INTRODUCTION

The genus *Campylobacter* belongs to the family of *Campylobacteraceae* and includes some of the most important intestinal pathogenic bacteria. The bacteria from this genus cause diarrhea and systematic diseases; they are important infectious agents in children less than 5 years of age and in young adults in both developed and developing countries (1,2). Within the genus *Campylobacter jejuni* is the most common pathogenic species in humans and is one of the most important causative agents of acute gastro-enteritis. This bacterium is transmitted to humans via consumption of contaminated foods and contact with warm-blooded animals, especially poultry, making epidemiological studies and source tracking imperative (3,4).

DNA sequencing techniques and the availability of sequence data of pathogenic bacteria via online databases allow researchers to perform comparative genomics studies. The sequence-based methods have high sensitivity, rapidity, discriminatory power, and reproducibility, and provide the interpretation and standardized nomenclature of the subtypes, which permit inter-laboratory comparisons and electronic distribution (5–7). Several methods have been used for *C. jejuni* and *Campylobacter jejuni* genotyping, including pulsed field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and amplified fragment length polymorphism (AFLP). Flagellin gene restriction fragment length polymorphism (*flaA*-RFLP) has achieved both epidemiological and strain discrimination goals in outbreak analyses (8–10).

*flaA* sequence typing is a genotyping method that is increasingly used in epidemiological investigations of thermo-tolerant *Campylobacter* species (11,12). However, for *C. jejuni*, RFLP may be just as discriminatory as PFGE and MLST; moreover, it is assuredly more convenient than the latter 2 techniques. The New England Biolab (NEB) cutter online software can be used for computer-simulated RFLP analysis and virtual gel-plotting of *flaA* alleles. To the best of our knowledge, there are no data on the molecular epidemiology of *C. jejuni* based on *flaA* gene sequencing analyses among clinical *C. jejuni* in Iran. This study will provide insight into the genetic relatedness of Iranian clinical *C. jejuni* isolates based on *flaA* sequence. We explored the use of DNA sequencing of the *flaA* gene and short variable region (SVR)-*flaA* together with computer-simulated RFLP analysis based on *flaA* sequence divergence as basis for subtyping of *C. jejuni* isolates of human origin.

MATERIALS AND METHODS

**Study design and data collection procedure:** Four hundred stool specimens were collected from diarrheal patients attending to 3 major children’s hospitals and medical centers in 8 different provinces of Iran between July 2012 and June 2013. Children under 5 years of age with acute diarrhea were included in this study; those
with persistent diarrhea or those who had received previous treatment with antibiotics in the previous 5 days were excluded. Acute diarrhea was defined as more than 3 loose episodes/day (13). All samples were transported to the laboratory in modified Cary-Blair transport medium with reduced agar content (1.6 g/L) (14). Identification of C. jejuni was performed by standard conventional biochemical tests and confirmed by PCR amplification of cadF, hipO, and aspA, specific for genus Campylobacter, C. jejuni and C. coli, respectively (15).

Amplification and sequencing of the flaA gene: In this study, a novel primer pair was designed based on the conserved sequences flanking the flaA locus of C. jejuni strain (GenBank accession no. AF050186.1), which encompass and amplify the entire flaA gene together with the flanking regions. A forward primer flaA-F (5′-TTTCTGATTAACACAAATGGTGCT-3′) and reverse primer flaA-R (5′-CTGTAGTAATCTTAACATTTTG-3′) were used, and the fragment between 2 primers was amplified in a 25 μL reaction mixture containing 10 ng of DNA template, 2.5 μL of the PCR buffer 10×, 200 μM of dNTPs, 5 mM of MgCl2, 0.1 μM of each primer, and 1 U of Taq DNA polymerase. The PCR products were sequenced (Genfanavar-Macrogen, Seoul, Korea) using ABI 3730XL capillary sequencer (Applied Biosystems, Foster City, CA, USA) after purification with a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany).

Computer analysis of the sequence: Bidirectional sequencing of amplified fragments was performed and analyzed using MEGA software version 6.0.6 (<http://megasoftware.net>). Aligned sequences were compared and a dendrogram was generated using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) method. A similarity cut-off value of 90% was used as the identity level.

GenBank accession numbers: The twenty nucleotide sequences obtained in this study were deposited in the GenBank database. The assigned GenBank accession nos. are KM396335 to KM396354.

In silico restriction analysis pattern: The aligned and trimmed 1,681 bp-fragment sequences were digested in silico with Ddel restriction enzyme (restriction enzymes are endonucleases that digest DNA at specific palindromic sites) and exported to the computer-simulated RFLP analysis and virtual gel plotting program (NEB cutter online software version: 1.0.0.4028 <http://www.neb.com>). The UPGMA dendrogram was drawn using GelCompar II software (Applied Maths, Sint-Martens-Latem, Belgium) with a similarity cut-off value of 90% as the identity level.

RESULTS

Bacterial strains: Twenty Campylobacter spp. were identified from a total of 400 clinical specimens taken from patients with diarrheal symptoms in Iran. PCR-based identification determined that all the Campylobacter isolates collected herein comprised C. jejuni.

