An Improved Culture Method for Selective Isolation of *Campylobacter jejuni* from Wastewater

Jinyong Kim¹, Euna Oh¹, Graham S. Banting¹,², Shannon Braithwaite¹,², Linda Chui²,³, Nicholas J. Ashbolt¹,², Norman F. Neumann¹,² and Byeonghwa Jeon¹*

¹ School of Public Health, University of Alberta, Edmonton, AB, Canada, ² Provincial Laboratory for Public Health, Alberta Health Services, Edmonton, AB, Canada, ³ Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, AB, Canada

*Correspondence:
Byeonghwa Jeon
bjeon@ualberta.ca

INTRODUCTION

*Campylobacter* is one of the leading foodborne pathogens worldwide. *C. jejuni* is isolated from a wide range of foods, domestic animals, wildlife, and environmental sources. The currently available culture-based isolation methods are not highly effective for wastewater samples due to the low number of *C. jejuni* in the midst of competing bacteria. To detect and isolate *C. jejuni* from wastewater samples, in this study, we evaluated a few different enrichment conditions using five different antibiotics (i.e., cefoperazone, vancomycin, trimethoprim, polymyxin B, and rifampicin), to which *C. jejuni* is intrinsically resistant. The selectivity of each enrichment condition was measured with C<sub>t</sub> value using quantitative real-time PCR, and multiplex PCR to determine *Campylobacter* species. In addition, the efficacy of *Campylobacter* isolation on different culture media after selective enrichment was examined by growing on Bolton and Preston agar plates. The addition of polymyxin B, rifampicin, or both to the Bolton selective supplements enhanced the selective isolation of *C. jejuni*. The results of 16S rDNA sequencing also revealed that *Enterococcus* spp. and *Pseudomonas aeruginosa* are major competing bacteria in the enrichment conditions. Although it is known to be difficult to isolate *Campylobacter* from samples with heavy contamination, this study well exhibited that the manipulation of antibiotic selective pressure improves the isolation efficiency of fastidious *Campylobacter* from wastewater.

Keywords: *Campylobacter jejuni*, wastewater, isolation, antibiotics, qRT-PCR
complication (Hughes and Cornblath, 2005). Among pathogenic Campylobacter species, C. jejuni and C. coli are most frequently associated with human infection (Kakoush et al., 2015). Thus far, the consumption of contaminated poultry is the primary cause of developing human campylobacteriosis (Whitey et al., 2013).

Despite the well-known fastidious nature of Campylobacter (Silva et al., 2011), Campylobacter is isolated from environmental sources, such as lake, river, sea, and sewage, suggesting that environmental water is a possible vehicle that transmits Campylobacter to humans (Jones, 2001). C. jejuni is the pathogenic species that is mainly related to water-borne campylobacteriosis worldwide (Pitkanen, 2013). In Canada, Campylobacter outbreaks caused by cross contamination related with meltwater and heavy rainfall are problematic to public health (Millson et al., 1991; Clark et al., 2003). However, the isolation of Campylobacter implicated in water-borne outbreak appear to be challenging, not only due to rapid loss in culturability of isolates from the environment (Wingerd and Flemming, 2011) and from stool samples (Bullman et al., 2012), but also due to the time gap between the initial infection and outbreak investigation (Hanninen et al., 2003; Jakopanec et al., 2008). Therefore, regular monitoring system of water resources by using culture-based methods is likely to underestimate the prevalence of Campylobacter spp. in the environment. This might mislead our understanding of the role played by the environmental sources in human infection and possibly the contamination of food chain by Campylobacter, even though Campylobacter is most frequently detected in animal fecal samples (29.7%), untreated human sewage (25.6%), and surface water (26.6%), according to a study in Alberta, Canada, among the three major foodborne pathogens, including Campylobacter, Salmonella, and Escherichia coli O157:H7 (Jokinen et al., 2011).

Various culture supplements have been examined to improve selective isolation of Campylobacter spp. (Corry et al., 1995). For example, ISO method 2005 has been applied for the detection of thermo-tolerant campylobacters from water, and alternative culture-based methods in combination with molecular end-point confirmation (Hokajarvi et al., 2013; Pitkanen, 2013). Sample volume, incubation time, enrichment volume, passage of enrichment, and PCR-primer specificity all play an important role (Levesque et al., 2011) and enrichment procedures as well (Rossef et al., 2001). Khan et al. (2009, 2013) compared two methods (i.e., centrifugation vs. membrane filtration) for the isolation and detection of Campylobacter from agriculture watersheds, and reported the effect of incubation temperatures and several antibiotics, to which C. jejuni is intrinsically resistant, we developed an improved enrichment method to recover culturable C. jejuni from wastewater samples. The efficiency of Campylobacter isolation was evaluated using quantitative real-time PCR (qRT-PCR) targeting genus-specific 16S rDNA primers, and a second end-point multiplexed PCR with species-specific primers. By using 16S rDNA amplicon sequencing, in addition, we identified the major bacteria in wastewater that compete with Campylobacter under the selective enrichment conditions.

