Resistance of *Campylobacter jejuni* Isolated from Layer Farms in Northern Jordan Using Microbroth Dilution and Disc Diffusion Techniques

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Abstract: *Campylobacter jejuni* is an important pathogen of significant public health importance. This pathogen is associated with human infection and has been isolated from mammals and birds. Ninety-two cloacal *C. jejuni* isolates were identified from 35 layer farms in Northern Jordan. Antimicrobial susceptibility was determined using minimal inhibitory concentration (MIC) and disc diffusion techniques with variable suggested breakpoints. Using MIC and EUCAST cut-off values, the study revealed a significantly high resistance level (100%) among the layers' isolates against ciprofloxacin and tetracycline. A relatively high resistance (41%) toward gentamicin and amoxicillin and low resistance to nalidixic acid (21%), erythromycin (14%), and florfenicol (6.5%) were also found. This high level of resistance may indicate abuses in the handling of antibiotics, which may require stricter control in the local layer industry. A good agreement between the 2 techniques used was demonstrated and the disc diffusion technique could be used as a rapid screening test for antimicrobial susceptibility of *C. jejuni* to many antibiotics using specific *Campylobacter* breakpoints.

Keywords: Antimicrobial susceptibility, *Campylobacter jejuni*, Jordan, Layer farms

Practical Application: Layer chicken meat and their eggs are subject to *Campylobacter* contamination and may present a source of infection to handlers and consumers. The abuse of antibiotics in layer chicken farms may lead to resistance of animal-origin human pathogens and potentially be reflected in human campylobacteriosis treatment. *C. jejuni* of chicken origin is quite resistant to most commonly used antibiotics and the careful selection of correct antibiotic treatment is necessary.

Introduction

Poultry flocks are considered natural hosts of Campylobacter spp. with worldwide prevalence ranging from 10% to 82%. *Campylobacter* spp. are a leading cause of bacterial gastroenteritis in humans with about 400 million cases of campylobacteriosis being reported annually worldwide by the European Food Safety Authority (EFSA) (EFSA 2006). However, due to underreporting, the true incidence is estimated to be 10 times higher than what is documented (Gibreel and Taylor 2006). The majority of campylobacteriosis cases are self-limiting; however, in prolonged cases or bacteremia, the macrolides, fluoroquinolones, or gentamicin are the drugs of choice. Resistance of *C. jejuni* to tetracycline, erythromycin, ciprofloxacin, kanamycin, nalidixic acid, and chloramphenicol has been reported (Luangtongkum and others 2007). Antibiotics resistance in *Campylobacter* isolates from both humans and animals against ciprofloxacin and fluoroquinolones is continuously increasing (de Jong and others 2009; Geenen and others 2011). In the Netherlands, approximately 50% of the *Campylobacter* isolates from humans were resistant to ciprofloxacin compared with 35% in the period 2002 to 2005 (Geenen and others 2011). In Europe, a steady increase in fluoroquinolone resistance in *Campylobacter* has been observed in many countries (de Jong and others 2009). Results from EU Member States show that the antimicrobial resistance of *C. jejuni* against fluoroquinolones in poultry ranges from 16.7% in Denmark up to 100% in Hungary and Latvia (Geenen and others 2011). In developing countries, the unrestricted use of antimicrobials in both human and veterinary medicine has initiated an ongoing debate regarding the use of these drugs in livestock. There is a scarcity of data on *C. jejuni* resistance rates in developing and Middle Eastern countries even though *Campylobacter* remains a major cause of acute enteric diseases in these countries (Moore and others 2006). Talhouk and others (1998) tested *Campylobacter* isolates from diarrheic human stools, chicken caeca, and raw chicken carcasses in Lebanon. Most of these isolates had high to moderate susceptibility to gentamicin (97%), amoxicillin clavulanate (95%), clindamycin (77%), chloramphenicol (77%), and ampicillin (69%). However, some isolates showed lower susceptibility against tetracycline (49%), erythromycin (47%), ciprofloxacin (39%), and norfloxacin (36%). In Jordan, *C. jejuni* was isolated from 0.9% of 1400 diarrheal Jordanian patients and 77% exhibited resistance toward cotrimoxazole (Battikhi 2002).

