



Assessment of Thermophilic *Campylobacter* Load in Surface Water Bodies of Uttarakhand Province of India Through Real-time Quantitative Polymerase Chain Reaction (qPCR) Assay

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ABSTRACT

Background: Being the leading cause of human diarrhoea globally, *Campylobacter* gained importance in developed and developing countries in the last few decades. It causes a foodborne illness that generally spreads through eating unprocessed foods of animal origin, contact with untreated water and animal feces. Contaminated water with *Campylobacter* can be a potential source of its spread into the human population. Therefore, it is of utmost importance to estimate the *Campylobacter* load of surface water bodies like rivers and lakes. The advancement in molecular techniques like quantitative real-time PCR (qPCR) has made it quite convenient to quantify the pathogen load by amplification of its genomic DNA isolated directly from an environmental sample. The present study aimed to assess the *Campylobacter* load in surface water bodies of the Uttarakhand Province of India using a qPCR assay.

Method: A total of 160 water samples were collected from different surface water bodies located in the human vicinity, including lakes, rivers, dams, industrial drains, irrigated fields and recreational waters from the Uttarakhand province of India. The *Campylobacter* spp. was isolated from the samples and characterized using biochemical methods and molecular confirmation using PCR. Further, the bacterial load of water samples collected from different sources was determined by SYBR dye-based Real-Time qPCR assay using absolute quantitation following the standard curve method.

Result: The biochemical and molecular investigations confirm the presence of *Campylobacter* spp. in the water samples. The bacterial load was found between a minimum of 7.7×10^2 cells to a maximum of 2.7×10^5 cells in water samples. The higher bacterial load in stagnant water sources near areas of human-animal interface suggests contamination of water bodies with human and animal fecal waste, posing a significant public health concern.

Key words: Bacterial load, Real-time qPCR, Surface water, Thermophilic *campylobacter*, Zoonotic pathogen.

INTRODUCTION

Although water is a necessity for all human beings, about half of the world's population experiences water scarcity, which intensifies with increasing demand and decreasing quality of water. The water bodies are becoming increasingly polluted and contaminated with a wide variety of pathogens posing a severe threat to human, animal and environmental health. The reasons for this can be the increasing human population and the rise in both industrialization and urbanization (Bhumbla *et al.*, 2020). Providing safe water is instrumental in ensuring One-health, toward which the World Health Organization (WHO) is leading several efforts to prevent the spread of waterborne infections. According to WHO, every year around 1.4 million people die because of inadequate drinking water, sanitation and hygiene (WHO, 2024a). A huge burden of water-borne pathogens is shared by zoonotic pathogens, among which Thermophilic *Campylobacter* spp. is one of the leading food-borne zoonotic bacterial pathogens causing gastroenteritis in humans worldwide (WHO, 2024b; EFSA ECDC, 2018). *Campylobacter* is common in natural water bodies like rivers and lakes because of the release of discharge from wastewater treatment plants into it and direct contamination by human and animal feces, including poultry and wild animals (Pitkanen, 2013; Olvera-Ramírez

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et al., 2023). However, the main source of human campylobacteriosis is broiler chicken and its products, but other sources like contaminated drinking water cannot be overlooked (Bobade *et al.*, 2023; Bobade *et al.*, 2024). The occurrence of Campylobacteriosis follows a spatial and seasonal variation as it depends on water origin, seasonality and physico-chemical properties of water (Ferrari *et al.*, 2019; Kuhn *et al.*, 2017; Strakova *et al.*, 2022; Djennad *et al.*, 2019). Though outbreaks of human campylobacteriosis through contaminated water have been

observed sparsely, the severity of illness caused by the organisms is a concern (CDC, 2024; Igwaran and Okoh, 2019; Zhang *et al.*, 2023a). Therefore, being important water-borne zoonotic pathogens, it is crucial to investigate the load of *Campylobacter* in surface water bodies to ensure one health.

