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RESEARCH ARTICLE

Virulence Repertoire and Antimicrobial Resistance of *Campylobacter jejuni* and *Campylobacter coli* Isolated from Some Poultry Farms in Menoufia Governorate, Egypt

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ABSTRACT

Poultry are considered the primary source of *Campylobacter* spp. infections in people. We aimed to detect various virulence factors of *Campylobacter* spp., using PCR and evaluation of antimicrobial resistance patterns, in a total of 350 samples collected from chickens: 300 samples from dead birds with postmortem lesions, 50 from normal birds. Overall, 170/350 (48.57%) were culture positive for *Campylobacter* spp. Among these, 25 (14.7%) isolates were identified as *C. jejuni* and five (2.94%) as *C. coli*. All 25 isolates of *C. jejuni* were confirmed by the presence of 23S rRNA and the species-specific gene *map*A; the five *C. coli* isolates were confirmed by the presence of *ceu*E. Simplex and multiplex PCR protocols were used to analyze the *C. jejuni* isolates for the presence of six putative virulence genes: the flagellum encoding gene *fla*A, the invasion-associated genes *iam*A and *vir*B11, and the cytotoxin genes *cdt*A, B and C. These were identified in 3/25 (12%), 2/25 (8%), 3/25 (12%), 25/25 (100%), 0/25 (0.0%), and 0/25 (0.0%), respectively. Among the five *C. coli* isolates, two (40%) harbored *vir*B11. The 30 *Campylobacter* isolates were classified into seven groups according to the exhibited antimicrobial resistance patterns, both species expressed high indices of antimicrobial resistance (0.67-0.89). The most effective antimicrobial against both species was amikacin while ciprofloxacin and doxycycline were effective against *C. jejuni*. Hence, both *C. jejuni* and *C. coli* isolated from diseased or healthy poultry constitute a public health concern because of the harbored virulence genes and high resistance to antimicrobials.

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INTRODUCTION

Campylobacter spp. are Gram-negative, slender, spirally curved (0.2-0.8 µm x 0.5-5 µm) bacteria. Although most of the species are microaerophilic, some can grow anaerobically or aerobically, living as commensal organisms in the gastrointestinal tract of many domestic and wild birds, as well as in mammalian hosts (Bolton, 2015: Skarp *et al*., 2016). Recently, there are described 25 *Campylobacter* species. Poultry are the main reservoir for *Campylobacter* and broiler meat is a major vehicle for transmission to human (Batz *et al*., 2012: Golz *et al*., 2014; Martinez-Anton *et al*., 2018).

The symptoms of *Campylobacter* infections in young birds range from no clinical signs to clear signs of diarrhea and weight loss while the PM examination characterized by distention of the jejunum, disseminated hemorrhagic enteritis, and in some cases, focal hepatic necrosis. In addition, human infection distinguished by the following symptoms; abdominal pain, fever, headache, nausea, vomiting, diarrhea which is frequently bloody. The previous symptoms could last for 3 to 6 days moreover other complications as bacteraemia, hepatitis, pancreatitis and miscarriage have been found. Furthermore, reactive arthritis and neurological disorders such as Guillain-Barré syndrome, neurological and

respiratory dysfunction from a polio-like form of paralysis are post-infection complications that also reported (Sahin *et al.*, 2015: Skarp *et al*., 2016).

The mechanism of *C. jejuni* immunopathogenesis is based on four main stages: adhesion to intestinal cells, colonization of the digestive tract, invasion of target cells, and production of toxins (Haddad *et al*., 2010). The flagellum encoding genes *fla*A and *fla*B control the major flagellin protein (Lertsethtakarn *et al*., 2011). The *Campylobacter* adhesion is controlled by many genes as the (*iam*A) which has been identified in some strains of *C. jejuni* and *C. coli* (Carvalho *et al*., 2001). The type IV secretion system, possibly involved in adhesion, is encoded by *vir*B11 (Dasti *et al*., 2010). Production of cytotoxins, especially the cytolethal distending toxins (CDTs), has been well investigated in *Campylobacter* species. Many Gram-negative bacteria have the ability to produce CDT (Ge *et al*., 2008), a tripartite toxin composed of three subunits encoded by genes *cdt*A, *cdt*B and *cdt*C. The iron uptake system is controlled by several genes including *ceu*E, which mediates a periplasmic binding protein. Mutants lacking these genes show a reduced ability to colonize chickens (Xu *et al*., 2010). The resistance of *Campylobacter* to multiple drugs, bile, and heavy metals is often mediated by active efflux pumps. Antimicrobial resistance in commensal bacteria develops on their exposure to specific antibiotics during carriage in broilers or other food animals or during infection in humans (Silva *et al*., 2011).

