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PREVALENCE AND ANTIBIOTIC RESISTANC PATTERNS OF *CAMPYLOBACTER* SPECIES ISOLATED FROM DIFFERENT SOURCES IN EYGPT

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ABSTRACT

Campylobacter food poisoning is understated in developing countries. To determine the occurrence and antimicrobial resistance of this pathogen in Zagazig City, Sharkia Governorate, Egypt, a total of 286 samples from chicken (195), cattle (47) and human (44) were collected. Bacteriological examination of the collected samples revealed high prevalence rates of *Campylobacter* species from human stool, chicken and raw milk samples (90.91%, 86.15% and 82.98%, respectively). *C. jejuni* was recognized as the most frequently recovered species (63.29%). There were no significant effects on the prevalence of *Campylobacter* spp. based on the sample type (p = 0.54). Thirty eight campylobacter isolates were confirmed molecularly using PCR amplification of 23S rRNA gene. Furthermore, PCR targeting *mapA* and *ceuE* genes were then applied for the confirmation of *C. jejuni* and *C. coli* isolates, respectively. The antibiotic resistance results showed that all isolates (100%) were resistant to ampicillin, amoxicillin and erythromycin. On the other hand, the lowest resistance rates were detected against amikacin, impenem and cefoxitin (28.74%, 32.39% and 47.77, respectively). In total, 207 (83.81%) campylobacter isolates were MDR, while 38 isolates (15.38%) and 2 chicken *C. jejuni* isolates (0.81%) were XDR and PDR, respectively. One hundred and forty five antimicrobial resistance profiles were generated with an MAR index of 0.45 or greater. Our results suggested that the presence of MDR campylobacters in chicken and raw milk, specifically to erythromycin and/ or ciprofloxacin aggravates the human health alarm and emphasizes the necessity to educate the consumers about the safeness and the goodness of such foods.

Keywords: *Campylobacter* spp., Prevalence, Multiple drug resistance, *mapA*, *ceuE*

INTRODUCTION

Thermophilic *Campylobacter* species, mainly *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli*), have been considered as major causes of acute bacterial gastroenteritis globally. They are microaerophilic microorganisms growing best in an atmosphere containing approximately 10% CO_2 and 5% O_2 at 41.5 °C (**Humphrey *et al.*, 2007**). Majority of human illness are attributed to *C. jejuni* (80-85%), while the residual cases are credited with *C. coli* (**Narvaez-Bravo *et al.*, 2017**).

Campylobacter species colonize the intestine of many farm animals (**Silva *et al.*, 2011**). The predominant reservoir of these pathogens is chicken and it carries them with no clinical signs (**Sahin *et al.*, 2002**). This bacterium is transmitted to humans via contaminated undercooked foods of animal origin, especially undercooked chicken meat and unpasteurized dairy products (**Gharbi *et al.*, 2018**).

With a low infective dose, *Campylobacter* species cause a gastrointestinal infection known as campylobacteriosis. Campylobacteriosis is an important zoonotic illness with a universal distribution (**Thomrongsuwannakij *et al.*, 2017**). In the most recent decades, there was an increase in the number of human infection cases in developing and industrialized countries with 96 million cases of acute gastroenteritis and 21 thousand deaths yearly worldwide (**WHO, 2015; Abulreesh *et al.*, 2017**). Actually, diarrheal disease has a great importance particularly in developing economies, where children < 2 years may die when infected with these microorganisms (**Saeed *et al.*, 2015**). Campylobacteriosis is characterized by inflammatory diarrhea (**Humphrey *et al.*, 2007**). In some cases, more severe complications like arthritis, septicemia and Guillain-Barré syndrome (GBS) can also occur when infection caused by either *C. jejuni* or *C. coli* (**Garcia-Sanchez *et al.*, 2018**).

Human campylobacteriosis is usually self-limiting. However, immunocompromised patients and young age with severe infections may need antimicrobial medications (**Skarp *et al.*, 2016**). Erythromycin is generally the first drug of choice, but fluoroquinolones and on a smaller scale tetracycline constitutes other options (**Szczepanska *et al.*, 2017**).

Campylobacters become more resistant to antimicrobials and many strains developed the multi-drug resistant (MDR) pattern to multiple medicaments (**Bouhamed *et al.*, 2018**). Multi-drug resistant campylobacter, particularly against quinolones and erythromycin, has increased globally and this has triggered worldwide alarms, as they are the main antibiotics used for the therapy of campylobacteriosis (**Zhou *et al.*, 2016**). Contaminated foods with MDR campylobacter strains harbor a significant hazard to the public health (**Szczepanska *et al.*, 2017**).

As the incidence of the campylobacter infections has increased, there is an urgent need to take measures to identify the source of the incriminated bacterium (**Eberle and Kiess, 2012**). In Egypt, several authors reported relatively high prevalence rate of *Campylobacter* spp. in human; 27.5% (**Abushahba *et al.*, 2018**), 9.37% (**Sainato *et al.*, 2018**) and 6% (**Ghoneim *et al.*, 2020**). At species level; *C. jejuni* and *C. coli* were previously recorded with prevalence rates 12.3% and 2.8%, respectively (**El-Tras *et al.*, 2015**). While, thirteen *C. jejuni* isolates (17.33%) were distinguished from 75 diarrheic persons (**GHONEIM *et al.*, 2020**).

To the best of our knowledge, the previously conducted studies in Zagazig (**Awadallah *et al.*, 2014; Abd El Tawab *et al.*, 2018; El-Hamid *et al.*, 2019**) stated limited data about the contribution of chicken and raw milk as potential sources of MDR *C. jejuni* and *C. coli* infections in humans. Therefore, the aim of the present investigation was to detect *Campylobacter* species, particularly *C. jejuni* and *C. coli* in chicken and raw milk as well as human samples using conventional and molecular tools in addition to identifying the antimicrobial resistance profiles of the recovered isolates against 10 different antimicrobial classes.

