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RESEARCH ARTICLE

Comparative Prevalence of Virulence Genes and Antibiotic Resistance in *Campylobacter jejuni* Isolated from Broilers, Laying Hens and Farmers

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ABSTRACT

Foodborne infections caused by bacterial pathogen *Campylobacter jejuni* (*C. jejuni*) are frequent throughout the globe. The primary objective of the present study is to examine the genetic factors responsible for the virulence and antibiotic resistance in *Campylobacter* strains isolated from broilers, laying hens, and farm workers. This investigation involved the collection of a total of 300 samples from broilers, laying hens, and farmers. The samples were processed for conventional isolation of *C. jejuni* and were confirmed through biochemical analysis and PCR amplification of the 16S rRNA gene. The isolated strains were processed for further screening to determine the presence of antimicrobial and virulence genes *tetO, gyrA, cdtA, cdtB, cdtC, virB11*, and *flaA*. A total of ten antimicrobials, ampicillin, ciprofloxacin, erythromycin, amoxicillin, tetracycline, azithromycin, streptomycin, levofloxacin, sulfamethoxazole-trimethoprim, and ceftriaxone were used for susceptibility testing in isolated isolates. *C. jejuni* was isolated from 25% of broilers, 17% layers, and 27% of farm workers. Moreover, *C. jejuni* isolates demonstrated high rates of resistance to ampicillin (69.6%), ciprofloxacin (68.1%), erythromycin (66.7%), amoxicillin (65.2%), tetracycline (63.8%), and azithromycin (63.8%). In contrast, the lower rates of resistance to several other antibiotics ranged from 34.8 to 47.9%. *C. jejuni* positive samples contained *tetO*, *gyrA*, *cdtA*, *cdtB*, *cdtC*, *virB11*, and *flaA* genes. The prevalence of virulence genes ranged between 55.1 to 79.7%. The study's findings emphasized the potential risk to consumer health by illustrating the possible transmission of antimicrobial-resistant bacteria to individuals via the food chain. Therefore, it may be advisable to enforce antimicrobial-use policies throughout the entire food manufacturing process.

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INTRODUCTION

The contamination of food with bacterial pathogens causes a wide range of diseases, ranging in severity from case to case. Foodborne infections are found throughout the world, which endangers the health of general public. The causes of foodborne pathogens can come from a variety of sources, including animals, the environment, human handlers and contaminated water used during the processing of food (Ali and Alsayeqh, 2022). *Campylobacter* has been isolated from poultry products, which have been identified as the principal sources of the pathogen in several recent studies (Hakeem and Lu, 2020). Campylobacteriosis is a significant global public health issue, causing approximately 400-500 million cases of diarrhea annually. *C. jejuni* is the primary cause of gastroenteritis worldwide, accounting for 80% of human campylobacteriosis cases (Tafa *et al.,* 2014; Tacconelli *et al.,* 2018). *C. jejuni* has been detected in poultry, small ruminants, cattle, dogs and cats. Even though *C. jejuni* can be found in the caecum of chickens at a concentration of up to 10^9 CFU/g of fecal matter, poultry rarely exhibit

clinical symptoms of the disease caused by this pathogen (Truccollo *et al*., 2021; Al Hakeem *et al*., 2022). Furthermore, chicken carcasses may serve as a significant reservoir for *C. jejuni* to humans. Individuals can become infected by encountering pathogen during processing of food / meat, preparation of food/meat and consuming contaminated food/meat (Kaakoush *et al.,* 2015; Al Syaad *et al.,* 2023; El-Saadony *et al*., 2023). Severe complications of *C. jejuni* infection include inflammatory bowel diseases and rare autoimmune illnesses such as Miller-Fisher syndrome and Guillain-Barré syndrome (Kaakoush *et al.*, 2015; Elsayed *et al*., 2019).

