Ann Microbiol (2011) 61:315–321
DOI 10.1007/s13213-010-0141-1

ORIGINAL ARTICLE

fla-typing, RAPD analysis, isolation rate and antimicrobial resistance profile of *Campylobacter jejuni* and *Campylobacter coli* of human origin collected from hospitals in Tehran, Iran

Mohammad Hamidian · Maryam Sanaei · Masoumeh Azimi-Rad · Mercedeh Tajbakhsh · Hossein Dabiri · Mohammad-Reza Zali

Received: 27 January 2010 / Accepted: 20 September 2010 / Published online: 3 November 2010
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Abstract The purpose of the current study was to investigate the isolation rate, antimicrobial resistance profile and molecular typing of *Campylobacter* spp. recovered from patients with diarrhea in hospitals in Tehran, Iran. Over a period of 13 months, from 562 diarrheal samples 49 (8.7%) *Campylobacter* spp. were isolated, of which 34 (69.5%) were *Campylobacter jejuni* and 12 (24.5%) were identified as *Campylobacter coli*. Antimicrobial susceptibility and typing of the *Campylobacter* spp. isolates was carried out using the Kirby–Bauer disk diffusion method, *fla*-typing and RAPD analysis. The highest resistance in the collected *Campylobacter* isolates was to ofloxacin (77.5%) followed by ciprofloxacin (73.4%), nalidixic acid (69.3%), ceftazidime (53%), cefotaxime (51%) and carbenicillin (40.8%) while all the isolates were susceptible to imipenem. The results of RAPD analysis and *fla*-typing showed a relative high diversity and weak clonality amongst *Campylobacter* spp. isolates from patients with diarrhea in two hospitals in Tehran, Iran. In addition, during the current study, *fla*-typing proved to be more reliable and reproducible for typing of isolates. To our knowledge, this is the first study providing structural data for RAPD analysis and *fla*-typing of *Campylobacter* spp. isolates recovered from Iranian patients.

Keywords *Campylobacter coli* · *Campylobacter jejuni* · Thermophilic *Campylobacter* · Acute gastro-enteritis

M. Hamidian (✉) · M. Sanaei · M. Azimi-Rad · M. Tajbakhsh · H. Dabiri · M.-R. Zali
The Research Institute for Gastroenterology and Liver Disease, Taleghani Hospital, Shaheed Beheshti University MC, 7th Floor, Parvane St, Yaman St, PO Box 1985717411, VelenjakTehran, Iran
e-mail: mohammad.hamidian@uni.sydney.edu.au

Introduction

Thermophilic *Campylobacter* spp. are considered as one of the major causative agents of acute gastro-enteritis in both humans and animals (Al-Mahmeed et al. 2006; Allos 2001; Corcoran et al. 2006; Feodoroff et al. 2009). The clinical manifestations of the enteritis caused by *Campylobacter* spp. range from asymptomatic infections to severe inflammatory bloody diarrhoea (Muller et al. 2006).

In recent years, many studies from different countries have increasingly reported *Campylobacter* spp. infections with resistance to commonly used antimicrobials, especially fluroquinolones (Corcoran et al. 2006; Feodoroff et al. 2009). Today, increasing levels of resistance to antimicrobials in *Campylobacter* spp. has become a major health problem in many countries (Corcoran et al. 2006).

A combination of phenotypic and molecular typing methods, e.g., antimicrobial susceptibility testing and *fla*-typing, has been used successfully in many epidemiological studies of *Campylobacter* isolates. These methods have proved reliable for investigation of diversity and relatedness, and to track sources of *Campylobacter* isolates (Corcoran et al. 2006; Ertaş et al. 2004; Wardak and Jagielski 2009).

To date, limited data focusing on isolation rate, antimicrobial resistance profile and epidemiology of *Campylobacter* spp. has been published in Iran (Hassanzadeha and Motamedifar 2007), where the isolation rate, significance and prevalence of *Campylobacter* spp. seem to be higher than previously thought (Feizabadi et al. 2007; Hassanzadeha and Motamedifar 2007; Jafari et al. 2008).

