


Original Article:**Molecular Identification of Members of Campylobacteriaceae Family in Gallstone**Neda Sheykh Zahed , Masood Ghane^{*} 

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^{*}Corresponding Author: email address: masoodghane@gmail.com (M. Ghane)**ABSTRACT**

Introduction: The role of Bacterial infections as one major cause of occurrence of gallstones has been admitted. Campylobacteraceae family consists of *Helicobacter*, *Campylobacter* and *Arcobacter* genus have been identified as significant bacteria in the appearance of gastric disorders. This study aimed to investigate the frequency of Campylobacteriaceae family bacteria in the gallstones of patients hospitalized in the surgery ward of Shahid Rajaei hospital in Tonekabon. **Materials and Methods:** Sample of gallbladder stone was collected from 36 patients. After culture in the BHI medium for the primary enrichment, DNA extraction was carried out and then, the presence of the desired bacteria was examined by PCR technique. The obtained data was analyzed by SPSS software (21) and Chi-square (χ^2) test. **Results:** Of total 36 samples of the studied gallstone, 3 samples (8.33%) were positive from viewpoint of presence of *Helicobacter*, 5 samples (13.88%) were positive in terms of presence of *Campylobacter* and only 1 sample (2.77%) was positive with respect to the presence of *Arcobacter*. No significant relationship was observed between type of stone and presence of these bacteria. **Conclusion:** The results achieved from this research show the presence of DNA belonging to the Campylobacteraceae family in the gallbladder stone, using PCR technique. These bacteria have an etiological significance in the formation of the gallstones. Therefore, more studies are required to determine the role of these bacteria in the formation of gallbladder stone.

Key words: Gallstone; *Helicobacter*; *Campylobacter*; *Arcobacter*; Polymerase Chain Reaction**INTRODUCTION**

Gastroenteritis is referred to as the inflammation of stomach, small and large intestines. Severe gastroenteritis is responsible for approximately 800,000 deaths annually through some bacteria (i.e. *Staphylococcus aureus*, *Escherichia coli*, *Clostridium difficile*, *Yersinia enterocolitica*, *Salmonella*, *Shigella*, *Campylobacter*, parasites (such as *Giardia*, *Cryptosporidium*), and viruses [1-3].

Gallstone is referred to as the most prevalent gall bladder disorder. It is believed that the disorder in performance and sediment of the biliary salts leads to its occurrence. Biliary salts can lead to inflammation, infection of gall bladder and infection of biliary tracts [4]. The rate of appearance of the gallstones in women is higher than men and has a direct relationship with aging [5]. Gallstones are classified into two classes of cholesterol stones and pigment

stones [6], which are mainly of cholesterol type in 75% of the patients [7]. In the scan via electronic microscope, it is shown that about 90% of the pigmented stones have been made out of the compacted compounds of bacteria along with pigmented solid materials. Such finding is indicative of the fact that bacteria play an important role in the formation of the pigmented stones and justifies the cause that those with pigmented stones suffer from the infection more than those with cholesterol stones [8-10].

As stated earlier, nowadays, bacterial infection has been accepted as a probable factor in the establishment of gallstones. Campylobacteraceae family consists of gram negative and microaerophilic bacteria in the curved and spiral forms. *Helicobacter Pylori* is the main factor of ulcer and inflammations in stomach and duodenum (gastritis, gastric ulcer

and duodenal ulcer) [11]. Out of the various species, *Campylobacter jejuni* and *Campylobacter coli* are regarded as the prevalent factors of diarrhea in human [12]. *Arcobacter Butzleri* has been identified as the significant bacteria in the appearance of gastric disorders [13]. Today, these bacteria are counted as the most prevalent infectious agents in the world [14].

The aim of this study is to measure the frequency rate of infection of these bacteria in the gallstones of the patients hospitalized in the surgery ward of the Shahid Rajaei hospital of Tonekabon, located in the northern part of Iran.

MATERIALS AND METHODS

Sample Collection

In this Cross sectional study, sample was collected from 36 patients with gallbladder stone hospitalized in the surgery ward of Tonekabon Shahid Rajaei hospital in 2016.

