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Original Research Paper

Chemotactic Behaviour of *Campylobacter* Strains as Function of Different Temperatures of 37°C and 42°C

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ABSTRACT

The chemotactic behaviour of *Campylobacter* strains was determined in presence of different amino acids at two different temperatures of 37°C and 42°C. Two strains of *Campylobacter,* catalase positive (*Campylobacter jejuni*) and catalase negative (*Campylobacter sputorum*) were isolated from river water in Tonekabon, Iran and identified by phenotyping and 16srRNA gene sequencing methods. Chemotactic responses of the isolates were assessed towards a variety of amino acids viz., L-cystine, L-asparagine, L-histidine, L-aspartic acid, L-serine, L-phenylalanine, L-leucine and L-tryptophan by disc and capillary methods at two temperatures 37°C and 42°C. *Campylobacter jejuni* showed positive chemotactic response towards L-cystine, L-tryptophan, L-phenylalanine, L-leucine, L-asparagine and L-serine at both, 37°C and 42°C, however, it was greater at 37°C. *Campylobacter sputorum* showed negative or weak response towards all the amino acids. In addition, *Campylobacter jejuni* illustrated strong chemotactic response to L-asparagine followed by L-serine, and weak chemotaxis response to L-phenylalanine and L-cysteine at 37°C. Overall, *Campylobacter jejuni* showed relatively strong chemotactic response to some amino acids and its stimulation was greater at 37°C. Hence, the human body temperature (37°C) in comparison to avian body temperature (42°C) probably promotes chemotactic response of *Campylobacter jejuni*, which might be a reason for causing diseases in human beings compared to avian species.

INTRODUCTION

Genus of *Campylobacter* belongs to gram negative, microaerophilic, curve, spiral and motile bacteria. These bacteria are divided into two groups based on catalase production and growth temperatures. However, pathogenic strains of C*ampylobacter* are catalase positive but, nonpathogenic strains belonged to the catalase negative (Blaser et al. 1984). Several reports indicated that most of the catalase negative *Campylobacter* have been isolated from the oral cavities of healthy humans (Macuch & Tanner 2000) whereas, catalase positive *Campylobacter* were isolated only from the enteritis patients (Park 2002).

Campylobacter infection ranges from mild to severe watery diarrhoea. Usually, the symptoms of the disease are abdominal pain, fever and general malaise, which progress to watery diarrhoea. The disease frequently is self-limiting but sometimes associated with late onset complications such as Guillain-Barre Syndrome (neurological) and Reiter's Syndrome (reactive arthritis) (Ang & Nachman 2003). Several invasive factors are considered for pathogenic *Campylobacter,* of which the bacterial motion is one of them. *Campylobacter* motility is an important character either for its identification (Baserisalehi et al. 2004) or for its chemotactic responses (Ketley 1997). Chemotaxis is a sensory control for swimming direction towards beneficial environments and away from unfavourable conditions. This phenomenon was best studied in *Escherichia coli* by Herrmann et al. (1983). According to their report, positive chemotactic of this bacterium toward urine causes urinary tract infection in human beings. Regarding to campylobacters, chemotactic response of *Campylobacter jejuni* seems to be prerequisite for pathogenesis in human disease (Ketley 1997). Hugdahl and his colleague in 1988 reported that presence of mucins, L-serine and L-fucose in tissue of human intestinal tracts induce movement of *Campylobacter jejuni* towards intestinal mucus layer and causes diarrhoea.

In contrast to pathogenic form of *Campylobacter* in humans, these bacteria are nonpathogenic in avian fauna (Kapperud & Rosef 1983, Newell & Fearnley 2003, Khanna et al. 2006). Although, the cause for this differential expression is not yet clear, induction of chemotactic behaviour at different temperatures might be considered as a reason. Therefore, based on forgoing evidence this study was conducted to investigate on chemotactic behaviour of *Campylobacter jejuni* and *campylobacter sputorum* in function of different temperatures (37°C and 42°C) in order to find maximum information concerning to why these bacteria are not pathogen for avian species, while some of them are pathogens for human beings.

MATERIALS AND METHODS

Isolation and identification of *Campylobacter*: Two strains of *Campylobacter* were isolated from the river water in Tonkabon, Iran. The isolates were characterized and identified using microscope examinations, Gram staining, oxidase and catalase tests. The isolates exhibiting characteristics typical of *Campylobacter* were characterized using standard *Campylobacter* phenotypic identification tests recommended by Atabay & Corry (1997). These tests included, H_2S lead acetate strip, nitrate reduction, growth in 1% glycine and 3.5% NaCl, growth at different temperatures, viz., 25, 37 and 42°C, and resistance to nalidixic acid (30 µg disc) and cephalothin (30 µg disc). Finally authentication of the isolates was done by gene sequence of 16SrRNA.

Authentication of *Campylobacter* isolates: Identification of the isolates was verified by gene sequencing of 16SrRNA. This method was carried out as follow: DNA was extracted from the isolates by standard kit (Roche-Germany). Then amplification of 16SrRNA gene was performed by PCR method and eventually the products were sent to Macrogen in South Korea (http://www.macrogen.com/) for DNA sequencing.

