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Triplex qPCR assay for *Campylobacter jejuni* and *Campylobacter coli* monitoring in wastewater

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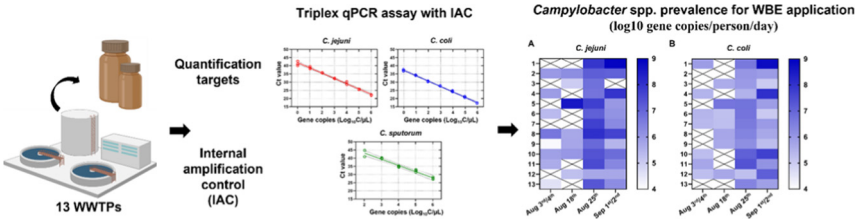
HIGHLIGHTS

- Triplex qPCR developed for *C. jejuni* and *C. coli* in wastewater surveillance applications.
- $ALOD_{100\%}$ and $PLOD_{80\%}$ are 10 gene copy/μL and 2 log10 cells/mL, respectively.
- *C. jejuni* and *C. coli* were determined in 52 samples collected over a month from 13 WWTPs.
- A peak of *C. jejuni* in wastewater was observed weeks before a reported outbreak.

GRAPHICAL ABSTRACT



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ABSTRACT

Campylobacter spp. is one of the most frequent pathogens of bacterial gastroenteritis recorded worldwide. *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli*) are the two major disease-associated species, accounting for >95 % of infections, and thus have been selected for disease surveillance. Monitoring temporal variations in pathogen concentration and diversity excreted from community wastewater allows the early detection of outbreaks. Multiplex real-time/quantitative PCR (qPCR) enables multi-target quantification of pathogens in various types of samples including wastewater. Also, an internal amplification control (IAC) is required for each sample when adopting PCR-based methods for pathogen detection and quantification in wastewater to exclude the inhibition of the wastewater matrix. To achieve reliable quantification of *C. jejuni* and *C. coli* towards wastewater samples, this study developed and optimized a triplex qPCR assay by combining three qPCR primer-probe sets targeting *Campylobacter jejuni* subsp. *jejuni*, *Campylobacter coli*, and *Campylobacter sputorum biovar sputorum* (*C. sputorum*), respectively. This triplex qPCR assay not only can directly and simultaneously detect the concentration of *C. jejuni* and *C. coli* in wastewater but also can achieve the PCR inhibition control using *C. sputorum* primer-probe set. This is the first developed triplex qPCR assay with IAC for *C. jejuni* and *C. coli*, to be used in the wastewater-based epidemiology (WBE) applications. The optimized triplex qPCR assay enables the detection limit of the assay ($ALOD_{100\%}$) and wastewater ($PLOD_{80\%}$) as 10 gene copy/μL and 2 log10 cells/mL (2 gene copies/μL of extracted DNA), respectively. The application of this triplex qPCR to 52 real raw wastewater samples from 13 wastewater treatment plants demonstrated its potential as a high-throughput and economically viable tool for the long-term monitoring of *C. jejuni* and *C. coli* prevalence in communities and the surrounding environments. This study provided an accessible methodology and a solid foundation for WBE-based

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monitoring of *Campylobacter* spp. relevant diseases and paved the road for future WBE back-estimation of *C. jejuni* and *C. coli* prevalence.

1. Introduction

Campylobacter, a kind of "S" or curve-shaped bacteria, is one of the four major causes of diarrhea, and it is also regarded as the most common cause of human gastroenteritis in the world. Diseases with *Campylobacter* are mainly caused by drinking contaminated water, raw milk, raw salads, and raw/undercooked poultry (Fravalo et al., 2021). Foodborne diseases, including Campylobacteriosis, cause 1 in 10 people to get sick and 33 million deaths worldwide each year (Burnham and Hendrixson, 2018; WHO, 2022). In Australia, Campylobacteriosis source of infection is mainly from eating chicken meat and contact with young pet dogs (Cribb et al., 2022). Although *Campylobacter* infections are usually mild, they can still be serious and even fatal in immunosuppressed individuals, young children, and the elderly. The minimal infectious dose of *Campylobacter* is usually reported as around 500 bacteria ingestion. However, studies have reported that very low doses (50 % infection dose, $\leq 10^2$ CFU) can cause infection and illness (Frirdich et al., 2017; Tribble David et al., 2010). Among the 17 species and 6 subspecies of *Campylobacter*, *C. jejuni*, and *C. coli* are the two species most frequently reported in human diseases, accounting for >95 % of illnesses (Leblanc-Maridor et al., 2011). Despite the high level of concern caused by *Campylobacter* infections, the true nature of the global incidence is largely unknown, as reports of *Campylobacter* outbreaks vary from country to country. Underreporting of *Campylobacter* infections, variances in reporting systems, challenges with diagnosis, and disparities in monitoring during outbreaks are factors contributing to the lack of actual prevalence of *Campylobacter* outbreaks (Hansson et al., 2018).

