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Original Research Article

ANTIMICROBIAL RESISTANCE, VIRULENCE-ASSOCIATED GENES, AND FLAGELLIN TYPING OF THERMOPHILIC *Campylobacter* SPECIES ISOLATED FROM DIARRHEIC HUMANS, RAW MILK, AND BROILER NICHES

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Abstract: Animal-to-human transmission is frequently linked to commensal bacteria in the gastrointestinal tract, as *Campylobacter* species. We provide a better insight into the existence of cytolethal distending toxin (*cdt*) and flagellinA (*flaA*) genes in different antimicrobial resistance patterns (pandrug-resistance (PDR), extensive drug-resistance (XDR) and multidrug-resistance (MDR)] in *Campylobacter* species recovered from chickens, milk, and human sources in Egypt. *Campylobacter* species isolation rate was 89.44%, being 79.50% and 20.50% for *C. jejuni* and *C. coli*, respectively. Animal samples (chickens and raw milk) showed a higher prevalence of *C. coli* (21.17%); whereas *C. jejuni* was highly documented in human samples (83.33%). Testing of antimicrobial susceptibilities revealed that none of the examined campylobacters was pansusceptible, while PDR (1.86%), XDR (65.53%), and MDR (32.61%) campylobacters were reported. Molecular analysis revealed that 50%, and13.16% of the isolated campylobacters were positive for the *cdt* and *flaA* genes, respectively. Interestingly, all *flaA*-positive isolates were *cdt*-positive *C. jejuni*. Restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) assay revealed a genetic diversity among *flaA* harboring isolates presented as three different RFLP patterns of varying sizes ranged from 112 to 647 bp, where *flaA*-RFLP pattern-I is overrepresented in *C. jejuni* isolates from various origins (human, milk, and chicken sources). Evidence from this study showed the possibility of treatment failure in campylobacteriosis due to the existence of resistant isolates to all antimicrobial drugs (PDR) and the marked genetic variability of *flaA*-RFLP pattern that is useful in the epidemiological issues.

Key words: *Campylobacter* species; antimicrobial resistance; virulence genes; flagellin typing

Introduction

Thermotolerant *Campylobacter* species, particularly *C. jejuni* and *C. coli* ranked the second most emerging bacterial zoonotic pathogens after *Salmonella* infection (1, 2). *Campylobacter* colonizes the gastrointestinal tract (GIT) of a broad range of domestic and wild animals, particularly chickens, turkeys, and pigs, which considered the main reservoirs of the bacteria (3). The foremost risk factors for getting human campylobacteriosis are unpasteurized milk and unprocessed poultry meat (4). Handling and utilization of contaminated undercooked poultry meat lasts the most common source of human *Campylobacter* infections (5).

A growing tendency of antimicrobial resistance was documented in *Campylobacter* species detected in the food chains and humans (6-9). Moreover, the alarming level of multidrug-resistance (MDR), extensive drug-resistance (XDR), as well as pandrug-resistance (PDR) in *Campylobacter* species is epidemiologically significant not only because of their resistance to numerous antimicrobial drugs, but also because they encompass the worrisome potential to be resistant to all of the authorized antimicrobial agents (10).

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In *Campylobacter* species, virulence features related to motility, colonization, intestinal adhesion, toxin generation, and invasion have been found. Carriage of these genes varies and helps to elucidate the virulence differences among isolates. The cytolethal distending toxin (cdt), a toxin that activates cell cycle arrest and subsequent death in sensitive eukaryotic cells, is one of the main well-studied virulence factors (11).

A broad genetic variability of *Campylobacter* populations are related to a lot of mutations in their surface antigens especially that gene encoding for flagella (12). Restriction fragment length polymorphism (RFLP) analysis of *C. jejuni* flagellin gene that contains two compartments (*flaA* and *flaB*) provides a great level of discrimination power for better understanding the epidemiology of campylobacteriosis (13,14). There were several reports studied the presence of *cdt* and *flaA* virulence determinants in MDR *Campylobacter* species (15-18). This is, however, the first study to look at *flaA* typing of MDR, XDR, and PDR *Campylobacter* species harboring *cdt* genes obtained from both animal and human origins in Egypt.

Material and methods

Samples

In the study, 360 samples of animal (n=308) and human (n=52) origins were examined between March 2017 and September 2019. Animal samples were attained from broiler chickens at slaughter age (6 weeks; n = 248) and raw unpasteurized milk (n = 60); those were gathered from Zagazig local pluck retail shops in Sharkia Governorate, Egypt. Chicken samples comprised breast meat (n = 55), cloacal swabs (n = 95), caecal parts (n = 48), and internal organs (liver and spleen, n = 25 each). The human stools were collected from diarrheic patients with gastroenteritis attending numerous laboratories located at Zagazig, Sharkia Governorate, Egypt. The samples were transferred in an icebox to the bacteriology laboratory without delay for the bacteriological analysis. The animal work was accredited by the committee of Animal Protection and Research Ethics, Faculty of Veterinary Medicine, Zagazig University. The human study was carried out in conformity with the World Medical Association's Declaration of Helsinki. Besides, an informed written consent was given by patients involved in the research study.