MATERIALS AND METHODS

Sampling

The study was conducted from March 2017 to September 2019. A total of 286 samples were collected from various sources; chickens (n = 195), cattle (n = 47) and human (n = 44) in Zagazig City, Sharkia Governorate, Egypt. The samples collected from broiler chickens included cloacal swabs (n = 75), breast meat (n = 40), caecal parts (n = 40) and liver (n = 40) were collected from retail outlets, while raw milk samples were collected from retail shops. Moreover, human stool samples were taken from gastroenteritis patients from clinical laboratories. Samples were collected in a sterile Bolton enrichment broth (Oxoid, UK) and transported in an ice box within 3 h to the laboratory for bacteriological analysis. The animal study was endorsed by the committee of Animal Welfare and Research Ethics, Faculty of Veterinary Medicine, Zagazig University. Regarding the human samples, it was approved by the research ethical committee of Faculty of Medicine, Zagazig University and the work was conducted in compliance with the Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans. Written informed permissions were taken from patients taking part in the research study after a complete explanation of the aim of the study.

Isolation and identification of thermophilic *Campylobacter* species

For isolation of *Campylobacter* species, the collected samples in Bolton enrichment broth were incubated at 41.5º C for 24 h in the culture vessel with headspace less than 1 cm and firmly capped lids. After enrichment, a loopful of the broth was streaked onto modified Cefoperazone Charcoal Deoxycholate agar, mCCDA (Oxoid, UK) prepared from Campylocater Blood-Free Selective Agar Base CM0739 and CCDA Selective Supplement SR155 (Oxoid, UK). The plate's incubation was done at 41.5ºC in darkness for 48 h under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2) using CampyGen sachets (Oxoid, UK) **(ISO, 2006)**. The presumptive identification of isolates as *C. jejuni* and *C. coli* was done by biochemical tests including catalase, oxidase, indoxyl acetate and hippurate hydrolysis and susceptibility to cephalothin and nalidixic acid (30 µg /disc, each) **(Quinn *et al.*, 1994).**

Molecular confirmation of *Campylobacter* species

A conventional PCR was used for the confirmation of biochemically identified campylobacter isolates. Bacterial DNA was extracted from fresh cultures using QIAamp DNA mini kit (Qiagen, USA) in accordance with the manufacturer's instructions. Oligonucleotide primers (Metabion, Germany) that specifically amplify target *Campylobacter* spp. genes were used for molecular identification (**Table 1**). The PCR was performed in a total reaction volume of 25µL using *Taq* DNA Polymerase Kit (Invitrogen, USA) in accordance with the manufacturer's instructions. The used concentration of each forward and reverse primers was 20 pmoL. The thermal profile comprised of an initial denaturation step at 94˚C for 5 min, followed by 35 cycles of denaturation at 94˚C for 30 sec, annealing at temperatures specific for each gene (**Table 1**) for 45 sec and extension at 72˚C for 45 sec and finally an extension step at 72˚C for 10 min. Reference strains of *C. jejuni* (NCTC11322) and *C. coli* (NCTC11366) were considered positive controls (the reference strains were kindly obtained from Biotechnology unit, Reference laboratory for Veterinary Quality Control on Poultry production, Animal Health Research Institute, Dokki, Giza, Egypt). PCR-grade water without template was served as a negative control.

Table 1 Oligonucleotide primers used for PCR amplification of *Campylobacter* specicc genes with their respective annealing temperatures

Specificity	Target gene	Oligonucleotide primer sequence (5'-3')	product (bp)	Annealing temperature	Reference
Genus *Campylobacter*	23S rRNA	F' TATACCGGTAAGGAGTGCTGGA R' ATCAATTAACCTTCGAGCACCG	650	55 ºC	**Wang *et al.*, 2002**
Campylobacter coli	*ceuE*	F 'AAT TGA AAA TTG CTC CAA CTA TG R' TGA TTT TAT TAT TTG TAG CAG	462	58 ºC	**Shin and Lee, 2009**
Campylobacter jejuni	*mapA*	F' CTA TTT TAT TTT TGA GTG CTT GT R' GCT TTA TTT GCC ATT TGT TTT ATT	589	55 ºC	

Antimicrobial susceptibility testing

Susceptibility of all campylobacter isolates to 22 antimicrobials representing ten different classes was tested using the following antimicrobial discs: β-lactams [ampicillin, AM (10 µg); amoxicillin, AX (25 µg); sulbactam-ampicillin, SAM (20 µg); amoxicillin-clavulanic acid, AMC (30 µg); cephalothin, KF (30 µg); cefoxitin, FOX (10 µg); cefepime, FEP (30 µg); impenem, IMP (10 µg) and azetronam, ATM (30 µg)], aminoglycosides [streptomycin, S (10 µg); gentamicin, CN (10 µg) and amikacin, AK (30 µg)], macrolides [erythromycin, E (15 µg) and azithromycin, AZM (15 µg)], quinolones [ciprofloxacin, CIP (5 µg) and nalidixic acid, NA (30 µg)], sulphonamides [trimethoprim-sulfamethaxole, SXT (25 µg)], phenicols [chloramphenicol, C (30 µg)], polypeptides [colstin, CT (10 µg)], oxazolidones [linezolid, LNZ (30 µg)], lincosamides [clindamycin, DA (2 µg)] and tetracyclines [doxycycline DO (30 µg)] (Oxoid, UK). This test was conducted following the Kirby-Bauer disk diffusion method (**Bauer *et al.*, 1966**). Each overnight culture of *Campylobacter* spp. was suspended in sterile normal saline and adjusted to a turbidity of a 0.5 McFarland standard. Each suspension was inoculated with a sterile swab on the entire surface of Mueller Hinton agar plates (Oxoid, UK) supplemented with 5% sheep blood. After drying the plates, the antimicrobial discs were aseptically placed on the plates. After incubation at 41.5ºC for 48 h under the microaerophilic condition, the inhibition zones were measured and interpreted on the Clinical Laboratory Standards Institute (CLSI) guidelines (**CLSI, 2016**) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (**EUCAST, 2017**). In the cases when CLSI recommendations were not available for campylobacters, the *Enterobacteriaceae* CLSI guidelines were tracked (**CLSI, 2013**). The MDR was identified as acquired resistance of a microorganism to at least one antibiotic in three or more antimicrobial categories, while extensively drug resistant (XDR) was identified as resistance of single bacterium to all antibiotics except two or fewer antimicrobial categories and pan drug resistant (PDR) was identified as resistance of a microorganism to all antibiotics in all antimicrobial categories (**Magiorakos *et al.*, 2012**).