Wastewater-based epidemiology (WBE) or wastewater surveillance is a transdisciplinary research field, that includes the analysis of markers such as chemicals, drugs, and microorganisms in wastewater (Choi et al., 2018; Gao et al., 2017; Rico et al., 2017). By analyzing the samples collected at the influent of a wastewater treatment plant or upstream sewers, and comparing the results over time and across locations, researchers could objectively map the trends and differences of the markers and obtain trends of public health (Lorenzo and Picó, 2019; Mao et al., 2020). Recently, wastewater surveillance of SARS-CoV-2 has achieved significant progress which further highlighted its importance for disease prevention and control (Li et al., 2021b; Zahedi et al., 2021). WBE-based disease surveillance could provide near real-time evidence that the infectious agent or its genetic component has entered the sewage system, sometimes even days before symptoms appear and often before an infected person has come into contact with a healthcare facility (Tiwari et al., 2023). The previous report has confirmed the feasibility and accuracy of WBE-based data-driven estimation of COVID-19 prevalence (Li et al., 2021a).WBE back-estimation is a reversal approach to reveal the disease prevalence by tracing the concentration of excreted biomarkers in influent wastewater. Different from clinical and other infection prevention surveillance, WBE-based monitoring is more convenient because the WBE back-estimation usually aims to estimate the overall prevalence of pathogenic targets rather than the active targets, thus does not require the differentiation of pathogen viability. WBE has been confirmed to enable the early warning of disease outbreaks for various human pathogens and the concentration peaks of pathogens in wastewater were usually observed weeks before the outbreaks (Diemert and Yan, 2019; Hellmér et al., 2014; Maida et al., 2022). *Campylobacter* is often reported to be detected in raw influent wastewater with a concentration between 10^2 and 10^5 CFU/100 mL (Chowdhari et al., 2022; Cui et al., 2019). Although the number is much lower than indicator organisms like *E. coli* and *Enterococci* (10^6–10^7 organisms/100 mL) (Jones, 2001; Matthews et al., 2010), it can still cause a public health threat because of its low infectious dose (<500 organisms can cause disease). This relatively low concentration, as well as the low recovery of pathogens in wastewater, largely challenges the detection and quantification of *Campylobacter*. Therefore, it is necessary and significant to develop a reliable analysis tool for the effective surveillance of *Campylobacter* using WBE (Pitkänen, 2013).

Existing methods for *Campylobacter* identification include culture-based methods, growth morphology, biochemical tests, and molecular techniques (Zhang et al., 2021). Since 1992, after the polymerase chain reaction (PCR) was first deployed for specific detection of *C. jejuni* and *C. coli*, it was regarded as a better alternative for the detection and quantification of these bacteria to species level and has been widely deployed to various types of samples (Banting et al., 2016; Igwaran and Okoh, 2019). Multiplex PCR-based methods, as a developed version of singular qPCR, enable multi-target detection and thus increase detection efficiency. To date, multiplex qPCR assays for specifically detecting various *Campylobacter* spp. have been reported mostly for clinical samples (Liu et al., 2017; Toplak et al., 2012; Vondrakova et al., 2014). However, their feasibility and the limit of detection and quantification for wastewater samples have hardly been identified or optimized. In addition, wastewater is a kind of complex matrix that contains various inhibiting agents for molecular methods. The qPCR detection of wastewater samples always requires an inhibition control test (internal amplification control, IAC) for each sample to exclude the effect of inhibitors on qPCR reactions (Hatt et al., 2013). This inhibition control test leads to additional consumption of qPCR reagents (primer-probe sets, buffers, and enzymes), times, and labor which increase the total cost of wastewater analysis.

This study established a triplex qPCR assay that can simultaneously quantify the concentration of *C. jejuni* and *C. coli* in wastewater while also achieving the qPCR inhibition control with *C. sputorum* in one tube (within the same test well). The linearity and the detection limit of assay (ALOD) and wastewater samples (PLOD) were identified and were compared to previous reports. Fifty-two raw influent wastewater samples were tested by this triplex qPCR assay. The results further confirmed the feasibility of the developed triplex qPCR method for wastewater analysis of *C. jejuni* and *C. coli*.

2. Materials and methods

2.1. Bacteria strains and cultures

The calibration curves of this triplex quantitative PCR assay were established by the following cultures. The *Campylobacter jejuni* subsp. *jejuni* (ATCC® 700819™), the *Campylobacter coli* (ATCC® 33559™), and the *Campylobacter sputorum biovar sputorum* (ATCC® 33562™) were purchased from the American Type Culture Collection (ATCC). According to the suggestion of ATCC product sheets, all these three strains were incubated on Trypticase Soy Agar (TSA) with 5 % Sheep Blood Agar Plates (Thermo Fisher Scientific, Australia) at 42 °C under microaerophilic conditions (Anaerocult® C for microbiology for the generation of an oxygen-depleted and CO_2-enriched atmosphere in an anaerobic jar, Merck, Australia) for 2–5 days.

2.2. Wastewater samples

Influent wastewater was sampled from 13 different wastewater treatment plants (Shoalhaven Water) in Australia on the 3rd, 4th, 18th, 25th of August, and 1st and 2nd of September 2020 in Illawarra Shoalhaven LHD, Australia. Details of the 13 different wastewater treatment plants are provided in Table S1 in the supplementary material. These samples were transported to the laboratory on ice and were stored in the fridge at 4 °C until further processing within 2–7 days. All these wastewater samples have the typical characteristics of pH at 7.2 ± 0.27(Avg ± std. dev), sulfate at 10–45 mg-S/L, dissolved oxygen (DO) of 0.67 ± 0.12 mg/L, total

suspended solids (TSS) at 200–600 mg/L, total chemical oxygen demand (TCOD) at 350–600 mg/L and soluble chemical oxygen demand (SCOD) at 50–150 mg/L (Shi et al., 2022).