We aimed to analyze the virulence genes and antimicrobial resistance patterns of *C. jejuni* and *C. coli* isolated from some of the poultry farms in Menoufia Egypt, to further understand the genotypic characters and pathogenesis of these notorious infectious agents.

MATERIALS AND METHODS

Sampling and isolation: A total of 300 samples (150 cecum, 100 duodenum, 50 bile) were collected from freshly dead chickens that had suffered distended gas-filled ceca, red spots on the duodenum wall, red-colored ingesta, and distended gallbladders. A further 50 samples (25 cecum, 25 duodenum) were collected from healthy birds without signs. Samples (1 g) were pre-enriched in Bolton broth and incubated for 24 h at 42ºC in microaerophilic conditions. A loopful of Bolton broth with culture was then inoculated onto charcoal-cefoperazone-deoxycholate agar (CCDA) plates and incubated in microaerophilic conditions (using Campy Gen gas-generating kit (Oxoid, UK). The isolates were cultivated in 20% glycerol broth and kept at -80ºc. The refreshed strains were identified on the basis of colony and cell morphology, motility, growth at 25ºC and 42ºC, catalase and oxidase tests, H_2S production on triple sugar iron agar slants, sensitivity to cephalothin and nalidixic acid, and sodium hippurate hydrolysis (in accordance with ISO 10272-1:2006 and ISO/TS 10272-2:2006 standards).

Molecular characterization of *Campylobacter* isolates and harbored virulence markers: The QIAamp DNA Mini Kit (Qiagen) with spin column was used in accordance with the manufacturer's instructions for extraction of DNA from the obtained *Campylobacter* isolates.

PCR protocols: In simplex PCR, primers for 23S rRNA, *Ceu*E, *map*A, *fla*A and *iam*A were used in a total reaction volume of 25 µl: Emerald Amp® GT PCR master mix (Takara, Japan) 12.5 µl, forward primer (20 pmol) 1 µl, reverse primer (20 pmol) 1 µl, template DNA 2 µl, and PCR-grade water 8.5 µl (Table 1).

In multiplex PCR, primers targeting the cytolethal distending toxins *cdt*A, B, and C were used in a total reaction volume of 50 µl: Emerald Amp® GT PCR master mix (Takara, Japan) 25 µl, forward primer (20 pmol) 2 µl, reverse primer (20 pmol) 2 µl, template DNA 8 µl, and PCR-grade water 13 µl PCR conditions were according to the references listed in Table 1.

Antimicrobial susceptibility testing: The bacterial counts were adjusted to a concentration of 1×10^6 colony forming units per 1 mL using sterile Muller–Hinton broth and a spectrophotometer at wavelength 660 nm. From the adjusted concentrations, 1 mL aliquots were spread onto Muller-Hinton agar plates, which were then dried at 40ºC for 20 min. Antibiotic discs (Oxoid) containing amikacin (AK) 30 mg, ampicillin (AM) 10 mg, ciprofloxacin (CIP) 5 mg, nalidixic acid (NA) 30 mg, lincomycin (MY) 10 mg, chloramphenicol (C) 30 mg, doxycycline (DO) 30 mg, cefotaxime (CTX) 30 mg, and trimethoprim/ sulphamethoxazole (SXT) 25 mg were distributed evenly on the agar surfaces and plates were incubated for 48 h under the same conditions conferred for isolation. The diameters of the inhibition zones were interpreted according to CLSI (2013).

