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Occurrence and Reduction of Shiga Toxin-Producing *Escherichia coli* in Wastewaters in the Kathmandu Valley, Nepal

Niva Sthapit [1], Bikash Malla [2], Sarmila Tandukar [2,3], Rajani Ghaju Shrestha [2,4], Ocean Thakali [1], Jeevan B. Sherchand [5], Eiji Haramoto [2,*] and Futaba Kazama [2]

1 Department of Engineering, University of Yamanashi, Yamanashi 400-8511, Japan; niva1180@gmail.com (N.S.); othakali@gmail.com (O.T.)
2 Interdisciplinary Center for River Basin Environment, University of Yamanashi, Yamanashi 400-8511, Japan; mallabikash@hotmail.com (B.M.); sar1234tan@gmail.com (S.T.); rajani_ghaju12@hotmail.com (R.G.S.); kfutaba@yamanashi.ac.jp (F.K.)
3 Policy Research Institute, Sano Gaucharan-5, Kathmandu 44600, Nepal
4 Division of Sustainable Energy and Environmental Engineering, Osaka University, 1-1 Yamadaoka, Suita 565-0871, Japan
5 Institute of Medicine, Tribhuvan University, Maharajgunj, Kathmandu 1524, Nepal; jeevanbsherchand@gmail.com
* Correspondence: eharamoto@yamanashi.ac.jp

Abstract: Inadequately treated effluents discharged from wastewater treatment plants (WWTPs) severely affect the environment and the surrounding population. This study analyzed the presence of the Shiga toxin-producing *Escherichia coli* (STEC) genes, *stx1*, and *stx2*, and the *E. coli* gene, *sfmD*, in municipal WWTP A (n = 11) and B (n = 11) where the reductions were also evaluated; hospitals (n = 17), sewage treatment plants (STPs) (n = 4) and non-functional WWTPs (not-working WWTPs) (n = 5) in the Kathmandu Valley, Nepal. The *sfmD* gene was detected in 100% of the samples in WWTPs, hospitals, and not-working WWTPs and 50% of STP samples. The highest detection of *stx1* and *stx2* was shown in the WWTP influents, followed by WWTP effluents, not-working WWTP wastewater, hospital wastewater, and STP wastewater. Log_{10} reduction values of *sfmD*, *stx1*, and *stx2* in WWTP A were 1.7 log_{10}, 1.7 log_{10}, 1.4 log_{10}, whereas those in WWTP B were 0.5 log_{10}, 0.6 log_{10}, 0.5 log_{10}, respectively, suggesting the ineffective treatment of STEC in the wastewater in the Kathmandu Valley. The high concentrations of the *stx* genes in the wastewaters suggest the increasing presence of aggressive STEC in the Kathmandu Valley, which should be a major public health concern.

Keywords: *Escherichia coli*; Nepal; pathogen; wastewater treatment

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1. Introduction

A primary public health concern is the contamination of environmental waters by fecal pathogens. Wastewater typically comprises high concentrations of microbiological contaminants that require treatment before being discharged into the environment [1]. Wastewater treatment plants (WWTPs) are commonly constructed to remove organic matter in wastewater, which enables the reduction in bacterial loads, subsequently decreasing environmental water contamination [2]. The treated effluent is sometimes reclaimed for several purposes, including irrigation, landscaping, and flushing toilets, because of the increase in global population and climate change causing further water crises [3,4]. However, wastewater in many developing countries, such as Nepal, is discharged into surface water bodies without adequate treatment, yet, is still used as a primary source for agricultural irrigation [5,6]. Inefficiently treated effluent is a threat to macro- and microflora and fauna of the surface water bodies and the supply of good water quality to the population. The increased microbial concentration in the inadequately treated effluent may also cause waterborne diseases, such as gastroenteritis [7].

The Shiga toxin-producing *Escherichia coli* (STEC) is an important cause of foodborne diseases; however, its source of contamination extends to leafy vegetables, water, and fecal-contaminated environmental water, too [8,9]. STEC infection is caused by the ingestion of the bacteria, for which the infective dose to induce a response by the body is fewer than ten cells, due to the presence of the *stx1* and/or *stx2* genes. The *stx1* and *stx2* genes are primary virulence factors that inhibit protein synthesis, which causes apoptosis and are responsible for STEC pathogenesis, whereas the *sfmD* gene encodes for the outer membrane usher protein in *E. coli* [10,11]. Diseases associated with STEC infection include dysentery, hemorrhagic colitis, and hemolytic uremic syndrome in humans [9,12,13]. STEC was first discovered in 1977 and was first isolated from water in 1989 [14,15]. Since then, there have been several reports of waterborne STEC-related outbreaks [16–19]. These outbreaks were caused by ruminant fecal contamination of surface water bodies leaching into the unconfined aquifer used by the city, insufficient filtration by water purifier and damaged connection between reservoir, and chlorine sterilization pipelines, contamination of the water distribution system by the sewage due to backflow at or near the leakage site, and the ingestion of lake water contaminated by feces. The low infective dose is attributed to the occurrence of waterborne STEC outbreaks, and the past studies illustrate the different methods of possibilities of STEC infection [20]. A study conducted in France showed STEC presence in slurry, wastewater, and river water surrounding and in the French slaughterhouses, highlighting the importance of proper treatment of wastewater to prevent contamination of food and the environment [21]. The remaining microbiome and nutrients in the effluent may also encourage the increase in pathogenic bacteria in the environment [22]. A previous study also detected the presence in groundwater, river water, feces, and manure in the Kathmandu Valley, and suggested the possibility of river water contamination from wastewater discharge and river water and groundwater interactions near the river channels contributing to groundwater contamination [23].

