Assessment of Antibiotics Effect on Planktonic and Biofilm Forms of *Campylobacter* Isolates

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ABSTRACT

Campylobacter spp. bacteria are one of the leading causes of food borne illness worldwide and has the ability to form biofilms. These biofilms have been reported to confer resistance against antibiotics. In this study, the effect of five different antibiotics has been compared on the planktonic and biofilm forms of *Campylobacter* isolates by determining their minimum inhibitory concentration (MICs). A total of 55 isolates (11 *Campylobacter jejuni* and 41 *Campylobacter coli*) were subjected to *fla*A typing and cluster analysis. On the basis of *fla*A typing, 23 isolates, comprising 17 *C. coli* and 6 *C. jejuni* representing each cluster were chosen and analyzed for their biofilm forming potential at two different temperatures (37°C and 42°C) under both aerobic and microaerobic conditions. The biofilm production was higher at 37°C in comparison to 42°C, and it was enhanced under aerobic conditions compared to microaerobic conditions at both temperatures. MICs of gentamicin, kanamycin, tetracycline, erythromycin and carbenicillin were determined for both planktonic and biofilm forms of campylobacter bacteria grown in 96 well microtitre plates containing Muller Hinton (MH) broth. All the isolates in the planktonic form showed absolute resistance against carbenicillin. The MIC values of gentamicin, kanamycin, tetracycline and erythromycin for planktonic form were found to be 0.032 µg/ml, 2 µg/ml, 0.1 µg/ml and 0.0125 µg/ml, respectively; while, for biofilm forms the same were found to be 1.025 µg/ml, 8 µg/ml, 0.8 µg/ml and 0.2 µg/ml, respectively. The findings revealed 32, 16, 8 and 4 fold higher resistance by biofilm associated campylobacter bacteria against gentamicin, erythromycin, tetracycline and kanamycin, respectively.

Keywords: *Campylobacter jejuni*; *Campylobacter coli*; *fla*A Typing; Biofilm; MIC; Antibiotic Resistance.

INTRODUCTION

Campylobacter spp. bacteria are among the leading cause of food borne illness worldwide (1). They have the ability to form biofilms not only in the intestinal tract of their hosts but also on industrial surfaces which provide them a favourable niche. These biofilms have also been reported to offer resistance against antibiotics and disinfectants (2). The two predominant causative agents of *Campylobacter* related in-

fections are *Campylobacter jejuni* and *Campylobacter coli* (3, 4), which reside asymptomatically as commensals in many animal species. Consumption of undercooked contaminated poultry or cross-contamination of other food products with raw poultry products is acknowledged to be a major source of infection. In spite of its fastidious laboratory growth requirements, *Campylobacter* acquire mechanisms allowing it to survive a range of *in vivo* and *ex vivo* conditions, one such mechanism being the ability to form biofilms.

Biofilms consist of growing microorganisms intimately associated with each other, producing an extracellular polymeric substance (ESP) of carbohydrate or exopolysaccharide adhering to synthetic or biological surfaces (5). Studies have shown that *Campylobacter* can survive in unfavorable conditions such as low level of nutrient and increased oxygenation when integrated with biofilms (6). Biofilms may provide the perfect niche for campylobacter survival as there are microenvironments in the biofilm that provide ideal conditions for its growth and maintenance (7). There exists a state of continuous attachment and detachment of bacteria from the matrix, making biofilms an enduring source of bacterial discharge. The presence of biofilms in water distribution system is thought to be a possible source for colonization of the bacteria in the poultry flocks (8). Grant *et al.* (9) demonstrated *ex vivo* adhesion of abundant campylobacter to the mucus overlying the human gut tissue. It has been suggested that different strains of *Campylobacter* differ in their survival in nutrient poor environments (10), ability of biofilm formation and structural and dynamic properties of biofilm (11).

The most important property of the biofilm forms in clinical medicine is the enhanced resistance to antimicrobial agents, through protection by the ESP (12), leading to multidrug resistance and therapeutic failure. Although the mechanisms are poorly understood, there is evidence that they may be related to the slow diffusion of drug and the modified nutrient environments leading to suppression of growth rate within the biofilm.

In view of the dearth of information on epidemiology of *Campylobacter* in relation to its survival in the environment, the present study was undertaken with the aim to study the biofilm forming ability among different strains of campylobacter and to compare the antimicrobial susceptibility of planktonic versus biofilm attached bacteria in terms of minimum inhibitory concentration (MIC) against a range of antibiotics.

MATERIALS AND METHODS

A total of 55 *Campylobacter* isolates comprising of 11 *C. jejuni* and 44 *C. coli* isolated from poultry caeca were used in this study. The isolates were maintained in 15% glycerol broth and stored at -80˚C. Bacteria were grown on Mueller Hinton agar for 48 h at 42˚C under microaerophilic conditions (BD GasPak™EZ, USA). The genomic DNA of the isolates was extracted using DNeasy Blood and Tissue Kit (Qiagen, USA) following the manufacturer's protocol. The *fla*A gene was amplified as per the protocol of CAMPYNET (13). The PCR was performed in a total volume of 50 µl, containing 4 µl of DNA, 5 µl of 10X PCR buffer, 20 ρmol of each forward and reverse primers, 5 µl of dNTP (2 mM), and 1 U of Dream Taq polymerase (ThermoFischer) and nuclease-free water to make up the final volume. Amplification was obtained with 35 cycles following an initial denaturation step at 94˚C for 5 min. Each cycle involved denaturation at 94˚C for 45 s, annealing at 45˚C for 45 s and extension at 72˚C for 105 s followed by a final extension step at 72˚C for 5 min. After amplification, 5 µl of the PCR product was electrophoresed with 2% agarose (SRL, India) in 0.5X Tris-borate-EDTA (TBE) buffer containing 0.5 µg of ethidium bromide/ml to determine the presence or absence of the *fla*A amplicon.

