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Phenotypic and genotypic characterization of some virulence genes of Campylobacter species isolated from fresh meat

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ABSTRACT

Campylobacter is the causative agent of the zoonotic disease Campylobacteriosis. It is transmitted by consuming or handling raw or undercooked foods of animal origin, resulting in gastroenteritis and diarrhea in humans. This search aimed to study the phenotypic and genotypic characterization of Campylobacter species isolated from fresh meat. So, seventy random samples of fresh cow meat were collected from different localities (slaughterhouses and butchers) in the Al-Gharbia governorate, Egypt. Campylobacter species were isolated on Preston Enrichment broth and Modified Charcoal Cefoperazone Deoxycholate agar, followed by identification using biochemical tests. The antimicrobial susceptibility was determined using the disk diffusion method, against 10 antimicrobials; amoxycillin, ampicillin, clindamycin, doxycycline, gentamicin, cefotaxim, norfloxacin, sulfamethoxazole, chloramphenicol, and ofloxacin. The existence of ciaB, cdtB and cadF virulence genes was screened in two *Campylobacter jejuni* and two *Campylobacter coli* isolates using PCR. The results showed that Campylobacter species were isolated from 38/70 samples (54.29%). They were identified as C. jejuni 30/38 (78.95%) and C. coli 8/38 (21.05%). The in-vitro sensitivity tests for all isolates revealed the highest resistance to amoxicillin (99%), followed by ampicillin (87%) and clindamycin (85.07%). The intermediate resistance was recorded against doxycycline (94.3%), chloramphenicol (93.5%) and gentamicin (90%). Meanwhile, the highest susceptibility was to cefotaxime (93.8%), norfloxacin (91%), sulfamethoxazole (90%) and ofloxacin (89.7%). The Virulence genes cdtB and cadF were detected in the four selected Campylobacter isolates, while CiaB was detected in one Campylobacter species, C. jejuni. In conclusion, detecting virulent Campylobacter spp. in fresh beef samples is motivating for applying good hygienic conditions during slaughtering, skinning, and evisceration.

1. INTRODUCTION

Campylobacter is a Gram-negative genus of microaerophilic spiral-shaped bacteria in the family *Campylobacteriaceae* that has a significant impact on human and animal health through its association with gastrointestinal infections. The genus Campylobacter has a diverse range of species, exceeding 20 species exhibiting diverse characteristics and environmental preferences, according to Facciolà et *al.* (2017). Distinguishing characteristics include thermophilic and non-thermophilic species, with the former growing at 42 °C, while the latter requires temperatures as low as 37 °C for in vitro growth (Sykes and Marks, 2014). Notably, thermophilic species such as *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter upsaliensis*, and *Campylobacter lari* are known to be the main causes of bacterial gastroenteritis worldwide. Among these, *Campylobacter jejuni* stands out as the predominant cause; accounting for approximately 90% of bacterial gastroenteritis cases worldwide (Facciolà et *al.*, 2017).

Thermophilic Campylobacter is widespread in poultry and livestock species, including cattle, swine, and sheep, with poultry identified as the primary source of infection for humans (Debelo et *al.*, 2022). This zoonotic foodborne disease poses a considerable health risk due to its low infectious dose and potential for serious sequelae (Al Amri *et al.*, 2007). Transmission occurs when contaminated water, milk, and raw or undercooked meat are consumed, with cross-contamination during food preparation or storage (Wieczorek et al., 2013).

In Egypt, Campylobacter has established itself as an endemic pathogen and a significant contributor to pediatric diarrhea (Helmy *et al.*, 2017).

Fluoroquinolones and macrolides are the main antibiotics used to treat Campylobacter infections because they are effective against this gram-negative pathogen (Da Silva et al., 2016). However, the emergence of resistance poses a significant challenge to the effectiveness of these antibiotics. Resistance to fluoroquinolones in Campylobacter species has been linked to several factors, such as mutations in the *gyr*A gene, which encodes the DNA gyrase subunit (Lucey *et al.*, 2002; Piddock *et al.*, 2003). Additionally, the involvement of major facilitator superfamily efflux pumps in fluoroquinolone resistance has been reported (Jeon *et al.*, 2009). As efflux pumps play a pivotal role in the intrinsic and acquired resistance of *Campylobacter jejuni* and *Campylobacter coli* to various antimicrobial agents, including both fluoroquinolones and macrolides (Luo *et al.*, 2003; Ge *et al.*, 2002).

