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Biofilm, Surface Hydrophobicity, Siderophore and Haemolytic Characterization of *Campylobacter jejuni* Isolated from Raw Duck Meat

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Abstract: In our earlier study we have reported the prevalence, antibiotic resistance and molecular characterization of *Campylobacter jejuni* (*C. jejuni*) isolated from raw duck meat. The present study aimed to assess biofilm production, cell surface hydrophobicity, siderophore production and haemolytic activity of forty *C. jejuni* strains. All 40 *C. jejuni* strains produced biofilm in Congo red agar method. Among them 10 (25%) strains exhibited strong biofilm production, 12 (30%) strains showed moderate biofilm production and 18 (45%) strains showed weak biofilm production while in test tube method, number of strains which scored 1 were 18 (45%), strains which scored 2 were 12 (30%) and strains which scored 3 were 10 (25%). Among the 40 *C. jejuni* strains, 10 (25%) strains showed cell surface hydrophobicity values above 80%, 12 (30%) strains exhibited cell surface hydrophobicity values ranging from 60-78% and 18 (45%) strains showed cell surface hydrophobicity values which ranged from 35-58%. In the siderophore study among 40 strains, sixteen strains produced (40%) siderophore and twenty four of strains (60%) were not able to produce siderophore. In the hemolytic activity among the 40 strains, fourteen strains (35%) showed strong positive beta haemolytic activity, ten strains (25%) showed moderate positive gamma haemolytic activity and sixteen (40%) strains showed weak positive alpha haemolytic.

Key words: Biofilm · Siderophore Production · Cell Surface Hydrophobicity · Hemolytic Activity · *Campylobacter jejuni*

INTRODUCTION

C. jejuni are gram-negative, thermophilic, obligate microaerophilic bacteria that are ubiquitous in temperate environments and colonize the intestinal mucosa of most warm-blooded hosts, including all food-producing animals and humans [1, 2]. The major gastrointestinal pathogen *C. jejuni* was shown to exist as three forms of mono species based on biofilm in liquid culture [3]. Biofilm is an assemblage of microbial cells that are associated with a surface and enclosed in a matrix of primarily polysaccharide materials and may contain non-cellular materials which are incorporated into the biofilms from the surrounding environment in which the biofilms are formed [4]. Biofilms formed by the human pathogen *C. jejuni* may arise in the gastrointestinal tract of animals, in water pipes and other industrial situations, leading to their possible transmission into the human food chain either directly or via farm animals [5, 6]. Biofilm adhere to surface in aqueous environment and begin to excrete a slimy, glucose like substance that can anchor them to all kinds of materials such as metals, plastics, soils particles, medical implant materials and tissues [7]. Once anchored to the surface, biofilm microorganism carry out a variety of beneficial reactions (by between standards) depending on the surrounding environmental conditions [8]. *C. jejuni* has been found in preformed biofilms of other bacterial species, postulated that *C. jejuni* in autochthonous biofilms had enhanced survival adherent to stainless steel coupons [9]. However, the biofilm formation by *C. jejuni* has not hitherto been properly demonstrated [10]. The selected *C. jejuni* mutants were tested for their ability to form biofilms to identify likely surface determinants that may play a role in biofilm formation [11]. It plays a critical