In the current study, we aimed to determine the isolation rate and antimicrobial resistance profile, and to investigate relationships between *Campylobacter jejuni* and *Campylo-*

bacter coli isolates from patients with diarrhea using *fla*-RFLPs and RAPD analysis during the period of study in order to gain further insight into the epidemiology of thermophilic *Campylobacter* spp. circulating in the hospitals of Tehran, Iran.

Material and methods

Study design, bacterial strains and data collection

During November 2008 to December 2009, stool samples of patients with acute diarrhea were investigated for *Campylobacter* spp. in two hospitals in Tehran, Iran. A diarrheal case was defined according to Sanders et al. (2005). All samples were transported to the laboratory of Department of Foodborne Diseases in Cary-Blair transport medium and microbiological analysis was carried out immediately. Demographic data of the patients were collected using questionnaires handed out to the personnel in hospitals responsible for data collection.

Isolation and microbiological identification of *Campylobacter* spp.

The collected samples were cultured in different media and an enrichment medium for optimal isolation as follows. Stool samples were transported to the laboratory in Cary-Blair transport medium and then enriched on Preston enrichment broth (Himedia, Mumbai, India) supplemented with polymixinB, rifampicin, trimethoprim lactate, amphotricinB, and 7% defibrinated sheep blood, and incubated under microaerobic conditions (85% N_2, 10% CO_2, 5% O_2) for 24 h at 42°C.

After enrichment, the samples were plated on Brucella agar base (Himedia, Mumbai, India) supplemented with the same antibiotic supplement for the selective isolation of *Campylobacter* spp. (Himedia, Mumbai, India) and 7% defibrinated sheep blood and incubated under microaerobic conditions (85% N_2, 10% CO_2, 5% O_2) for 48 h at 42°C (Feizabadi et al. 2007).

Colonies that resembled *Campylobacter* spp. morphologically were further identified by biochemical tests using standard microbiological and biochemical methods as previously described (Aarestrup et al. 1997; Engberg et al. 2000). *Campylobacter jejuni* ATCC 29428 was used as a positive control.

DNA isolation and detection of *Campylobacter* spp. at the molecular level

From the isolates positively identified as *Campylobacter* spp., whole-cell DNA was extracted as previously described (Jafari et al. 2008). To discriminate *C. jejuni* and *C. coli* from other *Campylobacter* spp. grown at 42°C, a specific conserved fragment of 16S rRNA in C. *jejuni* and *C. coli* was amplified using the following primers CCCJF: 5′-AATCTAATGGCTTAACCATTA-3′ and CCCJR: 5′-GTAACTAGTTTAGTATTCCGG-3′ (Linton et al. 1997) giving amplicons 852 bp in size. To distinguish between *C. jejuni* and *C. coli* isolates, a hippuricase gene (*hipO*) specific for *C. jejuni* was amplified using primers hipOF: 5′-GAAGAGGGTTTGGGTGGTG-3′ and hipOR: 5′-AGC TAGCTTCGCATAATAACTTG-3′, whereas a fragment including the 3′-end of an aspartokinase gene (*ask*) and a major part of an *orf* encoding a protein of unknown function located downstream from the *ask* gene is specific for *C. coli* (Linton et al. 1997). This latter segment was amplified using the primers ccF: 5′-GGTATGATTTCTAC AAAGCGAG-3′ and ccR: 5′-ATAAAAGACT ATCGTCGCGTG-3′ (Linton et al. 1997). The *hipO* gene- and the *ask* gene-associated fragments were 735 bp and 502 bp long, respectively.

Amplification was carried out in PCR buffer (SuperTaq, London, UK), 0.5 μM of each primer (all from Bioneer, Daejeon, Republic of Korea), and 1 U Super *Taq* DNA polymerase (SuperTaq). Reaction conditions and cycling programs are described in detail elsewhere (Linton et al. 1997). Molecular weight determination of the PCR products was performed by gel electrophoresis in 1.2% agarose using molecular weight standards (Mixed DNA Ladder; Fermentas, Ontario, Canada).

