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A Study on Relationship of Plasmid with Antibiotic Resistance in Thermophilic *Campylobacter* spp. Isolates from Environmental Samples

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Abstract: Susceptibility of thermophilic *Campylobacter* isolates from environmental samples to antibiotics was studied to investigate relation between occurrence of plasmid and antibiotic resistant character in *Campylobacter jejuni*. Antimicrobial susceptibility of environmental isolates of *Camp. jejuni*, *Camp. coli* and *Camp. lari* to antibiotics was assessed by disc diffusion method afterward, three strains of *Camp. jejuni* F44, P41 and W21 isolates were subjected to plasmid isolation and curing. The results on antibiotic susceptibility of *Campylobacter* isolates by disc diffusion method indicated that, all the isolates of *Campylobacter* were sensitive to ciprofloxacin and resistant to cefotaxime, cephalexin and ampicillin. In addition, more than 50% of the *Camp. jejuni* isolates were resistance to chloramphenicol and erythromycin. Plasmids were detected from 60, 50 and 80% of the *Camp. jejuni*, *Camp. coli* and *Camp. lari* isolates, respectively. The cured strain of *Camp. jejuni* F44 was sensitive to chloramphenicol and resistant to erythromycin. Hence, the most of *Campylobacter* isolates tested harbored plasmid and probably chloramphenicol resistant marker is plasmid mediated while, erythromycin resistant marker is chromosomally mediated. Therefore, probably gene resistant markers present in the plasmids can be transmitted among campylobacters in the environment and reach the human population by direct contact and via food products of animal origin.

Key words: *Campylobacter*, plasmid, antibiotic resistance, environment

INTRODUCTION

Members of the *Campylobacter* genus are gram-negative, curved and S-shaped microaerophilic bacteria. They are responsible for human gastroenteritis throughout the world and enteritis caused by them in some places is more important than that of Salmonella and Shigella (Scotter *et al*., 1993). Antimicrobial chemotherapy of patients with acute *Campylobacter* enteritis involves treatment with erythromycin, tetracyclines and fluoroquinolones (Alfredson *et al*., 2003). However, frequency of occurrence of antibiotics resistance *Campylobacter* increasing in developed and developing countries (Taylor and Courvalin, 1988), but antibiotics therapy still is final remedy in case of patient with acute *Campylobacter* enteritis. Resistance of *Campylobacter* spp. to a number of antibiotics such as tetracycline, erythromycin, ciprofloxacin, kanamycin nalidixic acid and chloramphenicol has been reported by Piddock *et al*. (2000). Furthermore, the rate of fluoroquinolone-resistant campylobacters in Thailand, from 0-84% increased during 1990-1995 (Isenbarger *et al*., 2002). Resistance of Emergence and dissemination of antibiotic resistance among *Campylobacter* spp. have been linked to the use of antibiotics in veterinary medicine and use as prophylactics and growth promoters in animal husbandry (Piddock *et al*., 2000). The increasing rate of human infections caused by antimicrobial-resistant strains of *Camp. jejuni* makes clinical management of cases of campylobacteriosis more difficult prolonging illness and compromising treatment of patients with bacteremia (Piddock, 1995).

Despite, deficiency of data in case of the molecular pathogenesis of campylobacters, many reports opined that antibiotic resistance markers in campylobacters could be chromosomally or plasmid-mediated (Taylor and Courvalin, 1988). For instance, tetracycline resistance in strains of *Campylobacter jejuni* and *Campylobacter coli* was mediated by plasmids (Taylor and Chau, 1996). Objectives of this study were assessed to determined susceptibility of thermophilic *Campylobacter* isolates to antibiotics. Afterward, plasmid isolation from pathogenic campylobacters was carried out to achieved information concerning to the presence of plasmid DNA in *Campylobacter* isolates and finally plasmid curing was done to seek correlation between resistance to antibiotics and plasmid occurrence in *Camp. jejuni*.

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MATERIALS AND METHODS

Organisms: Seventy isolates belonging to *Camp. jejuni*, *Camp. coli* and *Camp. lari* were isolated from environmental sources viz., animal feces (cow and horse) and sewage in Kazeroun, Iran, 2007. The method for isolation of the bacteria was prêt KB method (Baserisalehi *et al*., 2004). All these isolates were maintained in Burcella broth with 15% glycerol at -15°C.

Antibiotic susceptibility by disc diffusion method: The antimicrobial susceptibility pattern of the strains under study was studied by the disc diffusion method (Bauer *et al*., 1966). To perform the disc diffusion test, each culture was grown in 5 mL of Muller-Hinton broth until the turbidity corresponded to 0.5 MacFarland standard tube (1.5×10^8 cells mL^{-1}). The suspension was spread inoculated using sterile cotton swab onto Muller-Hinton agar plate and various antibiotic discs were placed on it. After incubating the plates at 37°C under microaerophilic conditions for 48 h the inhibition zones were recorded.