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role in the adherence of bacteria to the wide variety of surfaces [12]. Cell surface hydrophobicity (CSH) is a complex interaction between component of the surface of the bacteria and the surrounding environment [13]. It is also a significant determinant of adhesion and biofilm formation on polystyrene surfaces. *C. jejuni* is able to colonize the human intestinal mucosa and cause disease. For this reason, it is important to investigate mechanisms by which it adheres to epithelial cell and intestinal mucus cell [14]. Iron is essential for the growth of nearly all bacteria, but its availability is complicated by its extreme insolubility at neutral and alkaline pH. To acquire the necessary iron, aerobic microorganisms produce high-affinity compounds which bind and solubilize iron and transport it across the cell membrane. The iron-binding compounds are collectively termed siderophores. These are important because the growth of pathogenic bacteria *in vivo* depends upon their capacity to obtain the iron firmly held by substances such as transferrin and lactoferrin [15]. These abilities to produce siderophores and acquire iron permit bacterial multiplication in animals and may thus be regarded as a virulence factor [16]. *C. jejuni* has not been considered to be haemolytic on blood agar. However some *C.* spp. has been reported to produce a haemolysin, which may be related to virulence [17, 18]. *C jejuni* strains may be grouped into three autoagglutination (AAG) phenotypes. A variant derived from strain 81116 that is flagellate but immotile showed the strong AAG exhibited by the parent strain, suggesting that motility is not necessary for the AAG activity. AAG is correlated with both bacterial hydrophobicity and adherence to INT407 cells [19]. In our earlier study we reported the prevalence, antibiotic resistance and molecular characterization of *C. jejuni* isolated from raw duck meat [20]. The present study aimed to assess biofilm production, cell surface hydrophobicity, siderophore production and haemolytic activity of those *C. jejuni* strains.

MATERIALS AND METHODS

Biofilm/ Slime Production Test: Congo Red Agar, a solid medium, was prepared by adding Brain Heart Infusion Agar (BHIA) supplemented with 5% sucrose and Congo red dye [21]. Congo red was prepared as concentrated aqueous solution. Congo red dye solution and 5% sucrose were autoclaved separately and added to the BHIA at 55°C. The strains were streaked to a length of 1.5cm on Congo red Agar plate and incubated at 37°C for 24 hours and subsequently kept at room temperature. Black colonies were considered to be positive variants, while red colonies were considered to be negative.

Tube Method: Biofilm formation in test tube method was done by following Christensen *et al*. [22]. A loop full of test organisms was inoculated in 10 ml of trypticase soy broths with 1% glucose in test tubes. The test tubes were incubated at 37°C for 24 hours. After incubation, the test tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried. Tubes were then stained with crystal violet (0.1%). Excess stain was washed with deionized water. Tubes were dried in inverted position. The scoring for test tubes was done. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as 1-weak/none, 2-moderate and 3-high/strong.

Determination of Cell Surface Hydrophobicity: *C. jejuni* strains were examined for Cell surface hydrophobicity by following methodology of Rosenberg *et al*. [12]. About 1 ml of overnight broth culture was taken into 2ml of micro centrifuge tubes. The tubes were centrifuged at 10,000 rpm for 10 minutes. After centrifugation, the supernatant was discarded and the pellet was collected then washed in sterile phosphate-buffered saline (pH 7.1). The tubes were centrifuged at 10,000 rpm for 10 minutes, the supernatant was discarded and the pellet was suspended in the same buffer to an initial optical density (OD) of about 1.0 (A1) at 600 nm. Then, 300µl of xylene was added to 3ml of microbial suspension and vortexed for 2 minutes. After 10 min the O.D of the aqueous-phase was measured (A2) at 600 nm. The degree of hydrophobicity was calculated as (1-(A1/A0) 100%.

Determination of Siderophore Production: Strains were tested by chromo azural S (CAS) assay by adopting modified methodology of Schwyn and Neilands (1987) [23]. Nutrient agar 10ml was prepared, spread plated and incubated for 48 hours at 30°C. After incubation a thin layer of CAS reagent in 0.7% agar was spread on the bacterial growth and plates were again incubated for 24 hours at 30°C. Formations of yellow orange zone around the colonies indicate siderophore production. The positive indicated by formation of Orange/Yellow colour while negative indicated no colour change.

Determination of Virulence Factors

Assay of Hemolytic Activity: The haemolytic activities of all positive strains were determined on Blood agar assay [24]. The nutrient agar was prepared, sterilized and cooled then 5ml of human blood was mixed and poured into the plates. After solidification loopful of culture from all strains were streaked on the plates and incubated for 24 hours at 37°C. The presence of a clear colorless zone surrounding the colonies as α, β and γ were noted down.

