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Original Research Paper

Isolation, Identification and Characterization of *Campylobacter* Spp. Isolates from Environmental Samples in North Iran

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ABSTRACT

The major purpose of this study was isolation, identification and characterization of *Campylobacter* spp. from environmental samples viz., domestic animals (cow, sheep, horses), poultry, sewage and river water in north Iran. *Campylobacter* spp. were isolated using pret-KB method and then identified by phenotyping tests. Finally, the identification of strain was verified by 16S rRNA gene sequencing. In general, 64 strains of campylobacters were isolated from all the sources. Out of all isolates 48 strains were catalase positive and 16 strains were catalase negative. The highest isolation rate of *Campylobacter* spp. was recorded from river water (36.92%) followed by poultry (34.88%), cow (28.57%), horses (20%) and sheep (9%), while lowest isolation rate was recorded from sewage (7.4%). Overall, according to these data *Campylobacter* spp. exist with high frequency in north Iran. In addition, this bacterium was isolated from all sources (river water, sewage, domestic animals and poultry). Hence, based on foregoing evidence environments in north of Iran are vehicles of *Campylobacter* spp. and, therefore, the people who live in this area must respect to the personal hygiene in order to avoid from *Campylobacter* infection.

INTRODUCTION

Members of the *Campylobacter* genus are gram negative, curve, S-shaped, non-spore forming and microaerophilic bacteria commonly found in animal faeces (Baserisalehi et al. 2008). Campylobacters, based on catalase production, are divided into two groups, catalase positive and catalase negative. Although, catalase negative *Campylobacter* might be considered as non pathogenic, pathogenic C*ampylobacter* strains belong to the catalase positive group (Blaser et al. 1980). Of all catalase positive *Campylobacter* species, particularly *C. jejuni* is one of the most common causes of human bacterial diarrhoea in industrialized and developing countries (Cardinale et al. 2003). Infective dose of this bacterium is very small; it has been estimated that only 500 cells of *C. jejuni* can cause human illness (Black et al. 1989). This bacterium can enter into the human body through the consumption of contaminated water and animal food products viz., cow meat, milk and poultry (Aydin et al. 2007).

In 1999, the centre for disease control and prevention estimated that more than two million *Campylobacter* infections occurred annually in the US, which accounted for these bacteria as the most common cause of food borne illnesses (Mead et al. 1999). Extensive reports in developed countries pointed out that the consumption of contaminated poultry meat is major source of *Campylobacter* infection (Tauxe 1992, Rivoal et al. 1999). On the other hand, European Food Safety Authority's report in 2005 stated that during the last 30 years human campylobacteriosis has dramatically increased in industrialized countries (Heuer et al. 2001). The epidemiologic survey in developing countries illustrated different levels of isolation of *C. jejuni* from the samples in Bangkok, Thailand,

Nairobi, Kenya and India (Workman et al. 2006). On the other hand, similar to the developed countries, poultry was reported as major source of infection in those countries (Baserisalehi et al. 2007).

Therefore, based on foregoing evidence *Campylobacter* infections are considered a worldwide problem of economic burden and public health, causing considerable suffering. However, *C. jejuni* was isolated from domestic animals and poultry in south Iran (Baserisalehi et al. 2007), but not much information is available concerning to frequency of occurrence of this bacterium in the north of Iran. Hence, the present study was conducted to determine the rate of existence of this bacterium from domestic animals (cow, horse, sheep), poultry, sewage and river water in the north Iran in order to achieve maximum information concerning to the same.

MATERIALS AND METHODS

Sample collection: In total, 235 animal faeces (cow, horse, sheep), poultry, sewage, and river water samples were obtained from different areas of the north Iran (Mazandaran and Gilan provinces). The faecal sample was collected from each animal using sterile stick and polyethylene bag and transferred to the laboratory within one hour of sampling. The river water and sewage samples were collected in 500 mL sterile bottles and transported to the laboratory at ambient temperature and stored at 4°C before they were analysed within 2h. All the samples were subjected to detection of *Campylobacter* immediately upon arrival in the laboratory (Baserisalehi et al. 2004).

Sample processing and isolation: The pret-KB method was used for isolation of campylobacters (Baserisalehi et al. 2004). One gram of the collected fecal samples was emulsified in sterile phosphate-buffered saline (pH 7.0, 0.1 M) at 10% (w/v) concentration. The suspension was centrifuged at 8500 rpm for 10 min, and then kept at room temperature. After 10-15 min a loopful of supernatant was withdrawn and spread onto the mueller hinton agar. The plates were incubated at 37°C for 48 h under microaerophilic conditions and examined daily for 5 days.

