The frequency of virulence genes*: flaa, hipo and wlan* among *campylobacter jejuni* isolates obtained from clinical specimens in shiraz teaching hospitals.

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Abstract:

Summary: Some *Camplobacter* species, especially *Campylobacter jejuni* are major causes of human bacterial enteritis. This organism is Gram- negative rod with curved or S shape. They are catalase and oxidase- positive, microaerophilic, motile and are carried in the intestine of wild and domestic animals. In this study our aim was to detect the prevalence of campylobacter jejuni in diarrheal patients, and confirmation by PCR method based on three virulence gene *flaA* , *hipo* , *wlaN*. Although many genes are related to pathogenicity of these bacteria, in this study the presence of 3 pathogenic genes that are responsible for adhesion, colonization and invasion of this organism were examined. on 196 *C. jejuni* positive PCR sample isolated from human feaces, The *flaA* gene was present at rate of 100% cases. Detection rates for the *hipo* gene was 91% of samples.also 4.6% of positive *hipo* isolated was positive for *wlaN* gene .Finally the presence of C. *jejuni* confirmed as a considerable cause of gastroenteritis in Shiraz south- west Iran.

Keywords: Campylobacter Jejuni, Virulence Factors, Bacterial Adhesion, gastroenteritis, Guillain-barre syndrome (GBS)

Introduction

Foodborne infections are major cause of morbidity and mortality in worldwide, WHO estimated there are more than 2 million deaths annually (1, 2)*Campylobacter jejuni* is one of the most common causes of bacterial diarrhea in the world. The infection due to C. jejuni is called campylobacteriosis. Some patients may be asymptomatic or have moderate to severe symptoms such as diarrhea, that may be watery or bloody, abdominal pain, fever, headache, malaise and cramp, but vomiting is uncommon. Campylobacter infection does not commonly cause death. This infection occurs primarily in young children, elderly people and immunodeficient patients (1, 14). Campylobacter can be isolated during the year but highest isolation rates are in the late summer and early fall (6). Campylobacteriosis is generally a self-limited disease. Almost infected persons recover without any specific antibiotic treatment. Supportive measures, such as electrolyte and fluid replacement are the main therapies (1, 5). Erythromycin is the drug of choice for most C. jejuni infections. The consumption of undercooked poultry products, raw milk and unchlorinated water are the most sources of Campylobacter jejuni infection in humans (13-15). *C. jejuni* is a Gram-negative, curved slender, gull-wing shaped, non- spore forming, slow growing, fastidious, oxidase and catalase positive and microaerophilic (5% O2, 10% CO2) bacterium. These Organisms are usually motile by means of a single polar flagellum at one or both end and grows best at 42ºC (3-5). The pathogenesis of *C. jejuni* certainly involves both host(the health state of host and demographic factors)and pathogen-specific factors such as, adhesion properties, production of toxins and invasiveness of the strains.(6, 7). Some Studies suggest that campylobacter first colonizes in the mucus layer and M cells of the Peyer's patches. It penetrates in to mucus of the small bowel by using flagella-mediated motility and adheres to intestinal mucosa. Once established it elaborates other virulence factors and toxins to cause inflammation and epithelial damage with leakage of fluid(1, 8, 9). The flagellum encoding gene (*flaA*) seems to be necessary for the invasion to epithelial cells (10, 11). One of the other virulence markers is the hippuricase gene (*hipo*). It produces hippuricase or N- benzoyl glycine amidohydrolase that enable the bacteria to hydrolyze sodium hypurate. It has been shown that hippuric acid was not destroyed in any part of alimentary tract except the large intestine, where Campylobacter jejuni capable to degrading hippuric acid with hippuricase enzyme. However, the role of this gene in pathogensis of this bacterium is still controversial (5, 12, 13).*C .jejuni* and *C. coli* are very similar in diagnostic tests, except the sodium hippurate hydrolysis activity that does not exist in *C. coli*.

