Virulence Repertoire and Antimicrobial Resistance of Campylobacter jejuni and Campylobacter coli Isolated from Some Poultry Farms in Menoufia Governorate, Egypt

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A B S T R A C T

Poultry are considered the primary source of Campylobacter spp. infections in people. We aimed to detect various virulence factors of Campylobacter spp., using PCR and evaluation of antimicrobial resistance patterns, in a total of 350 samples collected from chickens: 300 samples from dead birds with postmortem lesions, 50 from normal birds. Overall, 170/350 (48.57%) were culture positive for Campylobacter spp. Among these, 25 (14.7%) isolates were identified as C. jejuni and five (2.94%) as C. coli. All 25 isolates of C. jejuni were confirmed by the presence of 23S rRNA and the species-specific gene mapA; the five C. coli isolates were confirmed by the presence of ceuE. Simplex and multiplex PCR protocols were used to analyze the C. jejuni isolates for the presence of six putative virulence genes: the flagellum encoding gene flaA, the invasion-associated genes iamA and virB11, and the cytotoxin genes cdtA, B and C. These were identified in 3/25 (12%), 2/25 (8%), 3/25 (12%), 25/25 (100%), 0/25 (0.0%), and 0/25 (0.0%), respectively. Among the five C. coli isolates, two (40%) harbored virB11. The 30 Campylobacter isolates were classified into seven groups according to the exhibited antimicrobial resistance patterns, both species expressed high indices of antimicrobial resistance (0.67-0.89). The most effective antimicrobial against both species was amikacin while ciprofloxacin and doxycycline were effective against C. jejuni. Hence, both C. jejuni and C. coli isolated from diseased or healthy poultry constitute a public health concern because of the harbored virulence genes and high resistance to antimicrobials.

INTRODUCTION

Campylobacter spp. are Gram-negative, slender, spirally curved (0.2-0.8 µm x 0.5-5 µm) bacteria. Although most of the species are microaerophilic, some can grow anaerobically or aerobically, living as commensal organisms in the gastrointestinal tract of many domestic and wild birds, as well as in mammalian hosts (Bolton, 2015: Skarp et al., 2016). Recently, there are described 25 Campylobacter species. Poultry are the main reservoir for Campylobacter and broiler meat is a major vehicle for transmission to human (Batz et al., 2012: Golz et al., 2014; Martinez-Anton et al., 2018).

The symptoms of Campylobacter infections in young birds range from no clinical signs to clear signs of diarrhea and weight loss while the PM examination characterized by distention of the jejunum, disseminated hemorrhagic enteritis, and in some cases, focal hepatic necrosis. In addition, human infection distinguished by the following symptoms; abdominal pain, fever, headache, nausea, vomiting, diarrhea which is frequently bloody. The previous symptoms could last for 3 to 6 days moreover other complications as bacteraemia, hepatitis, pancreatitis and miscarriage have been found. Furthermore, reactive arthritis and neurological disorders such as Guillain-Barré syndrome, neurological and
respiratory dysfunction from a polio-like form of paralysis are post-infection complications that also reported (Sahin et al., 2015; Skarp et al., 2016).

The mechanism of C. jejuni immunopathogenesis is based on four main stages: adhesion to intestinal cells, colonization of the digestive tract, invasion of target cells, and production of toxins (Haddad et al., 2010). The flagellum encoding genes flaA and flaB control the major flagellin protein (Lerstethakarn et al., 2011). The Campylobacter adhesion is controlled by many genes as the (iamA) which has been identified in some strains of C. jejuni and C. coli (Carvalho et al., 2001). The type IV secretion system, possibly involved in adhesion, is encoded by virB11 (Dasti et al., 2010). Production of cytotoxins, especially the cytolytic distending toxins (CDTs), has been well investigated in Campylobacter species. Many Gram-negative bacteria have the ability to produce CDT (Ge et al., 2008), a tripartite toxin composed of three subunits encoded by genes cdtA, cdtB and cdtC. The iron uptake system is controlled by several genes including ceeE, which mediates a periplasmic binding protein. Mutants lacking these genes show a reduced ability to colonize chickens (Xu et al., 2010). The resistance of Campylobacter to multiple drugs, bile, and heavy metals is often mediated by active efflux pumps. Antimicrobial resistance in commensal bacteria develops on their exposure to specific antibiotics during carriage in broilers or other food animals or during infection in humans (Silva et al., 2011).

