Comparative Immunology, Microbiology and Infectious Diseases 65 (2019) 194–200

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Antimicrobial resistances, and molecular typing of *Campylobacter jejuni* isolates, separated from food-producing animals and diarrhea patients in Iran

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ARTICLE INFO

Keywords:
Campylobacter jejuni
Diarrhea
MIC
Antimicrobial resistance gene
Fla-typing
RFLP

ABSTRACT

The aims of this study were to regain new epidemiology information about frequency, drug resistance rates, and typing of *Campylobacter jejuni* (*C. jejuni*) isolates, obtained from some poultry and cattle farms, slaughterhouses, and people with diarrhea. In this regard, Minimal Inhibitory Concentration (MIC) of several antibiotics and the associated antibiotic resistance genes, including *tetO*, *tetA*, *cmeB*, and $bla_{OXA\text{-}61}$ were evaluated. The isolates were also typed, using the *Fla*-RFLP method. Generally, between 233 food animal samples, 80 (34.33%) *C. jejuni* were isolated. Moreover, 20 out of 74 (27%) human specimens suspected to infectious diarrhea were *C. jejuni* positive. High frequencies of resistance to tetracycline (100%), ciprofloxacin (95%), and nalidixic acid (86%), and low frequencies of resistance to florfenicol (0%), erythromycin (5%), and gentamicin (8%) were observed. Furthermore, in the tetracycline-resistant isolates, the existences of *tetO*, *tetA*, and *cmeB* were 86%, 23%, and 48%, respectively. There was a significant correlation between the cluster types obtained from *Fla*-RFLP method and antibiotic resistance pattern. The results suggested that the genomic link between *Campylobacter* spp. should be always evaluated in each country to provide an insight about the *Campylobacter* spp., spread in the region, in order to implement the health-controlling programs efficiently.

1. Introduction

Campylobacter jejuni (*C. jejuni*), a gram-negative bacterium and food-borne pathogen, causes gastrointestinal (GI) diseases in human. The Center for Disease Control (CDC) and European-Food-Safety-Authority (EFSA) reports indicated that this bacterium along with *Salmonella* spp., are causing the greatest number of cases of bacterial GI diseases in the US and European Union. These two bacteria are located on the top of the list, in term of the incidence rate. The overall incidence of campylobacteriosis was 14 and 66.3 per 100,000 population in the US and European Union in 2016, respectively [1,2]. *C. jejuni* can colonize poultry, ruminant, and pig animals [3,4]; therefore it can be easily transmitted to humans through handling and consuming raw and semi-raw contaminated foods, as well as contaminated water [5,6]. The disease is self-limiting with diarrhea, abdominal pain, cramps, and fever symptom, which usually continues for about one week. However, sometimes the illness can become complex, especially in infants, geriatric patients, and people with immune deficiency and can lead to Guillain Barré and Miller Fisher syndromes [7]. In these cases, antibiotic therapy is essential. Macrolides, fluoroquinolones, and gentamicin are first-line treatment drugs [8]. However, during this decade, the increase in the emergence of new drug-resistant *C. jejuni* strains becomes a major global health challenge, which led the World Health Organization (WHO) to categorize *C. jejuni* as high drug-resistant bacteria [7]. Uncontrolled use of antibiotics such as macrolides and fluoroquinolones in food-producing animals and human medicine may have contributed to this increase [3,4]. Several mechanisms are proposed for antibiotic resistance in this bacterium. The main factor of resistance to tetracycline is the presence of *tetO/A* on the chromosome or plasmid of the bacterium, which has been found widely in isolates, recovered from various sources [9,10]. Furthermore, *Campylobacter* strains can produce β-lactamase enzymes due to the presence of $bla_{OXA\text{-}}$

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https://doi.org/10.1016/j.cimid.2019.06.001
Received 29 December 2018; Received in revised form 26 May 2019; Accepted 3 June 2019
0147-9571/

$_{61}$ in their chromosomes; hence, they are resistant to beta-lactam antibiotics [11]. It is proven that point mutations are involved in high levels of resistance to quinolones and fluoroquinolones [8]. In addition, in this strain, the presence of CmeABC as the active efflux pump can be a candidate for resistance to different classes of antibiotics, including fluoroquinolones, macrolides, tetracyclines, and beta-lactams [12].