Presently, only 88% of households in the Kathmandu Valley possess sewage facilities, and 11% of households still use a septic tank or pit [24]. There are currently five WWTPs in the Kathmandu Valley, of which only one WWTP was in operation (activated sludge system) and the remaining four WWTPs (aerated and non-aerated lagoons) were either under partial operation or non-functional during the time of sampling [5,23,25]. The status "partially operating" shows the inability to treat the wastewater as effectively as a fully functional treatment plant owing to poor operation, maintenance, and management [5]. In addition to WWTPs, wastewater is processed in sewage treatment plants (STPs) and hospitals before discharge.

Furthermore, the nutrient-rich environment and high density of cells in WWTPs facilitate horizontal gene transfer among microorganisms, which is also suggested to be involved in the evolution of some of the first STEC strains [26–28]. In addition to this, several studies have presented the inability of STEC to be removed entirely from wastewater effluents [21,29–31]. Previous studies in the Kathmandu Valley have examined the occurrence and reduction in pathogenic viruses and bacteria in WWTPs [32–34]. However, regular monitoring and surveillance of pathogens in the wastewater are not performed in the Kathmandu Valley. A contributing factor for this may include the cost of testing for pathogens on a regular basis. Since molecular methods in bacteriology typically initiate with DNA extraction for which several methods of varying advantages and disadvantages have been and are continuously developed, a cost-effective and time-efficient DNA extraction method that can be implemented in resource-poor countries is necessary to encourage regular pathogen surveillance [35]. One of these methods is the heat treatment or "boiling" method that enables bacterial DNA extraction by causing damage to the cell membranes to release the nucleic acid within to obtain the DNA extract cost-effectively and rapidly [35,36]. Due to the lack of surveillance of STEC in the wastewater, this study aimed to assess the applicability of heat-treated DNA extraction method for pathogen surveillance and analyzed the presence and reduction in STEC in two WWTPs of the Kathmandu Valley.

2. Materials and Methods

2.1. Collection of Wastewater Samples

Four types of wastewaters were obtained from different treatment plants and hospitals in the Kathmandu Valley between April and August 2018. Altogether, 48 wastewater samples, which comprised general hospital wastewater effluents that were untreated due to the absence of treatment systems (HWW) (n = 17), municipal WWTP (n = 22), STP influents (n = 4), and not-working WWTP (n = 5) samples were collected. The municipal wastewater samples were obtained by grab sampling from two WWTPs, WWTP A (n = 12) which used an oxidation ditch system and had a capacity of 17.3 million liters per day (MLD) designed for approximately 74,000 inhabitants and WWTP B (n = 10) which used stabilization ponds and had a capacity of 1.1 MLD designed for the population of eastern Lalitpur, for which the influents (n = 6 and 5 for WWTP A and B, respectively), and the effluents (n = 6 and 5) were obtained [25,37]. The water samples were collected in autoclaved 1 L plastic bottles and transported in an icebox to the laboratory within six hours of collection.

2.2. Enumeration of E. coli in Wastewater

As described in previous studies [23,38,39], the *E. coli* concentrations were determined in 100 mL wastewater for dilutions of 10^{-2}, 10^{-4}, and 10^{-6} using the Colilert Quanti-Tray 2000 (IDEXX Laboratories, Westbrook, USA). However, in this study, an additional bottle of 100 mL sample diluted to 10^{-2} was also directly incubated at 37 °C for 24 h. In the Quanti-Tray, the wells that fluoresced blue under the UV lamp were considered positive for *E. coli*. Based on the number of blue wells, the concentrations of *E. coli* were evaluated by referring to the most probable number (MPN) generating software (IDEXX Laboratories).

2.3. Performance of Heat and Kit DNA Extraction

Before performing DNA extraction of the WWTP samples, the performance of two different DNA extraction methods—heat DNA extraction and kit DNA extraction—were evaluated.

