



Study on antibiotic resistance profile and multiple antibiotic resistance index (MAR Index) in the *Campylobacter* spp. isolates from domestic animals and water

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ABSTRACT

Introduction: The purpose of this study was comparison study on antibiotic resistance profile and multiple antibiotic resistance index (MAR Index) in the *Campylobacter* spp. isolates from domestic animals and water.

Methods: The totally 364 fecal samples collected from poultry (182), sheep (41), cow (141) farms and drinking water (28) samples from animal farms were examined for isolation of *Campylobacter* spp. using pre-treatment-Kapandis Baseri (prêt KB) method. The isolates were confirmed by sequencing of *16srRNA* genes. Furthermore, *Campylobacter* isolates were evaluated for antibiotic resistance and multiple antibiotic resistance index (MAR Index) by disk diffusion method.

Results: The results indicated that *Campylobacter* spp. isolated from 50 samples. The isolation rate was highest in poultry (37/50) and lowest in goat (2/50). 36 and 14 isolates were identified as *C. jejuni* and *C. coli*, respectively. All of *C. jejuni* and *C. coli* isolates found resistant to amoxicillin/clavulanic, erythromycin and chloramphenicol and all sensitive to ciprofloxacin, kanamycin, gentamicin, streptomycin, tobramycin, tetracycline and imipenem. 36 and 14 percent of *C. jejuni* and *C. coli* (respectively) had multiple antibiotic resistance index 0.2 and upper.

Conclusion: Therefore, based on foregoing evidence, all of the isolates were resistant to antibiotics, therefore, human infection with *Campylobacter* spp. via utilization of animal origin products is possible.

Keywords: *Campylobacter*, antibiotic resistance profile, multiple antibiotic resistance index, domestic animals, water.

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INTRODUCTION

Members of *Campylobacter* genus are Gram-negative, microaerophilic, and motile curved bacilli, whose characteristics are similar to those of *Arcobacter*, and *Helicobacter* [1]. These bacteria are characterized by oxidase, catalase, hydrogen sulfide production, indoxyl acetate hydrolysis, sensitivity to nalidixic acid and cephalothin, resistance to triphenyltetrazolium chloride and hippurate tests. *Campylobacter* strain dose not oxidize and ferment

carbohydrates, they do not hydrolyze urea, but they reduce nitrate [2].

There are more than 30 species of *Campylobacter*. The species and subspecies that are most associated with human infections including *C. jejuni*, *C. coli*, *C. fetus*, *C. lari* [3]. *Campylobacter* spp., specifically *C. jejuni* and *C. coli*, cause campylobacteriosis in humans [4].

C. jejuni is the cause of watery diarrhea or bloody diarrhea. Different strains of *C. jejuni*, like enterotoxigenic strains of *Escherichia coli* and *Shigella dysentery*, produce heat-resistant

enterotoxin. Diarrhea-producing *C. jejuni* strains invade the intestinal epithelium with a method similar to *Shigella* infections. This bacterium is associated with Guillain-Barré syndrome. *Campylobacter* invades the lamina propria of the small and large intestine and causes cryptic abscesses. Unlike *C. fetus*, *C. jejuni* is very sensitive to the lysate and opsonic activity of antibodies and complement [4].

The most common disease caused by *Campylobacter* is acute enteritis. After 3 to 5 days of latent period, the disease starts with severe watery or bloody diarrhea. Fever and abdominal pain are also accompanied by diarrhea. In some cases, abdominal pain is so severe that it looks more like appendicitis or acute ulcerative colitis. Stools contain Vibrio-like bacteria and may contain blood. *Campylobacteriosis* is a mild enteritis and is a self-limiting disease that usually lasts one to seven days [5].

Campylobacter has been introduced as one of the main factors of sporadic food-borne enteritis in humans. [2] The transmission to individuals could take place by consumption of contaminated water, raw or unpasteurized milk, and contaminated food, especially poultry products. However, there are other sources like direct contact with domestic and wild animals [3].

Campylobacters gradually acquire resistance with respect to antibiotics and some of them have developed multidrug resistance [6]. For years, macrolides and fluoroquinolones were the first and the second selections for antimicrobial treatment of enteritis caused by *campylobacters* [7]. Use of fluoroquinolones as prophylaxis in poultry causes increased resistance in relation to fluoroquinolones [8, 9]. Chai *et al.* (2008) reported that the resistance of *C. jejuni* to the fluoroquinolones group of antibiotics is related to agricultural activities [6].