The use of antimicrobials in poultry-rearing systems can result in several concerns including emergence of resistant *C. jejuni* strains, influencing the properties of bacterial biofilms, and impact the survival of pathogens, ultimately leading to the formation of a mature and highly resilient biofilm in chickens (Melo *et al*., 2017; Kreling *et al*., 2020; Rossi *et al*., 2021). Recent molecular investigations have revealed the main genes responsible for pathogenesis of *C. jejuni* strains. These genes include that allow the bacteria to adhere to intestinal epithelial cells via the expression of the *cadF*, *racR*, *virB11* and *pldA* genes; invade the intestinal epithelial cells via the expression of the *ciaB* and *ceuE* genes; produce toxins via the expression of the *cdtA*, *cdtB* and *cdtC* genes (Kovács *et al*., 2020; Gharbi *et al*., 2022; Bunduruș *et al.*, 2023).

Although virulence factors of *C. jejuni* and mechanisms of pathogenesis are significant public health concern, but they have been inadequately investigated in Egypt. Therefore, this study was designed to investigate the antibiotic resistance genes and virulence factors of the *C. jejuni* strains from broilers, laying hens, and farm workers, to aid in assessing the severity of antimicrobial resistance and transmission of *C. jejuni* strains from poultry to humans.

MATERIALS AND METHODS

Study layout and selection: A total of 200 cloacal swabs (100 broiler and 100 laying hens), additionally 100 stool swab samples from workers on the same farms were gathered by Animal Reproduction Research Institute in the Giza region conducting routine surveillance analyses. The samples were transported in sterile plastic bags under refrigeration (4°C) and examined within a day. Human stool swab samples were collected from farm employees, segregated on the basis of their age as described in Table 1.

Identification and isolation of *Campylobacter* species: The swab samples were cultured at 41.5°C for 48 h in Bolton broth (9mL) supplemented with cefoperazone, trimethoprim, vancomycin, and amphotericin B for selected growth of *Campylobacter* species, under microaerophilic environment (85% N_2; 15% CO_2, and 5% O_2) using a gas pack jar system. After 48 h of incubation, 100 µL from each test tube were spread onto agar plate of modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) and were incubated at 41.5°C and for 48 h under same conditions. *Campylobacter* species suspected colonies exhibited grayish colonies, which were purified using blood agar plates (Wieczorek *et al*., 2018).

Identification by biochemical testing: The purified suspected colonies of *Campylobacter* species were subjected to biochemical testing using catalase, oxidase, Hippurate hydrolysis, 3.5% sodium chloride tolerance, and sensitivity to nalidixic acid and cephalothin discs.

Antibiotic sensitivity testing: *C. jejuni* isolates were subjected to disc diffusion antibiotic susceptibility testing following Clinical & Laboratory Standards Institute (CLSI) guidelines (CLSI, 2016) against Ampicillin (AM-10), Ciprofloxacin (CIP-5), Erythromycin (ERY-15), Amoxicillin (AX-30), Tetracycline (TE-30), Azithromycin (AZM-15), Streptomycin (S-15), Levofloxacin (LEV-5), sulfamethoxazole-trimethoprim (SXT-25), and Ceftriaxone (CRO-30). The recommendations for interpreting the data, both susceptible and resistant, issued by the European Committee on Antimicrobial Susceptibility Testing were followed.

Extraction of DNA: In the positive isolates, PCR was utilized to identify specific resistance and virulence genes. To extract DNA from samples, we used the QIAamp DNA Mini kit (Qiagen, Germany, GmbH). For 10 minutes at 56°C, 200 µl of sample suspension, 10 µl of proteinase K, and 200 µl of lysis buffer were incubated. The lysate was combined with 200 µl of 100% ethanol after incubation. After that, the samples were centrifuged and cleaned.

Molecular confirmation of *Campylobacter* species: Bacterial genomic DNA was extracted from the isolated cultures of *Campylobacter*. The samples were then processed using the High Pure PCR Template Purification Kit. A multiplex PCR assay was used to differentiate *C. jejuni* and *C. coli. Campylobacter* species were confirmed using 16S rRNA gene specific primers. For confirmation of *C. jejuni*, *hipO* gene was detected and for *C. coli ceuE* gene was detected (Han *et al.*, 2019).

