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
Campylobacter coli 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. All isolates 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 gyrA 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); gyrA

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 ºC 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 the organism 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 gyrA 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 and Fearnley, 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% \( \text{CO}_2 \), 5%\( \text{O}_2 \), 85% \( \text{N}_2 \)) 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 (mapA) of C. jejuni (Stucki et al, 1995) and siderophore binding protein, lipoprotein component of
enterocholin (ceuE) 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 gyrA as previously published (Lindmark et al, 2004). PCR products of the QRDR of gyrA 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.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Size (base pairs)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ceuE</td>
<td>CeuE F</td>
<td>AATTGAAAAATTGCTCAACTATG</td>
<td>462</td>
<td>Gonzalez et al. (1997)</td>
</tr>
<tr>
<td></td>
<td>CeuE R</td>
<td>TGATTATTATTATTATTAGTACGAG</td>
<td>462</td>
<td></td>
</tr>
<tr>
<td>mapA</td>
<td>MapA F</td>
<td>CTAATTATTATTATTATTAGTACGAG</td>
<td>589</td>
<td>Stucki et al. (1995)</td>
</tr>
<tr>
<td></td>
<td>MapA R</td>
<td>GCTTTATTGTACGAGTTTTATTATA</td>
<td>589</td>
<td></td>
</tr>
<tr>
<td>Tet(O)</td>
<td>DMT 1</td>
<td>GGCCTTTTGTATTGATGTCG</td>
<td>559</td>
<td>El-Adawy et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>DMT 2</td>
<td>ATGGACCAACCGACAGAAGG</td>
<td>559</td>
<td></td>
</tr>
<tr>
<td>QRDR</td>
<td>gyrA F</td>
<td>GATGGTTAAAAGGCGTTTAC</td>
<td>423</td>
<td>Lindmark et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>gyrA R</td>
<td>CGCCATACCTACAGCTATACC</td>
<td>423</td>
<td></td>
</tr>
</tbody>
</table>

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 ceuE and mapA 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 using PCR
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 gyrA gene as described below.

Results of Sequence and phylogenetic analysis
Sequences of the gyrA 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 gyrA 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 Campylobacter coli and jejuni

<table>
<thead>
<tr>
<th>antimicrobial Discs</th>
<th>C. coli No.</th>
<th>%</th>
<th>C. jejuni No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>2</td>
<td>6%</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>4</td>
<td>12%</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>5</td>
<td>15%</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>7</td>
<td>21%</td>
<td>2</td>
<td>67%</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>22</td>
<td>66%</td>
<td>2</td>
<td>67%</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>28</td>
<td>85%</td>
<td>3</td>
<td>100%</td>
</tr>
<tr>
<td>SXT</td>
<td>100</td>
<td>100%</td>
<td>3</td>
<td>100%</td>
</tr>
</tbody>
</table>
Photo. (1) agarose gel electrophoresis for tet (O) gene of 21 campylobacter spp. the positive amplification appeared 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 appeared at 559 bp, the lane no 11 represents the marker.

Fig. (1). Identity matrices of GyrA from selected Campylobacter strains isolated in this study (nucleotide/identical amino acid).
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; Mazì 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 gyrA, 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.

Acknowledgements
The authors would like to thank Dr E.M. Abdelwhab, Institute of Molecular Virology and Cell Biology, Friderich-Loeffler-Institut, InselRiems, Greifswald, Germany for supporting in writing the manuscript.

References


Lindmark, H., Harbom, B., Thebo, L., Andersson, L., Hedin,


الكشف عن جينات المقاومة للمضادات الحيوية بعض عترات ميكروب الكامبيلوباكتر المعزوله من البط المصرى.

المنى على عبد الحليم عبد الرحمن، ازهار جابر على، مى محمود مرسى، سعاد عبد العزيز ناصف، جينى أحمد حامد.

الملخص العربي:
ميكروب الكامبيلوباكتر كولاي والكامبيلوباكتر جيجوناي قد سببا اضطرابات في الجهاز الهضمي معاً.

الباحثون اعتمدوا على اختبارات تتابع (gyrA, ORDR) للتنتراسيكلين، حيث تم العثور على سبعة ظفرات جينية في كامبيلوباكتر كولاي والكامبيلوباكتر جيجوناي، وتشير هذه النتائج إلى أن فراخ البط قد تكون حاملة ميكروب الكلمبيلوباكترا المقاومة للمضادات الحيوية.