Multiple antimicrobial resistance indexing

The multiple antimicrobial resistances (MAR) indexing was used to quantify the multi-resistance of campylobacter isolates, as following:

MAR index = a/b

Where, “a” represents the number of antimicrobials to which the microorganism was resistant and “b” represents the total number of antimicrobials to which the microorganism was tested (**Krumperman, 1983**).

Statistical analysis

The results were analyzed using the SPSS v18.0 software (SPSS Inc., Chicago, IL, USA). The occurrence of *Campylobacter* spp. in different sources and variations in antimicrobial susceptibility between *C. jejuni* and *C. coli* were evaluated using chi square test. Fisher’s exact test was applied for smaller number of samples. To visualize the clustering pattern among campylobacter isolates from various hosts, non-metric multidimensional scaling was generated based on sorensen distance using the PC-ORD software.

RESULTS

Prevalence of thermophilic *Campylobacter* spp. in the examined samples

According to the phenotypic identification, *Campylobacter* spp. were isolated from 247 out of 286 of the examined samples (86.36%); 90.91% from human stool, 86.15% from chicken samples and 82.98% from raw milk (**Table 2**). Of note, each investigated sample contained only one identified campylobacter isolate. The results demonstrated a high isolation rate of chicken *Campylobacter* spp. from cloacal swabs (93.33%), followed by caecal parts (90%), chicken liver (80%) and breast meat (75%). Identification of campylobacters to the species level showed that *C. jejuni* was found to be the predominant species with an overall prevalence rate of 63.29%, while that of *C. coli* was 23.08%. The highest isolation rate of *C. jejuni* was detected in human stool (72.73%), followed by raw milk (70.21%). Meanwhile, the highest isolation rate of *C. coli* was observed in chicken (26.67%) (**Table 2**). Statistically, there were no significant effects on the prevalence of *Campylobacter* spp. based on the sample type ($p = 0.54$) and on the prevalence of both *C. jejuni* and *C. coli* from different sample types ($p = 0.08$).

Table 2 Prevalence of thermotolerant *Campylobacter* spp. in the collected samples

Sample type (No.)	Total No. of *Campylobacter* spp. (%)*	No. of *Campylobacter* spp. (%)*	
		C. jejuni	*C. coli*
Chicken samples (195)	168 (86.15)	116 (59.49)	52 (26.67)
Clocal swabs (75)	70 (93.33)	50 (66.67)	20 (26.67)
Breast meat (40)	30 (75)	22 (55)	8 (20)
Cecal parts (40)	36 (90)	24 (60)	12 (30)
Chicken liver (40)	32 (80)	20 (50)	12 (30)
Raw milk (47)	39 (82.98)	33 (70.21)	6 (12.77)
Human stool (44)	40 (90.91)	32 (72.73)	8 (18.18)
Total (286)	247 (86.36)	181 (63.29)	66 (23.08)

*The isolation rates were calculated in relation to the total number of the examined samples

Molecular confirmation of campylobacter isolates

Thirty eight campylobacter isolates (29 and 9 biochemically suspected *C. jejuni* and *C. coli* isolates, respectively) were confirmed by PCR amplification of 23S rRNA gene. All the isolates generated an amplicon of 650 bp size (**figure 1**). The results showed that all 29 *C. jejuni* isolates from chicken (n = 19), cattle (n = 5) and human (n = 5) and 9 *C. coli* isolates from chicken (n = 7), cattle (n = 1) and human (n = 1) those were identified phenotypically were confirmed by PCR ampilifications of *mapA* and *ceuE* genes with amplicons' sizes of 589 bp (**figure 2A**) and 462 bp (**figure 2B**), respectively.

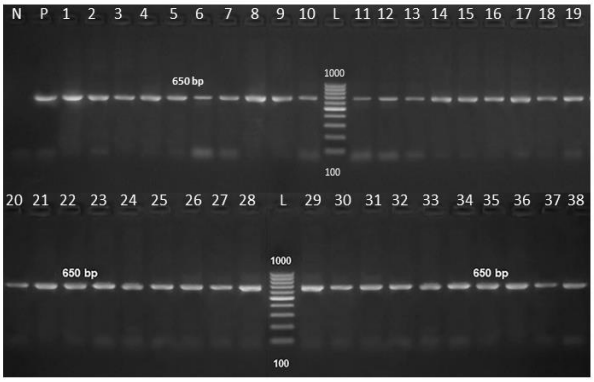

Figure 1 PCR amplification products of 23S rRNA gene of *Campylobacter* spp. (650 bp). Lane L: 100 bp DNA ladder "Marker", lanes 1-38: positive campylobacter isolates from poultry (lanes 1-26), cattle (lanes 27-32) and human (lanes 33-38) origins, lane P: positive control, lane N: negative control.

