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Original Article

Molecular Detection of *Arcobacter* in Human Stool Samples Using Housekeeping Genes

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Abstract

Background: *Arcobacter* is one of the most common bacteria in humans and livestock, leading to gastroenteritis in humans as well as genital and enteric diseases in animals. This bacterium is known to be the main cause of diarrhea. In molecular studies, the *16SrRNA* gene was primarily used as the standard gene for the determination of the *Arcobacter*. The purpose of this study was to investigate the molecular detection of *Arcobacter* using *glyA*, *atpA*, and *gyrA* genes compared to*16SrRNA*.

Methods: In this study, 61 samples of *Arcobacter* DNA isolated from fecal specimens of patients and healthy individuals in the sample bank were used. In order to detect *Arcobacter*, the intended primers for *16SrRNA* as well as *glyA*, *atpA*, and *gyrA* genes were used for polymerase chain reaction (PCR). The products obtained from the PCR were sequenced.

Results: The results of the proliferation reactions indicated the accuracy of the intended primers and the associated molecular experiments. Our results showed that 65.57% of the cases were detected to be positive for *Arcobacter* among 61 samples using the *glyA* gene. This percentage was higher compared to *16SrRNA* (42.62%), *gyrA* (42.62%), and *atpA* (24.59%). The analysis was statistically significant.

Conclusions: Given the presence of repetitive sequences in the *16SrRNA* in most bacteria, the interpretation of the results is likely to be difficult for researchers. The results of this study showed more sensitivity and accurate diagnosis of *Arcobacter* using the *glyA* gene than other studied genes. In diagnostic studies of *Arcobacter*, the *glyA* gene is proposed as an alternative to the *16SrRNA*.

Keywords: *Arcobacter,* Polymerase chain reaction, Diarrhea, *16SrRNA*, *glyA*

Background

Arcobacter, *Campylobacter*, and *Helicobacter* are members of the Campylobacter family (1). In 1978, *Arcobacter* spp. were first isolated from aborted bovine fetuses in England. To date, 22 species have been identified, including *Arcobacter butzleri*, *Arcobacter cryaerophilus*, and so on. They are clinically important and are related to human and pathogens of animals and have also been found in stools of people with diarrhea and samples of individuals with bacteremia, endocarditis, and peritonitis (2). A number of studies have also reported the isolation of *Arcobacter* from stool in healthy humans (3). *A. butzleri* is most commonly associated with human disease including enteritis or watery diarrhea (3,4). According to previous studies, in different countries such as South Africa, Belgium, and France, *A. butzleri* is one of the important pathogens in human stools (5,6). *Arcobacter* is the cause of persistent diarrhea (7,8). *Arcobacter* spp. have also been isolated from different biological samples of various animals (9-11). This bacterium can be transmitted through products of animal origin, water, and even oral-fecal contact with humans (12,13). The livestock and poultry carcasses can be contaminated with water, feces, and viscera infected with *Arcobacter* in slaughterhouses (14). This bacterium can be contaminated by contact with raw meat and even by eating unclean meat (15,16). Vegetables may also be contaminated if they are washed with contaminated water or in contact with contaminated feces (17). Animals are, therefore, significant reservoirs of *Arcobacter* spp. Although different methods and environments have been used to distinguish *Arcobacter* from different samples, a standard and accurate reference method has not been provided. Phenotypic and differential microbiological methods for confirmation of *Arcobacter* colonization, including oxidase, catalase, nitrate regeneration, hydrophilic hydrolysis, acetate indoxyl hydrolysis, growth in air and at 25°C, growth in 4% salt, growth in MacConkey medium, and resistance to cephoperazone (18). Due to phenotypic properties of *Arcobacter*, its detection by molecular methods is more accurate and realistic (19). Several molecular diagnostic methods have been developed to improve sensitivity and reduce the time needed to detect *Arcobacter* (20). However, molecular techniques are often difficult and costly. To date, no study has been conducted

on accurate molecular detection of *Arcobacter* spp. using the *glyA*, *atpA*, and *gyrA* genes simultaneously.

By targeting *16SrRNA* and *23SrRNA* genes, one-step PCR can be used to detect *A. butzleri*, *A. cryaerophilus*, and *A. skirrowi* at the same time (21). Other molecular methods have been proposed, including real-time PCR, DGGE, and AFLP (22). *16SrRNA* gene is significantly conserved within the species of the genus so that it can be used as a golden standard for bacterial diagnosis. Although *16SrRNA* gene is an indicator gene and the design of the primers is easy to amplify, it has many variables for the differentiation of microbial taxa. Almost all the studies in this field have been done on the molecular determination of *Arcobacter* using this gene. However, there are also some disadvantages. The *16SrRNA* nucleotide sequence has multiple copies and low molecular resolution and cannot be easily interpreted in a research framework (23,24). In this way, the use of other housekeeping genes such as *gyrA* (encoding DNA gyrase submit A), *glyA* (encoding serine transhydroxymethylase), and *atpA* (encoding the submission of F1 ATPase) may offer different potential benefits for the molecular detection of *Arcobacter* (25).