Several genes have been attributed to *Campylobacter* spp. virulence, but the most important is cytolethal distending toxin B (*cdt*B), which disrupts mucosal barriers by causing host cell death. Campylobacter adhesion fibronectin F gene (*cad*F) encodes a protein involved in the invasion and adhesion of *C. jejuni* (Miller *et al.*, 2010; Pickett *et al.*, 1996), and it has been reported to present at a high level in *C. jejuni* isolates. Also, heat survival and stress response

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proteins (*htr*B and *clp*P) are important for Campylobacter survival (Bowdre *et al.*, 1976; Konkelet *et al.*, 1999). Moreover, Campylobacter invasion antigen proteins, such as *Cia*B, *Cia*C, *Cia*I, provide effective invasion and colonization while also playing a role in intracellular survival (Eucker and Konkel 2012).

Cattle are considered an important source of Campylobacter infections in humans; in addition, water may be contaminated, further complicating the spread of antibiotic-resistant *Campylobacter* strains. This research aimed to study the phenotypic and genotypic characterization of virulence genes in *Campylobacter* species isolated from fresh meat, originating from cattle and to further identify the prevalence of antimicrobial resistance of *Campylobacter* species in Egypt, which helps guide effective strategies for treating and preventing *Campylobacter* infections linked to foodborne transmission.

2. MATERIALS AND METHODS

2.1. Ethical approval:

This search was ethically approved by the Ethical Approval Committee of the Faculty of Veterinary Medicine, Benha University, Egypt, under the Ethical Approval Number (BUFVTM 50-09-23).

2.2. Study Design:

The study was conducted from August 2022 to January 2023 to isolate, identify, and estimate the occurrence of Campylobacter in cattle meat samples from abattoirs, butcher shops, and supermarkets.

2.3. Sample Collection:

Seventy fresh cattle meat samples (n = 70) were randomly collected from various localities (slaughterhouses and butchers) in Al-Gharbia governorate. Samples were packed in sterile plastic bags, labeled, and promptly transferred in an ice box to the laboratory under sanitary conditions for further investigation.

2.4. Bacteriological Isolation and Identification:

2.4.1. Isolation of Campylobacter:

Ten grams of raw meat were collected and homogenized in 90 ml of Preston Enrichment (PE) broth (Nutrient broth No.2CM 67, oxoid, basing stoke UK) containing 5% lysed horse blood or sheep blood. After incubation at 42˚C for 48 hours in a micro-aerophilic condition (gas mix of 5% O2, 10% CO2, and 85% N2) done by using *Campylobacter* gas-generating kits (Oxoid, BR56) in conjugation with their catalyst-containing jars, samples were streaked onto Modified Charcoal Cefoperazone Deoxycholate (MCCDA) agar (CM739 plus SR155, oxoid, Basing Stoke, UK). Typical colonies (greyish, moistened, glossy flat in *C. jejuni* and creamy, greyish moisten with a slightly raised shiny surface in *C. coli*) were further identified through Gram staining and biochemical tests (Linton *et al.*, 1997; Persson *et al.*, 2005).

2.4.2. Identification of Campylobacter:

Presumptive identification of *Campylobacter* spp. was performed based on colonial appearance (Roop et al., 1984; Roberts and Greenwood, 2002), Gram staining, and biochemical tests. The biochemical tests included catalase, oxidase, urine, nitrate reduction, and glucose utilization tests (Baylis *et al., 2000;* Roberts and Greenwood, 2002). Sodium hippurate hydrolysis was used to distinguish between *Campylobacter jejuni* and *Campylobacter coli* (Harvey, 1980).

2.5. Testing of Antimicrobial Susceptibility:

Campylobacter spp isolates were submitted to in-vitro antimicrobial susceptibility testing using the standard agar disc diffusion method with ten antibiotic discs. Clear zones were measured, and results were categorized as susceptible (S), intermediate (I), or resistant (R) based on the CLSI 2014 standards (Table 1).