Identification of virulence and resistance genes: PCR was used to detect the virulence genes of *flaA* (located on flagellin), *virB11* (located on Type IV secretion system protein) and *cdt* clusters (*cdtA*, *cdtB*, and *cdtC*) located on cytolethal distending toxin in *Campylobacter* isolates. Moreover, in terms of the presence of the resistance genes including *gyrA* (conferring a point mutation leading to ciprofloxacin resistance) and *tetO* (conferring resistance to tetracycline) were detected using the primer sequences summarized in Table 2.

RESULTS

Identification and isolation of *C. jejuni*: A total of 200 cloacal swabs and 100 stool swab samples were subjected to cultivation of *C. jejuni.* In the current study, *C. jejuni* was identified in 25/100 (25%) of broiler chicken cloacal swabs and 17/100 (17%) of laying chicken cloacal swabs. While in the farmers workers, *C. jejuni* was found in

Table 1: Samples obtained from chickens and farm workers.

Type of samples	Chickens		Farm workers			
	Cloacal swabs		Adult stool		Young stool	
Source	Broiler chickens	Laying hens	Diarrheic	Apparently Healthy	Diarrheic	Apparently Healthy
Number of samples	100	100	20	40	25	15
Total	200		60		40	

Table 2: Primer's oligonucleotide, target genes, amplicon sizes, and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Reference
flaA	F: TGGGATTTCGTATTAACAC R: CTGTAGTAATCTTAAAACATTTTG	1713	(Lis and Connerton, 2016)
virB11	F: TCTTGTGAGTTGCCTTACCCCTTTT R: CCTGCGTGTCCTGTGTTATTTACCC	494	(Datta *et al*., 2003)
cdtA	F: GGAAATTGGATTTGGGGCTATACT R: ATCAACAAGGATAATGGACAAT	165	(Bang *et al.*, 2003)
cdtB	F: CAGAAAGCAAATGGAGTGTT R: AGCTAAAAGCGGTGGAGTAT	620	(Nahar and Rashid, 2018)
cdtC	F: TGGATGATAGCAGGGGATTTTAAC R: TTGCACATAACCAAAAGGAAG	555	(Bang *et al*., 2003)
tetO	F: GGCGTTTTGTTTATGTGCG R: ATGGACAACCCGACAGAAGC	559	(Gibreel *et al.,* 2004)
gyrA	F: TACACCGGTCAACATTGAGG R: CCGGATCGGTAAGCTTCTTCAAT	684	(Gibreel *et al*., 2004)

Table 3: Detection of *Campylobacter jejuni* by conventional method in chickens and farm workers

Type of samples	Chickens		Farmers workers			
	Cloacal swabs		Stool swabs (21 – 50 years)		Stool swabs (14 – 20 years)	
Source	Broiler chickens	Laying chickens	Diarrheic	Apparently Healthy	Diarrheic	Apparently Healthy
No. of positive samples	25/100	17/100	3/20	7/40	8/15	9/25
(%)	25%	17%	15%	17.5%	53.3%	36%
Total	42/200 (21%)		10/60 (16.6%)		17/40 (42.5%)	
			27/100 (27%)			

10/60 (16.6%) of adult males' stool swabs, 15% in diarrheic adult employees and 17.5% in apparently healthy adults. On the other hand, *C. jejuni* was found in 17/40 (42.5%) of young male stools, 53.3% of diarrheic stools, and 36% of apparently healthy. The prevalence results are summarized in Table 3.

Antibiotic susceptibility profiling *C. jejuni* isolates: The results of *C. jejuni* susceptibility to antibiotics in ten antimicrobial agents evaluated to establish the susceptibility of 69 isolates of *C. jejuni* are presented in Table 4. Out of the 69 isolates, 59 (85.5%) showed resistance to one or more antimicrobial agents. However, *C. jejuni* isolates had high rates of resistance to ampicillin (69.6%), ciprofloxacin (68.1%), erythromycin (66.7%), amoxicillin (65.2%), tetracycline (63.8%), and azithromycin (63.8%). Furthermore, the lowest rates of resistance to various antibiotics, on the other hand, ranged from 34.8% to 47.9%. Streptomycin (47.9%), levofloxacin (46.4%), sulfamethoxazole-trimethoprim (46.4%) and ceftriaxone (34.8%) resistance rates were recorded lower than other antibiotics. It's worth noting that every strain has an MDR (Multi-Drug Resistant) phenotype, exhibiting resistance to two or more than two classes of antibiotics. The most often shared resistance profiles were AM, CIP, CRO, SXT, TE and AZM.

