PFGE genotyping and molecular characterization of *Campylobacter* spp. isolated from chicken meat

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Summary

A total of 70 samples were collected from chicken meat obtained from 10 markets in Tehran, Iran from which 39 *Campylobacter coli* were isolated. Among 10 antibiotics used, maximum resistance was seen to trimethoprim-sulphamethoxazole (SXT) (97.36%), nalidixic acid (94.8%), ciprofloxacin (87.7%), streptomycin (89.72%), and tetracycline (97.4%). No resistance was to gentamycin was observed. None of the *Campylobacter* strains under study harbored integron, suggesting the involvement of other resistance mechanisms in emergence of multi drug resistance (MDR) phenotype among the isolates. Two major types (A and B) and 15 subtypes (A1-A8 and B1-B7) were identified. Pulsed-field gel electrophoresis (PFGE) analysis demonstrated a high degree of homogeneity while the majority of the isolates shared identical or very similar PFGE genotypes. Isolates with identical genotypes differed in their resistance profile, although all of them assigned to MDR phenotype. To our knowledge, this is the first molecular survey from Iran characterizing *Campylobacter* isolates from poultry, which adds to our knowledge the epidemiological linkage of *Campylobacter* isolates with MDR properties from different sources and emphasizes the need for cautious use of antimicrobials in different fields of food production chain.

Key words: Antimicrobial resistance, *Campylobacter* spp, Integron, Pulsed-field gel electrophoresis

Introduction

Campylobacter spp. is recognized as one of the most common causes of food-borne bacterial gastroenteritis in humans (Atabay *et al*., 1997). Enteritis due to *Campylobacter jejuni* and *Campylobacter coli* is the most important form of campylobacteriosis for public health (Friedman *et al*., 2004). Studies have shown that the main source of contamination of poultry carcasses with *C. jejuni* is their intestinal contents (Wedderkopp *et al*., 2000; Berrang *et al*., 2004). *Campylobacter* is widespread in the environment (Sahin *et al*., 2015) and a commensal of the intestinal tract of a wide range of domestic animals used for food production including birds and mammals (Franco, 1988). During slaughter the damage of intestinal tract integrity can lead to direct contamination of chicken carcasses (Kazwala *et al*., 1990). The consumption and handling of poultry and poultry products are a major source of human campylobacterial enteritis (Corry and Atabay, 2001; Modirrousta *et al*., 2016), In fact, identification, characterization and control of sources play an important role in preventing outbreaks due to strains of this bacterium (Newell and Fearnley, 2003).

Antibiotic resistance is an increasingly serious problem in some pathogens and the multidrug resistance is a public health concern (Fluit *et al*., 1999). The use of antimicrobial agents in food animals may result in the emergence and dissemination of antimicrobial resistant bacteria (Aarestrup and Engberg, 2001). Integrons are potentially a major agent in dissemination of multidrug resistance among gram-negative bacteria (Stokes *et al*., 1989; Hall *et al*., 1995). Integron structures are naturally occurring gene expression systems that can potentially capture and integrate one or more gene cassettes and convert them into functionally expressed genes that encode resistance determinants to several antimicrobial agents (Shane, 2000; Carattoli *et al*., 2001).

Several genetic typing methods have been developed for epidemiological and source tracking studies of bacterial infections (Wassenaar *et al*., 2000), among which pulsed-field gel electrophoresis (PFGE) is widely used for molecular typing of *Campylobacter* spp. (Hänninen *et al*., 2000; Kärenlampi *et al*., 2003). This typing method provides a satisfactory discriminatory power to characterize *Campylobacter* isolates of different origins.

The aim of the present study was to isolate and characterize the *Campylobacter* spp. of poultry origin and to assess the rate of antimicrobial resistance, integron content and genetic diversity of isolate using PFGE method.