With the advent of molecular techniques, it is no longer a challenge to detect the pathogen in various samples with the utmost sensitivity and specificity in the shortest possible time (Gerace *et al.*, 2022). In recent years, real-time quantitative Polymerase Chain Reaction (qPCR) has become the method of choice for determining bacterial load in environmental samples like water (Botes *et al.*, 2013; Kralik and Ricchi, 2017). qPCR relies on the real-time fluorescence-based detection of the template nucleic acid amplification subjected to the repeated steps of denaturation, annealing and extension (Mikael *et al.*, 2006; Kamboj *et al.*, 2014; Chaitanya *et al.*, 2018).

Bacterial culture, Conventional end-point PCR and Dead-end ultrafiltration (DEUF) methods have already been extensively used for *Campylobacter* detection in water samples, but the reports on the use of qPCR for estimation of bacterial load or pathogen copy number in water samples are still lacking. Therefore, the present study was undertaken to determine the bacterial load of *Campylobacter* spp. in water samples collected from surface water bodies of Uttarakhand using SYBR Green dye-based Real-Time qPCR.

MATERIALS AND METHODS

Study location and collection of samples

The present study was conducted in the Nainital and Udham Singh Nagar Districts of the Uttarakhand province of India (Fig 1). A total of 160 samples were collected from different surface water bodies located in the human vicinity, including lakes, rivers, dams, industrial drains, irrigated fields and recreational waters near human habitation. A

cross-sectional study for a particular area using random sampling was employed. Samples were collected in sterile Polyethylene sample containers (Abdos, India) and transported to the laboratory at low temperatures. The samples were kept at 4°C in a refrigerator for one week before processing.

Isolation and identification of *Campylobacter* from water samples

For enrichment, 1 ml of each water sample was added to 9 ml of Bolton Broth and incubated at 42°C for 24-48 hours with 5% CO₂. After enrichment, a loopful of the culture was inoculated on Blood Free *Campylobacter* Selectivity Agar (BFCSA) and incubated at 42°C for 48 hours with 5% CO₂. The colonies were sub-cultured multiple times using the streak plate method. Colony characteristics were observed and further confirmation was done by Gram staining and various biochemical tests, viz. Hippurate hydrolysis, oxidase, catalase, urease, TSI and latex agglutination test. The positive colonies were finally subjected to molecular confirmation using the Polymerase Chain Reaction (PCR).

Molecular detection using PCR

After biochemical identification the suspected samples were subjected to molecular confirmation using conventional end-point PCR targeting *cadF* gene specific for the *Campylobacter* genus. A commercial column-based Bacterial DNA purification kit (GeNei Laboratory Pvt. Ltd., Bangalore, India) was used for isolation of genomic DNA to be used as a template in PCR using the manufacturer's protocol. The isolated DNA was visualized by 0.5% agarose gel electrophoresis and checked for its concentration in terms of nanogram of DNA per µl of elute using Bio-Spectrometer (Eppendorf, Germany). Further, the purity of DNA templates was checked by estimating ratio of absorbance at 260 (A260) and 280 nm (A280). The PCR was run targeting *CadF* gene