To evaluate the performance of the heat DNA extraction method, 10^{-1} and 10^{-2} dilutions of *E. coli* K-12 (NBRC3301, National Institute of Technology and Evaluation, Kisarazu, Japan) were prepared in 100 mL autoclaved ultrapure water. Following the manufacturer's instructions, these samples were subjected to the Colilert-18 test kit (IDEXX Laboratories, Westbrook, ME, USA) [23,38,39]. On the Quanti-Tray 2000 (IDEXX Laboratories), wells that appeared yellow and blue under UV light were punctured using a micropipette to collect 500 µL from eight large positive wells and four small positive wells of the 10^{-1} tray and from eight large positive wells and six small positive wells of the 10^{-2} tray. In addition to this, 500 µL from four large negative wells and three small negative wells from the 10^{-1} tray, and from three small negative wells from the 10^{-2} tray were obtained. These cultured *E. coli* samples were stored in 1.5 mL microtubes with 500 µL 50% glycerol. During DNA extraction, 40 µL of the cultured *E. coli* in glycerol was transferred to a 0.2 mL PCR tube and heated at 95 °C for five minutes in the TaKaRa PCR Thermal Cycler Dice Touch (Takara Bio, Kusatsu, Japan). The resulting heated cultured *E. coli* in glycerol was used as the DNA extract.

Additionally, 200 µL bacterial concentrate of the corresponding samples were also subjected to a kit DNA extraction to obtain 200 µL bacterial DNA extract using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) following the provided instructions.

The performance of the two methods was evaluated based on the sensitivity, specificity, and accuracy obtained. The sensitivity was calculated as the number of true-positive samples divided by the sum of the true-positive samples and the false-negative samples. The specificity was calculated as the number of true-negative samples divided by the sum of the true-negative samples and the false-positive samples. The accuracy was calculated as the sum of the true-positive samples and the true-negative samples divided by the sum of the true-positive, false-negative, true-negative, and false-positive samples [39].

2.4. DNA Extraction and quantitative Polymerase Chain Reaction (qPCR)

One hundred mL of 10^{-2} dilution of the wastewater samples in autoclaved glass bottles was subjected to the Colilert test kit (IDEXX Laboratories) following the manufacturer's instructions and incubated at 37 °C for 24 h. As mentioned above, 500 µL positive samples of cultured *E. coli* were obtained from the bottle and stored with 500 µL 50% glycerol in 1.5 mL tubes. Forty microliters of this sample were used for heat DNA extraction following the thermal conditions as previously explained. Alternatively, 10 mL wastewater samples were concentrated using a nitrocellulose membrane filter (pore size, 0.22 µm; diameter, 47 mm; Nalgene, Tokyo, Japan), followed by DNA extraction using the Cica Geneus DNA Extraction kit to obtain a final volume of 300 µL of purified DNA (Kanto Chemical, Tokyo, Japan), as described previously [38,40,41].

The samples subjected to heat DNA extraction were referred to as cultured qPCR, and those subjected to kit DNA extraction were referred to as direct qPCR. The wastewater samples underwent qPCR of three assays, *stx1*, *stx2*, and *sfmD*, as shown in Table 1. A 25 µL qPCR reaction mixture, which comprised 12.5 µL Probe qPCR Mix (Takara Bio), 1.0 µL each of 10-pmol/µL of the forward and reverse primers [11,42], 1.0 µL of 5-pmol/µL TaqMan probe, 7.0 µL PCR-grade water, and 2.5 µL DNA extract was prepared. To generate a standard curve for the qPCR run, six tenfold serial dilutions of artificially synthesized plasmid DNA containing the amplification sequences (2.0×10^0–2.0×10^5 copies/µL; Takara Bio for *sfmD* and Eurofins Genomics, Tokyo, Japan for *stx1* and *stx2*) were added to each run. All samples were duplicated, including the standards and the negative controls. The qPCR was performed in the Thermal Cycler Dice Real-Time System TP800 (Takara Bio) using the following thermal conditions: hold at 95 °C for 30 s, followed by 2 step PCR of 45 cycles of 95 °C for five seconds and 60 °C for 30 s. The cut-off cycle threshold value was set to 35 for *sfmD* and 40 for *stx1* and *stx2*.

Table 1. Primer and probe sequences of the qPCR assays used in this study.

