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Original Article

Antimicrobial Resistance of *Campylobacter* Species Isolated from Broilers in Live Bird Markets in Shanghai, China

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Abstract

This study was conducted to determine the prevalence of antimicrobial resistance in *Campylobacter* spp. isolates from broilers in live bird markets (LBMs). A total of 209 *Campylobacter* spp. isolates (84 *Campylobacter jejuni*; 125 *Campylobacter coli*) were recovered from 364 broiler cecum samples collected from five LBMs in Shanghai, China. Minimum inhibitory concentrations of 13 antimicrobials were determined using agar dilution method. More than 96% of the *Campylobacter* spp. isolates were resistant to quinolones and tetracyclines. A high prevalence of macrolide resistance (erythromycin, 84.0%; azithromycin, 80.8%) was observed in *C. coli*, but not in *C. jejuni* (erythromycin, 6.0%; azithromycin, 2.4%). *C. coli* also showed significantly higher resistance than *C. jejuni* to clindamycin, gentamicin, and kanamycin. In contrast, *C. coli* isolates had lower resistance to florfenicol than the *C. jejuni* isolates. The majority of the *C. jejuni* (88.1%) and *C. coli* (97.6%) isolates exhibited multidrug resistance (MDR) to three or more classes of antimicrobials. All of the 208 ciprofloxacin-resistant *Campylobacter* spp. isolates were positive for the C257T mutation of the *gyrA* gene. In addition, the *tet*(O) gene was identified in all of the 202 doxycycline-resistant *Campylobacter* spp. isolates. Furthermore, 75.7% and 20.4% of the 103 azithromycin-resistant *Campylobacter* spp. isolates were positive for the A2075G mutation of the 23S rRNA gene and the presence of the *erm*(B) gene, respectively. Moreover, the *cat* gene was found in 14.3% (8/56) and 76.8% (73/95) of the chloramphenicol-resistant *C. jejuni* and *C. coli* isolates, respectively. To the best of our knowledge, this is the first report of the prevalence of antimicrobial resistance among *Campylobacter* spp. isolates originating from LBMs. The high prevalence of MDR *Campylobacter* spp. isolates in LBMs highlights the need to implement efficient intervention measures to control not only *Campylobacter* contamination in LBMs but also dissemination of antimicrobial resistance among *Campylobacter* spp. in poultry production.

Keywords: *Campylobacter*, antimicrobial resistance, broilers, live bird markets

Introduction

Campylobacter spp., especially *Campylobacter jejuni* and *Campylobacter coli*, are leading causes of bacterial gastroenteritis globally (Koluman and Dikici, 2013). Although usually self-limiting, severe or long-lasting *Campylobacter* spp. infections, especially of patients with compromised immune systems, often require antimicrobial therapy (McGill *et al.*, 2009). *Campylobacter* spp. are prevalent in food-producing animals, and the use of antimicrobials in food animal production has led to an increasing prevalence of antimicrobial-resistant *Campylobacter* spp. infection transmitted to humans through the food chain, thereby presenting a major threat to public health (Mäesaar *et al.*, 2016).

The handling and consumption of contaminated poultry, particularly broilers, seem to be the most common risk factor (Damjanova *et al.*, 2011). Broilers are regarded as a main reservoir of *Campylobacter* spp., and the colonization level of *Campylobacter* spp. in broiler ceca can reach 10^9 CFU/g of cecal content (Stern *et al.*, 2008). *Campylobacter* contamination often occurs during the slaughtering process and consumption of meat from a contaminated carcass is a significant source of human campylobacteriosis (Melero *et al.*, 2012).

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In China and other Asian countries, live bird markets (LBMs) are among the most important termini of the poultry industry, as people in these countries prefer to purchase live or freshly slaughtered poultry. Birds sold in LBMs are continuingly introduced from different sources and caged at high densities with those collected from other areas. These conditions provide an optimal environment for amplification and persistence of infectious agents. Hence, LBMs are potential reservoirs of poultry-associated pathogens, such as various avian influenza viruses that continue to cause human infection and death (Zhou *et al.*, 2009; Cowling *et al.*, 2013; He *et al.*, 2014). In addition, unlike other consummation termini of the poultry industry, consumers at LBMs are in direct and close contact with live or freshly slaughtered poultry. Together, the conditions of LBMs likely increase the risk for human *Campylobacter* spp. infection. However, studies concerning *Campylobacter* spp. from LBMs are very limited (Mansouri-najand *et al.*, 2012). Therefore, in this study, we investigated the prevalence and antimicrobial resistance of *C. jejuni* and *C. coli* isolates collected from five LBMs in Shanghai, one of the largest cities in China.