The evaluation of intra-species variation of an organism, using molecular typing techniques, is very crucial in the epidemiological studies. These methods can increase our knowledge about infectious diseases and association between strains, distributed in different sources and areas, in order to detect new outbreaks, the emergence of antimicrobial resistance, and new evolving pathogenic strains [2,13]. One of the most reliable *Campylobacter* typing methods is based on the *flagellin* gene, which is known as *fla*-typing. It is a fast, inexpensive, and simple method and has a high level of differentiation; hence, researchers widely use it [14,15]. It is noteworthy that another method, PFGE, has also high levels of differentiation, but it is a very expensive method and may not be available everywhere, therefore, in this research *fla*-typing method was used. In the *fla*-typing method, a restriction enzyme, such as *DdeI*, digest an amplified segment of the *flagellin* locus to generate fingerprints [16].

To monitor trends of increasing drug resistance in pathogens derived from infected patients and food products, more antimicrobial susceptibility testing should be updated every few years [8,17]. It is also suggested that evaluation of the origin of the resistant strains, using antimicrobial resistance and typing method, simultaneously, can help to understand the resistance mechanisms of the isolates and ways of circulation of the bacteria between different sources. It has been shown that the emergence of resistant strains of bacteria can be derived from different roots [5]. Updating information about this field will ultimately lead to better identification of treatment methods; therefore, to update this information, this study was conducted in two steps. First, the presence of the *C. jejuni* contamination in the poultry, cattle, and human was investigated, and then the antimicrobial susceptibility tests of the obtained isolates and the presence of some antimicrobial genes were determined. Subsequently, possible associations between clusters and resistant isolates were assessed, using *Fla*-typing generated fingerprints.

2. Material and methods

2.1. Study area and sample collections

Regarding the main aim of this study (found a possible association between the clusters of the *C. jejuni* isolates and antibiotic resistance pattern using the *Fla*-typing method), we preferred that the number of the isolates was equal in each source. Therefore, sampling was performed until 20 *C. jejuni* isolates were obtained from each source. The samples were collected from some poultry and cattle farms, and slaughterhouses located in Mazandaran province, Iran. These samples were collected during six months, from June to December of 2017, as follows: rectal swab samples from poultry and cattle animals located in different farms around Amol city, Iran; meat pieces from chicken and cattle carcasses in different poultry and ruminant slaughterhouses located in Amol, Iran. Each swab was placed in a bottle contained Stuart's transport medium (Difco Laboratories, Detroit, Michigan, USA). Each meat sample was cut aseptically with a sterile scalpel and approximately 25 g of the removed meat was tightly sealed in a sterile plastic wrap. All samples were kept cool in a cold container box, immediately after sampling, and transported to the microbiology laboratory within 4–8 hours.

To obtain human *C. jejuni* isolates, samples were taken from diarrhea patients, who had been suspected for infectious diarrhea, and referred to a private laboratory for sampling and diagnosis, located in Mazandaran province, Iran. At first, several clinicians examined the patients in their clinics and/or treatment centers such as hospitals. When they were suspected that patients might have infectious diarrhea, based on some clinical sign such as loose, watery, and bloodstained diarrhea and fever, they sent a sample to the lab to confirm their diagnosis. Every time a stool sample sent to the laboratory, we checked the patient's history. If there was a diagnosis of infectious diarrhea in the history of the patient, the sample was entered into the study. After obtaining informed consent from patients, the samples were used. Since the determination of the prevalence rate of *Campylobacter* infection in the patients was not the goal of this study, demographic information of the patients was not used in this study. The samples were collected using a standard fecal sample container and applied to the isolation of the bacteria.