Antimicrobial susceptibility testing

The collected isolates identified as *Campylobacter* spp. were tested for susceptibility by the Kirby–Bauer disk diffusion method on Muller-Hinton Agar (Oxoid, Basingstoke, UK) using ampicillin (10 μg), ceftazidime (30 μg), cefotaxime (30 μg), imipenem (20 μg), carbenicillin (100 μg), streptomycin (10 μg), nalidixic acid (30 μg), ofloxacin (5 μg), neomycin (30 μg), erythromycin (15 μg), gentamicin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), colistin (10 μg), and ciprofloxacin (5 μg) (all from MAST House, Merseyside, UK).

Interpretation of inhibition zones was carried out according to the criteria stipulated by the Clinical Laboratory Standards Institute, CLSI, guidelines (CLSI, Wayne 2006), and the resistance break points were those specified by the CLSI and reported either as sensitive or resistant based on standard break points. *Escherichia coli* ATCC 25922 was used as a control.

Restriction fragment length polymorphism

To detect restriction fragment length polymorphism (RFLP) patterns of the *fla* gene of the *Campylobacter* spp., 10 μl of

the amplified PCR products were digested using 2.5 U *Dde*I restriction enzyme in a final reaction volume of 30 µl as previously described (Harrington et al. 2003). The digested fragments were then analyzed on 1.6% agarose gels. Reproducibility of the *fla*-typing results was verified by repeating RFLPs twice for all 46 *C. jejuni* and *C. coli* isolates. A dendrogram of the PCR-RFLP of *fla* gene was created using NTSYS Software (NTSYSpc; Version 2.01; http://www.exetersoftware.com/cat/ntsyspc/ntsyspc.html).

Random amplified polymorphism DNA analysis

The reaction mixture of the random amplified polymorphism DNA (RAPD) analysis was carried out in a total volume of 25 µl containing 40 ng total DNA of each strain, 1.36 pM OPA-11 primer (5′-CAA TCG CCG T -3′) (Bioneer), 1.6 U *Taq* DNA polymerase (Super Taq), 1.5 µl 500 mM $MgCl_2$, 0.7 µl 10 mM dNTPs in 1X PCR buffer (all from Super Taq). The samples were amplified through four primary cycles (4 min at 94°C, 4 min at 34°C and 4 min at 72°C) and then 40 cycles of denaturation (30 s at 94°C), annealing (1 min at 36°C), extension (2 min at 72°C), and a final step of extension (10 min at 72°C). The PCR product was then separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. RAPD analysis was carried out twice for all 46 *C. jejuni* and *C. coli* isolates to check reproducibility.

Results

During the period November 2008 to December 2009, a total of 562 stool samples were collected from patients with diarrhea from two hospitals in Tehran, Iran. The samples were subsequently transferred to the Laboratory of Department of Food Borne Diseases for further analysis. Of all 562 stool samples, 49 (8.7%) were recovered and identified as *Campylobacter* spp. using microbiological methods. Of all isolated *Campylobacter* spp., 34 (69.5%) were identified as *Campylobacter jejuni* and 12 (24.5%) as *Campylobacter coli*. Three (6.1%) belonged to other *Campylobacter* spp. and, interestingly, there were no seasonal differences in frequency of *Campylobacter* spp. isolation. Amongst the positive samples for *Campylobacter* spp., 31(63.26%) were recovered from children less than 5 years old; no association between sex and *Campylobacter* isolation rate was observed. The clinical data of patients from whom *Campylobacter* spp. were isolated are summarized in Table 1.

Antimicrobial susceptibility testing

Susceptibility tests using the Kirby–Bauer disk diffusion method demonstrated that the highest resistance rate among the identified *Campylobacter* spp. isolates was to ofloxacin 38 (77.5%), followed by ciprofloxacin 36 (73.4%), nalidixic acid 34 (69.3%), ceftazidime 26 (53%), cefotaxime 25 (51%), carbenicillin 20 (40.8%), tetracycline 15 (30.6), ampicillin 8 (16.3%), colistin 7 (14.2%), erythromycin 5 (10.2%), neomycin 5 (10.2%), streptomycin 4 (8.1%), chloramphenicol 4 (8.1%) and gentamicin 2 (4%), whereas all isolates were susceptible to imipenem (Table 2).