Table 1: Antibiotics used for antimicrobial susceptibility testing of *Campylobacter* isolates:

Antibiotic	Abbreviation	Concentration (µg)
Cefotaxime	CTX	30µg
Doxycycline	DO	30 µg
Gentamycin	GEN	10 µg
Sulfamethaxazole	SXT	25µg
Ampicillin	AP	10 µg
Clindamycin	CD	2 µg
Amoxycillin	AMC	30µg
Norofloxacin	NOR	10µg
Ofloxacin	OF	10µg
Chloramephenicol	C	30 µg

2.6. Molecular Characterization of *Campylobacter* virulence genes by PCR

Two *Campylobacter jejuni* and two *Campylobacter coli* isolates subjected to Polymerese Chain Reaction for detecting 3 virulence genes; cytolethal distending toxin B (*cdt*B), Campylobacter adhesion fibronectin F (*cad*F), Campylobacter invasion antigen proteins B*(cia*B).Chromosomal DNA was extracted using the QIAamp DNA Mini kit. PCR reaction and Cycling conditions of the specified primers targeting *cdtB*, *ciaB* and *cadF* (Table 2) during PCR were used according to Emerald Amp GT PCR master mix (Takara). Products of PCR separated by gel electrophoresis on a 1.5% agarose gel, and a 100 bp DNA ladder served as a molecular size marker. (Sambrook *et al.*, 1989).

Table 2: Primer sequences, expected product sizes and PCR Cycling conditions:

Primer	Sequence	Product (bp.)	Reference
cdtB			
F	CAGAAAGCAAATGGAGTGTT	620	Nahar and Bin Rashid, 2018
R	AGCTAAAAGCGGTGGAGTAT		
ciaB			
F	TGCGAGATTTTTCGAGAATG	527	Zheng *et al.*, 2003
R	TGCCCGCCTTAGAACTTACA		
cadF			
F	TTGAAGGTAATTTAGATATG	400	Al Amri *et al*., 2007
R	CTAATACCTAAAGTTGAAAC		

Cycling conditions of the primers during cPCR.

Gene	Primary Denature-tion	Secondary Denature-tion	Annealing	Extension	No. of cycles	Final extension
cdtB	94˚C 5 min.	94˚C 30 sec.	51˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.
ciaB	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.
cadF	94˚C 5 min.	94˚C 30 sec.	49˚C 40 sec.	72˚C 40 sec.	35	72˚C 10 min.

3. RESULTS

3.1. Prevalence of *Campylobacter* species in cattle meat samples

Bacteriological examination of the collected fresh meat samples (n=70), revealed identification of 38 *Campylobacter* species (54.28%) depending on colonial appearance, Gram-staining and biochemical reactions (Figure 1).

The suspected Campylobacter colonies were greyish, moisten, glossy flat spreading colonies in *C. jejuni*, while creamy greyish in color moisten with slightly raised shiny surface in *C. coli* on Modified Charcoal Cefoperazon Deoxycholate agar. Their microscopic examination by Gram stain showed typical gram negative curved, twisted bacilli.

Biochemically they were positive in catalase test and oxidase test. In addition sodium Hippurate hydrolysis test was positive in 30 out of the identified 38 *Campylobacter*

species which indicated *C. jejuni* (79%). The other 8 isolates were negative for sodium Hippurate hydrolysis test and identified as *C. coli* (21%) (Fig. 1).

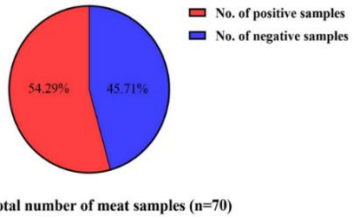
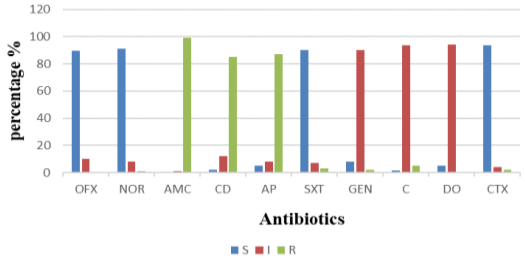


Fig1: Prevalence of *Campylobacter* species isolates in fresh meat samples (n=70).