Table 1: Primer types, sequences, and length of amplified products

Target agent	Target gene	Primer sequence (5'-3')	Amplicone size	References
Campylobacter	*23S rRNA*	F:TATACCGGTAAGGAGTGCTGGAG R:ATCAATTAACCTTCGAGCACCG	650 bp	Wang *et al*., 2002
C. coli	*Ceu*E	F:AATTGAAAATTGCTCCAACTATG R:TGATTTTATTATTTGTAGCAG CG	462 bp	Eunju and Lee (2009)
C. jejuni	*map*A	F:CTATTTTATTTTTGAGTGCTTGTG R:GCTTTATTTGCCATTTGTTTTATTA	589 bp	
Campylobacter	*fla*A	F:AATAAAAATGCTGATAAAACAGGTG R:TACCGAACCAATGTCTGCTCTGATT	855 bp	Datta e *al*., 2003
	*iam*A	F:GCGCAAATATTATCACCC R:TTCACGACTACTACTATGCGG	518 bp	Wieczorek *et al*., 2012
	*cdt*A	F:GNW:GGAAATTGGATTTGGGGCTATACT R:ATCAACAAGGATAATGGACAAT	165 bp	Bang *et al*., 2003
	*cdt*B	F:GTTAAAATCCCCTGCTATCAACCA R:GTTGGCACTTGGAATTTGCAAGGC	495 bp	
	*cdt*C	F:TGGATGATAGCAGGGGATTTTAAC R:TTGCACATAACCAAAAGGAAG	555 bp	

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Statistical analysis: The isolation rates of *Campylobacter*, the frequency of the obtained species and the sensitivity and resistance of isolates to antimicrobials were presented as percentages (%). The Multiple Antibiotic Resistance (MAR) Index was displayed as a percentage of effective antimicrobials to the total used types. The obtained isolates were classified into 7 groups according to the obtained Multiple Antibiotic Resistance (MAR) which ranged from 0.67 to 0.89.

RESULTS

Sample collection, isolation, and biochemical identification: Of the 350 collected samples, 170 isolates (48.57%) were gained on *Campylobacter* selective media. Of these, only 25/170 (14.7%) were biochemically identified as *C. jejuni* and 5/170 (2.94%) proved to be *C. coli*. While the remaining 140/170 (82.35%) isolates lacked the ability to be refreshed again (Table 2).

Molecular confirmation, virulence factors, and antimicrobial sensitivity patterns of the *Campylobacter* isolates: All the biochemically identified isolates 30/30 (100%) were confirmed as *Campylobacter* spp. from these isolates 25/25 (100%) were identified as *C. jejuni* after detection of the 23S rRNA and *map*A. Among the *C. jejuni* isolates, the major flagellin protein *fla*A was present with a rate of 3/25 (12%) and the invasion-associated marker *iam*A exhibited a distribution pattern of 2/25 (8%), (Table 3). In addition, the *vir*B11, which is associated with invasiveness, was detected in 3/25 (12%) of *C. jejuni*. Cytotoxin A (*cdt*A) showed the highest frequency among virulence genes and was present in all isolates of *C. jejuni* (100%). The five biochemically identified *C. coli* isolates were confirmed by the presence of the *ceu*E and harbored the *vir*B11 with a rate of 2/5 (40%).

The data presented in (Table 3) elucidated that all the 30 *Campylobacter* isolates were classified into seven groups (a-g) according to the obtained antimicrobial resistance patterns. The group (a) which contained 8 *C. jujeni* isolates those were sensitive to amikacin and expressed an intermediate response to ciprofloxacin, while the group (b) composed of 3 *C. jujeni* isolates which were sensitive to amikacin and ciprofloxacin but gave an intermediate response to doxycycline. Moreover, the group (c) included 5 *C. jujeni* isolates that were sensitive to amikacin, and group (d) contained 3 *C. jujeni* isolates which were intermediate to doxycycline. Furthermore, the group (e) composed of 3 *C. jujeni* isolates which expressed sensitivity to doxycycline while the group (f) contained 3 *C. jujeni* isolates which exhibited sensitivity to amikacin. Added to that, the group (g) composed of 5 *C. coli* isolates which were sensitive to amikacin. Isolates of both *C. jejuni* and *C. coli* exhibited high antimicrobial resistance indices that ranged from 0.67 to 0.89.

Ethical considerations: This study was performed according to the recommendations of the Guide to U.S. Government Principles dealt with the issue of care about and utilization of vertebrate animals in research and testing. The research protocol accepted by the Institutional Animal Care and Use Committee (IACUC) at the Faculty of Veterinary Medicine, University of Sadat City.

Table 2: Number of collected samples, obtained isolates on charcoal-cefoperazone-deoxycholate agar, and biochemical identification

Chickens	No. of cases	Collected organs	No. of samples	No. (%) of *Campylobacter* isolates	Biochemical confirmation		VBNC
					C. jejuni	*C. coli*	
Dead with PM lesions	300	Cecum	150	80 (53.3)	9	5	66
		Duodenum	100	45 (45)	3	0	42
		Bile	50	20 (40)	5	0	15
Cases without PM lesions	50	Cecum	25	20 (80)	6	0	14
		Duodenum	25	5 (20)	2	0	3
Total			350	170/ 350(48.57)	25/170(14.7)	5/170(2.94)	140/170(82.35)

* The other isolates were not refreshed due to the viable but non-culturable state (VBNC) due to unfavorable oxygen rich conditions.