In a local study on 38 broiler isolates, 21 isolates (55%) showed multidrug resistance and the remaining isolates (17%) were totally sensitive to the tested drugs (erythromycin, gentamycin, norfloxacin ciprofloxacin, doxycycline tetracycline, tilmicosin, amoxicillin, chlortetracycline, and trimethoprim). Of the 21 multidrug-resistant isolates, 2 were resistant to only 2 antibiotics (amoxicillin and trimethoprim), 3 were resistant to 9 antimicrobials, and the other 16 isolates were resistant to all tested antimicrobials (Osaili and others 2012).

Several comparisons between antimicrobial susceptibility testing methods, including broth microdilution, agar dilution, disc

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diffusion test, and the epsilometer test (*E*-test), have been reported to measure antimicrobial resistance in *Campylobacter* spp. with contraversal results (Ge and others 2002; McDermott and others 2005; Luangtongkum and others 2007; Miflin and others 2007; Lehtopolku and others 2012). The objectives of this work are to present the first results of an antimicrobial susceptibility assay of local *C. jejuni* isolated from layer farms and compare resistance patterns using both microbroth dilution and disc diffusion techniques with variable suggested breakpoints.

Materials and Methods

Campylobacter jejuni isolates

The method described by the International Organization for Standardization (ISO) 10272-1 (2006) was followed for the isolation and identification of *C. jejuni*. There are 35 separate layer chicken farms of mainly the Lohmann breed operating in Irbid Governorate (Northern Jordan), which comprise 43 houses. Cloacal swabs were collected from layers in all 43 houses, where each 1000 birds were represented by 5 swabs from 5 randomly selected birds. Samples were transported to our Food Research Laboratory under sterile cool condition and kept refrigerated (8 °C) to be examined within 6 to 8 h. Swabs were enriched using Bolton broth (Oxoid, U.K.) followed by plating on selective mCCD agar (Oxoid, U.K.). *C. jejuni* was confirmed morphologically and biochemically (catalase, oxidase, motility, microaerophilic growth at 25 °C, growth at 41.5 °C under aerobic conditions, DRYSPOT agglutination, and Hippurate hydrolysis) as described by Donnision (2003) and ISO (2006). Isolates were kept at −80 °C in nutrient broth (Oxoid, U.K.) with 15% glycerol and subcultured after thawing on Columbia Blood Agar (Oxoid, U.K.) as outlined by Gorman and Adley (2004). Subcultured isolates were stored at refrigerator temperature during processing and for no more than 3 d.

Ninety-two isolates were confirmed molecularly as *C. jejuni* using PCR techniques targeting the putative oxidoreductase subunit in the *C. jejuni* genome. The primers pair F (5′- CAA ATA AAG TTA GAG GTA GAA TGT-3′), R (5′- GGA TAA GCA CTA GCT AGC TGA T-3′) (Alpha DNA, Montreal, Canada) were used to amplify a 160 bp oxidoreductase DNA segment as described by Nayak and others (2005).

Antimicrobials susceptibility tests

The minimal inhibitory concentrations (MIC) of 9 antimicrobials were tested by the broth dilution method as described by the Clinical and Laboratory Standards Institute (CLSI 2008). Standardized concentrations of antimicrobial agents were added to sterile microarray wells and diluted in serial 2-fold dilutions across the plates. Stock solutions of antimicrobials were prepared at a concentration of 1280 μg/mL, or 40 times of the highest used concentration (CLSI 2008). Fifty microliters of the working dilution of each antimicrobial was serially diluted in 50 μL Mueller–Hinton Broth (Oxoid, UK) to obtain a 2-fold dilution series starting at 0.5 and up to 24 μg/mL. Cultures were prepared in saline to turbidity equal to McFarland 0.5 and then diluted onto Mueller–Hinton broth with 5% defibrinated horse blood (Oxoid, UK) to obtain an inoculum of approximately 10^5 CFU/mL and 100 μL of inoculum was pipetted into each well. MICs were defined as lowest concentrations in wells with no visual growth. Susceptibility profiles were determined by comparing the calculated MIC values to published breakpoints for each tested antimicrobial.

Table 1–Quality control ranges for *Campylobacter jejuni* ATCC 33560 grown at 42 °C for 24 h using Muller– Hinton broth with lysed horse blood.

