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EFFECT OF HEAVY METALS AND DETERGENTS ON THERMOPHILIC *CAMPYLOBACTER* SPP. ISOLATED FROM ENVIRONMENTAL SAMPLES

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ABSTRACT

Antimicrobial susceptibility of thermophilic *Campylobacter* spp., isolated from environmental samples, to heavy metals and detergents was evaluated in the present study. The antimicrobial susceptibility of thermophilic *Campylobacter* spp. viz., *Camp. jejuni*, *Camp. coli* and *Camp. lari* against heavy metals and detergents in term of minimal inhibitory concentration was assessed by E- test. The result obtained indicated that most of the heavy metals and detergents have an antimicrobial effect on the thermophilic *Campylobacter* spp. isolates, but minimal inhibitory concentrations of them were not similar. In general, the most *Campylobacter* strains were resistant to high concentration of molybdenum and the detergent Tween 80, while were sensitive to cadmium and cetrimide at less concentration. To interpret, it could be noted that flow of industrial sewage including different heavy metals and detergents into the environment reduces the population of thermophilic *Campylobacter*. Therefore, type of samples and sampling sites affect the isolation rate of thermophilic *Campylobacter* from environment.

INTRODUCTION

Campylobacters are now recognized as the most common cause of acute bacterial diarrhoea worldwide (Altekruse et al. 1998). The natural habitat for most *Campylobacter* spp. is the intestine of birds and warm-blooded animals, including sea gulls and several other wild birds (Kapperud & Rosef 1983). *Campylobacter* may enter the environment including water through the faeces of animals, birds or infected humans (Blaser et al. 1984). Several problems concerning to detection of *Campylobacter* from environment have been reported due to their slow growth character and small number in the environment (Rollins & Colwell 1986). To overcome, a variety of media and methods have been recommended for isolation of campylobacters from environment, but all of them are sub optimize, instance various selective culture media have been described for detection of *Campylobacter* spp. (Lauwers et al. 1982) incorporating different antibiotics as selective agents in order to achieve maximum isolation rates. Unfortunately, some strains of *Campylobacter* are sensitive to some of the antibiotics in the media (Corry et al. 1995). Recently, Baserisalehi et al. (2004a) recommended Kapadnis-Baseri device (KB device) and sample processing, Kapadnis Baseri medium (pret-KB method) (Baserisalehi et al. 2004b) for isolation of *Campylobacter* spp. from environmental samples without using antibiotics. The KB device was designed based on motility and activity of campylobacters at low temperature, which enable to isolate and enumerate *Campylobacter* spp. from the environmental samples. The pret KB method was recommended based on elimination of competing bacteria at the sample and culture levels. The pret minimizes most of the competing bacteria at the sample level and the KB medium without blood and antibiotic is selective and differential medium eliminate rest of the competing bacteria and differentiate *Campylobacter* from the other Gram negative bacteria at the culture level.

Although, water is main source of campylobacters in the environment (Blaser et al. 1984), it might be contaminated by industrial sewage including different heavy metals and detergents. To evaluate effect of these pollutants on campylobacters the present study was conducted to assess the effect of heavy metals and detergents on thermophilic *Campylobacter* spp. isolates from environmental samples in order to achieve maximum information regarding isolation of campylobacters from the environment.

MATERIALS AND METHODS

Isolation and identification of thermophilic *Campylobacter*: Twenty five strains of *Campylobacter* spp. were isolated from environmental samples, viz., river water (Pavana river in Pune, India) and sewage (Camp area Pune, India) using KB device and pret-KB methods (Baserisalehi et al. 2004a, 2004b). All suspected colonies grown on the KB medium were confirmed by typical morphology, darting motility, 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 mg disc) and cephalothin (30 mg disc). All thermophilic campylobacters, isolated from each sample, were confirmed using Hippurate hydrolysis, indoxyl acetate and urease tests.

Six thermophilic *Campylobacter* belonging to *Camp. jejuni*, *Camp. coli* and *Camp. lari* randomly were selected for further study. These strains were *Camp. jejuni* S1, *Camp. lari* S2 and *Camp. lari* S3, isolated from sewage, *Camp. jejuni* W1 and *Camp. coli* W2 and *Camp. coli* W3 isolated from the river water. All these isolates were maintained in Burcella broth with 15% glycerol at 15ºC.

Metals and detergents: All chemicals were from E. Merk, Darmastdt. The metal salts and detergents along with their concentrations are given in Table 1.

Table 1: Mertals and detergents used in the study.

