Campylobacter biofilm phenotype exhibits reduced colonization potential in young chickens and altered in vitro virulence

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ABSTRACT In this study, we evaluated the ability of different Campylobacter phenotypes (biofilm versus planktonic) to colonize young poultry. It has been suggested that a persistent Campylobacter biofilm reservoir may be involved in the initial contamination of poultry flocks. Campylobacter jejuni cultured adherent to agar was utilized as the biofilm model and C. jejuni cultured in broth was evaluated as the planktonic model. In 2 independent trials, 1-d-old broiler chicks were given 1 of 3 treatments: 1) 10⁵ cfu·mL⁻¹ of C. jejuni cultured in broth, 2) 10⁵ cfu·mL⁻¹ of C. jejuni cultured adherent to agar, or 3) no C. jejuni (negative control). Cecal contents of all birds were evaluated by culturing 12 d after the initial challenge with C. jejuni. In both trials, birds challenged with C. jejuni cultured in broth had approximately 3 to 4 log higher cecal Campylobacter concentration than birds challenged with C. jejuni cultured adherent to agar. Using 2 cell lines (INT 407 and DF1), virulence of C. jejuni cultured in broth versus adherent to agar also was evaluated by challenging monolayers of eukaryotic cells with 1 of 3 treatments: 1) 10⁵ cfu·mL⁻¹ of C. jejuni cultured in broth, 2) 10⁵ cfu·mL⁻¹ of C. jejuni cultured adherent to agar, or 3) no C. jejuni (negative control). The virulence study also showed differences of C. jejuni cultured in broth or agar in attachment and invasion abilities to tissue culture cells, but differences were not as consistent as with the chick colonization study. This study indicates that phenotype may play a role in colonization of chickens and virulence by C. jejuni.

Key words: Campylobacter jejuni, biofilm, colonization, chicken, virulence

INTRODUCTION

Campylobacter jejuni is a leading cause of foodborne illness with poultry and poultry products implicated as major sources of infection to consumers (Snelling et al., 2005). The colonization of poultry by C. jejuni has been well documented, yet much information concerning the process of colonization remains unknown (Sahin et al., 2002; Lee and Newell, 2006). In addition, a full understanding of the virulence mechanisms in human campylobacteriosis is lacking (Hu and Kopecko, 2000). Information concerning the process of colonization could greatly facilitate control of C. jejuni, which could potentially lead to a reduction of the incidences of campylobacteriosis.

A major question involved in colonization of poultry is the initial source of C. jejuni flock contamination. Epidemiological studies have been undertaken to identify risk factors and determine sources of infection. Poor cleaning, biosecurity breaches, poor poultry house maintenance, short empty periods, insects, and the presence of other animals on the farm have all been suggested as risk factors (Conlan et al., 2007). Detecting sources of Campylobacter infection to flocks may be difficult and, due to the susceptibilities and highly fastidious nature of C. jejuni, carry-over between flocks is difficult to explain (Newell and Fearnley, 2003). Yet, the majority of commercial flocks in the United States become colonized between 2 and 3 wk of age (Stern et al., 2001). Some studies suggest the risk factor for carry-over is relatively low and that a persistent reservoir, such as a biofilm, may be a more likely source (Shreeve et al., 2002). Many other bacteria and pathogens utilize a biofilm strategy, including Listeria monocytogenes, Salmonella, Shigella, Staphylococcus aureus, Escherichia coli, and Enterobacter, to survive inhospitable conditions and to cause disease (Bower and Daeschel, 1999; Stepanovic et al., 2004; Gunduz and Tuncel, 2006; Rode et al., 2007). However, whether C. jejuni biofilm phenotypes can even colonize chickens and exhibit altered virulence factors has not been inves-

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tigated and, therefore, was the objective of this study. In this research, *C. jejuni* cultured adherent to agar was utilized as a biofilm model and *C. jejuni* cultured in broth was utilized as a planktonic model because it has been previously reported that the genetic expression profile of *C. jejuni* grown on agar is similar to *C. jejuni* grown in a biofilm and both genetic expression profiles are very different than *C. jejuni* cultured in broth (Carriello et al., 2007).