The objective of this study is to identify *Arcobacter* in human stool samples using other housekeeping genes, including *gyrA*, *glyA*, and *atpA*, in order to develop target genes in addition to the *16SrRNA* gene for molecular detection.

Materials and Methods

Collection of Samples

In this study, we selected 61 genomic DNA samples extracted from human fecal specimens available from the DNA Bank of Infectious Diseases Research Center (Arak University of Medical Sciences). These DNA samples were extracted from stool samples and were enriched with special media (arco broth) containing antibiotics incubated at 28°C for 48 hours. Then, they were inoculated on Brucella agar medium followed by passive filtration of the broth through a 0.45 µm membrane filter placed on the blood agar medium (26). Among these DNA samples, 29 samples were from healthy people who were exposed to poultry meat, and 32 samples were from individuals with diarrhea.

Genus-Specific PCR

Genus-specific PCR has been used to detect *Arcobacter* at the level of the genus. Primers for *gyrA*, *glyA*, and *atpA* genes have been designed using specialized programs such as Primer Blast, Mega 4.0, Oligo 6.0, and Primer3. The sequences of *16SrRNA*-specific primers were provided by Gonzalez et al (15). The specifications of the used primers are shown in Table 1. The PCR reaction mix for each gene included 1.5 µL of extracted DNA (20-50 µg), 0.7 µL of each primer (Copenhagen, Denmark), 7.5 µL of 2x super master-mix (YTA, Iran), and 4.6 µL of DDW in the final volume of 15 µL. The amplification was performed with initial denaturation at 94°C (5 minutes) followed by 28 cycles of denaturation at 94°C (1 minute), annealing at specific temperatures (55 seconds), and extension at 72°C (55 seconds) (Table 1). Moreover, the final extension was carried out at 72°C (8 minutes). DNA extracted from the *Arcobacter* colonies and the water (no template) were considered positive and negative, respectively.

Gel Electrophoresis

Gel electrophoresis was used to evaluate the PCR results of each amplifier. The products of PCR reactions were loaded on a 1.3% agarose gel (Genefanavaran, Iran). The results were analyzed using the gel doc system (Quantum ST4, Germany).

Sequencing

PCR products were sequenced on an ABI automatic sequencer (Applied Biosystem Inc., CA, USA) using Macrogen (South Korea) facilities for confirmation of the amplification reaction.

Statistical Analysis

Statistical analyses were carried out using related software packages such as Excel 2007 and MedCalc 18.11.

Results

Evaluation of PCR Products for Electrophoresis

Figure 1 shows the PCR products electrophoresed on 1.3% agarose gel.

The sequencing results were analyzed using related software packages (Mega4 and Chromas). The amplicons

Table 1. Specifications of the Primers Used in the Study

Primer	Sequences (5 to 3)	Target Genes	Size of Products (bp)	Annealing °C
Arc1 Arc2	AGAACGGGTTATAGCTTGCTAT GATACAATACAGGCTAATCTCT	16SrRNA	181	52.7
GyrA F GyrA R	GAGATCAAGGAAGAAGTACAAG TGTATTTCTTCCTGCTTTTCTAATTG	*gyrA*	330	52.7
GlyA F GlyA R	AGCAGCTAATGAACATCCAAGT CCACCTTGAAGTCCTGGGAA	*glyA*	175	52.7
AtpA F AtpA R	TCAAGCTGGAGACGTTGC ATTGTGCAAACGCCTCAAGT	*atpA*	220	65

confirmed the data (Figure 2).

PCR Results

Of the 61 samples studied, 26 cases (42.62%) of *16SrRNA* gene PCR, 26 cases (42.62%) of *gyrA* gene PCR, 40 cases (65.57%) of *glyA* gene PCR, and 15 cases (24.59%) of *atpA* gene PCR were positive for *Arcobacter*, as detailed in Table 2.