PCR-RFLP analysis of the *flaA* gene

PCR amplicons of *flaA* (1,725 bp) were generated from all isolates as well as the reference strains *C. jejuni* ATCC 29428. After digestion with *Dde*I, three to seven restriction bands, ranging from 100- to 1,500-bp (Fig. 1), comprised of 38 different banding profiles were observed in the 46 isolates. In addition, five major clusters were identified at a within-cluster similarity level of approximately 80% despite the inconsistency of the RFLP bands at higher similarity levels (Fig. 2). All *Campylobacter* isolates were distinguishable by the *fla*-RFLP method. *fla*-typing of the all *C. jejuni* and *C. coli* isolates was demonstrated to be reproducible as the same patterns were seen in two repetitions.

RAPD analysis

Analysis of the 34 *C. jejuni* and 12 *C. coli* isolates by RAPD using the primer OPA-11 yielded 35 distinct band profiles. At a within-cluster similarity level of approximately 70%, five major clusters were identified while all the *C. jejuni* and *C. coli* isolates were completely distributed in all clusters. One *C. jejuni* isolate generated a quite different RAPD profile pattern and clustered as an isolate with less than 60% similarity level (Fig. 3).

Table 1 Clinical data of the 49 patients from whom *Campylobacter* spp. were isolated

Clinical manifestation	Patients < 5 years old	Vomiting	Nausea	Fever	Abdominal cramp	Bloody diarrhea	Weakness	Headache	Watery diarrhea	Mucosal diarrhea
Percentage (*n* = 49)	31 (63.26%)	18 (36.7%)	17 (34.6%)	17 (34.6%)	20 (40.8%)	12 (24.5%)	11 (22.4%)	1 (2%)	26 (53.06%)	24 (48.9)

Table 2 Antimicrobial resistance of *Campylobacter* spp. isolated from patients with diarrhea from Tehran, Iran. *AMP* Ampicillin, *OFX* ofloxacin, *CHL* chloramphenicol, *CIP* ciprofloxacin, *CAR* carbenicillin, *ERY* erythromycin, *GEN* gentamicin, *NAL* nalidixic acid, *NEO* neomycin, *COL* colistin, *STR* streptomycin, *TET* tetracycline, *CAZ* ceftazidime, *CTX* cefotaxime, *IMI* imipenem

Isolate	AMP	OFX	CHL	GEN	CIP	TET	NAL	STR	CAR	NEO	ERY	COL	CAZ	CTX	IMI
C. jejuni ($n = 34$)	6 (17.6%)	31 (91.2%)	3 (8.8%)	2 (5.8%)	29 (85.2%)	12 (35.2%)	27 (79.4%)	3 (8.8%)	16 (47%)	4 (11.7%)	4 (11.7%)	5 (14.7%)	21 (61.7%)	19 (55.8%)	0
C. coli ($n = 12$)	2 (16.6%)	7 (58.3%)	1 (8.3%)	0	6 (50%)	3 (25%)	6 (50%)	1 (8.3%)	4 (33.3%)	1 (8.3%)	1 (8.3%)	2 (16.6%)	5 (50%)	6 (50%)	0
Others ($n = 3$)	0	0	0	0	1 (33%)	0	1 (33%)	0	0	0	0	0	0	0	0
Total ($n = 49$)	8 (16.3%)	38 (77.5%)	4 (8.1%)	2 (4%)	36 (73.4%)	15 (30.6%)	34 (69.3%)	4 (8.1%)	20 (40.8%)	5 (10.2%)	5 (10.2%)	7 (14.2%)	26 (53%)	25 (51%)	0

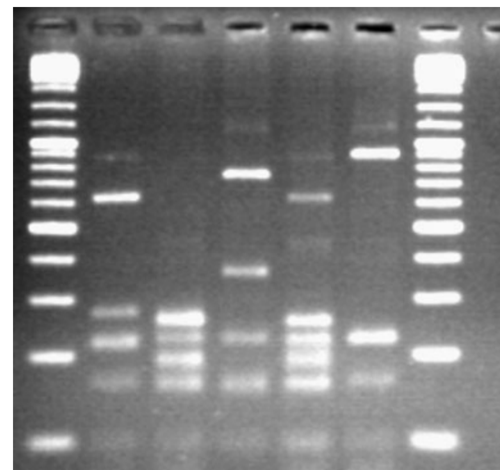

Fig. 1 *Dde*I *fla*A-restriction fragment length polymorphism (RFLP) patterns of Iranian *Campylobacter* spp. strains. Lanes: *1*, *7* Mixed ladder marker; *2–6* clinical isolates

