Reports of Biochemistry & Molecular Biology
Vol.9, No.3, Oct 2020

Original article

www.RBMB.net

Molecular Detection of *Campylobacter* Species: Comparision of *16SrRNA* with *slyD*, *cadF*, *rpoA*, and *dnaJ* Sequencing

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Abstract

Background: *Campylobacter* spp. are the main cause of human gastroenteritis. The *16SrRNA* sequencing is one of fast molecualr method to detect this fastidious. In this study, we compared the sequencing of *16srRNA* genewith four housekeeping genestodetect *Campylobacter* spp. in patients with diarrhea and healthy people.
Methods: 60 samples of *Campylobacter* DNA extracted from stool samples of 30 patients with diarrhea and 30 healthy people were used. In order to detect *Campylobacter*, we designed primers for proliferation of *16SrRNA*, *cadF*, *dnaJ*, *slyD*, and *rpoA* genes using Primer 3, Mega 4.0 and Blast software. Then the PCR products were sequenced using ABI system.
Results: The sequencing showed concordance of PCR-products with deposited sequences in the Gene Bank. Among diarrhea patients, 53.3% of samples were significantly ($p< 0.05$) positive for *slyD* and *cadF* genes and 50% of samples were positive using *16SrRNA*, *rpoA*, and *dnaJ* genes by PCR assay. The average of sensitivity and specificity were found 53.33% and 83.33%, respectively.
Conclusions: Due to various copies of repeated sequences *of 16SrRNA* gene, analyzing its amplicons on electrophoresis may be more difficult than the *slyD* and *cadF* genes. According to our results, among the 5 studied genes; the highest detection rate was related to *slyD* and *cadF* genes. Although, *dnaJ* and *rpoA* genes, instead of *16SrRNA* gene, can be considered as appropriate genes for molecular detection of *Campylobacter* bacteria.

Keywords: *cadF*, *Campylobacter*, Diarrhea, Molecular detection, *slyD*.

Introduction

Campylobacter spp. are gram-negative, spiral, and coma-shape bacteria. *Campylobacter* spp. require microaerophilic conditions (10% CO2, 5% O2) and also temperatures 37- 42 °C to grow (1). The size of *Campylobacter* genome is 1.6 to 7.1 MBp (2). Virulence factors of *Campylobacter* such as motility, adhesion, invasion to host cell, and production toxin contribute in pathogenicity and causing host cell necrosis (3, 4). Among virulence genes of *Campylobacter*, *flaA*, *flaB*, *racR*, *dnaJ*, and, *slyD* are known to be responsible for pathogenesis, while *ciaB* and *pldA* contribute in gene expression and colonization. Also, *cdtA*, *cdtB*, and *cdtC* genes are known to be responsible for production of cytotoxin (5). The *slyD* gene encodes Peptidyl Prolyl cis/ trans-isomerase which has role in the synthesis of amino acids(6). Small subunit ribosomal RNA (*16SrRNA)* gene is one of the most important target genes in molecular studies on the bacterial evolution and epidemiology (7). The *16SrRNA* gene is considered as a gold standard gene for estimating of phylogenetic diversity in microbial communities (8,9); Nevertheless, the widespread usage of *16SrRNA*, it can limit several aspects of the results as its high copy numbers in the genome, 1-15 or more copies (10). The copy numbers can

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Received: 19 May, 2020; Accepted: 8 Jun, 2020.

[DOI: 10.29252/rbmb.9.3.257]

differs between *Campylobacter* spp. and only a limited number of bacterial genomes have one copy of *16SrRNA* gene that its varies may be simultaneous with increasing number of copies (11). Also, the sequence of *16SrRNA* gene can be different among bacteria communities (12).

One of the main causes of diarrhea in humans is *Campylobacter* infection and its detection using bacterial culture is not easy. Therefore, accurate identification of this gene by molecular methods is very important.

The purpose of this study is to compare the detection of *Campylobacter* using the *16SrRNA* gene with *slyD*, *cadF*, *rpoA*, and *dnaJ* genes for determination in patients with diarrhea admitted to Amirkabir Hospital in Arak and healthy individuals people referred to the Arak health center in 2017-2018.

