Implication of Turkey broiler flocks in prevalence of antibiotic resistance *capmpylobacter spp*.

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Abstract: A total of 100 samples were collected from diseased fattening turkeys, samples included fecal swabs, liver, and intestine were subjected to conventional examination for *campylobacter species* identification, isolates were confirmed by PCR through the detection of *cad*F gene the conserved for genus *campylobacter*, *ceu*E gene specific for *campylobacter coli*, and *C*j gene specific for *campylobacter jejuni,* the results revealed that **16/100 (16%)** of samples were positive for *campylobacter species*, **9/16 (56.2%), 7/16(43.8%)** of isolates belong to *campylobacter jejuni, and campylobacter coli,* respectively. Phenotypic and genotypic antibiotic resistance attributes of isolates were studied by disc diffusion and PCR. The results revealed that **16/16 (100%)** of isolates showed antibiotic resistance patterns to ampicillin, tetracycline, and erythromycin. Resistance rates against cefotaxime and gentamycin were **(81.3%), (87.5, %),** respectively. Only **3/16 (18.8%)** of isolates showed resistance rate against imipenem, **16/16 (100%)** isolates demonstrated profiles of multidrug resistant strains. Studying the genetic antibiotic resistance attributes of isolates by PCR revealed that **10/16 (62.5%), 9/16 (56.2%)** of isolates have ***tet* O** gene for tetracycline resistance, and ***cme*B** gene for efflux pump, respectively. PCR failed to detect ***bla*OXA** gene for betalactams. The findings raised concerns due to the presence of circulating *campylobacter spp* in turkey farms that may impose a potential high public health risk caused by their zoonotic nature, furthermore disseminate antibiotic resistance genes against key antibiotics used in veterinary and human medicine.
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Introduction

Campylobacter spp are motile spirally curved, Gram negative bacteria that are commonly present in the intestinal tract of domestic and wild animals **(Blaser** and **Engberg, 2008)** *Campylobacter jejuni* and, *Campylobacter coli* are the most important pathogenic species, they grow in a microaerophilic atmosphere with 10% CO2 and 5% O2, at a narrow temperature range between 30∘C - 46∘C, and thus classified as thermophilic campylobacters **(Allos, 2001).**

Campylobacter is part of the normal flora living in the intestines of healthy chickens and other animals. During slaughtering and gutting chickens, the contents of intestines, including the *Campylobacter*, could contaminate raw chicken meat**.**

Many studies confirmed the risk of contamination of poultry carcass, meat and meat products at the time of slaughter and processing, in this regard, **(Alexandra, 2009)** concluded that *Campylobacter* is present in the crop at 10^4 and in the ceca at 10^7 CFU/g contents; while the estimated *Campylobacter* infectious dose for humans is 500 cells. **Viktoria *et al.,* (2007)** studied the prevalence of *Campylobacter* in samples collected from turkey carcasses at slaughter house they found that over one-quarter (29.2%) of the tested samples were *Campylobacter* positive**.**

Campylobacter can be easily spread from bird to bird through a common water source or through contact with infected feces. *Campylobacter* can also be present in the giblets, especially the liver **(CDC, 2015).**

Campylobacter bacteria are a major cause of foodborne diarrheal illness in humans and were the most common bacteria that cause gastroenteritis worldwide, in developed and developing countries. The high incidence, the disease course duration and the sequelae, makes campylobacteriosis highly important from a socio-economic perspective **(WHO, 2015).**

Campylobacteriosis most reported symptoms are diarrhea, cramping, abdominal pain, and fever within two to five days after exposure, bloody diarrhea accompanied by nausea and vomiting, the disease course lasts for about one week **(CDC, 2015).** In developing countries, infections are commonly detected in children younger than two years old,

sometimes resulting in death, *Campylobacter* species are prevalent in food animals such as poultry. The main route of transmission is believed to be foodborne via undercooked meat and meat products, often carcasses or meat are contaminated from feces during slaughtering **(WHO, 2015).**

Campylobacteriosis is estimated to affect over 1.3 million persons every year mainly in summer, although *Campylobacter* infection does not commonly cause death, but it has been estimated that approximately 76 persons with *Campylobacter* infections die each year **(CDC, 2015). Nachamkin, (1998**) concluded that *Campylobacter jejuni* not only is an important cause of bacterial gastroenteritis in humans but also has been associated with Guillain-Barré syndrome, which is an acute immune-mediated demyelinating disorder of the peripheral nervous system.