One of the most serious sequela of campylobacteriosis is the Guillain–Barré syndrome. Guillain–Barré syndrome (GBS) is an acute autoimmune disease that causes demyelination of the nerve in the peripheral nervous system. *C. jejuni* is thought to cause GBS through a mechanism called molecular mimicry. The antibodies generated against *C. jejuni* lipooligosaccharide (LOS) cross-react with gangliosides (GM1 and GD3) found in nerve tissue of GBS patients (2, 14). It is believed that *wlaN* gene is responsible for the biosynthesis of the LOS that carries a terminal galactose and mimics human ganglioside GM1, resulting in an increased risk for GBS in patients. The U.S. Centers for Disease Control and Prevention(CDC) estimates this complication may occur 1,000 per 100,000 cases of *C. jejuni* infections per year (15, 16). Despite the importance of these bacteria in the gastrointestinal diseases, unfortunately, few studies on the prevalence of bacteria and also frequency of virulence genes has been reported from Iran (17, 18). Our aim was to find

the frequency of virulence genes: *flaA*, *hipO* and *wlaN* among *Campylobacter jejuni* isolates from patient feaces in Shiraz teaching hospitals .

Materials and Methods

Patient samples: Nine hundred stool specimens (bloody, watery, mucoidy diarrhea) were collected from gastrointestinal patients in four Shiraz teaching hospital labratories from 2016 -2017. Samples were taken from patients who were not undergoing antibiotic therapy at the time of sampling. A swab was taken from each specimen and put in thioglycollate transport medium (Merck) and transferred to Microbiology laboratory immediately.

Isolation and identification of *C. jejuni* : Fecal swabs were inoculated onto Karmali agar selective medium with campylobacter selective supplement (Fluka) and incubated in a microaerophilic environment by using co_2 jar and Gaspack C (Merck). Plates were incubated at 42 ºC for 48-72 hours . Growth of *C. jejuni* appeared as gray and watery colonies on culture medium. Modify Gram staining (carbol fuchsin instead of safranin) were used to observe Gram negative gull-wings bacteria. Oxidase and catalase- positive bacteria were diagnosed as *C.jejuni*. DNA for PCR were extracted by the conventional boiling method .Fresh sample of bacteria were suspended in 300µl TE buffer and boiled at 95-100º c for 10 min, then centrifuged at 15000 r. p. m for 2 min .The supernatant were stored at -20 ºC for using as template DNA for PCR. Three sets of primers were used for detection of *flaA*, *hipo* and *wlaN* genes as described by Brooke(19) and Linton(20) [Table 1].

Table 1. PCR primers and annealing temperatures

Primer sequence 5 '- 3 '	Primer	Annealing temperature	Product size	reference
-ATAAAAATGCTGATAAACAGGTG	*flaA*	55 c·	750 bp	This study
R-TACCGAACCAATGTCTGCTCTG				
F-AATAGGAAAAACAGGCGTTG	*hipo*	56 c·	566 bp	Brooke R. Fitch
R-GTCCTGCATTAAAAGCTCCT				
F-TTAAGAGCAAGATAGAAGGTG	*wlaN*	52 c·	672 bp	Linton et al
-CCATTTGAATTGATATTTTTG				

All PCR amplifications were performed in 25 µl mixture, consisting of 2.5 µl PCR buffer (1x) 0.5 µl Taq (1v unit, Cinagen), 1 µl dnTp (200µM, Cinagen), 1.6 µl MgCl2 (1.5 Mm), 1 µl of a 35 pm solution of each primer and 2 µl extracted template, then nuclease free water was added to make the final volume to 25 µl and subjected to 40 cycles for amplification in a thermal cycler (Astec, Japan). The PCR program was as following, denaturation at 94º c for 1 min, annealing at a temperature specific to the primer pair for 1 min (Table 1), extension at 72º c for 1 min. The PCR product was analyzed by electrophoresis on 1%-1.5% agarose gel (Merck). DNA bands were stained with ethidium bromide and visualized with a UV trans illuminator and photographed.We had provide standard strain from Razi Vaccine and Serum Research Institute (code: RTCC 500203001) As positive control.

