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Campylobacter jejuni and *Campylobacter coli* in cecum contents of chickens of slaughter age: A microbiological surveillance

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Abstract. The presence of foodborne pathogens is a major concern for the food industry and increase in antibiotic resistance adds to the seriousness of this issue. Epidemiological studies have shown that there is little or no information from Iran on the prevalence of *Campylobacter* spp. in chickens of slaughter age. The aim of this study was to determine the prevalence, antibacterial susceptibility and type of *Campylobacter* species isolated from the cecum of chickens bred in Saqqez city, Kurdistan, western Iran. *Campylobacter* was isolated and identified by culture and molecular methods. Antibiotic susceptibility of *Campylobacter* species was performed by disk agar diffusion test and agar dilution methods. The bacterial isolates were typed by repetitive element sequence based polymerase chain reaction (rep-PCR) method. Fifty-five percent of the farms were found to be contaminated with *Campylobacter* spp. Gene amplification assay confirmed 67 isolates with *Campylobacter* spp., of which 57 (85.1%) were identified as *C. jejuni* and 10 (14.9%) as *C. coli*. Resistance to tetracycline was the most common finding (70.6%), followed by ciprofloxacin (63.7%) and amoxicillin (27.5%). All isolates retained their susceptibility towards gentamicin and meropenem. Results of MIC_{50} and MIC_{90} confirmed high resistance towards tetracycline and ciprofloxacin. Repetitive element sequence based-polymerase chain reaction (rep-PCR) placed *C. jejuni* in six profiles, while *C. coli* could not be separated as diverse clones. The present study focused on obtaining data regarding prevalence, antibiotic susceptibilities, genetic diversity at regular intervals and maintain and improve hygiene. The results of this study showed substantial genetic diversity of *C. jejuni* in chickens from western Iran.

INTRODUCTION

Consumption of chicken in comparison to other meat or its products is more prevalent worldwide due to its low-price, palatability, easy availability together with the presence of high quality protein, vitamins and minerals (Sallam, 2007). These factors have contributed to the flourishing of poultry farms and slaughterhouses. On the other hand, contamination of poultry meat and eggs with zoonotic pathogens has put a constraint in their consumption.

Campylobacter is one of the major zoonotic pathogens transmitted through uncooked or insufficiently cooked chicken and chicken products to humans leading to gastroenteritis (Epps *et al.*, 2013; McDowell

et al., 2008). Among the various *Campylobacter* species, *C. jejuni* and *C. coli* are the two most significant bacteria which apart from colonizing the colon and cecum of the digestive tract of chickens (Kittl *et al*., 2011; Luangtongkum *et al*., 2006), are also a major cause of human diarrhea in developing and developed countries. Information about the variability of these two species with respect to their behavior in the food chain, and their impact on treatment or virulence to humans is still lacking. These two species also differ in terms of conditions of growth from other pathogens associated with food-borne diseases. Almost 90.0% of the clinical cases are attributed to *C. jejuni* while, *C. coli* is comparatively lesser (approximately 10.0%) (Abdollahpour *et al*., 2015). While campylobacteriosis in a healthy person may not require antibiotic treatment, however the immunocompromised patients, elderly, infants, and acute cases require therapeutic interventions. Therapeutically, fluoroquinolone (ciprofloxacin) and macrolides (erythromycin) are drugs of choice (Jeon *et al*., 2010); however, their addition as additives in poultry feed has led to the emergence of antibiotic resistance strains causing serious problem for public health (Vandeplas *et al*., 2008). Currently no vaccine has been licensed for the prevention of campylobacteriosis. Quantitative risk assessment based on research data has concluded that there is a linear relationship between prevalence of *Campylobacter* in broiler flocks and public health risk (Hue *et al*., 2010).

Several typing methods have been used for clonal identification and detection on the source of *Campylobacter* spp. and amongst them, the rep-PCR technique has been favorably employed for clonal identification of *Campylobacter* isolates (Ahmed *et al*., 2012; Behringer *et al*., 2011).