2.3. Genomic template DNA extraction

DNA extraction for bacteria cultures was conducted with the GenElute™ Bacterial Genomic DNA Kits (Sigma-Aldrich, Australia). A volume of 1 mL of bacteria culture of each strain with a concentration of 10^8 cells/mL was transformed into 100 μL of extracted genomic DNA. The bacteria concentration was evaluated with the cell density meter (Biochrom, C08000), and was automatically converted from the unit of OD_{600} to cells/mL using the online cell density calculator (https://www.agilent.com/store/biocalculators/calcODBacterial.jsp). The final concentration of extracted DNA was adjusted to 20 ng/μL and was stored at −80 °C. The quality of the DNA was evaluated by NanoDrop 2000c (Thermo Fisher Scientific, Australia), and the DNA concentration was quantified by Qubit 4.0 Fluorometer with the Qubit™ 1X dsDNA High Sensitivity (HS) and Broad Range (BR) Assay Kits (0.1–120 ng; Thermo Fisher Scientific, Australia).

2.4. DNA genomic standards

According to the size of the genomic DNA of *C. jejuni* NCTC 11168 (1640 Kbp) and *C. coli* (1860 Kbps), the gene copies of 100 ng genomic DNA were estimably equal to 5.2×10^7 and 4.6×10^7 copies, respectively (Leblanc-Maridor et al., 2011). The extracted gDNA of *C. jejuni* and *C. coli* were adjusted to 20 ng/μL, which were 1.04×10^7 and 9.2×10^6 copies/μL, respectively. For generating the calibration curves, 10-fold gradient dilutions of each extract were produced in RNase- and DNase-free water, representing 10^0 to 10^6 genome copies/μL of templates. Since the *C. sputorum* strain was used as the qPCR inhibition control test and only the relative concentration of *C. sputorum* target is needed, the same calculations were applied for the production of the *C. sputorum* genomic standard, assuming a similar-sized genome with *C. jejuni* (5.2×10^7 gene copies/100 ng) (Pacholewicz et al., 2019). The genomic DNA of *C. sputorum* was also adjusted to 20 ng/μL, which was 1.04×10^7 copies/μL. The calibration curve of *C. sputorum* was also generated by using 10-fold gradient dilutions from 10^0 to 10^6 genome copies/μL of templates.

2.5. Triplex real-time qPCR assay

The sequence information of the three pairs of primer-probe sets was listed in Table S2 in the supplementary material. To establish this triplex qPCR assay, three pairs of primer-probe sets reported by previous studies were adopted for detecting *Campylobacter jejuni* subsp. *jejuni* (*C. jejuni*), *Campylobacter coli* (*C. coli*), and *Campylobacter sputorum biovar sputorum* (*C. sputorum*) respectively (Leblanc-Maridor et al., 2011; Pacholewicz et al., 2019). The original duplex qPCR assay targeting the N-benzoylglycine amidohydrolase hippuricase (*hipO*) gene of *C. jejuni* and the *glyA* gene (encoding serine hydroxymethyltransferase) of *C. coli* were reported to have high specificity and sensitivity to these *Campylobacter* species. In our study, we added one other qPCR assay as an internal amplification control (IAC) rather than for quantification purposes. The *Campylobacter sputorum* was chosen because it belongs to the same genus as the two target species. It might have a similar recovery with the two target species from wastewater samples, due to their similar bacterial structure and characteristics. It was expected that *C. sputorum* could be used as a control for both the recovery and internal amplification. In addition, it has been confirmed to have no effect on the specificity of the duplex-qPCR assay for *C. jejuni* and *C. coli* (Leblanc-Maridor et al., 2011). Therefore, this study didn't repeat the in vivo specificity evaluation and the comparison to the single qPCR test for our triplex-qPCR assay. However, in-silico PCR amplification was conducted for these three pairs of primers (Bikandi et al., 2004). The results showed that no bands were amplified and observed for any of the other *Campylobacter* species tested as well as for a range of bacteria, which could present in wastewater including *Helicobacter*, *Arcobacter*, *Escherichia*, *Enterococcus*, *Listeria*, *Enterobacter*, *Staphylococcus*, *Pseudomonas*, *Salmonella*. Several pre-testings were carried out to assess the feasibility of the triplex qPCR assay. It was found that the nonfluorescent QSY quencher modified original *C. jejuni* probe (FAM dye) didn't generate a fluorescence signal in this triplex assay. To eliminate the unexpected interaction between the reporters (FAM, VIC, and JUN) and the quenchers (QSY) of the three modified probes, the probe sequence targeting *C. jejuni* was modified by adding "CTTT" and "ATTTAGCAAGTCAG" sequences on the 5′ and 3′ ends, respectively. The probe was modified by only adding bases that complement the target gene on the 5′ and 3′ ends. Therefore, the length and sequence order of the qPCR products would not be affected. Based on the complementary base pairing rules, this modification will only increase the specificity of the detection. Sequence BLAST was conducted between modified *C. jejuni* hipO-probe and the NCBI database Nucleotide collection (nr/nt) (Fig. S1). The results showed high specificity to *C. jejuni*. The concentration of each primer and probe and the PCR run condition was evaluated and adjusted based on the original conditions in previous reports to achieve the best detection performance of this triplex qPCR assay.