A 10 µl sample of the PCR product was digested with 0.2 µl (2 U) of the restriction enzyme *Hpy*F3I (*Dde*I) and using 2 µl of 10X buffer, nuclease free water was used to make up the volume up to 30 µl. The reaction tubes were incubated at 37˚C for 4 h and stored at 4˚C till further examination. A 100 bp plus DNA ladder (Fermentas, USA) was used as the standard for molecular size determinations. Gels were run at 75 V for 90 min in 0.5X TBE buffer. The DNA bands were visualized and documented using the gel documentation system (UVP, USA)

Dendrograms were constructed on the basis of obtained RFLP patterns using GelCompar II (BioNumerics, Belgium) by the unweighted pair group mathematic average method (UPGMA). Similarities between the profiles based on the band patterns were derived using the Dice's coefficient (14).

The isolates to be assayed for biofilm forming ability were chosen after subjecting the isolates to *fla*A typing. One isolate was chosen from each cluster, formed at 70% similarity level. The protocol for bioassay formation was followed as per Merritt *et al.* (15) with slight modifications. Selected *Campylobacter* isolates were grown overnight in Mueller

Hinton broth at 42°C under microaerophilic conditions. The wells of 96 well microtitre plates (Nunc, Denmark) were inoculated with 200 µl of 48 h old cultures of *C. jejuni* and *C. coli* isolates with turbidity adjusted to 3 McFarland standard (~9 x 10^8 CFU) and sterile broth as negative control. Plates were incubated at 42°C and 37°C under microaerophilic as well as aerobic conditions for 48 h. Following incubation, the supernatant was removed and wells were dried for 30 min at 55°C. To the wells 125 µl 0.1% aqueous crystal violet (CV) was added and incubated for 10 min at room temperature. The unbound CV was removed, and the wells were washed twice with sterile distilled water. The wells were air dried and bound CV was extracted into 200 µl of 95% ethanol. One hundred twenty five microliters of this solution was removed from each well and placed in a new 96 well plate. The optical density was observed at 620 nm (OD_{620}) using a microplate reader to determine biofilm formation. By taking 42°C and microaerophilic conditions as standard, strains were classified into three categories: weak, moderate and strong biofilm producers as per Stepanovic *et al.* (16) as described in Table 1. Based on these criteria, optical density cut-off value (OD^c) is the average OD of negative control + 3 × SD (standard deviation) of negative control. Experiments were performed in triplicate.

Statistical analysis

The effect of each variable (species and atmosphere) was assessed using the student's t-test. Statistical significance was defined at $p<0.05$.

Table 1: Classification of isolates as per biofilm forming ability

Categories	Cut off level	*C. jejuni*	*C. coli*
Negative	OD ≤ OD^C	-	-
Weak	OD ≤ 2 X OD^C	1	1
Moderate	2 X OD^C ≤ OD ≥ 4X OD^C	5	14
Strong	OD ≥4X OD^C	-	2

OD^c = 3 SD above OD of Negative control; OD: Optical Density

Minimum inhibitory concentration

The minimal inhibitory concentrations (MICs) of gentamicin (32.8 µg/ml), kanamycin (128 µg/ml), tetracycline (25.6 µg/ml), erythromycin (25.6 µg/ml) and carbenicillin (408 µg/ml) for planktonic forms of *C. jejuni* and *C. coli* were determined using the broth microdilution assay. Two fold serial dilutions of antibiotics were prepared in Mueller Hinton (MH) broth. The wells of microtitre plates, each containing 100 µl of serially diluted antibiotics, were inoculated with 100 µl of overnight grown *Campylobacter* culture with an OD_{620} value of 0.046 and incubated at 42°C for 4 days. The growth was estimated by recording the OD_{620} at the end of 4 days incubation period. The MIC was the lowest concentration of antibiotic, for which no significant increase in OD_{620} was noted (17, 18).