Currently there are no information available on the prevalence and antibiotic susceptibility pattern of *Campylobacter* spp., in cecum of chickens of slaughter age from Iran. Given that Saqqez city is the major supplier of chickens for all over Iran, we selected this study to be conducted in chickens of this city with the following aims: i) investigate the prevalence of *Campylobacter* spp. phenotypically and genotypically in cecum of chickens of slaughter age, ii) study the antibiotic resistance pattern by disk agar diffusion and agar dilution, iii) evaluation of genetic similarity of *Campylobacter* isolates by rep-PCR.

MATERIALS AND METHODS

Sample collection and bacterial isolation
From July 2015 to February 2016, 200 poultry cecum contents were sampled from twenty farms in Kurdistan province, western Iran. The chickens were at 40 days of age or more. The cecum contents were grinded and added to Preston's enrichment broth base (M899) (Hi Media laboratories, Mumbai, India) supplemented with *Campylobacter* selective supplement IV (FD042) (Hi Media laboratories, Mumbai, India) and 5.0% defibrinated horse blood (Jamali *et al*., 2015). The culture was incubated at 37°C for 4h and then shifted to 42°C under microaerophilic conditions (85% N_2, 10% CO_2, 5% O_2) provided by commercial gas pack (Anaerocult C gas pack, Merck, Germany). After 24 h incubation, 0.1 ml of cultured enrichment broth was streaked on modified charcoal-cefoperazone-deoxycolate agar (mCCDA) (Scharlou, Spain) and incubated at 42°C for 48-72 h in the same condition. Colonies were examined morphologically and subcultured on blood agar (Hi Media laboratories, Mumbai, India) for further bacteriological and biochemical tests, including Gram's staining, oxidase and catalase production and nitrate reduction for identification of *Campylobacter* spp. The hippurate hydrolysis was used as a phenotypic test to differentiate between two major species, *C. jejuni* and *C. coli*. Finally, *Campylobacter* spp. colonies were stocked in Brain Heart Infusion (BHI) (Hi Media laboratories, Mumbai, India) with 15% glycerol (Merck, Germany) and 20% Fetal Bovine Serum (FBS) (Gibco) at -30°C for further analyses.

DNA extraction and Polymerase Chain Reaction (PCR) assay

DNAs of all samples were extracted from Preston's broth after enrichment step. Also, DNA was extracted from each sample of bacterial colonies for molecular confirmation. DNA extraction was performed by boiling method with a slight modification. Briefly, 300-µl overnight grown bacteria was first pelleted at 13,000 rpm, then the supernatant was discarded and 20-µl tissue buffer (0.25% SDS + 0.05M NaOH) was added. After thorough vortexing, samples were incubated at 95°C for 10 min followed by addition of 180-µl double-distilled water to the micro centrifuge tube. Three sets of primers were used for identification of *Campylobacter* spp., *C. jejuni* and *C. coli* based on the 16SrRNA for *Campylobacter* genus detection, *mapA* (outer membrane protein MapA) in *C. jejuni* and *ceuE*, (enterochelin uptake substrate-binding protein) in *C. coli* as described previously (Zendehbad *et al*., 2013) (Table 1). For genus identification, 12.5 µl of master mix (Amplicon, Denmark), 10 pmol each forward and reverse primer of 16SrRNA, 50ng DNA template were mixed and the final volume was adjusted to 25µl by distilled water. The fragments were amplified using the following thermal program: initial denaturation at 95°C for 4 min followed by 35 cycles, each with denaturation for 30s at 95°C, annealing for 45s at 59°C and extension for 50s at 72°C, and a final extension for 10 min at 72°C. Duplex PCR was performed for differentiating two species by the conditions described above except that the annealing temperature was reduced to 56°C. *C. jejuni* ATCC 33560 and *C. coli* ATCC 33559 were used as positive controls. Deionized water was used as negative control. The PCR products were electrophoresed on 1.5% agarose gel and bands visualized using SYBR Safe DNA gel stain.