Materials and methods

Primary isolation

This study was approved by the ethics committee of Arak Universityof Medical Sciences (Ethics code: IR.ARAKMU.REC.1397.229). We used 60 DNA samples available in DNA Bank of Infectious Diseases Research Center of Arak University of Medical Sciences, Iran. DNA samples were extracted from human stools and included 30 samples of patients with diarrhea and 30 fecal specimens from healthy individuals exposed to poultry meat. Because the chicken is a major host for the transmission of *Campylobacter* species.

Detection of Campylobactergenus by Polymerase chain reaction (PCR) and Sequencing

For each sample, five housekeeping genes, *16SrRNA*, *cadF*, *rpoA*, *dnaJ*, and, *slyD* were used in PCR. Properties of primers are shown in Table 1. The reaction mixture in final volume was 15 µl, contained 6.2 µl of Master mix (ID No: 5200300-1250, YTA, Iran), 50 ng genomics DNA, and 0.7 µl (10 pmol) of each primer (Copenhagen, Denmark). The PCR was performed in Thermocycler device (Eppendorf, Germany) under the following temperature conditions: denaturation at 95 ˚C for 5 min, 35 cycles including initial denaturation of 95 ˚C for 1 min, annealing 56.4 ˚C (*16SrRNA* gene) for 55 sec, Extension 72 ˚C for 1 min and final Extension 72 ˚C for 10 min. Annealing temperature for each gene is provided in Table 1. DNA extracted from *Campylobacter* colonies (from previous study Reference?) and distilled water were used as positive and negative control, respectively. The identification of *Campylobacter* at genus level from the extracted DNA of fecal specimen was done based on the size of amplicons on agarose 1% gel (Gene Fanavaran, Iran) in horizontal electrophoresis system (Padide Nozhen Pars, Iran) and gel documentation (Quantum ST4. Germany). Then, PCR products were sent to Macrogen company (South Korean) for sequencing and final confirmation by ABI Applied Biosystems 3730xl instrument.

Statistical Analysis

Statistical analyses were carried out using MedCal 18.11. P values less of 0.05 were considered as statistically significant.

Results

PCR results of housekeeping genes

Among of 60 samples, 21 samples (35%), 21 samples (35%), 20 samples (33.33%), samples (33.33%), 20 samples (33.33%), and 20 samples (33.33%) were positive for *Campylobacter* by *slyD*, *cadF*, *dnaJ*, *rpoA*, and *16SrRNA*, respectively Table 2).

Sequencing

Results of sequencing were analyzed by using Mega4 and Chromas software. Figure 1 (a-e) showed amplifications and sequencing results of different genes in present study. Data were compared with sequences of gene bank and confirmed statistically significant using BLAST software.

[DOI: 10.29252/rbmb.9.3.257]

Table 1. Properties of primers used to detect of *Campylobacter* genus.

Primers ID	Sequence (5' to 3')	Target gene	Size (bp)	Annealing temperature (˚C)
slyD-F *slyD-R*	GCGAAGGTGAAAATGGCGAA GATGATCGTGTCCATGTCCG	*slyD*	216	62
MD16S-F *MD16S-R*	ATCTAATGGCTTAACCATTAAAC GGACGGTAACTAGTTTAGTATT	*16SrRNA*	857	56.4
cadF-F *cadF-R*	TAAAAGCGGTGGATTTGGAC CAGGACATTTTGCTTGTGGA	*cadF*	218	54
rpoA-F *rpoA-R*	CGAGCTTGCTTTGATGAGTG AGTTCCCACAGGAAAACCTA	*rpoA*	121	52
dnaJ-F *dnaJ-R*	GGCAGGGGACAAGTAGGAA CCCCTATTGCCACTTTTGCT	*dnaJ*	227	52

Table 2. Results of amplifications for each group.

Genes / Group	*16SrRNA*	*slyD*	*cadF*	*rpoA*	*dnaJ*
	Positive number (Percentage)				
Healthy	5 (16.66%)	5 (16.66%)	5 (16.66%)	5 (16.66%)	5 (16.66%)
Patient	15 (50%)	16 (53%)	16 (53%)	15 (50%)	15 (50%)

Table 3. Sensitivity and specificity of PCR.