2.2. Isolation and identification of C. jejuni

The standard protocol of EN/ISO 10272-1 (2006), published by the International Organization for Standardization (ISO), was employed for the isolation of *C. jejuni* from all samples, sometimes with some modification [18]. Briefly, each fecal swap sample was put in Preston’s enrichment broth base (HiMedia Laboratories; Mumbai, India). Each meat sample was placed in a bottle containing 225 ml of 1% buffered peptone water (Difco, USA), and thoroughly mixed. Five milliliters of the homogenate solution was added to 45 mL of Preston’s broth. The campylobacter selective supplement IV (HiMedia Laboratories, Mumbai, India), as well as 5% (v/v) defibrinated sheep blood were added to all Preston’s broths. Then, they were incubated for 48 h at 42 ± 0.5 °C under microaerophilic atmosphere using Gas-pack C (Merck, Germany; 5% O_2, 10% CO_2, and 85% N_2) in an anaerobic jar. Subsequently, two drops from each broth were used to culture onto the chocolate Columbia Blood Agar (HiMedia Laboratories, Mumbai, India), supplemented with the same antibiotics. The plates were checked after 48 h incubation period at 42 ± 0.5 °C for any suspicious colony with small, gray, drop-like, and shiny properties. These suspected colonies were picked, and sub-cultured on the chocolate agar plates with 5% sheep blood, if necessary. *C. jejuni* were confirmed by conventional PCR assay, as elsewhere described [19]. A reference strain of *C. jejuni*, RTCC 1097, was used as a positive control.

2.3. Antibiotic susceptibility test

Evaluation of antibiotic susceptibility of the isolates was performed using in vitro broth microdilution assay according to the Clinical Laboratory Standards Institute (CLSI) guidelines and with a glance at the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standard [20,21]. The following antibiotics were used: nalidixic acid, ciprofloxacin, tetracycline, streptomycin, florfenicol, gentamicin, erythromycin, and ampicillin (Sigma–Aldrich®, Germany). Briefly, 100 μL of each antibiotic was added to the first well of a 96-well microplate, filled with 100 μL brain heart-infusion broth (BHI) supplemented with 0.5% yeast extract (Torlak, Serbia) and 10% horse serum (Gibco, New Zeland), and was serially diluted to achieve the concentration range from 128 to 0.0625 μg/mL. Each fresh culture of *Campylobacter* isolate was adjusted to 0.5 McFarland standard and 100 μL of approximately 10^5 CFU/mL of bacteria (after diluting in BHI) was pipetted into each well. The plates were incubated under a microaerophilic atmosphere with 60–70% humidity at 42 ± 0.5 °C for 24 h. The lowest concentration of each antibiotic that inhibits bacterial growth was determined, using a microplate reader (ELX800, BioTek, Vermont, USA) to define Minimal Inhibitory Concentration (MIC). The breakpoints of the antibiotics were chosen according to the EUCAST Standard [21]. Where it was not possible, the EUCAST Standard for Enterobacteriaceae was applied. The *C. jejuni* RTCC 1097 was used as quality control. Multidrug resistance (MDR) of the isolates was defined as resistance to three or more antimicrobial groups.

Table 1
Nucleotide sequences used as primers in the polymerase chain reaction for identification of *C. jejuni* and associated antibiotic resistance genes.

Target gene	Sequence (5' to 3')	Annealing temperature (°C)	Product size (bp)	Reference
mapA	CTATTTTATTTTTGAGTGCTTGTG GCTTTATTTGCCATTTGTTTTATTA	52	589	[19]
bla$_{OXA-61}$	AGAGTATAATACAAGCG TAGTGAGTTGTCAAGCC	54	372	[22]
cmeB	TCCTAGCAGCACAATATG AGCTTCGATAGCTGCATC	54	241	[22]
tetO	GCGTTTTGTTTATGTGCG ATGGACAACCCGACAGAAG	54	559	[22]
tetA	GTGAAACCCAACATACCCC GAAGGCAAGCAGGATGTAG	56	888	[10]
flaA	GGATTTCGTATTAACACAAATGGTGC CTGTAGTAATCTTAAAACATTTTG	45	1725	[23]

F: forward; R: reverse.