Antimicrobial susceptibility test (AST)

Antimicrobial susceptibility was performed by disk agar diffusion test and agar dilution method according to standard protocol of Clinical and Laboratory Standard Institute (CLSI, 2006). Muellar Hinton agar plates (MH-F) (Hi Media laboratories, Mumbai, India) were supplemented with 5% defibrinated horse blood and 20mg/L β-NAD (Sigma-Aldrich, USA). For disk agar diffusion test, 0.5 McFarland matched bacterial suspension was prepared from 18-24h culture, which was then inoculated onto MH-F plates followed by placing of the antibiotic disks. Finally, plates were incubated for 24h at 42°C in microaerophilic conditions. Antibiotic disks (Mast, Diagnostic, UK) included in the study were: erythromycin (15µg), tetracycline (15µg), ciprofloxacin (5µg), gentamicin (10µg), meropenem (10µg), amoxicillin (30µg) and ampicillin (30µg).

The antimicrobial susceptibility profile for all isolates was also determined by estimating Minimum inhibitory concentration (MIC) of three antibiotics using agar dilution method as per CLSI standards for erythromycin, tetracycline and ciprofloxacin (Sigma-Aldrich, USA). The MIC ranges for antimicrobial agents included: erythromycin:

Table 1. Primers used for PCR and rep-PCR

Organism	Primer	Product size (bp)	Sequence
Campylobacter spp.	*16crRNA*	857	*F 5'-ATC TAA TGG CTT AAC CAT TAA AC-3'* *R 5'-GGA CGG TAA CTA GTT TAG TAT T-3'*
C. jejuni	*mapA*	589	*F 5'-CTA TTT TAT TTT TGA GTG CTT GTG-3'* *R 5'-GCT TTA TTT GCC ATT TGT TTT ATT A-3'*
C. coli	*cueE*	462	*F 5'-AAT TGA AAA TTG CTC CAA CTA TG-3'* *R 5'-TGA TTT TAT TAT TTG TAG CAG CG-3'*
rep-PCR	ERIC	–	*ERIC1R 5'-ATG TAA GCTCCT GGG GAT TCA C-3'* *ERIC2 5'-AAG TAA GTG ACT GGG GTG AGC G-3'*

0.5 to 128µg/ml, tetracycline: 0.5 to 128µg/ml and ciprofloxacin: 0.25 to 64µg/ml. Briefly, a bacterial suspension equivalent to 0.5 McFarland standards was prepared from overnight cultures as described above for disk agar diffusion test. After adding inoculum according to CLSI guidelines on each plate containing antibiotic dilution, incubation was done as described for disk agar diffusion assay. All plates were monitored after 24 h. Isolates with insufficient growth were immediately re-incubated for 20-24h as described above as per CLSI guidelines. Antibiotic resistance was defined for the following antibiotics as follows: MIC≥32 µg/ml for erythromycin, MIC≥16µg/ml for ciprofloxacin, and MIC≥16µg/ml for tetracycline according to the CLSI recommendations.

Repetitive element sequence based-polymerase chain reaction (rep-PCR)

Bacterial DNA was extracted by Gene All Exgene Tissue SV mini kit (Gene All Biotechnology, Seoul, Korea) according to the manufacturer's instructions. Two primers were used for rep-PCR typing according to the previous study (Patchanee *et al*., 2012) (Table 1). The reactions were performed in 25µl reaction volumes containing 12.5µl commercial 2× Master mix (Ampliqon, Denmark), 800nM primers and 240ng of the DNA templates. The amplification cycles were set at initial denaturation at 95°C for 5 min, followed by inner denaturation, annealing and extension at 94°C for 45 s, 1 min for 52°C and 68°C for 10 min, respectively and final extension at 68°C for 20 min (Patchanee *et al*., 2012). The products were analyzed by electrophoresis on 1% agarose gel. Finally, dendogram was made based on the Dice-UPGMA method.

