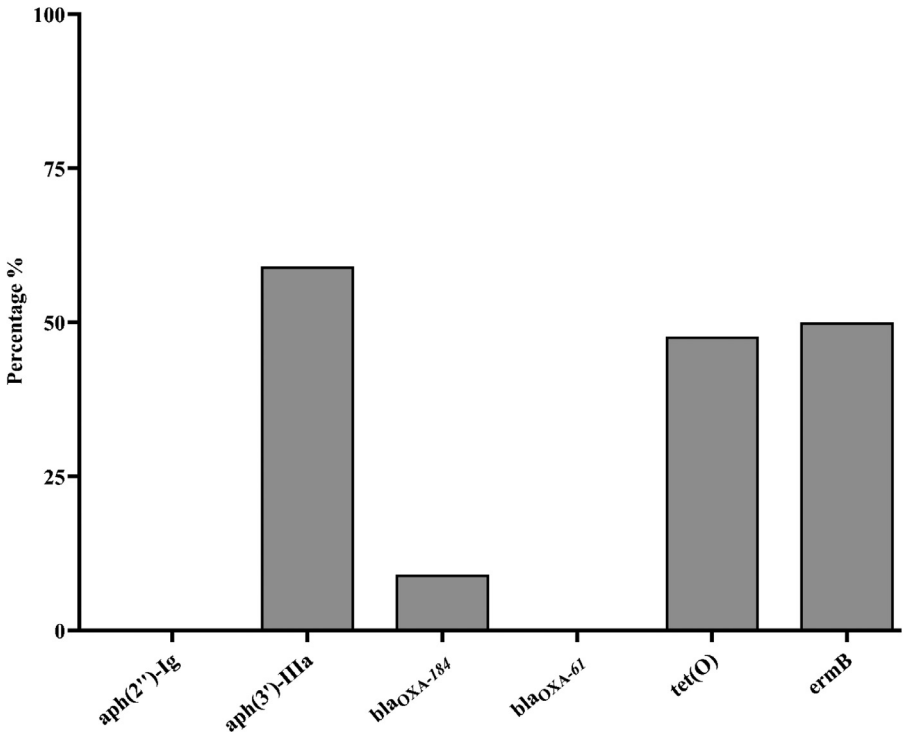

Figure 3. Percentage of antimicrobial resistance gene presence in *C. jejuni* isolates from NAE-raised broilers (n = 44). The bar plots illustrate the percentage of antimicrobial resistance gene presence in *C. jejuni* isolates obtained from the cloacal swab of NAE-raised broilers. Six antibiotic resistance genes [*aph(3′)-IIIa, aph(2″)-Ig, blaOXA-61, blaOXA-184, tet(O), and ermB*] associated with resistance against 5 antibiotic classes (aminoglycoside, β-lactamase, tetracycline, fluoroquinolones, macrolide) were tested.

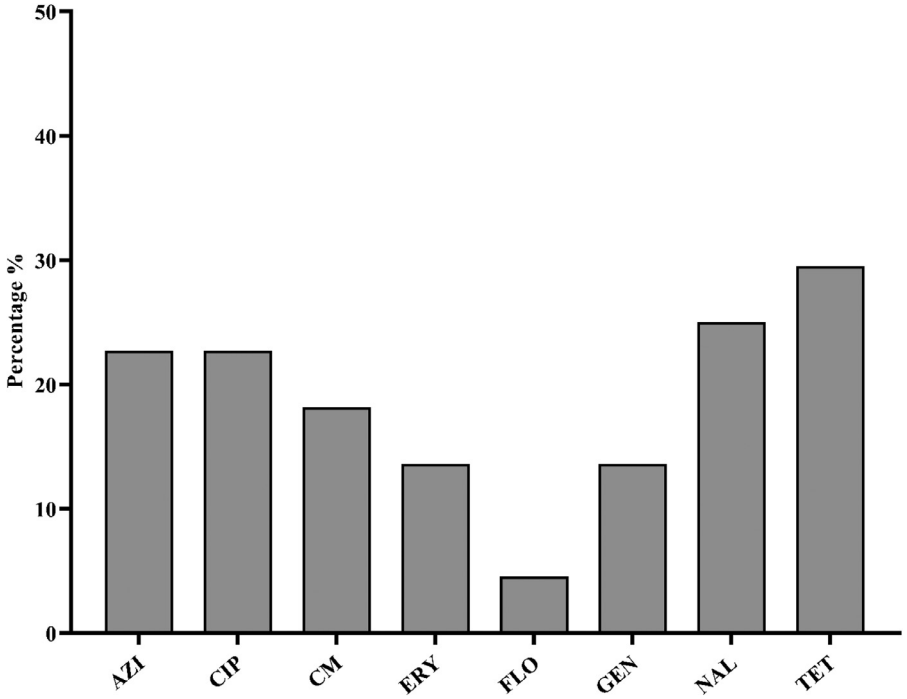

Figure 4. Phenotypic antibiotics resistance profile of *C. jejuni* isolated from NAE-raised broiler cloacal swabs (n = 44). The bar plots show the percentage distribution of antibiotic resistance among *C. jejuni* isolates derived from broiler cloacal swabs. The bar represents the percentage to specific antibiotics, including AZI: azithromycin, CIP: ciprofloxacin, CM: clindamycin, ERY: erythromycin, FLO: florfenicol, GEN: gentamycin, NAL: nalidixic acid, TET: tetracycline.

along with the *C. jejuni* colonization compared to a conventional system (Cox, 2005), our finding suggests that the prevalence of *C. jejuni* has not increased in NAE-raised broilers compared to conventionally raised birds. Potentially, the enhanced biosecurity and adoption of modern housing facilities in poultry farms might have played a significant role in maintaing similar levels of contamination. These modern houses can reduce the contact between commercial boilers with rodents, flies, cockroaches, beetles, and wild birds that may serve as a potential reservoir for bacteria transmission.