Metals				
Group	Metal	Metal salt	Salt conc./L	Metal conc./L
I	Copper	$CuCl_2.2H_2O$	166.5	63.5
I	Molybdenum	$Na_2MoO_4.2H_2O$	241.95	96
I	Zinc	$ZnCl_2$	136.29	65.4
I	Lead	$Pb(NO_3)_2$	331.21	207
II	Arsenic	$NaAsO_2$	129.91	75
II	Cadmium	$CdCl_2$	183.32	112.5
III	Mercury	$HgCl_2$	271.5	200.5
III	Silver	$AgCl_2$	169.8	107.8

Detergents		
Type	Name	Solubility
Anionic	Sodium lauryl sulfate	D/W
Noionic	Tween 80	D/W
Noionic	Tween 20	D/w
Noionic	Triton X 100	D/W
Cationic	Cetrimide	D/W

Minimal inhibitory concentrations of metals and detergents against *Campylobacter* isolates: The minimal inhibitory concentrations of metals and detergents for six isolates belonging to *Camp. jejuni, Camp. coli and Camp. lari* were determined by E-test (Baker 1992). E-test strip for each heavy metal and detergent was made using a piece of filter paper (Whatman No. 10, size1 × 7 cm). Each strip was divided into seven squares (1 × 1cm). The squares were labelled by respective concentrations in increasing order. The diluted heavy metals and detergents were dropped (10 µL) at the respective square of the strip and allowed to dry.

To perform the E-test, each culture was spread onto Mueller-Hinton agar plate separately. Then, two strips of two heavy metals or detergents were applied on each plate. The plates were incubated at 37°C for 48h under microaerophilic conditions and inhibitory concentration of each preservative was read at the point where the elliptical zone of inhibition intersected the E-test strip. Different concentrations of heavy metals and detergents were prepared as below:

a. Heavy metals: (10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10 and 100 mM)

b. Detergents (µg/mL): Cetrimide (0.67, 6.7, 6.7×10^1, 6.7×10^2, 6.7×10^3, 6.7×10^4 and 6.7×10^5), Triton-X 100 (0.106, 1.06, 1.06×10^1, 1.06×10^2, 1.06×10^3, 1.06×10^4 and 1.06×10^5), Tween 80 (0.1, 1.1, 1.1×10^1, 1.1×10^2, 1.1×10^3, 1.1×10^4 and 1.1×10^5), Tween 20 (0.1, 1.08, 1.08×10^1, 1.08×10^2, 1.08×10^3, 1.08×10^4 and 1.08×10^5) and SLS (0.1, 1, 10^1, 10^2, 10^3,10^4 and 10^5).

RESULTS AND DISCUSSION

Responses of thermophilic *Campylobacter* isolates to eight metals and five detergents are given in Tables 2 and 3. As shown in Table 2, out of all the groups of metals, group II and group I showed high and low toxicity against *Campylobacter* isolates respectively. This indicates that most of the isolates were relatively sensitive to group II and III metals, and relatively resistant to group I metals.

Varied MIC values of group I metals have been found against the isolates. All of the isolates were resistant to highest concentration of Mo tested. Although, some of the isolates were resistant to highest concentration of the Zn and Pb, some isolates were sensitive to them as indicated by their MIC values ranging from 10 to 100mM. Lowest MIC value of Zn was against *Camp. coli* W3, and highest against *Camp. jejuni* W1. Lowest MIC value of Pb was against *Camp. coli* W2 and highest against *Camp. lari* S2. MIC values of Cu against the isolates ranged from 10-100 mM. Lowest MIC value of Cu was against *Camp. lari* S2 and *Camp. coli* W3 and highest against remaining isolates.

In the metal ions group II, the MIC values of Cd against the isolates ranged from 0.01 to 1 mM. However, the MIC value of As against all *Campylobacter* isolates was 1 mM. Lowest MIC value of Cd was against *Camp. coli* W2, and highest against *Camp. lari* S3. Cadmium seems to be relatively more toxic to campylobacters as indicated by its lowest MIC value against them.

In the metal ions group III, MIC value of Ag against all of the isolates was 1 mM. The MIC values of Hg against *Campylobacter* isolates varied from 1 to 10 mM. Highest MIC value of Hg was against *Camp. lari* S2 and S3, and lowest against other isolates.

Varied range of MIC values of metals probably is due to varied response of species as well as different strains belonging to a species.

The range of MIC values of metals against thermophilic *Campylobacter* isolates are as follows:

Metals	*Cu*	*Pb*	*Mo*	*Zn*	*Cd*	*As*	*Hg*	*Ag*
MIC range (mM)	10-100	10-R*	R*	10-R*	0.01-1	1	1-10	1

*R, Resistant

The results obtained from the minimal inhibitory concentration of detergents against thermophilic *Campylobacter* are given in Table 3. However, SLS showed lowest MIC value (10 µg mL^{-1}) against *Camp. jejuni* W1. But the results indicated that in general, Tween-80 and cetrimide have respectively low and high antimicrobial effect. The MIC values of SLS against *Campylobacter* isolates varied from 10 to 10^4 µg m/L. Lowest MIC value of SLS was against *Camp. jejuni* W1, and highest against *Camp. lari* S2. The range of MIC values of cetrimide against the isolates was 6.7×10^1 – 6.7×10^2 µg m/L. Lowest MIC value of cetrimide was against *Camp. jejuni* S1, and highest against rest of the isolates.