RESULTS

Detection of *Campylobacter* species

Campylobacter spp. was detected in 11 (55.0%) cecum contents of twenty flocks by both conventional culture method and PCR. Figure 1 shows the prevalence of *Campylobacter* spp. collected from the cecum. By culture method, *Campylobacter* spp. was isolated from 58 (29.0%) samples including 50 (86.2%) *C. jejuni* and 8 *C. coli* (13.7%). All isolates were confirmed by PCR.

PCR was performed on 58 positive samples obtained by culture, all were positive for the 857bp band showing the presence of the genus *Campylobacter*. A total of 57

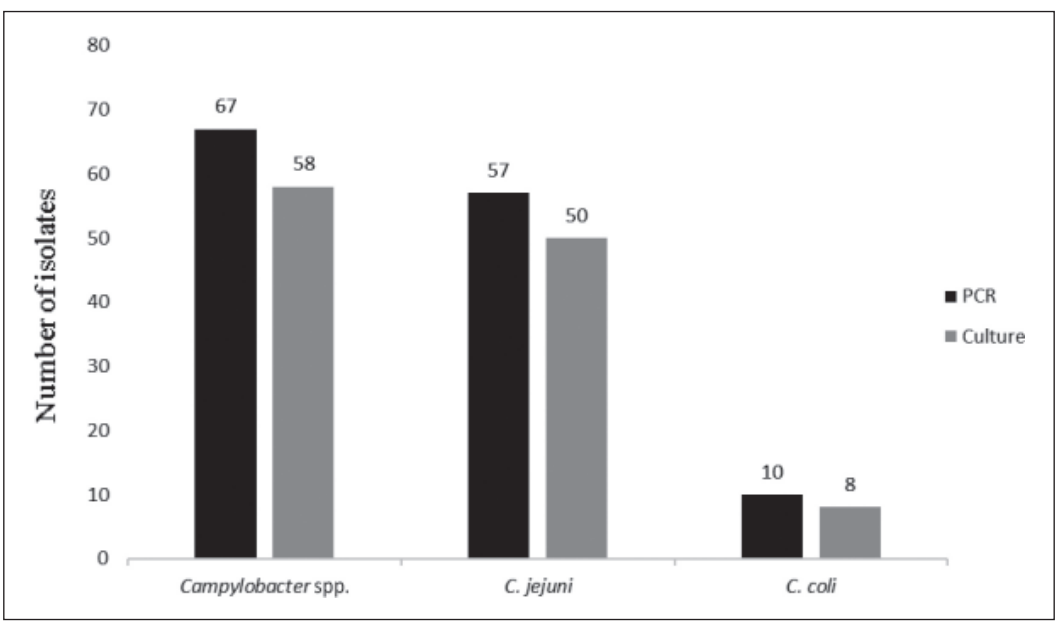

Figure 1. Comparison of phenotypic and molecular identification assay for *Campylobacter* spp.

cecum contents demonstrated the presence of 589bp band indicating the presence of *C. jejuni* and 10 samples produced 462 bp band indicating the presence of *C. coli*. These included nine samples which did not grow on culture; however, PCR from enrichment broth gave positivity for the presence of *Campylobacter* genus. Of these, 7 enrichment broths demonstrated the presence of *C. jejuni* and 2 demonstrated *C. coli*. Thus, in total, 67 cecum contents of chicken were positive for *Campylobacter* spp. The PCR had 100% sensitivity, 97.1% specificity, 86.5% positive predictive value and 100% negative predictive value over culture method; however, they were not statistically significant (χ^2=0.9425). Overall, *C. jejuni* was more prevalent than *C. coli* (85.1% vs. 14.9%).

Antibacterial susceptibility

Table 2 shows the pattern of non-susceptibility of *Campylobacter* species for seven antibiotics tested. On disk agar diffusion test, all isolates of *C. jejuni* and *C. coli* showed susceptibility towards gentamicin and meropenem. Amongst the most common therapeutic agents, 70.6 and 63.7% *Campylobacter* spp. isolates were resistant to tetracycline and ciprofloxacin, respectively, while only one isolate of *C. jejuni* showed resistant towards erythromycin.