MATERIALS AND METHODS

Bacterial Strains, Culture Conditions, and Primers

Campylobacter jejuni ATCC 33560 and NCTC 11168 were routinely cultured in Mueller Hinton (MH) media at 42°C under microaerobic conditions (5% O₂, 10% CO₂, and 85% N₂). The primers used in the study are described in Table 1.

Enrichment Conditions for Post Grit Samples from Wastewater Treatment Facilities

Raw sewage samples (post grit influent; PG) were collected from two different wastewater treatment facilities (Pine Creek and Bonnybrook) in Calgary, Alberta, in November and December, 2014. The samples were stored at 4–8°C and processed within 12 h after arrival. The wastewater samples (100 ml) were concentrated by centrifugation at 9000 rpm for 20 min at 4°C (Sorvall RC-5B), and pellets were resuspended in 4 ml of Bolton broth (Oxoid) for further enrichment process as described by Chenu et al. (2013) with minor modifications. Briefly, four different kinds of Bolton Broth (Oxoid) enrichment broth were prepared: (1) Bolton’s with Campylobacter-selective supplements [BN; cefoperazone 20 µg/ml, vancomycin 20 µg/ml, trimethoprim 20 µg/ml, and cycloheximide 50 µg/ml, Dalynn], (2) BN plus 10 µg/ml rifampicin [BNR], (3) BN plus 5 IU/ml polymyxin B [BNP], and (4) BN with both rifampicin and polymyxin B [BNRP]. Independently, 1 ml of pellet suspension was transferred to three wells in a 96-well plate and serially
diluted to determine most probable number (MPN). For the 1st enrichment procedure, the plates were incubated at 37 or 42°C for 40–48 h under microaerobic conditions. Then, the culture broths were transferred to a 2nd enrichment medium consisting of the same antimicrobial supplements with 150 μg/ml 2,3,5-triphenyl-tetrazolium chloride (TTC, Sigma) and incubated for 24 h. TTC is a color indicator to show metabolic activity, and the inclusion of the dye in the assay aids in detection of levels of growth (Gabrielson et al., 2002). The cultures were subject to qRT-PCR and multiplex PCR.

**Validation of C. jejuni Growth with Antibiotic Supplements**

*C. jejuni* ATCC 33560, which is a quality control (QC) strain for antimicrobial susceptibility testing of *C. jejuni* (Clinical and Laboratory Standards Institute [CLSI], 2010), and NCTC 11168 were employed to evaluate the growth capability of *C. jejuni* under different enrichment conditions. Four different kinds of enrichment broth were prepared as described above. *C. jejuni* ATCC 33560 and NCTC 11168 were cultured on MH agar plates at 42°C for 24 h and harvested with fresh MH broth. The bacterial suspension was adjusted to an OD$_{600}$ of 0.07 and incubated at 42°C with shaking at 200 rpm under microaerobic conditions. To determine the growth of *C. jejuni* strains, the samples were taken at 0, 3, 6, 12, and 24 h, and CFU and OD$_{600}$ values were measured.

**Confirmation of Campylobacter Growth using qRT-PCR**

To confirm if *Campylobacter* was successfully enriched, 50 μl of culture broth was transferred to 96 well PCR plates and heated to 95°C for 10 min to extract DNA. Quantitative PCR was performed using an ABI 7500 (Applied Biosystems) system with *Campylobacter* genus-specific 16S rDNA primers (de Boer et al., 2013). The internal control template (IAC) and primers were included in reaction mixtures to measure inhibitory effects in enrichment samples (Deer et al., 2010). Amplification was carried out with following conditions: 50°C for 2 min and 95°C for 30 s; 40 cycles at 95°C for 3 s and 60°C for 30 s. Ct values were evaluated to determine the growth of *Campylobacter* and 3-tube MPN estimates.

**Confirmation of Campylobacter spp. using Multiplex PCR**

To identify *Campylobacter* spp. in the enrichment broths, multiplex PCR was performed for 42°C enrichment broths as described elsewhere with primer sets for 16S rDNA and six species-specific primers (Yamazaki-Matsune et al., 2007). Same templates used in qRT-PCR were also employed for multiplex PCR. The amplification reaction was performed following conditions: 95°C for 15 min; 40 cycles at 95°C for 30 s, 58°C for 1 min and 30 s, 72°C for 1 min; 72°C for 7 min.