The antibiotic discs included: chloramphenicol (30 μg), norfloxacin (10 μg), kanamycin (30 μg), co-trimoxazole (25 μg), cefotaxime (30 μg), ampicillin (10 μg), ciprofloxacin (5 μg), tetracycline (30 μg), erythromycin (15 μg), gentamycin (10 μg), cephalexin (30 μg) (Hi Media Laboratories Limited, Mumbai).

Plasmid isolation: Plasmid was isolated using standard method recommended by Birnboim and Doly (1979). The quantitation and purity of DNA was done by determining 260/280 nm absorbance ratio spectra by spectrophotometer (UV-1601, Shimadzu).

Medium: Luria Bertani broth (LB) contained (g 100 mL^{-1}) Yeast Extract 0.5, NaCl 0.5 and Tryptone 1. The pH was adjusted to 7.2 and sterilized at 121°C for 20 min.

Reagents:

Solution I	:	50 mM Glucose, 10 mM Na_2EDTA, pH 8 and 25 mM Tris-Cl, pH 8
Solution II	:	0.2N NaOH and 1% SDS
Solution III	:	3M Sodium acetate, pH 4.8-5.3
TE buffer	:	10 mM Tris-Cl, pH 8 and 1mM Na_2EDTA, pH 8

Single colony of each isolate was inoculated into 2 mL LB medium separately and incubated overnight at 37°C under microaerophilic conditions. The cells were harvested by centrifugation at 5000 rpm for 5 min at 4°C. The cell pellet was resuspended in 100 μL of solution I. Then 200 μL of solution II was added and the suspension was mixed gently and incubated on ice for 10 min. The solution III (150 μL) was added to the mixture and incubated on ice for 10 min. The mixture was centrifuged at 11500 rpm, 15 min at 4°C and the supernatant from each tube was transferred to fresh Eppendroff tubes. The mixture of phenol and chloroform in 1:1 proportion (500 μL) was added in the tubes and mixed properly. In each case the phases were separated by centrifugation at 11500 rpm, for 15 min at 4°C. Aqueous layer from each tube was transferred to fresh set of Eppendroff tubes and 1000 μL of 70% ethanol was added into each tube and incubated at room temperature for 10 min. The suspensions were centrifuged at 11500 rpm for 15 min at 4°C. The supernatant was removed by aspiration and discarded. The precipitated plasmid DNA was dried at room temperature for 30 min and dissolved in 50 μL TE buffer.

Confirmation of plasmid DNA by agarose gel electrophoresis: Electrophoresis of plasmid DNA was performed using horizontal gel electrophoresis. Agarose gel (0.8%) was prepared in TAE buffer (40 mM tris-HCl, 50 mM Sodium acetate, 1 mM EDTA; pH 8). Gel was run for two and half hours at 50 volts, stained for 30 min with ethidim bromide (0.5 μg mL^{-1}). The plasmids were visualized using UV light in Alpha imager gel documentation system (Alpha Innotech Corp., USA).

Plasmid curing

Plasmid curing by chemical agents: Three *Camp. jejuni* isolates (F44, P41 and W21) randomly were selected and subjected to plasmid curing by chemical agents and elevated temperature. Chemical agents used for plasmid curing were, Acridine orange, Acriflavine, Ethidium bromide (Intercalating dyes) and Rifampin. To perform plasmid curing, stock solutions of curing agents were prepared in distilled water. The curing agents were serially diluted in LB broth (1600 to 1.6 μg mL^{-1}). Overnight growth (0.1 mL) (adjusted to No. 0.5 McFarland tube 1.5×10^8 cfu mL^{-1}) of each isolate was inoculated into each of the tubes containing 1 mL LB with increasing concentrations of curing agent and incubated at 37°C for 48 h. The Minimal Inhibitory Concentration (MIC) and the Minimal Bactericidal Concentration (MBC) were determined by observing, absence of growth in brain heart infusion broth and on nutrient agar at the lowest concentration of curing agent, respectively. The curing agents diluted to Subinhibitory Concentration (SIC) and were incorporated into the LB agar at SIC values. The overnight growth of resistant cultures was spread on LB agar with SIC of curing agent and incubated

at 37°C for 2 days. The isolated colonies were picked up by sterile toothpicks and inoculated on another LB agar plate (50 colonies/plate). The plates were incubated at 37°C for 48 h and used as master plates. The colonies from each master plate were replicated on Luria agar with 30 μg mL^{-1} chloramphenicol and on Luria agar with 15 μg mL^{-1} Erythromycin and incubated at 37°C for 48 h. Those colonies, which failed to grow on selective medium, were regarded as cured colonies.