Campylobacter species isolation and molecular confirmation

Campylobacter isolation was performed under microaerophilic conditions in accordance with a previously defined protocol (19). Enrichment of samples was done using Preston *Campylobacter* selective enrichment broth (Oxoid, Cambridge, UK), at 42 ºC for 48 h. The broth was cultivated on mCCDA, a modified charcoal-cefoperazone-deoxycholate agar (Oxoid, Cambridge, UK) then transmitted to the Oxoid`s Columbia agar (Oxoid, Cambridge, UK) complemented with 5% defibrinated sterile sheep blood. *Campylobacter* colonies have been confirmed by biochemical tests as catalase, oxidase, nitrate reduction, as well as assessing their resistance to cephalothin and nalidixic acid antibacterial agents (30 mg/disc, each) (20). The hydrolysis of hippurate was tested for biochemical identification of campylobacters into *C. coli* and *C. jejuni* (20). Using a QIAamp DNA Mini extraction kit (Qiagen GmbH, Hilden, Germany), the bacterial DNA was extracted following the manufacturer's guidelines. Amplifications of *Campylobacter`s* 23S rRNA gene (21), besides *C. jejuni (mapA*) and *C. coli* (*ceuE*) species specific genes (22) were conducted by conventional polymerase chain reactions (PCRs) using oligonucleotide primers mentioned in Table 1.

Antimicrobial susceptibility testing

Antimicrobial susceptibilities of the isolated campylobacters was performed on Mueller-Hinton agar media (Oxoid-CM0337B, Cambridge, UK) provided with defibrinated sterile sheep blood (5%) under microaerobic conditions adopting the agar disc diffusion method (23) in compliance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (24). Fourteen various antimicrobial classes including 24 antimicrobial discs (Oxoid, Cambridge, UK) were used including penicillins [ampicillin (AM; 10 µg) and amoxicillin (AX; 25 µg)], penicillin combinations [ampicillin-sulbactam (SAM; 20/10 µg) and amoxycillin-clavulanic acid (AMC; 20/10 µg)], cephalosporines [cephalothin (KF; 30 µg), cefoxitin (FOX; 30 µg), cefoperazone (CEP; 75 µg), and cefepime (FEP; 30 µg)], carbapenemes [meropenem (MEM; 10 µg)], monobactams [azetronam (ATM; 30 µg)], aminoglycosides [tobramycin (TOB; 10 µg), gentamycin (CN; 10 µg), and amikacin (AK; 30 µg)],

Table 1: Oligonucleotide primer sequences used in the study

Target gene	Primer name	Sequence of primers (5'→3')	Annealing temperatures (°C)	Amplicon (bp)	Reference
23S rRNA	23SF	TATACCGGTAAGGAGTGCTGGAG	55	650	(21)
	23SR	ATCAATTAACCTTCGAGCACCG			
*map*A	mapAF	CTATTTTATTTTTGAGTGCTTGTG	55	589	(22)
	mapAR	GCTTTATTTGCCATTTGTTTTATTA			
*ceu*E	ceuEF	ATTTGAAAATTGCTCCAACTATG	58	462	(22)
	ceuER	TGATTTTATTATTTGTAGCAGCG			
*fla*A	flaAF	TACTACAGGAGTTCAAGCTT	55	702	(28)
	flaAR	GTTGATGTAACTTGATTTTG			
cdt gene cluster	cdtF	CTTTATGACTGTTCTTCTAAATTT	42	2212	(29)
	cdtR	GTTAAAGGTGGGGTTATAATCATT			

F, forward; R, reverse; bp, base pair

macrolides [erythromycin (E; 15 µg), azithromycin (AZM; 15 µg), and clarithromycin (CLR; 15 µg)], quinolones [nalidixic acid (NA; 30 µg) and ciprofloxacin (CIP; 5 µg)], sulfonamides [sulfamethoxazole-trimethoprim (SXT; 23.75/1.25 µg)], amphenicols [chloramphenicol (C; 30 µg)], polypeptides [colistin (CT; 10 µg)], oxazolidones [lenzolid (LNZ; 30 µg)], lincosamides [clindamycin (DA; 2 µg)], and tetracyclines [doxycycline (DO; 30 µg)].

In order to identify *Campylobacter* isolates as susceptible, intermediate or resistant (25), the interpretive guidelines of CLSI (for most antimicrobials) (24) or the European Committee for Antimicrobial Susceptibility Testing (EUCAST) (for macrolides) were followed. As previously reported, multiple antibiotic resistance (MAR) indices were determined (26).The categorization of isolates as PDR, XDR, and MDR were detected as previously stated (27).