Prevalence of resistance and virulence genes in *C. jejuni*: Genes associated with antibiotic resistance identified in *C. jejuni* are presented in Table 5. According to the findings, *gyrA* had a prevalence of 75.4% and *tetO* of 65.2%. However, cytotoxin genes *cdtA*, *cdtB*, and *cdtC* were detected in 79.7% of *C. jejuni* isolates. In addition, the most common gene was the invasive gene *virB11* (63.8%) and the flagellar gene *flaA* was present in 55.1% of the isolates.

DISCUSSION

Campylobacter is a prevalent foodborne pathogen that can be found in various circumstances, such as humans, animals and the environment. The diverse disease patterns and the existence of antibiotic resistance pose challenges in the treatment of gastroenteritis caused by this pathogen. Antibiotic resistance patterns are identified using genomic, clinical, and epidemiological research methodologies. This has led to the emergence of multi-drug resistant (MDR) profiles in many isolates (Bunduruș *et al*., 2023). This research investigated the frequency of *C. jejuni*, antibiotic resistance and virulence genes in poultry and poultry farmers. The current study emphasized that the frequency of multidrug resistant *C. jejuni* may pose a concern for public health.

C. jejuni was found in 25% of broiler and 17% of laying chicken cloacal swabs. However, *C. jejuni* was identified in the stool of 16.6% of adults, 15% of diarrheic, and 17.5% of apparently healthy adult employees. Also, *C. jejuni* was identified in 36% of diarrheic stool and 53.3% of apparently healthy young farmer's employees. These findings correspond with (Salem *et al.*, 2019), which showed 27.6% of intestinal swabs positive for *Campylobacter* species. Furthermore, *C. jejuni* was isolated in 42.5% of young men, including 36% of youngsters who appear healthy and 53.3% of diarrheal persons, as depicted by (Elhadidy *et al*., 2020). There is little information about campylobacteriosis in humans, because wasn't examined extensively on diarrheal human (Igwaran and Okoh, 2019).

The emergence of antibiotic resistance in *C. jejuni* and its prevalence has increased globally (Luangtongkum *et al*., 2009), which threatens the health of the general public (Iovine, 2013). The current study showed 69.6% and 63.8% of *C. jejuni* isolates were resistant to ampicillin

Table 4: Susceptibility to 10 antimicrobial agents and biochemical tests for detection of *Campylobacter jejuni*

Susceptibility to 10 antimicrobial agents			Biochemical tests								
Antibacterial agent (Abbreviation, Concentration µg)	Class	Number of resistant strains (%)	Oxidase	Catalase	Nitrate reduction	Urease	Hippurate hydrolysis	Growth at 37°C	Growth at 43°C	Susceptibility Nalidixic acid	Susceptibility Cephalothin
Ampicillin (AM-10)	β-Lactam	48(69.6)	+	+	+	-	+	+	+	S[1]	R[2]
Ciprofloxacin (CIP-5)	Quinolones	47(68.1)	+	+	+	-	+	+	+	S	R
Erythromycin (ERY-15)	Macrolide	46(66.7)	+	+	+	-	+	+	+	S	R
Amoxicillin (AX-30)	Penicillin	45(65.2)	+	+	+	-	+	+	+	S	R
Tetracycline (TE-30)	Tetracycline	44(63.8)	+	+	+	-	+	+	+	S	R
Azithromycin (AZM-15)	Macrolides	44(63.8)	+	+	+	-	+	+	+	S	R
Streptomycin (S-15)	Aminoglycosides	33(47.9)	+	+	+	-	+	+	+	S	R
Levofloxacin (LEV-5)	Quinolones	32(46.4)	+	+	+	-	+	+	+	S	R
Sulfamethoxazole-Trimethoprim (SXT-25)	Sulfonamides	32(46.4)	+	+	+	-	+	+	+	S	R
Ceftriaxone (CRO-30)	cephalosporin	24(34.8)	+	+	+	-	+	+	+	S	R