Patients with intermittent abdominal pain, dyspepsia and the presence of gallstones in ultrasound were asked to give their written consent for sample collection. Demographic data, including age, gender, residential area and etc were collected from the patients through questionnaire. Patients with the history of chemotherapy, radiation therapy, abdominal surgery and cancer were excluded. From the viewpoint of apparent features and constituent compounds, the isolated stones were classified into 3 groups of cholesterol, mixed and pigmented ones. Following the surgery and under perfect sterile conditions in -4°C , the stone samples were transferred to laboratory. Then, the samples were ground into the sterile mortar, shattered into smaller pieces and inoculated onto BHI culture medium and placed into the incubator in 37°C for 72 hours. After the passage of incubation time, the samples were prepared for DNA extraction [9].

DNA Extraction

Extraction of DNA was implemented according to the instructions of the manufacturing company (Qiagene, Lot No: 11872534, Cat No: 51306). The purity of the extracted DNA was analyzed based on absorbance of the extracted DNA at 260 and 280 nm wavelengths by biophotometer (Eppendorf-Germany) and the presence of any bacterial DNA was verified through amplifying 16S rRNA universal gene according to the following method [15].

PCR Technique

In order to perform PCR, specific primer made by TAG Company (Copenhagen-Denmark) was used (Table 1). Each reaction was performed in a total volume of 25 μl , which contained 14.5 μl of molecular biology-grade water (Sigma Aldrich Company LTD., USA), 2.5 μl of 10 \times PCR buffer (Promega, USA), 1 μl of 10 pmol of forward and reverse PCR primers (for each microorganism separately) (table 1), 1 μl of 10 mM dNTPs (Promega, USA), 0.5 μl of smart Taq DNA polymerase (Promega, USA), 1 μl of 50 mM MgCl_2 (Promega, USA) and 5 μl of DNA template. The negative control tube contained the same PCR reagents as above but had 5 μl of water substituted for the DNA template. PCR amplification conditions on thermocycler (Biorad-Germany) with thermal and timing plan were performed according to table 2. An aliquot of all PCR products was run on a 1.5% (w/v) agarose gels with a 100 bp DNA ladder (Fermntas-Russia) and electrophoresed at 75 V for 40 min. The bands were visualized using ethidium bromide staining and photographed after UV treatment by a trans-illuminator (UV doc, England) [15].

Table 1. The specific primers was used

Primers name	gene name	5'→3'	length	References
27 F	16S rRNA gene	AGAGTTTGATCMTGGCTCAG	1450 bp	[15]
1492 R	universal	GGTTACCTTGTTACGACTT		
CS F	16S rRNA gene	GGAGGATGACACTTTTCGGAGCG	780 bp	[16]
CS R	<i>Campylobacter</i>	TCGCGGTATTGCGTCTCATTTGTATATGC		
miri F	16S rRNA gene	ATTCCACCTACCTCTCCCA	375 bp	[17]
miri R	<i>Helicobacter</i>	ACGGGTATCCGGCCTATG		
Arco F	16S rRNA gene	AGAGATTAGCCTGTATTGTAT	1100 bp	[18]
Arco R	<i>Arcobacter</i>	TAGCATCCCCGCTTCGAATGA		

Table 2. Steps, time span and temperatures used in the polymerase chain reaction (PCR)

Steps	16S rRNA			<i>Helicobacter</i>			<i>Campylobacter</i>			<i>Arcobacter</i>		
	temperature	time	cycle	temperature	time	cycle	temperature	time	cycle	temperature	time	cycle
Primary denaturation	95°C	5 min	1	94°C	5 min	1	95 °C	5 min	1	94°C	4 min	1
denaturation	95°C	45 sec		94°C	45 sec		95°C	45 sec		94°C	45 sec	
annealing	56.5 °C	45 sec	35	51 °C	30 sec	35	57.5 °C	60 sec	35	54 °C	45 sec	35
extension	72°C	75 sec		72°C	30 sec		72°C	60 sec		72°C	90 sec	
Final extension	72°C	5 min	1	72°C	4 min	1	72°C	10 min	1	72°C	10 min	1

Statistical Analysis

The obtained data were analyzed, using SPSS software (21) and Chi-square (χ^2) test with the rate of P value less than 0.05 as significant.

RESULTS

In this research, 36 patients with gallstones were studied. Six of the studied subjects (16.66%) were men and 30 (83.33%) were women. These patients were in an age range from 22 years to 82 years, with average age of 55 years. Among the samples of studied stone, 14 cholesterol stones (38.88%), 7 mixed stones (19.44%) and 15 pigmented stones (41.66%) were diagnosed.

Amplification of 16S rRNA Gene

In order to confirm the presence of DNA in all extracted samples, PCR technique was applied, using 16S rRNA universal primers. In all 36 extracted samples, fragment 1450 bp was observed, confirming the presence of 16S rRNA amplification gene.