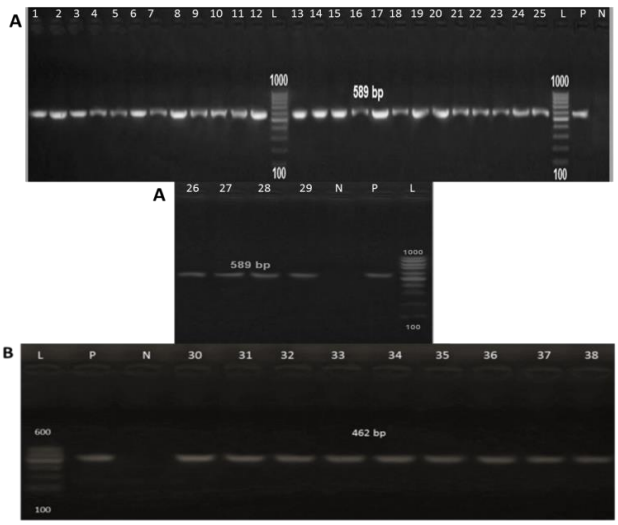

Figure 2 PCR amplification products of *mapA* gene specific for *C. jejuni* (589 bp) (A) and *ceuE* gene specific for *C. coli* (462 bp) (B). Lanes 1-19: *C. jejuni* from poultry, lanes 20-24: *C. jejuni* from cattle, lanes 25-29: *C. jejuni* from human, lanes 30-36: *C. coli* from poultry, lane 37: *C. coli* from cattle, lane 38: *C. coli* from human, lane L: 100 bp DNA ladder "Marker", lane P: positive control, lane N: negative control.

Antimicrobial susceptibility profile of the recovered *Campylobacter* spp. from different sources

Analysis of the antimicrobial resistance of 247 campylobacter isolates against the 22 tested antimicrobial agents demonstrated that all isolates (100%) were resistant to ampicillin, amoxicillin and erythromycin. Moreover, high resistance rates were observed against trimethoprim-sulfamethaxole (98.79%), followed by clindamycin (97.17%), cephalothin (96.76%), azithromycin (91.09%) and nalidixic acid (90.28%). Additionally, majority of campylobacter isolates were resistant to azetronam (81.78%), doxycycline (81.38%), amoxicillin-clavulanic acid (80.97%), cefepime (80.57%), chloramphenicol (80.16%), colistin (75.71%) and linezolid (73.28%). On the other hand, our results showed that amikacin, impenem and cefoxitin had the lowest resistance rates against the tested isolates (28.74%, 32.39% and 47.77%, respectively) (**Table 3**).

Based on *Campylobacter* species and regardless of the type of the collected samples, our results presented higher resistance rates in *C. jejuni* isolates than *C. coli* for the investigated antibiotics except for cephalothin, cefoxitin, impenem, streptomycin, gentamycin, ciprofloxacin, linezolid, colistin and doxycycline (**Figure 3**). Statistically, there was a significant effect ($P < 0.05$) on the resistance prevalence between *C. jejuni* and *C. coli* isolates against amoxicillin-clavulanic acid (84.53% and 71.21%), cefepime (83.98% and 71.21%), azithromycin (93.37% and 84.85%), chloramphenicol (83.43% and 71.21%) and colistin (72.38% and 84.85%), respectively. The resistance prevalence in *C. jejuni* and *C. coli* isolates was significantly higher for 3 of the tested antimicrobials; azetronam (86.19% and 69.69%) and streptomycin (58.01% and 77.27%), respectively ($P < 0.01$) and clindamycin (100% and 89.39%), respectively ($P < 0.001$). Meanwhile, there were no significant differences ($P > 0.05$) in the resistance of *C. jejuni* and *C. coli* isolates against the other antimicrobials; sulbactam-ampicillin, cephalothin, cefoxitin, impenem, gentamycin, amikacin, ciprofloxacin, nalidixic acid, trimethoprim-sulfamethaxole, linezolid and doxycycline (P values = 0.192, 0.911, 0.48, 0.27, 0.81, 0.99, 0.54, 0.78, .795, 0.59 and 0.22, respectively) (**Supplementary table 1**).

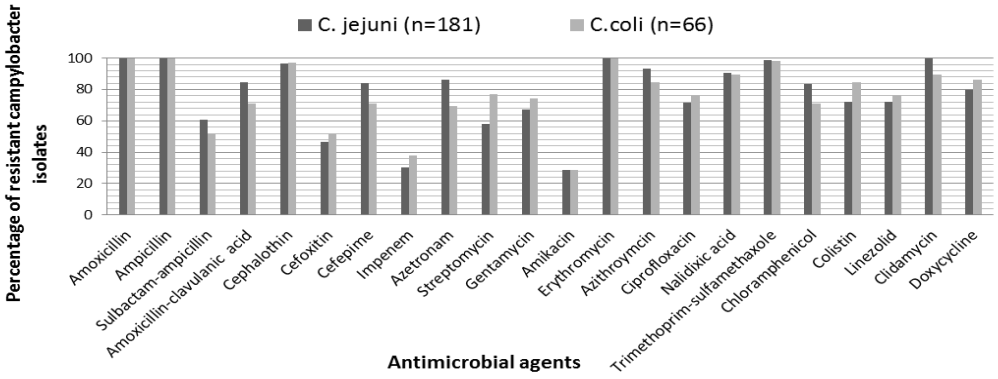

Figure 3 Prevalence of *Campylobacter* spp. resistance against 22 antimicrobial agents