We aimed to analyze the virulence genes and antimicrobial resistance patterns of C. jejuni and C. coli isolated from some of the poultry farms in Menoufia, Egypt, to further understand the genotypic characters and pathogenesis of these notorious infectious agents.

**MATERIALS AND METHODS**

**Sampling and isolation:** A total of 300 samples (150 cecum, 100 duodenum, 50 bile) were collected from freshly dead chickens that had suffered distended gas-filled ceca, red spots on the duodenum wall, red-colored ingesta, and distended gallbladders. A further 50 samples (25 cecum, 25 duodenum) were collected from healthy birds without signs. Samples (1 g) were pre-inoculated in Bolton broth and incubated for 24 h at 42°C in microaerophilic conditions. A loopful of Bolton broth and kept at 80°C. The refreshed strains were identified on the basis of colony and cell morphology, motility, growth at 25°C and 42°C, catalase and oxidase tests, H₂S production on triple sugar iron agar slants, sensitivity to cephalothin and nalidixic acid, and sodium hippurate hydrolysis (in accordance with ISO 10272-1:2006 and ISO/TS 10272-2:2006 standards).

**PCR protocols:** In simplex PCR, primers for 23S rRNA, CeuE, mapA, flaA and iamA were used in a total reaction volume of 5 µl: Emerald Amp® GT PCR master mix (Takara, Japan) 12.5 µl, forward primer (20 pmol) 1 µl, reverse primer (20 pmol) 1 µl, template DNA 2 µl, and PCR-grade water 5.5 µl (Table 1).

In multiplex PCR, primers targeting the cytolytical distending toxins cdtA, B, and C were used in a total reaction volume of 50 µl: Emerald Amp® GT PCR master mix (Takara, Japan) 25 µl, forward primer (20 pmol) 2 µl, reverse primer (20 pmol) 2 µl, template DNA 8 µl, and PCR-grade water 13 µl PCR conditions were according to the references listed in Table 1.

**Antimicrobial susceptibility testing:** The bacterial counts were adjusted to a concentration of 1x10⁶ colony forming units per 1 mL using sterile Muller–Hinton broth and a spectrophotometer at wavelength 660 nm. From the adjusted concentrations, 1 mL aliquots were spread onto Muller–Hinton agar plates, which were then dried at 40°C for 20 min. Antibiotic discs (Oxoid) containing amikacin (AK) 30 mg, ampicillin (AM) 10 mg, ciprofloxacin (CIP) 5 mg, nalidixic acid (NA) 30 mg, lincomycin (MY) 10 mg, chloramphenicol (C) 30 mg, doxycycline (DO) 30 mg, cefotaxime (CTX) 30 mg, and trimethoprim/ sulphamethoxazole (SXT) 25 mg were distributed evenly on the agar surfaces and plates were incubated for 48 h under the same conditions conferred for isolation. The diameters of the inhibition zones were interpreted according to CLSI (2013).

**Table 1: Primer types, sequences, and length of amplified products**

<table>
<thead>
<tr>
<th>Target agent</th>
<th>Target gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Amplicone size</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Campylobacter</td>
<td>235 RNA</td>
<td>F:TTATCCCGGTIAAGGAGTTGGCATTGGAAG R:ATCTAACTACCTTTGCAAGCACC</td>
<td>650 bp</td>
<td>Wang et al., 2002</td>
</tr>
<tr>
<td>C. jejuni</td>
<td>mapA</td>
<td>F:CTATTTATTTTGGTACGGTGGTGTG</td>
<td>589 bp</td>
<td></td>
</tr>
<tr>
<td>Campylobacter</td>
<td>flaA</td>
<td>F:AATAAATAATGGCTATAAAGACGGT GT:RATCGGAAACCAGTGTCGCTGTTGATT</td>
<td>855 bp</td>
<td>Datta et al., 2003</td>
</tr>
<tr>
<td>iamA</td>
<td>F:GCGGCAATAATTATCTACCC C:R TTCACCGACTACCTACTATGCCG</td>
<td>518 bp</td>
<td>Wieczorek et al., 2012</td>
<td></td>
</tr>
<tr>
<td>cdtA</td>
<td>F:GTTTAAATCCCGCTGCTATAACCC GT:RGTGGCCACTTGGAATTTTGCAAAGGC</td>
<td>165 bp</td>
<td>Bang et al., 2003</td>
<td></td>
</tr>
<tr>
<td>cdtB</td>
<td>R:GGTTGAGCAGCAGTGAGCAGTGATT</td>
<td>495 bp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cdtC</td>
<td>F:TTGATGATAGCAGGGGAGATTTTAAAC R:R TGACCAAAACCACAA</td>
<td>555 bp</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Sample collection, isolation, and biochemical identification: Of the 350 collected samples, 170 isolates (48.57%) were gained on *Campylobacter* selective media. Of these, only 25/170 (14.7%) were biochemically identified as *C. jejuni* and 5/170 (2.94%) proved to be *C. coli*. While the remaining 140/170 (82.35%) isolates lacked the ability to be refreshed again (Table 2).