**MATERIALS AND METHODS**

**Experimental Birds and Housing**

Day-of-hatch broiler chicks were obtained from a local hatchery (Cobb-Vantress, Fayetteville, AR). Birds were placed in cages measuring approximately 15 ft² (1.39 m²). Floors of the cages were wired with a catch pan located approximately 3 in. (7.62 cm) below wire floors. Birds had access to feed and water ad libitum for the duration of both experiments.

**Growth Conditions and Preparation of Bacteria**

Three strains of *C. jejuni* were utilized in these studies. *C. jejuni* NCTC strain 11168 was kindly donated by Qijing Zhang at Iowa State University (Ames), ATCC strain 1033431 was kindly donated by William Miller at Agricultural Research Service (Albany, CA), and strain 81-176 was kindly donated by Michael Johnson at the University of Arkansas (Fayetteville). For the bird challenge study, *C. jejuni* strain 11168 was used, whereas all 3 strains of *C. jejuni* were used for the tissue culture experiments. For growth in broth, a 10-μL loop of frozen culture was inoculated into 10 mL of Mueller-Hinton (MH) broth (Becton Dickinson, Franklin Lakes, NJ) and incubated at 42°C in microaerophilic conditions for 48 h. The culture was passed by vortexing to resuspend the cells and inoculating 10 μL of the suspended cells into fresh MH broth. For growth adherent to agar, a 10-μL loop of frozen culture was inoculated onto MH agar and incubated at 42°C in microaerobic conditions for 48 h. The culture was passed by collecting a 10-μL loop full of bacteria from the plate and inoculating onto fresh MH agar. All cultures were passed twice before use.

For use in challenge, bacteria grown in broth were collected by centrifugation at 8,000 × g for 5 min. Bacteria adherent to agar were collected with a 10-μL loop and placed into sterile Butterfield’s phosphate diluent (BPD; 6.8% KH₂PO₄; 7.2 pH) and centrifuged at 8,000 × g for 5 min to pellet the cells. All pelleted cultures were washed twice by resuspending pellets with vortexing, collecting by centrifugation, and resuspending in 10 mL of BPD. The concentration of the cultures was determined spectrophotometrically as described previously (Solis de los Santos et al., 2008). Cultures were serially diluted before use in the experiments and the initial concentration also was determined by plating aliquots using serial dilutions.

**Bird Challenge Experiment**

Day-of-hatch chicks (n = 60) were randomly assigned to 1 of 3 treatment groups (n = 20/pen). Treatment groups were: 1) negative control (no *C. jejuni*), 2) 10⁵ cfu·mL⁻¹ of *C. jejuni* cultured in broth, and 3) 10⁵ cfu·mL⁻¹ of *C. jejuni* cultured adherent to agar. All treatment groups were inoculated the day after hatch with 250 μL of bacteria suspended in BPD using a 1-mL syringe and stainless steel cannula. The entire trial was repeated in a second independent trial.

**Cecal Campylobacter Determination**

On d 12 postchallenge, all chicks were humanely killed using CO₂ asphyxiation and cecal contents were collected and placed in individual sterile plastic bags. *Campylobacter jejuni* concentrations were enumerated as described by Solis de los Santos et al. (2008). Briefly, cecal contents were squeezed into a sterile 15-mL centrifuge tube, weighed, and resuspended 1:10 in BPD. The suspensions were inoculated onto *Campylobacter* Line agar plates (Line, 2001) and incubated at 42°C for 48 h in microaerophilic conditions. Bacterial colonies were counted from the plates and converted to colony-forming units per gram (cfu·g⁻¹) of cecal content. The limit of detection using this method was 10⁵ cfu·mL⁻¹.