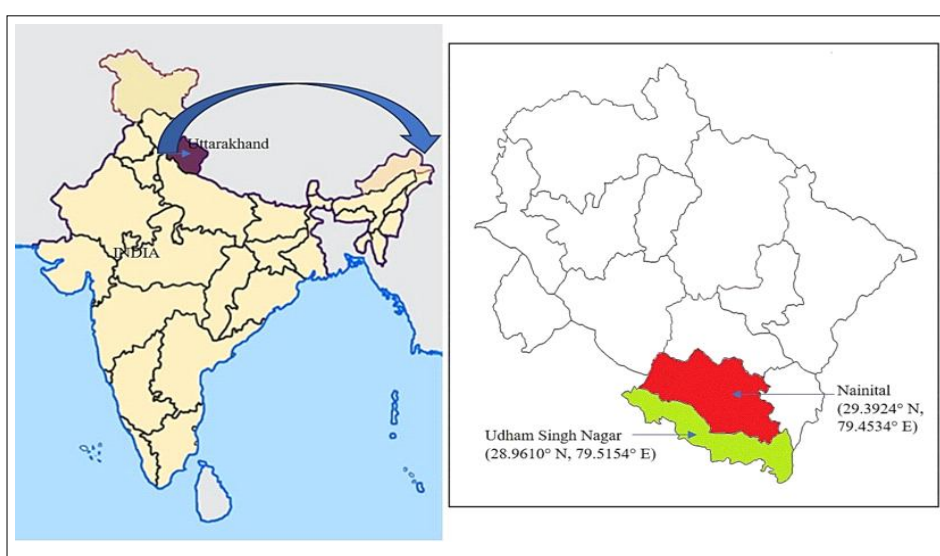


Fig 1: Location of study site of water sample collection.

specific for the *Campylobacter* genus. The sequence of forward (CadF-F) and reverse (CadF-R) primer was 5'TTGAAGGTAATTTAGATATG3' and 5'CTAATACCTAAAGTTGAAAC3' respectively (Konkel *et al.*, 1999). The PCR reaction was formulated containing 3 µl of genomic DNA (10 ng/µl), 12.5 µl of 2× PCR Master mix (GeNei Bangalore, India), 1 µl containing 20 picomol. each of forward and reverse primer in a total reaction volume of 25 µl. The initial denaturation was performed at 95°C for 5 minutes followed by 30 cycles of denaturation (94°C;30 sec.), annealing (50°C;30 sec.); extension (72°C; 60 sec.) and final extension at 72°C for 7 minutes. A positive control using commercially procured *Campylobacter* genomic DNA (HiMedia, Mumbai, India) and a negative control using nuclease-free water were also included in the PCR run in the PCR reaction. The PCR product with an expected amplicon length of 400 bp was visualized using 1% agarose gel electrophoresis.

Real-Time quantitative PCR (qPCR) assay

The bacterial load of water samples collected from different water bodies was estimated by SYBR dye-based Real-Time qPCR assay. The total DNA from 1.0 ml of each water sample was extracted using a miniprep spin column-based HiPurA® Water DNA Purification Kit (HiMedia, India) following the manufacturer's protocol which was used as template for qPCR assay. The DNA samples were eluted in a final volume of 50 µl and subsequently checked for concentration and quality.

The absolute quantification method was used for the quantification of *Campylobacter* in water samples. It was performed by plotting a standard curve of 10-fold serially diluted commercially acquired genomic DNA of *Campylobacter* (HiMedia, Mumbai, India). The copy number was calculated using the following formula:

$$\text{Copy number} = \frac{x \text{ (ng)} \times 6.022 \times 10^{23}}{N \times 10^9 \times 660}$$

Where

x = Amount of DNA (ng);

N = Length of amplicon and 660 is the average mass of 1bp of dsDNA in gm/mole.

The DNA samples were ten-fold serially diluted, ranging from a copy number of 3.25×10^{10} to 32.5 and used for plotting a standard curve (Ct vs copy number) by qPCR assay.

The SYBR dye based real-time PCR was performed in duplicate for all the unknown and control samples using the StepOne™ Real-Time PCR System (Applied Biosystems). The same *CadF* gene-specific primer set was used for qPCR. Each reaction was made in a final volume of 25 µl containing 1 µl of DNA template, 1 µl of each forward and reverse primer (10 picomole each) and 12.5 µl of 2× Hi-SYBR Master Mix (HiMedia, India). The Thermocyclic condition includes initial denaturation at 94°C for 10 min, followed by 40 cycles of denaturation (94°C, 30 sec),