The MICs of antibiotics for biofilm forms were determined against the aforementioned antibiotics using microtitre plate containing a 48 h preformed biofilm in each well. After aspiration of the culture supernatants, 200 µl of fresh MH broth containing two fold serially diluted antibiotics were added to each well and the plate was incubated for further 48 h at 42°C. The biofilm was then suspended by pipetting and growth was estimated by recording the OD_{620} at the end of the 48 h incubation period. The MIC was considered as the lowest concentration at which, no measurable growth was observed. All results were read as per "The Clinical & Laboratory Standards Institute" (CLSI), 2010 guidelines (19)

Scanning electron microscopy

The morphology of the *Campylobacter* biofilm was studied using scanning electron microscopy. The study was carried out using *C. jejuni* isolate C19 of poultry caecal origin. Biofilm for microscopy were cultured at 37°C under aerobic conditions. However, in this case, sterile coupons (3 mm diameter), cut out of polystyrene Petri dish were used as the surface for biofilm formation. Once the biofilm had been cultured, the coupons were removed from the growth media, washed and immediately fixed in 2.5% glutaraldehyde in phosphate buffer saline (pH 7.0) for 48 h at 4°C. The sample was dehydrated in a series (30%, 50%, 80%, 90% and 100%) of ethanol solutions. The samples were critical point dried with liquid carbon dioxide and sputter coated with a thin layer of gold using gold coater (JFC, 1600). Scanning electron microscope (Jeol, Japan) was used at high vacuum in the secondary electron imaging mode to observe the biofilms at 1600X, 3300X, 3500X and 4300X magnifications. Representative views of the biofilms were digitally imaged.

RESULTS

Out of the 55 isolates subjected to PCR assay for amplification of *fla*A gene of *Campylobacter* only 52 isolates (94.5%) produced a ~1725 bp amplicon. Appropriate amplification

was not observed for 3 *C. coli* isolates, which were considered as untypeable and were not included for further studies. The amplified *fla*A products of both *C. jejuni* and *C. coli* were subjected to restriction digestion with *Hpy*F3I (an isoschizomer of *Dde*I) restriction enzyme and banding patterns visualized on 2% agarose gel (Fig. 1). Out of 11 *C. jejuni* subjected to *fla*A typing, 9 *fla*A types (1-9) were found on the basis of number of bands obtained and their molecular weight (Fig. 2). Among 41 *C. coli* isolates, a total of 29 *fla*A types were observed (Fig. 3).

Selection of isolates for further study was done on the basis of percent similarity by *fla*A typing. A cut off level of 70% was set and one isolate from each cluster was selected. Those isolates which were not found to form any cluster were selected individually. Accordingly, 6 *C. jejuni* and 19 *C. coli* isolates were selected for further studies. Out of 19 *C. coli*, 2 isolates did not survive during the subculture, and only 17 *C. coli* were further characterized. Overall, a diverse profile was observed for *C. jejuni* isolates from similar and different geographical regions except for *fla*A types 5 and 6 (Fig. 3). Among *C. coli,* 5 *fla*A types (18, 20, 21, 24 and 28) revealed similar *fla*A genetic profiles irrespective of their location of isolation. However, 5 *fla*A types (9, 11, 13, 14 and 22) revealed similar genetic profiles of isolates from the same location of isolation. *C. jejuni* isolates obtained were genetically more diverse compared to that of *C. coli* based on the number of *fla* types obtained.

The biofilm forming ability of the isolates were evaluated under aerobic and microaerophilic conditions at two different temperatures i.e., 37°C and 42°C. The 6 *C. jejuni* isolates under study revealed significant influence of aerobic and microaerobic conditions on biofilm formation with all the 6 isolates showing higher O.D. under aerobic conditions. The two incubation temperatures 37°C and 42°C reflected minor variation and did not show any statistically significant effect ($p<0.05$) within the similar atmospheric conditions for *C. jejuni* (Fig. 4). Amongst the 17 *C. coli* isolates, a significant effect was observed for different strains of *C. coli* with regards to both atmospheric conditions as well as the temperatures of incubation. Among the isolates tested, 13 showed higher O.D. values at 37°C under aerobic conditions, while only 4 isolates showed highest O.D. values at 37°C under microaerobic conditions. It was observed that biofilm formation was higher under aerobic conditions as compared to microaerobic conditions and at 37°C as compared to 42°C by most of the isolates ($p>0.05$) (Fig. 5).

Out of 6 *C. jejuni*, 5 isolates (N1, A1, C19, PNC77 and C40) were found to be moderate biofilm producers and only 1 isolate (C7) was classified as weak biofilm producer. Out of 17 *C. coli* isolates, 14 (PNC49, PNC52, A5, B10, N8, B50, 207, A2, D12, E1, C35, C32, C37 and PNC50) were found to be moderate biofilm producers, 2 (C27, and B29) strong biofilm producers and only one (D7) weak biofilm producer (Table 1).

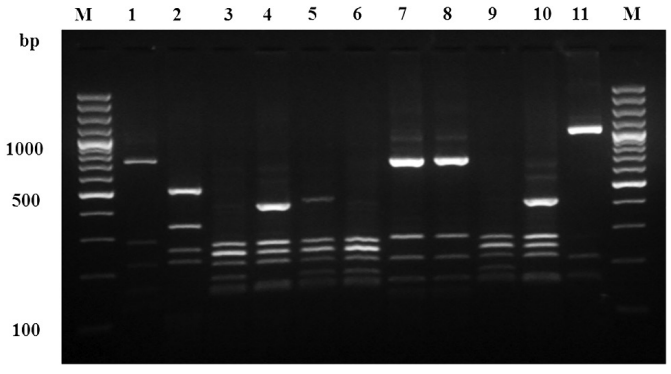

Figure 1: Agarose gel showing *fla* PCR-RFLP profiles of *Campylobacter* spp. using restriction enzyme *Dde*I

