Food Control 71 (2017) 94–100

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Biofilm formation of *Campylobacter* strains isolated from raw chickens and its reduction with DNase I treatment

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ARTICLE INFO

Article history:
Received 4 March 2016
Received in revised form
16 June 2016
Accepted 27 June 2016
Available online 29 June 2016

Keywords:
Campylobacter
Chicken
Biofilm
Motility
DNase I
Reduction

ABSTRACT

Campylobacter is a well-known bacterial agent that causes foodborne gastroenteritis. Its biofilm-forming ability is known to be important for its survival in harsh conditions. Poultry is a main reservoir of *Campylobacter*. In this study, we evaluated the biofilm-forming ability, motility, and antibiotic susceptibility of *Campylobacter* strains isolated from commercially purchased chickens of various sources in South Korea. From 37 (30%) out of 124 chickens, a total of 78 *Campylobacter* isolates were obtained, and the ability of these strains to form biofilms was studied on polystyrene surfaces. We identified seven biofilm-forming strains of *Campylobacter jejuni* and *Campylobacter coli*, respectively, out of 60 *C. jejuni* and 18 *C. coli* strains. Our study demonstrated that motility is inconsistent with biofilm-forming ability, suggesting that motility is not a single factor affecting biofilm formation of *Campylobacter*. Moreover, there was no clear relationship between antibiotic resistance and biofilm-forming ability. DNase I treatment significantly inhibited the biofilm formation or degraded the mature biofilms of 3 *C. coli* and 1 *C. jejuni* biofilm-forming strains ($p < 0.05$). It suggests that extracellular DNA plays a significant role in the biofilm formation of these strains. Collectively, our study demonstrated that biofilm-forming ability is not strongly correlated with motility and antibiotic resistance in *Campylobacter* and that extracellular DNA is required for biofilm formation of the isolated *Campylobacter* strains. It also suggests that DNase I is a potential method for the control of *Campylobacter* biofilms.

1. Introduction

Campylobacter spp. are one of the most common causes of food poisoning and acute bacterial gastroenteritis worldwide. In the United States, more than 1.3 million cases of *Campylobacter* infections occur every year (CDC, 2014). Approximately 3500 patients suffer from campylobacteriosis each year in South Korea (MFDS, 2015). The primary symptoms of campylobacteriosis are fever, diarrhea, vomiting, and headache. Illness associated with *Campylobacter* normally occurs after the consumption of undercooked poultry products, raw milk, or untreated tap water contaminated with *Campylobacter* (Jang et al., 2007; Little, Richardson, Owen, de Pinna, & Threlfall, 2008; Silva et al., 2011; Wei et al., 2014). A major source of *Campylobacter* is poultry, and chickens are a major reservoir (Beery, Hugdahl, & Doyle, 1988; Silva et al., 2011).

Biofilms are defined as an assemblage of microorganisms adhered to a surface and surrounded with a polymeric matrix (Shi & Zhu, 2009; Srey, Jahid, & Ha, 2013). As bacteria surrounded by biofilms are protected from environmental stresses, they tend to be more resistant to environmental stresses including antibiotics and disinfectants than planktonic cells (Hall-Stoodley, Costerton, & Stoodley, 2004; Reuter, Mallett, Pearson, & van Vliet, 2010; Simões, Simões, & Vieira, 2010; Srey et al., 2013). For this reason, biofilms can serve as an important source of cross-contamination in the food supply. Several reports have shown that Campylobacters are able to form biofilms in a variety of environments. Previous

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http://dx.doi.org/10.1016/j.foodcont.2016.06.038
0956-7135/

studies have shown that *Campylobacter* spp. can form biofilms under both microaerobic and aerobic conditions (Brown et al., 2014; Reuter et al., 2010). However, campylobacters are unable to grow under aerobic conditions. This fastidious nature of *Campylobacter* seemingly conflicts with the survival of the bacteria under environmental conditions outside of animal hosts and the subsequent high incidence of human infection. Considering the nature of biofilm often protecting the bacterial cells from the hostile environmental conditions, the ability to form biofilms may serve as a survival mechanism under environmental conditions such as aerobic conditions (Bronowski, James, & Winstanley, 2014; Reuter et al., 2010). This enhanced survival may contribute to the high incidence of infection by *Campylobacter* spp.

Factors such as food contact surfaces, pH, nutrient availability, and temperature affect the very complex processes of biofilm development (Shi & Zhu, 2009; Simões et al., 2010; Srey et al., 2013). Bacterial motility and surface properties affect bacterial attachment to the surfaces (Simões et al., 2010). The biofilm matrix builds an extracellular polymeric substance (EPS) while developing the biofilm; the EPS is composed of proteins, polysaccharides, lipids, and extracellular DNA (Hall-Stoodley et al., 2004; Tetz, Artemenko, & Tetz, 2009). The EPS diminishes the effect of antibiotics (Donlan, 2002; Taraszkiewicz, Fila, Grinholc, & Nakonieczna, 2013). In addition, extracellular DNA (eDNA) from lysed cells may be a component of EPS for biofilm formation and development (Tetz et al., 2009). DNase enzymes can degrade the eDNA and inhibit biofilm formation and maturation (Brown, Reuter, Hanman, Betts, & van Vliet, 2015b, 2015a; Tetz et al., 2009). Although eDNA has been recently shown to play an important role in the biofilm formation of *Campylobacter jejuni* (Brown et al., 2015b, 2015a; Svensson, Pryjma, & Gaynor, 2014), the role of eDNA in the biofilm formation of *Campylobacter* natural isolates from food sources is still poorly understood. Thus, the purpose of this study was to investigate the biofilm-forming ability of *Campylobacter* isolates from raw chickens on abiotic surfaces. Then, we investigated the associations between biofilm formation and motility, a common feature affecting the biofilm formation, and antibiotic resistance, a common public health concern. Finally, we evaluated the effect of DNase I treatment on the biofilms of *Campylobacter* isolates.