C. jejuni has many virulent factors, i.e., flagellar, adherence, toxins, invasion, and secretion. The virulence genes are the key to the pathogenesis of the bacteria. The current study tested 13 virulence genes associated with adhesion (*cadF, jlpA, pebA, porA, pldA*), toxin production (*cdtA, cdtB, cdtC*), invasion (*ciaB*), secretion (*virB9*), and motility (*flaAB, flgB*, and *flhB*). The cytolethal distending toxins (**CDT**) are a major heat-labile exotoxin produced by Gram-negative bacteria, which play a crucial role in the pathogenesis of *Campylobacter* (Jain et al., 2008). These toxins could interact with eukaryotic cells, leading to cell cycle arrest at the G2/M stage and ultimately resulting in cell death (Gargi et al., 2012; Bezine et al., 2014). In the present study, 65.9% of the *C. jejuni* isolates possessed all 3 cytotoxin subunit genes (*cdtA, cdtB,* and *cdtC*). The prevalence of *cdt* complex in our study was lower compared to previous studies, where the *cdtA, cdtB,* and *cdtC* were detected at frequencies 90-100% in *C. jejuni* isolated

Table 5. Antimicrobial resistance patterns of the tested *C. jejuni* isolates against 9 antimicrobial agents of 6 antimicrobial class.

Resistance pattern	Antimicrobial class	No of resistance isolates (%)
GEN, AZI, ERY, FLO, CIP, NAL, TET, CM,	Aminoglycoside, Macrolide, Phenicol, Quinolones, Tetracycline, Lincosamides	1 (2.27%)
GEN, AZI, ERY, FLO, CIP, NAL, TET,	Aminoglycoside, Macrolide, Phenicol, Quinolones, Tetracycline	1 (2.27%)
GEN, AZI, ERY, CIP, NAL, TET, CM,	Aminoglycoside, Macrolide, Quinolones, Tetracycline, Lincosamides	1 (2.27%)
GEN, AZI, CIP, NAL, TET, CM,	Aminoglycoside, Macrolide, Quinolones, Tetracycline, Lincosamides	1 (2.27%)
AZI, ERY, CIP, NAL, TET, CM,	Macrolide, Quinolones, Tetracycline, Lincosamides	2 (4.54%)
GEN, AZI, CIP, NAL, CM,	Aminoglycoside, Macrolide, Quinolones, Lincosamides	1 (2.27%)
GEN, AZI, CIP, NAL,	Aminoglycoside, Macrolide, Quinolones	1 (2.27%)
AZI, ERY, TET, CM,	Macrolide, Tetracycline, Lincosamides	1 (2.27%)
CIP, NAL, TET	Quinolones, Tetracycline	2 (4.54%)
AZI, CM	Lincosamides Macrolide	1 (2.27%)
TET	Tetracycline	4 (9.09%)
NAL	Quinolones	1 (2.27%)
CM	Lincosamides	1 (2.27%)
None	None	26 (59.09%)

Abbreviations: AZI, azithromycin; CIP, ciprofloxacin; CM, clindamycin; ERY, erythromycin; FLO, florfenicol; GEN, gentamycin; NAL, nalidixic acid; TET, tetracycline.

from human and poultry feces (Datta et al., 2003; Rozynek et al., 2005; Wieczorek et al., 2018). Similar to the *cdt* genes, the observed frequency of flagellar genes *flgB* (81.8%), *flaAB* (79.5%) had lower prevalence compared to *C. jejuni* isolated from the human, poultry feces, and poultry meat, where they detected approx. 100% occurrence (Datta et al., 2003; Quetz et al., 2012; Wieczorek et al., 2018; Melo et al., 2019; Sierra-Arguello et al., 2021). Additionally, in our study, 77.26% (combined hypermotile and motile) of isolated *C. jejuni* were motile, and there was a high correlation between the flagellar gene and the motility of *C. jejuni*. The primary role of flagella of the bacteria is to provide chemotactic motility and help them reach the favorable niche, adhesion, colonization, and invasion (Ottemann and Miller, 1997; Josenhans and Suerbaum, 2002; Lux and Shi, 2004; Haiko and Westerlund-Wikström, 2013; Chaban et al., 2018). Therefore, it has been postulated that the higher motility of *Campylobacter* means higher the risk of invading host-cell and higher the pathogenesis of bacteria (Hazell et al., 1986; Lertsethtakarn et al., 2011; Baldvinsson et al., 2014). Additionally, a positive correlation was observed between the motility and invasiveness of *C. jejuni* isolated from the poultry samples in human intestinal epithelial cells (Corcionivoschi et al., 2015). These findings suggest that the majority of *C. jejuni* isolated from NAE broilers sampled in this study possess greater colonizing and invasion potential but possess lower capabilities to produce cytolethal-distending toxins compared to human and other poultry meat *C. jejuni* isolates.