Assay	Function	Sequence (5′–3′) [a]	Reference
sfmD	Forward primer	ACTGGAATACTTCGGATTCAGATACGT	[11]
	Reverse primer	ATCCCTACAGATTCATTCCACGAAA	
	TaqMan probe	FAM-CAGCAGCTGGGTTGGCATCAGTTATTCG-TAMRA	
stx1	Forward primer	TTTGTYACTGTSACAGCWGAAGCYTTACG	[42]
	Reverse primer	CCCCAGTTCARWGTRAGRTCMACRTC	
	TaqMan robe	FAM-CTGGATGATCTCAGTGGGCGTTCTTATGTAA-TAMRA	
stx2	Forward primer	TTTGTYACTGTSACAGCWGAAGCYTTACG	[42]
	Reverse primer	CCCCAGTTCARWGTRAGRTCMACRTC	
	TaqMan probe	FAM-TCGTCAGGCACTGTCTGAAACTGCTCC-TAMRA	

Note: [a] FAM, 6-carboxyfluorescein; TAMRA, 5-carboxytetramethylrhodamine; Y denotes C or T; S denotes C or G; W denotes A or T; R denotes A or G; M denotes A or C.

2.5. Data Analysis

The χ2-test was used to compare the positive ratios between the direct qPCR and cultured qPCR and between assays in the influent and effluent samples of the WWTP. The McNemar χ2-test was performed to determine the relationship of the positive ratios between the *stx1* and *stx2* genes. The paired *t*-test analyzed the difference between the mean concentrations between assays and sample types. The $\log_{10}$ reduction value (LRV) was evaluated by subtracting the $\log_{10}$ concentration of the effluent from the influent; for the non-detects, the 1/10 of the limit of detection (LOD) value for each assay was used to compute. The Welch *t*-test was used to evaluate the difference in the LRV of the assays between the two WWTPs. The virulent *E. coli* ratio was calculated as the ratio of the *stx1* or *stx2* gene concentration to that of the *sfmD* gene using only the positive samples. A *p*-value of <0.05 was considered statistically significant in the conducted analyses.

3. Results

3.1. Performance of Heat and Kit DNA Extraction

Of the 26 samples that were Colilert positive and ten that were Colilert negative, the heat DNA extraction exhibited 100% sensitivity (26/26), 100% specificity (10/10), and 100% accuracy (36/36), whereas the kit DNA extraction expressed 96% sensitivity (25/26), 100% specificity (10/10), and 97% accuracy (35/36). The samples subjected to heat DNA extraction resulted in a mean Ct value of 23.1 ± 1.1, whereas those subjected to kit DNA extraction resulted in 21.5 ± 0.9. Statistically, the heat DNA extraction observed a significantly lower mean Ct value than that of the kit DNA extraction (paired *t*-test, $p < 0.05$); however, the purpose of the heat DNA extraction was to obtain qualitative data to determine the viability of typical *E. coli*. For this reason, the heat DNA extraction was used alongside direct qPCR using kit DNA extraction to verify *E. coli* viability later in the study.

3.2. Detection of E. coli Genes in Wastewater Samples

As indicated in Table 2, *sfmD* was detected in all wastewater samples (100%) using direct qPCR except for the STP (50%). Similarly, at least one of the two virulence genes was present in all except the STP (0%) using direct qPCR. In contrast, using the cultured qPCR, *sfmD* and at least one of the virulence genes were exhibited in all tested wastewater types. The *stx1* gene in WWTP A showed a significantly lower positive ratio in the effluent (17%) compared to in the influent (100%) ($\chi 2$ test, $p < 0.05$). The WWTP B effluent (80%) also expressed a lower positive ratio of the *stx1* gene to the influent (100%); however, unlike WWTP A, the difference was insignificant ($\chi 2$, $p > 0.05$). As for the *stx2* gene, only WWTP A exhibited a lower effluent positive ratio (67%) of the *stx2* gene compared to the influent (100%), although the difference was also insignificant ($\chi 2$, $p > 0.05$).

In addition to the difference between the influent and effluent, the difference in detection of the *stx1* and *stx2* genes was calculated. The HWW, not-working WWTP, WWTP A and B influents observed no significant difference in the positive ratio between the *stx1* and *stx2* genes (McNemar $\chi 2$, $p > 0.05$). Only the WWTP A and B effluents exhibited a significant difference between the *stx1* and *stx2*-positive ratios (McNemar $\chi 2$, $p > 0.05$). Since the *stx1* and *stx2* genes were undetected in the STP, the test was not applied to it.