*Campylobacter jejuni* colonies were confirmed by API Campy (Biomerieux, Durham, NC) and latex agglutination test (Panbio Inc., Columbia, MD).

**Mammalian and Avian Cell Line**

Mammalian cells (INT 407) utilized in this study were kindly donated by Jody Lingbeck at the University of Arkansas. Mammalian cells were cultured in Basal Medium Eagle (Sigma Chemical Co., St. Louis, MO) with 10% fetal bovine serum (Sigma) and 2 mM L-glutamine (Sigma) in 75-cm² tissue culture flasks. Avian cells (DF1; American Type Culture Tissue Collection, Manassas, VA) were cultured in Medium 199 (Invitrogen, Carlsbad, CA) with 9% fetal bovine serum and tryptose phosphate broth (Becton Dickinson) in 75-cm² tissue culture flasks.

**Adherence and Invasion Assays**

All cells were cultured in a 5% CO₂ atmosphere at 37°C. Cells were collected by pouring off culture medium and washing the monolayer with 2 mL of trypsin (0.25%; Sigma). After pouring off the wash trypsin, an additional 2 mL of trypsin was added to the flask and the flask was incubated under culture conditions for 15 min or until cells became unattached. The concentration of the cells was determined using a hemocytometer and cells were suspended in fresh medium to a final
concentration of $10^5$ cells·mL$^{-1}$. Cells were aliquoted into 24-well tissue culture plates (1 mL per well). All 24-well plates were incubated in a 5% CO$_2$ atmosphere at 37°C for 24 h to allow cells to attach.

After 24 h, all wells were washed 3 times with sterile PBS. Bacterial suspensions were used to fill the wells at 1 mL per well. Plates were incubated for 2 h to allow adherence and invasion of tissue culture cells. After 2 h, all plates were washed 3 times with PBS to remove any nonadhering bacteria before the addition of fresh medium containing 100 μg·mL$^{-1}$ gentamicin (to measure invasion alone) or, in the duplicate plates, PBS with 0.1% Triton X-100 (to measure adherence and invasion). Plates with media plus gentamicin were incubated in a 5% CO$_2$ atmosphere at 37°C for an additional 2 h, whereas plates with PBS plus 0.1% Triton were gently swirled for 30 min at room temperature to lyse the cells and release internalized bacteria. After the 2-h incubation of plates with media plus gentamicin, plates were washed 3 times with PBS before the addition of PBS with 0.1% Triton X-100. Cells were lysed using the same method as described above.

To determine the colony-forming units of bacteria adhering and invading the tissue culture models, 10-fold dilutions were made of the lysed cells in sterile PBS and plated onto MH agar. The colony-forming units of adhering bacteria were determined by using the formula: colony-forming units of nongentamicin-treated culture (adherent and invasive) − colony-forming units of gentamicin-treated culture (invasive) = colony-forming units of adherent bacteria.

**Statistical Analysis**

Cecal *Campylobacter* plate count data were logarithmically transformed before analysis to achieve homogeneity of variance (Byrd et al., 2001). All data were analyzed using ANOVA using the GLM procedure of SAS (SAS Institute, 2002). Statistically significant differences were reported at $P < 0.05$.

**RESULTS**

**Differences in Chick Colonization Depended on Growth Mode of *C. jejuni***

There was a statistical difference in the titer of *C. jejuni* cecal colonization numbers between the birds inoculated with $10^5$ cfu·mL$^{-1}$ of *C. jejuni* cultured in broth and $10^5$ cfu·mL$^{-1}$ of *C. jejuni* cultured adherent to agar for both independent trials ($P > 0.05$; Figure 1). For the first trial, the mean titer of *C. jejuni* cultured from the ceca was nearly 2 log higher than the initial level of inoculum for birds inoculated with $10^5$ cfu·mL$^{-1}$ of *C. jejuni* grown in broth. However, the final mean titer of *C. jejuni* cultured from the ceca was 3 log lower than the initial level of inoculum for birds dosed with $10^5$ cfu·mL$^{-1}$ of *C. jejuni* grown adherent to agar. In the second independent trial, the mean titer of cecal colonization was nearly the same as the initial inoculum for birds given $10^5$ cfu·mL$^{-1}$ of *C. jejuni* grown in broth (Figure 1B). However, the mean titer of *C. jejuni* cecal colonization was 4 log lower than the initial inoculum for birds dosed with $10^5$ cfu·mL$^{-1}$ of *C. jejuni* grown adherent to agar.