Materials and Methods

Sample collection

From November 2012 to April 2013, a total of 364 cecum samples from broilers were randomly collected from five LBMs located in five geographically separated districts (Minhang, Fengxian, Pudong, Songjiang, and Yangpu) of Shanghai (Table 1). For each LBM, 71–77 samples were collected at three to four time points between November 2012 and April 2013. Each cecum sample was collected from a different chicken during evisceration and put into a stomacher bag, and then rapidly transported on ice to our laboratory within 2 h.

Isolation and identification of Campylobacter *spp.*

A loopful of fecal content from each sample was directly streaked onto a *Campylobacter* spp. Selective Agar (Base) plate (Sigma, St. Louis, MO) containing 5% fresh sterile defibrinated sheep blood and *Campylobacter* supplement III (Sigma) (Ma *et al.*, 2014). The plates were incubated under microaerobic conditions (CO_2, 10%; O_2, 5%; N_2, 85%) at 42°C for 48 h. One presumptive colony from each plate was subcultured and identified by PCR strategies (Keramas *et al.*, 2003) and API-Campy kits (BioMérieux, Marcy l'Etoile, France). All the identified isolates were stored at −80°C in brain-heart infusion broth with 20% (v/v) glycerol.

Antibiotic susceptibility testing

The standard agar dilution method, as described by the Clinical Laboratory Standards Institute (CLSI, 2010), was employed to determine the susceptibility of *Campylobacter* spp. isolates to 13 antibiotic agents: nalidixic acid, ciprofloxacin, enrofloxacin, levofloxacin, erythromycin, tetracycline, doxycycline azithromycin, clindamycin, chloramphenicol, florfenicol, gentamicin, and kanamycin. These antimicrobials, with the exception of nalidixic acid (Sigma), were all obtained from the China Institute of Veterinary Drug Control (Beijing, China). Breakpoints for each antimicrobial agent and minimum inhibitory concentration (MIC) ranges for quality control strains *C. jejuni* ATCC 33560 and *Escherichia coli* ATCC25922 are shown in Table 2. A *Campylobacter* spp. isolate simultaneously resistant to three or more classes of antimicrobials was defined as multidrug resistant.

Detection of resistance determinants

The presence of genes involved in macrolide, tetracycline, and phenicol resistance was detected by PCR. Genomic DNA of *Campylobacter* spp. isolates was extracted using the TIANamp Bacterial DNA Kit (TIANGEN, Beijing, China), according to the manufacturer's directions, and used as templates for subsequent PCR reactions. The detection of the *tet*(O) (Qin *et al.*, 2011), *cat* (Wang and Taylor, 1990), *cfr* (Kehrenberg and Schwarz, 2004), *cmlA* & *floR* (Dai *et al.*, 2008), and *erm*(B) (Spiliopoulou *et al.*, 2004) genes was conducted by PCR analysis, as previously described. In addition, the mismatch amplification mutation assay (MAMA) PCR was used to detect the C257T mutation in the quinolone resistance-determining region (QRDR), which confers high-level quinolone resistance (Zirnstein *et al.*, 2000), and the mutations A2074C and A2075G in the 23S rRNA gene, which are associated with macrolide resistance (Alonso *et al.*, 2005). Moreover, the macrolide-resistant isolates were also screened for mutations in the L4 and L22 ribosomal protein genes (Cagliero *et al.*, 2006).