The generation of standard curves for the triplex qPCR assay was done in 20 μL PCR mixtures containing 10 μL of 2× TaqMan™ Multiplex Master Mix (Formulated to amplify up to 4 targets in a single reaction, Thermo Fisher Scientific, Australia), 400 nM of each primer of *C. jejuni* and *C. coli* assay (*hipO*-F and *hipO*-R for *C. jejuni*, *glyA*-F and *glyA*-R for *C. coli*,) and 200 nM of each probe of *C. jejuni* and *C. coli* assay (*glyA*-P and *hipO*-P respectively), 500 nM of each primer of *C. sputorum* assay, 100 nM of the probe of *C. sputorum* assay and 3 μL of template DNA (1 μL of each target). The thermal cycle protocol used was the following: activation of the Taq DNA polymerase at 95 °C for 10 min, then 50 cycles of 30 s at 95 °C and 60 s at 58 °C. Thermal cycling, fluorescent data collection, and data analysis were carried out with the Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories). The C_t value was determined by using the single threshold mode and was calculated automatically by the qPCR instrument. Data were collected ($C_t < 45$ cycles) only from the operation of the instrument in which the positive control was positive, and the non-template control was negative. The non-template control test was carried out by adding water instead of extracted DNA with three replicates for each instrumental run. Analytical efficiency (E) of the triplex qPCR assays were calculated by using the following formula:

$$E = 100 \times \left(10^{-1/slope} - 1\right)$$

2.6. C. jejuni and C. coli spiked wastewater

The wastewater for spiking mock tests was collected from a local wastewater treatment plant (WWTP) in Wollongong, Australia. This wastewater sample was confirmed as qPCR negative by using the developed triplex qPCR assay (Section 2.5). Three parallel extractions were conducted for this negative extraction control (NEC). Each extraction was tested with three triplex-qPCR assay repeats. The negative wastewater sample was spiked with *C. jejuni* and *C. coli* to generate the standard curves for the triplex qPCR assay. *C. jejuni and C. coli* cultures were spiked into 1 mL of wastewater to get a series of positive wastewater mocks with different final concentrations of 10^2, 10^4 and 10^6 cells/mL, respectively. Three parallel spiked wastewater mocks were extracted at each concentration. Then, the spiked wastewater samples were centrifugated at 12,000*g* for 5 min. After centrifugation, the supernatant was removed, and the solid particle was transferred into the Lysing Matrix E tube of the FastDNA™ SPIN Kit for Soil (MP Bio, Australia) for DNA extraction. The DNA extraction was strictly conducted following the instruction of the kit's manual. The final extracted DNA volume of the 1 mL wastewater sample was 50 μL. All extracted DNA was stored at −80 °C before analyzed by the triplex qPCR (Section 2.5). The difference is the 3 μL of template DNA used for building standard curves was replaced with 1 μL of extracted DNA, 1 μL of *C. sputorum* genomic template (0.2 ng/μL), and 1 μL of nuclease free water. After amplification, the C_t value of *C. sputorum* assay of each mock was within two cycles variation of the C_t values of the positive control wells, which indicated the absence of inhibition (Staley et al., 2012).

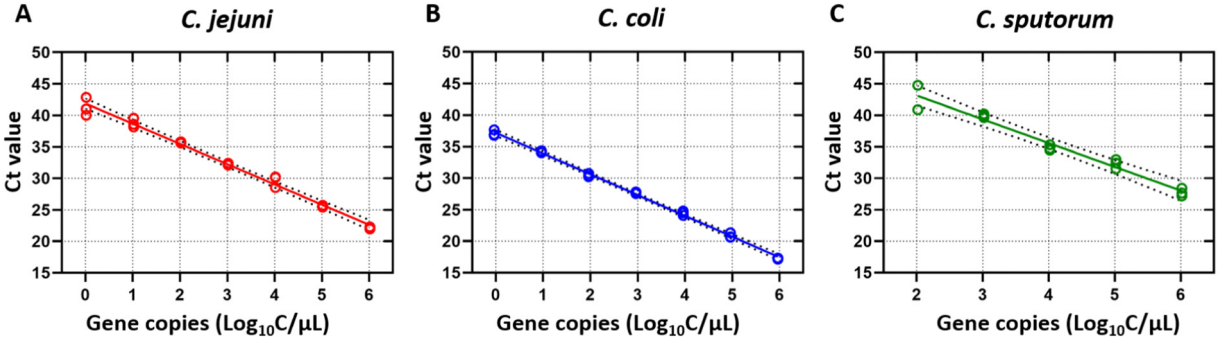

Fig. 1. Linear range and sensitivity of the triplex qPCR assay by using genomic standards. The empty symbols indicate each replicate of total three qPCR runs ($n = 3$). The solid lines represent the standard curves, and the dot lines represent the 95 % confidence bands.

Each parallel mock of each concentration was tested three times. Thus, a total of nine C_t values were generated at each concentration for each target. The DNA extraction and the triplex-qPCR preparation were carried out in different labs to exclude the potential contamination. The recovery of *C. jejuni* was reported as 22.05 ± 9.76 %, 10.65 ± 3.42 %, and 65.41 ± 48.41 % at concentrations of 10^6, 10^4, and 10^2 cells/mL, respectively (Zhang et al., 2023). *C. coli* had a recovery of 15.76 ± 6.48 %, 9.74 ± 2.44 %, and 10.58 ± 3.96 % at the concentration of 10^6, 10^4, and 10^2 cells/mL, respectively. *C. sputorum* had a recovery of 48.92 ± 12.07 % and 25.43 ± 18.56 % at 10^6 and 10^4 cells/mL, respectively. Significant differences between the recovery of *C. jejuni*/*C. coli* and *C. sputorum* were observed. Therefore, the *C. sputorum* assay was determined to be the control of internal amplification, not the recovery from wastewater samples.