According to the isolates' origins, the resistance levels of campylobacter isolates recovered from chicken, cattle and human samples were different (**Figure 4**). Statistically, there was a significant effect ($P < 0.05$) on the resistance rates between chicken, cattle and human isolates against impenem (36.9%, 15.38% and 30%) and nalidixic acid (94.05%, 84.62% and 80%), respectively. The difference in rates among chicken, cattle and human isolates were significantly higher for 11 of the tested antimicrobials; cefepime (78.57%, 69.23% and 100%), streptomycin (69.05%, 38.46% and 62.5%), trimethoprim-sulfamethaxole (100%, 100% and 92.5%) and clindamycin (98.81%, 100% and 87.5%), respectively ($P < 0.01$) and sulbactam-ampicillin (52.38%, 92.31% and 50%), amoxicillin-clavulanic acid (88.09%, 61.54% and 70%), azetronam (81.55%, 100% and 65%), gentamycin (76.19%, 35.89% and 72.5%), azithromycin (86.9%, 100% and 100%), chloramphenicol (75%, 100% and 82.5%) and linezolid (77.98%, 46.15% and 80%), respectively ($P < 0.001$). However, there is no significant effect ($P > 0.05$) on the resistance prevalence between campylobacter isolates from the three sources against the other antibiotics; cephalothin, cefoxitin, amikacin, ciprofloxacin, colistin and doxycycline ($P = 0.12, 0.24, 0.9, 0.22, 0.31$ and, 0.05, respectively) (**Supplementary table 2**). The clustering pattern of *Campylobacter* isolates were classified by their species and host. The distance among isolates is of the Sorensen type (**Figure 5**).

Table 3 Frequency of resistance of thermotolerant *Campylobacter* spp. recovered from different sources

Antimicrobial group	Antimicrobial	No. of resistant *Campylobacter* spp. from different sources (%)						Total (247)
		Chicken (168)		Cattle (39)		Human (40)		
		C. jejuni (n=116)	*C. coli* (n=52)	*C. jejuni* (n=33)	*C. coli* (n=6)	*C. jejuni* (n=32)	*C. coli* (n=8)	
Beta- lactams	Amoxicillin	116 (100)	52 (100)	33 (100)	6 (100)	32 (100)	8 (100)	247 (100)
	Ampicillin	116 (100)	52 (100)	33 (100)	6 (100)	32 (100)	8 (100)	247 (100)
	Sulbactam-ampicillin	64 (55.17)	24 (46.15)	31 (93.94)	5 (83.33)	15 (46.88)	5 (62.5)	144 (58.29)
	Amoxicillin-clavulanic acid	112 (96.55)	36 (69.23)	20 (60.61)	4 (66.67)	21 (65.63)	7 (87.5)	200 (80.97)
	Cephalothin	111 (95.69)	52 (100)	32 (96.97)	4 (66.67)	32 (100)	8 (100)	239 (96.76)
	Cefoxitin	54 (46.55)	22 (42.31)	14 (42.42)	4 (66.67)	16 (50)	8 (100)	118 (47.77)
	Cefepime	98 (84.48)	34 (65.38)	22 (66.67)	5 (83.33)	32 (100)	8 (100)	199 (80.57)
	Impenem	41 (35.34)	21 (40.38)	5 (15.15)	1 (16.67)	9 (28.13)	3 (37.5)	80 (32.39)
	Azetronam	101 (87.07)	36 (69.23)	33 (100)	6 (100)	22 (68.75)	4 (50)	202 (81.78)
Aminoglycosides	Streptomycin	76 (65.52)	40 (76.92)	12 (36.36)	3 (50)	17 (53.13)	8 (100)	156 (63.16)
	Gentamycin	90 (77.59)	38 (73.08)	11 (33.33)	3 (50)	21 (65.63)	8 (100)	171 (69.23)
	Amikacin	30 (25.86)	12 (23.08)	9 (27.27)	3 (50)	13 (40.63)	4 (50)	71 (28.74)
Macrolides	Erythromycin	116 (100)	52 (100)	33 (100)	6 (100)	32 (100)	8 (100)	247 (100)
	Azithroymcin	104 (89.66)	42 (80.77)	33 (100)	6 (100)	32 (100)	8 (100)	225 (91.09)
Quinolones	Ciprofloxacin	85 (73.28)	41 (78.85)	20 (60.61)	4 (66.67)	25 (78.13)	5 (62.5)	180 (72.87)
	Nalidixic acid	110 (94.83)	48 (92.31)	30 (90.91)	3 (50)	24 (75)	8 (100)	223 (90.28)
Sulphonamides	Trimethoprim-sulfamethaxole	116 (100)	52 (100)	33 (100)	6 (100)	30 (93.75)	7 (87.5)	244 (98.79)
Phenicols	Chloramphenicol	90 (77.59)	36 (69.23)	33 (100)	6 (100)	28 (87.5)	5 (62.5)	198 (80.16)
Polypeptides	Colistin	87 (75)	45 (86.54)	24 (72.73)	3 (50)	20 (62.5)	8 (100)	187 (75.71)
Oxazolidone	Linezolid	90 (77.59)	41 (78.85)	15 (45.45)	3 (50)	26 (81.25)	6 (75)	181 (73.28)
Lincosamide	Clindamycin	116 (100)	50 (96.15)	33 (100)	6 (100)	32 (100)	3 (37.5)	240 (97.17)
Tetracyclines	Doxycycline	90 (77.59)	48 (92.31)	22 (66.67)	5 (83.33)	32 (100)	4 (50)	201 (81.38)