Identification and biotyping of *Campylobacter* spp: *Campylobacter* identification was performed by subjecting all the suspected colonies to microscopic examination of wet mount under dark field microscopy, gram staining, glucose fermentation, oxidase and catalase test. The isolates exhibiting characteristic motility of *Campylobacter* were characterized by using standard *Campylobacter* phenotypic identification tests recommended by Atabay & Corry (1997). These tests included H_2S by lead acetate strip, nitrate reduction, growth in 1% glycine and 3.5% NaCl, growth at different temperatures (25, 37 and 42°C), hippurate hydrolysis, urease production, resistance to nalidixic acid (30 mg) and cephalothin (30 mg).

Authentication of campylobacters isolates: Seven *Campylobacter* strains were selected randomly and subjected to gene sequencing. Identification of these strains was verified by gene sequence of 16S rRNA according to the following method.

DNA extraction: DNA was extracted from all campylobacter isolates using high pure template DNA PCR kit (Roche-Germany) according to the supplier's instructions. The purity of the extracted DNA was assessed based on absorbance of the extracted DNA at 260 and 280 nm wavelengths by biophotometer (Eppendrof-Germany).

Amlification of 16S rRNA gener by PCR method: Universal primers produced by TAG kopenhagen (Denmark) were used to amplify 16S rRNA gene. The sequence of forward and reverse primers were 5'-AGGAGGTGATCCAACCGCA-3' and 5'-AACTGGAGGAAGGTGGGGA-3', respectively. Each reaction was performed in a total volume of 25 µL contained 14.5 µL of molecular biology-grade

water (Sigma Aldrich Company Ltd.), 2.5 µL of 10 × PCR buffer (Cinagen-Iran), 1 µL of 10 pmol each forward and reverse PCR primers, 1 µL of a 10 mM dNTPs (Cinagen-Iran), 0.5 µL of smar taq polymerase (Cinagen-Iran), 1 µL of 50mm $MgCl_2$ (Cinagen-Iran) and 5 µL of DNA template. Non-template control (NTC) tube contained the same PCR reagents as above but had 5 µL of water substituted for template. PCR amplification conditions on thermocycler (Eppendrof-Germany) were as follows: 95°C for 4 min, followed by 35 cycles of 95°C for 40 s, 56°C for 30 s, and 72°C for 40 s, with a final extension at 72°C for 5 min and storage at 4°C.

***Agarose gel electrophoresis*:** All PCR products were run on a 1.5% (w/v) agarose gels with a 100 bp DNA ladder (Fermntas-Russia). Aliquots of PCR products were electrophoresed at 75 V for 40 min; DNA was visualized using ethidium bromide and photographed after UV transillumination with Uvidoc (England).

16S rRNA gene sequencing: 16S rRNA PCR products were sent to macrogen in South Korea (http://www.macrogen.com/) for DNA sequencing.

Bioinformatics applications: All sequences data were subjected to BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/) to definitively identify each respective 16S rRNA gene amplicon.

RESULTS

Frequency of occurrence of campylobacters in different samples: The results obtained from isolation of campylobacters indicated that out of all the collected samples, 64 (27%) were positive for *Campylobacter.* This bacterium was detected with different levels in all the sources. As shown in Table 1, the frequency of occurrence of *Campylobacter* in river water was relatively high and in sewage was relatively low. In addition poultry, cow, horse and sheep were recognized as reservoirs of *Campylobacter* in the area of investigation.

Occurrence of catalase positive and negative campylobacters in different samples: A total of 64 *Campylobacter* isolates from different sources were evaluated for catalase production. The results obtained indicated that 48 and 16 isolates were catalase positive and negative respectively. Although, catalase positive *Campylobacter* were not detected from sheep, catalase negative *Campylobacter* strains were not recovered from the sewage (Table 2).

Identification and phenotyping of *Campylobacter* spp. isolates: All the isolates were subjected to identification using the recommended tests by Atabay & Corry (1997). As Shown in Table 3 frequency of occurrence of *C. jejuni* was relatively high, but for *C. gracilis* it was relatively low. Although *C. jejuni* was isolated from all the sources, *C. coli* was not detected from sewage. In addition *C. lari* was isolated from all the sources except sewage and horses. In this study *Arcobacter butzleri* was isolated from cow and river water while *C. gracilis* was isolated from river water.

16S rRNA PCR and sequencing analysis: The results obtained from 16S rRNA sequencing of PCR products was clear except one of the product (sample 2) which was unsequencable. There was 100% alignment agreement for all the samples by BLAST alignment program. As show in Table 4 BLAST analysis of the 16S rRNA sequence data for sample 1 indicated a 100% homology with the *C. gracilis* strain ATCC 33236. Sample 3 was identified by BLAST analysis of the sequencing data as *C. jejuni* strain SWUN0747 (99% homology). Sample 4 was identified by BLAST analysis of the sequencing data as *Aquaspirillum polymorphum* strain DSM 9160 (99% homology). Samples 5 and 7 indicated a 100% and 98% respectively, homology with the *A. butzleri* strain ED-1. Sample 6 was identified by BLAST analysis of the sequencing data as *C. jejuni* strain SWUN1206 (99% homol-

Table 1: Frequency of occurrence of campylobacters from different samples.