2.4. DNA extraction and PCR assays

DNA of each isolate was extracted using a gram-negative bacterial DNA extraction kit (CinnaPure DNA kit, Sinaclon, Iran), in accordance with the manufacturer's instructions. As mentioned in the isolation section, each *C. jejuni* isolate was confirmed using amplification of *mapA* by a PCR technique [19]. In addition, the presence of *tetO*, *bla*$_{OXA-61}$, and *cmeB*, involved in the antibiotic resistance mechanisms, were analyzed by a multiplex PCR [22]. The existence of *tetA*, a gene that is related to tetracycline resistance, was also screened using a PCR assay described by Maynard et al., published in 2003 [10]. The details of the primers used in this study are shown in Table 1. The PCR reaction mixtures were contained 12.5 μL of a PCR master mix (Sinaclon, Iran), 1 μL (0.5 μM) of both forward and reverse primers belonged to each gene (*mapA*, *tetA, tetO*, *bla*$_{OXA-61}$, and *cmeB*), and 2–4 μL of each DNA (40–60 ng/μL). The final volume of each mixture brought to 25 μL, using nuclease-free water. The PCRs were run in a PCR machine (Bio-Rad, iCycler, USA), with the following conditions: initial denaturation step at 95 °C for 5 min, 36 cycles with denaturation for 1 min at 94 °C, annealing shown in Table 1 for 1 min, extension for 1 min at 72 °C, and a final extension step at 72 °C for 10 min. For the amplification of the *tetA*, a protocol of 35 cycles was used. The tubes were kept in the machine at 4 °C until they were collected. The PCR products were stained with ethidium bromide (Sinaclon, Iran) in agarose gels (Sinaclon, Iran) and run for 45 min at 100 V, and then visualized under a UV light (BTS-20, Japan). Molecular size marker was a 100-bp DNA ladder (MBI, Fermentas).

2.5. Fragment length polymorphism analysis (Fla-RFLP)

The genetic similarity of the isolates was analyzed by *Fla*-RFLP technique as previously described [14,23]. In the first step, *flaA* was partially amplified, using FlaA-F and FlaA-R primers (Table 1). The PCR reaction mixture was prepared as stated in previous experiments. The program was run in the PCR machine as follows: initial denaturation was set at 94 °C for 4 min, followed by 36 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 40 s, extension at 72 °C for 90 s, and a final extension step at 72 °C for 10 min. In the second step, the PCR products were digested with *DdeI* restriction enzyme (NEB Biolabs, Hertfordshire, United Kingdom), as recommended by the manufacturer's instruction. Subsequently, the bands were separated on a 2.5% agarose gel, containing 0.5 X TBE (Tris-borate ethylenediaminetetraacetic acid) buffer and ethidium bromide (0.5 μg/mL) and run for 80 min at 100 V, and then photographed under UV illumination. Molecular size marker was a 100-bp DNA ladder (MBI, Fermentas).

2.6. Profile analysis

Gel images were saved in a tagged image files format (TIFFs) and loaded in BioNumerics version 6 (Applied Maths, Kortrijk, Belgium) for analysis. The programs normalized and adjusted each fingerprint; after that genetic similarity between the isolates was calculated using the Pearson correlation with 2% of the optimization tolerance and 4% of the position tolerance shift. The dendrogram of the isolates was also created by the Dice correlation coefficient and the unweighted pair group method with arithmetic averages (UPGMA) [24]. A cut-off of 80% was used to determine final groupings. Using Simpson's index diversity (SID), the discriminatory power of the method was determined [25].

2.7. Statistical analysis

The SPSS software, version 16.1 (SPSS, Inc.) was used to analyze any significant statistical differences in the results. The Pearson chi-squared and Fisher's exact two-tailed tests were used to evaluating any association between the clusters and the sources of the isolates and/or the clusters and the resistance to antibiotics. Fisher's exact two-tailed test was also employed to show any association between the results of MICs and resistance genes in the isolates. The significance of each test was set at $P \leq 0.05$.

3. Result

3.1. Frequency of the Campylobacter cases

To achieve 20 *C. jejuni* isolates from each group of samples (including the feces of poultry and cattle, as well as the carcasses of poultry and cattle in the slaughterhouses), 233 samples of food animals were collected and analyzed. Thus, the overall frequency of *C. jejuni* in food animals (without human samples) was 34.33%. In details, 20 out of 25 (80%) and 41 (48%) fecal samples from poultry and cattle were positive, respectively. In addition, 35% and 18% of raw meat obtained from poultry and cattle were contaminated with *C. jejuni,* respectively. In addition, 74 human specimens were entered into the study to isolate 20 *C. jejuni.*

3.2. The frequency of resistance among C. jejuni isolates

The details of the antibiotic susceptibility results, based on the origin of the samples are presented in Table 2. The pan-susceptible isolates, which were susceptible to all tested antibiotics, was observed in none of the isolates. All isolates were susceptible to florfenicol, whereas all of them were resistant to tetracycline. In addition, 95 (95%) and 86 (86%) out of them were also resistant to ciprofloxacin and nalidixic acid, respectively. The low frequency of resistance to gentamicin and erythromycin was observed in eight (8%) and five (5%) isolates, respectively.