The occurrence of high resistance to several antimicrobials, especially key drugs for the treatment of human campylobacteriosis, representing a potential risk for public health, also the emergence of antimicrobial resistance among *Campylobacter* isolates recovered from turkeys has increased dramatically, thus becomes a growing public health issue **(El-Adawy *et al.,* 2012).**

Poultry is widely recognized as a major reservoir in cases of Campylobacteriosis, due to symptomless carriage in the live bird. The problem is exacerbated by intensive rearing. Moreover, usage of antimicrobials in poultry production, for prophylactic, therapeutic or performance-enhancing purposes, contributes to the development of resistance in pathogens, which can have serious consequences for the treatment of human illness.

This study was aimed to investigate the prevalence of *Campylobacter spp* in turkeys and to assess the phenotypic and genotypic antimicrobial resistance (AMR) attributes of isolates.

2. Materials And Methods

2.1 Sampling

A total of 100 samples were collected from diseased turkeys with history of digestive symptoms (60 coloacal swabs, 20 liver, and 20 intestines) from Belbeis, Sharia governorate, Egypt in Summer 2017.

2.2 Isolation and Identification *Campylobacter species*

Isolation and identification of *Campylobacter spp* were applied according to (**ISO 10272-1 2006)**.

2.3 PCR technique for confirmation of genus Campylobacter and, Campylobacter species identification

2.3.1. Extraction of DNA: QIAamp DNA Mini Kit, catalogue no.51304 was used

2.3.2 PCR Master Mix: Emerald Amp GT PCR mastermix (Takara) code no. RR310A

2.3.3. Oligonucleotide primers: Metabion (Germany) with specific sequence for tested genes were used, primer sequences and thermal cycling condition as demonstrated in table (1).

2.4. Antibiogram of campylobacter isolates: All campylobacter isolates were tested for their susceptibility against 7 antibiotic agents' **ampicillin, imipenem, cefotaxime, cefoxitin**, **erythromycin**, g**entamycin**, and t**etracycline (Oxoid),** by disc diffusion method according to **(Quinn *et al.,* 1999)**.

2.5. PCR investigation of antibiotic resistance genotypic attributes: by using Oligonucleotide primers, Metabion (Germany), primer sequences and thermal cycling condition as demonstrated in table (1).

Table 1. Oligonucleotide sequences and thermal profiles used in PCR

Test target	Tested gene	Primer sequence (5´-3´)	Amplicon size	Thermal profile	Reference
Genus campylobacter	*CadF*	F: TGGAGGGTAATTTAGATATG R: CTAATACCTAAAGTTGAAAC	400 bp	94°C, 5 min; 35 cycles: 94°C, 1 min; 45°C, 1 min; 72°C, 3 min; And, 72°C, 10 min	*Konkel et al.,* (1999)
Campylobacter coli	*CeuE*	F: ATGAAAAAATATTTAGTTTTTGCA R: ATTTTATTATTTGTAGCAGCG	894 bp	94° C, 5min, 35 cycles:94° C, 1 min; 57°C, 1 min; 72°C, 1 min; And, 72°C, 10 min	Gonzalez *et al.,* (1997)
Campylobacter jejuni	*CJ*	F:- GAGTAAGCTTGGTAAGATTAAAG R: AAGAAGTTTTAGAGTTTCTCC	500 bp	94° C, 5min, 35 cycles:94° C, 1 min; 53°C, 1 min; 72°C, 1 min; And, 72°C, 10 min	Rantsioua *et al.,* (2010)
Tetracycline resistance	*tet O*	F: AACTTAGGCATTCTGGCTCAC R: TCCCACTGTTCCATATCGTCA	515 bp,	94° C, 5miN, 35 cycles:94° C, 1 min;56°C, 1 min; 72°C, 1 min; And 72°C, 10 min	Abdi-Hachesoo *et al.,* (2014)
Efflux pump	*cme* B	F: 5'-CCTACCTCCTATACCTGG-3' R: 5'-TTGAACTTGTGCCGCTGG-3'	515 bp	94° C, 5min,,35 cycles:94° C, 1 min;56°C, 1 min; 72°C, 1 min; And,72°C, 10 min	Pamela *et al.,* (2006)
βlactam resistance	*βla OXA*	F-TCGATGGATTGCTTTAATGG R- TTGTCAAGCCAAAAAGTATCG	564 bp	94° C, 5min; 35 cycles: 94° C, 1 min; 56°C, 1 min; 72°C, 1 min; And 72°C, 10 min	Alfredson *et al.,* (2005)