Lane M: 100 bp ladder; Lane 1: PNC52; Lane 2: PNC49; Lane 3: B31; Lane 4: C38; Lane 5: A2; Lane 6: B44; Lane 7: B45; Lane 8: B47; Lane 9: B43; Lane 10: C34; Lane 11: PNC50

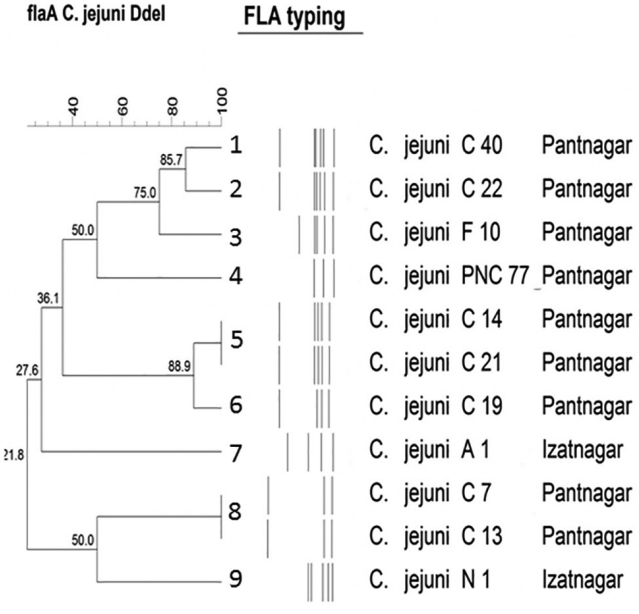

Figure 2: Dendrogram showing *fla*A PCR-RFLP profiles of *C. jejuni* using *Dde*I

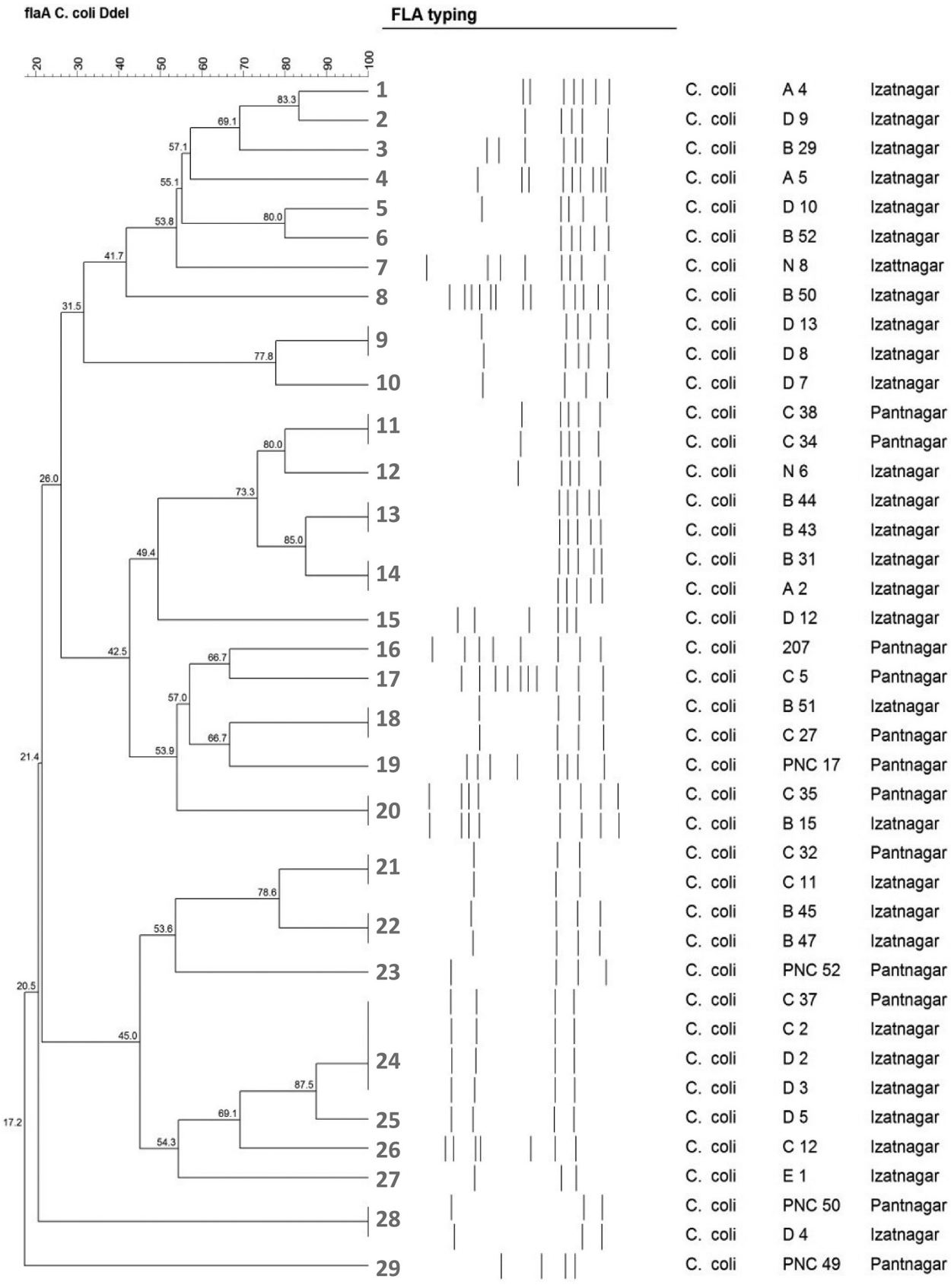

Figure 3: Dendrogram showing *fla*A PCR-RFLP profiles of *C. coli* using *Dde*I

Table 2: MIC values for different antibiotics on planktonic and biofilm bacteria.