Although the removal of the antibiotics can negatively impact the growth performance of birds, to address the increased problem of antibiotic resistance, the usage of antibiotics in poultry production has been reduced, and a "no antibiotic ever" program has been introduced (Gaucher et al., 2015; Cowieson and Kluenter, 2019; EFSA, 2019). When the birds are constantly exposed to antibiotics, bacteria present in poultry can develop resistance against treated antibiotics, and these antibiotic-resistance bacteria can spread to humans through the food chain (Dibner and Richards, 2005; Diarra et al., 2007; Diarra and Malouin, 2014). Antimicrobial resistance (**AMR**) is a major public health concern throughout the world, and antibiotic resistant *Campylobacter* spp. has been categorized as a serious public health threat (CDC, 2019). In this study, we analyzed the presence of AMR and AST on *C. jejuni* isolated from the cloacal swab of an NAE-raised broiler. In our study, 20.45% *C. jejuni* isolates were multidrug resistance (MDR), and isolates possess ARGs and show resistance against tetracycline, nalidixic acid ciprofloxacin, azithromycin, clindamycin, gentamicin and erythromycin at varying intensity. The European Union and the United States have banned fluoroquinolones since 2001 in poultry and have progressively removed the usage of other antibiotics from poultry diet since 2006 in the EU and 2013 in the United States (FDA, 2012; FDA, 2013; ESVAC, 2018; Page et al., 2021). The impact of these regulatory changes on antibiotic usage has been significant. In chickens, the usage of medically important antibiotics has decreased by 69% in total from 2016 to 2021 (FDA, 2021). Furthermore, aminoglycosides, macrolides, sulfonamides, and tetracyclines have seen reductions of 19%, 89%, 66%, and 59%, respectively, in chicken production (FDA, 2021). The prevalence of multidrug-resistant (**MDR**) *C. jejuni* reported in our study was higher compared to <3% reported by (Bailey et al., (2019), who collected samples in midwest USA. However, the prevalence of MDR obtained in this study was slightly lower then MDR reported (30%) by Hull et al., (2021), who collected samples from chicken in North Carolina. Additionally, the resistance of *C. jejuni* against the tested antibiotics falls within a similar range or even higher compared to previous studies in which *C. jejuni* was isolated from either conventionally raised birds or undefined poultry sources (Cody et al., 2012; Obeng et al., 2012; Giacomelli et al., 2014; Sifré et al., 2015; Sierra-Arguello et al., 2015; Narvaez-Bravo et al., 2017; Premarathne et al., 2017; Szczepanska et al., 2017; Khan et al., 2018; Schiaffino et al., 2019; NARMS, 2019; Varga et al., 2019; Rivera-Mendoza et al., 2020; Tang et al., 2020; Cobo-Díaz et al., 2021; Uddin et al., 2021; Liao et al., 2021). These results indicate that despite removing antibiotics from broiler production, antibiotic resistance to *C. jejuni* still prevails in broiler production system. The higher frequencies of resistance observed may be attributed to the previous availability of these antibiotics as growth promoters in poultry (Miranda et al., 2008; Apata, 2009; Elviss et al., 2009; Giacomelli et al., 2014; Diarra and Malouin, 2014; Ljubojević et al., 2017; Miranda et al., 2018) and *C. jejuni* was still maintaining it in their genome, as Erythromycin resistance gene was found to be stable in *C. jejuni* even after 33 passage in nonantibiotics medium (Caldwell et al., 2008).

Although in this study we did not test the presence of all the genes associated with resistance to the tested phenotypic antibiotics susceptibility, but among the tested there was higher ARGs compared to the showed phenotypic resistance. Aminoglycoside phosphotransferases (***aph***) is mainly coded by *aph(3′)-IIIa* and *aph(2″)-If*, which confers resistance against amikacin, gentamicin, and kanamycin (Fong and Berghuis, 2009; Yao et al., 2017). In our study aminoglycoside resistance genes *aph (3′)-IIIa* was present in 59.9% of isolates; however, only 13.63% isolates were resistance to Gentamicin. Similarly, a total of 50% *C. jejuni* isolated in this study possess *ermB* gene whereas only 13.6% isolates were resistance against erythromycin phenotypically. The potential reasons for the higher ARGs compared to the phenotypic resistant might associated with unexpression resistance genes due to potential mutation in the genes and other factors associated with the gene expression like temperature and growth medium (Ocejo et al., 2021).

In this study, 29.5% isolates were resistant to tetracycline and 47.7% isolates possess tetracycline resistance gene *tet(O)*. The results obtained for *tet(O)* gene was close to the results obtained by Cobo-Díaz et al. (2021),

who observed 36.4% isolates positive for *tet(O)* gene while analyzing 39,789 *C. jejuni* genome sequence available in the NCBI database. Furthermore, Abdi-Hachesoo et al. (2014) observed 51.8% *tet(O)* gene in *C. jejuni* isolated from poultry carcass. However, Wozniak-Biel et al. (2018) observed higher frequency (70-100%) of *tet (O)* gene in the broiler and turkey samples. The observation of a higher frequency of tetracycline resistance might be due to its availability as growth promoters in poultry (Ljubojević et al., 2017) and tetracycline accounts for 71% of total antibiotics used in USA up to 2015 . However, the use of tetracycline as a growth promoter was restricted in the USA from 1 January 2017, and its use was confined to therapeutic purposes only (US FDA, 209 Guideline). In our study, we observed moderate concordance ($\kappa = 0.68$) between genotypic and phenotypic resistance of tetracycline, which was lower compared to other studies that utilizes WGS to identify concordance (Marotta et al., 2019; Whitehouse et al., 2018; Painset et al., 2020). The lower concordance might be due to the robust ness of WGS compared to PCR for the accurate identification of target genes (Moran et al., 2011).