3. Results

Table 2. Prevalence rate of *Campylobacter spp* among examined samples

Sample		Positive Isolates	
Type	Number	Number	Prevalence
Fecal swabs	60	11	18.3%
Liver	20	2	10%
Intestine	20	3	15%
Total	100	16	16%

Table 3. Confirmation and Species Identification of *Campylobacter* Isolates by Conventional PCR

Target test	Tested genes	*Campylobacter* isolates	
		Number	Detection Rate
Campylobacter spp	*Cad* F	16	100%
Campylobacter coli	*Ceu*E	7	43.8%
Campylobacter jejuni	*C*j	9	56.2%

Table 4. Phenotypic antibiotic resistance profiles of Campylobacter isolates

Antibiotic Group		Antibiotic Agent	Abbrev.	Conc.	Resistant		Susceptible	
					NO	%	NO	%
βlactamins	Penicillins	Ampicillin	AM	10 µg	16	100%	0	0%
		Imipenem	IPM	10µg	3	18.8%	13	81.3%
	Cephalosporins	Cefotaxime	CTX	30 µg	13	81.3%	3	18.8%
		Cefoxitin	FOX	30 µg	10	62.5%	6	37.5%
Macrolydes		Erythromycin	E	15 µg	16	100%	0	0%
Aminoglycosides		Gentamycin	CN	10 µg	14	87.5%	2	12.5%
Tetracyclines		Tetracycline	TE	30 µg	16	100%	0	0%

Table 4-a. Phenotypic antibiotic resistance profiles of *Campylobacter coli* isolates

Antibiotic Group		Antibiotic Agent	Abbrev.	Conc.	Resistant		Susceptible	
					NO	%	NO	%
βlactamins	Penicillins	Ampicillin	AM	10 µg	7/7	100%	0	0%
		Imipenem	IPM	10µg	0	0%	7/7	100%
	Cephalosporins	Cefotaxime	CTX	30 µg	6/7	85.7%	1/7	14.3%
		Cefoxitin	FOX	30 µg	5/7	71.4%	2/7	28.6%
Macrolydes		Erythromycin	E	15 µg	7/7	100%	0	0%
Aminoglycosides		Gentamycin	CN	10 µg	6/7	85.7%	1/7	14.3%
Tetracyclines		Tetracycline	TE	30 µg	7/7	100%	0	0%

Table 4-b. Phenotypic antibiotic resistance profiles of *Campylobacter jejuni* isolates

Antibiotic Group		Antibiotic Agent	Abbrev.	Conc.	Resistant		Susceptible	
					NO	%	NO	%
βlactamins	Penicillins	Ampicillin	AM	10 µg	9/9	100%	0	0%
		Imipenem	IPM	10µg	3/9	33.3%	6/9	66.7%
	Cephalosporins	Cefotaxime	CTX	30 µg	7/9	77.8%	2/9	22.2%
		Cefoxitin	FOX	30 µg	5/9	55. 6%	4/9	44.4%
Macrolydes		Erythromycin	E	15 µg	9/9	100%	0	0%
Aminoglycosides		Gentamycin	CN	10 µg	8/9	88.9%	1/9	11.1%
Tetracyclines		Tetracycline	TE	30 µg	9/9	100%	0	0%

Table 5. Investigation of the presence of antibiotic resistance genes in isolated *campylobacter spp* by PCR

Antibiotic group	Tested genes	*Campylobacter* isolates		
		Campylobacter coli	*Campylobacter jejuni*	Total
Tetracycline	*tet* O	6/7 (85.7%)	4/9(44.4%)	10/16(62.5%)
Efflux pump	*cme* B	4/7(57.1%)	5/9(55.5%)	9/16 (56.2%)
Penicillin	*βla* OXA	0	0	--

