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Prevalence and antimicrobial resistance of *Campylobacter* isolates in broilers from China

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ABSTRACT

The prevalence and antimicrobial resistance of *Campylobacter* spp. in broiler chickens were determined in Shandong Province, China. In total, 275 *Campylobacter* isolates were obtained from 767 broiler cecal samples, including 208 *Campylobacter jejuni*, 53 *Campylobacter coli*, and 14 unidentified *Campylobacter* isolates. Minimal inhibitory concentrations of 11 antimicrobial agents were determined using the agar dilution method recommended by CLSI. More than 98% of the tested *Campylobacter* isolates were resistant to quinolones (nalidixic acid, ciprofloxacin and enrofloxacin) and tetracyclines (tetracycline and doxycycline). The *C. jejuni* isolates also exhibited a high rate of resistance to phenicol antibiotics and a moderate rate of resistance to macrolides and gentamicin. On the contrary, the *C. coli* isolates showed a high-level resistance to macrolides and gentamicin and little resistance to phenicol antibiotics. The vast majority of the *Campylobacter* isolates were classified as multidrug resistant. These findings reveal a broad extent of antimicrobial resistance in *Campylobacter* isolates from poultry in China and underline the need for prudent use of antibiotics in poultry production to minimize the spread of antibiotic resistant *Campylobacter*.

1. Introduction

Thermophilic *Campylobacter*, including *Campylobacter jejuni* and *Campylobacter coli*, is a main bacterial cause of acute gastroenteritis in humans in both developing and developed countries (Blaser, 1997; Englen et al., 2007). *Campylobacter* infection is also associated with the development of Guillain-Barré syndrome, a neurological disorder affecting the peripheral nervous system (Yuki, 2001; Leonard et al., 2004). For clinical treatment of campylobacteriosis, macrolide and fluoroquinolone antibiotics are often prescribed, however, *Campylobacter* resistance to both classes of antibiotics is on the rise (Payot et al., 2006; Gibreel and Taylor, 2006).

As a foodborne pathogen, *Campylobacter* is transmitted to humans via contaminated food and water (Allos, 2001). Particularly, the chicken is a natural host of *C. jejuni* and serves as a major reservoir for this pathogenic organism (Sahin et al., 2002; Lee and Newell, 2006). Contamination of chicken carcasses by *Campylobacter* often occurs during the slaughtering process and consumption of chicken meat is a significant source of human *Campylobacter* infections (Humphrey et al., 2007). Thus control of *Campylobacter* in poultry should yield a positive impact on improving food safety.

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For modern poultry production, antimicrobial agents have been widely used for growth promotion and disease control. Many of the antimicrobials used for animal agriculture are also used for human medicine. Thus, agricultural use of antibiotics poses a risk for selecting antibiotic resistant pathogens that can be potentially transmitted to humans and may compromise clinical treatment. Indeed, previous studies have shown that use of certain antimicrobials in chickens, especially fluoroquinolones, rapidly select for antibiotic-resistant *Campylobacter* (McDermott et al., 2002; Luo et al., 2003). Many studies have reported the prevalence of antimicrobial-resistant *Campylobacter* in animal reservoirs in different countries (Bachoual et al., 2001; Gibreel and Taylor, 2006; Alfredson and Korolik, 2007; Hariharan et al., 2009; Luangtongkum et al., 2009). However, little information is available on the prevalence and antimicrobial resistance of *Campylobacter* from poultry in China, where poultry production represents an important sector of animal husbandry and consumption of poultry meat is significant. In this study, we surveyed several broiler slaughter houses in five different regions of Shandong Provinces and determined the prevalence and antimicrobial resistance of *Campylobacter* in multiple chicken flocks.

2. Materials and methods

2.1. Isolation and Identification of Campylobacter

Campylobacter strains were isolated from cecal contents of broiler chickens, which were selected randomly from five different geographical areas in Shandong Province, China. All samples were collected in June 2008 from five slaughterhouses located in the southeast (Linyi), north (Zouping and Penglai), northwest (Longkou), and west (Shenxian) of Shandong Province. The samples were collected from 45 flocks (Table 1). From each flock, 15 up to 20 samples were collected.