Krumperman reported the usefulness of the Multiple Antibiotic Resistance Index for the identification of bacterial isolates from high-risk environments or contaminated stools [10].

The studies on the two species *C. jejuni* and *C. coli* show that antimicrobial resistance (AMR) genes can rapidly transmit between animal and human strains [5, 9]. The transmission of this resistant campylobacter to human can complex the treatment of *Campylobacteriosis* in human

and transfer resistant genes to gastrointestinal microflora of human.

Therefore, the present study conducted to compare the antibiotic resistance pattern and multiple antibiotic resistance index (MAR Index) in the *Campylobacter* spp. isolates from animals and farm waters, in order to find alternative drugs, promising source of pharmaceutical agents, guiding therapy and epidemiological monitoring of resistance.

MATERIALS AND METHODS

Sample collection

This study was approved from respective Ethics Committee of Behbahan branch, Islamic Azad University (IR.BEH.EC. 12387). In all, 392 fecal and 28 drinking tap water samples were collected from 28 animal farms of Behbahan city (Khuzestan Province, Iran). From these, 364 fecal samples were collected from poultry, cow, sheep and goat and 28 samples were collected from drinking tap water of animal farms. There was no history of previous disease outbreak in these farms. The Sampling was achieved between Jan and Sep 2021.

The samples were collected on sterile bags and falcon tube and examined for *Campylobacter* detection.

Sample processing and isolation

In this study, the isolation of *Campylobacter* was achieved according to pre-treatment-Kapandis Baseri (prêt-KB) technique by antibiotic free Kapandis Baseri (KB, Merk, Germany) medium. For this, the fecal and water samples were suspended at 10% sterile phosphate-buffered saline (0.1 mol L⁻¹, pH = 7). The suspension was centrifuged at 8500 rpm for 10 min and 0.1 ml supernatant from the prepared samples was cultured on the KB. The water samples were mixed with 10% sterile phosphate-buffered saline (0.1 mol L⁻¹, pH = 7) and centrifuged in 4000 rpm within 10 minutes; then, 0.1 ml supernatant from the tube was cultured on the KB [11].

All plates were incubated in microaerophilic conditions at 37°C for 48 h. All cultures examined daily for 5 days.

Identification and confinement of *Campylobacter* spp.

All suspected colonies grew on the KB were confirmed by typical morphology, motility, gram staining, catalase and oxidase tests. The isolates were examined for standard *Campylobacter* phenotypic identification that recommended by Atabay and Corry (1997) [12]. These tests included H₂S by lead acetate strip, nitrate reduction, growth in 1% glycine and 3.5% NaCl, at temperatures 25°C, 37°C, and 42°C. Furthermore, indoxyl acetate hydrolysis, hippurate hydrolysis, urease production, and resistance to nalidixic acid (30 µg) and cephalothin (30 µg) was examined. The Additional tests including oxidative-fermentative test, alkaline phosphatase production, and glucose fermentation for identification of *Campylobacters* were carried out.

At the end, the PCR method was utilized to confirm the phenotyping results.

DNA extraction and PCR method

DNA was extracted from suspected colony using phenol chloroform method. The PCR method was achieved in 25 ml of volume with 1µg of template DNA, 1 µmol L⁻¹ universal primer (27F/1492R) or specific primer (*Campylo* 16SrRNAF/*Campylo* 16SrRNAR), 3.2 m mol L⁻¹ MgCl₂, and 2 U/ 50µl of Taq DNA polymerase. The characteristics of primers were illustrated in table 1. All items used in the PCR were purchased from Yekta Tajhiz Azma (Tehran, Iran). PCRs were performed on a veriti 96-well fast thermal cycler (Applied Biosystems, Singapore), with an initial denaturation step of 94°C for 1 minute followed by 30 cycles of 94°C for 20 s, 54°C for 30 s (for 27F/1492R at 55°C for 30 s), and 72 °C for 40 s,

along with a final extension step at 72 °C for 2 minutes. PCR products were electrophorized using 1% agarose gel at 80 V for 60 minutes. The amplified genomes were visualized with UV transilluminator (Heidolph, Germany) after staining ethidium bromide (CinnaGen, Iran). A 100-bp DNA ladder (Yekta Tajhiz Azma, Tehran, Iran) was used as a DNA molecular ladder. The PCR products sent to Macrogen Company (Macrogen Inc. Seoul, Republic of Kore) for sequencing. The Basic Local Alignment Search Tool (BLAST) alignment tool (<http://www.ncbi.nlm.nih.gov/BLAST/>) was used to identify sequences in GenBank with the highest similarity to the isolated bacteria.