**Isolation of Campylobacter spp. and Identification of Non-Campylobacter Isolates by 16S rRNA Sequencing from Wastewater**

To isolate *Campylobacter* spp. from enrichment cultures, wells showing the lowest Ct value in qRT-PCR results in the 2nd enrichment plate were selected. The cultures were prepared with 10-fold serial dilutions and sub-cultured on Bolton agar plates with Bolton supplement (BB, Dalynn) or Bolton agar plates with Preston supplement (BP, Oxoid). Following 2–3 days incubation at 42°C under microaerobic conditions, several colonies showing different shape, color, and transparency were randomly picked and transferred to the same fresh broth. After 2 days incubation, 50 μl of the cultures was harvested and boiled at 95°C for 10 min. Genus-specific 16S rDNA PCR amplification was carried out to distinguish between *Campylobacter* and non-*Campylobacter* (Linton et al., 1996). Amplification was performed following conditions: 94°C for 1 min; 35 cycles at 94°C for 30 s, 52°C for 30 s, 72°C for 1 min; 72°C for 5 min. PCR amplicons were visualized using 2% agarose gel with SYBR safe DNA gel stain solution (Invitrogen). To identify non-*Campylobacter* competitors growing in the selective enrichment conditions, 16S rDNA was amplified with universal bacterial domain primers (27F and 1492R) for 100 *Campylobacter* genus-specific 16S rDNA negative isolates (Weisburg et al., 1991). Amplification was conducted following conditions: 94°C for 1 min; 35 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 1 min and 30 s; 72°C for 5 min. The amplified PCR products were purified and commercially.

---

**Table 1 | Primers used in this study.**

| Primer       | Sequence (5’→ 3’)       | Reference                           |
|--------------|-------------------------|-------------------------------------|
| *CampyLv1-16S-F* | CCT GAG GCA GCA AC G C   | Weisburg et al., 2013               |
| *CampyLv1-16S-R* | GGG AGT TAG CGG GTG ATT   |                                    |
| *CampyLv1-16S-P* | CTC GGA AAA GTG TCA TCC T |                                    |
| **CampyYM-16S-F** | GGA TCA CAC TTT TCG GAG C | Yamazaki-Matsune et al., 2007       |
| **CampyYM-16S-R** | CAT TGG AGC AGG GT TGC   |                                    |
| **C. hyointest-23S-F** | ATA ATC ATG GTG AGA ATC CTA G |                                    |
| **C. hyointest-23S-R** | GCT TCG CAT AGC TAA CAT |                                    |
| **C. coli-ask-F** | GGT ATG ATT TCT ACA AAG GGA G |                                    |
| **C. coli-ask-R** | ATA AAA GAC TAT CGT GCG GTG |                                    |
| **C. fetus-cstA-F** | GGT AGC GGC AGC TGC TAA GAT |                                    |
| **C. fetus-cstA-R** | ACG CAG TAA GCG ATA TTA TAG TAG |                                    |
| **C. lari-glyA-F** | TAG AGA GAT AGC AAA AGA GA |                                    |
| **C. lari-glyA-R** | TAC ACA TAA TAA TCC CAC CC |                                    |
| **C. jejuni-c0414-F** | CAA ATA AAG TTA GAG GTA GAA TGT |        |
| **C. jejuni-c0414-R** | CGA TAA GCA CTA AGC TGA T |                                    |
| **C. uspal-lpxA-F** | GCA TGG TGT GCA AAT TGA AGC |                                    |
| **C. uspal-lpxA-R** | TTC TAG CCC CTT GTC TGA T |                                    |
| **IAC-F** | CTA ACC TTC GTG ATG AGC AAT CG | Deer et al., 2010                   |
| **IAC-R** | GAT CAG CTA CGT GAG GTG TCT C A |                                    |
| **IAC-P** | ACG TAG TCG ATG CAC TCC AGT |                                    |
| 27F | AGA GTT TGA TCM TCG GTC AG | Weisburg et al., 1991              |
| 1492R | TAC GGG TAC CTT CGT AGC ACT T |                                    |

*Primer used in qRT-PCR to detect Campylobacter genus, **Primer used in qRT-PCR to detect Campylobacter species, ***Primer used as Internal Control template (IAC).*
sequenced by Sanger sequencing method (Macrogen, Inc., South Korea), and the results were analyzed by using Blastn1.

RESULTS

_Campylobacter jejuni_ Growth in the Presence of Additional Antibiotic Supplements

To improve the frequency of _C. jejuni_ isolation from wastewater samples that are heavily contaminated with various microorganisms, we decided to increase antibiotic selective pressure by using different combinations of multiple antibiotics to which _C. jejuni_ is intrinsically resistant (Taylor and Courvalin, 1988; Corry et al., 1995). For the growth testing, we used _C. jejuni_ ATCC 33560, a QC strain for antibiotic susceptibility testing (Clinical and Laboratory Standards Institute [CLSI], 2010), and _C. jejuni_ NCTC 11168, the first genome-sequenced _Campylobacter_ strain (Parkhill et al., 2000). Whereas the Bolton selective supplement (BN) consists of three antibiotics, including cefoperazone, vancomycin, and trimethoprim, the Preston _Campylobacter_-selective supplement contains polymyxin B, rifampicin, and trimethoprim. The two selective supplements for _Campylobacter_ isolation commonly contain trimethoprim. In the experiment, BN was used as basic antimicrobial supplements, and polymyxin B and/or rifampicin were added to BN to increase antibiotic selective pressure. The addition of either polymyxin B or rifampicin to BN did not affect the growth. The supplementation with both rifampicin and polymyxin B slightly reduced the OD$_{600}$ at 12 h; however, there was no significant difference in growth in the four different enrichment conditions (Figure 1). The results indicate that _C. jejuni_ can grow in the presence of combinations of the multiple antibiotics to which _C. jejuni_ is naturally resistant.