Table 2. Frequency of Positive Samples in Molecular Detection by Each Gene

atpA	*gyrA*	*16SrRNA*	*glyA*	Samples
9 (14.75%)	9 (14.75%)	9 (14.75%)	19 (31.14%)	Healthy people
6 (9.83%)	17 (27.86%)	17 (27.86%)	21 (34.42%)	Patient

Discussion

The increased isolation of *Arcobacter* from clinical samples and healthy people has increased its importance in general health (27). Due to the existence of *Arcobacter* in food products containing animal resources, vegetables, and water, this bacterium is introduced as a food-borne pathogen (28). In addition, *Arcobacter* can cause gastroenteritis in human and genital diseases in animals, which can confirm the need to detect *Arcobacter* (29). Because molecular detection methods are faster and more accurate than cultural ones, we used molecular methods to identify *Arcobacter* in fecal samples of healthy individuals and patients. In previous studies, the *16SrRNA* gene is mostly used for the diagnosis of the *Arcobacter* gene. Due to the existence of different bacterial genomes in stool samples as well as repeated nucleotide sequences in the *16SrRNA* gene of some bacteria, the detection of *Arcobacter* probably cannot be accurate (25). Therefore, in this study, we evaluated the molecular detection of *Arcobacter* using housekeeping genes such as *gyrA*, *glyA*, and *atpA* genes compared to *16SrRNA* genes. Based on the suggestions of other studies, these genes have been selected. To date, no study has been conducted in our study environment reporting similar prevalence rates for *Arcobacter* using these genes. To the best of our knowledge, no previous study has used four housekeeping genes (*gyrA*, *glyA*, *atpA*, and *16SrRNA*) simultaneously in the stool samples to differentiate *Arcobacter* species. Luis Collado et al proposed the use of the *gyrA* gene in the PCR-hybridation method (30). William G Miller et al reported the application of the *glyA* gene for genotyping *Arcobacter* spp. using the MLST technique (4). In a study conducted by Miller et al,

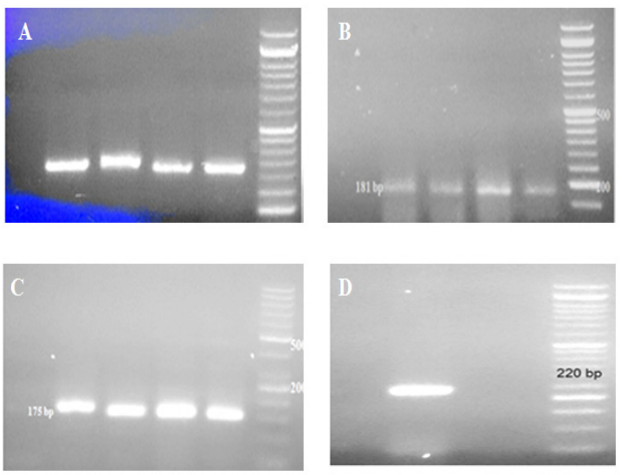

Figure 1. Electrophoresis of PCR Products of *gyrA* (A), 16*SrRNA* (B), *glyA* (C), and *atpA* (D) on 1.3% Agarose Gel. Product size: 330 bp, 181 bp, 175 bp, and 220 bp, respectively. L lane: ladder with size of 50 bp (YTA Co.). C- Lane: negative control. C+ Lane: positive control.

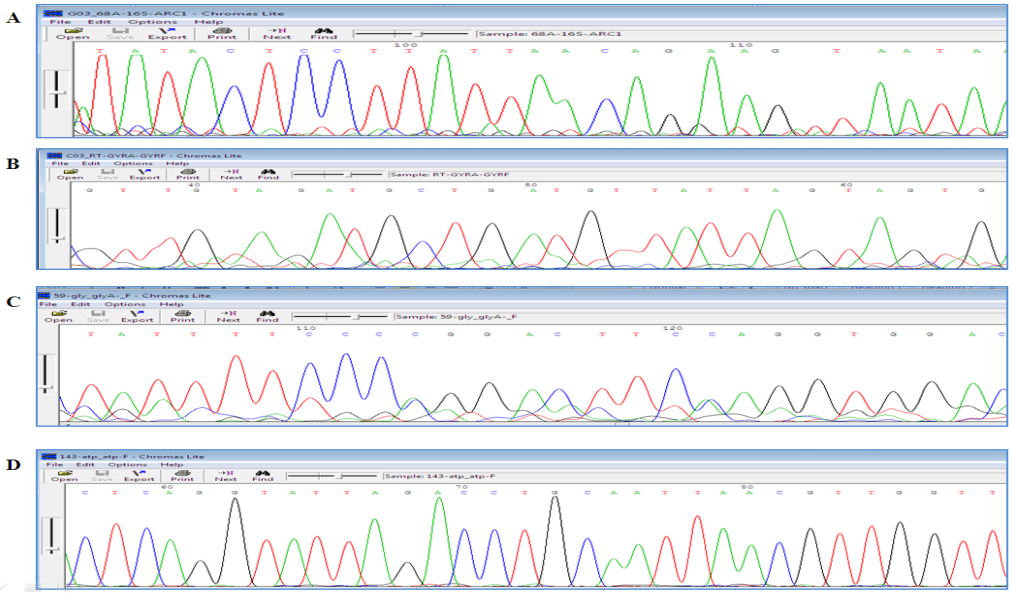

Figure 2. The Results of Sequencing of Genes: (A) *16SrRNA* (max score: 186, QC: 80%, Percent Identity: 91.34%, E value: 1e-46), B) *gyrA*, (max score: 223, QC: 89%, Percent Identity: 95.45%, E value: 4e-58), (C) *glyA*, (max score: 196, QC: 53%, Percent Identity: 95.20%, E value: 8e-50), (D) *atpA*, (max score: 322, QC: 96%, Percent Identity: 96.95%, E value: 8e-88).