RESULTS

Detection of Biofilm/Slim Production by Congo red Agar Method: In this study, positive 40 strains were subjected to Congo red agar medium. Slime producing strains appeared as rough, dry and black colonies and non-slime producing strains as pinkish red and smooth colonies. All 40 *C jejuni* strains were producing biofilm, among them 10 (25%) strains exhibited black colour that indicated strong positive strains, 12 (30%) strains showed wet, black, non-crystalline which indicated moderate positive colonies. Whereas 18 (45%) strains showed light black colouration which indicated weak positive strains.

Detection of Biofilm/Slim Production by Tube Method: Biofilm formation was confirmed positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as 1 for weak or none, 2 for moderate and 3 for high or strong biofilm producers. Among 40 strains of *C. jejuni*, the numbers of strains scored 1 were 18 (45%), strains scored 2 were 12 (30%) and strains scored 3 were 10 (25%). From the present investigation it was clear that the *C jejuni* strains were responsible for the production of biofilm on the test tubes.

Determination of Cell Surface Hydrophobicity (CSH): Cell surface hydrophobicity was determined based on the difference of the OD (A1, A2) of bacterial culture before and after adsorption using the formula as 1-(A1/A2)100%. Among the 40 positive strains, 10 (25%) strains showed CSH values above 80%, 12 (30%) strains showed CSH values ranging 60-78% and 18 (45%) strains showed CSH values which ranged from 35-58% (Table 1).

Determination of Siderophore Production: The production of siderophore was recognized by the yellow colour pigment produced by the bacteria on the medium. All the 40 positive strains were selected for the siderophore production. Among them sixteen strains produce (40%) siderophore and twenty four of strains (60%) not able to produce siderophore (Table 2).

Table 1: Determination of cell surface hydrophobicity (CSH) producing *Campylobacter jejuni* isolated from the raw duck meat

S.No	Strains Name	O.D values A2	O.D values A1	Percentage of CSH [1-(A1/A2)100%]	S.No	Name of the Strains	O.D values A2	O.D values A1	Percentage of CSH [1-(A1/A2)100%]
1	DF1	1.02	0.61	40.5%	21	DIn1	1.02	0.56	45.0%
2	DF2	1.12	0.24	78.5%	22	DIn2	1.06	0.60	43.3%
3	DF3	1.02	0.62	39.2%	23	DIn3	1.04	0.12	88.5%
4	DF4	1.02	0.30	70.5%	24	DIn4	1.06	0.12	88.6%
5	DF5	1.06	0.60	43.3%	25	DIn5	0.95	0.31	67.3%
6	DS1	1.02	0.32	68.6%	26	DIn6	1.10	0.19	82.8%
7	DS2	1.01	0.46	54.4%	27	DIn7	1.19	0.38	68.1%
8	DLi1	1.03	0.52	49.5%	28	DIn8	1.02	0.17	83.3%
9	DLi2	1.19	0.19	84.0%	29	DFe1	1.20	0.14	88.3%
10	DLi3	1.50	0.32	78.6%	30	DFe2	1.40	0.16	88.5%
11	DA1	1.06	0.12	88.6%	31	DFe3	1.04	0.67	35.5%
12	DA2	0.92	0.42	54.3%	32	DFe4	1.19	0.32	73.1%
13	DA3	1.09	0.36	66.9%	33	DFe5	0.98	0.36	63.3%
14	DA4	1.35	0.19	85.9%	34	DFe6	1.01	0.60	40.5%
15	DA5	1.02	0.17	83.3%	35	DFe7	1.03	0.59	42.7%
16	DBk1	1.07	0.54	49.5%	36	DFd1	1.02	0.46	54.9%
17	DBk2	1.20	0.43	64.2%	37	DFd2	1.18	0.26	77.9%
18	DBk3	1.06	0.30	71.6%	38	DFd3	1.01	0.63	37.6%
19	DN1	1.12	0.69	38.3%	39	DFd4	0.90	0.38	57.7%
20	DN2	1.04	0.58	44.3%	40	DFd5	1.01	0.46	54.4%