Molecular confirmation, virulence factors, and antimicrobial sensitivity patterns of the *Campylobacter* isolates: All the biochemically identified isolates 30/30 (100%) were confirmed as *Campylobacter* spp. from these isolates 25/25 (100%) were identified as *C. jejuni* after detection of the 23S rRNA and mapA. Among the *C. jejuni* isolates, the major flagellin protein flaA was present with a rate of 3/25 (12%) and the invasion-associated marker iamA exhibited a distribution pattern of 2/25 (8%), (Table 3). In addition, the virB11, which is associated with invasiveness, was detected in 3/25 (12%) of *C. jejuni*. Cytotoxin A (cdtA) showed the highest frequency among virulence genes and was present in all isolates of *C. jejuni* (100%). The five biochemically identified *C. coli* isolates were confirmed by the presence of the *cenE* and harbored the virB11 with a rate of 2/5 (40%).

The data presented in (Table 3) elucidated that all the 30 *Campylobacter* isolates were classified into seven groups (a-g) according to the obtained antimicrobial resistance patterns. The group (a) which contained 8 *C. jejuni* isolates those were sensitive to amikacin and expressed an intermediate response to ciprofloxacin, while the group (b) composed of 3 *C. jejuni* isolates which were sensitive to amikacin and ciprofloxacin but gave an intermediate response to doxycycline. Moreover, the group (c) included 5 *C. jejuni* isolates that were sensitive to amikacin, and group (d) contained 3 *C. jejuni* isolates which were intermediate to doxycycline. Furthermore, the group (e) composed of 3 *C. jejuni* isolates which expressed sensitivity to doxycycline while the group (f) contained 3 *C. coli* isolates which expressed sensitivity to amikacin. Added to that, the group (g) composed of 5 *C. coli* isolates which were sensitive to amikacin. Isolates of both *C. jejuni* and *C. coli* exhibited high antimicrobial resistance indices that ranged from 0.67 to 0.89.

Ethical considerations: This study was performed according to the recommendations of the Guide to U.S. Government Principles dealt with the issue of care about and utilization of vertebrate animals in research and testing. The research protocol accepted by the Institutional Animal Care and Use Committee (IACUC) at the Faculty of Veterinary Medicine, University of Sadat City.

### Statistical analysis:

The isolation rates of *Campylobacter*, the frequency of the obtained species and the sensitivity and resistance of isolates to antimicrobials were presented as percentages (%). The Multiple Antibiotic Resistance (MAR) Index was displayed as a percentage of effective antimicrobials to the total used types. The obtained isolates were classified into 7 groups according to the obtained Multiple Antibiotic Resistance (MAR) which ranged from 0.67 to 0.89.

### RESULTS

#### Table 2: Number of collected samples, obtained isolates on charcoal-cefoperazone-deoxycholate agar, and biochemical identification

<table>
<thead>
<tr>
<th>Chickens</th>
<th>No. of cases</th>
<th>Collected organs</th>
<th>No. of isolates</th>
<th>Biochemical confirmation</th>
<th>VBNC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>350</td>
<td></td>
<td>170 (48.57%)</td>
<td>25/25 (100%)</td>
<td>140/170 (82.35%)</td>
</tr>
<tr>
<td>Dead with PM lesions</td>
<td>300</td>
<td>Cecum</td>
<td>150</td>
<td>80 (53.3)</td>
<td>9</td>
</tr>
<tr>
<td>Cases</td>
<td>50</td>
<td>Duodenum</td>
<td>25</td>
<td>20 (80)</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>350</td>
<td></td>
<td>170 (48.57%)</td>
<td>25/25 (100%)</td>
<td>140/170 (82.35%)</td>
</tr>
</tbody>
</table>

The other isolates were not refreshed due to the viable but non-culturable state (VBNC) due to unfavorable oxygen rich conditions.