Total Sample	Gene Diagnostic	Result	Person	Percentage	Diagnostic analysis	Chi-square analysis	*p* value	DF
30 samples Patient + 30 samples healthy people	Detection With ***16SrRNA*** gene	**20 positive**	**15 Patient**	**25%**	Sensitivity: **50.00%** (31.297%-68.703%) Specificity: **83.33%** (65.279%-94.358%) Positive Predictive Value: **75.00%** (55.529%-87.816%) Negative Predictive Value: **62.50%**(52.967%-71.153%) Disease prevalence: **50.00%**(36.806%-63.194%)	**7.375**	**0.006**	**1**
			5 healthy	**8.3%**				
		40 Negative	**15 Patient**	**25%**				
			25 healthy	**41.7%**				
30 samples Patient + 30 samples healthy people	Detection With ***cadF*** gene	**21 positives**	**16 Patient**	**26.7%**	Sensitivity: **53.33%** (34.326%-71.658%) Specificity: **83.33%** (65.279%-94.358%) Positive Predictive Value: **76.190%** (57.341%-88.396%) Negative Predictive Value: **64.103%**(54.119%-72.998%) Disease prevalence: **50.00%** (36.806%-63.194%)	**8.717**	**0.0032**	**1**
			5 healthy	**8.3%**				
		39 Negative	**14 Patient**	**23.3%**				
			25 healthy	**41.7%**				
30 samples Patient + 30 samples healthy people	Detection With ***slyD*** gene	**21 positive**	**16 Patient**	**26.7%**	Sensitivity: **53.33%** (34.326%-71.658%) Specificity: **83.33%** (65.279%-94.358%) Positive Predictive Value: **76.190%** (57.341%-88.396%) Negative Predictive Value: **64.103%** (54.119%-72.998%) Disease prevalence: **50.00%** (36.806%-63.194%)	**8.717**	**0.0032**	**1**
			5 healthy	**8.3%**				
		39 Negative	**14 Patient**	**23.3%**				
			25 healthy	**41.7%**				
30 samples Patient + 30 samples healthy people	Detection With ***rpoA*** gene	**20 positive**	**15 Patient**	**25%**	Sensitivity: **50.00%** (31.297%-68.703%) Specificity: **83.33%** (65.279%-94.358%) Positive Predictive Value: **75.00%** (55.529%-87.816%) Negative Predictive Value: **62.50%** (52.967%-71.153%) Disease prevalence: **50.00%** (36.806%-63.194%)	**7.375**	**0.006**	**1**
			5 healthy	**8.3%**				
		40 Negative	**15 Patient**	**25%**				
			25 healthy	**41.7%**				
30 samples Patient + 30 samples healthy people	Detection With ***dnaJ*** gene	**20 positive**	**15 Patient**	**25%**	Sensitivity: **50.00%** (31.297%-68.703%) Specificity: **83.33%** (65.279%-94.358%) Positive Predictive Value: **75.00%** (55.529%-87.816%) Negative Predictive Value: **62.50%** (52.967%-71.153%) Disease prevalence: **50.00%** (36.806%-63.194%)	**7.375**	**0.006**	**1**
			5 healthy	**8.3%**				
		40 Negative	**15 Patient**	**25%**				
			25 healthy	**41.7%**				

[DOI: 10.29252/rbmb.9.3.257]