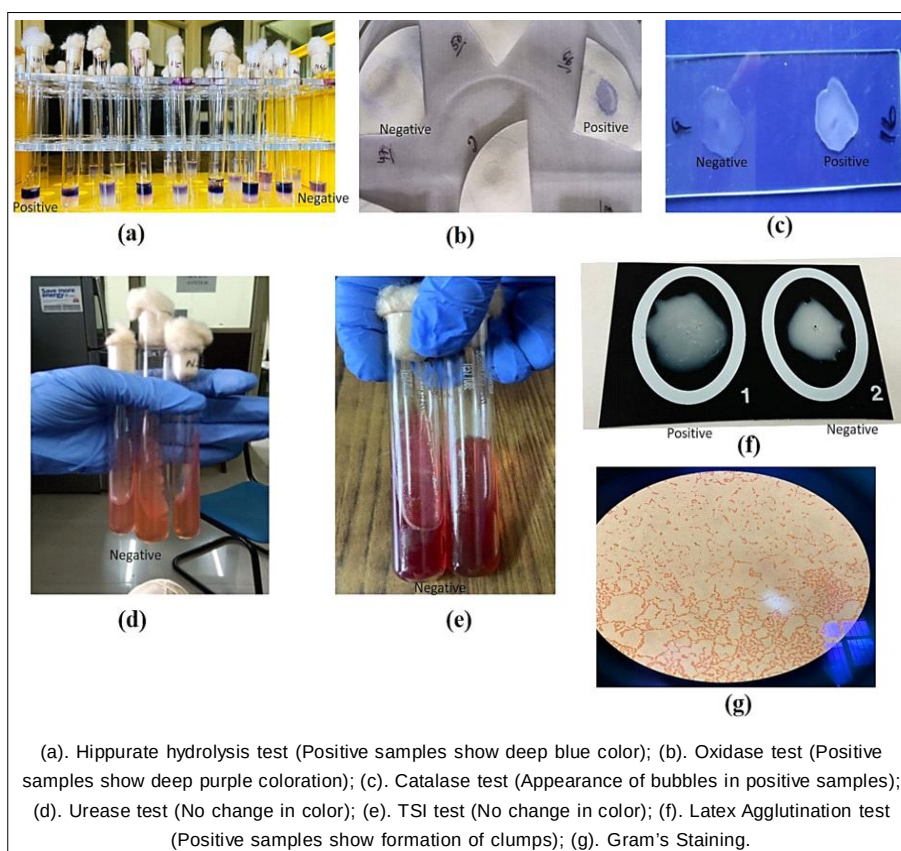


Fig 2: Biochemical confirmation of *Campylobacter*.

annealing (50°C, 30 sec) and extension (72°C, 30 sec), subsequently followed by a melt curve analysis step. The fluorescence for each cycle was acquired during the extension step and a no-template control (NTC) and positive template control (PTC) were also included in each run. The analysis of qPCR result was done by calculating the mean cycle threshold (Ct mean), standard deviation (Ct SD), coefficient of variation (CV%) for each dilution and a standard curve was plotted for the estimation of copy number in the unknown water samples using absolute quantification.

RESULTS AND DISCUSSION

Isolation and biochemical characterization of campylobacter

The typical small, pinpoint, sticky and slightly raised greyish colonies on BFCSA were found, which were suspected to be *Campylobacter* spp. The Gram's staining showed pink

coloured Gram-negative curved rod-shaped bacteria. Hippurate hydrolysis showed deep blue coloration in some reaction tubes, which suggests the presence of *Campylobacter jejuni*. The oxidase test showed purple coloration on rubbing the colonies on oxidase-soaked filter paper, whereas the Catalase test showed an appearance of bubbles on the slide consisting of 3% H₂O₂. In the Urease and TSI test where no change in colour was found. These results were indicative of the presence of *Campylobacter*. Latex agglutination showed formation of clumps with the reaction mixture, which confirms the presence of *Campylobacter*. The results of isolation and biochemical identification are shown in Fig 2. The results of the biochemical test were also in accordance with the previous studies conducted on *Campylobacter* by different groups (Garhia *et al.*, 2017; Singh *et al.*, 2023; Ansari *et al.*, 2025).

