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Short Communication

# Growth in the presence of specific antibiotics induces biofilm formation by a *Campylobacter jejuni* strain sensitive to them but not in resistant strains

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ABSTRACT

*Objective:* *Campylobacter jejuni* (*C. jejuni*) are among the most frequently identified bacteria associated with human gastroenteritis worldwide. Exposure to antibiotics may induce or inhibit biofilm formation in some bacterial species. Little work has been reported on the influence of antibiotics on biofilm formation by *C. jejuni*.
*Methods:* This study investigated the effect of six different classes of antibiotics with different modes of action (ampicillin, ciprofloxacin, erythromycin, nalidixic acid, rifampicin and tetracycline) on biofilm formation in vitro by seven *C. jejuni* from poultry with different antibiotic resistance profiles.
*Results:* The results indicated that in the presence of most of the tested antibiotics, biofilm formation by *C. jejuni* strains, which are resistant to them, was reduced but biofilm formation in sensitive strains was increased.
*Conclusion:* The ability of certain antibiotics to induce biofilm formation by a tested *C. jejuni* strain is of concern, with respect to the effective control of disease caused by this pathogen; however, further work is required to confirm how widespread this feature is.


## 1. Introduction

*Campylobacter jejuni* (*C. jejuni*) is commonly found in the gastrointestinal tract of poultry and is also frequently associated with human gastroenteritis worldwide [1]. *Campylobacter jejuni* is fastidious, and requires specific atmospheres and temperatures to grow [2]. Biofilm formation has been suggested to facilitate its survival and growth in its hosts and the environment [3].

The widespread use of antibiotics to fight infectious disease has led to the emergence of many antibiotic-resistant bacteria, which have become a global problem. Studies have shown that there is a significant increase in antibiotic-resistant *C. jejuni* strains isolated from humans and poultry, and this has been suggested to be associated with the treatment of poultry with antibiotics [4].

Previous studies have reported that the presence of certain antibiotics will influence bacterial biofilm formation by either inducing or inhibiting it [5]. For example, tetracycline has been shown to induce biofilm formation in *Escherichia coli* [6] and *Pseudomonas aeruginosa* [7]. Rifampicin and erythromycin have been shown to induce biofilm formation in *Staphylococcus epidermidis* [8]. Ciprofloxacin and ampicillin have been shown to induce biofilm formation in *Staphylococcus intermedius* [9]. On the other hand, the presence of sub-minimum inhibitory concentrations (MICs) of ciprofloxacin has been reported to inhibit biofilm formation by *Pseudomonas aeruginosa* and *Salmonella enterica* serovar typhimurium [10]. Since antibiotics are commonly used to treat and prevent infections in poultry, large amounts of antibiotics may be released in natural ecosystems and persist in poultry-related environments [11]. This might in turn affect the biofilm formation by the bacteria found in these environments, including *C. jejuni*. Previous studies that have examined the effects of antibiotics at sub-MICs on biofilm formation have largely focused on *Staphylococcus epidermidis, Staphylococcus aureus* and *Pseudomonas aeruginosa* [5].

Little work has been reported on the influence of antibiotics on biofilm formation by *C. jejuni*. An effect of antibiotics at sub-MIC levels on biofilm formation by resistant *C. jejuni* was noted as part

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of a broader phenotypic study on this pathogen [12]. The current study investigated the effect of different concentrations of antibiotics with different modes of action on biofilm formation by selected antibiotic-resistant *C. jejuni* isolated from poultry.

## 2. Material and methods

### 2.1. Bacterial strains and growth conditions

Seven *C. jejuni* strains (2862, 2863, 2865, 2866, 2868, 2869 and 2871) independently isolated from poultry obtained from retail outlets in Malaysia [13] and *C. jejuni* ATCC 33291 (Manassas, USA) were used in this study. Whole genome sequences of three of the *C. jejuni* strains (2865, 2868 and 2871) have been previously reported [14]. All the strains were maintained and resuscitated as previously described [15].