Table 1. The Prevalence of *Campylobacter* Species in Cecum Samples Collected from LBMs in Shanghai, China

District	*Nos. of cecum samples*	*No. of* Campylobacter jejuni[a]	*No. of* Campylobacter coli[a]	*No. of total isolates from each district*[a]
Minhang	71	20 (28.2)	47 (66.2)	67 (94.4)
Fengxian	77	16 (20.8)	22 (28.6)	38 (49.4)
Songjiang	73	12 (16.4)	34 (46.6)	46 (63)
Putuo	71	20 (28.2)	8 (11.3)	28 (39.4)
Yangpu	72	16 (22.2)	14 (19.4)	30 (41.7)
Total (%)	364	84 (23.1)	125 (34.3)	209 (57.4)

[a]Numbers in parentheses indicate percentages.
LBM, live bird market.

Table 2. Antimicrobial Susceptibility of 84 *C. jejuni* and 125 *C. coli* Strains Isolated from LBMs in Shanghai, China

Antimicrobial agent	MIC QC range (μg/mL)[a]		MIC breakpoint (μg/mL)[b]			C. jejuni		C. coli	
	ATCC33560	ATCC25922	S	I	R	MIC_{50}/MIC_{90} (μg/mL)	Resistant isolates (%)[c]	MIC_{50}/MIC_{90} (μg/mL)	Resistant isolates (%)[c]
Nalidixic acid	4–16	—	≤16	32	≥64	256/256	84 (100)	256/256	125 (100)
Ciprofloxacin	0.06–0.5	—	≤1	2	≥4	128/256	83 (98.8)	32/128	125 (100)
Enrofloxacin	—	0.008–0.03	≤0.5	1–2	≥4	16/128	83 (98.8)	8/16	122 (97.6)
Levofloxacin	0.03–0.25	—	≤2	4	≥8	32/256	83 (98.8)	16/32	122 (97.6)
Erythromycin	1–4	—	≤8	16	≥32	2/8	5 (6.0)	256/256	105 (84.0)
Azithromycin	0.03–0.12	—	≤2	4	≥8	0.125/1	2 (2.4)	256/256	101 (80.8)
Clindamycin	0.12–0.5	—	≤2	4	≥8	2/8	14 (16.7)	64/256	106 (84.8)
Chloramphenicol	1–4	—	≤8	16	≥32	32/64	56 (66.7)	32/64	95 (76.0)
Florfenicol	0.5–2	—	≤4	8	≥16	32/64	67 (79.8)	4/8	8 (6.4)
Tetracycline	0.25–1	—	≤4	8	≥16	256/256	83 (98.8)	256/256	122 (97.6)
Doxycycline	0.25–2	—	≤2	4	≥8	64/128	81 (96.4)	64/128	121 (96.8)
Gentamicin	0.5–4	—	≤2	4	≥8	0.25/256	15 (17.9)	256/256	117 (93.6)
Kanamycin	—	1–4	≤16	32	≥64	8/256	23 (27.4)	256/256	116 (92.8)

[a]The QC ranges of *C. jejuni* ATCC 33560 were directly adopted from CLSI (2010). Due to the lack of QC ranges of *C. jejuni* ATCC 33560 for enrofloxacin and kanamycin, we used *E. coli* ATCC 25922 as QC strain for these two antimicrobial agents (CLSI, 2010).

[b]MIC breakpoints for nalidixic acid, ciprofloxacin, erythromycin, azithromycin, clindamycin, florfenicol, tetracycline, doxycycline, and gentamicin are those recommended by the CLSI (2010). Since standardized MIC breakpoints for enrofloxacin, levofloxacin, chloramphenicol, and kanamycin are not available for *Campylobacte*r spp., we used the breakpoints of *Enterobacteriaceae* for these four antimicrobial agents as recommended by CLSI (2010).

[c]Numbers in parentheses indicate percentages.

I, intermediate; QC, quality control; R, resistant; S, susceptible.

Statistical analysis

The resistance frequencies for each class of antimicrobial agents and multidrug resistance (MDR) rates between *C. jejuni* and *C. coli* isolates from LBMs were compared with the chi-square test using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL). Differences were considered significant at $p < 0.05$.