\[\text{Ct Values of qRT-PCR in Campylobacter Detection under different Enrichment Conditions}\]

The Ct values of qRT-PCR for the detection of _Campylobacter_ varied depending on the antimicrobial enrichment. The addition of one of the antibiotics (i.e., either rifampicin or

---

1http://blast.ncbi.nlm.nih.gov/Blast.cgi

**FIGURE 1** | Growth of _Campylobacter jejuni_ ATCC 33560 and NCTC 11168 in four different antimicrobial enrichment conditions at 42° C. Measurement of OD$_{600}$ and CFU counting in _C. jejuni_ ATCC 33560 (A,C) and _C. jejuni_ NCTC 11168 (B,D); BN, Bolton broth with Bolton _Campylobacter_-selective supplement; BNR, BN supplemented with rifampicin; BNP, BN supplemented with polymyxin B; BNRP, BN supplemented with rifampicin and polymyxin B.
polymyxin B) significantly decreased the C_i value, meaning that *Campylobacter* population was increased by the selective enrichment. Furthermore, supplementation of both antibiotics showed the lowest C_i value compared to the other enrichment conditions (Figure 2), indicating that the increased antibiotic selective pressure enhanced the enrichment of *Campylobacter* in raw sewage samples. Positive samples were more frequently detected at 42° C than 37° C, and non-interpretable results, where C_i values could not be determined, were sometimes observed at 37° C (data not shown). This suggests that contaminating bacteria cannot be effectively inhibited at 37° C.

**Multiplex PCR Detection of *Campylobacter* spp. under different Enrichment Conditions**

In addition to qRT-PCR detection, multiplex PCR was performed to determine the species of *Campylobacter* isolates. The results of multiplex PCR demonstrated that the primary *Campylobacter* spp. were *C. jejuni* and *C. coli* (Table 2). *C. jejuni* and *C. coli* were more frequently detected by the addition of rifampicin compared to polymyxin B. In many cases, positive results were discrepant between qRT-PCR and multiplex PCR (54% in qRT-PCR in comparison with multiplex PCR). For example, the same sample that was *Campylobacter*-negative based on qRT-PCR was shown to be positive by multiplex PCR (data not shown).

**Enhanced *Campylobacter* Isolation from Raw Sewage by Increased Antibiotic Selective Pressure**

The frequency of *Campylobacter* isolation from raw sewage was determined under the four different antibiotic enrichment conditions. To examine the effect of agar media on the *Campylobacter* isolation, we plated the enrichment cultures on Bolton and Preston agars, common culture media for *Campylobacter*. Consistent with the qRT-PCR results, the addition of rifampicin, polymyxin B, and both antibiotics significantly increased the isolation frequency for *Campylobacter* and decreased the isolation frequency of non-*Campylobacter* (Figure 3). In particular, BNRP showed the highest isolation rate of *Campylobacter*, whereas BN did not recover any *Campylobacter* spp. (Figure 3). Whereas the antibiotic enrichment significantly affected the isolation frequency, Bolton and Preston agar media did not make any differences in the isolation frequency (Figure 3). Morphologically, small pinkish or transparent colonies usually turned out to be *Campylobacter* (data not shown). To identify the major non-*Campylobacter* populations growing on the selective enrichment media, we randomly selected 100 colonies based on colony morphologies and performed 16S rDNA amplicon sequencing. The major non-*Campylobacter* spp. included *Enterococcus, E. coli, Klebsiella, Proteus, and Pseudomonas* (Table 3). The supplementation of additional antibiotics, either single (i.e., BNR and BNP) or both (i.e., BNPR), suppressed the growth of other bacterial populations. However, *Enterococcus* spp., such as *Enterococcus durans* and *Enterococcus faecium*, were still isolated in BNPR (Table 3). Importantly, increased antibiotic selective pressure improved the frequencies of isolating *Campylobacter* from wastewater (Table 3).

**DISCUSSION**

In this study, we improved the efficacy of *C. jejuni* isolation from wastewater by increasing antibiotic selective pressure in the enrichment step. The addition of rifampicin, polymyxin B, or both to the enrichment media affected the C_i values of qRT-PCR results (Figure 2). According to the distribution of C_i values, the addition of the antibiotic(s) decreased C_i values, meaning that antibiotic supplements improved the growth of *Campylobacter*. In particular, rifampicin significantly reduced C_i values (Figure 2). A few studies have thus far reported that increased selective pressure enhances *Campylobacter* isolation from food. Yoo et al. (2014) reported that the addition of
rifampicin (10 µg/ml) or polymyxin B (5 IU/ml) to Bolton agar (Bolton agar with Bolton supplement) restrained the growth of non-Campylobacter without any inhibition of C. jejuni and C. coli in fresh produce foods. Chon et al. (2013) demonstrated that the addition of high concentrations of polymyxin B to the mBolton supplement in enrichment procedure improved the efficiency of C. jejuni and C. coli recovery and suppressed background competing bacteria. Consistently, our results showed that the supplementation with additional antibiotics improved the efficacy of C. jejuni isolation even from heavily contaminated wastewater samples. In addition, we also identified bacterial populations that compete with Campylobacter under the four different selective enrichment conditions. The inputs of Campylobacter entering the influent of wastewater treatment facilities in this study would be primarily from sewage effluent in Calgary and also possibly from wildlife, such as migrating birds (Cody et al., 2015). Depending on the treatment procedure, the incidence rate of Campylobacter in sewage effluent can be altered, and cross contamination between water resources and sewage is associated with water-borne Campylobacter outbreaks (Jones, 2001; Pitkanen, 2013).