Molecular confirmation using PCR

The suspected isolates were tested for confirmation by PCR targeting the *CadF* gene, which is a single-copy and eminently conserved gene responsible for adhesion of bacteria to the host cell, thus causing infection. The quality and quantity of DNA isolation were satisfactory as revealed by gel electrophoresis and spectrophotometric quantification. The concentration lies between 1500 to 2000 ng/μl and the A260/280 ratio was between 1.6 to 1.8. Due to the thermophilic and thermoresistant nature of *Campylobacter*, it needs competitively harsh treatment for disruption of the cell wall, hence causing difficulty in isolation and low yield of genomic DNA (Singh *et al.*, 2022). On PCR amplification, a specific band of 400 bp was seen on agarose gel, confirming the presence of the genus *Campylobacter* (Fig 3). The *CadF* gene has a significant diagnostic importance and has also been extensively used for *Campylobacter* detection (Singh *et al.*, 2022; Klena *et al.*, 2004; Nayak *et al.*, 2005).

Real-time qPCR-based quantification

The quality and quantity of DNA isolated from water samples were fairly well, with a concentration and A260/280 ratio between 1.5 and 1.9, respectively. To plot the standard curve, the copy number of serially diluted commercial *Campylobacter* genomic DNA was calculated and found between 32.25×10^1 and 3.25×10^{10} . For each dilution that was run in duplicate, the mean Cycle threshold (Ct mean), Standard deviation (Ct SD) and Coefficient of variation (CV%) were obtained (Table 1). The amplification plots for each dilution were obtained as typical sigmoid curves without any background signal (Fig 4) and a standard curve was plotted as a straight line (Fig 5.) The mean $\pm$ SD and CV% were found to be 0.764 and 2.30%, respectively, for the assay. A single sharp peak was observed on the melt curve analysis representing specific amplification (Fig 6).

The copy number of *Campylobacter* was estimated using the standard curve in unknown water samples collected from rivers and natural and artificial lakes. It was observed that a significant variation was found in bacterial load among the water samples, which ranges between a

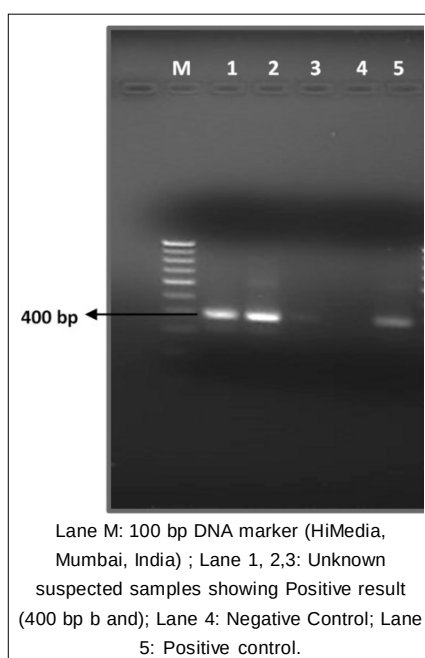


Fig 3: Molecular confirmation of *Campylobacter* by PCR.

Table 1: Ct mean, Ct SD and CV% for different dilutions of *Campylobacter* DNA.

Copy number	Ct mean	Ct SD	CV (%)
3.25×10^{10}	15.82	0.068	0.43
3.25×10^9	19.758	0.074	0.38
3.25×10^8	23.201	0.092	0.39
3.25×10^7	26.99	0.177	0.66
3.25×10^6	30.717	0.118	0.38
3.25×10^5	32.761	0.629	1.92
3.25×10^4	33.248	1.289	3.88
3.25×10^3	33.648	0.568	1.69
3.25×10^2	34.419	1.76	5.11
3.25×10^1	35.145	2.866	8.15

minimum bacterial load of 7.7×10^2 cells to a maximum of 2.7×10^5 cells, as listed in Table 2. It is evident from the above data that the highest load of *Campylobacter* is observed in the sample drawn from the Nainital district from a barrage built on the Gola River. Also, a higher load of *Campylobacter* was found in bodies where water is stagnant and highly polluted, contrary to running water, which can be correlated to the multiple ways of effluent entry to the stagnant water bodies, like barrages. The major source of *Campylobacter* contamination in the environment is through animal and poultry husbandry practices. Places where there is a higher human-animal interface are suspected to be more likely contaminated with *Campylobacter* (Taha-Abdelaziz *et al.*, 2023; Ogden *et al.*, 2009). Thermophilic *Campylobacter* can adapt to stressful

