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Evaluation of selected phenotypic features among *Campylobacter* sp. strains of animal origin

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ABSTRACT

A total of 43 *Campylobacter* isolates from poultry, cattle and pigs were investigated for their ability to form biofilm. The studied strains were also screened for motility, adhesion and invasion of Caco-2 cells as well as extracellular DNase activity. The relation between biofilm formation and selected phenotypes was examined. Biofilm formation by the tested strains was found as irrespective from their motility and not associated with colonization abilities of human Caco-2 cells. Results of our study show that *Campylobacter* isolates from various animal sources are able to form biofilm and invade human Caco-2 cells *in vitro*.

1. Introduction

It is commonly considered that in nature most bacteria exist in the form of biofilm rather than as free-swimming (planktonic) cells (O'Toole et al., 2000). Biofilm production significantly increases the ability of bacteria to survive in extreme conditions and is a possible means of their persistence and transmission (Haddock et al., 2010). Organisms within biofilm structures exhibit changes in metabolic activity and increased tolerance to various stress factors, including enhanced resistance to antimicrobial agents (Murphy et al., 2006; Reuter et al., 2010).

Campylobacter can form biofilm in most settings: natural, industrial and under laboratory conditions (Reeser et al., 2007). In laboratory conditions, *C. jejuni* was shown to form monospecies biofilm in liquid culture (Joshua et al., 2006). Such biofilm was determined to attach to a variety of surfaces, including stainless steel, glass, nitrocellulose membranes and various plastic surfaces (Teh et al., 2010). Haddock et al. (2010) showed that *C. jejuni* 81–176 strain can form microcolonies as well as biofilm on human intestinal tissues. It was suggested that this property may be an essential step in the ability of the strain to cause diarrhea in humans (Haddock et al., 2010). The primary source of human infection is considered to be consumption or handling of contaminated chicken. In poultry houses, *C. jejuni* was reported to be present in the biofilm formed in drinking vessels, which may be a potential route of bird infection (Zimmer et al., 2003). It is believed that *Campylobacter* persistence, possible way of transmission and survival outside the host is facilitated by attachment to pre-established biofilms (Hanning et al., 2008). Gastrointestinal tract of other food animal species has also been shown to be colonized with *C. jejuni* and *C. coli*. The digestive tract of healthy cattle has been demonstrated to be a significant reservoir of a number of *Campylobacter* species; yet, pig carcasses have been shown to be more frequently contaminated than beef (Moore et al., 2005; Nesbakken et al., 2003). In Poland, the prevalence of *Campylobacter* was detected in 14.9% of cattle and 29.9% of pig meat samples (Wieczorek and Osek, 2013).

The aim of this study was to investigate motility, biofilm formation and DNase production of *Campylobacter* strains isolated from various animal sources. Differences in phenotypes between strains as well as animal source were evaluated. Moreover, we selected a group of strong and moderate biofilm producing strains and another group comprising DNase positive strains. Correlations between biofilm formation and motility, adhesion or invasion of Caco-2 cells by the chosen *Campylobacter* strains were also investigated.

2. Materials and methods

2.1. Bacterial strains

A total of 43 *Campylobacter* strains isolated from cattle, pigs and poultry were tested. Among them, 9 (21%) were isolated from pigs, 10 (23%) were isolated from cattle and 24 (56%) from poultry; 14 strains (33%) belonged to *Campylobacter coli*, 29 (67%) to *Campylobacter jejuni* species. As control strain, the 81176 *Campylobacter jejuni* human isolate was used.

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Strains were grown under microaerobic conditions at 37 °C on Columbia agar containing 5% (v/v) sheep blood.

2.2. Motility assay

Campylobacter culture (3 µl) at OD_{600} 0.5 was spotted onto Mueller-Hinton (MH) medium with 0.4% agar, left to dry and then incubated under microaerobic conditions for 48 h at 37 °C.

2.3. Biofilm assay

C. jejuni strains were grown overnight in Mueller-Hinton broth (MHB) at 37 °C under microaerobic conditions. Wells of the 24-well polystyrene plates (BectonDickinson) containing 1 ml of MHB were inoculated with *Campylobacter* culture at OD_{600} 0.02. After incubation, the medium was removed, wells were washed and dried for 30 min at 55 °C, and 1 ml of 0.1% crystal violet (CV) was added and kept for 5 min at room temperature (RT). Then, the unbound CV was removed, wells were washed twice with H_2O, and dried at 55 °C for 15 min. Bound CV was decolorized with 96% ethanol. The absorbance at 570 nm (A_{570}) was measured using a BioMate spectrophotometer (Thermo Scientific) to determine biofilm formation. Each strain was tested in triplicate on three separate occasions. Control wells without bacterial cell culture were treated as described above. Results obtained for each strain were interpreted as follows: non-biofilm producers ($A_{570} \leq A_{570control} - 0.2$); weak biofilm producers ($A_{570control} < A_{570} \leq 2 \times A_{570control}$); moderate biofilm producers ($2 \times A_{570control} < A_{570} \leq 4 \times A_{570control}$); strong biofilm producers ($4 \times A_{570control} < A_{570}$) (Darwish and Asfour, 2013).