The collected ceca were individually packed and transported on ice to the laboratory within 5 hours of collection. For each cecum, a loopful of the fecal content was directly streaked onto *Campylobacter* Selective Agar (Base) (Oxoid Ltd., Basingstoke, England) containing 5% fresh sterile defibrinated sheep blood and *Campylobacter* supplement III (Sigma, St. Louis, MO, USA) for primary isolation. The plates were incubated in an environment of 10% CO_2, 5% O_2 and 85% N_2 at 42 °C for 36–48 h. One suspected colony was isolated from each cecal sample.

The isolates were identified to the genus/species level by multiplex PCR with three pairs of primers amplifying the 16S rRNA gene specific for the genus of *Campylobacter* (Linton et al., 1997), the *HipO* gene specific for *C. jejuni*, and the CC amplicon (located in the 16S–23S rRNA region) specific for *C. coli* (Keramas et al., 2003). The primers were listed in Table 2.

For PCR, crude chromosomal DNA of the isolates was prepared by boiling as described previously (Bachoual et al., 2001). The PCR mixture consisted of 10 μL of 2× PCR MasterMix (TIANGEN, Beijing, China), 0.4 μL of 10 nmol/L of each primer, 1 μL of chromosomal DNA template, and 6.6 μL of sterile distilled water. The PCR was carried out in a Veriti 96 well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with the following cycling conditions: heat denaturation at 95 °C for 5 min, 35 cycles at 95 °C for 35 s, 56.5 °C for 40 s, 72 °C for 45 s, and a final extension at 72 °C for 7 min. The *C. jejuni* ATCC 33560™ and *C. coli* ATCC 33559™ strains obtained from the American Type Culture Collection (Manassas, VA, USA) were used as the positive control.

2.2. Antimicrobial susceptibility testing of Campylobacter isolates

The agar dilution method was used to determine the susceptibility of *Campylobacter* isolates to 11 antimicrobial

Table 1
Campylobacter isolates obtained from broiler cecal samples.

Region	Number of samples	Number of flock	Number of positive flocks[a]	Number of *C. jejuni*[a]	Number of *C. coli*[a]	Number of unidentified *Campylobacter* species[a]	Total number of positive samples from each region[a]
Zouping	82	4	3 (75)	12 (14.6)	0 (0)	0 (0)	12 (14.6)
Linyi	370	23	16 (70.0)	100 (27)	3 (0.2)	0 (0)	103 (27.8)
Shenxian	185	11	9 (81.8)	71 (38.4)	16 (8.6)	7 (3.8)	94 (50.8)
Penglai	86	4	4 (100)	19 (22.1)	32 (37.2)	7 (8.1)	58 (67.4)
Longkou	44	3	3 (100)	6 (13.6)	2 (4.5)	0 (0)	8 (18.2)
Total number (%)	767	45	35 (77.7)	208 (27.1)	53 (6.9)	14 (1.8)	275 (35.9)

[a] Numbers in parentheses indicate the percentages.

Table 2
Primers used for PCR identification in this study.

Primer	Primer Sequence (5′–3′)	Amplified gene	Position	Size (bp)
16S rRNAf	GCGAAGAACCTACCYGGRCTTGATA	16S rRNA; genus-specific	nt 948–1244 of the 16S rRNA gene	314
16S rRNAr	TCGCGRTATTGCGTCTCATTGTATATG			
hipOf	GTACTGCAAAATTAGTGGCG	*hipO* of *C. jejuni*	nt 1478–1513 of the *hipO* gene	149
hipOr	GCAAAGGCAAAGCATCCATA			
CCf	GTTAAGAGTCACAAGCAAGT	Intergenic region between 16S and 23S rRNA; *C. coli* specific	nt 488–523 of the *C. coli* 16S and 23S rRNA intergenic spacer region	194
CCr	CTAAAAATATCTAAACTAAGTCG			

Table 3
Antimicrobial test ranges, MIC QC ranges, and breakpoints used for antimicrobial susceptibility testing by agar dilution.