Sr. No.	Isolate	Species	Gentamicin		Kanamycin		Tetracycline		Erythromycin	
			P	B	P	B	P	B	P	B
1	N1	*C. jejuni*	0.513	4.1^{C}	4	8^{A}	0.1	1.6^{D}	0.025	0.2^{C}
2	A1	*C. jejuni*	1.025	1.025	4	8^{A}	0.2	3.2^{D}	0.025	1.6^{F}
3	C19	*C. jejuni*	0.513	2.05^{B}	4	16^{A}	0.2	1.6^{C}	0.05	0.2^{B}
4	C7	*C. jejuni*	0.513	4.1^{C}	8	16^{A}	0.2	3.2^{D}	0.05	0.2^{B}
5	PNC77	*C. jejuni*	1.025	2.05^{A}	4	8^{A}	0.4	1.6^{B}	0.0125	0.2^{D}
6	C40	*C. jejuni*	1.025	2.05^{A}	4	8^{A}	0.4	0.8^{A}	0.1	1.6^{D}
7	C27	*C. coli*	1.025	2.05^{A}	8	16^{A}	0.2	1.6^{C}	0.1	0.8^{C}
8	PNC49	*C. coli*	1.025	4.1^{B}	4	8^{A}	0.2	3.2^{D}	0.2	0.4^{A}
9	PNC52	*C. coli*	0.257	1.025^{B}	8	16^{A}	0.1	0.8^{C}	0.1	0.4^{B}
10	B29	*C. coli*	1.025	2.05^{A}	2	8^{B}	1.6	3.2^{A}	0.0125	0.8^{F}
11	A5	*C. coli*	1.025	4.1^{B}	8	16^{A}	0.1	0.8^{C}	0.2	1.6^{C}
12	D10	*C. coli*	0.513	1.025^{A}	8	16^{A}	0.1	0.8^{C}	0.1	0.2^{A}
13	N8	*C. coli*	0.032	2.05^{F}	4	8^{A}	0.1	0.8^{C}	0.05	0.2^{B}
14	B50	*C. coli*	1.025	2.05^{A}	8	32^{B}	0.8	1.6^{A}	0.0125	0.2^{D}
15	207	*C. coli*	1.025	4.1^{B}	4	16^{B}	0.2	3.2^{D}	0.1	1.6^{D}
16	A2	*C. coli*	1.025	4.1^{B}	4	8^{A}	0.1	0.8^{C}	0.1	1.6^{D}
17	D12	*C. coli*	0.257	2.05^{C}	8	16^{A}	0.2	0.8^{B}	0.05	1.6^{E}
18	D7	*C. coli*	0.513	2.05^{B}	8	32^{B}	0.1	0.8^{C}	0.05	0.4^{C}
19	E1	*C. coli*	0.257	1.025^{B}	4	8^{A}	0.8	1.6^{A}	0.0125	0.2^{D}
20	C35	*C. coli*	0.257	2.05^{C}	8	16^{A}	0.1	0.8^{C}	0.0125	1.6^{G}
21	C32	*C. coli*	1.025	2.05^{A}	4	16^{B}	0.4	1.6^{B}	0.1	0.4^{B}
22	C37	*C. coli*	0.513	2.05^{B}	8	16^{A}	0.2	1.6^{C}	0.1	1.6^{D}
23	PNC50	*C. coli*	1.025	2.05^{A}	4	8^{A}	0.4	1.6^{B}	0.1	0.8^{C}

P: Planktonic cell; B: Biofilm; Superscript denotes increase in concentration:
A (2-fold), B (4-fold), C (8 fold), D (16 fold), E (32 fold), F (64 fold), G (128 fold)