2.7. Analysis of real raw wastewater samples

A total of 52 influent domestic wastewater samples (Section 2.2) were tested in this study. The DNA extraction of domestic influent wastewater samples was carried out by the following procedures: A volume of 50 mL of wastewater was first centrifuged at 3260*g* for 30 min. To increase the wastewater volume for DNA extraction, after removing the supernatant, another volume of 50 mL of wastewater was directly added into the same tube and centrifuged at 3260*g* for a further 30 min. In total, 100 mL of wastewater was centrifuged for further DNA extraction. After centrifugation, the supernatant was removed, and the solid particle was transferred into the Lysing Matrix E tube of the FastDNA™ SPIN Kit for Soil (MP Bio, Australia) for DNA extraction. The DNA extraction was conducted strictly following the instructions of the kit's manual. The final extracted DNA volume of the 100 mL wastewater sample was 100 μL. All the extracted DNA was stored at −80 °C. The triplex qPCR assay described above (Section 2.6) was adopted to test all extracted DNA with three replicates. Only the results with a C_t value of *C. sputorum* assay between 31 and 33 (less than two cycles changes compared to positive control) were regarded as reliable results. The negative sample (1 out of 52 wastewater samples, 1 out of total 156 qPCR runs) with a C_t value of *C. sputorum* assay out of this range was tested again with a 10-fold dilution of the extracted DNA. The sample was confirmed as negative by using the 10-fold diluted targets. The bacteria load of *C. jejuni* and *C. coli* (log10 gene copies/person/day) was calculated by using the following equation: bacteria load (log10 gene copies/person/day) = $\log10[(C_{DNA} * Q_{daily}) / P]$. C_{DNA} is the *Campylobacter* concentration (gene copies/L) in wastewater. Q_{daily} is the daily flow rate (L/day) of each WWTP. P is the treatment capacity (equivalent persons) of each WWTP. The normalized bacterial load, by considering the catchment population and daily flow rate of each WWTP, could be used to determine how it coincides with the infections.

3. Results

3.1. Linear range and sensitivity of the triplex qPCR assay

The standard curves were built by using serial 10-fold dilutions (1– 10^6 gene copies/μL) of genomic templates extracted from each strain's cultures and were presented in Fig. 1. The characteristics of each standard curve were listed in Table 1.

The R^2 values of three individual qPCR assays were all equal to 0.99, which showed a strong linear correlation between the template concentrations and the C_t values. The mean slope of *C. jejuni* and *C. coli* was −3.21 and −3.29, and the efficiency was 104.9 % and 101.3 %, respectively. The detection limit of the assay (ALOD) was determined as 1 gene copy/μL of the genomic template ($ALOD_{83.3\%}$ five positive results out of six qPCR runs, Table 2) for both the *C. jejuni* and *C. coli* assays. Moreover, this triplex qPCR assay is reliable with an $ALOD_{100\%}$ of 10 gene copies/μL for the samples containing both *C. jejuni* and *C. coli* genomic templates and for 6 successive qPCR runs. The slope and efficiency of *C. sputorum* assay were −3.78 and 83.9 %, which was slightly out of the normal range of an efficient qPCR assay (−3.58 to −3.1; 90 %–110 %). The ALOD of the *C. sputorum* assay was 10^2 gene copies/μL, which is much higher than *C. jejuni* and *C. coli* assays. However, this will not affect the detection performance of the triplex qPCR assay because the *C. sputorum* assay was only used to conduct the PCR inhibition control testing rather than bacteria quantification.

3.2. Feasibility of the triplex qPCR assay for the analysis of spiked wastewater mocks

To evaluate the feasibility of this triplex qPCR assay in the application of influent wastewater, negative wastewater confirmed by this triplex qPCR assay was employed to make bacteria cultures-spiked wastewater mocks. The detection results of these serial wastewater mocks were presented in Fig. 2.

Table 1
Characteristics of triplex qPCR standard curves for *C. jejuni* and *C. coli* quantification in bacteria culture and in spiked wastewater mocks.

Primer-probe sets		Efficiency (%)	Linearity (R^2)	Slope [95%CI]	Y-intercept [95%CI]
C. jejuni	SC	104.9	0.99	−3.21 [−3.43 to −2.99]	41.87 [41.07 to 42.68]
	WSC	119.4	0.94	−2.93 [−3.29 to −2.57]	47.87 [45.9 to 49.83]
C. coli	SC	101.3	0.99	−3.29 [−3.43 to −3.15]	37.18 [36.69 to 37.68]
	WSC	97.2	0.98	−3.39 [−3.58 to −3.2]	46.15 [45.19 to 47.10]
C. sputorum	SC	83.9	0.99	−3.78 [−4.43 to −3.13]	50.7 [47.92 to 53.47]

SC: Standard curve; WSC; Standard curve of wastewater mocks.

Table 2
The ALOD (genomic templates) and PLOD (spiked wastewater) of the triplex and simplex qPCR assay.

Primer-probe sets		Genomic templates		Spiked wastewater
		1 copy/μL	10 copies/μL	10^2 cells/mL (2 gene copies/μL)[a]
Triplex qPCR assay	*C. jejuni*	5/6	6/6	5/15 (4/5*)
	C. coli	5/6	6/6	15/15 (5/5*)
Simplex qPCR assay	*C. jejuni*	6/6	6/6	7/15 (4/5*)
	C. coli	6/6	6/6	15/15 (5/5*)

* Number of positive mocks/Number of total mocks.
[a] Equivalent number of gene copies/μL by supposing no DNA was lost during the extraction.