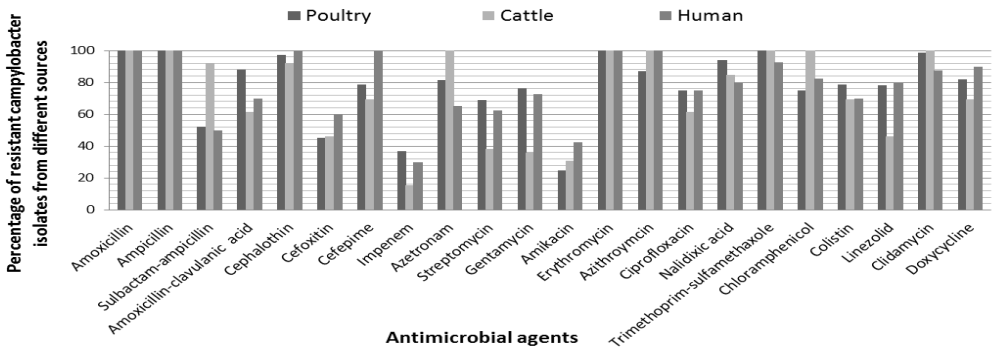

Figure 4 Prevalence of resistance in campylobacter isolates from different sources against 22 antimicrobial agents

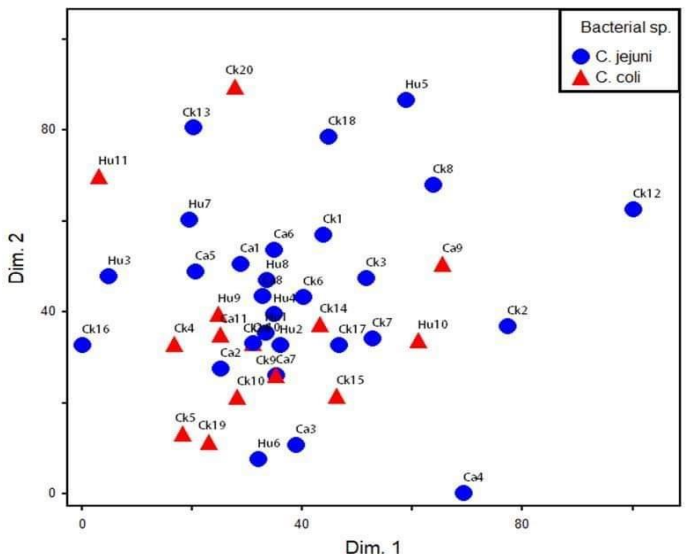

Figure 5 Non-metric multidimensional scaling plot showing the clustering pattern of *Campylobacter* isolates classified by their species (color and shape of symbol) and host (text above each dot). Each dot shows one isolate (n = 40). (Ck: chicken, Ca: cattle, Hu: human)

Antimicrobial resistance profiles and multiple antimicrobial resistance indices of campylobacter isolates

The obtainable results showed that in chicken isolates, *C. jejuni* isolates were resistant to 7 (10.35%), 8 (21.55%), 9 (35.34%) and 10 classes (32.76%) of the tested antimicrobial agents while, *C. coli* isolates were resistant to 7 (5.77%), 8 (19.23%), 9 (30.77%) and 10 classes (44.23%) of the investigated antimicrobial agents. Besides, in cattle isolates, *C. jejuni* isolates were resistant to 7 (18.18%), 8 (30.30 %), 9 (33.33%) and 10 classes (18.18 %) and in *C. coli* isolates were resistant to 7 (16.67%), 8 and 9 (33.33%, each) and 10 classes (16.67%) of the tested antimicrobial agents. Additionally, in human isolates, *C. jejuni* isolates were resistant to 8 (28.13%), 9 (40.63%) and 10 classes (31.25%) of the antimicrobial agents and in *C. coli* isolates were resistant to 7 (50%), 9 (37.5%) and 10 classes (12.5%) of the antimicrobial agents (**Table 4**).

In total, the prevalence of resistance to seven and eight antimicrobial classes was higher in cattle campylobacter isolates (17.95% and 30.77%, respectively) than chicken and human campylobacter ones. Meanwhile, the resistance to nine antimicrobial classes was the highest in human isolates (40%) and that to ten classes was the highest in chicken isolates (36.31%) (**Table 4**).

Interestingly, it was noticed that 207 (83.81%) isolates of campylobacter were MDR (resistant to 3-8 antimicrobial classes). Additionally, our results identified 38 XDR isolates (15.38%); 10 (25%), 26 (15.5%) and 2 (5.1%) were recovered from human, chicken and cattle samples, respectively. It is important to note the presence of 2 (0.81%) chicken *C. jejuni* those were PDR (**Table 4**).

Estimating the MAR indices for campylobacter isolates from different sources revealed that all tested chicken isolates presented an MAR index of 0.45 or greater. Moreover, human isolates had an index of 0.59 or greater and cattle isolates revealed an index of 0.63 or greater indicating a high risk source of contamination, where the antibiotics were often used.

Table 4 Multiple antimicrobial resistance index (MAR) of thermotolerant *Campylobacter* spp. recovered from different sources

MAR index	No. of antimicrobials to which the isolates were resistant (22)	No. of AMC*	No. of resistant *Campylobacter* spp. from different sources (%)						Total (247)	Character of resistant strains
			Chicken		Cattle		Human			
			C. jejuni (116)	*C. coli* (52)	*C. jejuni* (33)	*C. coli* (6)	*C. jejuni* (32)	*C. coli* (8)		
0.45	10	7	-	1 (1.92)	-	-	-	-	1 (0.40)	MDR
0.5	11	8	-	1 (1.92)	-	-	-	-	1 (0.40)	
0.55	12	7	1 (0.86)	1 (1.92)	-	-	-	-	2 (0.81)	
0.59	13	8	4 (3.44)	2 (3.85)	-	-	4 (12.5)	-	10 (4.05)	
0.64	14	7, 8, 9, 10	5 (4.31)	3 (5.77)	5 (15.15)	-	3 (9.38)	-	16 (6.48)	
0.68	15	7, 8, 9	18 (15.52)	1 (1.92)	4 (12.12)	2 (33.33)	1 (3.13)	-	26 (10.53)	
0.73	16	7, 8, 9, 10	11 (50)	3 (5.77)	7 (21.21)	2 (33.33)	6 (18.75)	3 (37.5)	32 (12.96)	
0.77	17	7, 8, 9, 10	22 (18.97)	17 (32.69)	10 (30.30)	-	4 (12.5)	2 (25)	55 (22.27)	
0.82	18	8, 9, 10	15 (12.93)	12 (23.08)	5 (15.15)	2 (33.33)	5 (15.63)	-	39 (15.79)	
0.86	19	7, 8, 9, 10	19 (16.32)	4 (7.69)	-	-	1 (3.13)	1 (12.5)	25 (10.12)	
0.91	20	9, 10	10 (8.62)	5 (9.62)	-	-	6 (18.75)	2 (25)	23 (9.31)	XDR
0.95	21	9, 10	9 (7.76)	2 (3.85)	2 (6.06)	-	2 (6.25)	-	15 (6.07)	
1	22	10	2 (1.72)	-	-	-	-	-	2 (0.81)	PDR