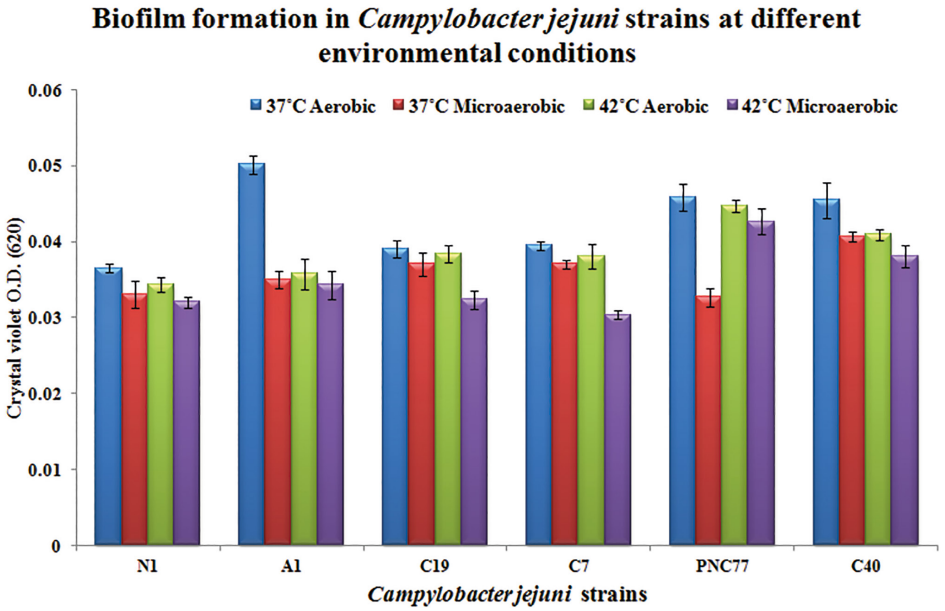

Figure 4: Graph showing biofilm formation by *Campylobacter jejuni* isolates
C. jejuni biofilm formation under aerobic/microaerophilic conditions at either 37°C/42°C.
Error bars represent one standard deviation from the mean.

Table 3: Number of times of occurrence of MIC values (µg/ml)

Conc. (µg/ml)	Gentamicin		Kanamycin		Tetracycline		Erythromycin	
	P	B	P	B	P	B	P	B
0.0125							5	
0.025							2	
0.032	1							
0.05							4	
0.1					8		9	
0.2					8		2	8
0.257	4							
0.4					4			4
0.513	6							
0.8					2	9		3
1.025	12	4						
1.6					1	9		8
2.0			1					
2.05		13						
3.2						5		
4.0			12					
4.1		6						
8.0			10	10				
16.0				11				
32.0				12				

P: Planktonic cell; B: Biofilm; R: Resistant

MICs of gentamicin, kanamycin, tetracycline, erythromycin and carbenicillin on planktonic *C. jejuni* and *C. coli* were determined. MICs of gentamicin, kanamycin, tetracycline and erythromycin for planktonic *Campylobacter* were found to be 0.032 µg/ml, 2 µg/ml, 0.1 µg/ml and 0.0125 µg/ml, respectively. However, all the isolates showed resistance against carbenicillin even at the highest concentration. The number of isolates showing particular MIC values are presented in table 3. All the isolates were found sensitive against gentamicin, kanamycin, erythromycin and tetracycline while all were resistant to carbenicillin (CLSI, 2010) (Table 2).

The MICs of the listed antibiotics were determined for *Campylobacter* biofilms using microtitre plate containing a 48 h preformed biofilm in each well. The different antibiotics showed significant effect on biofilm forms. The results are presented in table 2. MICs of gentamicin, kanamycin, tetracycline and erythromycin for *Campylobacter* in biofilm were found to be 1.025 µg/ml, 8 µg/ml, 0.8 µg/ml and 0.2 µg/ml, respectively. MIC for carbenicillin was not recorded, as all the isolates showed resistance for the highest concentration. The distribution of MIC pattern is presented in table 3.

Scanning electron microscopy

Scanning Electron Microscopy was undertaken to examine the biofilm microstructure. The *C. jejuni* isolate C19 produced

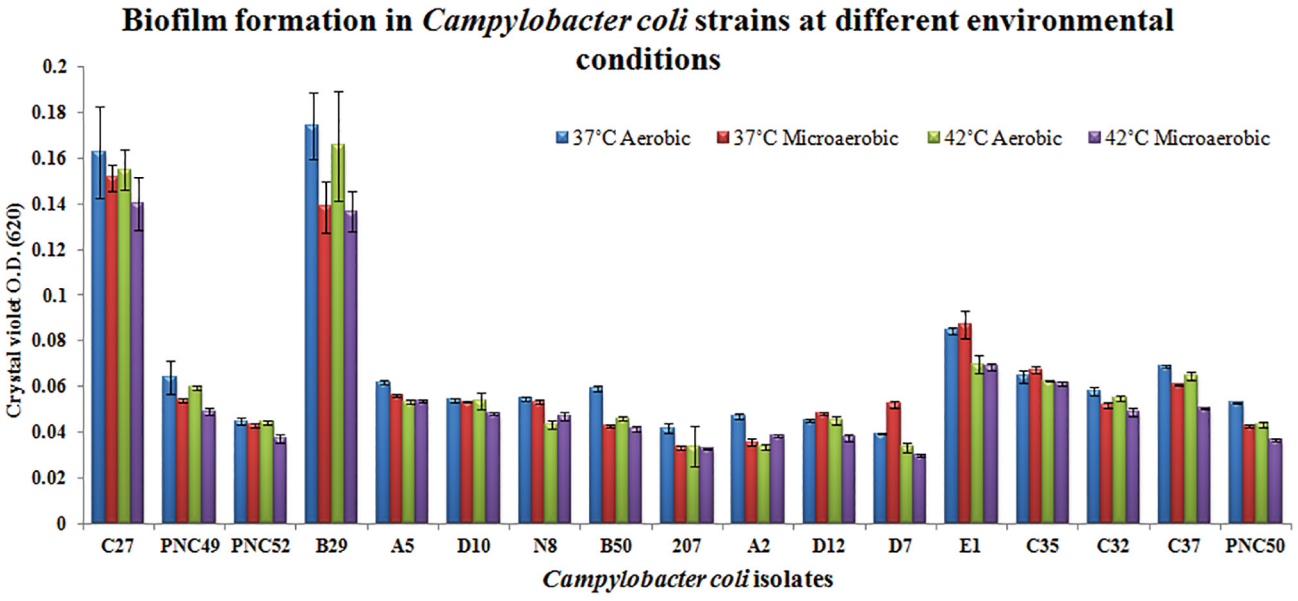

Figure 5: Graph showing biofilm formation by *Campylobacter coli* isolates

C. coli biofilm formation under aerobic/microaerophilic conditions at either 37°C/42°C. Error bars represent one standard deviation from the mean.

