Detection of antibiotic resistant genes of some *Campylobacter species* isolated from Egyptian ducks

Engy A. Hamed*, Mona A. A. Abdel Rahman, Azhar G. Shalaby, Mai M. Morsy and Soad A. Nasef.

National Laboratory for Quality Control on Poultry production, Animal Health Research Institute, Ministry of Agriculture, Nady El-Seid Street, Dokki P.O. Box246, Giza 12618, Egypt

Abstract

Campylobactercoli and *Campylobacter jejuni* may cause gastrointestinal disorders with or without necrotic hepatitis in poultry and serious foodborne enteritis with sometimes fatal consequences in humans. Little is known about the prevalence of *Campylobacter* spp. in ducks, particularly young ducklings.In this study, 36 (24%) isolates of *Campylobacter*spp. were isolated from 150 samples of 1-day-old ducklings in Egypt. Using biochemical tests and specific PCR, 33 *C. coli* and 3 *C. jejuni* were identified. Allisolates were sensitive to chloramphenicol and amikacin but resistant to sulfonamethoxazole-trimethoprim (SXT) using antibiotic disc-diffusion test. The majority of isolates were susceptible to tetracycline and erythromycin, meanwhile the resistance to ofloxacin and ciprofloxacin was relatively high. Nine out of 33 *C. coli* were positive for the tetracycline resistance gene *tet* (O), although only two out of them were resistant to tetracycline. A polymorphism in the quinolone resistance-determining region (QRDR) of *gyr*A gene from *C. coli*and *C. jejuni* isolates was identified by direct sequencing. These findings indicated that ducklings may be a source for antibiotic resistant *Campylobacter* spp. with potential poultry and public health hazards.

Keywords: *Campylobacter* spp.; Ducklings; Antibiotic resistance; *tet*(O); *gyr*A

Introduction

Thermophilic *Campylobacter* spp. has Gram negative cell wall structures with capsule and flagella. The bacteria are slender, curved rod to small spiral in shape with 0.2-0.5 µm width and 0.5-5.0 µm length. They need microaerophilic atmosphere at 37-42 °C for 48 ± 4 hours for optimal growth ***(Shane and Harrington, 1998).*** *Campylobacter jejuni*and *Campylobacter coli* are isolated from domestic and wild birds. The bacterium colonises the intestinal tract of healthy birds and it may

cause gastrointestinal disorders with or without necrotic hepatitis (avian vibrionic hepatitis). In chickens, although theorganism was isolated from 1-day-old chicks, it begins colonisation of the intestine from 2-3 weeks of age and peaks at the time of slaughtering ***(Zhang, 2008)***. Thus, the contamination of carcasses in slaughterhouses is common; nevertheless, hatcheries are a potential source for the infection of one-day old chicks by what is called by false vertical transmission due to the external contamination of egg shell. Meanwhile, vertical transmission of the bacterium from hens to the progeny is still debatable ***(Zhang, 2008).*** The rate of isolation of *Campylobacter* in chickens were higher than in ducks, although ducks were found to be frequently contaminated with *Campylobacter* spp. ***(Boonmar et al, 2007; Colles et al, 2011).***

In human beings, *C. jejuni*and C. *coli* are among the most important causes of foodborne gastroenteritis ***(Alfredson and Korolik, 2007),*** which mostly occurs due to consumption and/or mishandling of contaminated raw or undercooked poultry meat and products.Generally, the infection is self-limiting; however, serious complications (arthritis and Guillain-Barré syndrome) may happen, particularly in children, pregnant women, the elderly and immunocompromised patients ***(Gormley et al, 2008)***.