Materials and Methods

Samples

A total of 70 chicken meat samples were randomly purchased from the retail market during summer 2012 in

Tehran, Iran. All samples were immediately transported to laboratory in a commercially available Cary-Blair transport medium (Micromedia™, India) supplemented with 10 g/L sodium pyrovate and low agar content (0.5 g/L) on ice.

Isolation and identification of *Campylobacter* spp.

Of each sample, 25 g was homogenized in a stomacher Lab Blender 400 (Seward, London, England) with 225 ml of *Campylobacter* enrichment broth base (Preston enrichment broth base, Biomark™) supplemented with *Campylobacter* selective supplement IV (HIMEDIA, Mumbai, India, FD158) containing polymyxin B, 2500 IU; rifampicin, 5.0 mg; trimethoprim lactate, 5.0 mg, and amphotericin B, 5.0 mg. Incubation was done at 42°C for 48 h in a microaerophilic condition (85% N_2, 10% CO_2, 5% O_2) provided by gas pack type C (Merck™ Anaerocult C) and a total of 0.1 ml of the enrichment broth was then streaked onto charcoal cefoperazone deoxycholate agar (CCDA), a *Campylobacter* blood-free selective agar, supplemented with amphotericin B (10 mg/L) and cefoperazone (32 mg/L) for selective isolation of *Campylobacter* species (Merck™, Germany).

One presumptive *Campylobacter* colony from each selective agar plate was selected and subjected to identification according to standard microbiological and biochemical tests including Gram staining, production of catalase, oxidase, hippurate hydrolysis. The identity of isolates was confirmed by polymerase chain reaction (PCR) using primers specific for *cadF*, *asp* and *hipO* genes which specifically identify *Campylobacter* spp., *C. coli*, and *C. jejuni*, respectively (Kolackova and Karpiskova, 2005). The *C. jejuni* ATCC 29428 and *C. coli* ATCC 43478 strains were used as controls in each PCR assay.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was done using disk diffusion method with Muller-Hinton agar supplemented with 5% defibrinated sheep blood. The following antibiotic impregnated disks were used: nalidixic acid (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), tetracycline (15 µg), streptomycin (10 µg), gentamicin (10 µg), amoxicillin (30 µg), ampicilin (10 µg), cotrimoxazole (25 µg), and chloramphenicol (30 µg). After 48 h incubation at 42°C in a microaerophilic condition (85% N_2, 10% CO_2, 5% O_2), the results were interpreted according to interpretive criteria provided by CLSI (2008). *Escherichia coli* strain ATCC 25922 was used as control in antimicrobial susceptibility assay.

Distribution of class 1 and 2 integrons among the isolates

Class 1 integrons were investigated among the isolates using int-F/int-R primers which specifically amplify the conserved integrase gene (*int*) of this class of integrons. The class 2 integrons were explored by hep-F/hep-R primers which amplify the entire integrated resistance gene cassettes. The primer sequences and their designation are depicted in Table 1.

Pulsed-field gel electrophoresis

The genotyping of isolates was performed according to a standard protocol which was recommended by CDC for *Campylobacter* spp. (www.cdc.gov/pulsenet). Briefly, the bacteria were grown at 42°C for 48 h on *Brucella* agar with 5% (vol/vol) defibrinated sheep blood under microaerophilic conditions (10% CO_2, 5% H_2, and 85% N_2) and suspended in 0.85% NaCl as cell suspension buffer. Each cell suspension was adjusted to 0.57-0.82 absorbance at 610 nm wavelength. The plugs were prepared by addition of 20 µL of Proteinase K (20 mg/ml stock), 400 µL of adjusted cell suspensions and 400 µL SeaKem Gold (SKG) agarose in TE buffer (10 mM Tris, 1 mM EDTA, pH = 8.0) and dispensed into the wells of reusable plug molds. Lysis of cells in plugs was performed in cell lysis buffer (50 mM Tris, 50 mM EDTA [pH = 8.0], 10% sarcosine, 0.1 mg of proteinase K/ml) at 54°C. After washing steps, restriction digestion of plugs was performed in 1 µL of *Sma*I (40 U/µL) at 37°C. The plug slices were allowed to stand at room temperature for 5 min, after which they were loaded into the appropriate wells of a 1% SKG agarose gel.