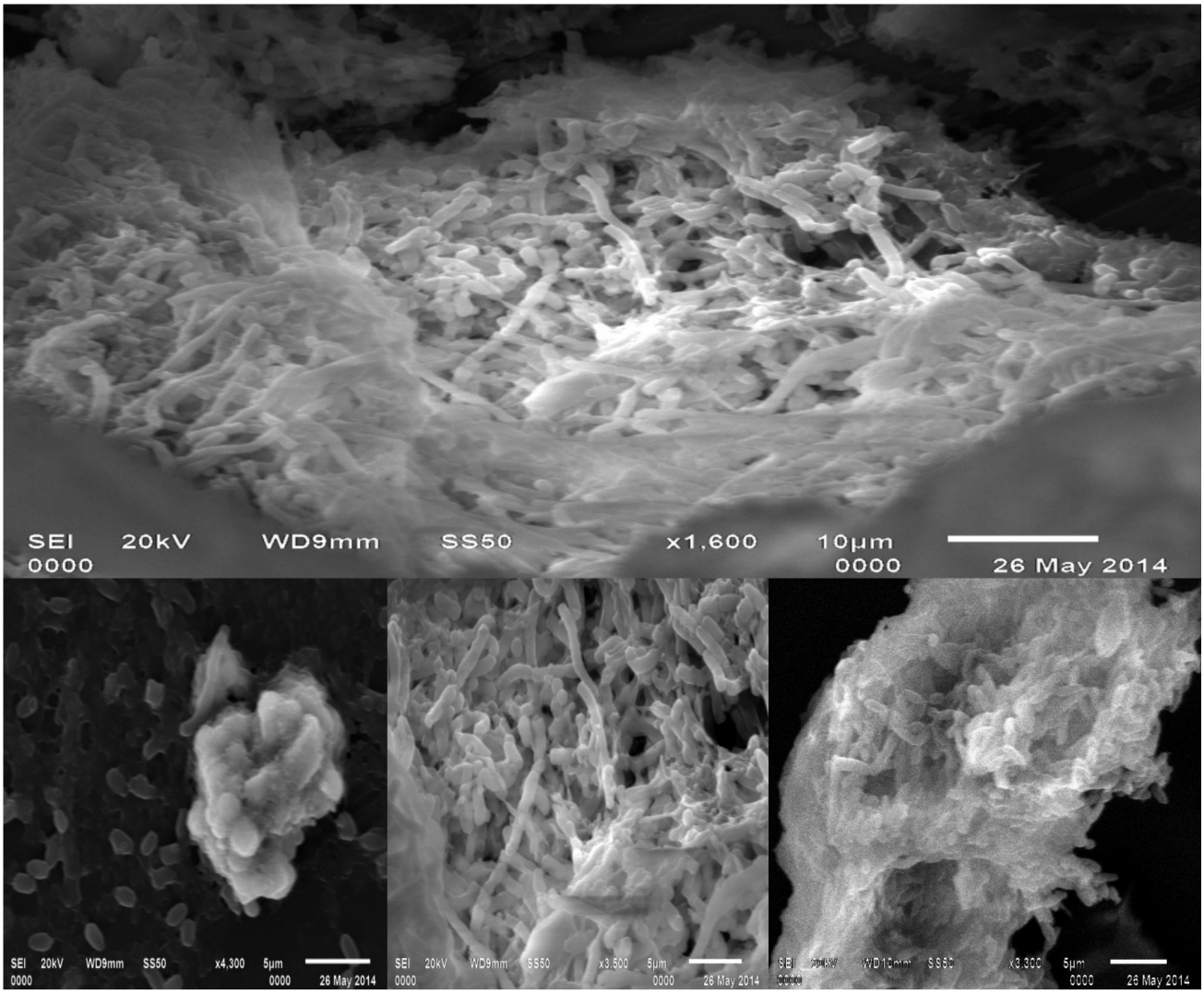

Figure 6: Scanning electron micrograph of *Campylobacter jejuni* biofilm

a mature biofilm in which a large number of cell clusters were entwined to form a dense net and the organism was found to be embedded in extensive matrix. Clusters were found enclosing abundant numbers of bacterial cells intimately associated with a fibrous cord like material. Morphology of the bacteria comprising the biofilm was relatively homogeneous, with the bacteria present as spiral or curved rod forms (Fig. 6).