Antibiotic	*C. jejuni* ATCC 33560
Amoxicillin	0.25–2.0
Ampicillin	1.0–4.0
Florfenicol	0.5–2.0
Ciprofloxacin	0.25–1.0
Doxycycline	0.12–0.5
Erythromycin	0.25–2.0
Gentamicin	0.25–2.0
Nalidixic acid	0.25–1.0
Tetracycline	0.25–1.0

The EUCAST disc diffusion methodology for *C. jejuni* was also followed to determine the susceptibility or resistance level. For the disc diffusion method, the inoculum equivalent to McFarland 0.5 turbidity (Oxoid, U.K.) was transferred onto Mueller–Hinton plates with 5% defibrinated horse blood using cotton-tipped swabs to produce a confluent lawn of bacterial growth. Once the inoculum was dried on the plates, antibiotic discs were distributed over them. Then, the plates were incubated at 42 °C for 24 h under microaerobic conditions. The zone diameter was measured with slipping calipers. MIC and disc diffusion tests of each antimicrobial were performed 3 times for each antimicrobial and isolate. For comparison, *C. jejuni* ATCC 33560 was used as the control organism.

The antimicrobials examined represent those commonly used in Jordan's poultry industry: ampicillin, erythromycin-SCN, gentamicin sulfate, ciprofloxacin, doxycycline HCl, tetracycline HCl, amoxicillin $3H_2O$, florfenicol, and nalidixic acid. These antibiotic powders were obtained from Tocelo Chemicals (Holland) through Arab Veterinary Industries Company (AVICO, Jordan), and nalidixic acid from Oman Biochem and Pharmaceuticals LLC (Oman) through Dar Al Dawa Company (Jordan).

All antibiotics discs were obtained from Arab Company for Medical Diagnostics, Jordan (ARCOMEX), except for nalidixic acid (Bioanalyse, Turkey).

Quality control and antimicrobial breakpoints

The quality control ranges of antibiotic resistance of the reference strain *C. jejuni* ATCC 33560 used in this study are presented in Table 1. From EUCAST (2012, 2016), the proposed zone diameters for defining the disc diffusion breakpoints for *Campylobacter* are only available for ciprofloxacin, erythromycin, tetracycline, and doxycycline. For the other antimicrobials, breakpoint values of the Antibiogram Committee of the French Society for Microbiology (Comite De L'antibiogramme De La Societe Francaise De Microbiologie Recommendations 2012) were used (Table 2). The EUCAST (2012, 2016) cut-off values for the microdilution method used in this study are also presented in Table 2.

Statistical analysis

The correlation and level of agreement between the standardized broth dilution method and the agar disc diffusion method were calculated using percentage agreement and kappa statistic (Viera and Garrett 2005) using IBM SPSS Statistics.

Results

Using the microdilution test, no significant variations were observed between the 3 replicates for each antibiotic or isolate. Coefficient of variation was 0.0% to 4.0%. For disc diffusion readings for all compounds or strains there was a substantial variation

Table 2–Antibiotic concentration and sensitivity breakpoints according to French Antibiogram committee and EUCAST.

	Sensitivity breakpoint according to French Antibiogram Committee[a]		Cut-off values according EUCAST[b]	
Antibiotic concentration	**MIC (μg/mL)**	**Disc (mm)**	**MIC (μg/mL)**	**Disc (mm)**
Amoxicillin(25 μg)	≤4	≥21	≤16	–
Ampicillin (10 μg)	≤4	≥19	≤8	–
Florfenicol (30 μg)	≤8	–	≤4	–
Ciprofloxacin (5 μg)	≤0.5	≥25	≤0.5	≥26
Doxycyclin (30 μg)*	≤4	≥19	≤0.5	≥30
Erythomycin (15 μg)	≤1	≥20	≤4	≥22
Gentamicin(10 μg)	≤2	≥18	≤2	–
Nalidixic cid (30 μg)	≤8	≥20	≤16	–
Tetracycline(30 μg)	≤4	≥19	≤2	≥30

*General values, not specific for *Campylobacter* (Soussy and others 1994) (–): Not available
[a]Comite De L'antibiogramme De La Societe Francaise De Microbiologie Recommendations (2012).
[b]EUCAST (2012,2016).

