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Persistence and Biofilm Assessment of *Campylobacter Jujeni* in Poultry Abattoir

*Balogu[1,2], T.V., Nwaugo[3], V.O. and Onyeagba[3], R.A.

ABSTRACT

Persistence of *Campylobacter* sp and its biofilm forming ability was assessed in two poultry abattoirs at two weeks intervals. Average prevalence (63.75%) of *Campylobacter* spp. was observed on assessing a total of 160 samples collected from the surfaces of packaging table (80%), dressing table (75%), floor source (70%) and washing table (30%). Biofilm assessment formed by *Campylobacter jejuni* within 5-days at 37^0C were in decreasing order of washing table> packaging table> dressing table > floor. An average rate (19.6%) of isolates to develop biofilm observed in both sites was considered relatively low. Absorbance value (Optical Density-OD_{590nm}) of formed biofilms ranged from 0.483 – 0.952. Wastewater from the facilities showed higher TDS (643 – 820 mgl^{-1}), TSS (1200 – 1775 mgl^{-1}), COD (152 – 141 mgl^{-1}) and BOD (30.3 – 32.5mgl^{-1}) than the WHO standards of 500 mgl^{-1}, 100 mgl^{-1}, 10 mgl^{-1} and 6 mgl^{-1} respectively. This is a clear indication of heavy microbial presence in the wastewater. Total bacterial count (TBC) was slightly higher in site A (4.4×10^5 CFU/ml) than site B (3.5×10^5 CFU/ml). Efficiency index ratio (≈/>1) observed in all tested drugs suggests their effectiveness in campylobacteriosis management. Decreasing drug sensitivity pedigree was observed with streptomycin> erythromycin & gentamincin > tetracycline & neomycin > penicillin> riphapicin & ampicillin > norflaxicin & cephalexin. These results of frequency and biofilm forming tendencies of *Campylobacter* spp. observed in this study can be of value in checkmating campybacteriosis outbreak from poultry abattoir facility.

Keywords: *Campylobacter jejuni,* biofilm, poultry, abattoir.

Introduction

Public health concern has been drawn to the sporadic cases of *Campylobacteriosis*. Universally, *Campylobacter* spp have recorded high prevalence in poultry and its products (Mackiw and Szponar 2007; Shane 2000). C. *jejuni*, has the highest prevalence in outbreaks of *Campylobacteriosis* and thus serves as prototype for *Campylobacters* (Tauxe, 1992). Mboto *et al.*, (2012) report state that *C. jejuni* is among the emerging food-related pathogens. Ability of this pathogen (*C. jejuni*) to grow optimally at 37^0 to 42^0C (Nachamkin, 2003), accounts for its high prevalence on the skin surface of poultry with approximate temperature of 41 – 42^0C. Processes at the abattoirs are mainly aimed at reducing the bioload of pathogens present on the carcass. However, Stern *et al.,* (1995) reported that processing of poultry in abattoirs have increased the bioload of *C. jejuni* in 10 – 1000 folds. Atuanya *et al.,* (2012) and Hinton *et al.* (2000) are of the opinion that slaughtering of animals in most abattoirs of developing countries are carried-out in unsuitable buildings by untrained slaughter-men and butchers that are unaware of

1 Department of Microbiology, Ibrahim Badamasi Babangida University, Lapai
2 Department of Food Science and Technology, Ibrahim Badamasi Babangida University, Lapai
3 Department of Microbiology, Abia State University, Uturu, Nigeria
* corresponding author: tovin2009@yahoo.com; +234(0)8034609137

sanitary principles. Assessment on necks and breast of birds (Samelis and Metaxopoulos, 1999), the handlers and poultry environment (Clouser, 1995) were noted as sources of abattoir contamination.

Formation of biofilm within the abattoir environment has enhanced bacterial persistence and bioload. This interactive matrix of aggregate microbes encased in an exopolymer compounds limits the destruction of these microbes by antibiotics, desiccators, sanitizers, mechanical damage and protozoan predators (Mosterller and Bishop, 1993), Ultra violet (UV) rays and heat treatments (Hall-stoodley *et al.*, 2004). *Campylobacter jejuni* was found to form biofilm in both monoculture and mixed culture media (Teh *et al.*, 2010; Joshua *et al.*, 2006) and this ability is enhanced in aerobic condition (Reuter *et al.*, 2010). Though, Wirtanen and Salo (2005) and Hall-stoodley *et al.*, (2004) argues that every microorganisms have varying ability to form biofilm under favourable conditions (moist surface, nutrient and physiological environments). Selective surfaces prone to form biofilm that were assessed by this study include dressing tables, packaging tables, washing tables and the facility floor. Other surfaces include joints, valves, cutting utensils and several dead ends (Ellebroek, 1997). Persistence of *C. jejuni* would be greatly enhanced by its ability to form biofilm on surfaces of food and processing environments. This poses a difficult challenge for campylobacteriosis management. Thus, this paper assessed the prevalence of *Campylobacter* spp. in two indigenous poultry abattoirs and ability of the isolates to develop biofilm within 5 days.