DNA extraction and amplification of 16SrRNA gene: DNA was extracted from the isolated strains 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) and then the purity was calculated based on absorbance ratio 260/280nm. The extracted DNA with ratio (260/280 nm) 1.9 ≤ corresponding to 121 µg DNA m/L was used for amplification of 16S rRNA by PCR.

Amplification of 16S rRNA was performed using universal primers produced by TAG Kopenhagen (Denmark). The sequence of forward and reverse primers were 5'-AGGAGGTGATCCAACCGCA-3' and 5'-AACTGGA-GGAAGGTGGGGA-3', respectively. Each reaction was performed in a total volume of 26.5 µL containing 14.5 µL of molecular biology-grade water (Sigma Aldrich Company Ltd.), 2.5 µL of 10 × PCR buffer (Cinagen-Iran), 1 µL each of 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.

PCR amplification conditions on an Eppendrof thermocycler 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. All PCR products obtained were run on a 1.5% (w/v) agarose gel with a 100 bp DNA ladder (Fermntas-Russia). PCR products were electrophoresed at 75V for 20 min; DNA was visualized using ethidium bromide and photographed by Uvidoc (England). After visualization of pure DNA bands, the PCR products have been sent to Macrogen in South Korea for DNA sequencing.

All the 16S rRNA sequenced data for bioinformatic applications were subjected to BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/) in order to identify each respective 16S rRNA gene amplicon.

Chemicals: Amino acids L-cystine, L-histidine, L-aspartic acid, L-serine, L-phenylalanine, L-leucine, L-tryptophan and L-asparagine were used in this study (HiMedia, Mumbai, India).

Amino acid suspensions were prepared in phosphate buffered saline (PBS, pH 7.4) containing NaCl (8g), K_2HPO_4 (1.2g) and KH_2PO_4 (0.34g) per litre of distilled water and then filter sterilized (pore size 0.45µm) after the pH adjusted to 7 with HCl or NaOH. All amino acids were prepared at a concentration of 0.1 mol/L (Khanna et al. 2006). All the amino acid suspensions were used to evaluate chemotactic response of *Campylobacter jejuni* and *Campylobacter sputorum* by disc and capillary method.

The effect of temperature on chemotactic response in *Campylobacter jejuni* and *Campylobacter sputorum* was evaluated by disc and capillary methods.

Five mL of the culture was added into each of two flasks (250 mL) containing 100 mL LB broth. After 24 hours incubation at 37°C, the contents of the flasks were centrifuged for 10 minutes at 28°C. The pellet of each centrifuged tube was suspended in two mL of PBS. The suspensions were pooled to a final volume of 25 mL and adjusted with MaCcfarland tube No. 5 (cell concentration approximately 10^9 cfu m/L).

***Chemotaxis assay*:** To perform disk method, 500mL of the suspended culture was placed on the base of each sterile Petri plate (diameter = 9cm), then 10mL of molten PBS agar (0.4% agar) at approximately 40-45°C was poured over the culture and the medium was gently swirled until total mixing of the cells with the medium. Sterile filter paper discs (diameter = 2mm), impregnated with the amino acid suspensions were placed on the center of solidified cell-agar mixture. The plates were incubated at 37°C and 42°C under microaerophilic conditions and the results were recorded after 14 hours. Diameter of cell accumulation around a disc was measured and recorded (Hazeleger et al. 1998). The experiment was performed in duplicate for each amino acid at each temperature. Eventually, the attractant amino acids resulted from disc method were used to perform capillary method.

Capillary method was carried out after preparation of sterile capillaries. Each capillary (90mL, Top-Tech Biomedicals, India), was filled with 30mL of amino acid suspension and sealed from one of the ends by flame. Then the tube was placed into 50mL of the cell suspension in clean glass tubes (1cm × 8 cm) with the open end of the capillary in contact with the cells. For each amino acid, two sets were prepared; one was incubated microaerobically at 37°C and another was incubated at 42°C in same conditions.

After 45 minutes, the sealed end of each capillary was broken then 10mL of the liquid was withdrawn for counting *Campylobacter* cells by plate count agar method and the results were recorded (Paster & Gibbons 1986).

Statistical analysis of the data was performed on the average of total viable count of *Campylobacter jejuni* per mL for each amino acid at each incubation temperature and the significant differences ($P \leq 0{\cdot}05$) between the averages at each temperature were determined by the Student's *t*-test.

RESULTS

Isolation and identification of *Campylobacter*: Preliminary identification of the isolates recognized them as *Campylobacter jejuni* and *Campylobacter sputorum.* The identified isolates were authenticated by DNA sequencing (16srRNA).

The isolates were assessed for their chemotactic response toward a variety of amino acids by disc method. Positive chemotactic response was recorded by observation of visible dense circular ring of bacterial accumulation around the disc.