Table 3. Statistical Value of *glyA* Gene Compared With *16SrRNA*

Diagnosis Analyses	n=61	Percent	Group	Result	Gene
Sensitivity: 52.830% (38.636-66.68%)	32 Patients 29 Healthy people	27.86	17 Patients	26 Positive	Identification by *16SrRNA*
		14.75	9 Healthy people		
Specificity: 68.750% (53.749-81.340%		24.59	15 Patients	35 Negative	
		32.78	20 Healthy people		
Sensitivity: 66.038% (51.733-78.480)	32 Patients 29 Healthy people	34.42	21 Patients	40 Positive	Identification by *glyA*
		31.14	19 Healthy people		
Specificity: 34.043% (20.864-49.31%)		18.03	11 Patients	21 Negatives	
		16.39	10 Healthy people		

Chi-squared (trend), 5.754; DF, 1; Significance level, P =.0165.

the PCR-RFLP technique was used for proliferation and sequencing of *atpA* to differentiate Campylobacteraceae and helicobacteraceae families (31). Al Rashid et al detected *Campylobacter jejuni*, *C. coli*, *C. lari*, *upsaliensis*, *Arcobacter butzleri* species by the *glyA* gene. In their study, a PCR-hybridization method was developed in which primers are used to amplify *glyA* fragments. Evaluation of this strategy with genomic DNA from different strains has shown that the above-mentioned method is specific and sensitive (32). Therefore, in the genes used in our study, *glyA* was also selected. Abdelbaqi et al studied the development of real-time PCR for investigating the quinolone resistance-determining regions in the *gyrA* gene of *Arcobacter* spp. in France (33). According to their study, the nucleotide sequences of the *gyrA* genes of *A. butzleri*, *A. cryaerophilus*, *A. cibarius*, and *A. skirrowi* were determined. Phylogenetic analysis of *gyrA* sequences provides results similar to phylogenetic analysis of the *16SrRNA* gene sequence and allows for differentiation between *A. butzleri* species (33).

In our study, in addition to the *16SrRNA* gene, we used other in-house genes (*gyrA*, *glyA*, and *atpA)* to detect *Arcobacter* at the molecular level. As shown in Table 2, 19 (31.14%), 9 (14.75%), 9 (14.75%), 9 (14.75%) of healthy people, who were exposed to poultry meat, and 21 (34.42%), 17 (27.86%), 17 (27.86%), 6 (9.83%) of patients with diarrhea were detected positive using proliferation of *glyA*, *16SrRNA*, *gyrA*, and *atpA* genes, respectively, among a total of 61 samples. Recently, it has been shown that *Arcobacter* can be better identified by *glyA* gene than other genes in both groups. Although this bacterium has been detected more frequently in the patient group than in healthy people, *glyA* may be useful for the identification of *Arcobacter* in both groups (P=0.01). In addition, according to statistical data, the sensitivity of the use of the *glyA* gene to detect the *Arcobacter* is higher compared to the 16SrRNA gene. As shown in Table 3, the difference in the prevalence between groups was statistically significant (P<0.05). Therefore, the proliferation of *glyA* gene by designing correct primers, which can be attached to genomes of different species of the *Arcobacter,* may be more useful than other studied genes for the detection of *Arcobacter*. In this study, due to limited financial resources, we did not examine all the housekeeping genes. We used only 4 genes for screening, and in future studies, other housekeeping genes can also be used. Despite the above discussion, it is recommended that this study should be carried out with a larger sample size and that the bacterial load of *Arcobacter* be studied in fecal samples of both healthy people and patients.

Conclusions

To date, *Arcobacter* has not been detected using *gyrA*, *atpA*, and *glyA* genes in clinical samples. The results of this study have shown that the *glyA* gene is more acceptable than other used housekeeping genes for molecular detection of this bacteria. Proliferation of the *glyA* gene may be considered as an alternative to the *16SrRNA* gene to detect *Arcobacter* genus.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Ethical Approval

This study was derivate from research project of Arak University of medical science with IR.ARAKMU.REC.1397.228 ethical code.

Authors' Contribution

AK: performed the experiments and manuscript preparation; AA: designed study, interpreted data, manuscript preparation and approved final manuscript; MA: designed study and interpretation of data; MA: clinical sample preparation and analyzed data; EGM: performed the experiments and interpretation of data

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