The results showed that this triplex qPCR assay has good linearity for both *C. jejuni* and *C. coli* quantification from about 10^2 to 10^7 cells/mL of wastewater mocks. The mean standard curves were $y = -2.93x + 47.87$ and $y = -3.39x + 46.15$ for *C. jejuni* and *C. coli*. The R^2 and efficiency were 0.94 and 0.98, and 119.4 % and 97.2 % of *C. jejuni* and *C. coli* assay, respectively. To evaluate the detection limit of wastewater mocks (PLOD), five parallel wastewater mocks with a concentration of 10^2 cells/mL of both strains were tested with the triplex qPCR for a total of fifteen qPCR repeats and the results were provided in Table 2. For the *C. jejuni* assay, four out of five parallel mocks (80 %) were detected as positive, with five out of fifteen qPCR repeats being positive. Although only 1/3 of the fifteen qPCR repeats were positive, the $POLD_{80\%}$ of the *C. jejuni* assay was defined at 10^2 cells/mL (2 gene copies/μL of extracted DNA) in this study because more than half of the mocks (80 %) were positive. All the five parallel mocks and fifteen qPCR repeats for the *C. coli* assay were positive, indicating a reliable $PLOD_{100\%}$ of the *C. coli* assay at 10^2 cells/mL (2 gene copies/μL of extracted DNA). ALOD and PLOD were also evaluated for each simplex assay targeting *C. jejuni* and *C. coli* (Table 2). This triplex qPCR assay showed comparable sensitivity to each simplex assay, especially for wastewater mocks (four out of five parallel mocks (80 %) were detected as positive). However, the higher positive ratio of each simplex assay at 1 copy/μL of genomic templates indicates that the *C. sputorum* assay slightly compromised the sensitivity of the triplex assay.

3.3. Application of the triplex qPCR assay to real domestic wastewater samples

To evaluate the application of this triplex qPCR assay on real domestic wastewater samples, a total of 52 wastewater samples collected from 13 WWTPs between August and September of 2020 were tested. The heat maps of the bacteria load of 13 WWTPs during a month were displayed in Fig. 3.

The concentration of *C. jejuni* and *C. coli* in these collected positive domestic wastewater samples ranged from 10 to 10^6 gene copies/100 mL. This range was consistent with the limit of quantification ($ALOQ_{83.3\%}$ = 3.3 gene copy/μL; $ALOQ_{100\%}$ = 33 gene copy/μL) approximated by multiplying the ALOD by 3.3 (Lister, 2005). The positive ratios of *C. jejuni* and *C. coli* in 52 wastewater samples were both 76.9 % (40/52) and most of the negative samples were collected on the 3rd/4th and 18th of August. Except for site 5, all the wastewater samples collected from other WWTPs had an obvious increase in *C. jejuni* load at the end of August 2020 (Fig. 3. A). The *C. jejuni* load of site 5 increased from the 18th of August and consequently decreased in early September. A similar increase in *C. coli* load was also observed during the same period (Fig. 3. B). Additionally, the *C. coli* load was frequently lower than the *C. jejuni* of each WWTP. The average *C. jejuni* load of 13 WWTPs was 5.51 ± 0.81, 6.06 ± 1.17, 7.23 ± 0.63, and 6.72 ± 1.03 log10 gene copies/person/day on each sampling date, and the *C. coli* load was 5.49 ± 0.24, 5.69 ± 0.49, 6.38 ± 0.59, and 6.35 ± 1.2 log10 gene copies/person/day. A peak of average *C. jejuni* load of the 13 WWTPs samples was observed on the 25th of August 2020. Meanwhile, the average *C. coli* load had an increase from the 25th of August and kept this level until early September.

4. Discussion

The triplex qPCR assay developed and modified in this study provided a rapid, sensitive, and reliable quantification tool for detecting *C. jejuni* and *C. coli* in domestic influent wastewater samples. Meanwhile, this triplex qPCR can also achieve the qPCR inhibition control testing within the same tube by *C. sputorum* assay, which significantly reduces the time, chemicals, and labor consumption of the detection. The primer-probe sets used for targeting *C. jejuni* and *C. coli* were designed by Leblanc-Maridor et al. in 2011, which were reported with high specificity for each species in various types of samples, thus the specificity of this triplex qPCR assay was not further evaluated (Leblanc-Maridor et al., 2011). In their study, the duplex qPCR assay could achieve an ALOD of 10 gene copies/reaction and a PLOD of 2.5 × 10^2 CFU/g of feces, 1.3 × 10^2 CFU/g of feed, and 1.0 × 10^3 CFU/m^2 for the environmental samples, respectively. In our study, the $ALOD_{83.3\%}$ and the $PLOD_{80\%}$ of wastewater sample were identified as one gene copy/reaction ($ALOD_{100\%}$ = 10 gene copies/reaction) and 10^2 cells/mL (2 gene copies/reaction of extracted DNA from wastewater mocks), respectively. They were similar to the ALOD and PLOD of the duplex qPCR assay developed for *C. jejuni* and *C. coli* (Leblanc-Maridor et al., 2011). The LODs of our triplex qPCR assay were equal to or even lower than the $LOD_{95\%}$ (between 2 and 65 copies/reaction) of the single or multiple *Campylobacter*-targeting qPCR assays evaluated by Banting et al. (2016). In addition, the correlations between the C_t values and the concentration of *C. jejuni* and *C. coli* cultures spiked wastewater were also identified at 0.94 and 0.98, respectively, indicating its good linearity and stability for wastewater analysis. Bonetta et al. (2016) developed a triplex PCR assay that can simultaneously detect pathogenic *Campylobacter*, *E. coli* O157:H7, and *Salmonella* spp. in wastewater (Bonetta et al., 2016). The PLOD of this triplex PCR is as low as ~2 CFU/100 mL for all the targeted pathogens in raw sewage. However, this method can only detect the presence of targets rather than provide exact concentrations in