Erythromycin, fluoroquinolones (FQ), gentamicin and tetracycline are clinically effective in treatment of *Campylobacter* spp. infections ***(Allos, 2001; Godschalk et al, 2004).*** Nevertheless, the misuse of antibiotics in poultry may lead to the emergence of antibiotic resistant strains **(Aarestrup and Engberg 2001).**Poultry treated with erythromycin, ciprofloxacin, nalidixic acid and tetracycline play an important role in transmission of resistant *Campylobacter* spp. strains to human beings ***(Gupta et al, 2004).*** Likewise, poultry are the most important source of human FQ-resistant *Campylobacter* spp. ***(Smith, 2009).***

Resistance of *Campylobacter* spp. to tetracycline is encoded by the tetracycline resistant gene *tet*(O), which can rapidly be transferred to tetracycline-sensitive strains ***(Avrain et al, 2004; Pratt and Korolik, 2005)***. Mutations in the quinolone resistance-determining region (QRDR) of the gyr*A* gene so presence of mutation especially in position of Thr-86-Ile (ACA ATA) has also been linked to the resistance of *Campylobacter* spp. to FQ (Hakanen et al., 2002). While isolation of *Campylobacter* spp. from 1-day-old chicks has been reported in several countries, such data on ducks are scarce ***(Newell andFearnley, 2003; Zhang, 2008).***Therefore, the aim of this study was to provide information about antibiotic resistance of

Campylobacter spp. in 1-day-old ducklings in Egypt.

Materials and methods

Bacterial isolation

Meconium samples (*n* = 150) from 1-day-old ducklings in Egypt were collected in 2011-2012. All samples were collected from the duckling which submitted to reference laboratory for veterinary quality control on poultry production for routine examination and about one gram of meconium was putted on 9ml Bolton broth (Oxoid) and incubated in microaerophilic condition (10% CO_2, 5%O_2, 85% N_2) for 24 h at 42 °C, then streaked on *Campylobacter* spp. blood free selective media (CCD agar and Karmali agar; Oxoid) according to the International Standards Organisation ***(ISO) 10272-1 (2006).*** Cell morphology test and motility test was done by using Gram stain and hanging drop technique using microscope Biochemical identification was done by oxidase, catalase, nalidixic acid sensitivity test and sodium hippurate hydrolysis test.

Antibiotic sensitivity test

The antibiogram of *Campylobacter* spp. was done by disc-diffusion test against nine antibiotics (Oxoid): ampicillin, tetracycline, erythromycin, ciprofloxacin, ofloxacin, sulfonamethoxazole-trimethoprim (SXT), gentamicin, amikacin and chloramphenicol according to the Clinical and Laboratory Standards Institute ***(formerly National Committee for Clinical Laboratory Standard, CLSI/NCCLS, 2009).*** Pure *Campylobacter* colonies were selected and put on 2 mL Muller Hinton broth in test tube. The test tubes were incubated at 42 °C in microaerophilic condition for slight turbidity compared against 0.5 McFarland tube. Muller Hinton agar plate with 5% defibrinated sheep blood was inoculated with previously prepared culture using sterile bacterial swabs in three different directions. The plate was incubated in 42°C for 24-48 h as previously described. Inhibition zones were measured to detect the resistant isolates.

PCR technique

DNA extraction

DNA extraction from positive samples was performed using the QIAamp DNA Mini kit (Qiagen) with slight modifications; 200 µL of the sample suspension was incubated with 20 µL proteinase K and 200 µL lysis buffer at 56 °C for 10 min. After incubation, 200 µL of 100% ethanol was added to the lysate, then the sample was washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µL elution buffer provided in the kit.

All isolates were confirmed by PCR targeting the gene encoding the membrane associated protein A (*map*A) of *C. jejuni* ***(Stucki et al, 1995)*** and siderophore binding protein, lipoprotein component of

enterocholin (*ceu*E) of *C. coli* ***(Gonzalez et al, 1997).*** The reference strains *C. jejuni*(WHO C 10-1) and *C. coli* (WHO C 10-2) provided by the External Quality Assurance Services (EQAS) were used as positive controls. The detection of the *tet*(O) in all *Campylobacter* spp. isolates in this study was done according to ***El-Adawy et al (2012).*** The oligonucleotide primers used in this study were purchased from Metabion International AG (Table 1).

A volume of 25 µL PCR reaction containing 12.5 µL Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µL of each primer of 20 pmol concentrations, 4.5 µL of water and 6 µL of template was used in a Biometra thermal cycler. The PCR products were separated by electrophoresis on 1-2% agarose gel (Applichem) in 1x TBE buffer at room temperature. A 100 base pair DNA Ladder (Qiagen) was used to determine the fragment size. The gel was photographed using a gel documentation system (Alpha Innotech, Biometra) and the data were analysed through computer software.