The MIC distributions of *C. jejuni* and *C. coli* are summarized in Table 2. On agar dilution, similar results were observed for *C. jejuni* towards three antibiotics, MIC_{50} and MIC_{90} for tetracycline being ≥32µg/ml and ≥64µg/ml, respectively, and for ciprofloxacin MIC_{50} and MIC_{90} were ≥8µg/ml and ≥32µg/ml, respectively, while isolates retained their susceptibility towards erythromycin (MIC_{50} being ≥2µg/ml and MIC_{90} ≥4µg/ml). Analogous to *C. jejuni*, *C. coli* isolates showed non-susceptibility to ciprofloxacin (MIC_{50} and MIC_{90} being ≥8µg/ml and ≥64µg/ml, respectively), tetracycline (MIC_{50} and MIC_{90} being ≥128µg/ml and ≥128µg/ml, respectively), while erythromycin was the most susceptible therapeutic

Table 2. Pattern of non- susceptibility of *Campylobacter* spp. towards therapeutic agents

Antibiotic agent	Resistant breakpoint	*Campylobacter*[a] spp. (%)	*C. jejuni*[a] (%)	*C. coli*[a] (%)
Disc diffusion method				
Erythromycin	≤13mm	1(1.7)	1 (2.0)	0
Tetracycline	≤14mm	41(70.6)	34 (68.0)	7 (87.5)
Ciprofloxacin	≤15mm	37(63.7)	31 (62.0)	6 (75.0)
Gentamycin	≤12mm	0 (0)	0 (0)	0 (0)
Meropenem	≤19mm	0 (0)	0 (0)	0 (0)
Ampicillin	≤13mm	10 (17.2)	5 (10.0)	5 (62.5)
Amoxicillin	≤13mm	16 (27.5)	10 (20.0)	6 (75.0)
Resistant to 1 antibiotic	–	18 (31.0)	18 (36.0)	0 (0)
Resistant to 2 antibiotics	–	27 (46.5)	26 (52.0)	1 (12.5)
Resistant to ≥3 antibiotics	–	10 (17.2)	3 (6.0)	7 (87.5)
Agar dilution method				
MIC Erythromycin	≥32µg/ml	1 (1.7)	1 (2.0) MIC* MIC_{90} (≥4)	0 (0) MIC_{50} (≥2) MIC_{90} (≥4)
MIC Tetracycline	≥16µg/ml	41 (70.6)	34 (68.0) MIC_{50} (≥32) MIC_{90} (≥64)	7 (87.5) MIC_{50} (≥128) MIC_{90} (≥128)
MIC Ciprofloxacin	≥4µg/ml	37 (63.7)	31 (62.0) MIC_{50} (≥8) MIC_{90} (≥64)	6 (75.0) MIC_{50} (≥8) MIC_{90} (≥32)

[a] Number and percentage of isolates.
* MIC as µg/ml.

Table 3. Non-susceptibility pattern of *C. jejuni* and *C. coli* towards multiple antibiotics

Antibiotic[a] resistance profile	*C. jejuni*[b]	*C. coli*[b]
ER + CIP	1	0
TET + CIP	21	5
TET + AMP	3	4
TET + AMOX	5	6
AMP + AMOX	0	3
CIP + AMP	2	3
CIP + AMOX	6	4
CIP + AMP + AMOX	0	1
TET + CIP + AMP	2	2
TET + CIP + AMOX	2	3
TET + AMP + AMOX	0	3
TET + CIP + AMP + AMOX	0	1

[a] ER – Erythromycin; TET – Tetracycline; CIP – Ciprofloxacin; AMP – Ampicillin; AMOX – Amoxicillin.
[b] Figures represent number of resistant isolates towards the various pattern of antibiotics

agent (MIC_{50} being ≥2µg/ml and MIC_{90} ≥4µg/ml, respectively).