Antimicrobial agent	MIC QC range (μg/mL)[a]	Test range (μg/mL)	MIC breakpoint (μg/mL)[b]		
			S	I	R
Nalidixic acid	4–16	1–512	≤16	32	≥64
Ciprofloxacin	0.06–0.5	0.03–64	≤1	2	≥4
Erythromycin	1–8	0.5–256	≤8	16	≥32
Azithromycin	0.03–0.12	0.03–64	≤2	4	≥8
Clindamycin	0.12–0.5	0.015–64	≤2	4	≥8
Florfenicol	0.5–2	0.25–64	≤4	8	≥16
Tetracycline	0.25–1	0.06–64	≤4	8	≥16
Doxycycline	0.5–2	0.06–64	≤2	4	≥8
Gentamicin	0.5–4	0.06–32	≤2	4	≥8
Enrofloxacin	N/A[c]	0.03–64	≤0.5	1–2	≥4
Chloramphenicol	1–4	0.5–64	≤8	16	≥32

[a] Agar dilution QC ranges of *C. jejuni* ATCC 33560™ approved by CLSI (2008).
[b] MIC breakpoints for nalidixic acid, ciprofloxacin, erythromycin, azithromycin, clindamycin, florfenicol, tetracycline, doxycycline, gentamicin are those recommended by the CLSI (2008); since standardized MIC breakpoint for enrofloxacin and chloramphenicol are not available for *Campylobacter*, we used the breakpoint for *Enterobacteriaceae* for enrofloxacin and the breakpoint for organisms other than *Streptococci* for chloramphenicol as recommended by CLSI (2008). S, Susceptible isolates; I, intermediate isolates; R, resistant isolates.
[c] N/A, No data available.

agents: nalidixic acid, ciprofloxacin, enrofloxacin, erythromycin, clindamycin, azithromycin, chloramphenicol, florfenicol, tetracycline, doxycycline, and gentamicin. All the antimicrobial agents were obtained from China Institute of Veterinary Drug Control (Beijing, China) except nalidixic acid which was obtained from Sigma. The susceptibility testing was performed according to the guideline of Clinical and Laboratory Standards Institute (CLSI) (2008). Fresh bacterial colonies taken directly from agar plates incubated for 24 h were re-suspended in sterile Mueller–Hinton broth to obtain a suspension of 0.5 McFarland turbidity. Two microliters of the bacterial colony suspension was inoculated onto the plates containing the antimicrobial agents. *C. jejuni* ATCC 33560™ was included on every plate as a quality control. The results were judged by minimum inhibitory concentration (MIC), which was defined as the lowest concentration of antimicrobial agent that prevented visible bacterial growth on the testing plates. For the accuracy of the test, the MICs of all the strains were repeated three times. Test concentrations and breakpoint for each antimicrobial agent, and quality control (QC) MIC ranges with *C. jejuni* ATCC33560™ are shown in Table 3. An isolate resistant to three or more classes of antimicrobial agents was considered to be multidrug resistant.

3. Results

3.1. Campylobacter prevalence

Of the 767 samples, 275 (35.9%) *Campylobacter* isolates were obtained, among which 208, 53, and 14 were identified as *C. jejuni*, *C. coli*, and unidentified *Campylobacter* species, respectively (Table 1). Of the 45 sampled flocks, 35 (77.8%) were positive for *Campylobacter*, with a flock prevalence for different regions ranging from 75.0% to 100%. The isolation rate varied greatly from region to region, ranging from 14.6% to 67.4%. Interestingly, the majority of the *C. coli* isolates were from two regions including Penglai and Shenxian, while the isolates from Linyi and Zouping were predominately *C. jejuni*.

3.2. Antimicrobial resistance of the Campylobacter isolates

Seven isolates did not grow during the antimicrobial susceptibility testing, so their data were not available in this study. Antimicrobial susceptibility test was performed on with 202 *C. jejuni* and 52 *C. coli* isolates. The MICs of all 11 antimicrobial agents tested against *C. jejuni* ATCC 33560™ (quality control) were within the CLSI 2008-defined QC ranges, indicating the good quality of the MIC testing plates. The prevalence of antimicrobial resistance in the *C. jejuni* and *C. coli* isolates are presented respectively in Tables 4 and 5. All the isolates were resistant to tetracycline, and the vast majority were also resistant to fluoroquinolones (>98.0%) and doxycycline (>98.1%). Notably, the majority of the *C. jejuni* isolates had ciprofloxacin MICs ≥128 μg/mL, indicating the high-level resistance to fluoroquinolones. For quinolones (ciprofloxacin, enrofloxacin and nalidixic acid), the overall resistance rates were similar between *C. jejuni* and *C. coli*, but it appeared that the resistance levels (reflected by the MIC values) in *C. jejuni* were higher than in *C. coli*. Compared to other antimicrobial agents, the resistance rates to macrolides (erythromycin and azithromycin) and clindamycin were relatively low in *C. jejuni* (8.9%, 26.7%, and 13.9%, respectively), however, the *C. coli* isolates were highly resistant to these antibiotics (100%, 98.1%, and 100%, respectively). Interestingly, the prevalence of florfenicol and chloramphenicol resistance was significantly higher in *C. jejuni* (79.2% and 30.7%, respectively) than in *C. coli* (1.9% and 3.8%, respectively). Another notable observation of this study was the high prevalence of gentamicin resistance, especially in the *C. coli* isolates (92.3%).