Sequence and phylogenetic analysis

Three *C. jejuni* isolates and nine *C. coli* isolates were randomly selected for partial amplification and sequencing of the QRDR of *gyr*A as previously published ***(Lindmark et al, 2004)***. PCR products of the QRDR of gyr*A* were purified using QIAquick PCR Product Extraction Kit (Qiagen). Sequencing reactions were done using BigDye Terminator v3.1 Cycle Sequencing Kit on an automatic sequencer (ABI-3130; Applied Biosystems). The generated sequences were assembled and query sequences were retrieved from the public GenBank database. Nucleotide and deduced amino acid sequences were aligned and compared with the closely related sequences using BioEdit version 7.1.7 ***(Hall, 1999).*** The phylogenetic relationship of all genes was inferred using the neighbour-joining and maximum likelihood methods implemented in MEGA5 software ***(Tamura et al, 2011).*** The phylogenetic trees were mid-point rooted and bootstrap values of all branches were obtained after 1000 replicate resampling. The generated sequences in this study have been deposited in GenBank under the accession numbers KJ735384 to KJ735395.

Table 1 Oligonucleotide primers used in this study.

Target gene	Primer	Sequence (5'-3')	Size (base pairs)	Reference
ceuE	CeuE F	AATTGAAAATTGCTCCAACTATG	462	Gonzalez et al. (1997)
	CeuE R	TGATTTTATTATTTGTAGCAGCG		
mapA	MapA F	CTATTTTATTTTTGAGTGCTTGTG	589	Stucki et al. (1995)
	Map A R	GCTTTATTTGCCATTTGTTTTATTA		
Tet(O)	DMT 1	GGCGTTTTGTTTATGTGCG	559	El-Adawy et al. (2012)
	DMT 2	ATGGACAACCCGACAGAAGC		
QRDR	gyrA F	GATGGTTTAAAGCCTGTTCAT	423	Lindmark et al. (2004)
	gyrA R	CGCCATACCTACAGCTATACC		

Results

Thirty-six (24%) *Campylobacter*isolates were recovered from 150 samples of one-day-old ducklings. All isolates were identified biochemically as *C. coli* (33 isolates) or *C. jejuni*(3 isolates) and the same results were confirmed by PCR targeting the *ceu*E and *map*A genes.

Antibiotic sensitivity test

Resistance of *C. coli* isolates to tetracycline, gentamicin, erythromycin, ampicillin, ciprofloxacin, ofloxacin and SXT were 6%, 12%,15%, 21%, 66%, 85% and 100%, respectively (Table 2). All isolates were sensitive to amikacin and chloramphenicol,

On the other hand, all *C. jejuni* isolates were resistant to ofloxacin and SXT, and sensitive to gentamicin, erythromycin, tetracycline, amikacin and chloramphenicol. Meanwhile, two isolates (67%) were resistant to ampicillin and ciprofloxacin (Table 2).

Results of detection of *tet*(O)gene by usingPCR

Nine of 33(27.3%) *C.* coli isolates were positive for the *tet*(O) gene, while all *C jejuni* isolates were negative for *tet*(O) gene. The results are shown in **Photo. 1 and 2**.

Nine *C. coli* had been selected randomly with different sensitivities to FQ (ofloxacin and ciprofloxacin) and three *C. jejuni* strains and were designated as AzEg1 to AzEg12, those 12 isolates were subjected to sequence analysis to detect possible mutations in the gyr*A*gene as described below.

Results of Sequence and phylogenetic analysis

Sequences of the *gyr*A gene of *C. coli* isolates showed high variability among different isolates. Nucleotide and amino acid identities ranged from 78.4 to 100% (Fig. 1). Likewise, *C. jejuni* isolates had 79.2-98.7% and 80-97.6% nucleotide and amino acids identities, respectively. Meanwhile, similarity between *C. coli* and *C. jejuni* was 78.9-98.2% for the nucleotide and 78.7-98.4% for the amino acids (Fig. 1).