The overall prevalence of multidrug resistance (MDR), defined as resistance to three or more antibiotics was 17.2% (Table 2 and 3). Presence of MDR in *C. coli* was much more in comparison to *C. jejuni* (87.5% versus 6.0%). The most common antibiotic resistance pattern was TETRA-CIPRO followed by TETRA-AMOX (Table 3).

rep-PCR fingerprinting

When rep-PCR was performed on DNAs extracted by boiling method, two to four fragments were generated for each bacterial isolates ranging from 100 to 500bp. Nevertheless, commercial DNA extraction kit was found to be superior to boiling method in terms of enhanced number and size of the fragments. All *C. jejuni* generated six to eight fragments (except one sample that had one amplicon) ranging from 150 to 2000bp. The *C. jejuni* and *C. coli* isolates could be differentiated as distinct profiles (Figure 2). In the present investigation, *C. jejuni* produced six different profiles. The most frequent profile (pK) included 32.0% of isolates. However, all *C. coli* isolates were located as one profile (pM). Figure 3 demonstrates the number of isolates in each profile.

DISCUSSION

Campylobacter is an important pathogen that causes bacterial enteric disease in humans worldwide. Prevalence of *Campylobacter* in diarrheic samples may be more than *Salmonella* (Zendehbad *et al.*, 2015). Chicken and its products are important sources of campylobacteriosis. Our study showed a prevalence of 33.5% in poultry by PCR, while conventional culture method detected only 29.0%. Previous studies conducted in Iran showed higher prevalence ranging from 40.0% to 70.0% in chicken meat (Taremi *et al.*, 2006, Rahimi & Tajbakhsh, 2008, Hassanzadeh & Motamedifar, 2007). These variations in the prevalence are related to the differences in samples tested, weather and climatic conditions, methods of skinning and kind of additives used in farms. At slaughtering process, *Campylobacter* diffuses leading to more contamination (Hue *et al.*, 2011; Tadesse *et al.*, 2011). Thus, chicken products in markets are more contaminated compared to chickens at farm. The rate of flocks' contaminated in our study was less (55.0%) than another study conducted in Shiraz (76.0%) (Ansari-Lari *et al.*, 2011), though the study did not state the rate of chicken contamination. Frequent use of acidifiers and cold weather in Saqqez city

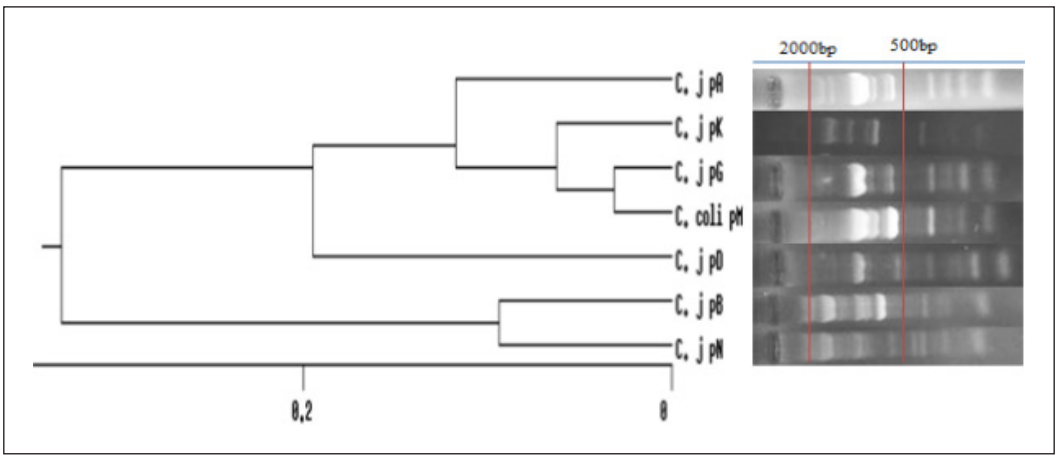

Figure 2. Profile analysis of *Campylobacter* spp. isolates based on ERIC fingerprints.