Identification of Infectious Agents

In order to diagnose the presence of DNA of the *Helicobacter*, *Campylobacter* and *Arcobacter* in the samples of gall bladder stone, PCR technique was used through application of the specific primers. Observation of fragment 375 bp, 780 bp, and 1100 bp suggest amplification of DNA in the genera of *Helicobacter*, *Campylobacter* and *Arcobacter*, respectively (figure 1).

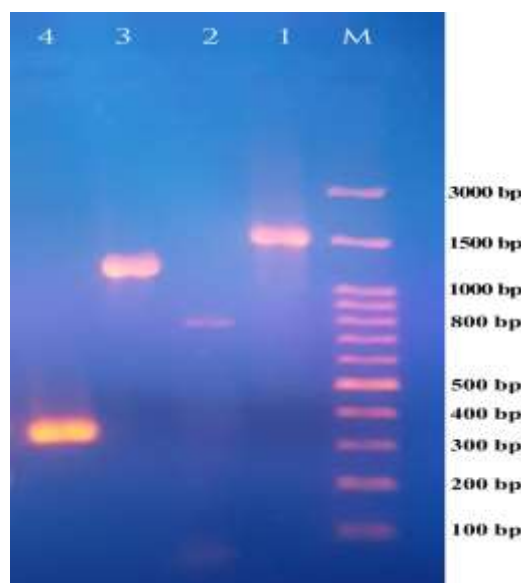


Figure 1. Agarose gel electrophoresis of PCR results from samples. Lane M: Size marker (100 bp), Lane 1: amplification of 16S rRNA universal gene (1450 bp), Lane 2: amplification of *Campylobacter* gene (780 bp), Lane 3: amplification of *Arcobacter* gene (1100 bp), Lane 4: amplification of *Helicobacter* gene (375 bp)

Of 36 samples of the studied gall bladder stone, 3 samples (8.33%) had *Helicobacter* infection. All of these individuals were placed in the age group higher than 40 years, and 3 patients were women from view point of gender and resided in village. From the perspective of type of stone among the patients, 1 individual (7.14%) had cholesterol stone, 1 individual (14.28%) had mixed cholesterol stone and 1 individual (6.66%) had pigmented stone.

Moreover, 5 samples (13.88%) were positive in terms of presence of *Campylobacter*. Of these 5 samples, 1 individual was male (16.66%) and 4 individuals (13.33%) were female. One of the individuals (20%) was placed in an age range from 30 to 40 years and 4 individuals (14.81%) were placed in an age range higher than 40 years, residing in the rural areas. Meanwhile, 1 patient (7.14%) had a gallbladder stone of cholesterol type, 3 patients (42.85%) had a mixed cholesterol stone and 1 patient (6.66%) had a pigmented stone.

Of total 36 samples of the studied biliary stone, only one sample (2.77%) was positive in terms of presence of *Arcobacter*. This patient was in

the age group higher than 40 years and women in terms of gender and had a rural living. The patient's stone type was cholesterol one (table 3).

With regard to the usage of chi-square test (X^2), it was dealt with studying of independence between positiveness of each one of infectious factors by each one of the propounded variables which, considering the obtained p value all of which are higher than 0.05, we conclude that, in the assurance level of 95%, there is not any relationship between existence of infection of *Campylobacteraceae* family bacteria and factors, including age, gender, living type and stone type.

Table 3. Distribution of *Campylobacteraceae* family by demographic variables in patients

Demographic variable	Status	Number (%)	<i>Helicobacter</i> Positive (%)	<i>Campylobacter</i> positive (%)	<i>Arcobacter</i> positive (%)
gender	women	30(83.33%)	3 (10%)	4 (13.33%)	1 (3.33%)
	men	6 (16.66%)	-	1 (16.66%)	-
Age	> 30 years	4 (11.11%)	-	-	-
	30-40	5 (13.88%)	-	1 (20%)	-
	< 40 years	27 (75%)	3 (11.11%)	4 (14.81%)	1 (3.7%)
Place of residence	city	14 (38.88%)	-	-	-
	Village	22 (61.12%)	3 (13.63%)	5 (22.72%)	1 (4.54%)
Type of stones	cholesterol	14 (38.88%)	1 (7.14%)	1 (7.14%)	1 (7.14%)
	Mixed	7 (19.44%)	1 (14.28%)	3 (42.85%)	-
	pigmented	15(41.66%)	1 (6.66%)	1 (6.66%)	-
total		36 (100%)	3 (8.33%)	5 (13.88%)	1 (2.77%)