Results and Discussion

Incidence of Campylobacter *spp.*

Overall, *Campylobacter* spp. were isolated in 209 (57.4%) of the 364 broiler cecum samples. *Campylobacter* spp. isolation rates varied from 39.4% to 94.4% across the five surveyed LBMs (Table 1). Among these samples, the prevalence of *C. jejuni* and *C. coli* was 23.1% and 34.3%, respectively, which corresponds well with previous reports on the greater proportion of *C. coli* than *C. jejuni* in broilers (Henry *et al.*, 2011; Ma *et al.*, 2014). However, the observation that *C. coli* was the predominant *Campylobacter* spp. in broilers was contrary to other reports (Chen *et al.*, 2010; Anderson *et al.*, 2012). It was consistent with the speculation that *C. coli*, instead of *C. jejuni*, is becoming the predominant *Campylobacter* spp. in chickens in China, which might be attributed to the fact that *C. coli* is intrinsically more adaptable to antimicrobial treatment, and the extensive use of antimicrobials in broiler production may have favorably selected *C. coli* over *C. jejuni* (Ma *et al.*, 2014; Wang *et al.*, 2016).

Quinolone resistance

High prevalence of quinolone resistance was observed among the 209 *Campylobacter* spp. isolates (nalidixic acid, 100%; ciprofloxacin, 99.5%; enrofloxacin, 98.1%; levofloxacin, 98.1%) (Table 2). These results are similar to previous observations in China that almost 100% of *Campylobacter* spp. strains isolated from chickens were resistant to quinolones (Chen *et al.*, 2010; Ma *et al.*, 2014), which might be caused by the extended use of these antimicrobials in chicken production. In contrast, a very low incidence of quinolone-resistant *Campylobacter* spp. has been reported in Canada (Agunos *et al.*, 2013), the United States (Ge *et al.*, 2013), and France (Guyard-Nicodème *et al.*, 2015), which may be benefited from prudent use policy of antimicrobials in these countries.

Quinolone resistance in *Campylobacter* spp. is usually conferred by point mutations in the QRDR of DNA gyrase A (GyrA), and the C257T mutation, leading to the Thr-86-Ile substitution in the gyrase, is the most frequently detected (Hormeño *et al.*, 2016). All of the 208 *Campylobacter* spp. (*C. jejuni* = 83; *C. coli* = 125) isolates resistant to ciprofloxacin were positive for the C257T mutation in the QRDR of *gyr*A. However, some *Campylobacter* spp. isolates examined in this study showed a high level of fluoroquinolone resistance. For example, 21 of 84 *C. jejuni* strains exhibited ciprofloxacin MICs of ≥256 μg/mL. However, the C257T mutation is not sufficient to confer such a high level of fluoroquinolone resistance in *Campylobacter* spp.; thus other mechanisms, such as additional mutations the QRDR of *gyr*A and enhanced activity of the MDR efflux pump CmeABC, may be involved (Guo *et al.*, 2010; Dai *et al.*, 2015).

Tetracycline resistance

High tetracycline (tetracycline, 98.1%; doxycycline, 96.7%) resistance rate was also present in the *Campylobacter*

spp. isolates of this study, which was consistent with other investigations performed in China (Chen *et al.*, 2010; Ma *et al.*, 2014). Tetracycline resistance in *Campylobacter* spp. is mainly conferred by the ribosomal protection protein gene *tet*(O), which is located either on the chromosome or a transmissible plasmid (Gibreel *et al.*, 2004; Abdi-Hachesoo *et al.*, 2014). In this study, all the 202 doxycycline-resistant *Campylobacter* spp. strains (81 *C. jejuni* and 121 *C. coli*) were positive for the *tet*(O) gene. Interestingly, three doxycycline-sensitive and *tet*(O)-negative *Campylobacter* spp. isolates (2 *C. jejuni* and 1 *C. coli*) exhibited high-level resistance to tetracycline (MIC, ≥256 μg/mL), indicating the possible existence of an unknown tetracycline resistance mechanism in these trains.

Macrolide and lincosamide resistance

The prevalence of macrolide and lincosamide resistance in *C. coli* was significantly higher than in *C. jejuni* (erythromycin, 84.0% vs. 6.0%; azithromycin, 80.8% vs. 2.4%; clindamycin, 84.8% vs. 16.7%, respectively) (Table 2) ($p < 0.01$). Resistance rates of *C. coli* (84.0%) to erythromycin were lower than that reported in previous studies performed in China (Chen *et al.*, 2010; Ma *et al.*, 2014), but higher than in Australia (Obeng *et al.*, 2012) and Poland (Wieczorek *et al.*, 2013). Furthermore, the incidence of erythromycin-resistant *C. jejuni* (6.0%) was comparable to some other findings (Gblossi Bernadette *et al.*, 2012; Torralbo *et al.*, 2015), but higher than that reported in most previous studies (Obeng *et al.*, 2012; Shin *et al.*, 2012).