The prevalence of multidrug resistance in the *Campylobacter* isolates is shown in Table 6. Over 90% of the *C. jejuni* (188 out of 202 isolates) and all the *C. coli* isolates were resistant to multiple antimicrobial agents. The most common multidrug resistant pattern in *C. jejuni* was the resistance to fluoroquinolones, tetracyclines and chloramphenicol/florfenicol. In *C. coli*, most of the isolates (45 out of 52 isolates) showed resistance to all classes of

Table 4
Distributions of MICs of 11 antimicrobial agents for 202 *C. jejuni* isolates.

Antimicrobial agent	MIC (μg/mL)												Susceptible isolates[b]	Intermediate isolates[b]	Resistant isolates[b]
	≤0.25	0.5	1	2	4	8	16	32	64	128	256	≥512			
Nalidixic acid	[c]	-	0	0	2	0	0	[a]0	[a]0	2	65	133	2 (1.0)	0 (0)	200 (99.0)
Ciprofloxacin	0	0	0	1	0	2	0	7	12	180 (≥128 μg/mL)			0 (0)	1 (0.5)	201 (99.5)
Enrofloxacin	2	1	0	1	1	19	138	39	1	-	-	-	3 (1.5)	1 (0.5)	198 (98.0)
Erythromycin	4	28	26	36	6	23	25	23	4	9	0	18	123 (60.9)	25 (12.4)	54 (26.7)
Azithromycin	179	2	0	2	1	0	0	0	0	18 (≥128 μg/mL)			183 (90.6)	1 (0.5)	18 (8.9)
Clindamycin	15	74	44	25	16	6	11	4	7	-	-	-	158 (78.2)	16 (7.9)	28 (13.9)
Florfenicol	2	2	6	6	8	18	122	37	1	-	-	-	24 (11.9)	18 (8.9)	160 (79.2)
Chloramphenicol	-	0	6	4	6	9	115	51	1	10 (≥128 μg/mL)			25 (12.4)	115 (56.9)	62 (30.7)
Tetracycline	0	0	0	0	0	0	0	4	9	189 (≥ 128 μg/mL)			0 (0)	0 (0)	202 (100)
Doxycycline	0	0	0	0	2	3	7	18	0	172 (≥128 μg/mL)			0 (0)	2 (1.0)	200 (99.0)
Gentamicin	78	35	20	1	13	11	15	8	21 (≥ 64 μg/mL)				134 (66.3)	13 (6.4)	55 (27.2)

[a] Thin vertical lines indicate the breakpoint between susceptible and intermediate isolates. Thick vertical lines indicate the breakpoint between intermediate and resistant isolates.
[b] Numbers in parentheses indicate the percentages.
[c] no data available.

antimicrobial agents tested except to the chloramphenicol/florfenicol class. Overall, the *C. coli* isolates were resistant to more antimicrobial agents than the *C. jejuni* isolates (Table 6).

4. Discussion

In this study, *Campylobacter* spp. were isolated from 35.9% of chicken cecal samples and most of the isolates were *C. jejuni*. This finding is consistent with previous reports that *C. jejuni* is the predominant *Campylobacter* species isolated from chicken intestinal tracts (Newell and Wagenaar, 2000; Sahin et al., 2002). However, the isolation rates of *C. jejuni* and *C. coli* varied among the five regions surveyed in this study. For example, most of the *C. coli* isolates were from Penglai and Shenxian, while samples from the other three regions mainly yielded *C. jejuni*. The reasons for the variations are unknown and could be attributable to differences in production practices and environments. Similarly, reports by other investigators also reported wide variation in the prevalence of *Campylobacter* in other countries. A 50% isolation rate of *C. jejuni* from chickens was reported in South Africa (Bester and Essack, 2008), compared to 20.8% in Grenada (Hariharan et al., 2009). *C. coli* is often detected in pigs (Varela et al., 2007), but a high prevalence (44.4%) in broiler chickens was also reported in Grenada (Hariharan et al., 2009).