(up to 7%) between the repetitions. The mean value of the coefficient of variation for each tested antimicrobial was close to 10%.

Based on results from broth microdilution technique and EUCAST sensitivity cut-off values (≤ mg/L), all 92 isolates (100%) were completely resistant to ampicillin, tetracycline, and ciprofloxacin, and 95% were resistant to doxycycline. In addition, 41% of the isolates were also resistant to amoxicillin and gentamicin and 21% to nalidixic acid while 14% and 6.5% were resistant to erythromycin and florfenicol, respectively (Table 3). EUCAST-Disc diffusion cut-off values are given for the 4 antibiotics (tetracycline, doxycycline, ciprofloxacin, and erythromycin) and their calculated resistance percentages for the studied isolates were 100, 100, 100, and 66, respectively. Highly significant statistical correlations were calculated for tetracycline and ciprofloxacin, but not for erythromycin, between the 2 techniques (Table 3).

Results of disc diffusion, MICs techniques along with the French breakpoints are presented in Table 4. Using the breakpoint values of the disc diffusion method, 100% of the isolates were resistant to ampicillin, ciprofloxacin, and nalidixic acid and 95% were resistant to tetracycline. However, only 16% were resistant to doxycycline; 63% to erythromycin; 53% to amoxicillin; and 22% to gentamicin. Using the broth dilution technique, there was 100% resistance to ampicillin, ciprofloxacin, and tetracycline, with 70% resistance to erythromycin and 50% to amoxicillin suggesting good agreement between the 2 techniques. However, higher resistance values were found for doxycycline (41%), gentamicin (38%), and nalidixic acid (63%) using MIC, with no significant correlation between the 2 techniques (Table 4). All isolates tested in the current study are multidrug-resistant (resistant to ≥3 antibiotics).

Discussion

This study revealed a significantly higher level of resistance of local *C. jejuni* isolates to most antibiotics under study. These resistance levels recorded for layer isolates are much higher than those reported in a previous local study of broiler isolates (Osaili and others 2012). This might be related to the longer life span of layers and, thus, an increased probability of exposure to antimicrobials. It also corroborates other observations, where significant differences in MIC_{50} of gentamycin, ciprofloxacin, and tetracycline between broiler and layer *Campylobacter* isolates are reported (Bester and Essack 2008).

Several countries have reported increasing resistance rates for fluoroquinolones, but not for macrolides or tetracycline (Gaudreau and Gilbert 2003). In France, however, the resistance of human isolates to fluoroquinolones has decreased in association with similar trends noticed in poultry and pig isolates (Gallay and others 2007). The rapid emergence of fluoroquinolones-resistant *Campylobacter* worldwide may be partially attributed to the enhanced fitness of this pathogen and related to the broad use of fluoroquinolones in veterinary medicine, especially in poultry. The resistance patterns for fluoroquinolones and tetracyclines reported in this study are significantly higher than antimicrobial resistance reported in the European Union (EFSA 2006). Specifically, EFSA recorded *Campylobacter* isolated from 20% to 64% of poultry, other meats, and animals were resistant to fluoroquinolones. Broad spectrum and inexpensive tetracyclines are extensively used in poultry, livestock, and human medicines. Therefore, it is not surprising to find high resistance rates in Jordan (this study), the United Kingdom (Piddock and others 2008), the United States (Son and others 2007), Spain and Japan (Itoh and others 1984), and Australia (Pratt and Korolik 2005). All isolates tested in this study exhibit resistance to tetracyclines (doxycycline, tetracycline). High incidences are generally recorded in the United States for conventionally and organically grown chickens and in Canada, where more than 50% of *C. jejuni* isolates from chickens were resistant to tetracycline (Deckert and others 2010). In addition, the high resistance to enrofloxacin (100%) reported here does not correlate with the low incidence of resistance (7%) reported in Sweden (Lindmark and others 2004) or 29.5% in Japan (Haruna and others 2012).