Samples	No. of samples tested	No. of *Campylobacter* isolates	Percentage of isolates
Cow	63	18	28.67
Sheep	22	2	9.00
Horses	15	3	20.00
Poultry	43	15	34.88
River water	65	24	36.92
Sewage	27	2	7.40
Total	235	64	27.23

Table 2: Occurrence of catalase positive and negative campylobacters in different samples.

Sample	No. of positive samples tested	No. of catalase positive	No. of catalase negative	Percentage of catalase positive	Percentage of catalase negative
Cow	18	15	3	23.80	4.77
Sheep	2	0	2	0	9.0
Horses	3	2	1	13.33	6.67
Polultry	15	12	3	27.90	6.98
River water	24	17	7	26.15	10.27
Sewage	2	2	0	0	7.40
Total	64	48	16	20.42	6.80

Table 3: Frequency of occurrence of *Campylobacter* species isolates in different samples.

Samples	No. of Catalase Positive	*C. jejuni*	*C. coli*	*C. lari*	*C. gracilis*	*A. butzleri*
Cow	15	7	4	3	0	1
Horses	2	1	1	0	0	0
Poultry	12	4	3	5	0	0
River water	17	7	5	2	1	2
Sewage	2	2	0	0	0	0
Total	48	21	13	10	1	3

Table 4: Comparison of phenotyping and genotyping identification.

Number of samples	Phenotyping detection	Genotyping detection
1	*C. gracilis*	100% homology with the *C. gracilis* strain ATCC 33236
2	*C. coli*	No clear
3	*C. jejuni*	99% homology with the *C. jejuni* strain SWUN0747
4	*C. lari*	99% homology with the *Aquaspirillum polymorphum* strain DSM 9160
5	*A. butzleri*	100% homology with the *A. butzleri* strain ED-1
6	*C. jejuni*	99% homology with the *C. jejuni* strain SWUN1206
7	*A. butzleri*	98% homology with the *A. butzleri* strain ED-1

ogy). These data regarding to comparison of phenotyping and genotyping identification of the isolates indicated that almost genotyping identification verified our phenotyping identification except sample 4, while gene sequencing result was differed from our phenotyping identification.

DISCUSSION

Campylobacters is established zoonoses infection and these organisms can be transmitted to human being via food viz., meat and milk, water and through contact with farm animals (Coker et al. 2000). A number of potential risk factors related to campylobacteriosis is untreated water, poor food hygiene and handling practices (Mc Mahon & Mahmood 1993). In order to find out the likely sources of *Campylobacter* it is necessary to characterize strains, which are commonly isolated from food chain and environment, and to identify these strains in the human infections.

In this study, biochemical assays were used to detect *Campylobacter* spp. In addition, 16S rRNA gene sequencing was used to confirm phenotyping identification. Several studies were supported our procedure regarding to verify the identification of *Campylobacter* using gene sequencing method. Mateo et al. (2006) identified *C. jejuni* and *C. coli* in retail poultry products using PCR method. In addition Morris et al. (2008) identified campylobacters by 16S rRNAs gene sequencing. Our data regarding to isolation and phenotyping identification was verified with approximately 86% by genotyping method.

On the other hand, out of all the sources, poultry is most important source of *Campylobacter* infection (Kapperud et al. 1993). In addition, distribution of *Campylobacter* spp. in chicken and meat was similar to that seen in humans, suggesting that both of these food sources play a significant role in human infection (Baserisalehi et al. 2007).

On the basis of foregoing evidence, domestic animals and poultry could be considered as a link between natural habitat of campylobacters and human beings. Hence, the present study was conducted to isolate these bacteria from fecal samples (domestic animals and poultry), river water and sewage. Although frequency of occurrence of campylobacters in river water was relatively high, *C. jejuni* was most important foodborne bacterium, detected from poultry with high frequency. Several studies parallel to these findings have shown that poultry is a major source of *C. jejuni* and chicken meat is predominantly associated with *Campylobacter* infection in man (Harrios et al. 1986, Humphery et al. 1993).

Baserisalehi et al. (2007) stated that incidence of *Campylobacter* in the environment of each geographical area is depended on its climate conditions. It means frequency of existence of *Campylobacter* in dry climate conditions compared to humid climate conditions is low. These data also concerning to the level of occurrence of *Campylobacter* in the environment supported their report. It is because, climate conditions in Mazandaran and Gilan provinces in Iran are humid and temperature range is 12 to 39°C, and the climatic conditions and temperatures in north Iran totally differ from the south, so accordingly frequency of incidence of *Campylobacter* in the north of Iran was relatively high.

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