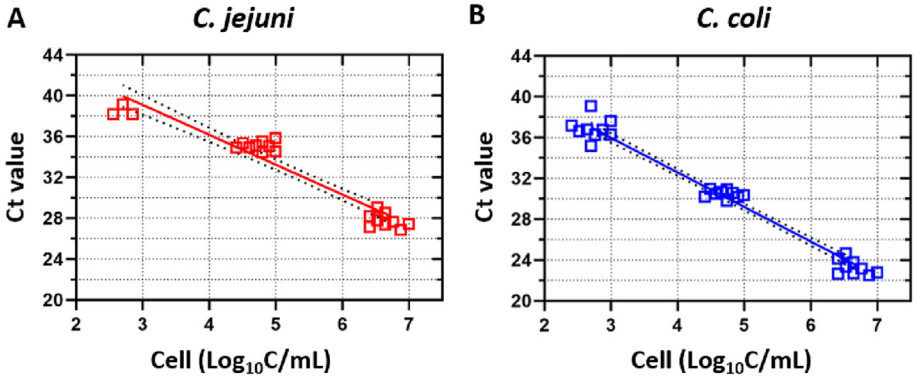

Fig. 2. Linear range and sensitivity of the triplex qPCR assay in analyzing spiked wastewater mocks (n = three parallel mocks × three qPCR replicates = 9). The empty symbols indicate each replicate qPCR run. The solid lines represent the standard curves, and the dot lines represent the 95 % confidence bands.

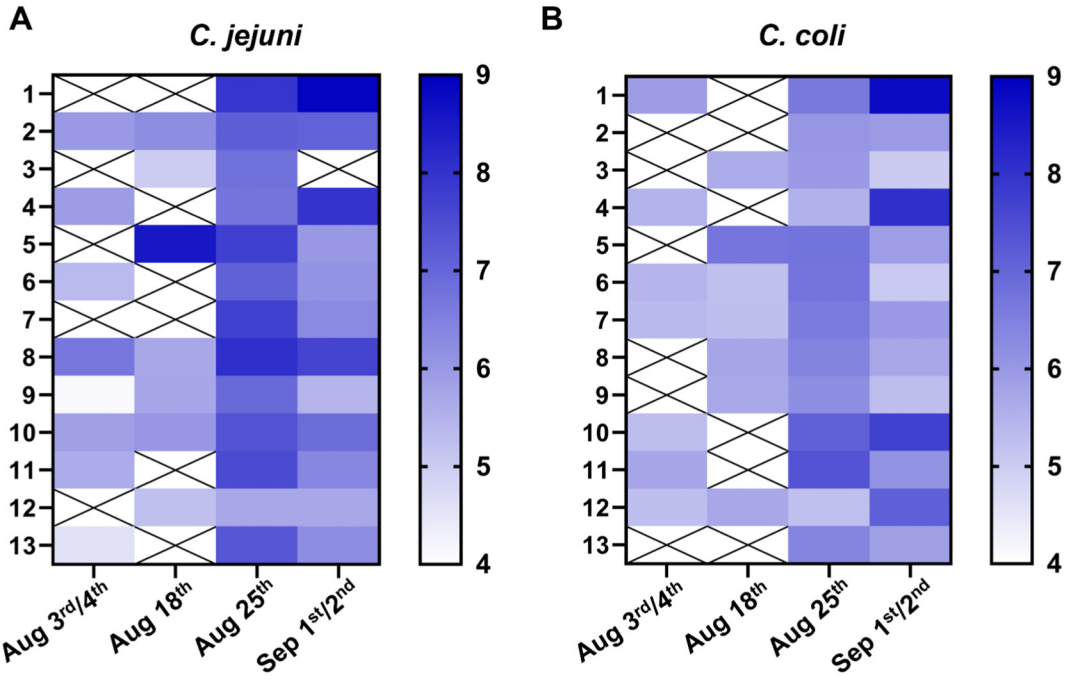

Fig. 3. Detection results of *C. jejuni* and *C. coli* in wastewater samples collected from 13 WWTPs in Australia by using the triplex qPCR assay. The color bar represents the bacteria load of *C. jejuni* and *C. coli* (log10 gene copies/person/day) of each WWTP. The crossed boxes represent the negative results.

wastewater samples which inhibits its application in wastewater-based disease surveillance. In addition, this method requires filtration and enrichment steps of wastewater samples which largely extend the detection time. The total volume of each wastewater mock in our study was 1 mL which is not consistent with the volume of raw wastewater (50 mL) used for field study. Therefore, the standard curves generated based on wastewater mocks only evaluated the linearity and sensitivity of this triplex qPCR assay for wastewater samples without considering the DNA recovery of different sample processing methods.