AMC*: antimicrobial classes, MDR: multidrug resistant, XDR: extensively drug resistant, PDR: pan drug resistant

DISCUSSION

Human campylobacteriosis is caused primarily due to consuming chicken meat, raw milk and inadequately pasteurized milk. The presence of campylobacter pathogens in food animals is particularly worrying for human health and controlling them has a significant implication on health of the public. The current study includes more completed and updated information about the frequency and the antimicrobial resistance of *C. jejuni* and *C. coli* from different sources in Zagazig.

Our results demonstrated that the occurrence of *Campylobacter* spp. in samples obtained from broilers was 86.15% (168 campylobacter isolates out of 195 samples). These results are consistent with previous reports in Poland (87.2%) (**Wieczorek *et al.*, 2012**) and Algeria (85%) (**Messad *et al.*, 2014**). Moreover, higher prevalence rates of *Campylobacter* spp. were reported in Italy (100%) (**Giacomelli *et al.*, 2014**) and Algeria (96%) (**Guessoum *et al.*, 2016**). On the other hand, low prevalence rates of avian *Campylobacter* spp. were previously documented in Harare (60.2%) (**Simango, 2013**) and India (44.9%) (**Vaishnavi, 2015**). In Egypt, in Giza and Cairo Governorates, 7 out of 360 chicken cloacal

swabs collected from different households, farms and shops were positive for *Campylobacter* spp. (1.9 %) (**Ghoneim *et al.*, 2020**). In Assiut Governorate, 24% of 104 chicken carcasses from two slaughterhouses contained *Campylobacter* spp. (**Abushahba *et al.*, 2018**).

In regard to *Campylobacter* spp. distribution, *C. jejuni* was the most common isolated distribution is comparable to other previous reports in Ireland, 68.9% and 32.4% and Austria, 65.1% and 33.3% for *C. jejuni* and *C. coli*, respectively (**EFSA, 2010**). A recent studies also documented that *C. jejuni* was more often isolated from chicken in Tunisia (**Gharbi *et al.*, 2018**) and Egypt (**Ghoneim *et al.*, 2020**). However, another study conducted in Italy reported that *C. coli* was the prevailing *Campylobacter* spp. among the isolates of chicken origin (**Nobile *et al.*, 2013**). Generally, the variations in *Campylobacter* spp. isolation rates between different studies could be attributed to different reasons such as the type of examined samples, location, climate factors, hygienic measures and isolation as well as techniques of identification (**Jorgensen *et al.*, 2011; Chatur *et al.*, 2014**).

Herein, *Campylobacter* spp. was isolated from 82.98% of raw milk samples, which is higher than levels obtained by El-Kholy et al., (13%) (**El-Kholy *et al.*, 2016**) and El-Zamkan and Hameed (22%) (**El-Zamkan and Hameed, 2016**) in Egypt, Kashoma et al., (13.4%) in Tanzania (**Kashoma *et al.*, 2016**) and Andrzejewska et al., (11.8%) in Poland (**Andrzejewska *et al.*, 2019**). *C. jejuni* were identified in the current study from 70.21 % of the examined raw milk samples. However results obtained by Hussain et al. in Pakistan, showed that the incidence rate of *C. jejuni* was 92.4% (**Hussain *et al.*, 2007**).

The more frequent second source of campylobacteriosis is raw milk (**Kashoma *et al.*, 2016**). Popularly, consuming organic and raw food has been increased, so consumers need to be aware of the danger related to consumption of unpasteurized milk (**Mie *et al.*, 2017**). The high occurrence of *Campylobacter* spp. in raw milk in the current study could be due to environmental contamination of milk during or after milking with infected animal wastes or from contaminated external surface of the teats, unsanitary equipment or hands of workers and storage practices (**Saad *et al.*, 2007**).

The occurrence of *Campylobacter* spp. in human stool samples was 90.91%. This result was higher than those reported Giza (16.7%) (**Hassanain, 2011**) and Assuit Governates (27.5%) (**Abushahba *et al.*, 2018**) in Egypt. Also higher than in other countries; Ethiopia (72.7%) (**Ewnetu and Mihret, 2010**), Nigeria (62.7%) (**Gwimi *et al.*, 2015**), Ghana (20.3%) (**Karikari *et al.*, 2017**) and Poland (9.6%) (**Szczepanska *et al.*, 2017**). *C. jejuni* were identified in the current study from 72.73 % of the examined human stool samples. This result were nearly similar to 69.3% and 76.9% reported in Romania (**Sorokin *et al.*, 2007**) and Egypt (**Abd El Tawab *et al.*, 2018**), respectively. The significantly higher percentage of *Campylobacter* spp. from human stool samples in our study could be ascribed to incorporation of stool samples mainly obtained from individuals with diarrhea rather than inspection of campylobacteriosis in population in general.