Repetition of the RAPD-PCR revealed that seven (15%) isolates produced different RAPD patterns when repeated with the same primer (OPA-11), the same reagent concentrations and the same cycling conditions. Furthermore, four isolates (three *C. jejuni* and one *C. coli*) did not produce any distinguishable RAPD bands.

Discussion

The current study documents new insights into the isolation rate, antimicrobial resistance profile and epidemiology of *Campylobacter* spp. clinical isolates in Tehran, Iran. This study also represents the diversity and relatedness of the isolates using RAPD analysis and *fla*-typing, in combination with antimicrobial susceptibility testing, which have already been proved to be simple and reliable for typing of *Campylobacter* spp. clinical isolates (Corcoran et al. 2006; Ertaş et al. 2004).

At first glance, the results show a relative higher percentage (8.7%) of *Campylobacter* spp. in patients with diarrhea in the hospitals of Tehran compared with our previous study (Jafari et al. 2008). The increasing incidence of *Campylobacter* spp. accentuate on the significance of *Campylobacter* spp., despite the lack of recognition in the country as, an important cause of diarrhea in Iran like industrialized countries (Wadl et al. 2009). The isolation of *Campylobacter* spp. in patients with diarrhea in consecutive studies during the 3 years (Baserisaleh et al. 2007; Feizabadi et al. 2007; Jafari et al. 2008) suggests that *Campylobacter* spp. can be considered as a major diarrheal pathogen, at least in Tehran. In addition, 31 (63.26%) of the *Campylobacter* spp. in the current study were isolated from patients less than 5 years old, revealing the higher incidence of this pathogen in Iranian children compared with older individuals.