PCR-based determination of virulence characteristics and flaA-RFLP assays

Campylobacter isolates showing variable antimicrobial resistance profiles (PDR, XDR, and MDR) were exposed to DNA extraction, using the above mentioned QIAamp DNA Mini kit as recommended by the manufacturer. These isolates were examined for virulence genes, namely cytolethal distending toxins (*cdt*) and flagellin A (*flaA*), using cdt and flaA primer sets (28, 29) with nucleotide sequences listed in Table 1. For *flaA*-RFLP analysis, each *flaA* amplicon (10 µL) was digested for 1h at 37ºC in a thermoshaker (Analytik Jena, Biometra) using 1µL Fast Digest *Mbo*I restriction enzyme (ThermoFisher, Germany) prepared in 2 µL of 10X FastDigest Green buffer. Nuclease-free water was added to a 20 µL final reaction volume. The restriction fragments were separated using 1.5% agarose gel (Starlab GmbH, Hamburg, Germany) in tris boric EDTA buffer at 200 V for 1 h, stained with ethidium bromide and visualized under ultra violet light. Documentation was done using a Bio Imaging System (Syngene, Cambridge, UK).

Statistical analysis

To determine if there were significant differences between *C. jejuni* and *C. coli* in terms of antimicrobial resistance rate, we run Fihser's exact test using GraphPad Prism version 8.0 (GraphPad Software Inc., San Diego, California,USA) and *P*-values were recorded based on a cutoff level of 0.05.

Results

Occurrence of Campylobacter species in animals and humans

Campylobacter species isolation rate was 89.44%, being 79.50% for *C. jejuni* and 20.50% for *C. coli*. Animal samples showed an increased prevalence of *C. coli* (21.17%) than human samples (16.67%). However, human samples showed an elevated prevalence of *C. jejuni* (83.33%) than animal samples (78.83%).

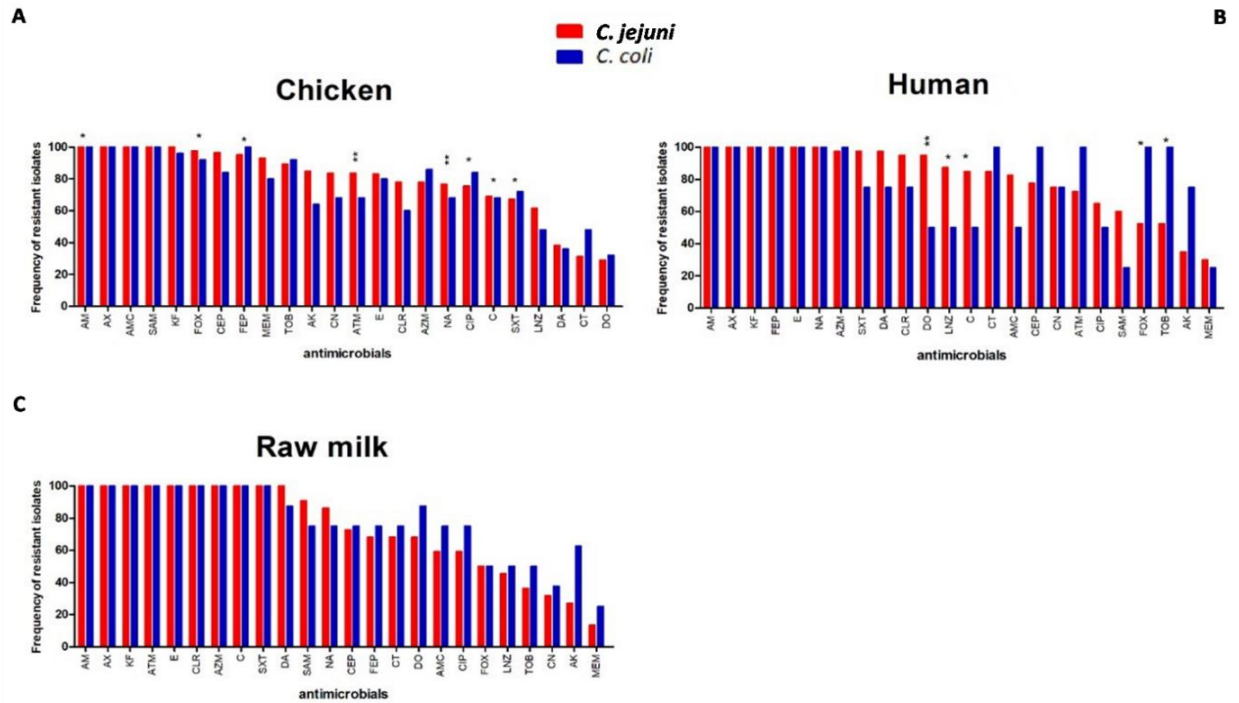

Figure 1: Frequency of antimicrobial resistance of *Campylobacter* species isolated from various sources (chickens, human and raw milk)