CONCLUSION

The present study showed that the prevalence of *C. jejuni* has not increased in NAE-raised broilers. However, *C. jejuni* isolated from cloacal swabs of NAE-raised broilers possessed a higher resistance to clinically important antibiotics (tetracycline, ciprofloxacin, and azithromycin), which are commonly used during human clinical cases of campylobacteriosis. Additionally, majority *C. jejuni* isolated in this study were hypermotile and possessed virulence genes that can play a crucial role in the pathogenesis of campylobacteriosis in human. The observed resistance to clinically important antibiotics and the presence of virulence genes indicates that the *C. jejuni* from NAE-raised broilers still pose a public health threat even though there was a shift in the poultry production system from the conventional to NAE.

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DISCLOSURES

The authors have declared no conflict of interest.

REFERENCES

Abdi-Hachesoo, B., R. Khoshbakht, H. Sharifiyazdi, M. Tabatabaei, S. Hosseinzadeh, and K. Asasi. 2014. Tetracycline Resistance genes in campylobacter jejuni and c. coli isolated from poultry carcasses. Jundishapur J. Microbiol. 7:e12129.

Allos, B. M. 2001. *Campylobacter jejuni* infections: Update on emerging issues and trends. Clin. Infect. Dis. 32:1201–1206.

Apata, D. 2009. Antibiotic resistance in poultry. Int J. Polt. Sci. 8:404–408.

Asakura, M., W. Samosornsuk, A. Hinenoya, N. Misawa, K. Nishimura, A. Matsuhisa, and S. Yamasaki. 2008. Development of a cytolethal distending toxin (cdt) gene-based species-specific multiplex PCR assay for the detection and identification of Campylobacter jejuni, Campylobacter coli and Campylobacter fetus. FEMS Immunol. Med. Microbiol. 52:260–266.

Bailey, M. A., R. M. Taylor, J. S. Brar, S. C. Corkran, C. Velásquez, E. Novoa Rama, H. F. Oliver, and M. Singh. 2019. Prevalence and antimicrobial resistance of Campylobacterfrom antibiotic-free broilers during organic and conventional processing. Poult. Sci. 98:1447–1454.

Baldvinsson, S. B., M. C. H. Sørensen, C. S. Vegge, M. R. Clokie, and L. Brøndsted. 2014. *Campylobacter jejuni* motility is required for infection of the flagellotropic bacteriophage F341. Appl Environ. Microbiol 80:7096–7106.

Beery, J., M. Hugdahl, and M. Doyle. 1988. Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. Appl. Environ. Microbiol. 54:2365–2370.

Bezine, E., J. Vignard, and G. Mirey. 2014. The cytolethal distending toxin effects on mammalian cells: A DNA damage perspective. Cell 3:592–615.

Caldwell, D. B., Y. Wang, and J. Lin. 2008. Development, stability, and molecular mechanisms of macrolide resistance in Campylobacter jejuni. Antimicrobial. Agent. Chemother. 52:3947–3954.

CDC, Centers for Disease Control and Prevention. 2019. Antibiotic resistance threats in the United States. Centers for Disease Control and Prevention, Atlanta, GA, 1–113. Accessed Oct. 2024 https://www.cdc.gov/drugresistance/biggest_threats.html.

Cecchini, M., J. Langer, and L. Slawomirski. 2015. Antimicrobial resistance in G7 countries and beyond: economic issues, policies and option for action. Organization for Economic Co-operation and Development, Paris.

Chaban, B., I. Coleman, and M. Beeby. 2018. Evolution of higher torque in *Campylobacter*-Type bacterial flagellar motors. Sci. Rep. 8:1–11.

Cheng, Y., W. Zhang, Q. Lu, G. Wen, Z. Zhao, Q. Luo, H. Shao, and T. Zhang. 2020. Point deletion or insertion in cmeR-box, A2075G substitution in 23s rRNA, and presence of *erm(B)* are key factors of erythromycin resistance in *Campylobacter jejuni* and *Campylobacter coli* isolated from central China. Front. Microb 11:203.

Cobo-Díaz, J. F., P. González del Río, and A. Álvarez-Ordóñez. 2021. Whole resistome analysis in *Campylobacter jejuni* and *C. coli* genomes available in public repositories. Front. Microbiol. 12:1–16.

Cody, A. J., N. M. McCarthy, H. L. Wimalarathna, F. M. Colles, L. Clark, I. C. J. W. Bowler, M. C. J. Maiden, and K. E. Dingle. 2012. A longitudinal 6-year study of the molecular epidemiology of clinical *Campylobacter* isolates in Oxfordshire, United Kingdom. J. Clin. Microbiol. 50:3193–3201.

Collins, J. P. 2022. Preliminary incidence and trends of infections caused by pathogens transmitted commonly through food—foodborne diseases active surveillance network, 10 US Sites, 2016 −2021. MMWR. Morb Mortal Wkly Rep. 71:1260–1264.

Corcionivoschi, N., O. Gundogdu, L. Moran, C. Kelly, P. Scates, L. Stef, A. Cean, B. Wren, N. Dorrell, and R. H. Madden. 2015. Virulence characteristics of hcp+ *Campylobacter* jejuni and *Campylobacter* coli isolates from retail chicken. Gut. Pathog. 7:1–11.

Cowieson, A., and A. Kluenter. 2019. Contribution of exogenous enzymes to potentiate the removal of antibiotic growth promoters in poultry production. Anim. Feed Sci. Technol. 250:81–92.