Material and Methods

Sample collection

A total of 160 samples were collected from two indigenous poultry abattoirs in Port Harcourt metropolis, Rivers State, located within the longitude $7^0$9'E and latitude $4^0$4'N. Two (2) samples (before and after poultry processing) were collected each from the surfaces of dressing table, packaging table, washing tubs and the facility floor. Samplings were replicated ten (10) times on both sites at two weeks interval. These surfaces were swabbed diagonally in opposite direction with commercial swab sticks and kept in ice bath ($<4^0$C) while on transits to the laboratory for analysis.

Isolation and identification

Each Swab sample was pre-enriched in 10 ml of Hunt Enrichment broth (Becton and Dickinson, Oxoid) for 24 h at 37^0C. Filter membranes with pore size of 0.6 μm and 50 mm diameter (Schleicher and Schuell ME 26) were placed on Tryptose Blood Agar plates (CM 233, Oxoid) containing 10% unlysed horse blood. Few drops (2 – 3) of pre-enriched samples were placed onto filters and left for 30 minutes. The filters were then removed and the plates were incubated at 37^0C for 48 h in a microaerophilic atmosphere (5% O_2, 10% CO_2, and 85% N_2) using Oxoid gas pack (BR 38) (Le Roux and Lastovica, 1990). *C. jejuni* was isolated in a selective medium using Skirrow's medium at 42^0C to inhibit other members of *Campylobacters* (which normally grow at 36 – 37^0C). Isolates were then gram stained for the Gram negative gull wing shaped rods (Nachamkin, 2003).

Determination of total viable bacterial count (TVBC)

Total bacterial counts of wastewater samples from the two sites were determined by the method described by Adesemoye *et al.* (2006). The waste samples were serially diluted to 10^{-4} and 0.1 ml was aseptically inoculated on a sterile nutrient agar plates and incubated at 30^OC for 24 h. Plates with distinct colonies were counted, and total bacteria was estimated and recorded as colony forming units per ml.

Biofilm assessment

Five glass slides (6 x 2 cm^3) were aspetically placed inside a jar containing 500 ml of Tryptic Soy Broth TSB (CM 129, Oxoid). Exactly 5 ml of 18 – 24 h old broth culture of the isolates were inoculated into TSB and incubated for 5 days at 37^0C. After this period, the glass slides were treated with phosphate buffered saline to remove unattached cells, followed by air drying and later heat fixed to avoid mechanical damage during staining. Crystal

violet (0.1%) was used to stain (flood) the glass slides for 5 min, and then rinsed with phosphate buffered saline (Balogu *et al.*, 2010; Adetunji and Adegokes, 2008). Only stained glass slides were graded by microscopic assessment.

Biofilm formed on the glass slide were further quantified by determining its optical density (OD) using spectrophotometer (NOPVASPEC II). Glass slides that retained the stains were washed and solubilised in 25 ml of 95% ethanol. Out of it, 200 µm was collected and the absorbance (OD_{590nm}) was determined. The absorbance value of each sample was subtracted from the constant (standard) absorbance value (OD control) measured at 590 nm to obtain the actual $OD_{590\ nm}$ value of the samples. Triplicates of the process were done and the mean value was computed and recorded (Balogu *et al.*, 2010; Stepanovic *et al.*, 2004; Prouty *et al.*, 2002).

Assessment of physicochemical parameter of poultry wastewater

Wastewater samples were analyzed for Total Suspended Solids (TSS), Total Dissolved Solid (TDS), Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD). These physico-chemical parameters were evaluated using standard methods (APHA, 1998; Adesomeye *et al.*, 2006; Ademoroti, 1996).

Antibiotic sensitivity assay

Standard inoculation method was used. A sterile loop was used to touch the top of a pure colony of isolates and pre-enriched in 2 ml of Hunt Enrichment broth, for 2 h at 37^{0}C and was spread on freshly prepared nutrient agar plates. Eleven commercial antibiotics disc (Bactron-Dickson antibiotic disc, USA) of known concentration were aseptically placed on the cultured agar plates and incubated at 37^{0}C for 18 – 24 h. Zones of complete inhibition was measured and translated into susceptible and resistance categories based on the interpretation table (Bactron, Dickson Microbiology Company, USA) modified by Balogu *et al.*, (2010).