AM, ampicillin; AX, amoxicillin; AMC, amoxycillin-clavulanic acid; SAM, ampicillin-sulbactam; KF, cephalothin; FOX, cefoxitin; CEP, cefoperazone; FEP, cefepime; MEM, meropenem; ATM, aztreonam; TOB, tobramycin; AK, amikacin; CN, gentamicin; E, erythromycin; CLR, clarithromycin; AZM, azithromycin; NA, nalidixic acid; CIP, ciprofloxacin; C, chloramphenicol; SXT, sulfamethoxazole-trimethoprim; LNZ, lenzolid; DA, clindamycin; CT, colistin; DO, doxycycline. * Significant; ** high significant

Campylobacter isolates were integrated in 222 chicken samples (89.52%), represented as 77.48% *C. jejuni* and 22.52% *C. coli*. An equal isolation rate of *C. jejuni* was found in human stools and chicken breast muscles (83.33% each), followed by nearly equal to 72% in each of chickens' internal organs, cecal parts, and cloacal swabs. Whereas the isolation percentage of *C. coli* from chicken breast muscles was 16.67% but from internal organs, cecal parts, and cloacal swabs sources was close to 22% each. Moreover, *C. jejuni* was recovered from 44 of 52 (84.62%) raw milk, while *C. coli* was recorded by a lower percentage (15.38%). The isolated campylobacters were genotypically confirmed by PCR-based detection of the 650 bp amplicon for 23S rRNA gene then further verified as *C. jejuni* and *C. coli* by yielding 589 bp amplicons for *mapA* and 462 bp amplicons for *ceuE* genes, respectively.

Antimicrobial resistance patterns of Campylobacter species

The antimicrobial susceptibility testing of 322 *Campylobacter* isolates including 256 *C. jejuni* and 66 *C. coli* against 24 antimicrobial agents representing14 antimicrobial classes are presented in Figure 1.

The results showed that all campylobacters isolated from human and animal origins were resistant to amoxicillin, ampicillin, erythromycin, and cephalothin (100% each). Furthermore, close to 90% of *Campylobacter* isolates showed resistance to azithromycin, clarithromycin, nalidixic acid, sulfamethoxazole-trimethoprim, and clindamycin. Ciprofloxacin resistance was common in a large proportion of *C. jejuni* and *C. coli* (72.66% and 60.61%, respectively). Conversely, lower resistance percentages were reported for amikacin (29.69% and 40.91%) and cefoxitin (37.89% and 54.55%) against *C. jejuni* and *C. coli* isolates, respectively. Interestingly, alarming meropenem resistance was observed in one third population of *C. jejuni* and *C. coli* isolates (32.81% and 33.33%, respectively). Statistical analysis showed non-significant ($P > 0.05$) variations in the resistance of either *C. jejuni* or *C. coli* recovered from chickens (Figure 1A), humans (Figure 1 B), and raw milk

(Figure 1 C) to most of the examined antimicrobials.

Campylobacter isolates showed PDR, XDR, and MDR patterns. Of note, no examined isolate was pansusceptible, and 1.86% (6/322) of *Campylobacter* isolates showed resistance to all antimicrobial agents tested being a PDR pattern including cloacal swabs (n = 2) and human stools (n = 4). Extremely increased percentage of *Campylobacter* isolates (65.53%; 211/322) showed XDR profiles. However, 32.61% (105/322) of the isolates presented MDR patterns.

Regarding the isolation source, close to half population of both *Campylobacter* species isolated from raw milk were MDR. While 74.42% *C. jejuni* and 66% *C. coli* of a chicken source were XDR. Also, 65% of *C. jejuni* and 75% of *C. coli* originated from human stools exhibited XDR, and MDR patterns, respectively.

A distinct 160 antimicrobial resistance patterns with MAR indices ranged from 0.5 to 1.00 were extracted from the antibiogram analysis of *Campylobacter* isolates. The most pronounced antibiotype numbers among the 105 MDR *Campylobacter* isolates were 22, 26, and 34 (n= 4; 3.81% each). While, the most recurrent XDR profile was for antibiotype 134 that was observed in 3.32% of these strains (n = 7) and identified in *C. jejuni* isolated from chicken cloacal swabs followed by antibiotypes 127, 151, and 159 (n = 6; 2.84% each) (Table 2).

Distribution of cdt and flaA virulence genes and characterization of flaA-RFLP patterns among Campylobacter species

Thirty-eight *Campylobacter* isolates (29 *C. jejuni* and 9 *C. coli*) representing all sample origins [chickens (27), raw milk (5), and humans (6)] resistant to at least 14 antimicrobial agents and categorized as MDR (n = 6), XDR (n = 30) and PDR (n = 2) were examined for the existence of *cdt* and *flaA* virulence genes using PCR assays. As shown in Table 2, half of the examined isolates gave positive results for the *cdt* gene cluster (19/38 = 50%); those exhibiting PDR, XDR, and MDR patterns. Also, most of *cdt*-positive isolates were *C. jejuni* (78.95%) and originated from a chicken origin (73.68%).