The electrophoresis conditions consisted of an initial switch time of 0.5 s and a final switch time of 25 s and a gradient of 6 V/cm for 20 h in 0.5 X TBE on CHEF Mapper XA System (Bio-Rad™).

Computer analysis of PFGE patterns

The PFGE patterns were analyzed using Gel Compare II version 4.0 software (Applied Maths, Sint-Matenslatem, Belgium). Standard *Salmonella brandreup* H9812 strain digested by XbaI restriction enzyme was

Table 1: Primers used in this study

Organism	Primer	PCR product (bp)	Sequence (5′ to 3′)	Reference
Campylobacter spp.	cadF	400	TTGAAGGTAATTTAGATATG CTAATACCTAAAGTTGAAAC	Nayak *et al*. (2005)
Campylobacter jejuni	hipO	735	GAAGAGGGTTTGGGTGGTG AGCTAGCTTCGCATAATAACTTG	Linton *et al*. (1995)
Campylobacter coli	asp	500	GGTATGATTTCTACAAAGCGAG ATAAAAGACTATCGTCGCGTG	Linton *et al*. (1995)
Integron class 1	int1	900	TGCGTGTAAATCATCGTCGT CAAGGTTCTGGACAGTTGC	Adabi *et al*. (2009)
Integron class 2	hep2	1500	GATGCCATCGCAAGTACGAG CGGGATCCCGGACGGCATGCACGATTTGTA	White *et al*. (2001)

used as DNA size marker. Matching the types and portrayal of dendrogram was performed by Dice coefficient and UPGMA (unweighted pair group method with arithmetic averages) clustering.

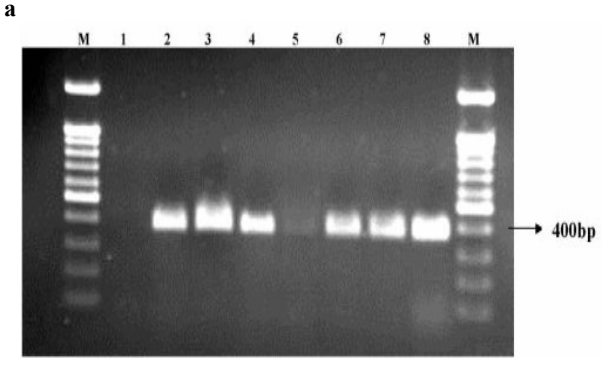
Results

A total of 39 *C. coli* were isolated according to the conventional biochemical tests. An amplification band of 400 bp was obtained for all isolates using *CadF* oligonucleotides as primer which confirms that the isolates belong to *Campylobacter* spp (Fig. 1a). The *C. coli* isolates were further confirmed at the genus level by PCR amplification of *asp* gene which produced a 500 bp band for all the isolates (Fig. 1b). None of the isolates except for *C. jejuni* ATCC 29428 produced 735 bp band of *hipO,* which is the specification of *C. jejuni* (Fig. 1c).

Among 10 antibiotics used, the maximum resistance was seen to tetracycline (97.4%), co-trimoxazole (97.36%), nalidixic acid (94.8%), streptomycin (89.72%), and ciprofloxacin (87.7%), respectively. No resistance was seen to gentamicin. The results of antimicrobial resistance of *Campylobacter* isolates are shown in Table 2. In total, 18 resistance profiles were identified, though all of the isolates assigned to MDR phenotype because of resistance to more than 3 antimicrobial agents.

Attempts for detection of class 1 and 2 integrons showed that none of the *Campylobacter* strains under study harbored the integrons (Figs. 1d-e). Consequently, no resistance gene cassettes were characterized in relation to integrons.