The 16S rDNA amplicon sequencing analysis of individual colonies from the enrichment plates revealed that Escherichia, Pseudomonas, Klebsiella, and Enterococcus were the major competing bacteria in C. jejuni isolation from wastewater (Table 3). Baylis et al. (2000) identified competitor organisms in foods by using Preston and Bolton selective supplement media, showing that Yersinia, Enterobacter, Escherichia, Enterococcus, Pseudomonas, and Klebsiella are representative competitors. This is quite similar to our results from the BN enrichment conditions. Escherichia were frequently isolated in BN (Table 3), presumably because extended-spectrum beta-lactamase (ESBL)-producing E. coli may reduce the selectivity of Bolton supplement and consequently E. coli growth would suppress Campylobacter (Moran et al., 2011). Although the supplementation of additional antibiotic(s) suppressed the overgrowth of competing bacteria and enriched Campylobacter, Enterococcus survived well in the presence of five different antibiotics as it was frequently isolated with Campylobacter (Table 3). The survival of Enterococcus in the presence of vancomycin (20 µg/mL) in Bolton supplement indicated that Enterococcus isolated from the enrichment broth is vancomycin-resistant enterococci (VRE), a drug-resistant strain of serious public health concern (Cetinkaya et al., 2000). This study aimed at developing an improved culture method to isolate Campylobacter from wastewater, and we used the influent samples, not the effluent, since the influent is more contaminated than the effluent. Therefore, the results do not provide the information about the level of Campylobacter contamination in the effluent that may have a direct impact on public health compared to the influent data.

In this study, we demonstrated that antibiotic selective pressure and culture temperature are the critical factors for C. jejuni isolation from raw sewage. The BN, BNR, BNP, and BNRP conditions showed similar MPN values at 42°C; however, only BNR showed reasonable MPN values and BNR and BNRP showed relatively lower MPN numbers at 37°C compared to those at 42°C (data not shown). The results exhibited that

![FIGURE 3](image-url) Percentage distribution of Campylobacter and non-Campylobacter isolates in four different enrichment conditions at 42°C. After antimicrobial enrichment, strains were isolated by growing on Bolton agar plates supplemented with Bolton selective supplement (BB; A) and Bolton agar plates supplemented with Preston selective supplement (BP; B). The results are based on PCR detection with primers for Campylobacter 16S rDNA. The number of isolates in BB is as follows; BN 18, BNR 17, BNP 22, and BNRP 25. The number of isolates in BP is as follows; BN 18, BNR 18, BNP 24, and BNRP 25.

![Table 3](image-url) Distribution of Campylobacter and non-Campylobacter strains in four different enrichment conditions at 42°C.

| Species          | BN   | BNR  | BNP  | BNRP |
|------------------|------|------|------|------|
| E. coli          | 9 (25%) | 10 (28.6%) | 1 (2.2%) | 0    |
| E. fergusonii    | 3 (8.3%) | 4 (11.4%) | 0      | 0    |
| E. durans        | 0    | 5 (14.3%) | 7 (15.2%) | 8 (16%) |
| E. faecium       | 4 (11.1%) | 6 (17.1%) | 6 (13%) | 4 (8%) |
| P. aeruginosa    | 5 (13.9%) | 3 (8.6%) | 5 (10.9%) | 0    |
| P. penneri       | 0    | 0    | 1 (2.2%) | 0    |
| P. mirabilis     | 0    | 0    | 4 (8.7%) | 0    |
| K. pneumoniae    | 15 (41.7%) | 0 | 0 | 0    |
| Campylobacter    | 0    | 7 (20%) | 22 (47.8%) | 38 (76%) |
| Total number of isolates | 36 (100%) | 35 (100%) | 46 (100%) | 50 (100%) |
culture temperature also plays an important role in the selective enrichment of \( C. \) jejuni. Humphrey et al. showed the effect of antibiotics and temperature on the recovery rate in cold-damaged \( C. \) jejuni. The sub-lethally injured cells are more sensitive to antibiotics in 43°C than 37°C, affecting the restoration of \( C. \) jejuni (Humphrey, 1986). In previous studies, Humphrey et al. also suggested that pre-incubation at 37°C for 4–18 h followed by 42°C or 37°C incubation for 48 h would be beneficial to the recovery of \textit{Campylobacter} in comparison with 42°C (Humphrey, 1989; Humphrey and Muscat, 1989). Whereas Khan et al. (2013) demonstrated that the detection frequency of \textit{Campylobacter} spp. was higher at 37°C in BN than at 42°C, \( C. \) jejuni was detected more frequently at 42°C than at 37°C. Consistently, our results suggested that 42°C seems to enhance \( C. \) jejuni growth in raw sewage samples.