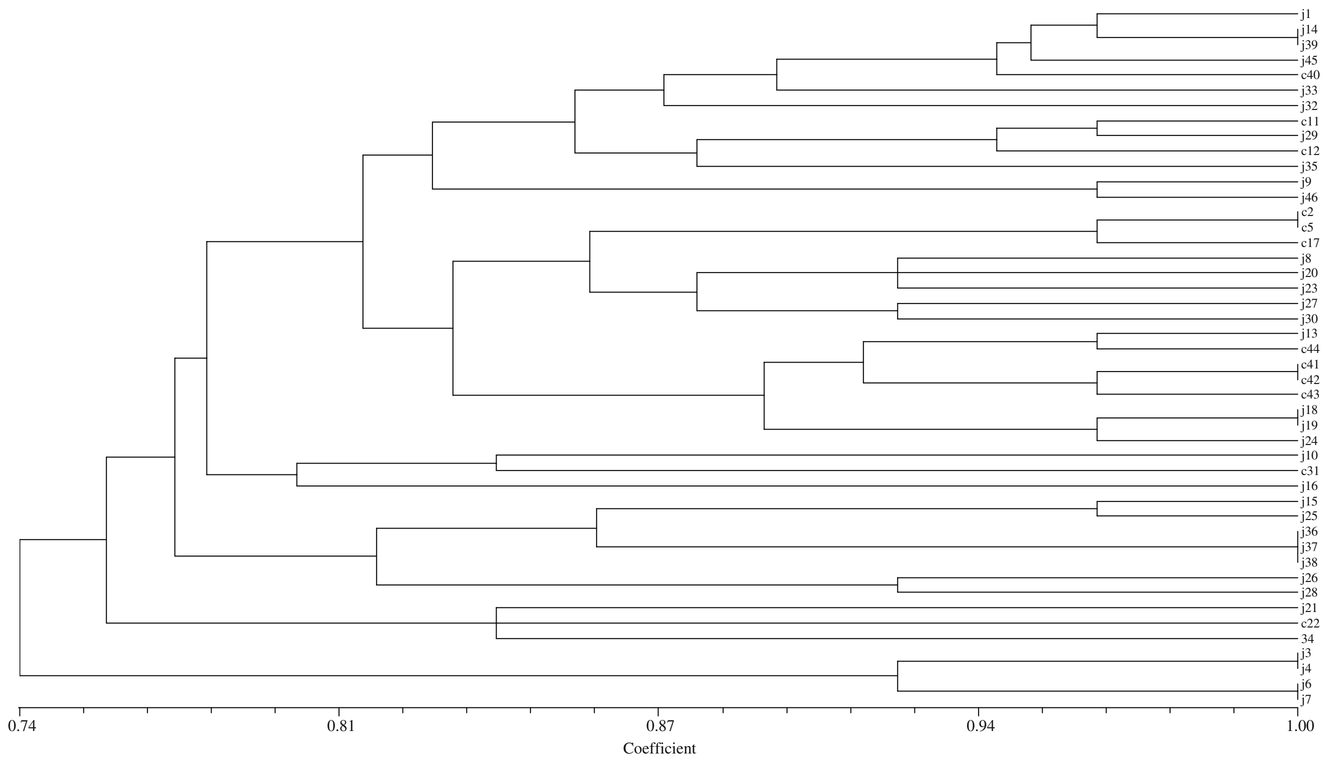

Fig. 2 *fla*-RFLP dendrogram of *Campylobacter coli* (*c*) and *Campylobacter jejuni* (*j*) isolates, collected from patients with diarrhea from Tehran, Iran

During the last decade, an increasing proportion of fluoroquinolone-resistant *Campylobacter* isolates have been reported around the world (Gupta et al. 2004); our study reports a similar trend (Table 2), as all the *C. jejuni* and *C. coli* isolates were resistant to at least one antimicrobial agent, with the highest resistance rate being observed for ofloxacin 38 (77.5%) followed by ciprofloxacin 36 (73.4%) and nalidixic acid 34 (69.3%). Overall, the present study