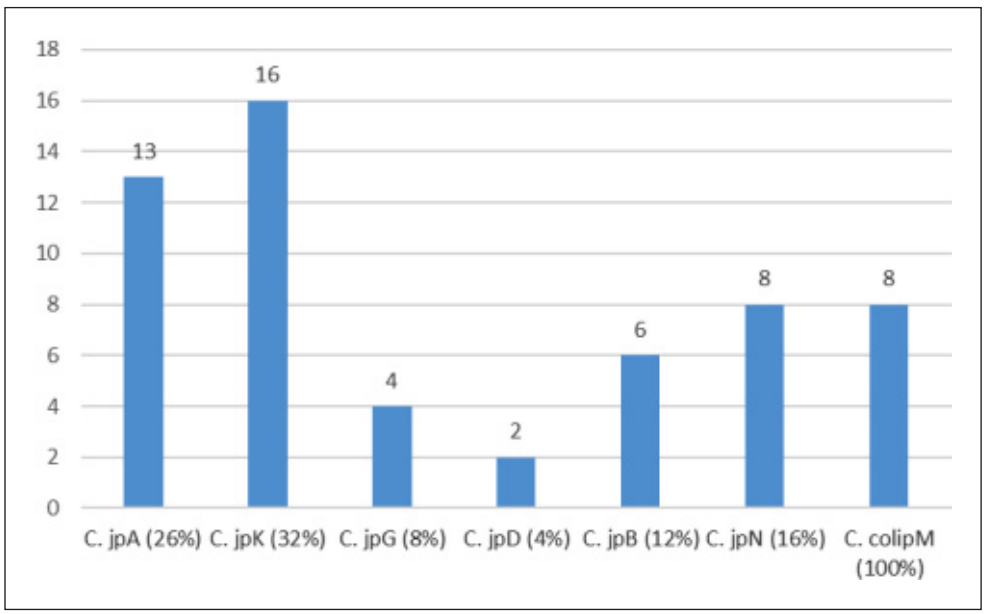

Figure 3. Number and percent of isolates located in different profiles of *C. jejuni* and *C. coli*. The generated profiles do not have any relationship with the location and resistance profiles of isolates.

in comparison with Shiraz may be the reason for less contamination of flocks. Result of flock contamination in current study is closer to a study conducted in Czech Republic (50.0%) which is less than that that reported from Italy and Spain (72.0%), France (82.0%) had a higher prevalence rate than from Denmark and Germany (23.0%–30.0%) (Bardon *et al*., 2009).

C. jejuni was the major etiological factor of contamination in chickens in our study. Similar results have been documented in earlier studies (Allain *et al*., 2014, Bardon *et al*., 2009, Rahimi and Tajbakhsh, 2008). On the other hand, other studies show *C. coli* to be more prevalent (Ansari-Lari *et al*., 2011; Khoshbakht *et al*., 2015; Wieczorek *et al*., 2012). Swine and rodents are the major

source and reservoirs to *C. coli*, respectively (Tadesse *et al.*, 2011; Wysok *et al.*, 2015). Due to the restriction placed on swine meat in Iran, it is common that prevalence of *C. coli* is lower than reported in other studies. In addition, control and prevention of rodents at farms help in declining the rate of contamination (Allain *et al.*, 2014).