The additional antibiotic(s) plus an increased incubation temperature (i.e., 42°C) improved the isolation rates of \( C. \) jejuni and \( C. \) coli from heavily contaminated raw sewage samples. The addition of rifampicin and polymyxin B specifies the selective enrichment of thermo-tolerant \textit{Campylobacter} spp., such as \( C. \) jejuni and \( C. \) coli, the major human pathogenic species (Kaakoush et al., 2015). Based on our findings, increased antibiotic selective pressure and culture temperature are the key parameters impacting the success in \( C. \) jejuni isolation from heavily contaminated wastewater samples. Additionally, rifampicin appears to be effective in improving the selectivity of \textit{Campylobacter} enrichment for PCR-based quantification methods, whereas both rifampicin and polymyxin B are required to suppress competing bacterial growth and improve the selectivity of \( C. \) jejuni isolation with culture-based methods.

**AUTHOR CONTRIBUTIONS**

Design of the project: JK, NA, NN, and BJ; Performance of the experiments: JK, EO, GB, and SB; Data analysis: JK, EO, GB, SB, LC, NA, NN, and BJ; Writing of the manuscript: JK, NA, and BJ.

**FUNDING**

This study was supported by Alberta Innovates-Energy and Environment Solutions (AI-EES). The laboratory facilities were supported by the Canada Foundation for Innovation (CFI).