### 2.2. Antibiotic susceptibility test

Antibiotic susceptibility of the seven *C. jejuni* strains from poultry against the antibiotics listed in Table 1 has previously been reported in studies in which the MIC was determined using the Sensititre *Campylobacter* MIC plate following manufacturer's instructions (Trek Diagnostic Systems, East Grinstead, UK) at 37 °C [13]. Antibiotic susceptibility of *C. jejuni* ATCC 33291 was determined using the paper disk agar diffusion method on Mueller-Hinton agar (Oxoid, UK) [16]. Antibiotic susceptibility testing against rifampicin was not performed as *C. jejuni* is intrinsically resistant to this antibiotic, which is often used as a selective agent in culture medium for isolating *C. jejuni* [17].

### 2.3. Assessment of biofilm formation

The ability of the *C. jejuni* strains to form biofilms in the presence of different antibiotics was determined in 96-well polystyrene microtiter plates, as previously described with slight modifications [15]. Briefly, the strains were grown as sessile cultures under microaerobic conditions for 48 h at 37 °C. After incubation, the colonies on the agar plates were harvested by suspending in 5 mL of Mueller-Hinton broth (MHB) (Oxoid, UK). The cells were then diluted to a concentration of ~$10^7$ CFU/mL in MHB containing antibiotics at different concentrations and a 200 μL aliquot was transferred to a microtiter plate well. Six wells were used for each treatment and a further six wells were filled with uninoculated medium, which served as negative control, and inoculated medium without antibiotic, which served as positive control. The list of antibiotics and their respective tested concentrations is shown in Table 1. The plates were then incubated at 37 °C for 4 or 6 days statically under microaerobic conditions. After incubation, optical density (OD) of cell growths was measured using a microplate reader (Tecan, Switzerland) at 600 nm. The supernatant was removed and biofilm formation examined using crystal violet staining, with the absorbance determined at 550 nm using a microplate reader. Biofilm formation was reported as specific biofilm formation (SBF) = (AB − CW)/G, where AB is the $OD_{550nm}$ of attached and stained bacteria, CW is the $OD_{550nm}$ of stained control wells containing bacteria-free medium, and G is the $OD_{600nm}$ of cells growth in broth [18].

### 2.4. Statistical analysis

All experiments were performed in triplicate with independently grown cultures. All statistical analysis was performed using SPSS 18 software (PASW Statistics 18; SPSS Inc.). One way ANOVA with Tukey's post hoc test at a 95% confidence level was performed on all data sets.

## 3. Results and discussion

Antibiotic resistance profiles of *C. jejuni* strains used in this study are shown in Table 2. From the obtained results, all seven *C. jejuni* strains isolated from poultry were resistant to all tested antibiotics except strains 2862 and 2865, which were sensitive to erythromycin (ERY). *Campylobacter jejuni* ATCC 33291 was sensitive to four out of the six tested antibiotics, except rifampicin (RIF) and ampicillin (AMP). Previous studies have shown that some antibiotics can significantly induce biofilm formation in some bacteria when present at concentrations below the MIC [5]. Since all the seven *C. jejuni* strains used in this study were resistant to most of the six tested antibiotics, concentrations of antibiotics that represented the CLSI guideline MIC interpretive criteria [16,19] were used along with $^1/_2$ MIC and 2 × MIC to investigate the effect of different antibiotics concentrations on biofilm formation (Table 1).

In a preliminary study (data not shown), biofilm formation by all of the *C. jejuni* strains at 2 days, 4 days and 6 days of growth were

**Table 2**
Antimicrobial resistance profile of *Campylobacter jejuni* strains used in this study.

| Bacterial strains | Antimicrobial resistance profile | | | | | |
|---|---|---|---|---|---|---|
| | TET | RIF | CIP | AMP | NAL | ERY |
| 2862 | R | R | R | R | R | S |
| 2863 | R | R | R | R | R | R |
| 2865 | R | R | R | R | R | S |
| 2866 | R | R | R | R | R | R |
| 2868 | R | R | R | R | R | R |
| 2869 | R | R | R | R | R | R |
| 2871 | R | R | R | R | R | R |
| ATCC 33291 | S | R | S | R | S | S |

R represents resistant and S represents sensitive for antimicrobial agents: tetracycline (TET), rifampicin (RIF), ciprofloxacin (CIP), ampicillin (AMP), nalidixic acid (NAL), and erythromycin (ERY).