Among 39 isolates from rectal swabs, 30 (76.9%) isolates were typeable by PFGE while 9 isolates were excluded because their PFGE patterns were not distinguishable after multiple attempts. Tenover (1995) guidelines were applied for allocation and elucidation of PFGE banding patterns (Tenover 1997). Accordingly, two major clusters were obtained from total isolates. The most similarity among the isolates was 100% and the least similarity was 26%. Two major types (A and B) and 15 subtypes (A1-A8 and B1-B7) were identified which is indicative of a high level of homogeneity among the isolates (Fig. 2). Isolates with identical genotypes differed in their resistance profile, although all of them were categorized in MDR.



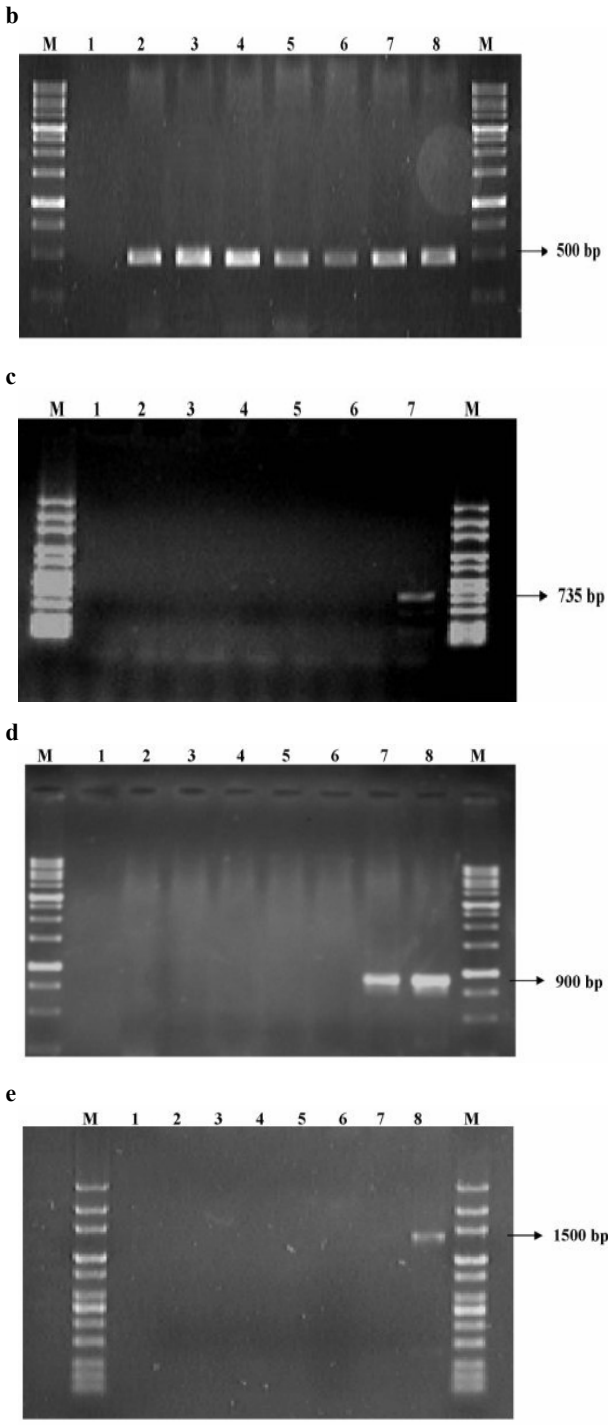


Fig. 1: a) PCR amplification of *cadF* gene (400 bp). M: Mid-Range DNA ladder. Lane 1: Negative control, Lanes 2-7: Isolates under study, and Lane 8: Positive control, **b**) PCR amplification of *asp* gene (500 bp). M: Mid-Range DNA ladder. Lane 1: Negative control, Lanes 2-7: Isolates under study, and Lane 8: Positive control, **c**) PCR amplification of *hipO* gene (735 bp). M: Mid-Range DNA ladder. Lane 1: Negative control, Lanes 2-6: Isolates under study, and Lane 7: Positive control, **d**) PCR amplification of class 1 integron *int1* gene (900 bp). M: Mid-Range DNA ladder. Lane 1: Negative control, Lanes 2-6: Isolates under study, Lanes 7 and 8: Positive controls, and **e**) PCR amplification of class 2 integron using *hep2* primers (1500 bp). M: Mid-Range DNA ladder. Lane 1: Negative control, Lanes 2-7: Isolates under study, and Lane 8: Positive control