Biofilms were visualized in independent experiments using Field Emission Scanning Electron Microscopy (FE SEM). Briefly, *Campylobacter* strains were grown on Columbia agar plates for 24 h. Cells were harvested, suspended in 5 ml BHI broth (OD_{600} 0.05) and cultivated for 48 h at 37 °C in 5% CO_2 on cover slides placed in Petri dishes. Biofilms were fixed for 24 h in 0.1 M cacodylate buffer (pH 7.3) with 3% glutaraldehyde followed by 60–min wash in cacodylate buffer without glutaraldehyde, and four further 30–min washes in fresh buffer, followed by subsequent dehydration for 6 h in 96% ethanol and drying at RT. Finally, biofilms were plasma coated with gold-palladium (circa 2–4 nm thick) and analyzed at nanometer image resolution by FE SEM (MERLIN Carl Zeiss Germany) at 2–5 kV range accelerating voltage.

2.4. Culturing of Caco-2 cells

Caco-2 cells, derived from a human colonic carcinoma, were obtained from the American Type Culture Collection (ATCC HTB-37). Cells were routinely cultured in Eagle's minimum essential medium with Earle's salts and 2 mM L-glutamine, 20% fetal bovine serum, 0.1 mM nonessential amino acids and 1 mM sodium pyruvate at 37 °C in a 5% CO_2, 95% air atmosphere.

2.5. Adhesion and invasion assays

Campylobacter strains were grown in Columbia agar containing 5% (v/v) sheep blood under microaerophilic conditions at 37 °C. Caco-2 cells were seeded into a 24-well tissue culture dish and grown overnight, as described above, to a cell density of ca. 10^5 cells per well. Bacteria were added into the wells at MOI of 100 bacteria to one epithelial cell. Infected monolayers were incubated for 2 h to allow adhesion and for invasion to occur. Monolayers were then washed three times with sterile PBS and incubated for 2 h with MEM supplemented with 100 µg ml^{-1} gentamicin to kill extracellular bacteria. All studied strains were sensitive to gentamicin (MIC ≤ 2 µg ml^{-1}). Other monolayers containing MEM without gentamicin served as control wells indicating the total number of *Campylobacter* strains both adhering to and invading the epithelial cell monolayers. Subsequently, monolayers were washed as described above and lysed with 0.1% Triton X-100 in PBS for 15 min at room temperature. Adherent and invasive bacteria were enumerated by colony number on BHI agar cultured under microaerophilic conditions.

2.6. DNase activity assay

DNase activity was determined using the DNase Test Agar (Graso). Positive results were visible as a clear zone around bacterial growth.

In the assay, 0.5 µg of *Campylobacter* chromosomal DNA were added to 40 µl of filter-sterilized supernatant from bacterial cultures. Samples were incubated 1 h at room temperature and visualized on agarose gels (0.8%).

2.7. DNA preparation and PCR

Chromosomal DNA was extracted from the tested *Campylobacter* isolates using Genomic Mini (A&A Biotechnology). The *C. jejuni CJE0256*, *CJE0566* and *CJE1441* genes were amplified by PCR using CJE256r 5′-ATAAGCGTTTTAATACTTGCT-3′, CJE256f 5′-TCATCCATA GGGTATTGTTT-3′, CJE566r 5′-TTGTTATCCACTCTAGCTTT- 3′, CJR566f 5′-TCACAATCAATCTTATATTTTCT-3′, CJR1441r 5′-CTTTAT TATCCACTCTA-3′ and CJR1441f 5′-GTCACATTTTACTTTAATATT CTT-3′ primers. PCR was performed using standardized cycling parameters: 95 °C for 1 min initial denaturation followed by 25 cycles of denaturation at 95 °C for 30 s, variable annealing (45 °C - *CJE0256*, *CJE0566* genes and 47 °C - *CJE1441* gene) for 30 s, primer extension step at 72 °C for 30 s, and final extension step 72 °C for 5 min. Results were visualized on agarose gels (0.8%).

2.8. Statistical analysis

Statistical significance of the data was calculated using the Student *t*-test, assuming equal variance. Results were considered to be statistically significant when P was < 0.05.

3. Results

3.1. Biofilm formation by Campylobacter isolates

To identify biofilm-forming strains among *Campylobacter* strains of various animal origin, the CV biofilm assay was performed and absorbance measurements were compared with the median values of the negative control sample (MH broth). Based on obtained results, strains were divided based on Darwish and Asfour (2013) into groups comprising: weak, medium and strong biofilm producers. In total, 43 strains from various sources, including cattle (n = 10), poultry (n = 24) and pigs (n = 9), were analyzed. Overall, 95% (41/43) of the tested *Campylobacter* strains were found to produce biofilm. Most of them (67%; 29/43), were weak biofilm producers, while for 16% (7/43) and 12% (5/43) of the strains moderate or strong biofilm production was observed, respectively (Fig. 1). In terms of the analyzed *Campylobacter* species, 57% (8/14) of *C. coli* strains and only 14% (4/29) of *C. jejuni* strains were moderate or strong biofilm producers ($P \leq 0.01$). Among *C. jejuni,* strains isolated from cattle ($P \leq 0.01$) were the most efficient biofilm producers, while no statistically significant differences among *C. coli* strains isolated from poultry and pigs were noted. Out of the 41 biofilm producing strains, 40% (4/10) of the strains isolated from cattle, 55% (5/9) of the strains isolated from pigs, and only 12.5% (3/24) of poultry strains were found to be moderate or strong biofilm producers (Fig. 1).

Field Emission Scanning Electron Microscopy (FESEM) allowed for a more precise visualization of biofilm formation by *Campylobacter* cells. Both weak, moderate and strong biofilm producer strains possessed flagella and adhered to the tested surface (Fig. 2ABCDEF).