Most *C. jejuni* usually produces β-lactamases; therefore, it is traditionally not treated with β lactams. The 100% resistance to ampicillin found in our study agrees with that found in clinical and environmental isolates in developing countries (Baserisalehi and others 2005; Khorsavi and others 2011). Lower resistance is cited in developed locations, such as Australia, Germany, Canada, and Ireland (46%, 9.1%, 22%, and 33%, respectively) (Oza and others 2003; Guévremont and others 2006; Unicomb and others 2006; Döhne and others 2012). High resistance rates are generally reported toward amoxicillin (Bester and Essack 2008) which is close to the 40% to 50% resistance reported in this study.

Worldwide, resistance to aminoglycosides is generally very low because gentamicin is rarely used in the poultry industry and, hence, resistance to gentamicin has not been reported in many countries (Wardak and others 2007; Deckert and others 2010; Zhao and others 2010). However, gentamicin is used locally in veterinary medicine practices and, therefore, this study reports a 20% to 40% resistance rate, which agrees with the 19% and 26% reported in South Africa and India, respectively (Baserisalehi and others 2005; Bester and Essack 2008).

Campylobacter resistance to macrolides is of public health importance because erythromycin is the drug of choice for treating human campylobacteriosis. High resistance to erythromycin is usually linked to high levels of tylosin used in the poultry industry (Iovine and Blaser 2004), and this may explain the 14% to 70% resistance level (depending on the method used) observed in our isolates. High resistance percentages were also reported by Bester and Essack (2008) and Baserisalehi and others (2005), although recent reports from developed countries show complete susceptibility toward erythromycin (Wardak and others 2007; Deckert and others 2010; Döhne and others 2012).

Table 3–Agreement between the MIC and disc diffusion antimicrobial resistance values of 92 *Campylobacter jejuni* isolates using EUCAST breaking points values.

Antibiotic	Resistant by both methods	Susceptible by both methods	No. (%) of resistance by disc diffusion	No. (%) of resistance by MIC	No. of isolates with agreement in both methods	% Agreement between both methods	Kappa[a]
Amoxicilin	–	–	–	37 (40.6)	–	–	–
Ampicillin	–	–	–	92 (100)	–	–	–
Florfenicol	–	–	–	6 (6.5)	–	–	–
Ciprofloxacin	92	0	92 (100)	92 (100)	92	100	1
Doxycycline	–	–	92 (100)	87 (95)	87	94.7	0.90
Erythromycin	13	31	61 (66.3)	13 (14.1)	44	47.8	0.15
Gentamicin	–	–	–	38 (41)	–	–	–
Nalidixic Acid	–	–	–	19 (21)	–	–	–
Tetracycline	92	0	92 (100)	92 (100)	92	100	1

(–): Not calculated (no breaking points available).
[a]The magnitude of kappa indicates the level of agreement between the 2 tests as follows: 0.01 to 0.20 slight agreement, 0.21 to 0.40 fair agreements, 0.41 to 0.60 moderate agreement, 0.61 to 0.80 substantial agreement, 0.81 to 0.99 almost perfect agreement.

Table 4–Agreement between the MIC and disc diffusion antimicrobial resistance values of 92 *Campylobacter jejuni* isolates using French breaking points.

Antibiotic	Number of isolates susceptible by both methods	Number of isolates resistant by both methods	No. (%) of resistance by disc diffusion	No. (%) of resistance by MIC	No. of isolates with agreement in both methods	% agreement between both methods	Kappa[a]
Amoxicilin	43	46	49 (53)	46 (50)	89	96.7	0.93
Ampicillin	0	92	92 (100)	92 (100)	92	100	0.97
Florfenicol	–	–	–	87 (94.6)	–	–	–
Ciprofloxacin	0	92	92 (100)	92 (100)	92	100	0.97
Doxycycline	54	15	15 (16)	38 (41.3)	69	75	0.43
Erythromycin	28	58	58 (63)	64 (69.6)	86	93.5	0.85
Gentamicin	57	20	20 (22)	35 (38)	77	83.7	0.62
Nalidixic acid	0	58	92 (100)	58 (63)	59	64	0.63
Tetracycline	0	87	87 (94.6)	92 (99.6)	87	94.6	0.95

(–): Not calculated (no breaking points available).
[a]The magnitude of kappa indicates the level of agreement between the 2 tests as follows: 0.01 to 0.20 slight agreement, 0.21 to 0.40 fair agreements, 0.41 to 0.60 moderate agreement, 0.61 to 0.80 substantial agreement, 0.81 to 0.99 almost perfect agreement.