**Differences in Virulence Depended on Growth Mode but Varied by Strain and Cell Line**

Differences in *C. jejuni* cultured adherent in broth and adherent to agar were seen but were strain-dependent and cell line-dependent (Figure 2). *Campylobacter jejuni* strain 11168 cultured in broth had a greater ability to attach to INT 407 cells than *C. jejuni* cultured adherent to agar ($P < 0.05$; Figure 2A). However, there were no statistical differences in the abilities of *C. jejuni* cultured in broth versus *C. jejuni* cultured adherent to agar to attach to INT 407 cells for strains 43431...
importation, and oxidative stress defense shown when comparing *C. jejuni* grown in an adherent mode versus those grown as planktonic cells in broth could possibly explain the differences in colonization seen in this study (Dykes et al., 2003; Sampathkumar et al., 2006). Specifically, Sampathkumar et al. (2006) showed that motility was downregulated in *C. jejuni* NCTC 11168 grown adherent to agar. Because motility is a key factor in colonization (Jones et al., 2004), it was not surprising that *C. jejuni* grown adherent to agar did not colonize as fast or as well as *C. jejuni* cultured in broth. Dykes et al. (2003) showed that *C. jejuni* grown adherent to glass beads had a different stress protein profile than planktonic cells and the adherent cells may be deficient in a complex stress response. This could indicate that the cells grown adherent to agar in our study were less able to survive the stresses associated with passage through the chicken gastrointestinal tract.

A complete understanding of the molecular mechanisms of disease produced in humans by *C. jejuni* is lacking. However, adherence and invasion of intestinal epithelial cells are well known virulence factors. Campylobacteriosis can be caused by the following: 1) adherence to epithelial cells resulting in colonization, 2) adherence and invasion of intestinal cells resulting in damage to cells, and 3) invasion and extraintestinal translocation with migration via the lymphatic system (Hu and Kopecko, 2000). Like chicken colonization, the initial stages of adherence and invasion of human epithelial cells are highly dependent on motility (Hu and Kopecko, 2000; Jones et al., 2004). However, from this study, it is not apparent if there is any difference between growth mode and virulence as measured in human intestinal cells (Figure 2).

For poultry, initial inoculation would most likely occur in the adherent (e.g., attached to litter) as opposed to the free-swimming state. When *Campylobacter* was evaluated grown under these conditions in avian fibroblast cells, inconsistent or no differences were observed (Figure 3). Therefore, it can be concluded from these results that the growth mode has a greater effect on initial chicken colonization than to changes in virulence.

Importantly, research has indicated that differences initially thought to be dependent on growth mode (e.g., adherent versus free-swimming) were actually differences in bacterial expression dependent on growth phase (e.g., lag versus log phase growth; Lazazzera, 2005). Therefore, to eliminate this potential problem, we used the *C. jejuni* strain NCTC 11168 for the bird challenge portions of our studies. Previous research by Sampathkumar et al. (2006) demonstrated no differences in growth curves between agar and broth culturing for the NCTC 11168 *C. jejuni* strain. Because differences in colonization ability, colonization dynamics, and in vitro virulence are reported for different strains (Young et al., 1999; Zheng et al., 2006; Coote et al., 2007; Gilbert and Slavik, 2007; Gilbert et al., 2008), careful interpretation should be taken when applying the results of the current study to other strains of *C. jejuni*.