Only five of the screened isolates had *flaA* gene (5/38 = 13.16%), including one MDR isolate from a human source, and four XDR isolates originated from chicken, and milk sources (2 for each). All *flaA*-positive isolates were *C. jejuni* and possessed the *cdt* genes.

The PCR-RFLP analysis of *flaA* gene of the five isolates revealed three different RFLP patterns of varying sizes ranged from 112 to 647bp (Figure 2). *Campylobacter* isolates exhibiting each *flaA*-RFLP pattern is presented in Table 2. *FlaA*-RFLP pattern-I corresponding to similar banding pattern of ~565 bp and ~202 bp was existed in three *C. jejuni* isolates with a relationship among the isolates of various sources (human, milk, and chicken sources) indicating the possibility of having the same origin. Further types (*flaA*-II and III) corresponding to ~647 bp and ~112 bp bands, and 361 bp and 235 bp bands, respectively were detected only in one *C. jejuni* isolate originated from chicken, and milk sources, respectively.

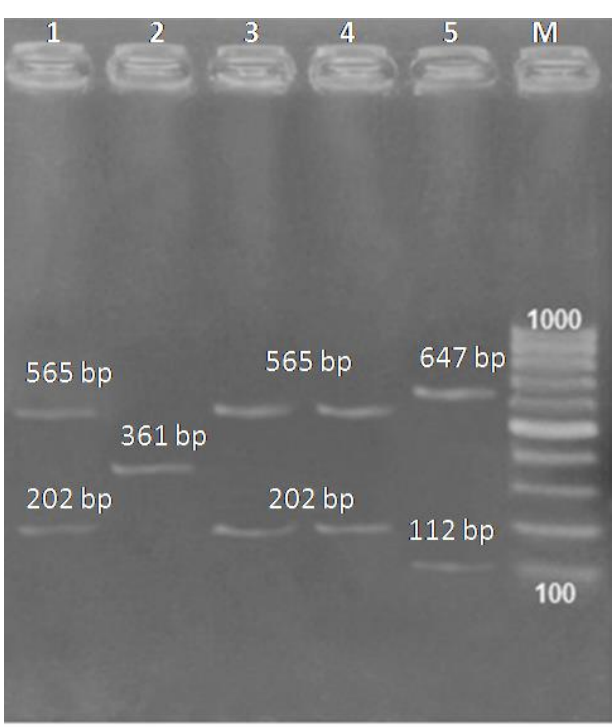

Figure 2: Agarose gel electrophoresis showing *flaA*-RFLP-PCR patterns of *C. jejuni* isolates. PCR amplicons revealed three different RFLP patterns of varying sizes

Table 2: Antimicrobial resistance patterns and multiple antibiotic resistance indices of *Campylobacter* species isolated from human and animal sources