Antibiotic susceptibility by disc diffusion method

The antimicrobial susceptibility pattern of the strains under study was studied by the disc diffusion method designed by Bauer *et al.* (1966) [15]. To performing the disc diffusion method, each culture was grown in 5 mL of Muller-Hinton broth until the turbidity corresponded to 0.5 MacFarland standard tube (1.5×10⁸ cells mL⁻¹). The suspension was spread inoculated using sterile cotton swab onto Muller- Hinton agar plate and 14 types of antibiotics that are frequently used in clinical and agricultural practices were placed on it. After incubating, the plates at 37°C under microaerophilic conditions for 48 h, the inhibition zones were recorded.

Table 1. The characteristics of primers

Name of primers	Sequence (5 to 3)	Target gene	Annealing temperature (°C)	Amplicon size	Reference
27F	AGAGTTTGATCMTGGCTCAG	16SrRNA	55	1490	[13]
1492R	CGGTTACCTTGTTACGACTT				
<i>Campylo</i> 16SrRNAF	CCGGGAACGTATTCACCG	16SrRNA	54	1232	[14]
<i>Campylo</i> 16SrRNAR	TGCCCTACACAAGAGGACAAC				

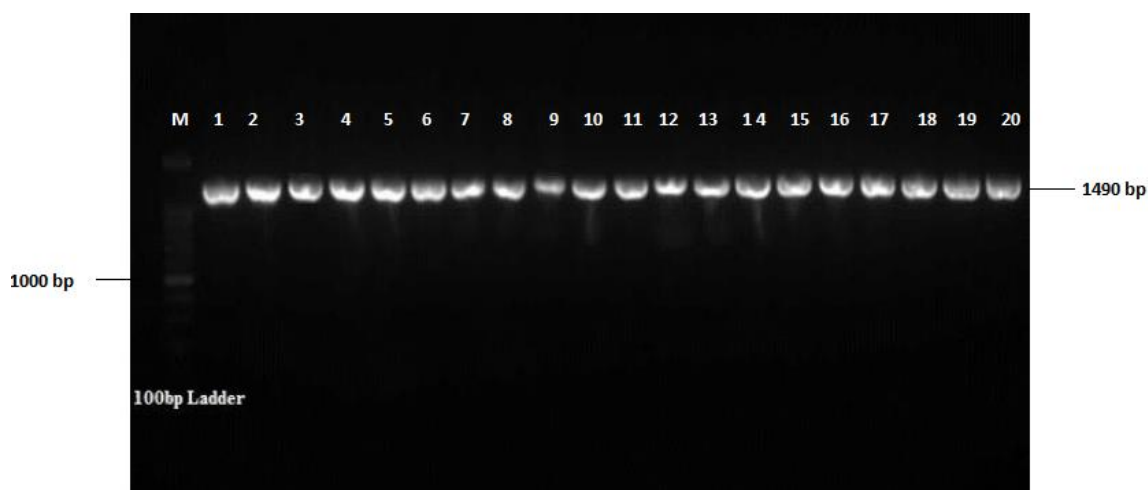


Figure 1. PCR product electrophoresis (M: Marker, 1: positive control, 2 to 20: positive samples).

The antibiotic discs included: ampicillin (10µg), amoxicillin/clavulanic ac. (20/10 µg), cephalotin (30 µg), cefotaxime (30 µg), imipenem (10 µg), streptomycin (10IU), gentamicin (15µg (10 IU)), kanamycin (30IU), tobramycin (10 µg), erythromycin (15 IU), nalidixic acid (30 µg), ciprofloxacin (5 µg), tetracycline (30 IU), and chloramphenicol (30 µg) (Padtanteb, Iran) [16].

Multiple antibiotic resistance index (MAR index)

The MAR index of the isolates was determined as a/b, where 'a' represents the number of multiple antibiotics to which the specific isolates are resistant, and 'b' represents the number of multiple antibiotics to which the specific isolates are exposed [10].

RESULTS

The fragment of 1490 bp was amplified in poultry, cow, sheep, and water samples (Figure

1). The highest and lowest frequency of *Campylobacter* species were isolated from poultry and sheep samples, respectively. The total of 50 *Campylobacter* isolates were identified in PCR method. The frequency of *Campylobacter* isolates was summarized in table 2.

In phenotyping evaluation and molecular confirmation, 36 strains of *C. jejuni* (72%) and 14 strains of *C. coli* (28%) were identified.