Campylobacter jejuni showed positive chemotaxis response towards L-cystine, L-tryptophan, L-phenylalanine, L-leucine, L-asparagine and L-serine at both, 37°C and 42°C but, it was greater at 37°C (Table 1). The results obtained from chemotactic behaviour of *Campylobacter sputorum* illustrated that approximately negative or weak response has been shown towards all amino acids.

The investigation was continued by performing capillary method using the attractant amino acids. The results obtained indicated that *Campylobacter jejuni* cells move greater towards all the amino acids at 37°C than that at 42°C. In addition, strong chemotactic response was found towards L-asparagine follow by L-serine and weak chemotaxis response was found towards L-phenylalanine and L-cysteine.

No chemotactic response was observed towards L-phenylalanine and L-cystine at 42°C (Table 2).

Table 1. Chemotaxis response of *Campylobacter* isolates by disc method.

L-amino acids (0.1 mol/L PBS)	*C. jejuni*		*C. sputorum*	
	37°C	42°C	37°C	42°C
Cystine	++	+	-	-
Histidine	-	-	-	-
Tryptophan	++	+	-	-
Aspartic acid	-	-	-	-
Serine	++	+	-	-
Phenyl alanine	++	+	-	-
Leucine	++	+	-	-
Asparagine	++	+	+	-

Each data point represents average of three replicates.
++ Diameter of migrating ring < 1cm; + Diameter of migrating ring > 1 cm
- Absence of migrating ring

DISCUSSION

In overall, movement of the bacterial cells in aquatic environments is not in random directions. Motile bacteria could find favorite direction with a relatively complex process that involves sensing of chemicals in the environment and transfer a signal across the plasma membrane. This signal transfers inside of the cell and regulate the flagella rotation (Barton 2005).

Campylobacters are microaerophilic, nonproteolytic, nonlipolytic and nonsaccharolytic. They neither ferment nor oxidize carbohydrates. Hence, they obtain energy only from oxidation of amino acids or tricarboxylic acids (Grau 1991). In general, colonization of bacteria in intestine requires capability to move into the mucus layer covering the intestinal cells. In this regard cork-screw'form motility of *Campylobacter* allows them to penetrate into mucus barrier. In fact, motility character along with chemotactic behaviour culminates colonization of *Campylobacter jejuni* in intestine, while non-chemotactic mutants were unable to colonize in animal models (Takata et al. 1992). As mentioned above, *Campylobacter* achieve energy from amino acids or tricarboxylic acids hence, their chemotactic behaviour helps them to get amino acids.

Campylobacter genus contains 16 species and 6 subspecies (On 2001), which have different abilities to produce catalase and survive at discrepancy of temperatures. Although, some of these bacteria are pathogens, nonpathogenic strains are included in this genus.

To achieve maximum information regarding to chemotactic responses of pathogenic and nonpathogenic *Campylobacter* at different temperatures, the present study was conducted to isolate and identified two species of *Campylobacter* from environmental samples. The results obtained from phenotyping and genotyping (16srRNA) of the isolates recognized them as *Campylobacter jejuni* and *Campylobacter sputorum*. *Campylobacter jejuni* was cata-

Table 2: Chemotaxis response of *Campylobacter jejuni* by capillary method. Amino acids (0.1 mol/L PBS).

L-Amino acids	*C. jejuni* TVC m/L at 37°C	42°C
Asparagine	3.33×10^6	1.33×10^6
Serine	1.27×10^6	1×10^6
Tryptophan	1×10^4	10^2
Phenylalanine	10^3	-
Cystine	10^3	-
Leucine	10^4	10^2

Each data point represents average of three replicates

lase positive and categorized in thermophilic *Campylobacter* group while *Campylobacter sputorum* was catalase negative and does not categorize in thermophilic *Campylobacter* group. These bacteria have shown different chemotactic responses towards amino acids.

Campylobacter jejuni showed positive chemotactic towards L-cystine, L-tryptophan, L-phenylalanine, L-leucine and L-asparagine at both 37°C and 42°C, but it was greater at 37°C. Negative or weak response towards all the amino acids was observed for *Campylobacter sputorum.*

On the other hand, the results obtained from capillary assay indicated that the number of *Campylobacter jejuni* towards all the amino acids was greater at 37°C. In addition, no chemotaxis was observed towards L-phenylalanine and L-cystine at 42°C. Parallel to our data Khanna et al. (2006) opined that growth rate as well as chemotactic response of *Campylobacter jejuni* was greater at 37°C than that of at 42°C.

In fact, many bacteria sense their population density to express virulence factors; this phenomenon is recognized as quorum sensing (Moat 2002). *Campylobacter jejuni* move towards some amino acids viz., L-cystine, L-tryptophan, L-phenylalanine, L-leucine and L-asparagine and accordingly its population reach to optimum density for expressing its virulence factors, however, *Campylobacter sputorum* was unable to do the same. Therefore, it can be concluded that quorum sensing in *Campylobacter jejuni* was more compared to *Campylobacter sputourm* and this phenomenon was greater at 37°C.

Although, observable fact is not possible yet, we conclude that probably *Campylobacter jejuni* lead to disease in human compared to avian species due to human body's temperature and intestinal tissue components.

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