Cox, L. A. 2005. Potential human health benefits of antibiotics used in food animals: A case study of virginiamycin. Environm. Int. 31:549–563.

Dadgostar, P. 2019. Antimicrobial resistance: implications and costs. Infect. Drug Resist 12:3903–3910.

Datta, S., H. Niwa, and K. Itoh. 2003. Prevalence of 11 pathogenic genes of *Campylobacter jejuni* by PCR in strains isolated from humans, poultry meat and broiler and bovine faeces. J. Med. Microbiol. 52:345–348.

Diarra, M. S., and F. Malouin. 2014. Antibiotics in Canadian poultry productions and anticipated alternatives. Front. Microbiol 5:282.

Diarra, M. S., F. G. Silversides, F. Diarrassouba, J. Pritchard, L. Masson, R. Brousseau, C. Bonnet, P. Delaquis, S. Bach, B. J. Skura, and E. Topp. 2007. Impact of feed supplementation with antimicrobial agents on growth performance of broiler chickens, *Clostridium perfringens* and *Enterococcus* counts, and antibiotic resistance phenotypes and distribution of antimicrobial resistance determinants in *Escherichia coli* isolates. Appl. Environ. Microbiol 73:6566–6576.

Dibner, J., and J. D. Richards. 2005. Antibiotic growth promoters in agriculture: history and mode of action. Poult. Sci 84:634–643.

Elviss, N. C., L. K. Williams, F. Jørgensen, S. A. Chisholm, A. J. Lawson, C. Swift, R. J. Owen, D. J. Griggs, M. M. Johnson, T. J. Humphrey, and L. J. V. Piddock. 2009. Amoxicillin therapy of poultry flocks: effect upon the selection of amoxicillin-resistant commensal *Campylobacter* spp. J. Antimicrob. Chemother 64:702–711.

European Food Safety Authority and European Center for Disease Prevention and Control. 2019. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2017. EFSA J. 17:5598.

European Food Safety Authority, and European Centre for Disease Prevention and Control. 2021. The European Union One Health 2019 Zoonoses Report. EFSA J. 19:6406.

European Medicines Agency (ESVAC). 2018. Sales of veterinary antimicrobial agents in 31 European countries in 2018: Trends from 2010 to 2018 (Tenth ESVAC Report, Ema/184855/2017). European Medicines Agency, Amsterdam, Netherlands. Accessed Oct. 2024 https://www.who.int/foodsafety/publications/antimicrobials-.

FDA. 2012. Guidance for Industry #209: The judicious use of medically important antimicrobial drugs in food-producing animals. U. S. Food and Drug Administration, Silver Spring, MD.

FDA. 2013. Guidance for Industry# 213: new animal drugs and new animal drug combination products administered in or on medicated feed or drinking water of food-producing animals: recommendations for drug sponsors for voluntarily aligning product use conditions with GFI# 209. U.S. Food and Drug Administration, Silver Spring, MD.

FDA, 2021. 2020 Summary report on Antimicrobials sold or distributed for use in food-producing animals.

Fong, D. H., and A. M. Berghuis. 2009. Structural basis of *aph(3′)-IIIa*-mediated resistance to n1-substituted aminoglycoside antibiotics. Antimicrob. Agents Chemother. 53:3049–3055.

Ford, L., M. Kirk, K. Glass, and G. Hall. 2014. Sequelae of foodborne illness caused by 5 pathogens, Australia, circa 2010. Emerg. Infect Dis 20:1865–1871.

Francois Watkins, L. K., M. E. Laughlin, L. A. Joseph, J. C. Chen, M. Nichols, C. Basler, R. Breazu, C. Bennett, L. Koski, M. P. Montgomery, M. J. Hughes, S. Robertson, C. G. Lane, A. J. Singh, D. Stanek, E. Salehi, E. Brandt, G. McGillivary, J. Mowery, J. DeMent, R. D. Aubert, A. L. Geissler, S. de Fijter, I. T. Williams, and C. R. Friedman. 2021. Ongoing outbreak of extensively drug-resistant *Campylobacter jejuni* infections associated with us pet store puppies, 2016-2020. JAMA Netw. Open 4: e2125203.

Gargi, A., M. Reno, and S. R. Blanke. 2012. Bacterial toxin modulation of the eukaryotic cell cycle: Are all cytolethal distending toxins created equally. Front. Cell. Infect. Microbiol 2:124.

Gaucher, M., S. Quessy, A. Letellier, J. Arsenault, and M. Boulianne. 2015. Impact of a drug-free program on broiler chicken growth performances, gut health, *Clostridium* perfringens and *Campylobacter jejuni* occurrences at the farm level. Polt. Sci 94:1791–1801.

Giacomelli, M., C. Salata, M. Martini, C. Montesissa, and A. Piccirillo. 2014. Antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* from poultry in Italy. Microb. Drug. Resist 20:181–188.

Golden, C. E., and A. Mishra. 2020. Prevalence of *Salmonella* and *Campylobacter* spp. In alternative and conventionally produced chicken in the United States: A systematic review and meta-analysis. J. Food Prot. 83:1181–1197.

Haiko, J., and B. Westerlund-Wikström. 2013. The role of the bacterial flagellum in adhesion and virulence. Biology 2:1242–1267.

Hazell, S. L., A. Lee, L. Brady, and W. Hennessy. 1986. *Campylobacter* pyloridis and gastritis: association with intercellular spaces and adaptation to an environment of mucus as important factors in colonization of the gastric epithelium. J. Infect. Dis. 153:658–663.