Of the seven antibiotics tested, we found 70.6% isolates with non-susceptibility towards tetracycline. Scientific reports available from Iran shows tetracycline resistance in *Campylobacter* spp. to range from 31.0% to 90.0 % (Dallal *et al.*, 2010; Hue *et al.*, 2010), while in other countries, it varies from 33.0-85.0% (Nobile *et al.*, 2013; Zendehbad *et al.*, 2013). Our finding is in agreement with previous studies that reported tetracycline resistance to be more prevalent in *Campylobacter* (Mirzaie *et al.*, 2011; Nobile *et al.*, 2013; Tang *et al.*, 2009; Zendehbad *et al.*, 2015). Contrary to other reports, ciprofloxacin resistance was found to be more prevalent amongst other antibiotics in *Campylobacter* spp. (Dabiri *et al.*, 2014; Hassanzadeh and Motamedifar, 2007; Jamali *et al.*, 2015; Kittl *et al.*, 2011; Taremi *et al.*, 2006; Zendehbad *et al.*, 2015). Probably, traditional use of enrofloxacin for the treatment of *E. coli* infection resulted in upsurge of ciprofloxacin resistance (Luangtongkum *et al.*, 2006). Doxycycline, an antibiotic used in the treatment of fowl cholera is available at feed farms in this region, which may be the reason for the increased resistance towards tetracycline resistance.

Most of the studies conducted in Iran shows rare resistance to erythromycin (Dabiri *et al.*, 2014; Jamali *et al.*, 2015; Taremi *et al.*, 2006). Because macrolide group antibiotics, especially erythromycin is not advised or rarely used at poultry farms. This might probably be the reason of rare resistance in *Campylobacter*. In parallel to other studies, all isolates in our investigation were susceptible to gentamicin and meropenem (Dallal *et al.*, 2010; Hassanzadeh and Motamedifar, 2007; Jamali *et al.*, 2015; Kittl *et al.*, 2011; Ledergerber *et al.*, 2003; Luangtongkum *et al.*, 2006). Although the use of this antibiotic at farms is routine, it is given in early hatchery period, when *Campylobacter* does not colonize chickens (Luangtongkum *et al.*, 2006). Thus, resistance to gentamicin does not emerge in this period.

Resistance to ampicillin and amoxicillin in *Campylobacter* isolates was 17.2 and 27.5%, respectively in our study. However, this prevalence has been reported as 86.0%, 80.0% and 61.0% in other studies conducted in Malaysia, Iran and Italy, respectively (Mirzaie *et al.*, 2011; Nobile *et al.*, 2013; Tang *et al.*, 2009). Moreover, the presence of MDR *Campylobacter* spp. in our investigation has been lower than that reported from other studies conducted in Iran and Algeria (Luangtongkum *et al.*, 2006). Our data confirms the earlier stated hypothesis regarding farm's management, where it was postulated that antibiotic selection fulfills diverse purpose, including additive or control determination, as well as the treatment of the disease, which could be a strong reason for the emergence of existence of resistant and MDR isolates (Vandeplas *et al.*, 2008).

Several methods have been attempted for *Campylobacter* clonal identification and amongst them, rep-PCR is an inexpensive typing technique. Like other studies, low number of generated profiles contribute to genetic similarity in farms with the same management and rearing methods (Wilson *et al.*, 2009). In addition, generated profiles in rep-PCR do not have any relationship with the location and resistance profiles of isolates (Behringer *et al.*, 2011). The *Campylobacter* spp. is competent bacteria thus, genetic diversity is a usual phenomenon. However, the result of the present investigation demonstrated that this diversity is limited by similar management and environmental conditions.

CONCLUSION

This study was the first of its kind investigation on the *Campylobacter* contamination from west of Iran. Results showed that contamination in conventional poultry farms of Saqqez city was lower than that reported from other regions of Iran. This low prevalence rate is associated with mountainous and

relative cold environmental conditions of western regions, including Saqqez in comparison to other regions in Iran and frequent usage of acidifiers. Thus, this region can retain the potentiality of conventional chicken production however, with stringent monitoring of foodborne campylobacteriosis. Usage of tetracycline and ciprofloxacin should be constrained on the farms as well as in community. The surveillance of antibiotic resistance in *Campylobacter* species can decrease the selective pressure of development of antibiotic resistant bacteria. Although due to genetic diversity, location and source tracing of *Campylobacter* spp. is difficult, additional studies at molecular level are required to validate this.

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