In *Campylobacter* spp., macrolide resistance is often mediated by point mutations in the 23S rRNA gene, especially A2074C and A2075G (Lehtopolku *et al.*, 2011). In this study, no A2074C mutation was detected among the 103 azithromycin-resistant *Campylobacter* spp. isolates (2 *C. jejuni* and 101 *C. coli*), while 75.7% (78/103) of these strains, except 25 *C. coli*, harbored the A2075G point mutation as determined by MAMA PCR. Screening of the *erm*(B) gene and mutations in the L4 and L22 genes in the 25 *C. coli* isolates without the 23 s rRNA mutations revealed that 21 harbored the *erm*(B) gene, but no L4 or L22 mutations were found. The *erm*(B)-positive rate in LBM-origin *Campylobacter* spp. isolates (10.0%, 21/209) was significantly higher than that in *Campylobacter* spp. strains from other sources (3.7%, 58/1554) (Wang *et al.*, 2014). The high density and continued introduction of broilers in LBMs may provide optimal conditions for the spread and persistence of *erm*(B)-carrying *Campylobacter* spp. isolates, finally resulting in the high incidence of the *erm*(B) gene. Notably, there were four *C. coli* isolates demonstrating high-level macrolide resistance that did not contain the 23 s rRNA mutations or the *erm*(B) gene. This may be attributed to the enhanced activity of the CmeABC efflux pump and/or other unknown mechanisms, thus further studies are warranted (Guo *et al.*, 2010; Qin *et al.*, 2014).

Phenicol resistance

Interestingly, the florfenicol resistance rate of *C. jejuni* was significantly higher compared with *C. coli* (79.8% vs. 6.4%, respectively) (Table 2) ($p < 0.01$), but the *Campylobacter* spp. with the higher chloramphenicol resistance rate was *C. coli*, rather than *C. jejuni* (76.0% vs. 66.7%, respectively). 29.2% *Campylobacter* spp. strains (*C. jejuni*, 54; *C. coli*, 7) were resistant to both phenicols. Low chloramphenicol resistance rates in *Campylobacter* spp. isolates were found in investigations conducted in Spain (Perez-Boto *et al.*, 2013), Japan (Ozawa *et al.*, 2012), and Iran (Chakeri *et al.*, 2012). However, it is worth noting that the incidence of chloramphenicol resistance among *Campylobacter* spp. isolates was very high in China (Chen *et al.*, 2010) and Brazil (de Moura *et al.*, 2013).

In this study, of the 151 (*C. jejuni*, 56; *C. coli*, 95) chloramphenicol-resistant *Campylobacter* spp. isolates, 81 (*C. jejuni*, 8; *C. coli*, 73) were positive for the *cat* gene, but not *cmlA*. In addition, the *floR* and *cfr* genes, which confer florfenicol resistance, were not detected in any of the 75 (*C. jejuni*, 67; *C. coli*, 8) florfenicol-resistant *Campylobacter* spp. isolates.

Aminoglycoside resistance

A very high prevalence of gentamicin (93.6%) and kanamycin (92.8%) resistance was observed among the *C. coli* isolates, but the corresponding resistance rates of *C. jejuni* were only 17.9% and 27.4%, respectively (Table 2). In addition, as mentioned above, the resistance rates of the *C. coli* isolates to azithromycin and clindamycin were also significantly higher than those of the *C. jejuni* isolates. Some previous studies also reported that resistance to some antimicrobial classes (i.e.,

Table 3. Antimicrobial Resistance Patterns of *Campylobacter* Isolates from Five LBMs in Shanghai, China