Isolate No.	*Campylobacter* species	Source	*cdt* genes	*flaA*/ RFLP pattern	Antimicrobial resistance pattern	MAR index	Antimicrobial resistance type
1	*C. jejuni*	Chicken muscle	-	-	AX, AM, SAM, AMC,KF, FEP, CEP, TOB, CN ,E, AZM, CLR, CIP, NA, SXT, C, CT, LNZ, DA	0.79	MDR
2	*C.j ejuni*	Chicken muscle	-	-	AX, AM, AMC, KF, TOB, CN, E, AZM, CLR, CIP, NA, SXT, C, CT, LNZ, DA, DO	0.71	XDR
3	*C. jejuni*	Chicken cloacal swab	-	-	AX, AM, SAM, AMC , KF,CEP, ATM, TOB, CN , E, CLR, CIP, NA, SXT,C, CT, DA , DO	0.75	XDR
4	*C. jejuni*	Chicken organs	+	-	AX, AM, AMC, KF, FOX, FEP, ATM, TOB, E, AZM, CLR, CIP, NA, SXT, CT, LNZ, DA, DO	0.75	XDR
5	*C. coli*	Chicken muscle	-	-	AX, AM, SAM ,KF, FEP, ATM, TOB, CN, AK, E, AZM, CLR, NA, SXT, CT, LNZ, DA, DO	0.75	XDR
6	*C. coli*	Chicken muscle	+	-	AX, AM, SAM ,KF,FEP, ATM, TOB, CN, AK, E, AZM, CLR, NA, SXT, CT, LNZ, DA, DO	0.75	XDR
7	*C. jejuni*	Chicken muscle	-	-	AX, AM, SAM, AMC ,KF, FOX, ATM, CN, E, AZM, CLR, CIP, NA, SXT, C, LNZ, DA, DO	0.75	XDR
8	*C. jejuni*	Chicken muscle	+	-	AX, AM, AMC, KF, FEP, CEP, ATM, AK ,CN, E, AZM, CLR, CIP, NA SXT, C, CT, LNZ, DA	0.79	XDR
9	*C. jejuni*	Chicken muscle	+	-	AX, AM, AMC, KF, FEP, CEP, TOB, CN, E, AZM, CLR, CIP, NA, SXT, C, CT, LNZ DA, DO	0.79	XDR
10	*C. jejuni*	Chicken cloacal swab	+	-	AX, AM, SAM, AMC, KF, CEP, CN, AK, E, AZM, CLR, CIP, NA, SXT, C, CT, LNZ, DA, DO	0.79	XDR
11	*C. jejuni*	Chicken muscle	-	-	AX, AM, SAM, AMC, KF, FOX, FEP, ATM, TOB, CN, AK, E, AZM, CLR, CIP, NA, SXT, C, LNZ DA, DO	0.88	XDR
12	*C. coli*	Chicken caecal part	+	-	AX, AM, SAM, AMC, KF, FOX, FEP, CEP, IMP, ATM, TOB, CN, E, AZM, CLR, CIP, NA, SXT, CT, DA, DO	0.88	XDR
13	*C. jejuni*	Chicken cloacal swab	+	-	AX, AM, AMC, KF, CEP, FEP, IMP, ATM, TOB, CN, E, CLR, CIP, NA, SXT, C, CT, DA ,DO	0.79	XDR
14	*C. coli*	Chicken cloacal swab	+	-	AX, AM, SAM, AMC, KF, FOX, FEP, ATM, TOB, CN, E, CLR, NA, SXT, C, CT, LNZ, DA, DO	0.79	XDR
15	*C. jejuni*	Chicken cloacal swab	+	-	AX, AM SAM,AMC, KF CEP, FEP, IMP, ATM, CN, AK, E, AZM, CLR, NA, SXT, C, LNZ DA, DO	0.83	XDR
16	*C. coli*	Chicken cloacal swab	-	-	AX, AM, AMC, KF, CEP, FEP, ATM, CN, AK, E, AZM, CLR, CIP, NA, SXT, C, CT, LNZ, DA, DO	0.83	XDR
17	*C. jejuni*	Chicken muscle	-	-	AX, AM, AMC, SAM, KF, FEP, CEP, ATM ,CN , E, AZM, CLR, CIP, NA, SXT,C, CT, LNZ, DA, DO	0.83	XDR
18	*C. jejuni*	Chicken muscle	+	-	AX, AM, SAM, AMC, KF, FEP, ATM, TOB, CN, E, AZM, CLR, CIP, NA, SXT, C, CT, LNZ, DA, DO	0.83	XDR
19	*C. jejuni*	Chicken organ	+	-	AX, AM, SAM, AMC, KF, FEP, CEP, ATM, TOB, AK, E, AZM, CLR, CIP, NA, SXT, C, CT, LNZ, DA, DO	0.88	XDR
20	*C. coli*	Chicken cloacal swab	-	-	AX, AM, SAM, AMC, KF, FEP, IMP, ATM, TOB, CN, AK, E, AZM, CLR, CIP, NA, SXT, C, CT, LNZ, DA	0.88	XDR
21	*C. coli*	Chicken organ	-	-	AX, AM, SAM, AMC, KF, FOX, CEP, FEP, IMP, ATM, TOB, CN, AK , E, AZM, CLR, NA, SXT, C, CT, DA, DO	0.92	XDR
22	*C. jejuni*	Chicken cloacal swab	+	+/647, 112	AX, AM, AMC, KF, CEP, FEP, IMP, ATM, CN , E, AZM, CLR, NA, SXT, C, CT, LNZ, DA, DO	0.79	XDR
23	*C. coli*	Chicken caecal part	-	-	AX, AM, AMC, KF, FOX, CEP, IMP, ATM, TOB, CN, E, AZM, CLR, CIP, NA, SXT, C, CT, LNZ, DA, DO	0.88	XDR
24	*C. jejuni*	Chicken organ	+	+/565, 202	AX, AM, SAM, AMC, KF, FEP, IMP,ATM, TOB,CN, E, AZM, CLR, CIP, NA, SXT, C, CT, LNZ, DA, DO	0.88	XDR
25	*C. jejuni*	Chicken caecal part	-	-	AX, AM, AMC, KF,CEP, FEP, IMP, ATM, TOB, CN, AK, E, AZM, CLR, CIP, NA, SXT, C, CT, LNZ, DA, DO	0.92	XDR
26	*C. jejuni*	Chicken muscle	-	-	AX, AM, SAM ,AMC, KF, FOX, FEP, CEP, IMP, ATM, TOB, E, AZM, CLR, CIP, NA , SXT, C, CT, LNZ ,DA, DO	0.92	XDR
27	*C. jejuni*	Chicken cloacal swa	+-	-	AX, AM, SAM,AMC, KF, FOX, CEP, FEP, IMP, ATM, TOB, CN, AK , E, AZM, CLR, CIP, NA SXT, C, CT,LNZ, DA, DO	1	PDR
28	*C. jejuni*	Raw milk	+	+/565, 202	AX, AM, SAM, KF, FOX, CEP, FEP, ATM, E, AZM, CLR, NA, SXT, C, CT, LNZ, DA, DO	0.75	XDR
29	*C. jejuni*	Raw milk	+	+/361, 235	AX, AM, SAM, AMC, KF, CEP, FEP, ATM, TOB, E, AZM, CLR, NA, SXT, C, CT, DA, DO	0.75	XDR
30	*C. jejuni*	Raw milk	-	-	AX, AM, SAM, AMC, KF, FOX, CEP, FEP, ATM, E, AZM, CLR, NA, SXT, C, CT, LNZ, DA, DO	0.79	XDR
31	*C. jejuni*	Raw milk	+	-	AX, AM, SAM, AMC, CEP, KF, ATM, FOX, AK, E, AZM, CLR, NA, SXT, C, CT, LNZ, DA, DO	0.79	XDR
32	*C. jejuni*	Raw milk	-	-	AX, AM, SAM, AMC, KF, FOX, CEP, FEP, IMP, ATM, TOB, CN, E, AZM, CLR, CIP, NA, SXT, C, CT, LNZ, DA	0.92	XDR
33	*C. jejuni*	Human stool	-	-	AX, AM, AMC, KF, FEP, CN, E, AZM, CLR, CIP, NA, SXT, C, CT	0.58	MDR
34	*C. coli*	Human stool	+	-	AX, AM, KF, FOX, CEP, FEP, ATM, TOB, AK, E, AZM, NA, SXT, CT, DA	0.63	MDR
35	*C. jejuni*	Human stool	-	-	AX, AM, AMC, KF, FEP, CN, E, AZM, CLR, CIP, NA, SXT, C, CT, LNZ, DA, DO	0.71	MDR
36	*C. jejuni*	Human stool	+	+/565, 202	AX, AM, KF, FOX, CEP, FEP, ATM, TOB, CN, E, AZM, CLR, NA, CT, LNZ, DA, DO	0.71	MDR
37	*C. jejuni*	Human stool	-	-	AX, AM, SAM, KF,FOX, CEP, FEP, ATM, TOB, E, AZM, CLR, NA, SXT, C, LNZ, DA, DO	0.75	XDR
38	*C. jejuni*	Human stool	-	-	AX, AM, SAM, AMC, KF, FOX, CEP, FEP, IMP, ATM, TOB, CN, AK, E, AZM, CLR, CIP, NA, SXT ,C, CT,LNZ, DA, DO	1	PDR