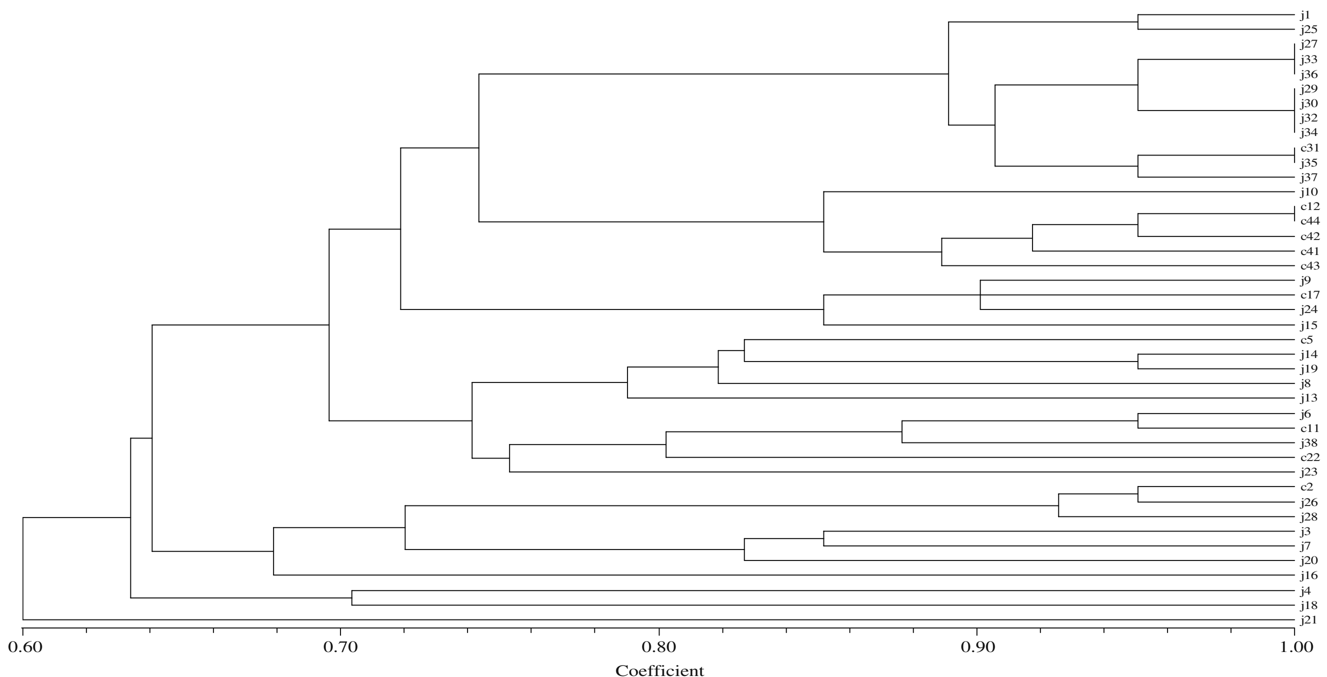

Fig. 3 Random amplified polymorphism DNA (RAPD) analysis dendrogram of *C. coli* (*c*) and *C. jejuni* (*j*) isolates collected from patients with diarrhea from Tehran, Iran

shows a higher resistance rate towards all of the commonly used antimicrobials compared with previous studies from Iran and other countries (Feizabadi et al. 2007; Gupta et al. 2004). Other antimicrobials, such as ceftazidime, cefotaxime, carbenicillin and tetracycline, had resistance rates of 53%, 51%, 40.8% and 30.6, respectively, which are also higher than in the previous study (Feizabadi et al. 2007).

To date, several phenotypic and molecular typing techniques have been employed successfully for characterization, molecular typing and epidemiology of *Campylobacter* spp., although each of them also has its advantages and constraints (Corcoran et al. 2006; Ertaş et al. 2004; Wardak and Jagielski 2009; Wassenaar and Newell 2000). However, despite some limitations, analysis of *Campylobacter* spp. isolates using RAPD analysis and *fla*-typing has proved to be useful for rapid, preliminary characterization of strains (Fitzgerald et al. 2001). Surprisingly, in the current study, *C. jejuni* and *C. coli* isolates were distributed randomly in all clusters (Fig. 2), and we could not distinguish and/or separate our clinical isolates based on the source, RFLP patterns, RAPD bands or antimicrobial profile. In fact, the RFLP patterns showed a high diversity and weak clonality either in *C. jejuni* or *C. coli* isolates. Interestingly, in the current study, *fla*-typing proved to be more reliable, simpler and more reproducible than OPA-11-RAPD analysis as in some (ca. 15%) cases RAPD analysis was not reproducible and generated different patterns for the same isolate when repeated. Furthermore, four isolates (three *C. jejuni* and one *C. coli*) did not generate distinguishable RAPD bands while all isolates produced interpretable *fla*-RFLP bands. These findings are in agreement with the results of Corcoran et al. (2006) and Ertaş et al. (2004), and contradict the findings of Madden et al. (1998) and Nielsen et al. (2000) who described RAPD analysis as being more discriminatory than *fla*-typing.

It should be noted that discrepancies in prevalence, antimicrobial resistance and typing results of *Campylobacter* spp. in the current study compared with other studies may be due to the use of different sampling techniques, susceptibility testing, analytical methods, typing techniques, geographical and/or seasonal differences, and it is thus very hard to compare and judge between findings made under different conditions (Ge et al. 2002).

To put the issue into prospective, the findings of the current study revealed *fla*-typing to be a reliable and reproducible typing method for our isolates compared with RAPD analysis. The results also revealed in diarrheal patients a relative high percentage of *Campylobacter* spp. with weak clonality and resistance to most antibiotics, especially fluroquinolones. Further investigations on the isolation rate, antimicrobial resistance profile and molecular epidemiology of *Campylobacter* spp. isolates are required to provide additional insights and comprehensive information on the extent of occurrence and epidemiology of this pathogen in Iran.

Acknowledgments The authors thank Dr. Feizabadi, Dr. Alebouyeh, Ms. Mehrnoosh Enjelasi and staff members of the Department of Food Borne Diseases in the Research Institute for Gastroenterology and Liver Disease, Shaheed Beheshti University M.C., whose technical assistance made this work possible.

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