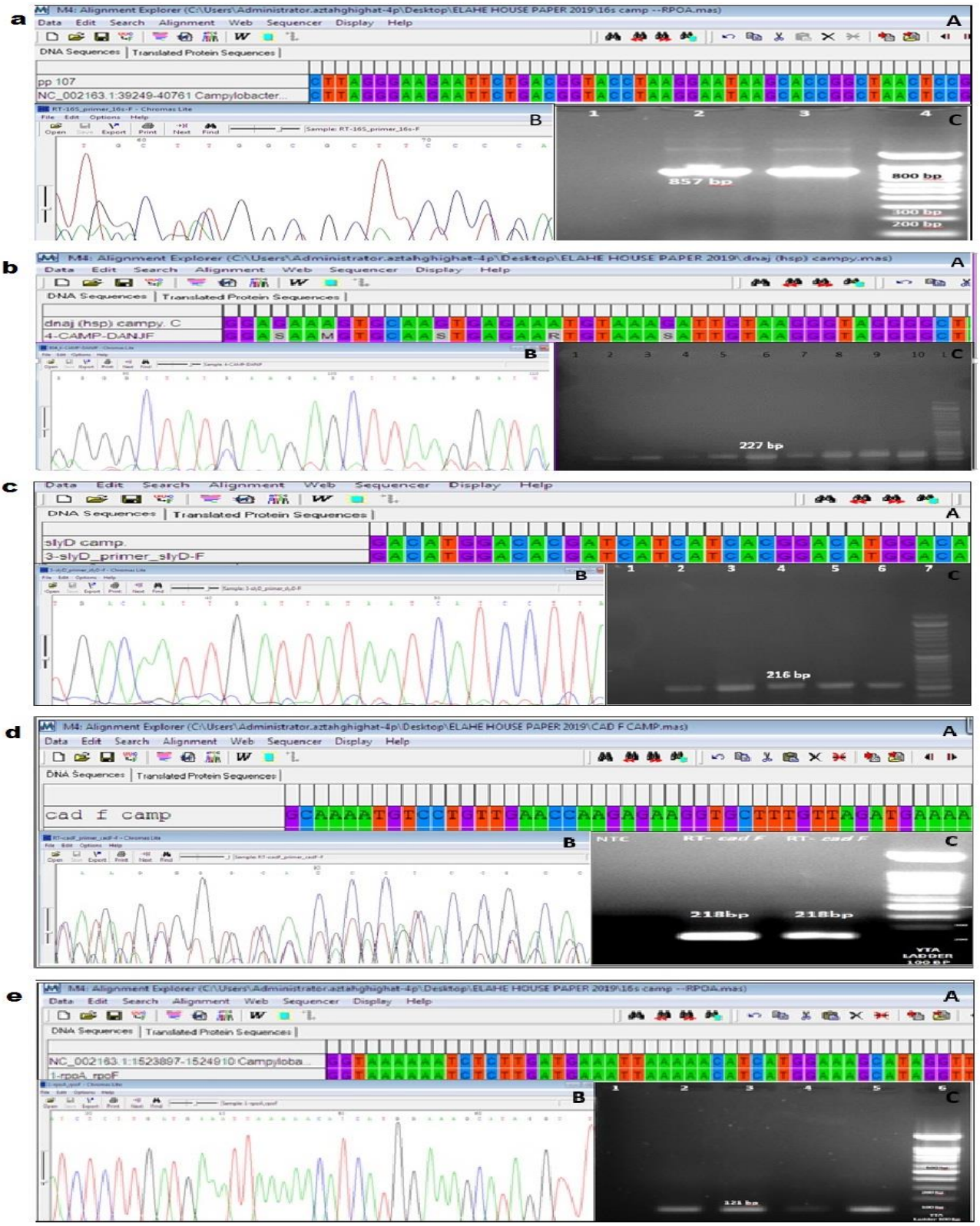

Fig. 1. (A, B) a-e: Sequencing results in Mega4 and chromes software for *16SrRNA* {max score: 67.1, QC: 40%, total score: 67.1, Percent Identity: 82.54 %, E value: 1e-09)}, *dnaJ* {max score: 301, QC: 92%, total score: 301, Percent Identity: 94.09%, E value: 5e-80}, *slyD* {max score: 234, QC: 79%, total score: 270, Percent Identity: 87.13%, E value: 7e-60}, *cadF* {max score: 301, QC: 92 %, total score: 301, Percent Identity: 94.09%, E value: 5e-80} and *rpoA* {max score: 115, QC: 76%, total score: 115, Percent Identity: 97.10%, E value: 4e-2}, (C) a-e: Results of PCR product for *16SrRNA*, *dnaJ*, *slyD*, *cadF* and *rpoA* on 1.3% agarose gels. Product size: 857, 227, 216, 218 and 121bp.

[DOI: 10.29252/rbmb.9.3.257]

Discussion

The increasing isolation of *Campylobacter* from clinical specimens and healthy people has increased its importance in public health (13). Due to the presence of *Campylobacter* in food products with the animal origin, vegetables, and water, this bacterium has been identified as a food-born pathogen (14). In addition, *Campylobacter* can cause gastrointestinal diseases in humans (gastroenteritis) and abortions in animals (15). Since molecular methods are faster and more accurate than microbial culture methods, in this study we compared molecular diagnostic assay using different genes to detect *Campylobacter* in human fecal samples.