[1] S = Susceptible: [2] R = Resistant

Table 5: Prevalence of resistance and virulence genes in *C. jejuni*

Detected genes		*C. jejuni* (n=69)	
		No.	(%)
Tetracycline	*tetO*	45	65.2 %
Ciprofloxacin	*gyrA*	52	75.4%
Cytolethal distending toxin genes	*cdtA*	55	79.7 %
	cdtB	55	79.7%
	cdtC	55	79.7 %
Invasive gene	*virB11*	44	63.8 %
Flagellar gene	*flaA*	38	55.1%

and tetracycline, respectively. According to Ge *et al*. (2013), ampicillin and tetracycline resistance was found to be present in less than 50% of *C. jejuni* isolates. In the present study, the isolates of *C. jejuni* had a significant level of ciprofloxacin resistance (68.1%). According to Elhadidy *et al*. (2020), 55.8% of the *C. jejuni* isolates were resistant to ciprofloxacin. Variations of the ciprofloxacin resistant *C. jejuni* were first identified by Ge *et al*. (2013). This investigation showed that *C. jejuni* isolates exhibited resistance to erythromycin (66.7%), amoxicillin (65.2%), and azithromycin (63.8%). This finding corresponds to Bunduruș *et al*. (2023) and Wardak *et al*. (2007) that showed high levels of in vitro activity against *C. jejuni* isolates with erythromycin, tetracycline, gentamicin, and ciprofloxacin. Furthermore, the lowest rates of resistance to various antibiotics, ranged from 34.8% to 47.9% (Streptomycin, Levofloxacin, Sulfamethoxazole-trimethoprim and Ceftriaxone).

Our findings depicted that 75.4% of the *C. jejuni* isolates were positive for presence of *gyrA* genes, responsible for ciprofloxacin resistance. Moreover, 65.2% of isolates were positive for *tetO*. Moreover, isolated *C. jejuni* strains obtained from this investigation are carried cytolethal distending toxin (CDT) *cdtA*, *cdtB* and *cdtC* genes (79.7%) were seen in the isolates. However, virulence genes CDT, they are connected to pathogen adhesion, colonization and invasion *C. jejuni* strains, were frequently found (Wieczorek *et al*., 2018). According to the findings, the invasive gene *virB11* (63.8%) and the flagellar gene *flaA* were detected in 55.1% of the isolates (Bardy *et al*., 2002). Also, it was common to find the virulence genes *flaA*, and *virB11* which are linked to pathogen adherence, colonization, and invasion (Wieczorek *et al*., 2018; Sierra-Arguello *et al*., 2021).

Conclusion: The existence of antibiotic resistant *Campylobacter* strains identified from laying hen and broiler farm employees may suggest a severe threat to public health. This study discovered drug resistance with a substantial link to pathogenic factors. More research will help to establish the possible causes of the emergence of AMR and failure of antibiotics during clinical treatment of campylobacteriosis. It is vital to evaluate novel therapeutic options as well as the most recent antibiotic generation. Cutting-edge molecular tools like proteomics and genomics are expected to shed fresh light on the molecular processes that lead to *Campylobacter* antibiotic resistance.

Ethics approval statement: The experiments were reviewed and approved by the Zoonoses Department Committee, Faculty of Veterinary Medicine, Benha University (no. BUFVM 08-09-23). *C. jejuni* strains were isolated and characterized at the Animal Reproduction Research Institute's department of reproductive diseases laboratory, Giza, Egypt.

Author's contribution: ASh, and MA Conceptualization, data curation, and methodology. MWA, YSM, and SAA software, data analysis and original draft. AEA, and AAA reviews, editing. MEA writing, reviews, editing, and performed statistical analyzes. There is no conflict of interest among authors.

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