Discussion

In the present study a total of **100** samples were collected from fattening turkeys at the slaughter age between **150** to **160** day old, samples were examined for *Campylobacter spp* isolation by using conventional bacteriological methods, the results revealed that **16/100 (16%)** of samples were positive for *Campylobacter spp* with a prevalence rate of **(16%).** PCR for the detection of ***cad*F** gene which is a genus specific conserved gene for *campylobacter* was applied in order to confirm the positivity of isolates, PCR targeting ***cad*F** for detection of genus *campylobacter* was also used **by (Nayak*, et al.*, 2005).** In the same regard, almost similar prevalence rate was reported by (**Carmelo *et al.*, 2013)** who detected *Campylobacter spp* from poultry samples with a prevalence rate of **(20.7%)** meanwhile, higher prevalence rate was reported by **(Korsak *et al.*, 2015)** who reported a prevalence rate of **(41.1%)**.

Humans often become infected by zoonotic pathogens as *Campylobacter coli*, and *Campylobacter jejuni* by ingesting contaminated food or water, in this instance raw or uncooked meat, like poultry meat, and contact with animals stand for the main transmission roots (**Blaser** and **Engberg, 2008).** In the current study the identified species were confirmed by using PCR for detection of ***ceu*E**, and ***C*j** genes which are specific for *Campylobacter coli, and Campylobacter jejuni*, respectively. In this instance (**Nayak *et al.*, 2005)** applied PCR for detection of ***ceu*E** gene and the specific undefined gene for species identification of both *Campylobacter coli* and *Campylobacter jejuni* species. PCR results of our investigation demonstrated that **7/16 (43.8%)**, and **9/16 (56.2%)** of isolates were *Campylobacter coli*, and *Campylobacter jejuni*, respectively. In this instances, nearly similar detection rates of *Campylobacter species* were reported by **(Carmelo *et al.*, 2013**) who reported detection rates of **(48.2 %, and 51.8%)** for *Campylobacter coli*, and *Campylobacter jejuni,* respectively. Variable detection rates of *Campylobacter spp* were recorded by different researchers as **(Engy *et al.*, 2015)** who recorded that **(91.7%)** of the total **36** detected isolates were identified as Campylobacter *coli* and **(8.3%)** *Campylobacter jejuni*. Furthermore, **(Kashoma *et al.*, 2014)** who confirmed that **(72.3%)** of the detected isolates were *campylobacter coli,* **(5.3%)** of isolates were *campylobacter jejuni,* and that **(22.5%)** of isolates as other *Campylobacter spp.*

An emergence of multiple resistance patterns of *Campylobacter species* to several antibiotic classes has been observed globally, the most common antimicrobial agents Macrolides, as erythromycin which is commonly used in the treatment of Campylobacter infections, tetracycline is considered an alternative choice. However, campylobacter resistance to fluoroquinolones, macrolides, aminoglycosides, and beta-lactams have been developed. (**Hindawi, 2013).** Furthermore, concerns of the demonstrated resistance of *Campylobacter* to the fluoroquinolones that has limited their use as drugs of choice in human medicine and the increasingly detected resistance to macrolides (erythromycin) as an alternative choice, beside the increasingly demonstrated resistance to aminoglycosides, and beta lactams including, penicillin, cephalosporin are increasing in medical, veterinary and scientific domains (**Giacomelli *et al.*, 2014**).

In the current study, **16/16 (100%)** of isolates showed phenotypic resistance patterns against at least one antimicrobial agent that is classified in three or more antimicrobial group, as **(100%)** of isolates showed resistance against penicillin, erythromycin, and tetracycline, also **(87.5%)**, and **(81.3%)** of isolates showed resistance against gentamycin, and cefotaxime, respectively, consequently the isolates can be considered multidrug resistant strains as defined by **(EUCAST, 2014)** this result demonstrated the potential high public health risk imposed by these isolates, similar finding was also reported by (**Aarestrup *et al.*, 2011)** who concluded the association of emergent campylobacter resistant strains in human clinical samples with the emergence of antimicrobial resistance observed in animals, the same result was also found by (**Pérez *et al.*, 2013)** who described (10.3%) of their studied isolates as pan-susceptible campylobacter populations, they also reported that multidrug resistance isolates were observed in *Campylobacter coli* compared with *Campylobacter jejuni* **(33.3%** vs. **11.9%),** they also raised their concerns from the public health risk imposed by those populations as they demonstrated resistance against fluoroquinolone, macrolide, and tetracycline.