Horrocks, S., R. Anderson, D. Nisbet, and S. Ricke. 2009. Incidence and ecology of *Campylobacter jejuni* and *C. coli* in animals. Anaerobe 15:18–25.

Hull, D. M., E. Harrell, A. H. M. van Vliet, M. Correa, and S. Thakur. 2021. Antimicrobial resistance and interspecies gene transfer in Campylobacter coli and Campylobacter jejuni isolated from food animals, poultry processing, and retail meat in North Carolina, 2018−2019. PLoS One 16:e0246571.

International Food Safety Analysis Collaboration (IFSAC), 2021. Foodborne illness source attribution estimates for 2019 for Salmonella, Escherichia coli O157, Listeria monocytogens, and Campylobacter using multi-year outbreak surveillance data, United States.

Jain, D., K. N. Prasad, S. Sinha, and N. Husain. 2008. Differences in virulence attributes between cytolethal distending toxin positive and negative *Campylobacter jejuni* strains. J. Med. Microbiol. 57:267–272.

Josenhans, C., and S. Suerbaum. 2002. The role of motility as a virulence factor in bacteria. Int. J. Med. Microbiol. 291:605–614.

Kaakoush, N. O., N. Castano-Rodriguez, H. M. Mitchell, and S. M. Man. 2015. Global epidemiology of *Campylobacter* infection. Clin. Microbiol. Rev 28:687–720.

Khan, J. A., R. S. Rathore, H. H. Abulreesh, F. A. Qais, and I. Ahmad. 2018. Prevalence and antibiotic resistance profiles of *Campylobacter jejuni* isolated from poultry meat and related samples at retail shops in Northern India. Foodborne Pathog. Dis 15:218–225.

Lertsethtakarn, P., K. M. Ottemann, and D. R. Hendrixson. 2011. Motility and chemotaxis in *Campylobacter* and *Helicobacter*. Annu. Rev. Microbiol 65:389–410.

Liao, Y. S., B. H. Chen, R. H. Teng, Y. W. Wang, J. H. Chang, S. Y. Liang, C. S. Tsao, Y. P. Hong, H. Y. Sung, and C. S. Chiou. 2021. Antimicrobial resistance in *Campylobacter coli* and *Campylobacter jejuni* from human campylobacteriosis in Taiwan, 2016 to 2019. Antimicrob. Agents Chemother. 66:e0173621.

Linton, D., M. Gilbert, P. G. Hitchen, A. Dell, H. R. Morris, W. W. Wakarchuk, N. A. Gregson, and B. W. Wren. 2000. Phase variation of a β-1,3 galactosyltransferase involved in generation of the ganglioside GM1-like lipo-oligosaccharide of Campylobacter jejuni. Mol. Microbiol. 37:501–514.

Ljubojević, D., M. Pelić, V. Radosavljević, and M. Ćirković. 2017. Food safety hazards related to fish produced in aquaponicsEuropean Aquaculture Society, Dubrovnik, Croatia, 676–677.

Lux, R., and W. Shi. 2004. Chemotaxis-guided movements in bacteria. Crit. Rev. Oral Biol. Med. 15:207–220.

Mackinnon, A. 2000. A spreadsheet for the calculation of comprehensive statistics for the assessment of diagnostic tests and inter-rater agreement. Comput. Biol. Med. 30:127–134.

Marotta, F., G. Garofolo, L. Di Marcantonio, G. Di Serafino, D. Neri, R. Romantini, L. Sacchini, A. Alessiani, G. Di Donato, and R. Nuvoloni. 2019. Antimicrobial resistance genotypes and phenotypes of *Campylobacter jejuni* isolated in Italy from humans, birds from wild and urban habitats, and poultry. PloS One 14:e0223804.

Melo, R. T., A. L. Grazziotin, E. C. V. Júnior, R. R. Prado, E. P. Mendonça, G. P. Monteiro, P. A. B. M. Peres, and D. A. Rossi. 2019. Evolution of *Campylobacter jejuni* of poultry origin in Brazil. Food Microbiol 82:489–496.

Miranda, J., B. Vázquez, C. Fente, J. Barros-Velázquez, A. Cepeda, and C. Franco. 2008. Evolution of resistance in poultry intestinal *Escherichia coli* during three commonly used antimicrobial therapeutic treatments in poultry. Polt. Sci 87:1643–1648.

Miranda, C. D., F. A. Godoy, and M. R. Lee. 2018. Current status of the use of antibiotics and the antimicrobial resistance in the Chilean salmon farms. Front. Microbiol. 9:1284.

Moran, L., C. Kelly, M. Cormican, S. McGettrick, and R. Madden. 2011. Restoring the selectivity of Bolton broth during enrichment for Campylobacter spp. from raw chicken. Lett. Appl. Microbiol. 52:614–618.

National Antimicrobial Resistance Monitoring System (NARMS). (2015). NARMS 2015 human isolates surveillance report. Centers for Disease Control and Prevention. Accessed June 2022. https://www.cdc.gov/narms/reports/annual-human-isolates-report-2015.html.

National Antimicrobial Resistance Monitoring System (NARMS). (2019). NARMS Update: Integrated report summary 2016-2017. U.S. Food and Drug Administration. Accessed June 2022. https://www.fda.gov/animal-veterinary/national-antimicrobial-resistance-monitoring-system/2019-narms-update-integrated-report-summary.