Total flaA and flaA-SVR nucleotide and amino acid sequence analysis: The designed PCR primers used in this study amplified 1,743 bp of flaA gene in all (100%) of our Campylobacter spp. isolates. After splicing the low-quality sequences at the ends, a region of 1,681 bp harbored the entire flaA sequence and produced 560 amino acids was subjected to analysis. The flaA sequences of 20 isolates were aligned and compared with that of C. jejuni strain NCTC11168, the complete genome of which is available in GenBank (Accession no. AF050186.1). The nucleotide composition of 1,681 bp flaA segments showed an average G + C content of 37.4%.
Fig. 2. UPGMA dendrogram made from 20 clinical C. jejuni isolates based on the nucleotide similarity of 1,681 nucleotides from total flaA sequences. C. jejuni strain NCTC11168 (GenBank accession no. AF050186.1) was used as a control. The data are depicted in relation to location, date of isolation, sequence type (CT, common type; ST, single type), and related Accession no. Horizontal axis of dendrogram shows the similarity index among the isolates.

Phylogenetic relationships in the population: A UPGMA dendrogram was created based on the sequence homology of the flaA gene in the isolates. Two major clusters were obtained (A and B) with 4 and 6 members, respectively. All members in cluster A were composed of identical sequences, whereas differences in 8 nucleotide positions, resulting in 5 amino acid changes, were present in the isolates in cluster B (Fig. 2). Interestingly, all isolates from Tehran North and Tehran South were included in clusters A and B, respectively, despite discrete isolation dates. The most parsimonious relationships were analyzed; the level of discrimination that could be discerned from the entire sequence of the total flaA gene was compared with the flaA-SVR and the results were very similar (Fig. 3). In silico flaA analysis with the Ddel restriction enzyme using NEB cutter online software showed 6–11 fragments. The UPGMA dendrogram from virtual gel plotting among the isolates showed very similar clustering (Fig. 3).

All three methods (flaA sequence typing, flaA-SVR sequence typing, and PCR-RFLP of the flaA sequence) produced identical numbers of profiles (10 profiles), common types (5 CT), and Simpson Diversity indices (Di: 0.86).

DISCUSSION

In the current study, 2 molecular typing methods,
flaA sequencing and in silico PCR-RFLP, were used to compare relative discriminatory power and typability for differentiation in 20 C. jejuni isolates of clinical origin. A DdeI restriction enzyme was used for in silico restriction digestion of the complete flaA gene; DdeI is supposed to provide distinguishable banding patterns in comparison with other restriction enzymes including Sau3A I and AluI (16,17).

The flagellum is a target for the human immune system, and any mutation event in the flaA locus that engenders positive selection increases the growth or survival of the bacterium (18). Comparison of a 1,681-bp trimmed nucleotide sequence of the flaA gene from 20 isolates of C. jejuni showed 2 highly variable regions; one at positions 539 to 689 designated the flaA-SVR; and a large variable region (flaA-LVR), at approximately positions 700 to 1,600. The SVR region of the flaA gene has been previously described by Khawaja et al. and Meinersmann et al. at base positions 450 to 600 (19,20). This region showed a more narrow range of diversity in the flaA-SVR among the Campylobacter population under study. The sequence data of the LVR showed similar results to those of the SVR; owing to the length of the sequence and the complexity of LVR data analysis, this region is not suitable for molecular epidemiological studies of Campylobacter infections.

The average G + C content of the flaA 1,681-bp segments was 36.8%, which is consistent with the equivalent data reported by Khawaja et al. (36.4% G + C content of the flaA gene in C. jejuni TGH9011 (19)). This phenomenon shows that, despite continuous point mutations occurring within the coding sequence of the flaA gene, the overall nucleotide composition of the gene has not substantially changed among different populations. The flaA amino acid sequence contained 2 perfect repeat boxes (GSGFSA/SGS and GSGFSSQ/GS) in all of our C. jejuni isolates. To the best of our knowledge, although repeat boxes have been introduced previously, boxes with such sequences were introduced for the first time in this study.

A total of 22% nucleotide variable sites versus 16% amino acid variable sites were obtained among the isolates under scrutiny which shows that 6% of the mutations are silent. Moreover 2.5% singleton sites were detected in the nucleotide sequences, all of which were effective in changing the related amino acid sequences. In silico RFLP analysis of the flaA gene according to sequence data obtained through total flaA sequencing generated a UPGMA dendrogram similar to that gener-
ated following analysis of the complete \textit{fla}A gene sequence. This suggests that PCR-RFLP analysis can be considered an effective genotyping tool in epidemiological investigations where financial resources are limited or in large-scale population surveillance. Parsimony analysis of the \textit{fla}A-SVR sequences also generated a UPGMA dendrogram that was closely related to that generated by analysis of the complete \textit{fla}A gene sequence. To the best of our knowledge, this study is the first report to compare 3 \textit{fla}A typing methods. Accordingly, comparison of \textit{fla}A-SVR sequencing and \textit{fla}A-RFLP typing methods suggests that, although the 2 methods discriminate slightly differently among \textit{Campylobacter} populations, both are effective for outbreak analyses and source-tracking studies (8).

In contrast to the 2 ends of the flagellin gene of \textit{C. jejuni}, the sequence of the related protein within its internal regions was highly conserved. Such patterns were also obvious in the flagellin sequence of \textit{C. coli} and other bacteria such as \textit{Bacillus subtilis}, \textit{Salmonella typhimurium}, and \textit{Escherichia. coli}. It can be proposed that the mutational and/or recombination events at the ends of the \textit{fla}A gene, occur more frequently, which accounts for the antigenic variation of this region (21–23).

In conclusion, this study showed; i) analysis of the \textit{fla}A-SVR yielded very similar results to those obtained by sequencing the entire gene; ii) \textit{fla}A-SVR typing is a cheap, rapid, and reliable method for epidemiological study of \textit{C. jejuni} isolates of clinical origin.

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Conflict of interest None to declare.

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