Among them, 93 isolates (93%) were MDR. The most frequent MDR

Table 2
Percentage of the *Campylobacter jejuni* isolates resistant to various antibiotic drugs and the associated resistance encoding genes.

Biological origin	Florfenicol	Erythromycin	Gentamicin	Streptomycin	Ampicillin	Nalidixic acid	Ciprofloxacin	Tetracycline	bla_{OXA-61}	*cmeB*	*tetO*	*tetA*
Poultry (N = 20)	0	1	1	8	11	16	20	20	11	10	19	4
Poultry meat (N = 20)	0	2	2	7	12	17	20	20	12	13	17	6
Cattle (N = 20)	0	2	2	5	9	18	18	20	10	9	17	3
Cattle meat (N = 20)	0	0	2	9	7	16	20	20	9	9	17	4
Human (N = 20)	0	0	1	6	6	19	17	20	7	7	16	6
Total (N = 100)	0	5	8	35	45	86	95	100	49	48	86	23

Table 3
The percentage of multidrug resistance (MDR) isolates separated from different sources and types of clusters of these isolates in association with the MDR patterns.

Number of MDR[a]isolates	Source of isolates (N = 20 in each source) and the number of resistance isolates					
	Poultry	Poultry meat	Cattle	Cattle meat	Human	Type of clusters[b]
MDR to three drugs (N = 37)	9	6	5	8	9	2/3/4/5/6/7/8/9/10/11/14
MDR to four drugs (N = 35)	6	7	10	5	7	3/4/6/7/8/9/10/15
MDR to five drugs (N = 15)	4	4	1	4	2	5/6/7/8/9/11/12/13
MDR to six drugs (N = 6)	1	2	2	1	0	8/9/11/12
Total (N = 93)	20	19	18	18	18	–

[a] Multidrug resistance.
[b] This represents the number of clusters that are related to the MDR isolates.

isolates, which included 39 isolates (39%), were resistant to three different antibiotics, which had four different resistance patterns. Resistance to four, five, and six antibiotics were also observed in 35, 15, and 6 isolates. Table 3 shows the resistance patterns of the MDR isolates.

3.3. The occurrence of the antibiotic resistance genes

Among all tetracycline-resistant isolates, 97 isolates (97%) had one, two or all three *tetO*, *tetA*, and *cmeB* genes. *TetO*, *tetA*, and *cmeB* were observed in 86 (86%), 23 (23%), and 48 (48%) tetracycline-resistant isolates, respectively. Nine isolates (9%) possessed all three genes. Seven isolates (7%) harbored two *tetO* and *tetA*, simultaneously. Furthermore, 36 *C. jejuni* isolates (36%) had both *tetO* and *cmeB*. Notably, three isolates (3%), resistant to tetracycline, did not carry any of the genes; among those, two were isolated from cattle and one from a human. All bacteria that were phenotypically resistant to ampicillin were also positive for the bla_{OXA-61}. In addition, three isolates carry the bla_{OXA-61}, but the MIC for them was 4 µg/mL, which were located in the sensitive category.

3.4. Fla-RFLP typing

Overall, *Fla*-RFLP could type 99% of the isolates (Fig. 1), and using this approach the isolates were divided into 15 different groups, named C1 to C15 (SID = 0.7863). Among these groups, five distinct clusters (C9, C8, C3, C4, and C5) contained 75% of the isolates. The most prevalent cluster type was C9 (n = 42), which overlapped between *C. jejuni* belonged to all sources. Five clusters were unique and had only one isolate, including C1 and C15 that had an isolate from cattle source, and C2, C4, and C14 that had a bacterium isolated from cattle meat. In total, the isolates with cattle origin were more variable than the others and scattered between clusters. The isolates with cattle origin had also a lower prevalence in cluster eight (C8), in comparison to the other isolates (Fisher's $p < 0.05$). Furthermore, the isolates derived from cattle were more likely to locate in cluster four (Fisher's $p < 0.05$). Interestingly, all human-associated isolates were grouped in clusters, where certainly isolates from other sources were also present.

3.5. Association between antibiotic resistances, sources, and clusters in the isolates

No significant associations, based on the source of the isolates were observed for the different antimicrobial agents; however, the human isolates were less resistant, in comparison to the other *C. jejuni* isolates. There was a significant correlation between cluster types and antibiotics resistance patterns. The isolates from the cluster 11 and 12 had significantly higher ($P < 0.01$) resistance to some antibiotics than the other tested isolates. No significant associations were observed in the MIC of tetracycline-resistance isolates, based on the number of the genes involved in the development of resistance to tetracycline.