**Table 1**
Types of antibiotics and their respective concentrations used in the study.

| Class | Antibiotic | Modes of antimicrobial action | MIC interpretive criteria (μg/mL)[a] | Concentrations used (μg/mL) |
|---|---|---|---|---|
| Tetracyclines | Tetracycline (Sigma-Aldrich, USA) | Interferes with protein synthesis by binding to 30S ribosomal subunit | R: ≥16 | 8, 16, 32 |
| Rifamycins | Rifampicin (Nacalai Tesque, USA) | Interferes with bacteria RNA synthesis | R: ≥4 | 2, 4, 8 |
| Fluoroquinolones | Ciprofloxacin (LKT Laboratories, USA) | Interferes with bacteria DNA synthesis | R: ≥4 | 2, 4, 8 |
| β-lactams | Ampicillin (Sigma-Aldrich, USA) | Interferes with cell wall synthesis | R: ≥32 | 16, 32, 64 |
| Fluoroquinolones | Nalidixic acid (Acros Organics, Belgium) | Interferes with bacteria DNA synthesis | R: ≥32 | 16, 32, 64 |
| Macrolides | Erythromycin (Sigma-Aldrich, USA) | Inhibits protein synthesis by binding to 50S ribosomal subunits | R: ≥32 | 16, 32, 64 |

[a] MIC interpretive criteria is based on CLSI guidelines [16,19] and R represents resistance.

examined. The results showed that there was no biofilm formed after 2 days, while higher levels of biofilm formation were observed in most of the strains (seven of the eight tested strains) after 6 days of incubation as compared with 4 days of incubation. Therefore, in this experiment, biofilm formation by the *C. jejuni* strains in the presence of different antibiotics was assessed after 4 days and 6 days of incubation. Biofilm formation by the *C. jejuni* strains in the presence of different antibiotics at various concentrations are shown in Figs. 1 and 2. The specific biofilm formation (SBF) index was used, as it incorporates bacterial growth and biofilm formation in a single parameter [18]. This is an appropriate formula with which to evaluate biofilm formation by the strains in response to different antibiotics with various concentrations that may affect their cell growth. Biofilm formation by most of the seven strains isolated from poultry was significantly lower in comparison with the control in the presence of AMP, RIF, tetracycline (TET), ciprofloxacin (CIP) and nalidixic acid (NAL) after either 4 or 6 days of incubation at one or more of the different tested antibiotic concentrations (Figs. 1 and 2). Although some of these antibiotics have been shown to induce biofilm formation in some bacteria, they also inhibit biofilm formation in others. Sub-MICs of certain antibiotics, especially those acting on the cell wall or on ribosomes, can inhibit biofilm formation by disrupting the adhesion capacity of the bacteria [20]. Biofilm formation by *Pseudomonas aeruginosa* and *Salmonella enterica* serovar typhimurium was reported to be inhibited in the presence of sub-MICs of ciprofloxacin [10].

The current results showed that these antibiotics do not have any effect on biofilm formation by certain strains (TET and NAL on strain 2863; CIP on strains 2863 and 2866; RIF on strain 2868; and AMP on strain 2865). These results suggest that the effect of these antibiotics on inhibiting biofilm formation may be strain dependent. It is interesting to note that ERY did not affect biofilm formation by strains 2863, 2865 and 2866, but reduced biofilm formation by strains 2869, 2868 and 2871 at lower concentrations only. Conversely, biofilm formation by strains 2862 and 2871 was significantly increased as compared with the control in the presence of higher concentrations of ERY (64 μg/mL). The method used to determine sensitivity to the antibiotics indicated that strain 2862 was sensitive to 32 μg/mL of ERY. The formation of biofilm was therefore surprising, but was verified a number of times. A reason for this has been suggested in previous studies, which have proposed that some antibiotics can act as antagonists of biofilm formation (and growth) at low concentrations, agonists at higher concentrations, and antagonists at still higher levels [5].