MDR, multiple drug-resistance; XDR, extensively drug-resistance; PDR, pan drug-resistance; AMA, antimicrobial agent; AM, ampicillin; AX, amoxicillin; AMC, amoxycillin-clavulanic acid; SAM, ampicillin-sulbactam; KF, cephalothin; FOX, cefoxitin; CEP, cefoperazone; FEP, cefepime; MEM, meropenem; ATM, aztreonam; TOB, tobramycin; AK, amikacin; CN, gentamicin; E, erythromycin; CLR, clarithromycin; AZM, azithromycin; NA, nalidixic acid; CIP, ciprofloxacin; C, chloramphenicol; SXT, sulfamethoxazole-trimethoprim; LNZ, lenzolid; DA, clindamycin; CT, colistin; DO, doxycycline

Discussion

Currently, an emerging problem among campylobacters is the elevating resistance to major antibiotics in use, particularly macrolides and fluoroquinolones (30, 31). *Campylobacter* species transmission to humans is owing to consumption of improperly cooked poultry products and raw unpasteurized milk (32, 33). Several studies reported the *cdt* and *flaA* virulence determinants in *Campylobacter* species (15-18). However, no reports investigated their existence in different antimicrobial resistant patterns (PDR, XDR, and MDR) among *Campylobacter* species recovered from chicken, milk, and human origins in Egypt.

Previous studies showed the association of *C. jejuni* only with poultry and human samples without detection of *C. coli* (16). Conversely, we detected both *Campylobacter* species in humans and animals with a higher occurrence rate of *C. jejuni* (79.50%) than that of *C. coli* (20.50%). Overall, most isolated campylobacters were recovered from chicken samples (89.52%), while previous studies reported varying rates of *Campylobacter* prevalence in chickens ranging from 24 to 62% (34, 35).

Unneglectable percentage of campylobacteriosis in human cases (23%) in Egypt is due to consumption of unpasteurized milk; also, cross-contamination with campylobacters could occur during milking of cattle (36). Previously, 82.86 % of *Campylobacter* isolates were reported in raw milk (37); thus, raw milk was considered as a second main source of *Campylobacter* infections (38). We reported *C. jejuni* prevalence rate of 84.62% in raw milk samples, which have been previously documented with a nearly equal prevalence rate (74.28%) (37), while a lower prevalence rate (34%) was detected previously (39).