Note where: D- Duck, DF- Duck Feather, DS- Duck Skin, DLi- Ducing Liver, DA-Duck Anus, DBk- Duck Beaks, DN- Duck Nail, DIn- Duck Intestine, DFe- Duck Feces, DFd- Duck Feed

Table 2: Determination of Siderophore Producing *Campylobacter jejuni* isolated from the raw duck meat

S. No	Name of the strains	Siderophore Production	S. No	Name of the strains	Siderophore Production
1	DF1	-	21	DIn1	-
2	DF2	+	22	DIn2	-
3	DF3	-	23	DIn3	+
4	DF4	-	24	DIn4	+
5	DF5	-	25	DIn5	-
6	DS1	-	26	DIn6	+
7	DS2	-	27	DIn7	-
8	DLi1	-	28	DIn8	+
9	DLi2	+	29	DFe1	+
10	DLi3	+	30	DFe2	+
11	DA1	+	31	DFe3	31
12	DA2	-	32	DFe4	32
13	DA3	-	33	DFe5	33
14	DA4	+	34	DFe6	34
15	DA5	+	35	DFe7	35
16	DBk1	-	36	DFd1	36
17	DBk2	-	37	DFd2	37
18	DBk3	+	38	DFd3	38
19	DN1	-	39	DFd4	39
20	DN2	-	40	DFd5	40

Note where: D- Duck, DF-Duck Feather, DS-Duck Skin, DLi-Duck Liver, DA-Duck Anus, DBk-Duck Beaks, DN-Duck Nail, DIn-Duck Intestine, DFe-Duck Feces, DFd-Duck Feed

Table 3: Prevalence of haemolytic producing *Campylobacter jejuni* isolated from the duck meat

S.No	Name of the strains	Positive Strains	S.No	Name of the strains	Positive Strains
1	DF1	Alpha haemolytic	21	DIn1	Alpha haemolytic
2	DF2	Alpha haemolytic	22	DIn2	Gamma haemolytic
3	DF3	Alpha haemolytic	23	DIn3	Beta haemolytic
4	DF4	Gamma haemolytic	24	DIn4	Beta haemolytic
5	DF5	Alpha haemolytic	25	DIn5	Gamma haemolytic
6	DS1	Alpha haemolytic	26	DIn6	Beta haemolytic
7	DS2	Alpha haemolytic	27	DIn7	Alpha haemolytic
8	DLi1	Alpha haemolytic	28	DIn8	Beta haemolytic
9	DLi2	Beta haemolytic	29	DFe1	Beta haemolytic
10	DLi3	Beta haemolytic	30	DFe2	Beta haemolytic
11	DA1	Beta haemolytic	31	DFe3	Gamma haemolytic
12	DA2	Gamma haemolytic	32	DFe4	Beta haemolytic
13	DA3	Alpha haemolytic	33	DFe5	Alpha haemolytic
14	DA4	Beta haemolytic	34	DFe6	Gamma haemolytic
15	DA5	Beta haemolytic	35	DFe7	Gamma haemolytic
16	DBk1	Alpha haemolytic	36	DFd1	Gamma haemolytic
17	DBk2	Gamma haemolytic	37	DFd2	Beta haemolytic
18	DBk3	Beta haemolytic	38	DFd3	Alpha haemolytic
19	DN1	Alpha haemolytic	39	DFd4	Alpha haemolytic
20	DN2	Alpha haemolytic	40	DFd5	Gamma haemolytic

Note where: D- Duck, DF- Duck Feather, DS- Duck Skin, DLi- Duck Liver, DA- Duck Anus, DBk-Duck Beaks, DN-Duck Nail, DIn-Duck Intestine, DFe-Duck Feces, DFd-Duck Feed

Determination of Virulence Factors

Haemolytic Assay: The results of Hemolytic activity percentage among the 40 strains, revealed 14 (35%) had strong positive beta hemolytic activity, 10 (25%) had moderate positive gamma hemolytic activity and 16 (40%) had weak positive alpha hemolytic (Table 3).