The highest and lowest frequency of *C. jejuni* and *C. coli* was isolated from poultry and sheep fecal samples, respectively. The frequency of *C. jejuni* and *C. coli* isolates in poultry, cow and sheep fecal samples and water samples was presented in Table 3.

As shown in Table 4, all the isolates of *Campylobacter* were sensitive to ciprofloxacin, kanamycin, gentamicin, streptomycin, tobramycin, tetracycline, imipenem and resistant to amoxicillin/clavulanic, erythromycin and chloramphenicol. Amongst *C. jejuni* isolates,

Table 2. Frequency of *Campylobacter* spp. isolated from domestic animal feces and water

Samples	No. of samples	No. of <i>Campylobacter</i> spp. isolated	Occurrence of <i>Campylobacter</i> spp. isolated(%)
Poultry feces	182	37	20.3
Cow faeces	141	8	5.7
Sheep feces	41	2	4.9
Water	28	3	10.7
Total	392	50	12.8

Table 3. Frequency of *C. jejuni* and *C. coli* isolated from domestic animal feces and water samples

Samples	No. of <i>Campylobacter</i> spp. isolated	No. of <i>C. jejuni</i> isolated (%)	No. of <i>C. coli</i> isolated (%)
Poultry feces	37	29(78.4)	8(21.6)
Cow feces	8	4(50)	4(50)
Sheep feces	2	1(50)	1(50)
Water	3	2(66.67)	1(33.34)
Total	50	36(72)	14(28)

91.7 of them were sensitive to cephalotin. Less than 50% of the *C. jejuni* and *C. coli* isolates were sensitive to nalidixic acid, ampicillin and cefotaxime. Amongst *C. coli* isolates, 85.7 of them were sensitive to cephalotin.

The antimicrobial resistance profile and MAR index of the *Campylobacter* isolates are shown in Table 5. All of the isolates were resistant to

multiple antibiotics, with a multiple antibiotic resistance index (MARI) ≥ 0.2 . The 36 *C. jejuni* strains exhibited 9 different antibiogram patterns. The majority of the *C. jejuni* strains (15 isolates) were resistant to six antibiotics (MAR index of 0.43) with the resistant pattern NAL AMC AMP CTX ERY CHL (13 isolates) dominating the group. The 14 *C. coli* strains showed 6 different

Table 4. susceptibility of environmental *Campylobacter*'s isolates from domestic animal feces and water samples by disc diffusion method

Antibiotics	<i>C. jejuni</i> (36)	<i>C. coli</i> (14)
*CIP	100	100
NAL	5.6	14.3
KAN	100	100
GEN	100	100
STR	100	100
TOB	100	100
AMC	0	0
AMP	36.1	8.6
CEF	91.7	85.7
CTX	36.1	7.1
ERY	0	0
TET	100	100
IPM	100	100
CHL	0	0

*CIP: Ciprofloxacin, NAL: Nalidixic acid, KAN: Kanamycin, GEN: Gentamicin, STR: Streptomycin, TOB: Tobramycin, AMC: Amoxicillin/clavulanic ac., AMP: Ampicillin, CEF: Cephalotin, CTX: Cefotaxime, ERY: Erythromycin, TET: Tetracycline, IPM: Imipenem, CHL: Chloramphenicol

Table 5. Antibiotic resistance profile and multiple antibiotic resistance index of *C.jejuni* and *C.coli* from poultry, cow and sheep and goat and water samples.

<i>Campylobacter</i> species	Antimicrobial Resistance Pattern	Antimicrobial Classes	No. of isolates (%)	MAR index
<i>C.jejuni</i>	AMC, AMP, ERY, CHL	4	1(2.8%)	0.29
	NAL, AMC, ERY, CHL	4	5(13.9%)	0.29
	AMC, AMP, CTX, ERY, CHL	5	1(2.8%)	0.36
	NAL, AMC, CTX, ERY, CHL	5	7(19.4%)	0.36
	NAL, AMC, AMP, ERY, CHL	5	6(16.6%)	0.36
	NAL, AMC, CEF, CTX, ERY, CHL	6	1(2.8%)	0.43
	NAL, AMC, AMP, CTX, ERY, CHL	6	13(36.1%)	0.43
	NAL, AMC, AMP, CEF, ERY, CHL	6	1(2.8%)	0.43
	NAL, AMC, AMP, CEF, CTX, ERY, CHL	7	1(2.8%)	0.5
<i>C.coli</i>	AMC, AMP, ERY, CHL	4	1(7.1%)	0.29
	AMC, AMP, CTX, ERY, CHL	5	1(7.1%)	0.36
	NAL, AMC, CTX, ERY, CHL	5	4(28.6%)	0.36
	NAL, AMC, AMP, CTX, ERY, CHL	6	4(28.6%)	0.43
	NAL, AMC, CEF, CTX, ERY, CHL	6	2(14.3%)	0.43
	NAL, AMC, AMP, CEF, CTX, ERY, CHL	7	2(14.3%)	0.5