The data are analyzed using SPSS21 software and OLAP program in Excel 2013. Chi square test was used to determine the statistical difference between the groups.

Results

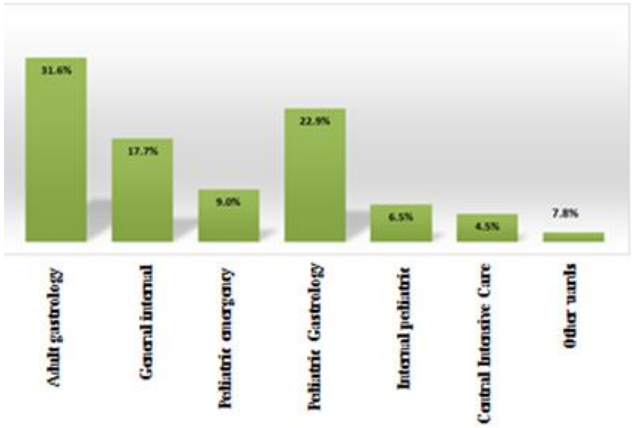
Of the 900 stool samples collected during the course of one year, 196 Campylobacter were isolated from the diarrhea patients referred to educational hospitals in Shiraz. Of the total positive samples, 119 cases of Campylobacter were isolated from male samples (60.7%) and 77 cases (39.28%) from female samples. the incidence of Campylobacter in men is higher than that of women. but there is no significant relationship. All samples that were detected in terms of phenotypic Campylobacter, according to biochemical tests and growth at 42 ° C, belonged to the jejuni species.The number of positive samples separated by age is given in Table (2). According to these statistics, this relationship has been significant ($p< 0.05$). The highest proportion of positive samples was from the age group above 60 years (33.3%) and under 5 years old (29.1%).[Table 2]

Table 2: Positive Sample rate due to the Age

		age.code2					
		Under 5	5-15	15-30	30-60	Above 60	Total
positive	Count	67	17	29	39	44	196
	% within age.code2	34.2%	8.7%	14.8%	19.8%	22.4%	21.6%
negative	Count	162	76	93	303	70	704
	% within age.code2	70.9%	81.3%	76.2%	87.3%	66.7%	78.4%
Total	Count	229	93	122	342	114	900
	% within age.code2	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%

In some studies, it is noted that in developing countries, as opposed to industrialized countries, where Campylobacter is more prevalent in the late summer and early autumn, Campylobacter prevalence does not follow a specific time pattern. According to this chart, the prevalence of Campylobacter in the spring (48%) was higher than the rest of the seasons.Also, based on the statistical analysis and according to Figure1, the number of positive samples isolated from the sections of the study was significant. The most positive samples of Campylobacter were from patients admitted to the Adult gastro logy ward (31%) and pediatric gastro logy ward (22.9%), ($P = 0.04$). [Figure1]

Figure 1: Distribution of percentage of positive samples isolated by wards



The number of positive samples isolated from various types of samples (682 watery samples, 177 mucosal specimens and 41 blood samples) is presented in Table (3). According to these statistics, the most infected samples were isolated from watery samples (682 samples). That is was significant relationship between type of sample and present of *C.jejuni*.($p< 0.05$).[Table3]