MIC value of Triton X 100 against all of the *Campylobacter* isolates was 10^5 µg m/L. While, the range of MIC values of Tween 20 against the isolates ranged from 1.1×10^3 – 1.1×10^5 µg m/L. Lowest MIC value of Tween 20 was against *Camp. coli* isolates, and highest against *Camp. jejuni* S1. Although most of the isolates were resistant to Tween 80, MIC value of Tween 80 against the sensitive isolates was 1.08 × 105µg m/L.

In general, cationic agents showed more toxicity, and nonionic agents less toxicity against thermophilic *Campylobacter* isolates.

The range of MIC values of detergents against thermophilic *Campylobacter* isolates are as follows:

Detergents	*SLS*	*TRI X 100*	*T-20*	*T-80*	*Cetrimide*
MIC range (µg mL^{-1})	$10\text{-}10^4$	1×10^5	$1.1\times10^3\text{-}1.1\times10^5$	1.08×10^5-R*	$6.7\times10^1\text{-}6.7\times10^2$

*R, Resistant

Table 2: MIC values* of metals against thermophilic *Campylobacter* isolates*, by E-test.

Isolate	MIC (mM)							
	Cd	Cu	Mo	Pb	Ag	As	Hg	Zn
Camp. jejuni S1	0.1	100	R**	R	1	1	1	R
Camp. jejuni W1	0.1	100	R	R	1	1	1	100
Camp. lari S3	1	100	R	R	1	1	10	R
Camp. lari S2	0.1	10	R	100	1	1	10	R
Camp. coli W2	0.01	100	R	10	1	1	1	R
Camp. coli W3	0.1	10	R	R	1	1	1	10

*Incubation at 37°C under microaerophilic conditions for 48 h. Cu, $CuCl_2.2H_2O$; Mo, $Na_2MoO_4.2H_2O$; Zn, $ZnCl_2$; Pb, $Pb(NO_3)_2$, As, $NaAsO_2$; Cd, $CdCl_2$; Hg, $HgCl_2$; Ag, $AgCl_2$.
**R, Resistant

Table 3: MIC values* of detergents against thermophilic *Campylobacter* isolates.

Isolate	MIC (µg/mL)				
	SLS	CET	TRI	T-20	T-80
Camp. jejuni S1	10^3	6.7×10^1	1.06×10^5	1.1×10^5	R*
Camp. jejuni W1	10	6.7×10^2	1.06×10^5	1.1×10^4	R
Camp. lari S3	10^3	6.7×10^2	1.06×10^5	1.1×10^4	R
*Camp. lari S*2	10^4	6.7×10^2	1.06×10^5	1.1×10^4	1.08×10^5
Camp. coli W2	10^3	6.7×10^2	1.06×10^5	1.1×10^3	1.08×10^5
Camp. coli W3	10^3	6.7×10^2	1.06×10^5	1.1×10^3	R

SLS, Sodium lauryl sulfate; T 80, Tween 80; T 20, Tween 20; TRI, Triton X 100; CET, Cetrimide
* R, Resistant

The results obtained from minimal inhibitory concentration of heavy metals against thermophilic *Campylobacter* isolates demonstrated that most of the isolates were relatively sensitive to group II and III metals, while most of them were resistant to group I metals. Although, most of the enteric bacteria viz., *Shigella* spp., *Salmonella* spp., *Yersinia* spp., *Vibrio* spp., and *Escherichia coli* are resistant to cadmium chloride (Kazmi et al. 1985), thermophilic *Campylobacter* isolates in this study were sensitive to cadmium.

Regarding antimicrobial effect of silver on *Campylobacter* isolates, Spacciapoli et al. (2001) have reported that the *Camp. gracilis* and *Camp. rectus* are sensitive to silver nitrate. Parallel with this report our findings indicate that thermophilic *Campylobacter* spp. isolates in this study were sensitive to silver. Therefore, the existence of most heavy metals in the environmental water culminated in significant diminution of campylobacter population. On the other hand, cadmium and silver chloride can be considered as antimicrobial agents against *Campylobacter* and may possess advantages over traditional disinfectants and antibiotics.

The results obtained from minimal inhibitory concentration of detergents against thermophilic campylobacters indicated that, out of all the detergents, cationic agent viz., cetrimide showed more effect against *Campylobacter* isolates while, noionic agents such Triton X 100 and Tween 80 showed low effectiveness. Although, Scott & Bloomfield (1993) reported that detergents and water alone were ineffective in removing contamination, Dawkins et al. (1984) reported that detergents and hot water could remove *Campylobacter* from surface areas. Our observation illustrated that detergents with high concentrations had antimicrobial effect against thermophilic *Campylobacter* isolates.

In different aspects, several papers have been published regarding special techniques and media for isolation of campylobacters (Baserisalehi et al. 2005), whereas, none of them considered suitable sample and sampling sites for isolation of campylobacters from environment. It means, in order to achieve maximum isolation rate of campylobacters from environment and to avoid doubt concerning to frequency of occurrence of these bacteria in the environment, type of samples and sites of collection must be considered as critical factors. It is because flow of industrial sewage including heavy metals and detergents into the environment could affect population of campylobacters.

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