2. Materials and methods

2.1. Chicken sample collection

A total of 124 raw chickens were purchased from grocery stores in packaged form from 2 different manufacturers or from 3 traditional markets in unpackaged form located in South Korea from December 2013 to March 2014. The samples were transported to the laboratory at room temperature or on an ice pack (4 °C) and analyzed immediately or stored at 4 °C and analyzed within 2 days.

2.2. Isolation of Campylobacter from raw chickens

A previous protocol was followed with modifications to isolate *Campylobacter* strains from raw chickens (Chon, Hyeon, Park, Song, & Seo, 2012). Each raw chicken sample (800–1100 g) was placed in a sterile poultry rinse bag (390 × 520 cm) (Nasco, Fort Atkinson, USA), and 400 ml of buffered peptone water (Difco, Sparks, USA) was added. The bags were shaken for 1 min, then 25 ml of chicken rinse was added to 25 ml of 2× Bolton broth (Oxoid, Basingstoke, England). Then, it was supplemented with laked horse blood (2.5%), cefoperazone (20 μg/ml), vancomycin (20 μg/ml), trimethoprim (20 μg/ml), and cycloheximide (50 μg/ml) in a 250 ml sterile culture flask (SPL, Pocheon, Korea). The samples were enriched at 42 °C for 48 h under microaerobic conditions (5% O_2, 5% CO_2, 85% N_2) using AnaeroPack-MicroAero (Mitsubishi Gas Chemical Co., Tokyo, Japan) in anaerobic jars. After incubation, one loopful of the enrichment broth was streaked on preston agar, Campylobacter Agar Base (Oxoid) supplemented with laked horse blood (5%), polymyxin B (5 IU/ml), rifampicin (10 μg/ml), trimethoprim (10 μg/ml), and amphotericin B (10 μg/ml). The plates were incubated under microaerobic conditions at 42 °C for 48 h. Presumptive *Campylobacter* isolates were subjected to a microscopic analysis, Gram staining and oxidase tests (Sigma-Aldrich, St. Louis, USA). In addition, no growth was confirmed under aerobic conditions. The isolates that appeared spiral under the microscope, Gram stained negative, and tested oxidase positive were confirmed to be *Campylobacter*. The confirmed *Campylobacter* isolates were individually stored in glycerol stocks at −80 °C for further analyses. To differentiate between *C. jejuni* and *C. coli*, hippurate hydrolysis tests (Hardy Diagnostics, Santa Maria, CA, USA) were conducted. The isolates tested hippurate hydrolysis positive were determined to be *C. jejuni*, while the isolates tested hippurate hydrolysis negative were determined to be *C. coli*. When the results were unclear, a PCR analysis was performed using primers specific for the *ceuE* gene (Gonzalez, Grant, Richardson, Park, & Collins, 1997). The PCR primers used for *C. jejuni* detection were JEJ1 (5′-CCTGCTACGGTGAAAGTTTTGC-3′) and JEJ2 (5′-GATCTTTTTGTTTTGTGCTGC-3′) and the PCR primers for *C. coli* detection were COL1 (5′-ATGAAAAAATATTTAGTTTTTGCA-3′) and COL2 (5′-ATTTTATTATTTGTAGCAGCG-3′). The PCR program was 1 cycle of 94 °C for 2 min, then 35 cycles of 94 °C for 45 s, 57 °C for 45 s, 72 °C for 1 min, and finally 1 cycle of 72 °C for 7 min.

2.3. Biofilm assay

The ability of *Campylobacter* isolates to form biofilms was determined as previously described using crystal violet staining of the 96-well polystyrene microtiter plates (SPL) (Kim, Park, & Kim, 2015). Briefly, *Campylobacter* strains were grown on tryptic soy agar supplemented with 5% sheep blood (TSAB) at 37 °C for 48 h from −80 °C stock cultures. The cultures grown on TSAB were suspended in Mueller-Hinton Broth (MHB) at OD_{600} 0.1–0.2 using sterile cotton swab, spread at 100 μl on TSAB, and incubated at 37 °C for 14–15 h under microaerobic conditions (5% O_2, 5% CO_2, 85% N_2). Then, the grown cells were suspended in MHB at OD_{600} 0.01 using cell scrapers, aliquoted at 100 μl in the 96-well plates, and incubated at 37 °C under microaerobic conditions. After 72 h incubation, the cell suspensions were removed after pipetting up and down 4 times. Then, the wells were rinsed twice with 150 μl of sterile water by pipetting up and down 4 times for each rinse. The plates were completely dried at 37 °C, and the biofilms were stained with 100 μl of 1% crystal violet solution (Sigma-Aldrich) at room temperature for 30 min. After staining, the plates were washed in slowly running tap water, rinsed with water and dried, and the bound crystal violet was solubilized in 100 μl solution of 30% methanol and 10% acetic acid per well. Then, the optical density was measured at 590 nm using a microplate reader (Infinite M200 Pro NanoQuant, Tecan, Switzerland).

2.4. Motility assay

Motility was examined using a previously described method (Kim et al., 2012). Briefly, *Campylobacter* strains were grown on TSAB for 48 h at 37 °C under microaerobic conditions. Grown cells were suspended in MHB (OD_{600} 0.1), then 200 μl sterile pipette tips were dipped in the cell suspension and then stabbed into the center of a 0.4% Mueller-Hinton soft agar plate. Plates were incubated in the upright position for 48 h at 37 °C under microaerobic conditions. Motility was measured by measuring the diameter of motility halos.