The nucleotide sequence alignment is shown in Fig. 2 while the amino acid sequence alignment is shown in Fig. 3. All isolates, regardless of the resistance to FQ, possessed the

resistant allele marker 86 isoleucine (86I) (Fig. 3). Nevertheless, a silent mutation (ATA to ATT) in residue 257 (coding for 86I) was observed in sequences from *C. coli* isolates AzEg6 and *C. jejuni* AzEg5 and AzEg10 (Fig. 3). Phylogenetic analysis of the generated sequences in this study indicated two genetic groups, designated here as clades A and B (Fig. 4). Clade A had eight *C. coli* isolates (AzEg 1, 2, 3, 4, 7, 8, 11, 12) and one *C. jejuni* (AzEg 9). This cluster was genetically close to *C. jejuni* and *C. coli* (>99% nucleotide identity) from commercial poultry flocks in Spain and *C. jejuni* from diarrheic patients in Japan (Fig. 4). Clade B had only one *C. coli* (AzEg 6) and two *C. jejuni* (AzEg 5, 10), and was genetically close to *C. coli* (>99% nucleotide identity) from human and domestic chickens in India, Japan and Italy (Fig. 4).

Three *C. jejuni* strains and 9 *C. coli* strains were randomly selected for sequencing of the QRDR of gyr*A* and compared with the closely related sequences using BioEdit version 7.1.7 ***(Hall, 1999)***. Maximum Likelihood mid-point rooted phylogenetic trees were generated using MEGA5 software ***(Tamura et al, 2011)***. The bootstrap values are shown on the node of each branch.

Table (2) *Number of resistance isolates of Campylobactercoli and jejuni*

antimicrobial Discs	**No. of resistance isolates**			
	C. coli		***C. jejuni***	
	No.	**%**	**No.**	**%**
Amikacin	0	0%	0	0%
Chloramphenicol	0	0%	0	0%
Tetracycline	2	6%	0	0%
Gentamicin	4	12%	0	0%
Erythromycin	5	15%	0	0%
Ampicillin	7	21%	2	67%
Ciprofloxacin	22	66%	2	67%
Ofloxacin	28	85%	3	100%
SXT	100	100%	3	100%

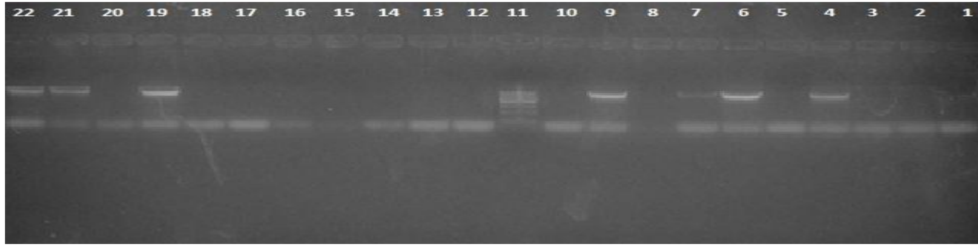

Photo. (1)agarose gel electrophoresis for tet (O) gene of 21 campylobacter spp. the positive amplification appeated at 559 bp, the lane no 11 represents the marker

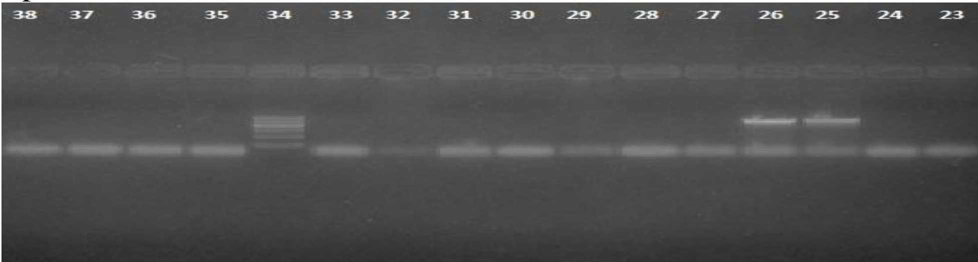

Photo. (2)agarose gel electrophoresis for tet (O) gene of 15 campylobacter spp. the positive amplification appeated at 559 bp, the lane no 11 represents the marker.