Narvaez-Bravo, C., E. N. Taboada, S. K. Mutschall, and M. Aslam. 2017. Epidemiology of antimicrobial resistant *Campylobacter* spp. isolated from retail meats in Canada. Int. J. Microbiol. 253:43–47.

Obeng, A. S., H. Rickard, M. Sexton, Y. Pang, H. Peng, and M. Barton. 2012. Antimicrobial susceptibilities and resistance genes in *Campylobacter* strains isolated from poultry and pigs in Australia. J. Appl. Micrbiol. 113:294–307.

Ocejo, M., B. Oporto, J. L. Lavín, and A. Hurtado. 2021. Whole genome-based characterisation of antimicrobial resistance and genetic diversity in *Campylobacter jejuni* and *Campylobacter coli* from ruminants. Sci. Rep 11:8998.

On, S. L. W., and P. J. Jordan. 2003. Evaluation of 11 PCR assays for species-level identification of Campylobacter jejuni and Campylobacter coli. J. Clin. Microbiol. 41:330–336.

Ottemann, K. M., and J. F. Miller. 1997. Roles for motility in bacterial-host interactions. Mol. Microbiol 24:1109–1117.

Page, E.T., Short, G., Sneeringer, S., & Bowman, M. (2021). The marker for chicken raised without antibiotics 2012-17. United States Department of Agriculture, Economic Research Service. Accessed Oct. 2021. https://www.ers.usda.gov/publications/pub-details/?pubid=102186.

Painset, A., M. Day, M. Doumith, J. Rigby, C. Jenkins, K. Grant, T. J. Dallman, G. Godbole, and C. Swift. 2020. Comparison of phenotypic and WGS-derived antimicrobial resistance profiles of *Campylobacter jejuni* and *Campylobacter coli* isolated from cases of diarrhoeal disease in England and Wales, 2015−16. J. Antimicrob. Chemother. 75:883–889.

Pascoe, B., L. K. Williams, J. K. Calland, G. Meric, M. D. Hitchings, M. Dyer, J. Ryder, S. Shaw, B. S. Lopes, and C. Chintoan-Uta. 2019. Domestication of *Campylobacter jejuni* NCTC 11168. Microb. Genom. 5:e000279.

Pope, J. E., A. Krizova, A. X. Garg, H. Thiessen-Philbrook, and J. M. Ouimet. 2007. *Campylobacter* reactive arthritis: a systematic review. Semin. Arthritis Rheum 37:48–55.

Poudel, S., T. Li, S. Chen, X. Zhang, W.-H. Cheng, A. T. Sukumaran, A. S. Kiess, and L. Zhang. 2022. Prevalence, antimicrobial resistance, and molecular characterization of *Campylobacter* isolated from broilers and broiler meat raised without antibiotics. Microbiol. Spectr. 10:e00251-22.

Poultry Health Today. 2020, Nearly 60% of the US broilers now raised without antibiotics, but that number may have peaked. Accessed May 2020. https://poultryhealthtoday.com/nearly-60-of-us-broilers-now-raised-without-antibiotics-but-that-number-may-have-peaked/

Premarathne, J. M. K. J. K., A. S. Anuar, T. Y. Thung, D. A. Satharasinghe, N. N. Jambari, N. A. Abdul-Mutalib, J. T. Yew Huat, D. F. Basri, Y. Rukayadi, Y. Nakaguchi, M. Nishibuchi, and S. Radu. 2017. Prevalence and antibiotic resistance against tetracycline in Campylobacter jejuni and C. coli in cattle and beef meat from Selangor, Malaysia. Front. Microbiol. 8:2254.

Quetz, d. J. S., I. F. N. Lima, A. Havt, M. M. G. Prata, P. A. Cavalcante, P. H. Q. S. Medeiros, D. A. C. Cid, M. L. Moraes, L. C. Rey, A. M. Soares, R. M. S. Mota, B. H. Weigl, R. L. Guerrant, and A. A. M. Lima. 2012. *Campylobacter jejuni* infection and virulence-associated genes in children with moderate to severe diarrhoea admitted to emergency rooms in northeastern Brazil. J. Med. Microbiol. 61:507–513.

Rivera-Mendoza, D., I. Martínez-Flores, R. I. Santamaría, L. Lozano, V. H. Bustamante, and D. Pérez-Morales. 2020. Genomic analysis reveals the genetic determinants associated with antibiotic resistance in the zoonotic pathogen *Campylobacter* spp. distributed globally. Front. Microbiol. 11:2231.

Rosenquist, H., L. Boysen, A. L. Krogh, A. N. Jensen, and M. Nauta. 2013. *Campylobacter* contamination and the relative risk of illness from organic broiler meat in comparison with conventional broiler meat. Int. J. Food Microbiol 162:226–230.

Rozynek, E., K. Dzierzanowska-Fangrat, P. Jozwiak, J. Popowski, D. Korsak, and D. Dzierzanowska. 2005. Prevalence of potential virulence markers in Polish *Campylobacter jejuni* and *Campylobacter coli* isolates obtained from hospitalized children and from chicken carcasses. J. Med. Microbiol. 54:615–619.

SAS Institute. 2013. SAS Institute SAS Proprietary Software Release 9.4. SAS Inst. Inc., Cary, NC (2013).

Scharff, R. L. 2020. Food attribution and economic cost estimates for meat- and poultry-related illnesses. J. Food Prot 83:959–967.