Statistical tool (efficiency index ratio)

Efficiency index ratio (E_I) of antibiotics tested was estimated to determine the significance of the drugs (Balogu *et al.,* 2013).

$$E_1 = \frac{[0.1\ (S_i - 1) + 1]}{[0.1\ (R_1 + 1) + 1]} = \frac{[S_i + 9]}{(R_1 + 11]}$$

Where S_i = Sensitivity rate (%); $R_i = 100 - S_i$

$E_I \geq 1$ = significant; $E_I < 1$ = not significant

Results

Prevalence of *Campylobacter jejuni* in the two poultry abattoirs was only persistent in 102 among the 160 samples assessed (Table 1). Overall frequency of isolates was 63.75%, in which site A was 57.5% and site B was 70% (Fig. 1). Prevalence of *Campylobacter* was high in samples from packaging table (80%), dressing table (75%) and floor surface (70%) while the least, washing tubs (30%) was significantly different ($p < 0.05$) (Fig. 2).

Table 1: Prevalence of *Campylobacter* sp in the various sampled sources

Source	Site A		Site B		OP
	NE	PC (%)	NE	PC (%)	
Dressing table	20	10 (50)	20	20 (100)	30
Packaging table	20	14 (70)	20	18 (90)	32
Washing table	20	6 (30)	20	6 (30)	12
Floor surface	20	16 (80)	20	12 (60)	28
Total	**80**	**46**	**80**	**56**	**102**

NE = Number examined; PC = Positive cases; OP = Overall positive

Among 102 isolates of C. *jejuni* assessed for biofilm formation, only 20 of them developed biofilm within 5-day period at 37^{0}C. Results in Table 2 showed that *Campylobacter* spp. isolated from washing tubs have the highest mean optical density

$(OD)_{590nm}$ value of 0.952, followed by isolates from packaging table (0.601), dressing table (0.598) and the least was floor surface (0.483).

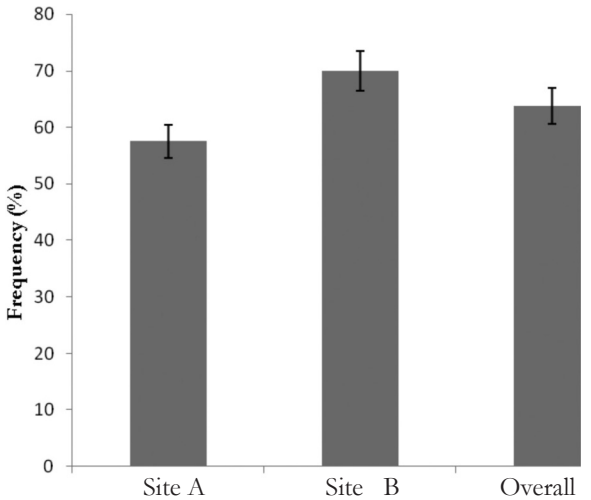

Fig. 1: Prevalence of *Campylobacter* spp in the sites studied

Total bacterial count (TBC) of wastewater was slightly higher in site A (4.4×10^5 CFU/ml) than site B (3.5×10^5 CFU/ml) (Fig. 3). All selected physicochemical parameters of wastewater assessed in this study, showed higher values of TDS (643 – 820 mgl^{-1}), TSS (1200 – 1775 mgl^{-1}), COD (152 – 141 mgl^{-1}) and BOD (30.3 – 32.5 mgl^{-1}) than the WHO standards of 500 mgl^{-1}, 100 mgl^{-1}, 10 mgl^{-1} and 6 mgl^{-1} respectively. This is a clear indication of heavy microbial presence in the wastewater (Table 3).

Drug sensitivity assay revealed that the isolates were highly susceptible to streptomycin (100%), erythromycin (90%), gentamincin (90%), tetracycline (80%), neomycin (80%) and penicillin (70%). Others that are moderately sensitive include riphapicin (60%), ampicillin (60%), norfloxicin (50%), cephalexin (50%) and kanamycin (50%) (Table 4).