3.3. Concentrations of E. coli and Its Genes in Wastewater Samples

The concentrations using direct qPCR for *sfmD*, *stx1,* and *stx2,* as well as the concentration of *E. coli* using the Colilert method for all wastewater samples are shown in Table 3. The concentrations of *E. coli* using the Colilert method and direct qPCR ranged from 6.3 ± 0.8 $\log_{10}$ copies/L to 8.9 ± 0.3 $\log_{10}$ copies/L and from 9.1 ± 0.1 $\log_{10}$ copies/L to 11.0 ± 0.1 $\log_{10}$ copies/L, respectively. Alternatively, the *stx1* and *stx2* concentrations ranged from < LOD to 7.1 ± 0.3 $\log_{10}$ copies/L and from < LOD to 7.7 ± 0.3 $\log_{10}$ copies/L, respectively. The mean concentration of *sfmD* in all wastewater samples was significantly greater (2.2 $\log_{10}$) than for the Colilert method (paired *t*-test, $p < 0.05$). Between the *stx1* and *stx2* genes, the *stx2* gene is significantly greater in concentration than the *stx1* gene in the HWW, WWTP A influent and effluent, and WWTP B influent and effluent samples (paired *t*-test, $p < 0.05$). However, there is no significant difference in concentrations between the *stx1* and *stx2* in the STP and not-working WWTP.

Table 2. Detection of *E. coli* genes in wastewater samples.

Wastewater Type	No. of Tested Samples	*sfmD*				*stx1*				*stx2*			
		Direct qPCR		Cultured qPCR		Direct qPCR		Cultured qPCR		Direct qPCR		Cultured qPCR	
		No. of Positive Samples (%)	Ct (Mean ± SD)	No. of Positive Samples (%)	Ct (Mean ± SD)	No. of Positive Samples (%)	Ct (Mean ± SD)	No. of Positive Samples (%)	Ct (Mean ± SD)	No. of Positive Samples (%)	Ct (Mean ± SD)	No. of Positive Samples (%)	Ct (Mean ± SD)
HWW	17	17 (100)	28.3 ± 3.9	17 (100)	22.2 ± 2.1	6 (35)	37.7 ± 0.9	8 (47)	34.9 ± 2.1	6 (35)	37.0 ± 0.9	9 (53)	32.4 ± 3.0
STP	4	2 (50)	31.3 ± 0.4	4 (100)	22.1 ± 0.8	0 (0)	NA	0 (0)	NA	0 (0)	NA	1 (25)	36.3
Not-working WWTP	5	5 (100)	27.3 ± 2.6	5 (100)	22.7 ± 1.2	3 (60)	38.2 ± 1.2	5 (100)	36.5 ± 2.7	3 (60)	37.5 ± 1.5	5 (100)	35.5 ± 3.1
WWTP A influent	6	6 (100)	25.3 ± 0.4	6 (100)	21.7 ± 1.0	6 (100)	36.5 ± 0.9	6 (100)	33.9 ± 1.8	6 (100)	35.5 ± 1.2	6 (100)	31.8 ± 2.2
WWTP A effluent	6	6 (100)	30.5 ± 1.6	6 (100)	21.9 ± 0.6	1 (17)	38.8	6 (100)	33.5 ± 1.1	4 (67)	38.9 ± 0.3	6 (100)	31.8 ± 1.7
WWTP B influent	5	5 (100)	24.9 ± 0.5	5 (100)	21.6 ± 0.8	5 (100)	35.7 ± 0.9	5 (100)	32.9 ± 1.3	5 (100)	34.7 ± 0.9	5 (100)	31.3 ± 2.0
WWTP B effluent	5	5 (100)	26.4 ± 2.2	5 (100)	21.5 ± 0.9	4 (80)	36.6 ± 1.3	5 (100)	32.6 ± 1.0	5 (100)	36.0 ± 1.3	5 (100)	31.1 ± 1.6
Total	48	46 (96)	27.6 ± 3.2	48 (100)	22.0 ± 1.5	25 (52)	36.8 ± 1.3	35 (73)	34.1 ± 2.1	29 (60)	36.3 ± 1.7	37 (77)	32.3 ± 2.6

Note: SD, standard deviation; NA, not applicable.

Table 3. Concentrations of *E. coli* and its genes in wastewater samples.

Wastewater Type	No. of Tested Samples	Colilert	*sfmD*	*stx1*	*stx2*
		Conc. (log_{10} MPN/L) (Mean ± SD)	Conc. (log_{10} Copies/L) (Mean ± SD)	Conc. (log_{10} Copies/L) (Mean ± SD)	Conc. (log_{10} Copies/L) (Mean ± SD)
HWW	17	7.5 ± 1.4	10.0 ± 1.2	6.5 ± 0.3	6.9 ± 0.3
STP	4	6.3 ± 0.8	9.1 ± 0.1	<LOD [a]	<LOD [b]
Not-working WWTP	5	8.2 ± 0.8	10.3 ± 0.8	6.3 ± 0.3	6.7 ± 0.6
WWTP A Influent	6	8.7 ± 0.2	10.9 ± 0.1	6.8 ± 0.2	7.5 ± 0.3
WWTP A Effluent	6	7.3 ± 0.3	9.2 ± 0.6	5.2 ± 0.4	6.1 ± 0.6
WWTP B Influent	5	8.9 ± 0.3	11.0 ± 0.1	7.1 ± 0.3	7.7 ± 0.3
WWTP B Effluent	5	8.6 ± 0.7	10.5 ± 0.8	6.5 ± 0.9	7.3 ± 0.4

Note: MPN, most probable number; SD, standard deviation; LOD, limit of detection; [a] 6.3 log_{10} copies/L; [b] 6.6 log_{10} copies/L.