DISCUSSION

The *fla*A-RFLP typing revealed 94.5% typeability as reported by Harrington *et al.* (20) who found 93% typeability of PCR-RFLP using *Dde*I enzyme. The *C. jejuni* isolates obtained were genetically more diverse compared to that of *C. coli* based on the number of *fla*A types obtained, as reported by Khoshbakht *et al.* (21) and Rajagunalan *et al.* (22). Overall, *fla*A typing was able to discriminate *C. jejuni* and *C. coli* isolates from similar as well as different geographical regions and sources. *Dde*I proved to be useful for the initial grouping of the strains (23).

It has been reported that under stressful conditions *Campylobacter* spp. undergo biofilm formation to remain viable in the environment and serve as a continuous source of contamination to the flocks in poultry houses as well as to the food in processing plants (24). The production of biofilms by most of the isolates was seen at 37°C under aerobic conditions followed by 42°C under aerobic, 37°C under microaerobic and at 42°C under aerobic conditions. These findings in this study were in accordance with Sulaeman *et al.* (25) and Reuter *et al.* (26) who reported that oxygen rich

conditions promote the biofilm formation by microaerophilic campylobacter bacteria. But on the contrary, Reeser *et al*. (27) has reported a higher biofilm production by campylobacter bacteria under microaerophilic conditions.

According to Stepanovic *et al*. (16) classification of biofilm producing ability of microorganisms, most of the isolates of *Campylobacter* were found to produce moderate biofilms with a few isolates showing strong and weak biofilm production. Out of 6 *C. jejuni* isolates, 5 (83.3%) were classified as moderate biofilm producers and the remaining isolate were weak biofilm producers. However, out of 17 *C. coli* isolates, 14 (82.3%) were moderate, 2 (11.7%) were strong and one (5.9%) isolate was a weak biofilm producer. Comparable findings were reported by Teh *et al.* (28), who reported that most *Campylobacter* spp. produce moderate biofilm with a few strains showing strong biofilm producing ability. Biofilm forming abilities of different bacterial strains have been reported to exhibit considerable difference when incubated at same temperature and atmospheric conditions (29, 30).

Bacteria in a biofilm are relatively resistant to antimicrobial agents and host immune response (31). Sessile bacteria have proved to be less susceptible to antimicrobial agents as compared to their non-attached planktonic counterparts (32). In the present study, bacteria in biofilm were found to be several fold more resistant to antibiotics than planktonic bacterial cells. MIC of gentamicin for biofilm was 2-4 fold higher than MIC for planktonic cells. Sepandj *et al*. (33) observed only 12.5% sensitivity of gentamicin for biofilm associated bacteria. Low penetrating ability of gentamicin for biofilm may be a reason for increased MIC values. Gentamicin has been shown to have less penetrating ability in biofilm since less than 25% of this antibiotic was found in biofilm after 24 h of incubation (34).

In case of kanamycin, a four-fold increase in MIC was observed for the biofilm forms. Although kanamycin is known to rapidly kill growing cells, sessile cells in biofilm are perhaps the reason for its resistance (35). MIC of tetracycline was found to increase by 8 fold in biofilm associated bacteria, tetracycline is found to show good penetration for biofilm (36) and possibly there are other unknown reasons for its resistance. The MIC value for biofilm associated bacteria for erythromycin, considered as drug of choice against campylobacter bacteria, increased by 16 folds. In our study, planktonic as well as biofilm bacteria were found to have absolute resistance against carbenicillin. Findings of Spoering and Lewis (37) are in agreement with the results of this study, showing 100% resistance of biofilm bacteria for carbenicillin. Production of β-lactamases by bacteria or presence of slow growing bacteria in biofilm may possibly be the one of the reasons for its tolerance.

Chen *et al*. (38) has reported several fold increase in MIC value of biofilm compared to planktonic bacteria. Comparable results were presented by Grenier *et al*. (17) who have reported an increase in MIC of various antibiotics by 32 to 256 fold. Sepandj *et al*. (33) stated that the gram negative bacteria in biofilm state are much less susceptible to the antibiotics than their planktonic counterparts.

Scanning electron microscopy was performed to understand the morphology of biofilm grown on polystyrene coupons. *Campylobacter* spp. has been reported to develop biofilm on various surfaces *viz*. glass fibre (39), glass coverslips (40), plastic coupons (41) and microtitre plates (27). Reeser *et al*. (27) has reported a higher degree of biofilm formation on polystyrene surface. Similar coupons used in the present study gave comparable results. Biofilm was observed with large number of cell clusters intertwined to form a thick mesh like structure. In an earlier study, similar results for campylobacter biofilm formation were reported by Joshua *et al*. (11). Bacteria comprising the biofilm were spiral or curved, comparable to the results of Svensson *et al*. (42), indicating the preservation of normal morphological features in biofilms during the normal incubation period of 48 hours. On the contrary, Gunther and Chen (30) reported both spiral and coccoid forms of the bacteria in roughly similar proportions. The variation in findings may be due to difference in the incubation time and other related parameters.

This study concluded that under aerobic or stressful conditions, *Campylobacter* spp. adapted to a biofilm lifestyle, allowing survival under detrimental conditions. The biofilm leads to increased resistance for antibiotics, and can function as a reservoir of viable planktonic cells in environment. The increased level of biofilm formation under aerobic conditions is likely to be an adaptation contributing to the infectiious existence of *Campylobacter* species in the environment.

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