Table 2: Antimicrobial resistance among *C. coli* strains isolated from chicken carcasses

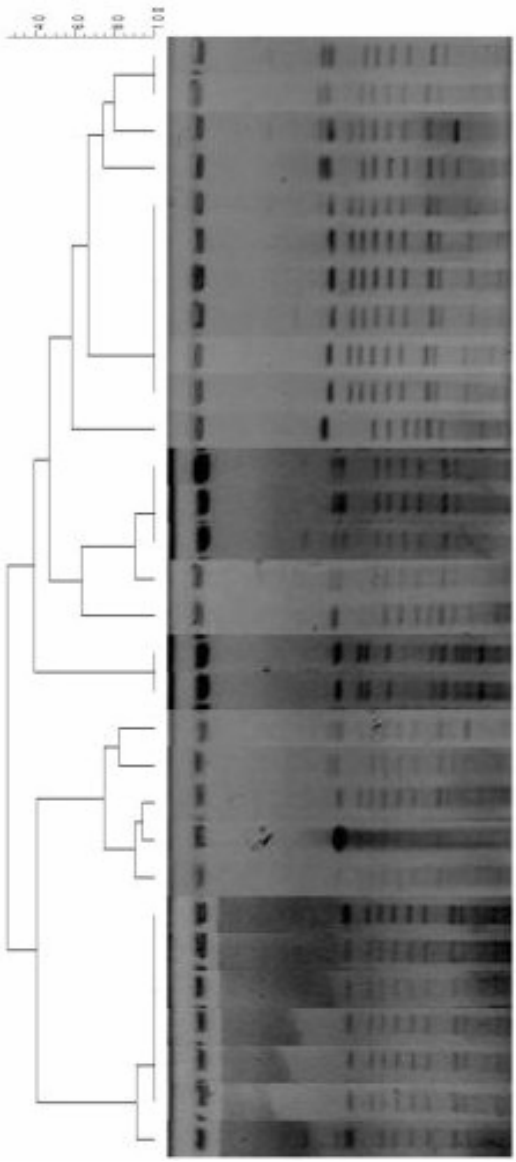
Antimicrobial drug	Number (%)
Ampicillin	32 (82.1%)
Amoxicillin	30 (79.5%)
Cotrimoxazol	31 (97.36%)
Streptomycin	35 (89.72%)
Gentamicin	0 (0%)
Erythromycin	33 (84.4%)
Ciprofloxacin	34 (87.7%)
Nalidixic acid	36 (94.8%)
Chloramphenicol	27 (69.3%)
Tetracyclin	38 (97.4%)

Discussion

A total of 39 *C. coli* were isolated from 55% of the chicken meat. A few previously performed studies in Iran have reported the isolation rate of *Campylobacter* spp. from chicken meats to be 56.1% and 63% in Isfahan and Tehran, respectively (Taremi *et al*., 2006; Rahimi and Tajbakhsh, 2008). In a report by Rahimi *et al*. (2010) from Khuzestan, *Campylobacter* spp. was isolated from 60% of poultry meat. The results emphasize the thermophilic nature of *Campylobacter* spp. which increases the possibility of its isolation in warm seasons.

Many reports have shown the prevalence of *Campylobacter* spp. in chicken meat or by-products between 32.3% (South Africa) and 70.7% (USA) which reveals that the rate of isolation of *Campylobacter* spp. from poultry is higher in more developed countries (Zhao *et al*., 2001; Van Nierop *et al*., 2005).