Our findings revealed the high prevalence of fluoroquinolone-resistant *Campylobacter* in poultry in China. The prevalence of fluoroquinolone-resistant *Campylobacter* varies greatly between different countries. No fluoroquinolone-resistant isolates were detected in Norway (Norström et al., 2007), and a 9.4% ciprofloxacin resistance rate was reported in Grenada (Hariharan et al., 2009). In contrast to the low resistance reported in the above two studies, ciprofloxacin-resistant *Campylobacter* was high in India (77.1%) (Jain et al., 2005), the United Arab Emirates (85.4%) (Sonnevend et al., 2006), and South Africa (91%) (Bester and Essack, 2008). Our results were comparable with the findings in the latter countries. The high fluoroquinolone-resistance rates of *Campylobacter* in our study may be attributed to the widespread use of fluoroquinolones in poultry production in China. This class of antibiotics is used for both prevention and control

Table 5
Distributions of MICs of 11 antimicrobial agents for 52 *C. coli* isolates.

Antimicrobial agent	MIC (μg/mL)												Susceptible isolates[b]	Intermediate isolates[b]	Resistant isolates[b]
	≤0.25	0.5	1	2	4	8	16	32	64	128	256	≥512			
Nalidixic acid	-[c]	-	0	0	0	0	0	[a]0	[a]0	3	45	4	0 (0)	0 (0)	52 (100)
Ciprofloxacin	0	0	0	0	0	0	0	21	25	6 (≥128 μg/mL)			0 (0)	0 (0)	52 (100)
Enrofloxacin	0	0	0	0	0	27	24	1	0	-	-	-	0 (0)	0 (0)	52 (100)
Erythromycin	0	0	0	0	0	0	0	0	0	0	0	52	0 (0)	0 (0)	52 (100)
Azithromycin	0	0	1	0	0	0	0	0	0	51 (≥128 μg/mL)			1 (1.9)	0 (0)	51 (98.1)
Clindamycin	0	0	0	0	0	0	10	32	0	10 (≥128 μg/mL)			0 (0)	0 (0)	52 (100)
Florfenicol	0	1	24	13	12	1	0	0	1	-	-	-	50 (96.2)	1 (1.9)	1 (1.9)
Chloramphenicol	-	14	0	16	10	9	1	2	0	-	-	-	49 (94.2)	1 (1.9)	2 (3.8)
Tetracycline	0	0	0	0	0	0	0	1	2	49 (≥ 128 μg/mL)			0 (0)	0 (0)	52 (100)
Doxycycline	0	0	0	0	1	3	5	28	11	4 (≥128 μg/mL)			0 (0)	1 (1.9)	51 (98.1)
Gentamicin	3	0	1	0	0	0	6	17	25 (≥ 64 μg/mL)				4 (7.7)	0 (0)	48 (92.3)

[a] Thin vertical lines indicate the breakpoint between susceptible and intermediate isolates. Thick vertical lines indicate the breakpoint between intermediate and resistant isolates.
[b] Numbers in parentheses indicate the percentages.
[c] no data available.

of poultry diseases. It is well known that use of fluoroquinolones in poultry selects for fluoroquinolone-resistant mutants and leads to the emergence of fluoroquinolone-resistant *Campylobacter* in the treated birds (Luangtongkum et al., 2009). In addition, fluoroquinolone-resistant *Campylobacter* does not show a fitness cost and can effectively compete against fluoroquinolone-susceptible *Campylobacter* in chickens in the absence of antibiotics (Luo et al., 2005).