Table 2: Detection and quantification of biofilm formed after 5 days at 37^0C

Sample source	M_a	Sample size	Mean of $OD_{(590nm)}$	Range of $OD_{(590nm)}$
Dressing table	++(2)	6	0.598	1.80 – 0.74
Packaging table	+++(3)	8	0.601	1.96 – 0.34
Washing tub	+++(3)	4	0.952	2.01 – 0.54
Floor surface	++(2)	2	0.483	0.81 – 0.25

N = No of positive cases (those that retained the stain)
M_a = Microscopic grading of stained biofilm glass slide
+ = low; ++ = moderate; +++ = high

Table 3: Physicochemical properties of wastewater from Site A and Site B

Parameter measured	Site A	Site B	WHO Standard
TDS (Mg/L)	643 ± 7.3	820 ± 6.8	500
TSS (Mg/L)	1200 ± 31.3	1775 ± 22.5	100
COD (Mg/L)	152 ± 3.40	141.0 ± 4.5	10
BOD (Mg/L)	30.3 ± 2.88	32.5 ± 1.8	6

Values are means + SD of 3 triplicates

Discussion

Campylobacteriosis is recently on the global red alert list of food borne diseases. Perhaps the pathogen is evolving or stably adapting to the environment through biofilm (Shane 2000; Zhao *et al.*, 2001; Spencer and Guan, 2004; Ugboma *et al.*, 2013, Mboto *et al.*, 2012). High prevalence of 63.75% observed in this study, affirmed the reports of Willis *et al.*, (2000) and Mackiw and Szponar (2007), that Campylobacter persists in poultry environment at 100% and 75.4% respectively. However, Nicole (2005) reported a contrary prevalence of *Campylobacter* (8%) from a poultry processing facility. Different hygienic and HACCP measures, sampling sources and methodology used during sampling may as well account for variations in prevalence of bacterial contamination. Constant

Table 4: Antibiotics (drugs) susceptibility assay of *Campylobacter* spp

Antibiotics (µg)	No of isolates tested	Sensitivity rate (%)	Efficiency index ratio (E_I)
Streptomycin(10)	10	10(100)	9.91
Erythromycin(10)	10	9(90)	4.71
Tetracycline(30)	10	8(80)	2.87
Penicillin(10)	10	7(70)	1.93
Norfloxicin (10)	10	5(50)	0.97
Riphapicin (5)	10	6(60)	1.35
Neomycin (30)	10	8(80)	2.87
Cephalexin (30)	10	5(50)	0.97
Ampicillin (10)	10	6(60)	1.35
Kanamycin (20)	10	5(50)	0.97
Gentamicin (20)	10	9(90)	4.71

S = (>11 mm); R = (<11 mm except gentamicin & streptomycin at < 6 mm)

$E_I = (S_i + 9)/(R_i + 11)$; $E_I \geq 1$ = significant; $E_I \leq 1$= not significant

NB: S_i = sensitivity (%), R_i = resistant (100 –S_i)

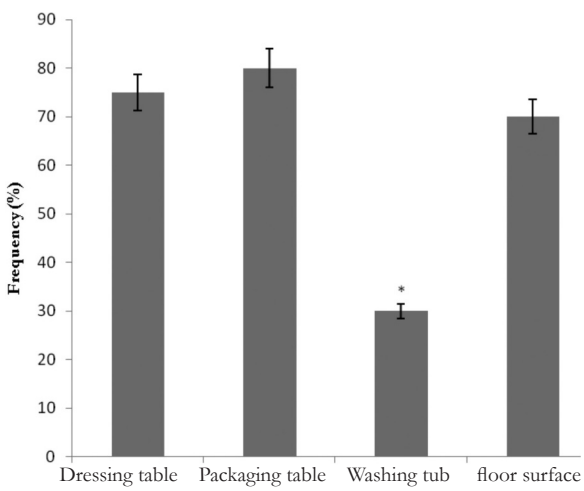

Fig. 2: Comparative prevalence of *Campylobacter* spp from different sample sources