DISCUSSION

Bacteria in a biofilm are relatively resistant to changes in environmental conditions, to antimicrobial agents and to host immune responses [4]. It is an attractive hypothesis, therefore, that *C. jejuni* cells form a biofilm to

survive adverse conditions in animal hosts. *C. jejuni* has been found to colonize biofilms of autochthonous bacteria [25, 26]. It has been found in preformed biofilms of other bacterial species. Its adherence to stainless steel coupons were assumed to have formed by biofilms [9]. Tube method (TM) and Congo Red Agar method (CRA) methods are recommended as a general screening method for detection of biofilm producing bacteria in laboratories [21, 22]. The results in this study indicated that distributions of 40 *C. jejuni* strains were producing biofilm. In which 10 (25%) strains showed black colouration indicating strong positive, 12 (30%) strains showed wet, black, non-crystalline colouration which indicatesed moderate positive and 18 (45%) strains showed light black colouration which indicated weakly positive when compared with previous literature [27]. Hydrophobicity and surface charge of clinical strains of *C. jejuni* strains were investigated by aqueous two-phase partitioning. There is a good correlation between the different physico-chemical methods reflecting the same bacterial property. Hydrophobic surface adhered better to human intestinal HT-29 cells than strains with less charge and a more hydrophobic surface [28]. The hydrophobicity of the microbial surface plays a critical role in the adherence of bacteria to the wide variety of surfaces [7] which was similar to present study. In this study, Cell Surface hydrophobicity was determined based on the difference of the OD (A1, A2) of bacterial culture before and after adsorption. Among the 40 positive strains, 10 (25%) strains were above 80%, 12 (30%) strains were above 60-78% and 18 (45%) strains were above 35-58% hydrophobicity. Iron is essential for the growth of nearly all bacteria. The availability of iron to potentially pathogenic bacterial strains is restricted by the iron-binding proteins of the host [29]. The 40 strains of *C.* species grown under iron-limiting conditions while the strains produced no detectable siderophores, all the strains freely utilized exogenous siderophores produced by other organisms as iron carriers. These data suggest that the use of an exogenous siderophore (either purified or present in a coinfecting microorganism) may be important in developing a suitable laboratory model for Ciosis [30]. In this present study, among the 40 positive strains were selected for the siderophore production using Chromo azurols plate. Sixteen strains (40%) produced siderophore and the 24 strains (60%) were not able to produce siderophore. There are many virulence genes such as *hip* O gene, *fla* A gene, *cad* F gene, *rac* R gene, *iam*, *vir* B11, *pld* A gene, *cdt* A gene, *cdt* B gene and *cdt* C gene which are responsible for expression of adherence, invasion, colonization and Cytotoxin production in *C. jejuni* [31- 33]. Different studies have produced conflicting results regarding the hemolytic activity and it has not been determined whether the haemolysin is secreted or cell associated. It was found that the alpha-like hemolytic organisms were micro aerobically cultured with high concentrations of CO2 in the gas mixture and beta-like hemolysis was observed after prolonged incubation [34, 35]. In this study, among the 40 positive strains (14)35% of beta-haemolysin was detected, another (16) 40% of alpha-like hemolysis and (10) 25% of gamma-like hemolysis were detected.

CONCLUSION

Human campylobacteriosis is one of main cause of food poisoning in most of the industrialized countries. The present study concluded raw duck meats which were containing *C. jejuni* had ability to produces biofilms, siderophores, shown cell surface hydrophobicity and haemolytic activity. These bacterial factors are important for upregulation and colonization of *C. jejuni.* Hence strategies done to eliminate and/or diminution of *C. jejuni* in the food chain, should be performed keeping all these in mind.

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