The internal amplification control (IAC) assay is always required to accompany the PCR assay for target detection in complex samples like stool, environment, and wastewater samples (Li et al., 2015; Yang et al., 2017; Zhi et al., 2022). This is because the extracted DNA quality of these complex samples is varied and might contain compounds including polyphenols and heavy metals that can inhibit PCR or reduce PCR efficiency. The triplex qPCR assay developed in this study adopted the *C. sputorum* assay, which belongs to the same genus of targets, as the internal amplification control that can directly reveal the presence/absence of PCR inhibitors in the same quantification well. This largely reduced the cost of PCR reagents and sample preparation time for qPCR detection and increased the reliability of detection results of wastewater samples. Furthermore, only the C_t values of wastewater samples, which are no more than two cycles different from the positive control, can be involved in the calculation of real gene copies (Mondal et al., 2021). This data selection can avoid the underestimation of gene concentrations in wastewater caused by outliers. In addition, a previous study has reported that the pathogen-specific internal amplification controls have higher accuracy than human gene (e.g., albumin gene) amplification for PCR inhibition diagnosis (Roux et al., 2019).

To further access the feasibility of this triplex qPCR assay in the application of wastewater-based surveillance for *C. jejuni* and *C. coli* monitoring, 52 raw influent wastewater samples were tested to map the variation of *C. jejuni* and *C. coli* load in wastewater during a month. This one-month surveillance recorded a peak of the average *C. jejuni* load of 13 WWTPs within the sampling area of Illawarra Shoalhaven LHD Australia. The average *C. jejuni* load of 13 WWTPs wastewater samples increased from around 5.5 log10 to 7.2 log10 gene copies/person/day at the end of August, then decreased to 6.7 log10 gene copies/person/day in early September. In the NSW OzFoodNet annual report of 2020, one foodborne disease outbreak induced by *C. jejuni* was reported in September of 2020 in Illawarra Shoalhaven LHD (CommunicableDiseasesBranch, 2022). In this outbreak, three people got an illness, one was confirmed as *C. jejuni* infection by the lab, and one was hospitalized. The *C. jejuni* load peak found in our study occurred weeks before the outbreak was officially reported, which is consistent with the findings of several earlier studies for the surveillance of other wastewater-based human diseases. Hellmér et al. evaluated the presence of eight pathogenic viruses in wastewater samples collected between January and May 2013 in Sweden (Hellmér et al., 2014). In their study, the highest concentration of noroviruses was detected 2 to 3 weeks before most of the patients were diagnosed with this infection. In another case, Solis-Moreira developed a new wastewater surveillance method that successfully detected the SARS-CoV-2 variants of concern up to 2 weeks before clinical diagnosis (Solis-Moreira, 2022). Although *Campylobacter* is a zoonotic pathogen, urban sewage systems may also involve *Campylobacter* of animal origins, leading to an overestimation of community *Campylobacter* prevalence based on WBE. However, from the perspective of long-term disease surveillance, periodic detection and quantification of major pathogenic *Campylobacter* in wastewater can still reveal the change in its prevalence in related communities and the surrounding environment, hence, to achieve the early warning and timely intervention of disease outbreaks. In addition, a previous study has reported that genomic analysis of wastewater *Salmonella* isolates can detect the clinically unreported salmonellosis outbreak (Diemert and Yan, 2019). Yan et al. (2018) monitored the concentration of *Salmonella* in wastewater from Honolulu, Hawaii, over a 54-week period. Their results showed that there was a positive and significant linear and rank correlation between the concentration of *Salmonella* in wastewater and the clinical salmonellosis case numbers over the same period. These successful wastewater surveillance studies on *Salmonella* supported the feasibility and usefulness of *Campylobacter* wastewater surveillance by linking the wastewater data to the clinical data.

5. Conclusions

In conclusion, the triplex qPCR assay developed and optimized in this study can successfully detect *C. jejuni* and *C. coli* at one gene copy/μL of genomic templates, and at 2 log10 cells/mL of wastewater samples. Meanwhile, the internal amplification assay targeting *C. sputorum* was also

involved to identify the presence of PCR inhibitors and exclude the underestimation of real gene copy numbers. By testing with the serial dilutions of bacteria cultures-spiked wastewater mocks, the characteristics of the standard curves for wastewater mocks indicated their good linearity and stability for analyzing wastewater samples. The results of 52 domestic wastewater samples further confirmed its feasibility in wastewater analysis and showed high potential for its application in wastewater-based epidemiology of *Campylobacter* outbreaks. However, to further deploy this triplex qPCR assay for wastewater surveillance, a series of concerns including the targets recovery of various sample processing methods, the results variation caused by sample volume, and the bacteria decay during in-sewer transport should be investigated to develop a standard workflow for the wastewater surveillance of *Campylobacter* disease. In addition, further specificity assessment may be conducted by using artificial wastewater spiked with various target and non-target bacteria, although the specificity of individual primer-probe set has been evaluated towards bacteria cultures and negative feces and environmental samples.

CRediT authorship contribution statement

Shuxin Zhang - Study design, conducting experiment, data analysis and writing – original draft.

Jiahua Shi - Wastewater sampling, writing – review & editing.

Xuan Li - Writing – review.

Stephen Luby - Writing – review.

Lachlan Coin - Writing – review.

Jake W. O'Brien - Writing – review & editing.

Muttucumaru Sivakumar - Data collection – Daily flow rate of each WWTP, Writing – review & editing.

Faisal Hai - Writing – review & editing.

Guangming Jiang - Supervision, Writing – review & editing.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Guangming Jiang reports financial support was provided by ARC Discovery project. Shuxin Zhang reports financial support was provided by University of Wollongong-PhD scholarship. Jake W. O'Brien reports financial support was provided by NHMRC Emerging Leadership Fellowship.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.164574.

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