At the same time, a control for curing of each marker was maintained by inoculating non-cured culture on selective media to have a check on spontaneous loss of resistant markers.

Characterization of cured and non-cured *Camp. jejuni* isolates: Morphology of the cured and non-cured *Camp. jejuni* was evaluated using phase contrast microscope (Nikon, Japan). They were subjected to phenotypic identification tests recommended by Atabay and 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). Additional tests were hippurate hydrolysis, indoxyl acetate hydrolysis, urease production, alkaline phosphotase production and Glucose fermentation.

RESULTS

Antibiotic susceptibility of *Campylobacter* isolates: The results on antibiotic susceptibility of thermophilic *Campylobacter* isolates by disc diffusion method indicated that all the isolates of *Campylobacter* were sensitive to ciprofloxacin and resistant to cefotaxime, cephalexin and ampicillin. Besides, all the *Camp. lari* isolates were resistant to co-trimoxazole. All isolates of *Camp. coli* were sensitive to tetracycline. Amongst *Camp. jejuni* isolates, 74 and 70% of them were sensitive to gentamicin and kanamycin while 59 and 55% of them were sensitive to erythromycin and norfloxacin respectively. Less than 50% of the *Camp. jejuni* isolates were sensitive to chloramphenicol, tetracycline and co-trimoxazole and less than 50% isolates of *Camp. coli* were sensitive to rest of the antibiotics except co-trimoxazole and chloramphenicol. The number of *Camp. coli* isolates sensitive to antibiotics was relatively less than that of *Camp. jejuni*. Besides, less than 50% of *Camp. lari* isolates were sensitive to chloramphenicol, gentamicin, norfloxacin, kanamycin and erythromycin except tetracycline (Table 1).

Plasmid isolation: A set of forty isolates of *Campylobacter* was randomly selected and subjected to detection of plasmid by alkali lysis method. As shown in Table 2, 60% *Camp. jejuni*, 50% *Camp. coli* and 80% *Camp. lari* isolates harboured plasmids with ≥21 kb in size. The results indicated that the frequency of occurrence of the plasmid in *Camp. lari* isolates was relatively high while in *Camp. coli* was relatively low. Based on these observations, no correlation has been found between sources of isolates and presence of plasmids in *Campylobacter* isolates. Purity of plasmid DNA was found to be between 1.4-1.9 corresponding to 74.2-121 μg DNA mL^{-1}.

Plasmid curing: Curing is to confirm whether the genes for resistance are encoded by genomic DNA or plasmid DNA. Here, attempt was made to cure antibiotic resistant marker from *Camp. jejuni* isolates using chemical agents and physical agent (elevated temperature).

Camp. jejuni isolates F44, P41 and W21 were subjected to plasmid curing by chemical agents. The results obtained indicated that MIC, SIC and MBC values of rifampicin were relatively high while, that of Ethidium bromide were relatively low. The frequency of plasmid curing induced by rifampicin was 4%. Plasmid curing was observed only in *Camp. jejuni* F44. Acridine orange, Acriflavine and Ethidium bromide could not cure *Camp. jejuni* (Table 3).

However, erythromycin and chloramphenicol were considered as resistant markers for plasmid curing. But all cured *Camp. jejuni* showed loss of resistance to chloramphenicol (Table 4).

Characterization of cured and original *Camp. jejuni* isolates: The results obtained from characterization of cured and original *Camp. jejuni* indicated that cured and original *Camp. jejuni* exhibited similar behavior regarding all tests as well as morphology.

Table 1: Susceptibility of environmental campylobacters by disc diffusion method

Campylobacter sp.	No. of isolates	Percentage of *Campylobacter* isolates sensitive to										
		Ch*	Ce*	No*	Ka*	Co*	Cf*	Am*	Ci*	Te*	Er*	Ge*
Camp. jejuni	27	33	0	55.5	70.3	18.5	0	0	100	33	59.2	74.0
Camp. coli	18	50	0	33.4	29.7	50.0	0	0	100	100	33.4	38.9
Camp. lari	25	36	0	24.0	32.0	0.0	0	0	100	76	44.0	32.0

*Ch: Chloramphenicol, Ce: Cephalexin, No: Norfloxacin, Ka: Kanamycin, Co: Co-trimoxazole, Cf: Cefotaxime, Am: Ampicillin, Ci: Ciprofloxacin, Te: Tetracycline, Er: Erythromycin, Ge: Gentamicin

Table 2: Plasmid isolation from *Campylobacter* isolates

Campylobacter sp.	No. of isolates	Isolates with plasmid (%)	Plasmid size (Kb)
Camp. jejuni	10	60	21-21<
Camp. coli	10	50	21
Camp. lari	10	80	21