4. Discussion

The current study includes more completed and updated information about the frequency, antimicrobial drug resistance, and genetic diversity of the *C. jejuni* isolates, obtained from different sources, during 2017 in Iran. Most of the studies and reports from researchers and international public health organizations have repeatedly emphasized that there is an increase in the spread of antimicrobial-resistant *Campylobacter* isolates in the world [2,7,26]. In this regard, studies must be conducted to find the reasons behind this increase. Determination of the genetic diversity, level of the genetic similarity, and the relationship of the isolates, as well as the ability of the isolates to infect and disseminate between different sources, can be helpful to find the reasons.

In this study, the overall presence of *C. jejuni* in food products was moderate and in accordance with some other studies. Nisar et al. in 2018 reported that 20% of their samples were positive for *C. jejuni* [27]. Consistent with the result of our study, Khan et al. and Raeisi et al. also reported the overall prevalence of *C. jejuni* in poultry as 30% and 23.46%, respectively [17,28]. The prevalence of *C. jejuni* has been reported highly variable in different sources, however, in most cases, including these two recent studies, poultry samples were always situated on the top of the list, whether in the feces or the meat samples. The reasons for the variation of the results, reported in these studies, have been reviewed elsewhere and suggested that differences in sampling design, testing methods, variations in geographical and seasonal