environmental conditions through various strategies, like changing its shape and transition into viable but non-culturable (VBNC). Environmental stressors like low temperature, chloride treatment and aerobic conditions trigger the bacteria to enter the VBNC state (Kim *et al.*, 2021; Li *et al.*, 2014). This makes its detection and quantification a tedious task, leading to its missed detection and false reporting in epidemiological surveys. However, molecular methods like PCR and qPCR make it more convenient through the detection of nucleic acid by overcoming the problems of bacterial isolation and culture. Wastewater-based epidemiology (WBE) of *Campylobacter* is an important tool for early prediction of outbreaks and analyzing its status in the food chain and qPCR-based methods are recommended for use in WBE due to their

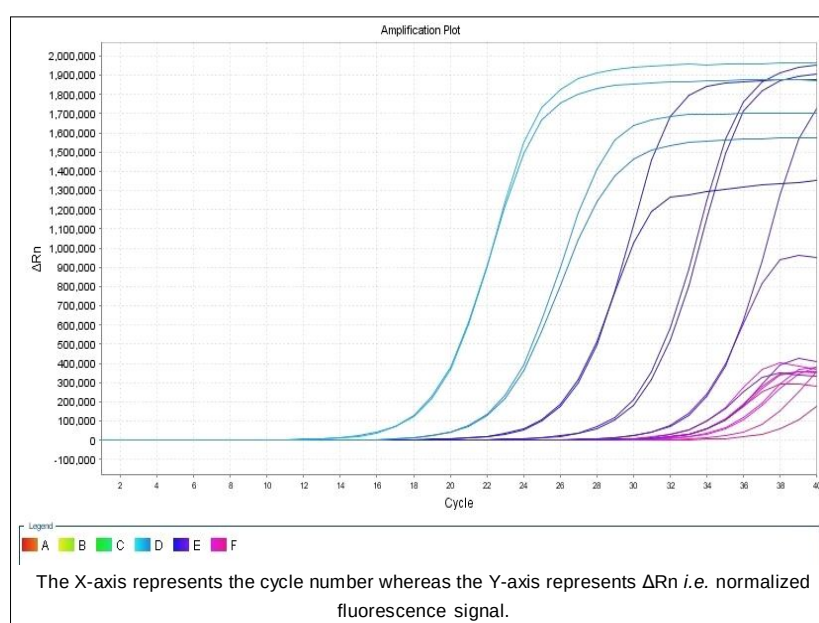


Fig 4: Amplification plots for different dilutions of *Campylobacter*.