Table 3: Molecular confirmation, virulence factors, and antimicrobial sensitivity patterns of 25 confirmed *C. jejuni* and five *C. coli* isolates

Groups	23S rRNA	*C. coli* *ceu*E	*C. jejuni* *map*A	Virulence genes						Antimicrobial resistance patterns	MAR index
				*fla*A	*iam*A	*Vir*B11	*cdt*A	*cdt*B	*cdt*C		
Group (a)	8/8 (100%)	0/8 (0.0%)	8/8 (100%)	3/8 (37.5%)	2/8 (25%)	0/8 (0.0%)	8/8 (100%)	0/8 (0.0%)	0/8 (0.0%)	AM, NA, MY, C, DO, CTX, SXT.	0.78
Group (b)	3/3 (100%)	0/3 (0.0%)	3/3 (100%)	0/3 (0.0%)	0/3 (0.0%)	1/3 (33.3%)	3/3 (100%)	0/3 (0.0%)	0/3 (0.0%)	AM, NA, MY, C, CTX, SXT.	0.67
Group (c)	5/5 (100%)	0/5 (0.0%)	5/5 (100%)	0/5 (0.0%)	0/5 (0.0%)	1/5 (20%)	5/5 (100%)	0/5 (0.0%)	0/5 (0.0%)	AM, CIP, NA, MY, C, DO, CTX, SXT.	0.89
Group (d)	3/3 (100%)	0/3 (0.0%)	3/3 (100%)	0/3 (0.0%)	0/3 (0.0%)	0/3 (0.0%)	3/3 (100%)	0/3 (0.0%)	0/3 (0.0%)	AK, AM, CIP, NA, MY, C, CTX, SXT.	0.89
Group (e)	3/3 (100%)	0/3 (0.0%)	3/3 (100%)	0/3 (0.0%)	0/3 (0.0%)	1/3 (33.3%)	3/3 (100%)	0/3 (0.0%)	0/3 (0.0%)	AK, AM, CIP, NA, MY, C, CTX, SXT.	0.89
Group (f)	3/3 (100%)	0/3 (0.0%)	3/3 (100%)	0/3 (0.0%)	0/3 (0.0%)	0/3 (0.0%)	3/3 (100%)	0/3 (0.0%)	0/3 (0.0%)	AM, CIP, NA, MY, C, DO, CTX, SXT.	0.89
Total	25/25 (100%)	0/25 (0.0%)	25/25 (100%)	3/25 (12%)	2/25 (8%)	3/25 (12%)	25/25 (100%)	0/25 (0.0%)	0/25 (0.0%)		
Group (g)	ND	5/5 (100%)	0/5 (0.0%)	0/5 (0.0%)	0/5 (0.0%)	2/5 (40%)	0/5 (0.0%)	0/5 (0.0%)	0/5 (0.0%)	AM, CIP, NA, MY, C, DO, CTX, SXT.	0.89