---

**REFERENCES**

Abulreesh, H. H., Paget, T. A., and Goulder, R. (2005). Recovery of thermophilic \textit{Campylobacters} from pond water and sediment and the problem of interference by background bacteria in enrichment culture. \textit{Water Res.} 39, 2877–2882. doi: 10.1016/j.watres.2005.05.004  
Baylis, C. L., Macphee, S., Martin, K. W., Humphrey, T. J., and Betts, R. P. (2000). Comparison of three enrichment media for the isolation of \textit{Campylobacter} spp. from foods. \textit{J. Appl. Microbiol.} 89, 884–891. doi: 10.1046/j.1365-2672.2000.01203.x  
Bullman, S., O’Leary, J., Corcoran, D., Sleator, R. D., and Lucey, B. (2012). Molecular-based detection of non-culturable and emerging campylobacteria in patients presenting with gastroenteritis. \textit{Epidemiol. Infect.} 140, 684–688. doi: 10.1017/S095026881100859  
Cetinkaya, Y., Falk, P., and Mayhall, C. G. (2000). Vancomycin-resistant enterococci. \textit{Clin. Microbiol. Rev.} 13, 686–700. doi: 10.1128/CMR.13.4.686-707.2000  
Chen, J. W., Pavic, A., and Cox, J. M. (2013). A novel miniaturized most probable number method for the enumeration of \textit{Campylobacter} spp. from poultry-associated matrices. \textit{J. Microbiol. Methods} 93, 12–19. doi: 10.1016/j.mimet.2013.01.013  
Chon, J. W., Kim, H., Yun, J. H., Park, J. H., Kim, M. S., and Seo, K. H. (2013). Development of a selective enrichment broth supplemented with bacteriological charcoal and a high concentration of polymyxin B for the detection of \textit{Campylobacter} jejuni and \textit{Campylobacter} coli in chicken carcass rinses. \textit{Int. J. Food. Microbiol.} 162, 308–310. doi: 10.1016/j.ijfoodmicro.2013.01.018  
Clark, C. G., Price, L., Ahmed, R., Woodward, D. L., Melito, P. L., Rodgers, F. G., et al. (2003). Characterization of waterborne outbreak-associated \textit{Campylobacter} jejuni, Walkerton, Ontario. \textit{Emerg. Infect. Dis.} 9, 1232–1241. doi: 10.3201/eid0910.020584  
Clinical and Laboratory Standards Institute [CLSI] (2010). \textit{Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria; Approved Guideline. M45-A2}, 2nd Edn. Wayne, PA: CLSI.  
Cody, A. J., Mccarthy, N. D., Bray, J. E., Wimalarathna, H. M., Colles, F. M., Jansen Van Rensburg, M. J., et al. (2015). Wild bird-associated \textit{Campylobacter} jejuni isolates are a consistent source of human disease, in Oxforshire, United Kingdom. \textit{Environ. Microbiol. Rep.} 7, 782–788. doi: 10.1111/1758-2229.12314  
Corry, J. E. L., Post, D. E., Colin, P., and Laisney, M. J. (1995). Culture media for the isolation of \textit{Campylobacters}. \textit{Int. J. Food. Microbiol.} 26, 43–76. doi: 10.1016/0168-1655(95)00044-K  
de Boer, R. F., Ott, A., Guren, P., Van Zanten, E., Van Belkum, A., and Koostra-Smid, A. M. (2013). Detection of \textit{Campylobacter} species and \textit{Arcobacter butleri} in stool samples by use of real-time multiplex PCR. \textit{J. Clin. Microbiol.} 51, 253–259. doi: 10.1128/JCM.01716-12  
Deer, D. M., Lampel, K. A., and Gonzalez-Escalona, N. (2010). A versatile internal control for use as DNA in real-time PCR and as RNA in real-time reverse transcriptase PCR assays. \textit{Lett. Appl. Microbiol.} 50, 366–372. doi: 10.1111/j.1472-765X.2010.02804.x  
Gabrielson, J., Hart, M., Jarelöv, A., Kühn, J., Mckenzie, D., and Möllby, R. (2002). Evaluation of redox indicators and the use of digital scanners and spectrophotometer for quantification of microbial growth in microplates. \textit{J. Microbiol. Methods} 50, 63–73. doi: 10.1016/S0167-7012(02)00011-8  
Hanninen, M. L., Haajanen, H., Pummi, T., Wermundsden, K., Katila, M. L., Sarkkinen, H., et al. (2003). Detection and typing of \textit{Campylobacter jejuni} and \textit{Campylobacter coli} and analysis of indicator organisms in three waterborne outbreaks in Finland. \textit{Appl. Environ. Microbiol.} 69, 1391–1396. doi: 10.1128/AEM.69.3.1391-1396.2003  
Hokajarvi, A. M., Pitkanen, T., Siiljanen, H. M., Nakari, U. M., Torvinen, E., Siitonen, A., et al. (2013). Occurrence of thermotolerant \textit{Campylobacter} spp. and adenosviruses in Finnish bathing waters and purified sewage effluents. \textit{J. Water Health.} 11, 120–134. doi: 10.2166/wh.2012.192  
Hughes, R. A., and Cornblath, D. R. (2005). Guillain-Barré syndrome. \textit{Lancet} 366, 1653–1666. doi: 10.1016/S0140-6736(05)67665-9  
Humphrey, J. T. (1986). Techniques for the optimum recovery of cold injured \textit{Campylobacter jejuni} from milk or water. \textit{J. Appl. Bacteriol.} 61, 125–132. doi: 10.1111/j.1365-2672.1986.tb04265.x  
Humphrey, J. T. (1989). An appraisal of the efficacy of pre-enrichment for the isolation of \textit{Campylobacter jejuni} from water and food. \textit{J. Appl. Bacteriol.} 66, 119–126. doi: 10.1111/j.1365-2672.1989.tb02461.x  
Humphrey, T. J., and Muscat, I. (1989). Incubation-temperature and the isolation of \textit{Campylobacter jejuni} from food, milk or water. \textit{Lett. Appl. Microbiol.} 9, 137–139. doi: 10.1111/j.1365-7265.1989.tb00308.x  
Jakopanec, I., Borgen, K., Vold, L., Lund, H., Forseth, T., Hannula, R., et al. (2008). A large waterborne outbreak of campylobacteriosis in Norway: the need to focus on distribution system safety. \textit{BMC. Infect. Dis.} 8:128. doi: 10.1186/1471-2334-8-128
Jokinen, C., Edge, T. A., Ho, S., Koning, W., Laing, C., Mauro, W., et al. (2011). Molecular subtypes of Campylobacter spp. Salmonella enterica, and Escherichia coli O157:H7 isolated from faecal and surface water samples in the Oldman River watershed, Alberta, Canada. Water Res. 45, 1247–1257. doi: 10.1016/j.watres.2010.10.002

Jones, K. (2001). Campylobacters in water, sewage and the environment. J. Appl. Microbiol. 90, 685–795. doi: 10.1046/j.1365-2672.2001.01355.x

Kaakoush, N. O., Castano-Rodriguez, N., Mitchell, H. M., and Man, S. I. M. (2015). Global epidemiology of Campylobacter Infection. Clin. Microbiol. Rev. 28, 687–720. doi: 10.1128/CMR.00006-15

Khan, I. U., Hill, S., Nowak, E., and Edge, T. A. (2013). Effect of incubation temperature on the detection of thermophilic Campylobacter species from freshwater beaches, nearby wastewater effluents, and bird fecal droppings. Appl. Environ. Microbiol. 79, 7639–7645. doi: 10.1128/AEM.02324-13

Khan, I. U., Gannon, V., Loughborough, A., Jokinen, C., Kent, R., Koning, W., et al. (2009). A methods comparison for the isolation and detection of thermophilic Campylobacter in agricultural watersheds. J. Microbiol. Methods 79, 307–313. doi: 10.1016/j.mimet.2009.09.024

Kirk, M. D., Pires, S. M., Black, R. E., Caipo, M., Crump, J. A., Develleischauwer, B., et al. (2015). World Health Organization estimates of the global and regional disease burden of 22 foodborne bacterial, protozoal, and viral diseases, 2010: a data synthesis. PLoS Med. 12:e1001921. doi: 10.1371/journal.pmed.1001921