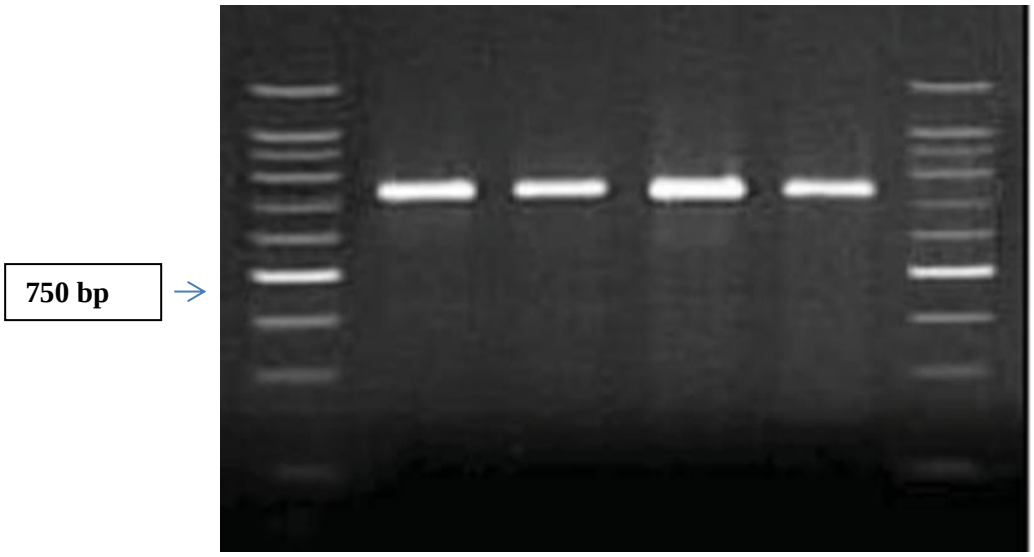
Table 3: Positive sample size by type of sample

Type of stool	Frequency	Percent	Valid Percent
watery	682	75.8	75.8
mocucy	177	19.7	19.7
blody	41	4.5	4.5
Total	900	100.0	100.0

In order to molecular diagnosis, DNA extraction was done for all isolated Campylobacters. The DNA of all Campylobacter specimens was extracted and then examined the presence or absence of *flaA*, *hipo*, and *wlaN* genes by PCR method.

PCR on DNA of all 196 samples that were phenotypically tested and their biocompatible Campylobacter tests confirmed to investigate the presence of the *flaA* gene. PCR results showed that all 196 samples had *flaA* gene (21.6%). [Figure2]

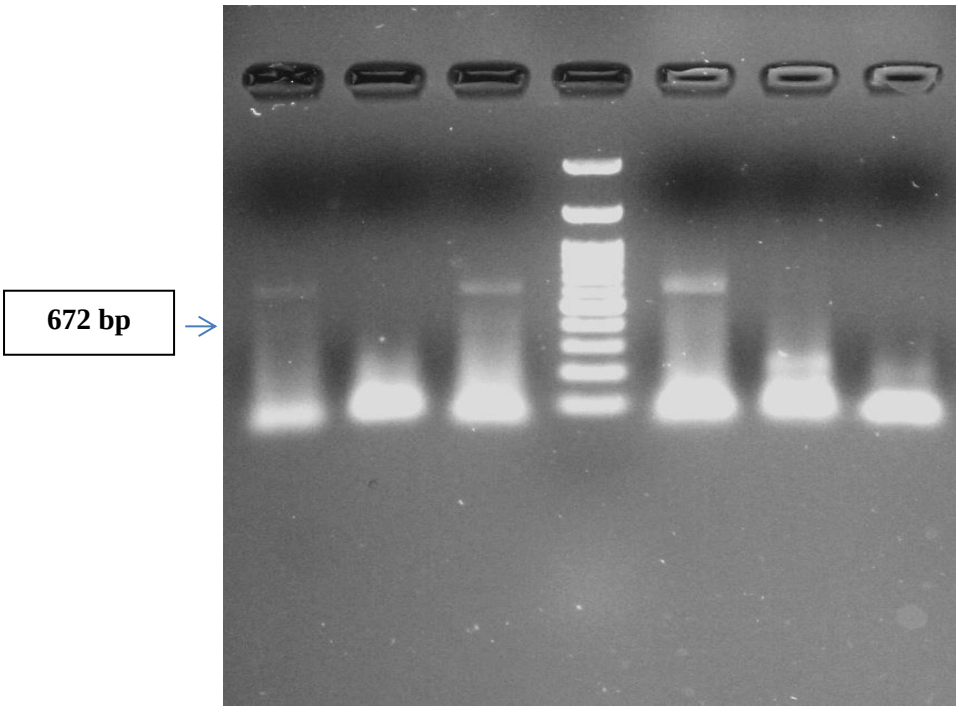
Figure2: *flaA* gel electrophoresis



PCR was performed on all 196 samples that were reported for *flaA* positive gene expression. In this experiment, 177 specimens contained *hipO* gene (91%) and were not able to detect the presence of the *hipO* gene for 19 (9%) specimens. Of these 19 samples, however, were positive for hyporate hydrolysis in biochemical tests.