DISCUSSION

Several reports suggest the outbreak of gall bladder stone disease (higher than 10%) in the western countries, and it is becoming more recurrent in 1 to 3% of the society's individuals every year [19-20]. In the developed countries, including U.S.A and Europe, outbreak of the gallstones has been reported to be 10% to 15% considering the race [21]. In Iran and other developing countries, gallbladder stone is not a prevalent disease among the middle-aged individuals, but it is becoming increasingly common among the elderly [22]. Several studies have confirmed that various bacteria can exist within the gallstones, where bacterial infection has been admitted as a causative factor of the gallbladder stone [23].

During the studies conducted by Ting et al. using PCR method in 1998, gallbladder and biliary stone of 30 patients was studied and analyzed, and DNA of the bacteria such as *E.coli* was identified in the stones of 8 patients, *Propionibacter* in 7 patients and *Streptococcus pyogenes* in 2 patients [24].

In another study carried out in the Oxford University in 2004, the bacteria isolated from biliary stones and salts of 65 patients included: Genera of *Enterococcus*, *Citrobacter*, *Pseudomonas*, *Entrobacter*, *Klebsiella*, *Salmonella*, *Acinetobacter*, *proteus*, *Escherichia coli* and *Staphylococcus aureus* [25].

In 2008, another study carried out on the biliary stones by Attasaranya et al., leading to isolation of the gram-negative organisms, including *Escherichia*, *Klebsiella* and *Entrococci* from the biliary salts. Anaerobic bacteria such as *Clostridium* and *Bacteroides* were isolated from the elderly individuals and patients with history of biliary operation as well [21].

In a study conducted by Eslami et al. on the biliary stone of 100 patients in Iran, they could identify various species of the bacteria, including 10 gram-positive bacteria such as *Entrococci* and *Staphylococci* and 63 gram-negative bacteria such as *Pseudomonas*, *Proteus*, *Citrobacter*, *Providencia*, *E.coli*, *Klebsiella*, *Entrobacter* and *Acinetobacter* [9].

In this research which is carried out to determine the rate of frequency of the bacterial infection of the gallbladder stones in the patients of Tonekabon with Campylobacteraceae Family, 3 genera of *Helicobacter*, *Campylobacter* and *Arcobacter* were studied through the use of specific primers and PCR technique. Of 36 samples of the studied stone in the patients, *Helicobacter*'s DNA was identified in 3 samples (8.33%). More than a half of world's population is infected with *Helicobacter pylori*, and an outbreak of this infection is higher in the developing countries; consequently, more than 80% of the population of the developing countries is infected with *Helicobacter pylori*.

Some studies have shown presence of *Helicobacter*'s DNA in the biliary system [26]. For example, Abayli et al. in 2005, Monstein et al. in 2002, Silva et al. in 2003, Farshad et al. in 2006 and Misra et al. in 2007 have reported the prevalence of this bacterium in the biliary stones to be 55%, 3.31%, 7.8%, 12%, and 73% respectively [8, 27-30].

As yet, a lot of reports have documented the presence of *Helicobacter pylori* in the biliary diseases, and this suggests high frequency of infection of the digestive system with genus of *Helicobacter* [26]. The findings achieved from this research is in line with the results obtained by other researchers.

Concerning identification of the *Campylobacter* in the samples of gallbladder stone, the only study which deals with surveying and identification of genus of *Campylobacter* in the biliary salts is the study of Harada et al. in 2000 where they were able to find a connection in the presence of *Campylobacter* in the gallstone [31]. In this study, they could identify the *Campylobacter* genus in 3 samples out of the biliary salts (21%) and show the existence of a pathogenetic relationship with infection of the *Campylobacter*. Considering that little studies have been carried out regarding the rate of frequency of this genus and its species in various countries, sufficient epidemiologic information is not accessible.

In this research, of total 36 gallstones of the patients, the genus of *Arcobacter* was found only in 1 case of the patients, which can be introduced as the first identification of *Arcobacter*'s DNA in the gallstones.

CONCLUSION

Since the detection of members of the family Campylobacteriaceae in the gallstones shows that these bacteria play a role in the formation of these lesions, correct diagnosis and antibiotic treatments can be used to prevent this disease. Based on the findings of the present study, using the multiplex PCR technique can be faster and simultaneously detect the presence of these bacteria. It is also suggested that research be done on more samples and other gastrointestinal diseases.

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"The authors declare no conflict of interest"

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