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DISCUSSION

C. jejuni is a normal inhabitant of the intestinal tract of a wide variety of wild and domestic animals. In poultry, contamination of retail products occurs through de-feathering, evisceration, and dipping during the slaughtering process (Di Giannatale *et al*., 2010). *C. jejuni* colonizes the mucus overlying epithelial cells primarily in the cecum and small intestine of chickens, but can also be recovered from elsewhere in the gut and from liver and spleen (Lamb-Rosteski *et al.*, 2008). From our data on isolation, biochemical testing, and molecular confirmation using 23S rRNA and *map*A, the commonest isolate was *C. jejuni* (25/170, 14.7%) similar to the findings of Van Deun *et al.* (2007) and El-Jakee *et al*. (2015) in Belgium and Egypt respectively. Furthermore, *C. jejuni* is considered a leading cause of enteric illness in many western countries, developing countries, and the European Union (Wagenaar *et al.*, 2013). Moreover, *C. jejuni* is strongly linked with Guillain–Barré syndrome, an autoimmune syndrome that may result in respiratory and severe neurologic dysfunction and could be fatal in the bad circumstances (Haddad *et al.*, 2010). Although, there is obtained 170 isolates which were kept at -80ºc, not all isolates were refreshed. There is a difference between the high rate of early isolation of *Campylobacter* on mCCDA medium and the lowered rate of biochemically identified isolates. This result could be regarded to the viable but non-culturable state (VBNC) of *Campylobacter* due to unfavorable conditions rich in oxygen, that lead to the failure of the culture techniques for refreshment of preserved isolates (Zhao *et al*., 2017). The virulence and survival factors of *C. jejuni* depend on motility, adhesion, colonization, invasion, toxin production, iron acquisition, and antimicrobial resistance. In this study, we used PCR based on known genetic sequences to explore a subset of putative *C. jejuni* virulence-associated genes that play a vital role in infection. The frequency of *fla*A, a major flagellin gene, was 3/25 (12%), lower than that determined by El-Jakee *et al*. (2015) in Egypt who found that the frequency of the *fla*A was 35.75%. Regarding *iam*A, which has been designated an invasion-associated marker in some *C. jejuni* and *C. coli* strains (Carvalho *et al*., 2001: Bolton, 2015), this gene was confirmed in 2/25 (8%) of *C. jejuni* isolates, lower than the findings of Wieczorek and Osek (2008). The occurrence of the *vir*B11 gene was a marker for the plasmid pVir, which is associated with invasiveness. It should be noted that the distribution pattern of *vir*B11 in the *C. jejuni* isolates was 3/25 (12%) which was higher than that determined by González-Hein *et al.* (2013) and closely similar to the findings of Van Deun *et al.* (2013). Concerning the existence of cytotoxin genes, our study confirmed that *cdt*A was present in all the *C. jejuni* isolates, confirming the widespread of cytotoxin genes in poultry isolates as demonstrated by Rozynek *et al*. (2005) and Van Deun *et al*. (2007). Moreover, their data suggested that the clinical outcome is dependent on production of cytotoxins and other virulence factors. The participation of the *cdt*A gene in the development of infection in chickens appears to be significant, due to its high existence rate (100%).

The serious problem of *Campylobacter* multidrug resistance is basically mediated by active multidrug efflux pumps (Silva *et al.*, 2011). From our results, it was clear that most of the *C. jejuni* isolates were characterized by the high resistance to ampicillin, cefotaxime, chloramphenicol, doxycycline, lincomycin, nalidixic acid, and trimethoprim/ sulphamethoxazole. The apparent MAR index was high in all seven groups of *Campylobacter*, according to the obtained resistance patterns with values ranged from 0.67 to 0.89. The high frequency of resistance to ampicillin and other beta lactame antimicrobials has been confirmed by Stef *et al*. (2013), who linked this type of resistance to the overproduction of beta-lactamases. Although, some groups of *Campylobacter* expressed sensitivity to amikacin, ciprofloxacin, and doxyxycline, there is encountered resistance to ciprofloxacin, nalidixic acid, and doxycycline in other groups which comes in consistence with Wieczorek *et al.* (2012). The prominent susceptibility to amikacin expressed in some groups agree with El-Jakee *et al.* (2015). The overall high frequency of antimicrobial resistance in *Campylobacter* spp. represents a serious public health concern. The rational interpretation of this crisis is the frequent encountering of specific antimicrobials during the commensal carriage of *Campylobacter* spp. in chickens and large animals or during human infections. There is strong evidence linking the uncontrolled use of antimicrobials in animal production with the emergence and widespread of resistance in *Campylobacter* spp. (Silva *et al*., 2011).

Conclusions: *Campylobacter* isolates from diseased and normal poultry cases harbored many virulence and cytotoxin genes that are crucial in the pathogenesis of this infectious agent. Our study demonstrated the high cytotoxicity and antimicrobial resistance of *C. jejuni* and *C. coli*, confirming that both species are serious and notorious infectious hazards of public health concern. Moreover, from our conclusions it was clear that there is an urgent need for implementation of stringent control, public health, and food protection strategies. Our results call for continuous monitoring and effective vaccine formulation strategies for lowering the excessive use of antimicrobials and reducing the problem of antimicrobial resistance.

Authors contribution: Mohamed Sabry Abd Elraheam Elsayed and Reda Tarabees were the leaders of this study they planned, monitored, and evaluated the research steps. They helped also in sampling, isolation, genotyping, antimicrobial susceptibility testing, writing, revising the manuscript, and data analysis. Ola Harb and Ahmed Sabry helped with sampling, isolation, and most of the genotyping. Awad Shehata helped with conceptualization of the study, provided some technical advice, and helped with data analysis.

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