The shortage of cleanliness in food processing can cause *Campylobacter* transmission to humans dealing with food-producing animals especially poultry and milk, the main reservoirs for *Campylobacter* species. Alarming elevated percentage of *C. jejuni* in human stools (83.33%) was found in this study that have been previously documented with a high prevalence rate of 66.67% (37). Conversely, lower prevalence of 27.5%, 16.66%, and 4.07% were reported in previously published researches in Egypt (34), (40), and (41), respectively.

The haphazard use of antimicrobials in veterinary and human medicine resulting in drug resistance (42). Here, all *Campylobacter* isolates from animal and human sources were resistant to erythromycin. Also, ciprofloxacin resistance was common in a large portion of isolates from animal and human sources. Furthermore, alarming meropenem resistance in one third population of *C. jejuni* and *C. coli* was found. This indicating an emerging situation especially as erythromycin, and ciprofloxacin are the mostly used for *Campylobacter* infections treatment in humans, and the meropenem is the last-resort for treating campylobacteriosis (43).

Counteract the extend of antimicrobial resistant campylobacters in humans and livestock animals is essential by continuing detection of the resistance rates (30). Herein, we detected 160 different drug resistance patterns while testing 24 antimicrobials among 14 antimicrobial categories (Table 2). Interestingly, all isolated campylobacters were classified into MDR, XDR, and PDR without detection of any isolate as a pansusceptible.

A disturbing circumstance has been considered worldwide due to the increase of MDR *Campylobacter* strains (44). Here, 32.61% of *Campylobacter* isolates recovered from humans and animals exhibited a MDR pattern, that is closely similar to previously reported studies (37, 45).

Furthermore, the most worrying issue is the beginning of recording resistance to all classes of antimicrobial agents except two or fewer (XDR), and resistance to all antimicrobial agents among all categories (PDR) as previously documented (37, 46). In this research, we detected a high percentage of XDR (65.53%) and alarming (1.86%) PDR *Campylobacter* isolates.

Remarkably, cdt and flagellin are important virulence factors in *Campylobacter* infection. Till date, several studies showed the relation between antimicrobial resistance and the *cdt* and *flaA* detection in MDR *Campylobacter* isolates (15, 16, 18). However, no reports detect the *cdt* and *flaA* in PDR or XDR *Campylobacter* isolates. Herein, we

reported that a half of analyzed *Campylobacter* isolates displayed MDR, XDR, and PDR patterns were *cdt*-positive. Conversely, previous data showing that the *cdt* gene cluster was highly abundant (90%–100%) if the isolate was of human or animal origin (47 - 50). Interestingly, the *cdt* gene cluster was discovered here for the first time in *C. coli*, while most of *cdt*-positive isolates were *C. jejuni* (78.95%). Previously, no *cdt* genes were reported in *C. coli* isolates (17).

Several studies reported *flaA* gene in 100 % of *Campylobacter* isolates (16, 17). Our study presented that only 13.16% of *Campylobacter* isolates had the *flaA* gene, all were MDR or XDR *C. jejuni cdt*-positive. While no *flaA* gene was found in *C. coli* or PDR isolates.

The PCR-RFLP analysis of *flaA* gene using *Mbo*I restriction endonuclease, revealed three different RFLP patterns indicating extensive genetic variability of *C. jejuni* isolates. *FlaA*-RFLP pattern-I (~565 bp and ~202 bp) was presented in three *C. jejuni* isolates with a relationship among the isolates of various origins (human, milk, and chicken), which is in accordance with a previous report (51). While, further types (*fLaA*-II, III) corresponding to ~647 bp and ~112 bp bands, and 361 bp, and 235 bp bands, respectively were detected only in *C. jejuni* isolates from chicken, and milk sources, respectively as reported in a previous study (52).

Conclusion

This report showed an alarming existence of PDR and XDR *Campylobacter* species in animals and humans. Moreover, it represented the relation between antimicrobial resistance and the harboring of *cdt* and *flaA* genes in PDR or XDR *Campylobacter* isolates that has not been reported previously rather than the MDR isolates. Furthermore, extensive genetic variability of *C. jejuni* was noticed among XDR and PDR isolates, while using *flaA*-RFLP assay.

Authors declare no conflict of interest.

E.Y.E and N.K.A. contributed equally to the conception and design of the study. A.H. carried out the classical microbiological techniques and molecular genetic studies. N.K.A. and A.H.S. participated in the analysis and interpretation of data and wrote the initial draft of the manuscript; A.A.H. participated in the design of the study and in the analysis and interpretation of data. All authors revised the manuscript critically for important intellectual content and gave the final approval of the version to be published.

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