Strain ATCC 33291 behaved differently to the other strains in this study (Figs. 1 and 2). Tetracycline, CIP, NAL and ERY, which resulted in a reduction or no significant changes in biofilm formation by other strains, were found to increase biofilm formation by ATCC 33291 (which is sensitive to all these antibiotics), as compared with the positive control. There was no significant difference in SBF by the strain in the presence of RIF and AMP, which the strain is resistant to, as compared with the positive control. Similarly, a previous study found that streptomycin induces biofilm formation in a streptomycin-sensitive *Escherichia coli* strain but not in a streptomycin-resistant strain [6]. These results suggest that the effect of antibiotics in inducing or inhibiting biofilm formation differs between antibiotic-sensitive and antibiotic-resistant strains. The exact mechanism responsible for biofilm inhibition and induction of *C. jejuni* in the presence of antibiotics remains to be characterised.

In conclusion, the current results show that most of the antibiotics tested in this study significantly reduce biofilm formation by *C. jejuni* strains, which are resistant to them. The results also suggested that it is possible that antibiotics may increase biofilm formation in the *C. jejuni* strains, which are sensitive to those examined in this study; however, further work is required to validate this. Interestingly, the presence of erythromycin at higher concentrations was also able to significantly increase biofilm formation by one of the tested resistant strains. The results reported in this study indicate that the presence of antibiotics as a result of their widespread use to treat or prevent diseases might

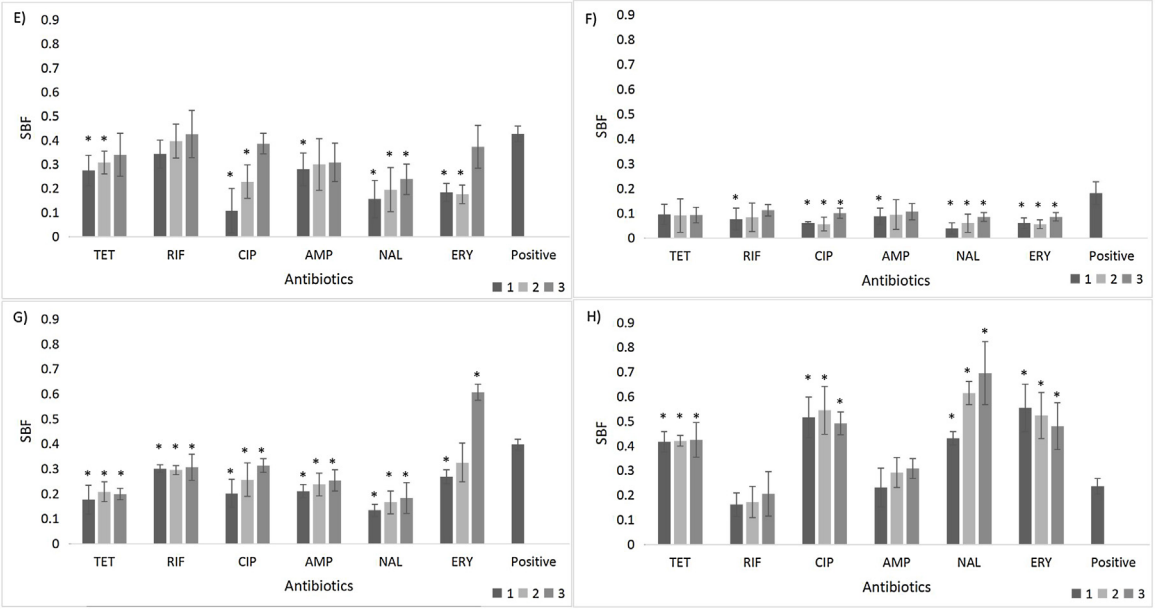


**Fig. 1.** Specific biofilm formation (SBF) by *Campylobacter jejuni* strains. (A) 2862; (B) 2863; (C) 2865; (D) 2866; (E) 2868, (F) 2869; (G) 2871; (H) ATCC 33291, in the presence of different antibiotics at different concentrations after 4 days of incubation.
All results are presented in mean ± SD where n = 3.
*Significant difference ($P < 0.05$) in specific biofilm formation as compared with positive control (biofilm formed in medium without antibiotics).
1, 2, 3 represents different concentrations of antibiotics used, as indicated in Table 1, where 1 represents 1/2 MIC, 2 represents MIC and 3 represents 2 × MIC.
SBF, specific biofilm formation; TET, tetracycline; RIF, rifampicin; CIP, ciprofloxacin; AMP, ampicillin; NAL, nalidixic acid; ERY, erythromycin.