AMC: Amoxicillin/clavulanic ac., AMP: Ampicillin, ERY: Erythromycin, CHL: Chloramphenicol, NAL: Nalidixic acid, CTX: Cefotaxime, CEF: Cephalotin

resistance patterns. The majority of the *C.coli* strains (6 isolates) were resistant to six antibiotics (MAR index of 0.43). The resistant patterns AMC AMP ERY CHL, AMC AMP CTX ERY CHL and NAL AMC AMP CEF CTX ERY CHL were shared by one *C.jejuni* and one *C.coli* strains while NAL AMC CTX ERY CHL was common to seven *C.jejuni* and four *C.coli* strains. The resistant pattern NAL AMC AMP CTX ERY CHL was shared by 13 *C.jejuni* and 4 *C.coli* strains while NAL, AMC, CEF, CTX, ERY, CHL was common to one *C.jejuni* and two *C.coli* strains.

DISCUSSION

Several researchers have stated that the use of antibiotics in animals and poultry as a growth supplement leads to antibiotic resistance and reduction in the effectiveness of these products in medicine and veterinary use. It is possible that during or after the antibiotic treatment, these resistant strains appear [17]. In the present study, all the isolated strains were sensitive to ciprofloxacin, tetracycline, imipenem, gentamicin, streptomycin, kanamycin, and tobramycin. Our study results for ciprofloxacin were similar to the findings by other researchers

[11, 18]. In the recent decades, some of the authors have reported the increasing trend of resistance to ciprofloxacin in some of the *Campylobacter* species, especially those that are isolated from poultry [9, 19, 20, 21]. In our study, the *Campylobacter* isolates were sensitive in relation to tetracycline; similar reports have been delivered by other researchers. However, a few authors also reported resistance to tetracycline [9, 19, 20]. In our research, all isolates were sensitive in relation to aminoglycosides (gentamicin, streptomycin, kanamycin and tobramycin). Similar results have been reported by some authors [18, 22, 23]. In opposition, others have reported few cases of resistance in relation to these antibiotics in the *Campylobacter* species [19, 21, 24]. In our research, all the *Campylobacter* isolates were resistant to amoxicillin/clavulanic acid, erythromycin, and chloramphenicol. Some of the isolates exhibited variable resistances in relation to nalidixic acid, ampicillin, cefotaxime, and cephalothin. In our research, 100% of the isolated *Campylobacter* species were resistant to erythromycin, which is supported by the findings of other researchers [18, 24]. Numerous authors have reported results similar to our research for the resistance of *Campylobacter* isolates in relation to amoxicillin/clavulanic acid, ampicillin, nalidixic acid, cefotaxime, cephalothin, and chloramphenicol [25, 26]. However, some of the authors have reported cases of *Campylobacter* isolates' sensitivity to amoxicillin/clavulanic acid, ampicillin, nalidixic acid, cefotaxime, cephalothin, and chloramphenicol [23, 24]. Our studies showed that all the *Campylobacter* isolates showed resistance to four to seven antibiotics. In our research, all isolates had multiple antibiotic resistance index 0.29-0.5.

CONCLUSION

The resistance of *C. jejuni* and *C. coli* to macrolides (erythromycin), amoxicillin/clavulanic acid, ampicillin, nalidixic acid, cefotaxime, cephalothin, and chloramphenicol was an alarming finding in this study since this resistance could result from the broad and non-methodical usage of these antibiotics in the veterinary field. Considering our research, ciprofloxacin is introduced as a selective antibiotic for

campylobacteriosis and aminoglycosides, tetracycline and imipenem as the alternate antibiotics in this geographical zone. In this study, the entire isolates resistance to antibiotic had an MRA Index of 0.29 to 0.5. Considering of this index, the consumption of sheep, cows, or poultry, and contaminated food derived from them can be a source of danger and potential contamination. This could impact human health as well as the state of the commercial industry that deals with such edibles.

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ETHICS

This study approved in Ethics Committee of veterinary medicine faculty of Behbahan Branch, Islamic Azad University, Behbahan, Iran (IR.BEH.EC. 12387)

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DECLARATIONS

The authors declare that they have no conflict of interest.

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