Similar to fluoroquinolones, the resistance to tetracyclines was also highly prevalent in the *Campylobacter* isolates obtained in this study. This finding is consistent with previously reported studies on tetracycline-resistant *Campylobacter* in other regions (Cui et al., 2005; Luangtongkum et al., 2006; Bester and Essack, 2008). With macrolides and clindamycin, the resistance rate of *C. coli* to these two classes of drugs was much higher than *C. jejuni* in this study. Since the majority of the *C. coli* isolates were from Penglai and Shenxian, we further compared the macrolide resistance rates of *C. coli* and *C. jejuni* from these two regions. For each of the two regions, the prevalence of resistance to macrolides and clindamycin was much higher than that for *C. jejuni* (data not shown), suggesting that the difference is likely due to the intrinsic ability of *C. coli* to develop resistance to the antibiotics. Similarly, the level of gentamicin resistance (Tables 4 and 5) was moderate for *C. jejuni* (27.2%), but very high for *C. coli* (92.3%). Overall, the gentamicin resistance rates identified in this study are much higher than reported in other studies (Trieber and Taylor, 2000). Further study is required to determine what contribute to the high-level resistance to macrolides, clindamycin and gentamicin in the *C. coli* isolates.

Another interesting finding of this study is the high rate of resistance in *C. jejuni* to the phenicols, especially to florfenicol (Table 4), which is in contrast to many previously reported studies in other countries (Trieber and Taylor, 2000; Hariharan et al., 2009). The high resistance rate to florfenicol was likely due to the long-term use of this antibiotic as a growth promoter for broilers in China prior to 2006. However, the usage of florfenicol in poultry production may not totally explain the high

Table 6
Multidrug resistance patterns of the *Campylobacters* isolates.

Campylobacter species	Number of resistant agents	Antimicrobial resistance patterns	Number of isolates
C. jejuni	2	Q, T	13
		M, T	1
	3	Q, L, T	4
		Q, **F**, **T**	**86**
		Q, T, G	1
		Q, M, T	5
	4	Q, M, T, G	3
		Q, **M**, **F**, **T**	**27**
		Q, L, F, T	6
		Q, **F**, **T**, **G**	**28**
		Q, M, L, T	3
	5	Q, M, F, T, G	10
		Q, M, L, T, G	10
		Q, M, L, F, T	2
		Q, L, F, T, G	1
	6	Q, M, L, F, T, G	2
C. coli	4	Q, M, L, T	4
	5	**Q**, **M**, **L**, **T**, **G**	**45**
	6	Q, M, L, F, T, G	3

Abbreviation of antimicrobial agents: Q, Fluoroquinolones (nalidixic acid, ciprofloxacin and enrofloxacin); M, Macrolides (erythromycin and azithromycin); L, Clindamycin; P, Phenicols (chloramphenicol and florfenicol); T, tetracyclines (tetracyclin and doxycycline); G, Gentamicin.
[a]Boldface indicates prevalent pattern.

resistance rate, because the *C. coli* isolates obtained in this study were basically susceptible to the phenicols despite the fact that they were also exposed to the same selection pressure. Other mechanisms might also have contributed to the prevalence of and difference in phenicol resistance in the *C. jejuni* and *C. coli* isolates.

A large number of *Campylobacter* isolates obtained in this study showed a multidrug resistance phenotype. Many of the *C. jejuni* isolates were multi-resistant to fluoroquinolones, tetracyclines and phenicols, while the majority of the *C. coli* isolates had multidrug resistance to all classes of test agents except to phenicols. Few reports observed such a high frequency multidrug resistance in *Campylobacter* as reported in this study. The multidrug resistance patterns along with the high MICs of various antibiotics may reflect the overuse of different antimicrobial agents in the poultry production. Once it is prevalent, removal of antibiotic selection pressure may not simply diminish antibiotic resistance.

In conclusion, antimicrobial resistance is highly prevalent in the poultry *Campylobacter* isolates from Shandong, China, and many of them are resistant to multiple antimicrobial agents with high MIC values. Although *Campylobacter* as a cause for foodborne diseases is still underestimated in China, the high prevalence of multidrug resistant *Campylobacter* in broilers is alarming, given the fact that contaminated poultry meat is the major source of human *Campylobacter* infections. Foodborne transmission of antibiotic-resistant *Campylobacter* to humans compromises the clinical treatment of human campylobacteriosis. Thus, prudent measures for antimicrobial usage and active surveillance should be established to reduce the prevalence and spread of antimicrobial resistant *Campylobacter*.

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