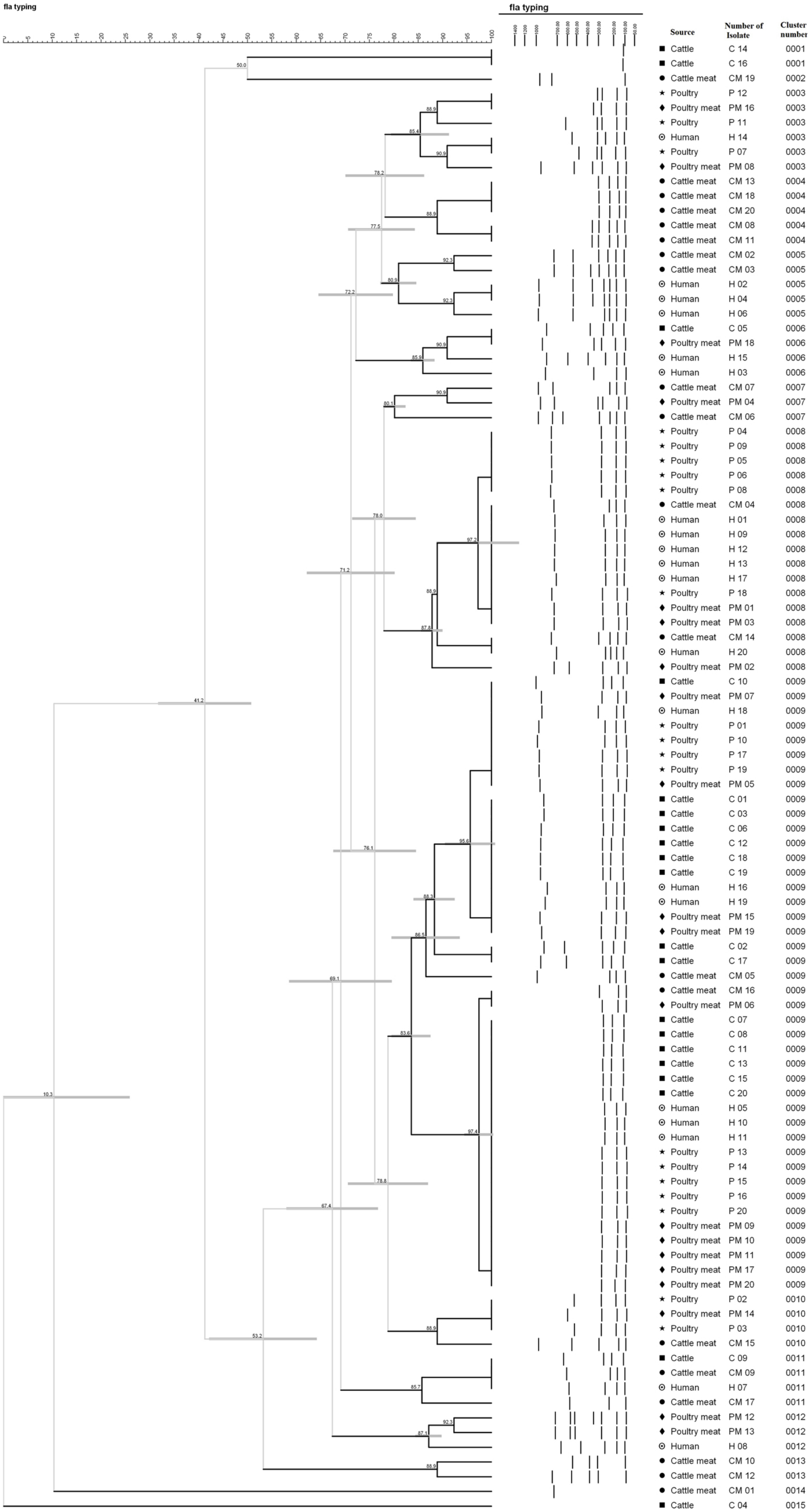

Fig. 1. *Fla*-typing dendrogram of *Campylobacter jejuni* isolates, collected from poultry, poultry meat, cattle, cattle meat, and human, using the UPGMA analysis. The *fla*-typing assay identified 15 different clusters.

factors, transport conditions, population, and differences in the standard of biocontrol protocols and sanitary conditions at the level of farms and slaughterhouses can be involved in this variation results [29]. Among these reasons, the most real and important reason may be sanitary conditions at the level of farms and slaughterhouses. It has been shown that the prevalence of the bacteria can be decreased by the implementation of a strict surveillance/monitoring program of hygiene practices in the European Union (EU) [30]. Therefore, finding a direct correlation among various studies might be difficult. Actually, the prevalence rate of the bacteria should not be compared together, but in general, the most contaminated resources in each region should be investigated and compared together, to find the most important source of the human infection.

Approximately a quarter of the suspected cases of gastroenteritis samples were positive for *C. jejuni*. As the data represented a highly selected population and it just indicated the proportion of the diarrhea cases in which *C. jejuni* is involved, the results were not compared to other studies. However, the high infection rate is alarming and is an indication of public health at risk, especially for children, and in this regard, more attention should be paid to developing countries. Moreover, the prevalence of this bacterium is important in children, especially in children under the age of two years, since *Campylobacter* infections are frequent in children in developing countries and can sometimes kill them [7]. Similar to the current research, in a study published in 2017, Carev et al. reported that about 30% of diarrheic stool samples from children were *C. jejuni* positive [31].

Similarly to the other investigation reports, high resistance to tetracycline, ciprofloxacin, and nalidixic acid was observed in this study [26,29]. Fluoroquinolones and tetracycline antibiotics have been frequently used in the livestock industry for many years [3]. Over these years, out of sight of regulatory systems, uncontrolled use of antibiotics has continuously occurred. Some researchers suggested that this overuse of antibiotics has been the cause of the emergence and development of resistant bacteria via the natural selection phenomenon, as well as genetic and spontaneous mutations [4]. However, other reasons can also be behind the scenes, which need further analysis. No statistical differences in the number of resistant isolates in different sources have been found; however, human isolates showed resistance to antimicrobial drugs at lower levels, in comparison to the others. Several factors may be the cause of this low resistance to antimicrobial drugs in human isolates, including differences in human intestinal microbiota in comparison to the other sources that can communicate differently with *C. jejuni* in the human intestinal environment. Another factor can be lower level of challenging of human *C. jejuni* isolates with various antibiotics [26]. It has been shown that some *C. jejuni* can produce betalactamase enzymes and use major efflux pump systems that lead to resistance to penicillin, amoxicillin, and the other similar antibiotics [11,12,32,33]. In agreement with other studies, less than half of the isolates were resistant to ampicillin and streptomycin, which confirms this ability of the bacteria [32]. Resistance against gentamicin and erythromycin were rare in this study. Khan et al. and Raeisi et al were also reported the same results [17,28]. Although the resistance rate of gentamicin and erythromycin is low, and this resistance has been observed only in little isolates in this study, but even the low rate is still alarming, because these drugs are the main drugs selected for treating *Campylobacter*-associated infections. Therefore, the clinicians should consider applying multi-drug treatment.