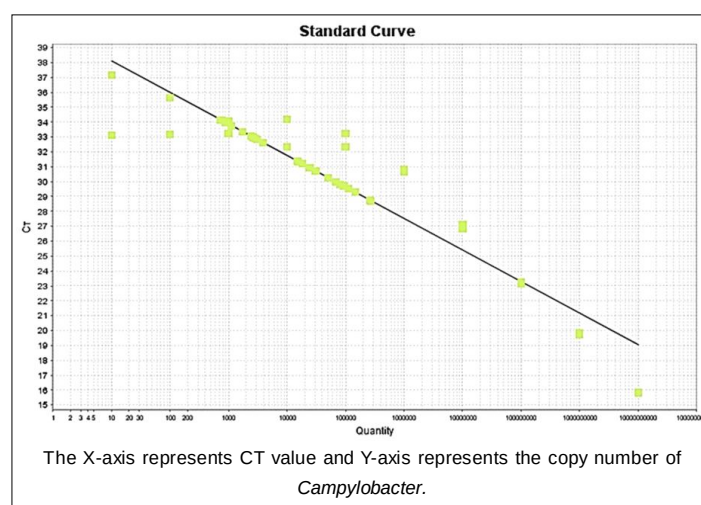
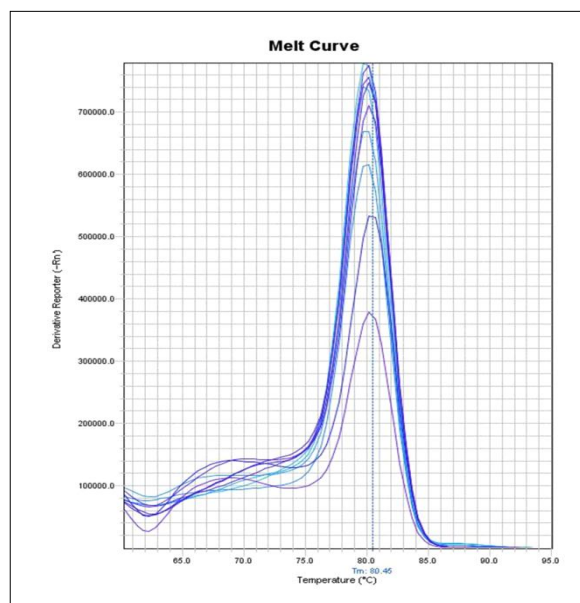


Fig 5: Standard curve plotted using different dilutions of *Campylobacter* DNA by Real-Time qPCR.

Table 2: *Campylobacter* load (Copy number) estimated by qPCR from different water samples.

Location	Sample I.D	Source	Quantity
Nainital	N103	River	6.7×10^4
	N104	River (Barrage)	2.7×10^5
	Lal	River	1.5×10^4
	Gola	River	3.8×10^3
	N44	Lake	4.9×10^4
	N42	Lake	2.5×10^5
	N3	River	1.1×10^3
	N29	Lake	7.3×10^3
	N13	River	2.4×10^4
	N23	Lake	9.04×10^2
	N53	Lake	7.9×10^4
	N88	Lake	3.08×10^4
	N43	Lake	2.4×10^3
	N51	Lake	8.7×10^2
	N26	Lake	2.4×10^4
	N45	Lake	3.04×10^3
Udham Singh Nagar	R28	River	1.1×10^5
	R22	River	2.6×10^4
	Chak	River	1.7×10^3
	R24	River	2.8×10^4
	R25	River	1.4×10^5
	6	River	1.8×10^4
	R20	River	1.4×10^4
	10	River	7.7×10^2
	Haladi	River	7.9×10^4
	R9	Others (Artificial lake)	9.3×10^4
	R17	River	2.6×10^3

**Fig 6:** The Melt curve for *Campylobacter* was plotted by increasing the temperature from 60°C to 95°C and taking readings between 60°C and 95°C in a continuous acquisition mode, which revealed a typical dissociation curve of SYBR Green.

higher sensitivity (Zhang *et al.*, 2023b; Chowdhari *et al.*, 2022). Indeed, due to the advent of molecular biology and genomics, more advanced and specific methods are now available, like metagenomics or direct sequencing of specific bacteria from the mixed environmental pools of samples (Zhang *et al.*, 2021, Pathak *et al.*, 2018). Assessing the pathogen load of the surface water bodies and taking appropriate steps for their treatment can help to limit infections like Campylobacteriosis to promote one-health, especially in developing countries like India with high population density (Bell *et al.*, 2021).

CONCLUSION

The quantification of *Campylobacter* in surface water bodies may provide substantial insights into improving surveillance and mitigation strategies to limit its spread in human and animal populations. qPCR presents an efficient way to assess bacterial load in water samples. The present study concludes that a higher *Campylobacter* load is expected in water bodies with a high human-animal interface in surrounding areas and the areas with stagnant water. For a developing country like India with the highest population in the world, it may be a critical finding that should be addressed and worked upon to ensure one-health.

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Conflict of interest

The authors declare no conflict of interest related to this work submitted for publication.

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