"*" = significantly different ($p < 0.05$) across bars

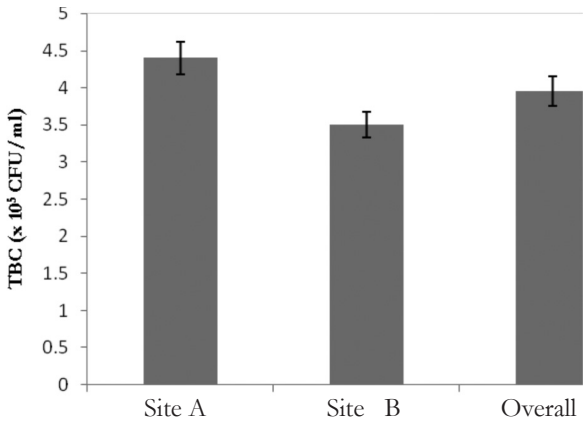

Fig. 3: Total bacterial count (TBC) of the samples from the sites

human-carcass contact rates during dressing and packaging explains the high prevalence observed in samples from packaging table (80%) and dressing table (75%). Contrary to expectation, a high a rate of 70% was observed in floor surface, regardless the regular use of recommended sanitizers. Perhaps, poultry droppings (good reservoir of *C. jejuni*) attached to foot wears are effectively transferred by human traffic. However, washing tubs had the least rate of 30%, probably due to the use of sanitizing agents and less human-carcass contacts. Wastewater from these washing tubs have been implicated as most potential reservoir of *Campylobacters* in poultry facility (Kazwala *et al.*, 1990; Jeffrey *et al.*, 2001; Guan and Holley 2003; Nicholson *et al.*, 2000).

Human-carcass-surface contacts, human traffic and poor sanitary conditions encouraged biofilm and persistence of campylobacter in poultry facility. Persistence of isolates in this study were not proportional to their biofilm formation as reported in the studies of Hood and Zottola (1995); Gulsun *et al.*, (2005); Adetunji and Adegoke, (2008). Based on the $OD_{(590nm)}$ values, isolates from WT (0.952) had the highest detected biofilm followed by PT (0.601), DT (0.598) and FS (0.483), which did not corresponding to the decreasing prevalence pedigree of PT>DT>FS>WT. Perhaps, some isolates are

late colonizers or require enough time (> 5-days) to develop detected biofilm. Wirtanen and Salo (2005) reported that two (2) days is enough for *Campylobacter* to form biofilms on steel and glass sheets; and is possible in both mixed and monoculture media (Teh *et al.*, 2010). This contradicted the low biofilm rate (19.6%) of *C. jejuni* observed in this study, and further portrays that surface material determines the rate of microbial biofilm formation.

Total bacterial count (TBC) of the wastewater was assessed as biomarkers to estimate the contamination level at the sites. The average total bacterial count (TBC) was high for samples from the two studied poultry abattoirs. Based on benchmark released by WHO (2002), any water contaminated at this level is neither good for domestic use nor it is supposed to be discharged directly into the environment without treatment. Saikia and Joshi (2010) observed TBC of 2.0 – 11 x 10^6 CFU/ml from different poultry sites and 5.0 x 10^5 – 4.1 x 10^7 CFU/ml from wash water of poultry processed parts (thighs, breasts, gizzard, tail, liver, wings and heart). Shane (2000) observed that poultry processing (dressing) and environmental factors aimed at reducing microbial load within the facility actually increases the bioload of *C. Jejuni* by 10 – 1000 folds. The reason may be connected to the changes in some physicochemical parameters (pH, temperature etc.) which optimally encouraged *C. jejuni* at 42^{O}C. It was observed that TDS and BOD of wastewater assessed in this study were higher than the WHO water standards. This is a clear indication of heavy microbial presence in the wastewater. Adesemoye *et al.* (2006) observed a similar result of 630 mgl^{-1}, 1800 mg^{-1}, 142 mgl^{-1} and 35 mgl^{-1} as the maximum values for TDS, TSS, COD and BOD respectively.

All tested drugs are recommendable for campylobacter infection management in poultry abattoirs. Among the highly efficient include streptomycin, erythromycin, gentamicin, tetracycline, neomycin and penicillin; while moderate drugs are riphapicin, ampicillin and norfloxicin; perhaps the expensive and rare use of these drugs as growth promoters in Nigeria Agriculture, accounts for its high susceptibility rate. However, cephalexin and kanamycin were found non-significant in treating *Campylobacter* spp. Consumer International (2003) reported sensitivity rate of 98% – 100% efficiency of similar drugs on *campylobacters* isolated from chicken and turkey sources. Feizabadi *et al.*, (2007) and Urumova *et al.*, (2008) affirmed that *Campylobacter* spp are highly susceptible to neomycin, erythromycin, gentamicin, streptomycin, norfloxacin at 84.6% – 100%. Efficiency index ratio statistically predicts the suitability level of these drugs to manage campylobacteriosis from poultry-related facilities. In spite of these drugs efficiency, *Campylobacter* spp still persist in poultry facility and a good biofilm former within 5 days. These pose a potent threat in poultry industry. Thus, the findings of this study can be of value in checkmating campybacteriosis outbreak in poultry abattoir facility.

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