#### Table 3: Molecular confirmation, virulence factors, and antimicrobial sensitivity patterns of 25 confirmed *C. jejuni* and five *C. coli* isolates

<table>
<thead>
<tr>
<th>Groups</th>
<th>235 rRNA</th>
<th>C. coli</th>
<th>C. jejuni</th>
<th>Virulence genes</th>
<th>AM, NA, MY, C, DO, CTX, SXT.</th>
<th>MAR index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group (a)</td>
<td>100% (0.0%)</td>
<td>100% (0.0%)</td>
<td>37.5% (25%)</td>
<td>100% (0.0%)</td>
<td>AM, NA, MY, C, DO, CTX, SXT.</td>
<td>0.78</td>
</tr>
<tr>
<td>Group (b)</td>
<td>100% (0.0%)</td>
<td>100% (0.0%)</td>
<td>33.3% (100%)</td>
<td>100% (0.0%)</td>
<td>AM, NA, MY, C, DO, CTX, SXT.</td>
<td>0.89</td>
</tr>
<tr>
<td>Group (c)</td>
<td>100% (0.0%)</td>
<td>100% (0.0%)</td>
<td>20% (100%)</td>
<td>100% (0.0%)</td>
<td>AM, NA, MY, C, DO, CTX, SXT.</td>
<td>0.89</td>
</tr>
<tr>
<td>Group (d)</td>
<td>100% (0.0%)</td>
<td>100% (0.0%)</td>
<td>0% (0.0%)</td>
<td>100% (0.0%)</td>
<td>AM, NA, MY, C, DO, CTX, SXT.</td>
<td>0.89</td>
</tr>
<tr>
<td>Group (e)</td>
<td>100% (0.0%)</td>
<td>100% (0.0%)</td>
<td>33.3% (100%)</td>
<td>100% (0.0%)</td>
<td>AM, NA, MY, C, DO, CTX, SXT.</td>
<td>0.89</td>
</tr>
<tr>
<td>Group (f)</td>
<td>100% (0.0%)</td>
<td>100% (0.0%)</td>
<td>0% (0.0%)</td>
<td>100% (0.0%)</td>
<td>AM, NA, MY, C, DO, CTX, SXT.</td>
<td>0.89</td>
</tr>
<tr>
<td>Total</td>
<td>25/25 (100%)</td>
<td>100% (12%)</td>
<td>12% (100%)</td>
<td>100% (0.0%)</td>
<td>AM, NA, MY, C, DO, CTX, SXT.</td>
<td>0.89</td>
</tr>
<tr>
<td>Group (g)</td>
<td>100% (0.0%)</td>
<td>100% (12%)</td>
<td>12% (100%)</td>
<td>100% (0.0%)</td>
<td>AM, NA, MY, C, DO, CTX, SXT.</td>
<td>0.89</td>
</tr>
</tbody>
</table>
C. jejuni is a normal inhabitant of the intestinal tract of a wide variety of wild and domestic animals. In poultry, contamination of retail products occurs through de-feathering, evisceration, and dipping during the slaughtering process (Di Giannatale et al., 2010). C. jejuni colonizes the mucus overlying epithelial cells primarily in the cecum and small intestine of chickens, but can also be recovered from elsewhere in the gut and from liver and spleen (Lamb-Rosteski et al., 2008). From our data on isolation, biochemical testing, and molecular confirmation using 23S rRNA and mAP4A, the commonest isolate was C. jejuni (25/170, 14.7%) similar to the findings of Van Deun et al. (2007) and El-Jakee et al. (2015) in Belgium and Egypt respectively. Furthermore, C. jejuni is considered a leading cause of enteric illness in many western countries, developing countries, and the European Union (Wagenaar et al., 2013). Moreover, C. jejuni is strongly linked with Guillain-Barre syndrome, an autoimmune syndrome that may result in respiratory and severe neurologic dysfunction and could be fatal in the bad circumstances (Haddad et al., 2010). Although, there is obtained 170 isolates which were kept at -80°C; not all isolates were refreshed. There is a difference between the high rate of early isolation of Campylobacter on mCCDA medium and the lowered rate of biochemically identified isolates. This result could be regarded to the viable but non-culturable state (VBNC) of Campylobacter due to unfavorable conditions rich in oxygen, that lead to the failure of the culture techniques for refreshment of preserved isolates (Zhao et al., 2017). The virulence and survival factors of C. jejuni depend on motility, adhesion, colonization, invasion, toxin production, iron acquisition, and antimicrobial resistance. In this study, we used PCR based on known genetic sequences to explore a subset of putative C. jejuni virulence-associated genes that play a vital role in infection. The frequency of flaA, a major flagellin gene, was 3/25 (12%), lower than that determined by El-Jakee et al. (2015) in Egypt who found that the frequency of the flaA was 35.75%. Regarding iamA, which has been designated an invasion-associated marker in some C. jejuni and C. coli strains (Carvalho et al., 2001; Bolton, 2015), this gene was confirmed in 2/25 (8%) of C. jejuni isolates, lower than the findings of Wieczorek and Osek (2008). The occurrence of the virB11 gene was a marker for the plasmid pVir, which is associated with invasiveness. It should be noted that the distribution pattern of virB11 in the C. jejuni isolates was 3/25 (12%) which was higher than that determined by González-Hein et al. (2013) and closely similar to the findings of Van Deun et al. (2013). Concerning the existence of cytotoxin genes, our study confirmed that cdtA was present in all the C. jejuni isolates, confirming the widespread of cytotoxin genes in poultry isolates as demonstrated by Rozynek et al. (2005) and Van Deun et al. (2007). Moreover, their data suggested that the clinical outcome is dependent on production of cytotoxins and other virulence factors. The participation of the cdtA gene in the development of infection in chickens appears to be significant, due to its high existence rate (100%).