PCR was performed on all 177 samples that were reported for hypo-positive gene expression. In this experiment, 39 samples had *wlaN* gene (4.6%), and in 138 cases, this gene was absent. [Figure3]

Figure3*: wlaN* gel electrophoresis



Discussions

Foodborne infections are major cause of morbidity and mortality in worldwide, WHO estimated there are more than 2 million deaths annually (1, 2)

The food-born pathogen *Campylobacter* is a leading cause of gastrointestinal human infection and it has been recognized as a major public health issue in various countries .Transmission of *C. jejuni* from animals to humans usually occurs through ingestion of contaminated food, water or raw milk. The incidence of sporadic infection follows a bimodal age distribution, with the highest incidence in young children and elderly people. Infected patients may present mild to severe symptoms include, abdominal pain, cramps, fever and watery to bloody diarrhea(3, 21). In our study the highest proportion of positive samples was from the age group above 60 years (33.3%) and under 5 years old (29.1%). In studies, it is noted that in developing countries, as opposed to industrialized countries, where Campylobacter is more prevalent in the late summer and early autumn, Campylobacter prevalence does not follow a specific time pattern (22), but the prevalence of Campylobacter in our area in spring was higher than the rest of the seasons (48%). According to these statistics, the most infected samples were isolated from watery samples (682 samples). That is was significant relationship between type of sample and present of C.*jejuni*.

In this one-year study the prevalence of *C. jejuni* in diarrheal patients in four teaching hospitals in Shiraz city, southwest Iran, was 21.6% .As we know this is the first report in this regard and also this is the first report that showed the prevalence of importance of *C. jejuni* virulence associated genes (*flaA, hipo and wlaN*), by a molecular detection technique (PCR). The *flaA* gene encodes for a flagella protein that is essential for motility, adhesion and colonization and invasion in the intestine(10). In the present study, the presence of *flaA* gene was observed in all clinical specimens (100%), which is similar to another studies conducted in the world. For example Datta study in Japan(23) Talukder in Bangladesh (24), Ripabelli in Italy (25). The *hipO* gene is essential for sodium hippurate hydrolysis and is specific for *C. jejuni* (12). In our study 91% of bacterial isolates contained *hipo* gene .In some studies, such as the study by Linton indicated that the prevalence of this gene in Campylobacter jejuni was 100%(20). In a study by Slater et al., The prevalence of this gene was 95%(26), which was close to our study. Other genes in the Campylobacter jejuni pathogen is the *wlaN* gene. As the Studies have shown that the *wlaN* gene is presenting in the Gelin-barre syndrome. Guillain–Barré syndrome (GBS) is an acute autoimmune disease that causes demyelination of the nerve in the peripheral nervous system. It is believed that wlaN gene is responsible for the biosynthesis of the LOS that carries a terminal galactose and mimics human ganglioside GM1(27, 28). In this study 4.6% of positive *hipO* isolates (39 samples)were positive for *wlaN* gene . In reports from eastern countries such as Japan, the *wlaN* prevalence in Campylobacter jejuni isolated from humans is 25%(23). In one study in Greece, the prevalence of the *wlaN* gene in Campylobacter jejuni isolated from humans was expressed 16%(29). Our results were lower than other studies in the world. According to our study,*C. jejuni* is cause of the 21.6% of clinical diarrhea cases in the studied patients .

Although Campylobacteria is a self-limiting illness and antibiotic resistance are increasingly developing too, and also this bacterium is associated with the relatively common Guillain-Barre syndrome, so further studies are needed.

Conflict of interest: The authors have no conflicts of interest in this study.

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