**DISCUSSION**

In this study, *C. jejuni* cultured in broth used to inoculate 1-d-old chicks had much higher (approximately 3 to 4 log$_{10}$ cfu·g$^{-1}$) cecal concentrations 12 d after inoculation than chicks given *C. jejuni* cultured adherent to agar (Figure 1). The differences in motility, metabolism, protein synthesis, iron uptake, membrane transport, and oxidative stress defense shown when comparing *C. jejuni* grown in an adherent mode versus those grown as planktonic cells in broth could possibly explain the differences in colonization seen in this study (Dykes et al., 2003; Sampathkumar et al., 2006). Specifically, Sampathkumar et al. (2006) showed that motility was downregulated in *C. jejuni* NCTC 11168 grown adherent to agar. Because motility is a key factor in colonization (Jones et al., 2004), it was not surprising that *C. jejuni* grown adherent to agar did not colonize as fast or as well as *C. jejuni* cultured in broth. Dykes et al. (2003) showed that *C. jejuni* grown adherent to glass beads had a different stress protein profile than planktonic cells and the adherent cells may be deficient in a complex stress response. This could indicate that the cells grown adherent to agar in our study were less able to survive the stresses associated with passage through the chicken gastrointestinal tract.

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**Figure 2.** A comparison of the abilities of 3 different strains of *Campylobacter jejuni* cultured in broth versus agar to attach (panel A) or invade (panel B) human intestinal epithelial cells (INT 407). Adherence and invasion are reported as log$_{10}$ cfu·mL$^{-1}$. a,bColumns with no common superscript within strain indicate the adherence or invasion abilities differ significantly ± SEM ($P < 0.05$).
It has been estimated that as much as 98% of the bacteria found in the environment are adherent to some surface as opposed to free-swimming planktonic cells (Dunne, 2002). Adherence to surfaces can provide bacteria with advantages that include increased resistance to sanitizers, increased resistance to stresses such as desiccation, and retention in optimal environments (Dunne, 2002). It is possible that long-term survival of Campylobacter in the environment is dependent upon biofilm formation. Results from this study indicate that young poultry contaminated with Campylobacter in the biofilm state have reduced colonization potential. This may be one explanation as to why it takes 2 to 3 wk for many poultry flocks to become contaminated with Campylobacter.

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REFERENCES


\[ C_{\text{jejuni}} \text{strain, log}_{10} \text{cfu·mL}^{-1} \]

\[ \text{Biofilm, pellicle and plate cultures: Multiple profiles of immobilized growth. Zoonoses Public Health 54(Suppl. 1):54.} \]


\[ \text{Campylobacter jejuni colonization and transmission in broiler chickens: A modelling perspective. J. R. Soc. Interface 4:819–829.} \]


\[ \text{Planktonic or biofilm growth affects survival, hydrophobicity and protein expression patterns of a pathogenic Campylobacter jejuni strain. Int. J. Food Microbiol. 89:1–10.} \]

\[ \text{Evaluation of attachment and penetration abilities of Campylobacter jejuni isolates obtained from humans and chicken carcasses during processing and at retail. J. Food Saf. 25:209–223.} \]

\[ \text{Biofilm formation in an ice cream plant. Antonie Van Leeuwenhoek 89:329–336.} \]


\[ \text{Adaptation of Campylobacter jejuni NCTC11168 to high-level colonization of the avian gastrointestinal tract. Infect. Immun. 72:3769–3776.} \]

\[ \text{Lessons from DNA microarray analysis: The gene expression profile of biofilms. Curr. Opin. Microbiol. 8:222–227.} \]

\[ \text{Filling an ecological niche. Avian Dis. 50:1–9.} \]

\[ \text{Development of a selective differential agar for isolation and enumeration of Campylobacter spp. J. Food Prot. 64:1711–1715.} \]

\[ \text{Sources of Campylobacter colonization in broiler chickens. Appl. Environ. Microbiol. 69:4343–4351.} \]


\[ \text{Sources of infection and modes of transmission. Anim. Health Res. Rev. 3:95–105.} \]