Schiaffino, F., J. M. Colston, M. Paredes-Olortegui, R. Francois, N. Pisanic, R. Burga, P. Penataro-Yori, and M. N. Kosek. 2019. Antibiotic resistance of *Campylobacter* species in a pediatric cohort study. Antimicrob. Agents Chemother 63:e01911–e01918.

Shane, A. L., R. K. Mody, J. A. Crump, P. I. Tarr, T. S. Steiner, K. Kotloff, J. M. Langley, C. Wanke, C. A. Warren, A. C. Cheng, J. Cantey, and L. K. Pickering. 2017. 2017 Infectious diseases society of America clinical practice guidelines for the diagnosis and management of infectious diarrhea. Clin. Infect. Dis 65:e45–e80.

Sierra-Arguello, Y. M., R. B. Morgan, G. Perdoncini, L. M. Lima, M. J. P. Gomes, and V. P. do Nascimento. 2015. Resistance to β-lactam and tetracycline in *Campylobacter* spp.isolated from broiler slaughterhouses in southern Brazil. Pesquisa Vet. Brasileira 35:637–642.

Sierra-Arguello, Y. M., G. Perdoncini, L. B. Rodrigues, L. Ruschel dos Santos, K. Apellanis Borges, T. Quedi Furian, C. T. Pippi Salle, H. L. de Souza Moraes, M. J. Pereira Gomes, and V. Pinheiro do Nascimento. 2021. Identification of pathogenic genes in *Campylobacter jejuni* isolated from broiler carcasses and broiler slaughterhouses. Sci. Rep. 11:1–8.

Sifré, E., B. A. Salha, A. Ducournau, P. Floch, H. Chardon, F. Mégraud, and P. Lehours. 2015. EUCAST recommendations for antimicrobial susceptibility testing applied to the three main *Campylobacter* species isolated in humans. J. Microbiol Methods. 119:206–213.

Singer, R. S., L. J. Porter, N. F. D. Schrag, P. R. Davies, M. D. Apley, and K. Bjork. 2020. Estimates of on-farm antimicrobial usage in broiler chicken production in the United States, 2013-2017. Zoon. Public Health 1:22–35.

Skarp, C., M.-L. Hänninen, and H. Rautelin. 2016. Campylobacteriosis: The role of poultry meat. Clin. Microbiol. Infect 22:103–109.

Szczepanska, B., M. Andrzejewska, D. Spica, and J. J. Klawe. 2017. Prevalence and antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* isolated from children and environmental sources in urban and suburban areas. BMC Microbiol 17:80.

Tack, D. M., L. Ray, P. M. Griffin, P. R. Cieslak, J. Dunn, T. Rissman, R. Jervis, S. Lathrop, A. Muse, and M. Duwell. 2020. Preliminary incidence and trends of infections with pathogens transmitted commonly through food-Foodborne Diseases Active Surveillance Network, 10 US Sites, 2016-2019. Morb. Mortal. Wkly Rep. 69:509.

Tang, M., Q. Zhou, X. Zhang, S. Zhou, J. Zhang, X. Tang, J. Lu, and Y. Gao. 2020. Antibiotic resistance profiles and molecular mechanisms of *Campylobacter* from chicken and pig in China. Front Microbiol 11:2722.

Uddin, M., S. B. Neogi, S. S. Islam, J. Ferdous, M. Khan, S. Rahman, S. Yamasaki, and S. Kabir. 2021. Occurrence and multidrug resistance of *Campylobacter* spp. at duck farms and associated environmental and anthropogenic risk factors in Bangladesh. BMC Infct. Dis. 21:1–17.

Varga, C., M. T. Guerin, M. L. Brash, D. Slavic, P. Boerlin, and L. Susta. 2019. Antimicrobial resistance in *Campylobacter jejuni* and *Campylobacter coli* isolated from small poultry flocks in Ontario, Canada: A two-year surveillance study. PLoS One 14:e0221429.

Whitehouse, C. A., S. Young, C. Li, C.-H. Hsu, G. Martin, and S. Zhao. 2018. Use of whole-genome sequencing for *Campylobacter* surveillance from NARMS retail poultry in the United States in 2015. Food Microbiol 73:122–128.

Wieczorek, K., T. Wolkowicz, and J. Osek. 2018. Antimicrobial resistance and virulence-associated traits of *Campylobacter jejuni*

isolated from poultry food chain and humans with diarrhea. Front. Microbiol 9:1508.

Wilson, D. J., E. Gabriel, A. J. H. Leatherbarrow, J. Cheesbrough, S. Gee, E. Bolton, A. Fox, P. Fearnhead, C. A. Hart, and P. J. Diggle. 2008. Tracing the source of Campylobacteriosis. PLoS Genet 4:e1000203.

World Health Organization. (2017). Campylobacter. WHO Fact Sheet. Accessed May 2020. https://www.who.int/news-room/fact-sheets/detail/campylobacter.

Wozniak-Biel, A., G. Bugla-Płoskońska, A. Kielsznia, K. Korzekwa, A. Tobiasz, A. Korzeniowska-Kowal, and A. Wieliczko. 2018. High prevalence of resistance to fluoroquinolones and tetracycline *Campylobacter* Spp. isolated from poultry in Poland. Microb. Drug Resist. 24:314–322.

Yao, H., D. Liu, Y. Wang, Q. Zhang, and Z. Shen. 2017. High prevalence and predominance of the aph (2 ″)-If gene conferring aminoglycoside resistance in *Campylobacter*. Antimicrobial. Agents Chemother 61:e00112–e00117.