3.2 Antimicrobial resistance of isolated *campylobacter* species:

Antimicrobial susceptibility of isolated *Campylobacter* spp. was evaluated by disc diffusion method. It came up with 99% of the isolates displayed the highest phenotypic resistance against amoxicillin, followed by ampicillin (87%) and clindamycin (85.07%) as the lowest detected level of resistance. While the isolated *Campylobacter* spp. showed intermediate resistance against doxycycline (94.3%) chloramphenicol (93.5%) and gentamicin (90%). On the other hand, the isolates were susceptible to cefotaxime (93.8), norfloxacin (91%), sulfamethoxazole (90%), and ofloxacin (89.7%), (Fig. 2).



Fig(2): Antimicrobial susceptibility of Campylobacter isolates
OFX (ofloxacin), NOR (norfloxacin), AMC (amoxicillin), CD (clindamycin), AP (ampicillin), SXT (sulfamethaxazole), GEN (gentamicin), C (chloramphenicol), DO (doxycycline), CTX (cefotaxime)

3.3. Molecular characterization of *Campylobacter* virulence genes

PCR demonstration of *cad*F, *cdt*B and *cia*B virulence genes in two *Campylobacter jejuni* and two *Campylobacter coli* isolates showed presence of *cad*F and *cdt*B in all isolates tested, while *cia*B gene was detected only in one isolate of *C. jejuni* (Figure 3).

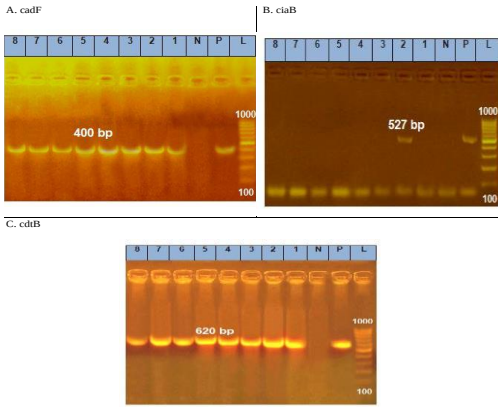


Fig (3): PCR amplification products of *Campylobacter* species virulence genes. a) Agarose gel electrophoreses showed amplification of cadF gene of both *Campylobacter jejuni* and *C.coli* , lane 4,5 showed positive amplification at 400 bp L: ladder (100-1000) , P: positive control. N: negative control, b) Amplication of *cia*B gene of *Campylobacter jejuni* lane ,2 showed positive amplification appeared at 572bp lane L:Ladder (100 – 1000) L: ladder , P: positive control. N: negative control, c) Amplification of *cdt*B gene of both *Campylobacter jejuni* and *C. coli*, lane 4,5, showed positive amplification appeared at 620 bp L:Ladder (100-1000) , P: positive control. N: negative control.
(620 bp), Lane L: 100bp ladder, lane P: positive control. Lane N: negative control, lanes 1-4.

4. DISCUSSION

Campylobacter species are of great public health concern, particularly due to zoonotic transmission through consumption of contaminated meat or water or undercooked poultry, or red meats (Havelaar *et al*., 2013; Sharma *et al.*., 2016).

This study revealed a high prevalence (54.28%) of species of *Campylobacter* in raw fresh beef samples (n = 70), which surpasses previous findings by Igwaran and Okoh (2020), who found that the occurrence rate of Campylobacter was 34% in the examined meat samples, emphasizing a contamination issue in meat products. The 38 *Campylobacter* isolates were then characterized into two species: *C. jejuni*, which had the highest prevalence rate (79%), followed by *C. coli (21*%). The presence of *C. jejuni* and *C. coli is* in line with what Ocejo et al. (2019) and Sulaiman et al. (2019) found, which is more proof that these species are important in foodborne infections.