Koenraad, P. M. F. J., Rombouts, F. M., and Notermans, S. H. W. (1996). Rapid identification by PCR of thermophilic Campylobacter. J. Microbiol. Methods 28, 687–720. doi: 10.1128/CMR.00006-15

Law, J. W.-F., Ab Mutalib, N.-S., Chan, K.-G., and Lee, L.-H. (2015). Rapid determination of the optimal culture conditions for detecting thermophilic Campylobacter in environmental water. J. Microbiol. Methods 105, 41–47. doi: 10.1111/j.1756-5508.2015.02000.x

Levesque, S., St-Pierre, K., Frost, E., Arbeit, R. D., and Michaud, S. (2011). Epidemiological aspects of thermophilic Campylobacter in water-related environments: a review. Water Environ. Res. 83, 52–63. doi: 10.2175/106143097x125182

Linton, D., Owen, R. J., and Stanley, J. (1996). Determination of the optimal culture conditions for detecting thermophilic Campylobacters in environmental water. J. Microbiol. Methods 28, 687–720. doi: 10.1128/CMR.00006-15

Linthorst, D., Owen, R. J., and Stanley, J. (1996). Rapid identification by PCR of the genus Campylobacter and of five Campylobacter species enteropathogenic for man and animals. Res. Microbiol. 147, 707–718. doi: 10.1016/S0923-2508(97)85118-2

Millson, M., Bokhout, M., Carlson, J., Spielberg, L., Aldis, R., Borczyk, A., et al. (1991). An outbreak of Campylobacter jejuni gastroenteritis linked to meltwater contamination of a municipal well. Can. J. Public Health 82, 27–31.

Moran, L., Kelly, C., Cormican, M., Mcgettrick, S., and Madden, R. H. (2011). Restoring the selectivity of bolton broth during enrichment for Campylobacter spp. from raw chicken. Lett. Appl. Microbiol. 52, 614–618. doi: 10.1111/l.1472-765X.2011.03046.x

Parkhill, J., Wren, B. W., Mungall, K., Kettle, J. M., Churcher, C., Basham, D., et al. (2000). The genome sequence of the food-borne pathogen Campylobacter jejuni reveals hypervariable sequences. Nature 403, 665–668. doi: 10.1038/3501088

Pitkanen, T. (2013). Review of Campylobacter spp. in drinking and environmental waters. J. Microbiol. Methods 95, 39–47. doi: 10.1016/j.mimet.2013.06.008

Roset, O., Rettedal, G., and Lageide, L. (2001). Thermophilic campylobacters in surface water: a potential risk of campylobacteriosis. Int. J. Environ. Health Res. 11, 321–327. doi: 10.1080/09603120081791

Schrader, C., Schielke, A., Ellerbroek, L., and Johne, R. (2012). PCR inhibitors - occurrence, properties and removal. J. Microbiol. Methods 113, 1014–1026. doi: 10.1111/j.1567-2672.2012.03584.x

Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs, P. A., and Teixeira, P. (2011). Campylobacter spp. as a foodborne pathogen: a review. Front. Microbiol. 2:200. doi: 10.3389/fmicb.2011.00200

Taylor, D. E., and Courvalin, P. (1988). Mechanisms of antibiotic-resistance in Campylobacter species. Antimicrob. Agents Chemother. 32, 1107–1112. doi: 10.1128/AAC.32.8.1107

Van Dyke, M. I., Morton, V. K., Mcclenan, N. L., and Huck, P. M. (2010). The occurrence of Campylobacter in river water and waterfowl within a watershed in southern Ontario, Canada. J. Appl. Microbiol. 109, 1053–1066. doi: 10.1111/j.1365-2672.2010.04730.x

Weisburg, W. G., Barns, S. M., Pelletier, D. A., and Lane, D. J. (1991). 16s ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173, 697–703.

Whiley, H., Van Den Akker, B., Giglio, S., and Bentham, R. (2013). The role of environmental reservoirs in human campylobacterioses. Int. J. Environ. Res. Public Health 10, 5886–5907. doi: 10.3390/ijerph10115886

Wingender, J., and Flemming, H. C. (2011). Biofilms in drinking water and their role as reservoir for pathogens. Int. J. Hyg. Environ. Health 214, 417–423. doi: 10.1016/j.ijheh.2011.05.009

Yamazaki-Matsune, W., Taguchi, M., Seto, K., Kawahara, R., Kawatsu, K., Kumeda, Y., et al. (2007). Development of a multiplex PCR assay for identification of Campylobacter cib, Campylobacter fetus, Campylobacter hyointestinalis subsp hyointestinalis, Campylobacter jejuni, Campylobacter lari and Campylobacter upsaliensis. J. Med. Microbiol. 56, 1467–1473.

Yoo, J. H., Choi, N. Y., Bae, Y. M., Lee, J. S., and Lee, S. Y. (2014). Development of a selective agar plate for the detection of Campylobacter spp. in fresh produce. Int. J. Food Microbiol. 189, 67–74. doi: 10.1016/j.ijfoodmicro.2014.07.032

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2016 Kim, Oh, Banting, Braithwaite, Chui, Ashbolt, Neumann and Jeon. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.