In the last few years, antimicrobial resistance in foodborne microorganisms involving campylobacters is considered one of the vitally important problems on public health (**Wieczorek and Osek, 2013; EFSA, 2015**). In clinical setting, macrolides (i.e erythromycin), fluoroquinolones (i.e ciprofloxacin) and tetracycline are widely used in the treatment of campylobacter infections because of their availability and low cost.

Some reports have demonstrated a slow increase in the resistance rate of campylobacters to macrolides, which are considered the first drugs of choice for treatment of campylobacteriosis, especially in paediatric patients (**Kurinčič *et al.*, 2007;Wieczorek and Osek, 2013**). In this study, all campylobacter isolates from chicken, human and cattle were resistant to erythromycin (100% each). This is in accordance with a recent report in Egypt, where 100% of campylobacter isolates from chicken were resistant to erythromycin (**Abd El Tawab *et al.*, 2018**). On the other hand, lower resistance rates to this drug were recorded for campylobacter isolates from milk samples in Iran (7.69%) (**Rahimi *et al.*, 2013**) and from human in China (21.8%) (**Pan *et al.*, 2016**). Ladely *et al*. reported that exposing campylobacters to macrolides for a long duration results in their resistance (**Ladely *et al.*, 2007**).

Our research demonstrated a high level of ciprofloxacin resistance among campylobacter isolates from chicken, human and cattle (75%, 75% and 61.54%, respectively). Similarly, a higher resistance rate (99.2%) was also reported for campylobacter isolates from chicken in Tunisia (**Gharbi *et al.*, 2018**). Using quinolones in veterinary fields leads to the emergency of ciprofloxacin resistance among campylobacters isolated from human and chicken (**Engberg *et al.*, 2004; Gupta *et al.*, 2004**). However, small number of campylobacter isolates was resistant to quinolones in some countries such as Australia, in which quinolones were not allowed to be used in chicken production (**Skarp *et al.*, 2016**). Due to the increased resistance to fluoroquinolones and quinolones, these agents became ineffective in campylobacteriosis treatment (**Han *et al.*, 2016**).

There were also high resistance rates to doxycycline in our campylobacter isolates from human, chicken and cattle (90%, 82.14% and 69.23% respectively). This is in accordance with a previous report carried out on campylobacter isolates from raw milk (71.43 %) in Poland (**Wysok *et al.*, 2011**). Due to the broad spectrum of activity and the low cost of tetracyclines, they have been widely used in the prophylaxis therapy of human and animal infections and as feed complements for chicken. These have resulted in the emergence of high resistant bacteria (**Hassanain, 2011**). Alarmingly, the increased resistance of campylobacter isolates to antimicrobials, particularly erythromycin, (fluoro) quinolones and tetracycline can result in failure in the treatment resulting in higher illness and death rates in humans (**Zhu *et al.*, 2006**).

The high resistance of campylobacter isolates to the antimicrobials in the present study might be due to the widespread and the uncontrolled use of these agents in veterinary medicine as growth promoters or in human and animal treatments. This gives a reflection about the extent of using these antimicrobials in Egypt and therefore proposes a challenge to the management of campylobacter infections.

Nowadays, the emergence of MDR campylobacter isolates is becoming a growing challenge as it can impair the effective therapy of campylobacter infections. In the current study, there were XDR and PDR campylobacter isolates especially to the antimicrobials those are used in the treatment of campylobacter infections leading to more difficulties in controlling these infections. It is interesting to note that all *Campylobacter* spp. in this study had an MAR index greater than 0.45, which indicates a high frequency of antibiotics usage in Egypt. This worrisome resistance rates were also recorded for campylobacter isolates from chicken in many countries like Italy (100%) (**Fraqueza *et al.*, 2014**), Algeria (100%) (**Messad *et al.*, 2014**) and Pakistan (90.4%) (**Nisar *et al.*, 2017**). The appearance of MDR can be attributed to the attainment of several resistance determinants in the same DNA molecule or individual determinants such as multiple drug pumps (**Levy, 200**2). The CmeABC (multi-drug efflux pump) has been concerned with the campylobacter resistance mechanisms to macrolides, fluoroquinolones and tetracyclines (**Ventola, 2015**).

In order to confirm the identification and discrimination of C. *jejuni* and *C. coli* conventional PCR has been used targeting *mapA* and *ceuE* genes, respectively (**Ghoneim *et al.*, 2020**). In the present work the conventional PCR results confirmed the identification of thirty eight campylobacter isolates (29 and 9 phenotypically suspected *C. jejuni* and *C. coli isolates*, respectively). Accordingly, the conventional culture methods and biochemical reactions were 100% in accordance with the results of PCR for identification and differentiation of *C. jejuni* and *C. coli*. The same results were reported in Egypt (**Girgis *et al.*, 2014**).

This study is limited by some factors, mainly related to lack of fund: first, the dependence on PCR for characterizing the bacterial species could be subjected to lack of specificity, and other techniques (e.g. MALDI-TOF) is recommended and will be taken into consideration in future studies. However, including control positive and negative compensated, to some extent, this limitation. Other shortcoming is that we depended on phenotypic approach to define the resistance to antimicrobials. Indeed, an ongoing project that involves this point on the same isolates is in plane for future publication. In addition, we were not able to include other sample types from “Cattle” (i.e. other than milk) due to unavailability and lack of access to the sampled animals.

CONCLUSION

Our data demonstrated that the relatively high isolation rate of campylobacters from chicken, raw milk and human stool samples in addition to the development of MDR strains to multiple antimicrobial classes, especially to macrolides, quinolones and tetracycline are alarming situations with potential serious consequences to the health of human. Therefore, there is a need to reduce using antimicrobial agents in food animals and to implement specific control procedures to decrease contamination levels by campylobacters to prevent resistant campylobacter strains from emerging and spreading.

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