3.4. LRVs of E. coli Genes in Municipal WWTP

Among the three assays tested in the two WWTPs, only the *stx2* gene showed a significant difference in mean concentrations between the influent and effluent in both WWTP A and B (paired *t*-test, $p < 0.05$). The *sfmD* and *stx1* genes only exhibited a significant difference in LRV between the influent and the effluent in WWTP A. In addition to this, the highest LRV of the *sfmD* (1.7 ± 0.6) and *stx1* (1.7 ± 0.5) genes were shown in WWTP A, as shown in Figure 1. The lowest LRV was observed for *sfmD* (0.5 ± 0.8) and *stx2* (0.5 ± 0.3) genes in WWTP B. Between the two WWTPs, a significantly greater LRV (1.1) was shown by the WWTP A, compared to the WWTP B (Welch *t*-test, $p < 0.05$) for all three genes tested.

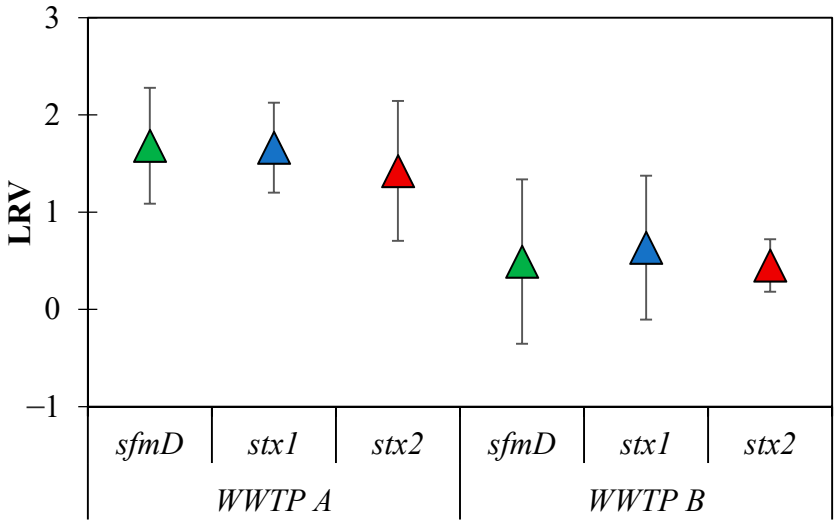

Figure 1. Log_{10} reduction values of *E. coli* genes in the wastewater treatment plants.

3.5. Ratio of Virulent E. coli Genes

The ratio of the virulence gene to *sfmD* gene ranged from 0.01% ± 0.01%–0.15% ± 0.13% for all samples, as presented in Table 4. The *stx1*/*sfmD* ratios were ranked as follows: WWTP A effluent > WWTP B influent > WWTP B effluent > HWW > WWTP A influent > not-working WWTP > STP, whereas the *stx2*/*sfmD* ratios were ranked as follows: WWTP A effluent > WWTP B effluent > WWTP B influent > WWTP A influent > HWW > not-working WWTP > STP. In addition to this, the ratio between the Colilert and *sfmD* gene results was determined. The highest Colilert/*sfmD* ratio (1.43% ± 2.14%) was exhibited by the WWTP A effluent, whereas the lowest ratio (0.60% ± 0.78%) was seen in the STP.

Table 4. Ratio of virulent *E. coli* genes.

Wastewater Type	No. of Tested Samples	Colilert/*sfmD* Ratio (%)	*stx1*/*sfmD* Ratio (%)	*stx2*/*sfmD* Ratio (%)
HWW	17	1.43 ± 2.14	0.01 ± 0.01	0.03 ± 0.02
STP	4	0.60 ± 0.78	NA	NA
Not-working WWTP	5	0.90 ± 0.36	0.004 ± 0.002	0.01 ± 0.01
WWTP A Influent	6	0.73 ± 0.36	0.01 ± 0.01	0.05 ± 0.03
WWTP A Effluent	6	1.77 ± 1.39	0.02	0.15 ± 0.13
WWTP B Influent	5	0.87 ± 0.51	0.01 ± 0.01	0.06 ± 0.04
WWTP B Effluent	5	1.17 ± 0.51	0.01 ± 0.01	0.09 ± 0.12