There was no significant difference in the demonstrated phenotypic resistance profiles observed in this study between the investigated *Campylobacter coli* and *Campylobacter jejuni* isolates, as **(100%)** of isolates from both species demonstrated resistance to penicillin, erythromycin and tetracycline. In the same regards, resistance rates demonstrated to gentamycin were **(88.9%)** and **(85.7%)** for *Campylobacter jejuni* and *Campylobacter coli*, respectively. Also, resistance rates demonstrated against cefotaxime, and cefoxitin were (**77.8%** and**, 55. 6%)** for *Campylobacter jejuni* and **(85.7%,** and **71.4%)** for *Campylobacter coli*. This result, differed from that recorded by (**Kashoma *et al.*, 2014)** who reported that *Campylobacter coli* isolates displayed a higher proportion of resistance than *Campylobacter jejuni* against most antimicrobials.

The results of this study, demonstrated that (**100%**) of isolates from both *Campylobacter coli*, and

Campylobacter jejuni were resistant to erythromycin, while this result was in agreement with that of **(Engy *et al.,* 2015)** who recorded the prevalence of erythromycin resistance among their isolates and (**Carmelo *et al.,* 2013)** who recorded that (**80.1%)** of their studied Campylobacter isolates demonstrated resistance to erythromycin, the result disagreed with that of (**El-Adawy *et al.,* 2015)** who reported that **(100%)** of *Campylobacter* isolates were susceptible to erythromycin.

Gibreel *et al.,* (2004) reported that both Kanamycin and tetracycline resistance is mediated by a plasmid that is transferred by conjugation between *Campylobacter strains*. In the current work, there was observed phenotypic resistance to gentamycin in **6/7 (85.7%)**, and **8/9 (88.9%)** of *Campylobacter coli*, and *Campylobacter jejuni*, respectively. While lower resistance rate was observed by **(Carmelo *et al.,* 2013)** who recorded a resistance rate of **(27.9%)** among the *Campylobacter spp* involved in their study**,** the present result was in contrast to the result reported by (**El-Adawy *et al.,* 2015)** who reported that **(100%)** of the studied *Campylobacter jejuni* isolates, and *Campylobacter coli* isolates were sensitive to gentamycin.

Luangtongkum *et al.,* (2006) reported that, since the use of tetracycline as feed additives in poultry production for both therapeutic and sub therapeutic purposes, it is possible that *campylobacter* may have evolutionally become resistant to tetracycline, leading to the widespread distribution of tetracycline-resistant *campylobacter* in animal reservoirs regardless of the production types, their finding agreed with the results recorded by this study as **(100%)** of tested *Campylobacter coli*, and *Campylobacter jejuni* isolates demonstrated phenotypic resistance patterns to tetracycline by disc diffusion test, this result agreed with that of (**Giacomelli *et al.,* 2014)** who reported a resistance rate of **(96%)**. Lower resistance rates were observed by **(El-Adawy *et al.,* 2015)** who observed resistance rates of **(44.0%,** and **51.3%)** *Campylobacter coli* and *Campylobacter jejuni,* respectively.

The resistance rate detected for ampicillin were **(100%)** for both **7**/*7 Campylobacter coli*, and **9/9** *Campylobacter jejuni*, while this result disagreed with that of (**Ewnetu** and **Mihret, 2010)** who detected **a** resistance rate of **(16.6%**) against ampicillin. Almost similar resistance rate was reported by **(Giacomelli *et al.,* 2014)** who recorded the prevalence of ampicillin resistant strains with a rate of **(88%)**.

Resistance rates demonstrated against cefotaxime, cefoxitin, and imipenem were (**77.8%, 55. 6%**, and **33.3%)** for *Campylobacter jejuni* and, were **(85.7%, 71.4%** and, **0%)** for *Campylobacter coli*, respectively. This result agreed with that reported by (**Giacomelli *et al.,* 2014)** who detected resistance rate of **(100%)** for at least three cephalosporin, the result also agreed with that recorded by **(Martin** and **Kaye, 2004)** who found that campylobacter strains can be considered resistant to beta lactams, as penicillin and narrow-spectrum cephalosporin but not to carbapenems.