	AzEg 2	AzEg 3	AzEg 9	AzEg 8	AzEg 7	AzEg 4	AzEg 11	AzEg 1	AzEg 12	AzEg 5	AzEg 6	AzEg 10
AzEg 2	ID	100	98.2	98.9	98.7	99.4	98.7	97.9	98.7	79.2	78.7	78.9
AzEg 3	100	ID	98.2	98.9	98.7	99.4	98.7	97.9	98.7	79.2	78.7	78.9
AzEg 9	97.6	97.6	ID	98.7	98.7	97.6	98.9	97.1	97.9	79.4	78.9	79.2
AzEg 8	98.4	98.4	98.4	ID	98.9	98.4	99.2	97.4	98.2	79.2	78.7	78.9
AzEg 7	97.6	97.6	97.6	98.4	ID	98.2	99.2	97.9	98.7	79.4	78.9	79.2
AzEg 4	99.2	99.2	96.9	97.6	96.9	ID	98.2	98.2	99.2	78.9	78.4	79.4
AzEg 11	98.4	98.4	98.4	99.2	98.4	97.6	ID	98.2	98.4	79.4	78.9	79.2
AzEg 1	96.9	96.9	95.3	96.1	96.9	96.9	96.9	ID	98.9	79.2	78.7	79.2
AzEg 12	97.6	97.6	96.9	96.9	97.6	98.4	96.9	97.6	ID	79.2	78.7	79.7
AzEg 5	80.7	80.7	80.7	80.7	81.5	80.7	80.7	80.7	81.5	ID	97.4	98.7
AzEg 6	78.4	78.4	78.4	78.4	79.2	78.4	78.4	78.4	79.2	95.3	ID	97.6
AzEg 10	80	80	80	80	80.7	80.7	80	80	81.5	97.6	96.1	ID

Fig. (1). Identity matrices of GyrA from selected *Campylobacter* strains isolated in this study ($^{nucleotide/}$identical$_{/amino\ acid}$)

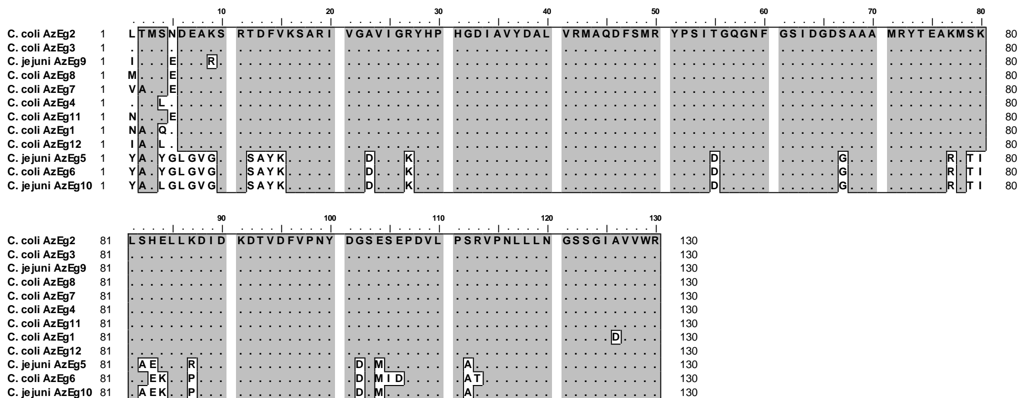

Fig. (2). Nucleotide sequnence alignment of gyrA gene from selected *Campylobacter* strainsisolated in this study

Fig. (3). Amino acid sequnence alignment of GyrA protein from selected *Campylobacter* strains isolated in this study