4. Discussion

4.1. Performance of DNA Extraction Methods

WWTPs in the Kathmandu Valley presently still lack routine monitoring and management and require further development to decrease the cause of different diseases [32]. This study analyzed the detection and reduction efficiency of STEC in several types of wastewaters in the Kathmandu Valley. Since the detection of the genes does not provide

information about the viability of the pathogens, the Colilert method was applied alongside the direct qPCR method. However, the process of DNA extraction using a commercial kit is costly, laborious, and time-consuming. It sometimes requires special equipment, such as a centrifuge, to perform the procedure, which may be unsuitable for application in developing countries. Simplified versions of DNA extraction have been previously performed by heating bacterial material without adding reagents, such as by heating bacterial colonies suspended in distilled water in test tubes on a hot water bath for 10 min; by heating the bacterial colonies suspended in distilled water in a microwave oven for 10 s followed by centrifugation; by incubating the bacteria at 99 °C for 15 min; by culturing the bacteria followed by centrifugation and heating the precipitate at 100 °C for 10 min; and by boiling the bacteria suspended in TE buffer for 15 min followed by centrifugation [35,36,43,44]. The *E. coli* K-12 samples cultured using the method in this study underwent DNA extraction using a kit and by heating and exhibited comparable results. The performance of the heat extraction method was higher than that of the kit extraction method; however, the Ct value of the latter was significantly greater than that of the former. Despite that, the difference in Ct values did not exceed two cycles. Several past studies concluded no significant difference between the heat extraction and the commercial kit extraction methods similar to that of this study and suggest the applicability of heating bacterial material for DNA extraction, too [35,36,43–45]. One study assessed the ability of the bacterial DNA extracted using heat treatment to perform PCR, Restriction Fragment Length Polymorphism (RFLP), and DNA sequencing and concluded comparable results to the more complicated DNA extraction methods of these processes and suggested its applicability on other biological samples, as well [35]. Similarly, another study compared the cost per sample and the average process duration among six different DNA extraction methods and found that the boiling method cost nothing to perform and averaged about 20 min to process, whereas the cost per sample of the remaining five methods ranged from €3.96 to €5.7 and the durations ranged from 30 min to >1 h [44]. For these reasons, the heat DNA extraction was implemented on the cultured samples to obtain at least the qualitative data from qPCR to verify the presence of viable *E. coli* in the wastewater samples quickly and efficiently.

4.2. Detection of E. coli Genes in Wastewater Samples

This study also observed higher detection of the *stx2* gene than the *stx1* gene, which is congruent with many past studies [1,21,30,46,47]. However, the positive ratios in this study for the *stx1* and *stx2* genes in the influent and effluent of WWTP A (100%, 100%; 17%, 67%) and WWTP B (100%, 100%; 80%, 100%) were much higher than those of the previous studies. The differences in the presence of *stx1* and *stx2* among the studies may be attributed to the different geographical locations and their varying fecal flora composition in the wastewater and the different methods performed and sampling sizes and strategies [1]. Another contributing factor to the higher STEC presence in this study is the treatment technology employed by the treatment plants. A study that analyzed the presence of virulent genes of *E. coli* in activated sludge-based treatment plants with various modifications determined that the prevalence of the *stx1* and/or *stx2* genes did not reduce after treatment in all modifications, and the most prevalent virulent gene in all wastewater samples analyzed was the *stx2* gene [47]. Similarly, a study in Brazil reported at least six strains of STEC in the raw and treated wastewater from aerated lagoons [48]. The results of this study were consistent with the previous studies and in addition to that, this study investigated an activated sludge-based treatment plant that lacked the disinfection process prior to discharge and a stabilization pond system that was improperly maintained, which may further justify these results.

The concentrations of the *stx2* gene in the HWW, WWTP A, and WWTP B were also significantly greater than those of the *stx1* gene, which agreed with studies conducted in Hawaii, USA, Queensland, Australia, and Saxony, Germany [30,49,50]. Studies have indicated that this is typically the case due to the different biology of the two *stx*-phages in the bacterial host [51,52]. The *stx1* and *stx2* phages harbor a late promoter that induces

phage lysis; however, there is a functional promoter containing a binding site for a protein that complexes with iron adjacent to the *stx1* gene. This indicates that the transcription of the *stx1* gene can be activated during low concentrations of iron and is not only dependent on the late promoter of the *stx1*-encoding phage for *stx1* production and release. In contrast, the *stx2* expression is highly dependent on phage-inducing agents that induce transcription of the late promoter of the *stx2*-encoding phage for *stx2* release [51,53,54]. Additionally, there are only two identified variants of *stx1*, whereas the *stx2* has at least 13 characterized variants that are more commonly associated with severe effects of STEC infection [10,54]. Based on the results obtained in this study, the higher concentrations of the *stx2* gene than the *stx1* in the wastewaters suggest the growing presence of aggressive STEC infection in the population of the Kathmandu Valley and should be a public health concern. This is especially true due to the trail of the wastewater effluent discharged into the river water, which is reclaimed for irrigational purposes and interacts with groundwater by the river banks used for household use too, as previously discussed [23].