A few reports from Iran and other countries have indicated that *C. jejuni* is more frequently isolated from



Pulsotype	Antibiotic Resistant	Resistance Profile
A_1	A-AP-CIP-C-E-NA-S -TS	1
A_1	A-AP-C-E-NA	2
A_2	A-AP-CIP-E-NA-S-T-TS	3
A_3	A-AP-NA-S-TS	4
A_4	CIP-C-E-NA-S-T-TS	5
A_4	A-AP-CIP-NA-S-T-TS	6
A_4	A-CIP-C-NA-S-T-TS	7
A_4	A-AP-CIP-C-NA-S-T-TS	8
A_4	AP-CIP-C-NA-S-T-TS	9
A_4	A-AP-CIP-E-NA-T-TS	10
A_5	CIP-C-E-NA-S-T-TS	5
A_6	A-AP-CIP-E-NA-T-TS	10
A_6	A-AP-CIP-C-NA-S-T-TS	8
A_6	A-AP-CIP-E-NA-S-T-TS	3
A_7	AP-CIP-C-NA-S-T-TS	9
A_8	A-AP-CIP-E-NA-S-TS	11
A_4	CIP-C-E-NA-S-T-TS	5
A_4	A-AP-CIP-C-E -NA-S-TS	1
B_1	A-AP-CIP-NA-T-TS	12
B_2	A-AP-CIP-E-NA-S-T-TS	3
B_3	A-AP-CIP-E-NA-S-T-TS	3
B_4	A-AP-CIP-C-E-NA-S-T-TS	13
B_5	A-NA-TS	14
B_6	A-AP-CIP-E-NA-S-T-TS	3
B_6	CIP-C-E-NA-S-T-TS	5
B_6	C-E-T	15
B_6	A-AP-E-S-TS	16
B_6	A-AP-CIP-C-E-NA-S-T-TS	17
B_6	A-AP-CIP-C-E-NA-S-T-TS	17
B_7	CIP-NA-S-T-TS	18

Fig. 2: The dendrogram of *SmaI* digested PFGE profile of 30 *Campylobacter* strains. A: Amoxicilin, AP: Ampicilin, C: Chloramphenicol, CIP: Ciprofloxacin, E: Erythromycin, G: Gentamycin, NA: Nalidixic acid, S: Streptomycin, T: Tetracyclin, and TS: Cotrimoxazol

poultry than *C. coli* (Ge *et al*., 2003; Rahimi and Ameri *et al*., 2011). This is in contrast with our results as all of our isolates were *C. coli* and no *C. jejuni* was isolated during our study period; however, the probability of clonal dissemination of *C. coli* strains in provider slaughterhouses from which the chicken meat was transported to the stores should not be ignored. In other words, *C. coli* strains may have become dominant and replaced the pre-existing *C. jejuni* strains in some time periods. This controversial observation is a frequent phenomenon in epidemiological and environmental studies which are performed in different times and places.

In our study the rate of multi drug resistance (MDR) phenotype was 100% among the total isolates. A few other studies have indicated the rate of MDR among *Campylobacter* isolates from poultry to be between 56.5% and 75% in different countries (Taremi *et al*., 2006; Han *et al*., 2007). Because of the increased reporting of multi-resistance in *Campylobacter* world-wide, attempts should be made to care for and control the antimicrobial usage in animal husbandry.

All of our isolates were *C. coli* and showed a high rate of resistance to erythromycin (84.4%).

In a study by Anonymous *et al*. (2005) from Estonia the lowest resistance (14%) was seen to erythromycin among *Campylobacter* isolates from chicken products and all the resistant isolates were *C. jejuni*. In a study by Ishihara *et al*. (2004) all of the *C. jejuni* isolates were susceptible to macrolide antibiotics, whereas 48.4% of the *C. coli* isolates were resistant. Overall, it can be deduced that probably *C. coli*, not *C. jejuni*, is intrinsically resistant to erythromycin. Different studies have also reported a low erythromycin resistance among their *Campylobacter* isolates which is consistence with the common strategy of erythromycin therapy as a first line choice for campylobacteriosis (Fallon *et al*., 2003; Ledergerber *et al*., 2003). This is the first time that a high rate of resistance to erythromycin is reported which requires considerable attention and monitoring.