Antimicrobial resistance patterns[a]	*No. (%) of* Campylobacter *strains*	
	C. jejuni *(n = 84)*[b]	C. coli *(n = 125)*[b]
Q	1 (1.2)	0
Q-A	0	1 (0.8)
Q-T	9 (10.7)	2 (1.6)
Q-M-T	0	1 (0.8)
Q-P-A	0	1 (0.8)
Q-P-T	**41 (48.8)**[c]	1 (0.8)
Q-T-A	4 (4.8)	6 (4.8)
Q-L-P-T	3 (3.6)	0
Q-L-T-A	1 (1.2)	3 (2.4)
Q-M-L-A	0	1 (0.8)
Q-M-P-T	1 (1.2)	0
Q-M-T-A	0	1 (0.8)
Q-P-T-A	12 (14.3)	6 (4.8)
Q-L-P-T-A	8 (9.5)	0
Q-M-L-P-T	1 (1.2)	1 (0.8)
Q-M-L-T-A	0	14 (11.2)
Q-M-P-T-A	2 (2.4)	0
Q-M-L-P-T-A	1 (1.2)	**87 (69.6)**[c]
MDR (%)[d]	74 (88.1)	122 (97.6)

[a]Abbreviations of antimicrobial agents: Q, quinolones (nalidixic acid, ciprofloxacin, enrofloxacin, and levofloxacin); P, phenicols (florfenicol and chloramphenicol); M, macrolides (erythromycin and azithromycin); T, tetracyclines (tetracycline and doxycycline); L, clindamycin; A, aminoglycosides (gentamicin and kanamycin).
[b]n, the number of *Campylobacter* isolates.
[c]The bold text indicates the major resistance patterns.
[d]The number (%) of multidrug-resistant *Campylobacter*.

aminoglycosides, macrolides, and lincosamides) was much higher in *C. coli* than *C. jejuni* (Ma *et al.*, 2014; Torralbo *et al.*, 2015). Further research is needed to better elucidate the molecular mechanism leading to the differences in antimicrobial resistance between *C. jejuni* and *C. coli*.

Multidrug resistance

As is shown in Table 3, all, but one, of the *Campylobacter* spp. isolates were resistant to two or more classes of antimicrobials. Overall, 196 (93.8%) of the 209 *Campylobacter* spp. isolates exhibited MDR. In addition, the MDR rate was significantly higher ($p < 0.05$) among *C. coli* isolates than *C. jejuni* (97.6%, 122/125 vs. 88.1%, 74/84, respectively). The most common antimicrobial resistance pattern in *C. jejuni* (48.8%, 41/84) was the combination of quinolones, phenicols, and tetracyclines. In *C. coli*, 87 of 125 (69.6%) isolates exhibited resistance to all of the antimicrobial classes tested. The difference in dominant MDR patterns between the *Campylobacter* spp. might be due to the intrinsic ability of *C. coli* to develop resistance to these antimicrobials (Chen *et al.*, 2010). Interestingly, a similar high MDR prevalence was also observed in the surveillance studies concerning human-origin *Campylobacter* spp. in China (Zhang *et al.*, 2014; Zhou *et al.*, 2016). The extremely high rates of antimicrobial resistance in *Campylobacter* spp. isolates from human and LBM origin suggested that some of the antimicrobial classes, such as quinolones and tetracyclines, may no longer be suitable for the treatment of human *Campylobacter* infection in China.

Conclusions

This is the first report addressing antimicrobial resistance of *Campylobacter* spp. isolated from LBMs in China. An extremely high rate of antimicrobial resistance was observed in the *Campylobacter* spp. isolates, which was likely due to the extensive use of various antimicrobials in poultry production. Furthermore, given the fact that consumers purchasing poultry from LBMs have direct contact with live or freshly slaughtered birds, the high prevalence of MDR *Campylobacter* spp. isolates in LBMs may pose a high risk factor for human *Campylobacter* spp. infection and treatment. The findings of this study highlight the need for efficient measures to control *Campylobacter* spp. contamination in LBMs, while the extensive antimicrobial resistance among *Campylobacter* spp. isolates examined in this study underlines the need for prudent use of antimicrobials in poultry production to minimize the emergence and spread of antibiotic-resistant *Campylobacter* spp. strains.

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Disclosure Statement

No competing financial interests exist.

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