In previous studies, *16SrRNA* gene was mainly used for molecular detection of *Campylobacter* species. Due to the existence of different types of bacterial genomes in the excreted DNA from the feces as well as repeated nucleotide sequences in the *16SrRNA* gene in most bacteria, the identification of the *Campylobacter* is not sufficiently certain by using only *16SrRNA* gene. Regarding the necessity of accurate detection of *Campylobacter,* this study examined the detection of these bacteria by using *cadF*, *rpoA*, *dnaJ*, and *slyD* genes compared with *16SrRNA* gene. Bang et al. in 2003 proposed *cadF*, *flaA*, and *ceuE* genes for determination of *Campylobacter* by PCR assay (16). Ritz et al. in 2009 suggested usage of *rpoA* gene for genotyping of *Campylobacter* species *by* Multilocus sequence typing *(*MLST*)* and evaluation of each gene by using the real time-PCR assay (6). Konkel ME et al. in 1999 used different genes to detect *Campylobacter*, and used *cadF* gene for reproduction and sequencing of gene, and according to this study, this gene can be very suitable for diagnosis of *Campylobacter* (17). Datta et al. in 2003 determinated *Campylobacter* spp. with 11 virulence gene by multiplex-PCR assay. Among 11 primers designed for virulence genes, *dnaJ*, *cadF*, and *cdtB* were present in all samples were positive for *Campylobacter* (18).

In this study, not only *16SrRNA* gene was used but also *rpoA, cadF, slyD,* and *dnaJ* genes were used as housekeeping genes for molecular detection of *Campylobacter*. Among of 30 samples of patients with diarrhea, 15 samples (50%) were positive for *Campylobacter* by *16SrRNA* gene which was the same with the results of *rpoA* and *dnaJ* genes, While the results of *slyD* and *cadF* genes in patients with diarrhea were 16/30 (53.33%), therefor, the results of these genes were more sensitivity than *16SrRNA, dnaJ,* and *rpoA* genes. Also among of healthy people samples, the results of all genes were consistent with each other, and among 30 healthy people samples, *Campylobacter* were identified in five healthy people samples by studied housekeeping genes. These data showed that *cadF* and *slyD* are probably the most suitable genes for accurate detection for *Campylobacter*. According to similar studies, *cadF* and *cdtB* have been identified as suitable genes for the detection this bacterium, and results of this gene are consistent with other studies. The *dnaJ* gene has been reported to be suitable for the diagnosis for *Campylobacter* and its results are consistent with the *16SrRNA* gene. The *rpoA* gene in this study was able to detect 90% of *Campylobacter* isolates, and this result is consistent with other studies. Here, total DNA was extracted from the stool so it is a mixture of genome of different organisms and can the inconsistency with other studies. As shown in Table 3, the sensitivity and specificity of *using cadF* and *slyD* gene for detection of *Campylobacter* by PCR were 53.33% and 83.33%, respectively. The sensitivity and specificity of using these genes have been reported more than *16SrRNA*, *rpoA*, and *dnaJ* housekeeping genes. Therefore, the amplification of the *slyD* and *cadF* genes with specific primers, can be adequate for identification of *Campylobacter* in both groups, patients with diarrhea and healthy people.

In this study, we showed that *Campylobacter* can be detected from a mixture of different genomes with the highest accuracy using housekeeping genes, including *cadF*, *slyD*, *dnaJ*, and *rpoA*, without using the *16srRNA* gene. Probably, replication *of cadF* and *slyD* genes can be considered as a alternative for *16SrRNA* in diagnosis of *Campylobacter,* however research in a larger statistical society is suggested.

The application of PCR molecular method is a more accurate method for diagnosis of *Campylobacter* bacteria in genomic DNA extracted

[DOI: 10.29252/rbmb.9.3.257]

from the feces. The diagnostic standard in different laboratories is based on the usage of the *16SrRNA* gene. One of important implications of our study is molecular detection of *campylobacter* within template DNA that has been purified from the stool with mixture of genomes of different organisms. In this study, we showed that *Campylobacter* can be detected from a mixture of different genomes with the highest accuracy using housekeeping genes, including *cadF*, *slyD*, *dnaJ* and *rpoA*, Apart from the *16srRNA* gene. Probably, *replication of cadF* and *slyD* genes can be considered as an alternative for *16SrRNA* in molecular detection of *Campylobacter*.
Campylobacter species.

Acknowledgment
This study is performed in Arak University of Medical Sciences. We are thankful to the staff of Infectious Diseases Research Center of Arak University of Medical Sciences for her technical assistance.

The authors declare they have no conflict of interests.

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[DOI: 10.29252/rbmb.9.3.257]

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[DOI: 10.29252/rbmb.9.3.257]