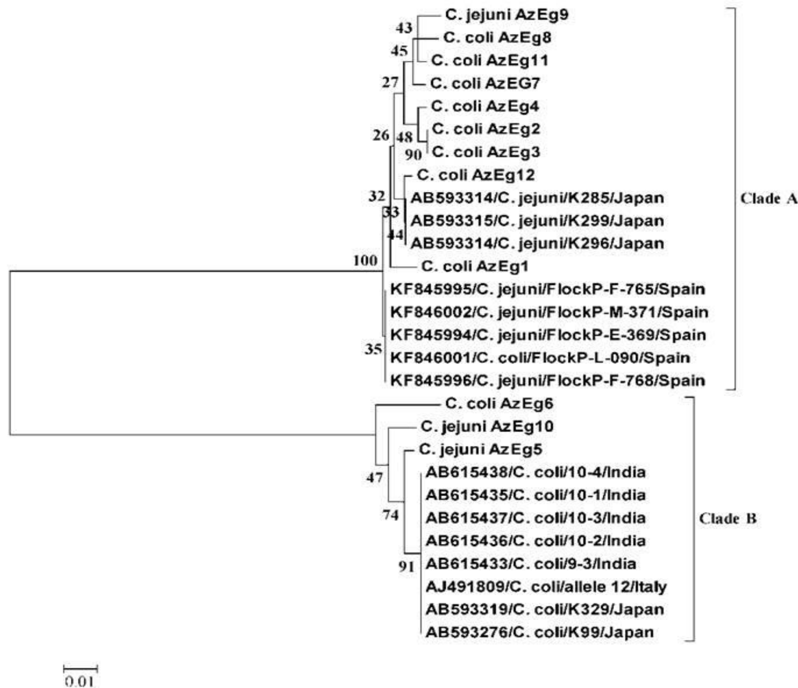

Fig. (4). Phylogenetic relatedness of *Campylobacter* spp. isolated from one-day old ducklings in Egypt

Discussion

In this study 150 meconium samples were collected from 1-day-old ducklings and examined for the presence of *Campylobacter* spp.; 36 (24%) samples were positive, which is similar to results obtained from ducks in slaughterhouses in Thailand ***(Boonmar et al, 2007).*** We also observed that the prevalence of *C. coli* was higher than *C. jejuni*, which is in accordance with those findings reported in commercial farmed ducks in Malaysia ***(Nor Faiza et al, 2013)*** but in contrast to the prevalence of *Campylobacter* spp. in domesticated ducks in the United Kingdom ***(Colles et al, 2011).*** *Campylobacter* infections in this study may have occurred due to vertical transmission or external contamination of eggs ***(Zhang, 2008).***

The majority of the Egyptian isolates tested herein were sensitive to amikacin, chloramphenicol, tetracycline, gentamycin and erythromycin, which is in accordance with previous reports on

poultry and human origin *Campylobacter* spp. isolates ***(Luber et al, 2003; Tsai and Hsiang, 2005; Luangtongkum et al, 2007; Wardak et al, 2007; Gu et al, 2009; Nonga and Muhairwa, 2010)***. Susceptibility of *C. coli* to FQ (ciprofloxacin and ofloxacin) was relatively low and all Egyptian isolates were resistant to SXT, which is similar to previous studies in Germany and Taiwan ***(Luber et al, 2003; Tsai and Hsiang, 2005).*** Moreover, 2/3 (67%) *C. jejuni* isolates were resistant to ampicillin, whereas in the USA 16.4% were resistant ***(Luangtongkum et al, 2007).***

Distribution of antimicrobial resistance, as well as putative antibiotic-resistance genetic markers, may be useful in the epidemiology of *Campylobacter* spp. infections ***(Randall et al, 2003).*** Increased antibiotic resistance is being reported in *C. jejuni*, particularly tetracycline and ciprofloxacin resistance ***(Nachamkin et al, 2000)***. The most important mechanism of tetracycline resistance in *Campylobacter* is the plasmid-mediated transfer of the *tet*(O) gene, which encodes the ribosomal protection protein ***(Gibreel et al, 2004; Mazi et al, 2008).*** By PCR screening, nine isolates possessed the *tet*(O) gene although only two isolates were resistant to tetracycline. The *tet*(O) gene has been reported in tetracycline sensitive *Campylobacter* spp. isolates in Canada ***(Gibreel et al, 2004).***