Zhang and **Plummer, (2008)** concluded that campylobacter resistance to tetracycline can be attributed to its ability to undergo spontaneous mutations and also its ability to acquire resistance determinants by natural transformation, transduction, or conjugation, as in case of conjugation of $tet(O)$-carrying plasmids. **Connell, (2003)** concluded that resistance of *Campylobacter jejuni* and *Campylobacter coli* to tetracycline is attributed mainly to the acquisition of *tet* (O) gene which encodes ribosomal protection proteins (RPPs). In the present study, PCR technique was applied to investigate the genetic attributes of isolates for tetracycline resistance by detection of ***tet* (o)** gene, the results of PCR was in accordance with those revealed by disc diffusion, in this regard **10/16 (62.5%), 6/7 (85.7%),** and **4/9 (44.4%)** of Campylobacter isolates, *Campylobacter coli*, and *Campylobacter jejuni*, respectively. These results are in agreement with that reported by **(Abdi-Hachesoo *et al.,* 2014)** who recorded detection rates for *tet* (o) gene as followed: **(83.1% 92.5,** and **74.4%)** for the studied Campylobacter isolates, *Campylobacter coli,* and *Campylobacter jejuni,* respectively. **Engy *et al.,* (2015)** also recorded that **9/33 (27.3%)** *Campylobacter coli* isolates were positive for the tetracycline resistance gene *tet (O)*, although only two of these were resistant to tetracycline in the disc diffusion test.

Macrolides are of the safest and most effective antimicrobial drugs used against most of Gram-positive and the Gram-negative microorganisms, including Campylobacter, their mode of action is to interrupt protein synthesis in bacterial ribosome resulting in inhibition of bacterial RNA-dependent protein synthesis (**Poehlsgaard** and **Douthwaite, 2005).** Conformational changes in the ribosome subsequently, termination of the elongation of the peptide chain is caused by binding of macrolide to the target site in the bacterial 23S rRNA (**Pfister *et al.,* 2004).** The resistance to macrolides can also be mediated by modifications of the ribosomal proteins L4 and L22, resistance to macrolide in *Campylobacter speices* is also commonly mediated by efflux pump, in this instance, (**Cagliero *et al.,* 2006)** reported that at least eight efflux systems are identified of which is *cme*ABC multidrug efflux pump that works in synergy with mutations. Furthermore, **(Hindawi, 2013)** mentioned that *cme* ABC multidrug efflux pump are the major efflux mechanism causing macrolides

antimicrobial resistance in campylobacters. Resistance rates recorded by disc diffusion for *Campylobacter coli,* and *Campylobacter jejuni* were (**88.9%** and **85.7%),** respectively, this result was in accordance with the result of PCR for detection of ***cme*B** gene which mediates the efflux pump mechanism and mainly mediates macrolide resistance, as **9/16 (56.2%), 4/7(57.1%),** and **5/9(55.5%)** of *Campylobacter isolates, Campylobacter jejuni*, and *Campylobacter coli,* respectively. Furthermore, (**Cagliero *et al.*, 2006)** studied the resistance attributes of highly macrolides resistant *Campylobacter strains* with specific target site mutations, they found that inactivation of *cme*ABC resulted in reduced resistance to macrolides in addition, it leads to restored susceptibility to erythromycin, suggesting the significant synergistic function of efflux system with target mutations in acquiring and expression of macrolide resistance in campylobacter.

Martin and **Kaye, (2004)** confirmed that Beta lactams mode of action is through binding to penicillin binding proteins causing disruption of peptidoglycan crosslinking in bacterial cell wall leading to cell death. Interestingly, although results of disc diffusion applied in this study revealed that 16/16 **(100%)** of isolates are phenotypically resistant to ampicillin, PCR failed to detect ***βla* OXA** gene, the specific for penicillin resistance in the studied isolates. Studies and researches interpreted the resistance of campylobacter to beta lactams due to multiple mechanisms, in this regards (**Tajada *et al.*, 1996)** attributed beta lactams resistance in *Campylobacter jejuni* and *Campylobacter coli* to their ability to produce beta lactamases, meanwhile (**Lin *et al.*, 2002)** reported that beside the ability to hydrolyze beta lactam ring through production of beta lactamases, resistance in campylobacter strains can be attributed to the action of efflux pumps that is mediated by *cme*ABC genes in the resistant mutants.

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