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Increased Emergence of Fluoroquinolone-Resistant *Campylobacter jejuni* in Biofilm

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Campylobacter jejuni is a leading food-borne pathogen that annually causes approximately 400 million to 500 million human infection cases worldwide (1). As a zoonotic pathogen, *C. jejuni* colonizes the intestines of various animal species, particularly poultry, and is transmitted to humans via consumption of contaminated foods (1). Antibiotic treatment is warranted for serious cases of human campylobacteriosis (2); however, the increasing prevalence of *Campylobacter* isolates resistant to clinically important antibiotics, such as fluoroquinolones (FQs) and macrolides, has often been implicated in adverse patient outcomes and is considered a serious public health problem (3).

Enhanced mutability in biofilm has been reported to occur in some bacterial species, and this mutability changes affect the occurrence of spontaneous mutations associated with antibiotic resistance (4, 5). Although *C. jejuni* is known to form biofilm (6) and FQ resistance (FQ^R) is primarily caused by point mutations in DNA gyrase A (2), it has not been understood whether biofilm would have an impact on *C. jejuni*'s development of FQ^R. In this study, *C. jejuni* NCTC 11168 was maintained on Mueller-Hinton (MH; Oxoid) agar plates at 42°C microaerobically (5% O_2, 10% CO_2, and 85% N_2). *C. jejuni* biofilm formation was carried out according to a method described elsewhere (7). Briefly, overnight culture of *C. jejuni* was resuspended in MH broth to give an optical density at 600 nm (OD_{600}) of 0.07, and the bacterial liquid culture was grown with shaking (200 rpm) to dissociate bacterial cells to a planktonic stage prior to inoculation. After incubation with shaking for 4 h, the liquid culture was diluted with fresh MH broth, making the OD_{600} approximately 0.07. Sterile 24-well plates were inoculated with 1 ml of *C. jejuni* suspension and incubated for 24 h without agitation. The supernatant was used to determine the frequency of FQ^R development in planktonic cells. Biofilm formed in 24-well plates was washed with phosphate-buffered saline (PBS) three times, and 1 ml of fresh MH broth was added to the biofilm. By this procedure, *C. jejuni* cells that were released from the biofilm were allowed to grow in MH broth because *C. jejuni* biofilms continuously shed bacterial cells (6). After 24 h of incubation, supernatant was collected for measurement of the frequency of FQ^R development in *C. jejuni* cells dispersed from biofilm. Total bacterial count was determined by performing serial dilution and culturing on MH agar plates. MH agar plates supplemented with ciprofloxacin at 1×, 2×, and 4× the MIC (0.125 μg ml^{-1}, 0.25 μg ml^{-1}, and 0.5 μg ml^{-1}, respectively) were used to determine the counts of FQ-resistant cells. Resistant colonies were randomly selected and subjected to Etest (bioMérieux) to determine the level of FQ^R. The MIC of ciprofloxacin as determined by Etest is known to correlate with the MIC as measured with standard antimicrobial susceptibility testing, such as broth microdilution or agar dilution (8). The quality control strain *C. jejuni* ATCC 33560 was included in the test.

C. jejuni cells from biofilm demonstrated significantly ($P = 0.006$) higher frequencies of FQ^R than planktonic cells, approximately 3.2-fold, at 1× the MIC of ciprofloxacin (Table 1). The mean values of FQ^R frequency were also higher in biofilm than in planktonic cells at 2× and 4× the MIC (Table 1). Based on the results of the ciprofloxacin susceptibility test, however, biofilm did not make differences in the emergence of FQ^R exceeding the breakpoint MIC of ciprofloxacin (4 μg ml^{-1}) suggested by the Clinical and Laboratory Standards Institute (CLSI) (9), compared to planktonic cells (data not shown). *C. jejuni*'s ability to form biofilms is important for human infection and survival in harsh environments (10, 11). Interestingly, it has been reported that high numbers of *Campylobacter* spp. are isolated from naturally present biofilms (12). By extrapolation of previous reports and the results in this study, it can be speculated that *Campylobacter*-harboring biofilms may release *Campylobacter* cells with increased FQ^R.

TABLE 1 FQ^R frequencies in biofilms and planktonic cells of *C. jejuni*[a]

Culture	MIC (μg/ml)		
	1×	2×	4×
Biofilm	$(2.69 \pm 3.60) \times 10^{-7}$*	$(1.17 \pm 1.91) \times 10^{-7}$	$(6.39 \pm 3.84) \times 10^{-8}$
Planktonic	$(8.48 \pm 5.36) \times 10^{-8}$	$(1.85 \pm 0.97) \times 10^{-8}$	$(1.09 \pm 0.10) \times 10^{-8}$

[a] The MIC of ciprofloxacin is 0.125 μg ml^{-1}. FQ^R frequency was calculated by dividing the number of FQ-resistant colonies by the total bacterial count. The values shown are the means and standard deviations of results from five independent experiments. Statistical significance of FQ^R frequency between planktonic cells and biofilms was analyzed by using an unpaired Mann-Whitney two-sample test. *, $P < 0.01$.

In this study, we showed that *C. jejuni* biofilm is involved in enhanced FQ^R. Although the molecular details will be elucidated in future studies, the results successfully demonstrate that biofilm formation is implicated in the development of *C. jejuni*'s resistance to FQ, an antibiotic class of clinical importance.

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