The serious problem of Campylobacter multidrug resistance is basically mediated by active multidrug efflux pumps (Silva et al., 2011). From our results, it was clear that most of the C. jejuni isolates were characterized by the high resistance to ampicillin, cefotaxime, chloramphenicol, doxycycline, lincomycin, nalidixic acid, and trimethoprim/ sulphamethoxazole. The apparent MAR index was high in all seven groups of Campylobacter, according to the obtained resistance patterns with values ranged from 0.67 to 0.89. The high frequency of resistance to ampicillin and other beta lactame antimicrobials has been confirmed by Stef et al. (2013), who linked this type of resistance to the overproduction of beta-lactamases. Although, some groups of Campylobacter expressed sensitivity to amikacin, ciprofloxacin, and doxycycline, there is encountered resistance to ciprofloxacin, nalidixic acid, and doxycycline in other groups which comes in consistence with Wieczorek et al. (2012). The prominent susceptibility to amikacin expressed in some groups agree with El-Jakee et al. (2015). The overall high frequency of antimicrobial resistance in Campylobacter spp. represents a serious public health concern. The rational interpretation of this crisis is the frequent encountering of specific antimicrobials during the commensal carriage of Campylobacter spp. in chickens and large animals or during human infections. There is strong evidence linking the uncontrolled use of antimicrobials in animal production with the emergence and widespread of resistance in Campylobacter spp. (Silva et al., 2011).

Conclusions: Campylobacter isolates from diseased and normal poultry cases harbored many virulence and cytotoxin genes that are crucial in the pathogenesis of this infectious agent. Our study demonstrated the high cytotoxicity and antimicrobial resistance of C. jejuni and C. coli, confirming that both species are serious and notorious infectious hazards of public health concern. Moreover, from our conclusions it was clear that there is an urgent need for implementation of stringent control, public health, and food protection strategies. Our results call for continuous monitoring and effective vaccine formulation strategies for lowering the excessive use of antimicrobials and reducing the problem of antimicrobial resistance.

Authors contribution: Mohamed Sabry Abd Elraheem Elsayed and Reda Tarabees were the leaders of this study they planned, monitored, and evaluated the research steps. They helped also in sampling, isolation, genotyping, antimicrobial susceptibility testing, writing, revising the manuscript, and data analysis. Ola Harb and Ahmed Sabry helped with sampling, isolation, and most of the genotyping. Awad Shehata helped with conceptualization of the study, provided some technical advice, and helped with data analysis.

REFERENCES