The lowest resistance pattern recorded in this study was toward florfenicol (6.5%), which was calculated according to the EUCAST cut-off values. This trend parallels the very low resistance percentages (0.0 to 2.6) reported for chloramphenicol (Deckert and others 2010) and nonresistance to florfenicol (Zhao and others 2010).

Multidrug resistance among *C. jejuni* isolates from both animal and human sources is increasing. In addition to quinolones, isolates are commonly resistant to macrolides, tetracycline, and ampicillin. Results show that the *CmeG* gene functions as a multidrug efflux transporter, thus contributing to antibiotic resistance and oxidative defense in *Campylobacter* spp. (Jeon and others 2011). The general percentage of multidrug-resistant isolates has been reported as up to 15% to 43% (Hänninen and Hannula 2007; Bester and Essack 2008). Not surprisingly, all our tested isolates exhibited multidrug resistance, which indicates the widespread misuse of antibiotics in the local layer industry. Antimicrobials used in laying birds are usually deposited in the eggs (Goetting and others 2011). Therefore, the unwise use of antimicrobials in layer hens will affect public health through egg consumption or consumption of the birds at the end of laying cycle.

Using EUCAST breakpoints, this study demonstrates a perfect statistical agreement (kappa) between the 2 techniques (MIC compared with disc diffusion) for ciprofloxacin and tetracycline, but not for erythromycin. However, using French breakpoints, there was a highly statistical agreement between disc diffusion and broth dilution techniques for ampicillin, amoxicillin, erythromycin, ciprofloxacin, and tetracycline. Similarly, there was substantial agreement for gentamicin and nalidixic acid and moderate agreement for doxycycline as statistically calculated (kappa values). Similarly Luangtongkum and others (2007) reported a high level of agreement and correlation between the 2 methods for gentamicin, ciprofloxacin, norfloxacin, and nalidixic acid, but not for erythromycin. These apparent disagreements in determining the resistance values vary according to the investigated antimicrobial agent(s) and the cut-off values used (Ge and others 2002; Moore and others 2006).

The use of different techniques (MIC, disc diffusion, E-test, agar or broth dilutions) makes it difficult to compare otherwise similar studies on *Campylobacter* antimicrobial susceptibility. The use of different breakpoints is also confounding when comparing antimicrobial resistance. It is suggested by several authors (Moore and others 2006; Van der Beek and others 2010) that the MIC technique is the preferable method to test *Campylobacter* susceptibility. However, disc diffusion has also been suggested by reference laboratories, international organizations, and other researchers (EUCAST, CA-SFM, CLSI, BSAC, Ge and others 2002). The results of this study show perfect agreement between disc diffusion and broth microdilution methods for 6 out of the 9 studied antibiotics (Table 4), which agrees with other findings (Luangtongkum and others 2007; Miflin and others 2007). However, Lehtopolku and others (2012) considered disc diffusion a nonreliable tool for testing *Campylobacter* spp. Therefore, this study supports suggestions that qualified multilaboratory disc diffusion studies are being emphasized for the creation of international standardized breakpoints.

Conclusions

It appears that antibacterial resistance is a serious and challenging issue. Use of antibacterial should be limited to situations where the drugs are necessary for ensuring animal health and welfare and

done under veterinary guidance through efficient global and local monitoring systems. Variation in resistance detected by different geographical regions in the world necessitates continuous public health monitoring of *Campylobacter* spp. antimicrobial susceptibilities. The high levels of antimicrobial resistance found among *Campylobacter* isolates in this study require shared responsibility between farmers, veterinarians, and authorities in Jordan for the judicious use of antibiotics in poultry farms. The disc diffusion method is flexible, convenient, and not labor-intensive compared with the MIC technique. The breakpoints used for disc diffusion may not provide a very reproducible method for susceptibility testing of *C. jejuni*. This is a major concern due to the common use of this method in routine microbiology laboratories and in some research studies. Disc diffusion may be used as a rapid screening test and further studies must assess whether this method and corresponding breakpoints could be improved.

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