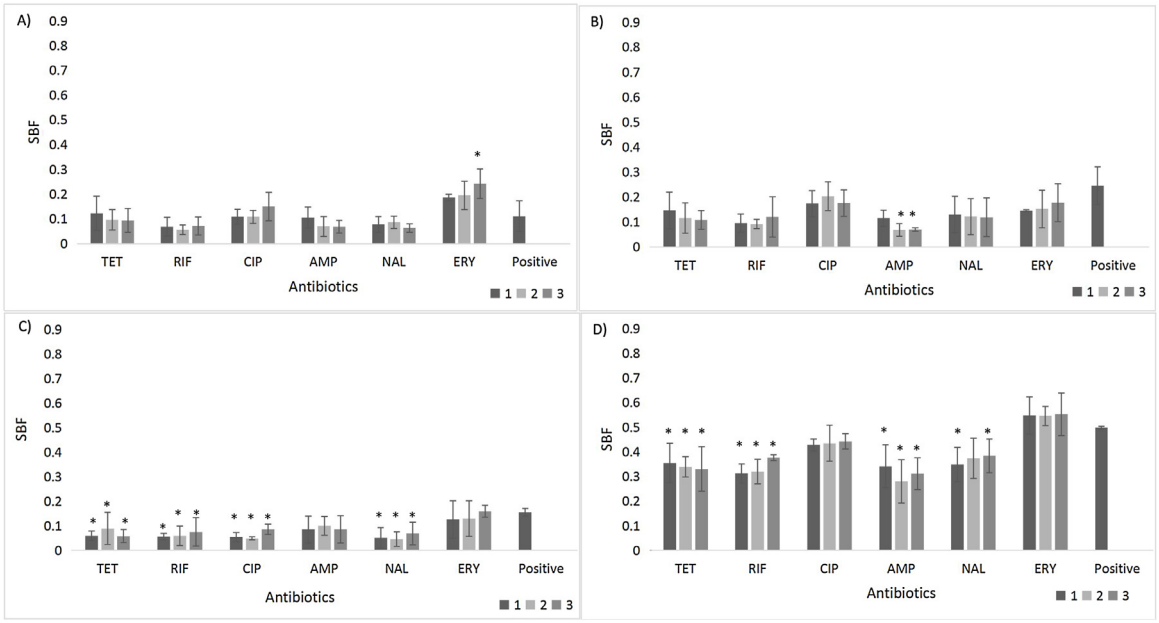


**Fig. 2.** Specific biofilm formation by *C. jejuni* strains: (A) 2862; (B) 2863; (C) 2865; (D) 2866; (E) 2868, (F) 2869; (G) 2871; (H) ATCC 33291 in the presence of different antibiotics at different concentrations after 6 days of incubation.
All results are presented in mean ± SD where n = 3.
*Significant difference ($P < 0.05$) in specific biofilm formation as compared with positive control (biofilm formed in medium without antibiotics).
1, 2, 3 represents different concentrations of antibiotics used, as indicated in Table 1, where 1 represents 1/2 MIC, 2 represents MIC and 3 represents 2 × MIC.
SBF, specific biofilm formation; TET, tetracycline; RIF, rifampicin; CIP, ciprofloxacin; AMP, ampicillin; NAL, nalidixic acid; ERY, erythromycin.

lead to induction of biofilm formation by some strains of *C. jejuni*, which may pose a threat to public health. Further studies need to be carried out in order to investigate the mechanism of biofilm inhibition or induction in the presence of antibiotics.

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## Competing interests

None declared.

## Ethical approval

Not required.

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