All of the isolates in our study belonged to *C. coli* species and the majority of them were resistant to all antimicrobials tested. Other investigators have also reported the increased resistance of *C. coli* isolates to mainstream antimicrobial agents under study rather than *C. jejuni* (Asai *et al*., 2007; Little *et al*., 2008) This emphasizes the intrinsic resistance mechanisms in *C. coli*; however, more studies are needed to recognize the exact mechanisms involved.

The campylobacteriosis is not included in the routine diagnostic schedule of medical laboratories in Iran and the high resistance rate among *Camylobacter* isolates of veterinary origin raises the worry of whether many undiagnosed diarrhea cases in medicine are due to MDR *Campylobacter* spp. with animal origin. In a recent research performed by our laboratory, MDR phenotype was also observed among *Campylobacter* spp. of clinical origin (data not published).

Until recently, integron structures were not widely identified in *Campylobacter* spp. and therefore there was little controversial knowledge regarding their potential contribution in antimicrobial resistance. In this study, no integron was observed among the *C. coli* examined. In a study by van Essen-Zandbergen *et al*. (2007) from the Netherlands, no class 1 or 2 integrons were detected in the *Campylobacter* isolates isolated from broilers. Lee *et al*. (2002) determined the presence of class 1 integron in 21% of 105 *C. jejuni* of poultry origin. It can be inferred that integrons have a very low distribution among the *Campylobacter* spp. and can only be found among the *C. jejuni* strains.

The absence of integrons among our isolates and the high resistance observed to many of antimicrobial agents under study, suggests that integrons do not play an important role in development of resistance among *Campylobacter* isolates. This raises the probability of involvement of other resistance mechanisms in emergence of MDR phenotype in this genus of human and veterinary interest. Identical or closely related genotypes with different resistance properties (i.e. pulsotypes 5 through 10) underline the role of mobile resistance elements in conferring resistance through horizontal gene transfer.

These data could contribute to better understanding of the resistance properties of *Campylobacter* spp. against different antimicrobial agents although the mechanisms are still under investigation.

Pulsed-field gel electrophoresis restriction pattern analysis of our isolates demonstrated a high degree of homogeneity among the isolates. This homogeneity may be due to

i) The clonal dissemination and persistence of a few genotypes in the provider slaughterhouses

ii) The potential of a few genotypes for maintenance and spreading through harsh environmental conditions of slaughterhouse

Moreover, it should not be ignored that utility of 2 restriction enzymes (*Sma*I and *Kpn*I) could provide a better discrimination among the isolates (Wassenaar and Newell, 2000). To our knowledge, this is the first survey from Iran characterizing *Campylobacter* spp. isolates from broilers. The absence of genetic information on *Campylobacter* spp. of poultry origin in Iran emphasizes the need to monitor their genotypes and potential relationship with medical isolates. A number of studies based on PFGE have confirmed a genetic relationship between *Campylobacte*r spp. of broilers and human origin. Previous studies have shown genetically related *Campylobacter* genotypes in poultry and human infections (Michaud *et al*., 2005; Wayne, 2008; Denis *et al*., 2009).

In conclusion, identical or very similar PFGE profiles of *Campylobacter* isolates from chicken meat suggests a clonal dissemination and emphasizes the need to monitor the provider slaughterhouses from which the chicken meat is transported the stores. The dominance of MDR strains in poultry could affect the clinical strains and make the patients treatment more problematic. High prevalence of MDR phenotype among *Campylobacter* spp. isolated from chicken meat and lack of contribution

of integrons, emphasizes the need for exploring the exact resistance mechanisms involved.

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Conflict of interest

None.

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