Resistance to FQ was mediated by the presence of one or more point mutations in the QRDR of *gyr*A, whereas most of these studies have analysed isolates mainly from chickens and human beings ***(Bachoual et al, 2001; Luo et al, 2003; Zhang and Plummer, 2008).*** In the current study, all randomly selected ducklings-isolates had isoleucine in position 86, commonly found in resistant *Campylobacter* spp. ***(Ruiz et al, 1998; Luo et al, 2003).*** Approximately all selected isolates but two were resistant to one or two of FQ; nevertheless two FQ-susceptible isolates also possessed the resistant marker, which was also found in a previous study (Bachoual et al., 2001). Other mutations infrequently linked to resistance to FQ (e.g. Thr86Lys, Ala70Thr, Asp90Asn, Val149Ile, Asn203Ser, Ala206Val and Ala206Thr) ***(Ruiz et al, 1998; Luo et al, 2003)*** were not observed in the current study. FQ-resistant *Campylobacter* carrying the resistant markers in the gyrA can be stably maintained in the absence of antibiotic selection pressure and may persist on poultry farms even after FQ withdrawal ***(Price et al, 2007).*** In ConclusionsOne-day-old ducklings are a potential source for antibiotic-resistant *Campylobacter* spp. Genetic markers linked to the antibiotic-resistance of *Campylobacter*, particularly to FQ,

may be useful but antibiogram is important.

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الكشف عن جينات المقاومه للمضادات الحيويه بعض عترات ميكروب الكامبيلوباكتر المعزوله من البطِ المصرى

انجى احمد حامد ، منى على عبد الحليم عبد الرحمن،ازهار جابر على ،مى محمود مرسى ، سعاد عبد العزيز ناصف

المعمل القومى للرقابه البيطريه على الانتاج الداجنى / معهد بحوث صحه الحيوان / وزاره الزراعه . دقى . جيزه . مصر

الملخص العربى

ميكروب الكامبيلوباكتركولاي والكامبيلوباكترجيجوناي قديسببا اضطرابات في الجهازالهضمي معاً وبدون التهاب الكبد الناخرفي الدواجن. ويسبب اضطرابات حاده في الجهازالهضمي لدي الانسان ايضا. ولايعرف سوى القليل عن انتشارميكروب الكامبيلوباكترفي البط وخاصة فراخ البط. في هذه الدراسة تم عزل ٣٦ (٢٤٪) عينه ايجابيه لميكروب الكامبيلوباكتر من اجمالي ١٥٠ عينة من فراخ البط عمريوم واحد في مصر. باستخدام الاختبارات البيوكيميائية و اختبار انزيم البلمره المتسلسله تم التعرف على ان ال٣٦ معزوله هى ٣٣الكامبيلوباكتر كولاي و ٣ والكامبيلوباكتر جيجوناي. وتم عمل اختبار الحساسيه للمعزولات باستخدام المضادات الحيوية باستخدام طريقه الانتشار (disk diffusion) ووجد انها كانت جميعها حساسة للكلورامفينيكول والأميكاسين ولكن مقاومة للسلفاميثازون-تراى ميثوبريم (SXT) . وكانت غالبية المعزولات مقاومه للتتراسيكلين والإريثروميسين،وفي الوقت نفسه كانت المقاومة لأوفلوكساسين وسيبروفلوكساسين عالية نسبيا. وتم التعرف على وجود جين tet(O) المسؤل عن مقاومه الميكروب للتتراسيكلين فى ٩ معزولات من اصل ٣٣ معزوله الكامبيلوباكتر كولاي ،على الرغم من اثنان فقط منها كانت مقاومة للتتراسيكلين.تحديدالمنطقة المقاومة للكينولون (QRDR) من gyrA من خلال اجراء اختبار تتابع الجينات وأظهرت النتائج ٦ طفرات جينية فى كامبيلوباكتر كولاى و كامبيلوباكتر جيجوناى والتي كانت مقاومة لكل من سيبروفلوكساسين و أوفلوكساسين.وتشيرهذه النتائج إلى أن فراخ البط قد تكون حامله ميكروب الكلمبيلوباكترالمقاومة المضادات الحيوية.