The antibiotic resistance profile of the isolated *Campylobacter* species against 10 antibiotics showed elevated resistance to amoxicillin (99%), ampicillin (87%) and clindamycin (85.07%). The isolates exhibited high susceptibility to cefotaxime (93.8), followed by norfloxacin (91%), sulfamethoxazole (90%), and ofloxacin (89.7%) (Fig. 2). These findings align with those of Igwaran and Okoh (2020), who reported that the highest phenotypic resistance displayed by *Campylobacter* isolates was against clindamycin (100%), with the majority of the tested isolates showing resistance to the test antibiotics; in addition to the reporting of multi-drug resistant isolates. Hagos et al. (2021) concurred with our findings, stating that 96.9%, 85.9%, and 50% of the isolates exhibited resistance to ampicillin, amoxicillin, and streptomycin, respectively.

Campylobacter pathogenesis relies on a variety of virulence factors that enable its survival and transmission. These virulence factors include adhesion factors, invasion factors, toxins, and surface structures (Kreling et al., 2020). This study demonstrated the detection of three virulence genes by PCR in two selected *C. coli* and two *C. jejuni* isolates. The first was the *cad*F gene, which contains Campylobacter's adhesion protein for fibronectin. It is responsible for binding to fibronectin in epithelial cells and allowing the delivery of Cia proteins to the cytosol of host cells (Monteville et al., 2003). The second was *Cia*B gene, which is one of the Campylobacter invasion antigen proteins. It is secreted by Campylobacter through the flagellar type III secretion system, "T3SS." *Cia*B proteins are potent entry and colonization factors that also play a role in intracellular survival (Eucker and Konkel, 2012; Kreling et al., 2020). Moreover, Konkel et al. (2001) illustrated that inoculation of piglets with *cia*B mutant *C. jejuni* did not cause diarrhea until 3 days' post-infection, and only mild histological lesions appeared in the intestine. Otherwise, a piglet inoculated with a wild *C. jejuni* strain developed diarrhea and severe histological lesions within 24 hours; shortening of the villi and production of exudate in the lumen (Konkel et al., 2001).

The third gene studied in this study was the *cdt*B gene (cytolethal distending toxin B). Cytolethal distending toxin is the only known Campylobacter toxin, and it is encoded by three genes: *cdt*A, *cdt*B, and *cdt*C. The subunit *cdt*B is the active toxic component of the toxin that causes direct DNA damage, inhibits cell division, and initiates apoptosis. The *cdt*A and *cdt*c subunits are responsible for binding to and internalization into the host cell (Abuoun et al., 2005).

The current results indicate the presence of *cad*F and *cdt*B in all isolates tested. Several studies, including; Ripabelli et al.

(2010), reported a high incidence of *cad*F in *Campylobacter* spp. isolates from various sources, and Andrzejewska et al. (2015) discovered the *cad*F gene in *C. jejuni* and *C. coli* isolated from foods, animal, human feces and/or poultry meat. Also, Melo et al. (2013) found *cad*F gene in 67.3% of *C. jejuni* strains isolated from chicken carcasses. Meanwhile, Igwaran and Anthony (2020) identified *cad*F gene in 37.25% of *Campylobacter* spp. isolated from meat samples (lamb, chicken, turkey, beef, and pork). Additionally, the *cdt*B gene was observed in the screened *C. jejuni* and *C. coli*, which is consistent with the findings of Ripabelli et al. (2010) and Andrzejewska et al. (2015), who detected a higher presence of *cdt*B gene in *C. jejuni* (100% and 93.3%, respectively) compared to *C. coli* isolates (97.2% and 89.6%, respectively). In contrast, Igwaran and Anthony (2020) reported the presence of *cdt*B gene in 5.88% of *C. jejuni* and 16.67% of *C. coli* isolates. In addition, results showed that *cia*B gene was detected only in one isolate of *C. jejuni* and not found in any *C. coli* isolates. Closely, Igwaran and Anthony (2020) didn't detect it in *C. jejuni* or *C. coli*, while Meloetal (2013) found the *cia*B gene in 37/55 (67.3%) *C. jejuni* strains.

5. CONCLUSIONS

This study revealed the presence of virulent Campylobacter species in fresh beef samples. As a result, there is a risk of infection from consuming raw or uncooked meat. So, the application of good hygienic conditions during slaughtering, skinning, and evisceration, regular monitoring and examination of meats, and avoiding cross-contamination during food preparation or storage are important to maintain food safety standards.

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