In this study, another worrying issue was a high prevalence of MDR isolates, which were even resistant to 5—6 drugs. The MDR *C. jejuni* isolates were separated from all sample types, without any statistical differences. Other researchers have also observed the MDR isolates in campylobacters [8,17]. All of these results indicate that factors involved in the resistance mechanism, including the existence and activity of cmeABC efflux pump, can be linked to multi-drug resistance [12]. In this regard, these factors can be shared between *C. jejuni* isolates, and unfortunately, in the near future, we may see the outbreak of pan-resistance isolates.

The prevalence of the genes involved in the tetracycline resistance was correlated well with the incidence of tetracycline resistance phenotypes of the isolates. In several studies, in line with the present data, most of the tetracycline resistance isolates have *tetO* [8]. Abdi et al suggested that some *Campylobacter* isolates that are resistant to tetracycline can have another gene, *tetA*, instead of the *tetO* or they even can carry both of them [9]. The present data also showed that some isolates harbored *tetA*. In this regard, half of the isolates had also an ability to use the efflux pump that also can cause resistance to tetracycline. Hartmann et al proposed that the plasmid-encoded *tetO* and active cmeABC efflux pump are involved in the tetracycline resistance in *Campylobacter* spp [34]. Some of these bacteria had all three genes, showing that these bacteria acquired resistance through different approaches, simultaneously. Researchers believed that one of these three factors must be present in the DNA of the bacteria, in order for the tetracycline resistance to occur, but in the present study, none of these genes were present in the three isolates. Therefore, other unknown factors such as the existence of another efflux pump system could also be involved, which should be further evaluated. There was a strong correlation between the existence of the bla_{OXA-61} and resistance to ampicillin. The same results were observed by Sierra-Arguello et al, in a study conducted in Brazil [35].

Fla-RFLP method exhibited an acceptable power typing in this study. Several studies used this method for the typing of *Campylobacter* spp. and obtained similar results. Behringer et al. and the authors of this investigation had also been evaluated the reproducibility of the *Fla*-RFLP method and observed 100% reproducibility [36]. The number of distinct types was also very close to each other. In addition, 95% of the *Campylobacter* isolates were typed in Behringer et al. study [36]. In general, because of the high specificity of the primers, the typing power of the method was lower than methods such as RAPD, PFGE or MLST. Compared to another study, which had been used the MLST method for typing of the *Campylobacter* spp., SID of the *fla*-RFLP method was less [36].

The human isolates were grouped in clusters with isolates from the other sources, indicating the spread and transfer of the bacteria between different sources. This result shows the importance of implementation of the best hygienic regulations in slaughterhouses and food chain companies. Moreover, the results showed a high diversity of the *C. jejuni* isolates. Rosef et al also observed a high genetic diversity among *Campylobacter* isolates obtained from food samples [37]. However, some clusters were related to the isolates from a specific source. In this study, some clusters were correlated with high antibiotic resistance rate. Fortunately, these clusters included a small number of bacteria; however, this is an important and alarming issue and may indicate the emergence of new resistant strains. A group of bacteria that located in a cluster and have a high antibiotic resistance rate can have the same genetic roots and can spread their genetic element. Hence, we should pay more attention to these bacteria.

5. Conclusion

Based on the results, attention should be drawn to the prevalence of the isolates with high antibiotic resistance rate and the associated resistance genes, as well as genetic similarity subscriptions fields between human isolates and others. The results showed this relationship is alarming, and such these results help the governments to conduct international standards and control measure strategies. Therefore, they can monitor and implement at the most detailed level, whether at the farm's level or at the level of food production and processing, to reduce the level of contamination and to effectively decrease *Campylobacter* infection, and dissemination of resistant isolates in human. In addition, the results of the present study using the updated information help the governments to prevent the emergence of resistant *C. jejuni* isolates, especially resistance to erythromycin and streptomycin, as these drugs are used as the main drug for the treatment of human campylobacteriosis. On the other hand, as a limitation in this study, sampling data, such as demographic information of the patients, the month of sampling, temperature, and animal husbandry control and health strategies were not available and used. These factors can be helpful and should be applied in future researches.

Acknowledgments

This study was supported by Islamic Azad University of Ayatollah Amoli branch and Amol University of Special Modern Technologies, Amol, Iran.

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