Plasmids isolated by Birnboim and Doly (1979) method

Table 3: Susceptibility of *Campylobacter jejuni* isolates to chemical curing agents

Camp. jejuni isolates	Curing agent	SIC*	MIC*	MBC*
		----------(μg mL^{-1})----------		
F44	Acridine orange	6.25	12.5	25
	Acriflavine	6.25	12.5	25
	Ethidium bromide	1.6	3.2	6.4
	Rifampine	80	160	320
P41	Acridine orange	6.25	12.5	25
	Acriflavine	12.5	25	50
	Ethidium bromide	1.6	3.2	6.4
	Rifampine	80	160	320
W21	Acridine orange	6.25	12.5	2.5
	Acriflavine	12.5	25	50
	Ethidium bromide	1.6	3.2	6.4
	Rifampine	80	160	320

*MIC: Minimal Inhibitory Concentration, *MBC: Minimal Bactericidal Concentration, *SIC: Subinhibitory Concentration

Table 4: Curing of plasmid in *Campylobacter jejuni*

Curing agent	Cured strain	Eliminated marker	Frequency of curing (%)
Acridine orange	-	-	0
Acriflavine	-	-	0
Ethidium bromide	-	-	0
Rifampine	F44	ch^r*	4
Elevated temperature	F44	ch^r	8

*ch^r: Chloramphenicol resistant marker, -: No strain was cured

DISCUSSION

Antimicrobial resistance property can come up through acquisition of genetic material encoding enzymes that inactivate a particular antibiotic (Hoffman, 1999). Gene resistance markers in campylobacters can be presented in plasmid, chromosome or both. For instance, erythromycin resistance has been reported previously as by chromosomal genes, whereas chloramphenicol resistance is plasmid encoded. It has been reported that resistance to chloramphenicol, kanamycin and tetracycline in *Camp. jejuni* is plasmid-mediated, while resistance to rest of the antibiotics is chromosomally mediated (Taylor and Courvalin, 1988; Taylor and Chau, 1996). On the other hand, several reports illustrated that some virulence factors of *Campylobacter jejuni* are associated with existence of the plasmid in the bacterium (Bacon *et al*., 2000; Tracz *et al*., 2005).

Present findings from this study indicated that out of all *Campylobacter* tested; plasmids were detected in 60% *Camp. jejuni*, 50% *Camp. coli* and 80% *Camp. lari* isolates with ≥21 kb in size. It means frequency of plasmid occurrence in *Camp. lari* is relatively high while, in *Camp. coli* was relatively low. Hence, it could be interpreted that occurrence of the plasmid in the *Camp. lari* isolates with high frequency might induce antibiotic resistant property to the bacterium.

Although *Campylobacter jejuni* is most important causes of bacterial diarrhea worldwide (Taylor, 1992), the details of its molecular pathogenesis are not well understood (Bacon *et al*., 2000). Thus, the present study was undertaken to carry out plasmid curing in order to achieve maximum information concerning to location of resistant markers in this bacterium.

Plasmid curing defined as a loss of plasmid from cell, which leads to loss of specific phenotypes such as drug resistance (Bouanchaud *et al*., 1969). To perform plasmid curing three strains of *Camp. jejuni* (F44, P41 and W21) isolates were subjected to plasmid curing to find out location of resistant markers of chloramphenicol and erythromycin. The result obtained indicated that the frequency of plasmid curing induced by elevated temperature and rifampicin was 8 and 4%, respectively while, Acridine orange, Acriflavine and Ethidium bromide could not cure *Camp. jejuni*. According to the data, only *Camp. jejuni* F44 was cured and the cured strain were sensitive to chloramphenicol and resistant to erythromycin. Therefore, it can be concluded that probably erythromycin resistant marker in *Camp. jejuni* is chromosomally mediated, while chloramphenicol resistant marker is plasmid mediated. This finding is supported by Wang and Taylor (1990). Although, according to present data chloramphenicol and erythromycin resistant markers in *Camp. jejuni* is plasmid and chromosomally mediated respectively, Dasti *et al*. (2007) reported, tetracycline resistance in *Camp. jejuni*, could be plasmid or chromosomally mediated.

The results obtained from characterization of cured and original strain of *Camp. jejuni* F44 using phenotypic identification tests indicated that no significant difference has been found between cured and original *Camp. jejuni* F44. Therefore, it can be concluded that the most of phenotypic characters of *Camp. jejuni* are associated with primary metabolism and its metabolites is chromosomally mediated.

Furthermore, present finding indicated that plasmid mediated of some antibiotic resistance markers in campylobacters cause transmission of the resistance markers among these bacteria and finally reach the human population by direct contact and via food products of animal origin.

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