Typically, a not-working WWTP is expected to show a higher concentration of *E. coli* genes in the effluent than in operating WWTPs since the influents in the not-working WWTPs are discharged without treatment as the effluent. However, in this case, a lower concentration of all three genes was observed only in the WWTP A effluent. In contrast, the WWTP A influent and WWTP B influent and effluent exhibited greater concentrations of all three genes than in the not-working WWTPs. The lack of difference between the operational/partially operational and the non-functional WWTPs suggests the inefficacy of the WWTPs being used in the Kathmandu Valley. As previously discussed, this may be induced by the lack of a disinfection step in the WWTP, proper and regular monitoring, and maintenance due to the complexity of the operation, costly materials and electricity, and the shortage of human resources [25,32].

4.3. Reduction in E. coli Genes in WWTPs

Furthermore, this study also reported LRVs of the *sfmD*, *stx1*, and *stx2* in WWTP A (1.7 ± 0.6, 1.7 ± 0.5, and 1.4 ± 0.7, respectively), and WWTP B (0.5 ± 0.8, 0.6 ± 0.7, and 0.5 ± 0.3, respectively). As mentioned above, this may be attributed to the wastewater treatment technique implemented on the influent. Studies have demonstrated that different treatment techniques remove the various microorganisms at different rates [4,55]. Oakely and Mihelcic discussed how the removal of one pathogen type, such as indicator bacteria, to meet the effluent standards may not indicate sufficient removal of another pathogen type. Previous studies on the reduction in *E. coli* in conventional activated sludge treatment plants have obtained a reduction ranging from 0.30–2, analogous to the LRVs acquired in this study [55]. Another study on the same WWTP obtained LRVs of 1.4 for *E. coli* [32], which is also congruent with this study. However, the LRVs of the indicator viruses (pepper mild mottle virus and tobacco mosaic virus) were 0.2 ± 0.6 and 0.2 ± 0.5, respectively, which were lower than the default virus LRV for well-managed and designed activated sludge plants. Unlike WWTP A, the LRVs of *E. coli* in this and previous studies were lower in WWTP B than in past studies and in addition to that, the LRVs of almost all tested viruses were inconsiderable [56,57].

4.4. Infectivity of Virulent E. coli

Additionally, as shown in Table 1, the infectivity of the pathogens was also evaluated by conducting cultured qPCR alongside direct qPCR. The combination of the two methods provided data on the gene concentration as well as the infectivity of the pathogens in the wastewater sample simultaneously in a shorter duration than typical culture-based methods followed by molecular methods. Subjecting the wastewater samples to both methods simultaneously also showed a more accurate depiction of the pathogen and gene presence in the sample since the culture-based methods alone don't reveal the viable but non-culturable, dormant, or injured cells, whereas the molecular method enables the detection of the pathogen regardless of viability [58]. In this study, the presence of the *sfmD*,

stx1, and *stx2* genes using cultured qPCR (100%, 73%, 77%) were all greater than those using the direct qPCR (96%, 52%, 60%), and the lower Ct values of the former (22.0 ± 1.5, 34.1 ± 2.1, 32.3 ± 2.6) than the latter (27.6 ± 3.2, 36.8 ± 1.3, 36.3 ± 1.7) confirmed growth of the pathogens in the sample, which indicates the presence of viable bacteria. This suggests that the virulence genes detected in this study may have been derived from infectious STEC circulating in the Kathmandu Valley, and the combination of the cultured and direct qPCR is a recommended method for application in developing countries.

Furthermore, this study also examined the Colilert/*sfmD* ratio as well as the virulent *E. coli* gene ratio, as shown in Table 3. Since the Colilert method was used to enumerate the *E. coli* in the wastewater samples immediately after collection, the ratio between the MPN concentration and the molecular concentrations were determined. In this study, the Colilert/*sfmD* ratio ranged from 0.60% ± 0.78% to 1.77% ± 1.39%, which may suggest that up to 2% of the *sfmD* genes may have been derived from viable *E. coli*. In addition to this, the *stx1*/*sfmD* ratio ranged from 0% 0.02%, and the *stx2*/*sfmD* ratio ranged from 0 0.15% ± 0.13%, suggesting that up to 0.2% of the *E. coli* genes may have comprised of virulence genes that, as previously discussed, was derived from infectious STEC. All the results combined suggest the inefficacy of the WWTPs in the Kathmandu Valley in eliminating pathogens from the wastewater before discharging it back into the environment. Therefore, it may be necessary to improve and update the policies for the wastewater management guidelines in the Kathmandu Valley.

5. Conclusions

Studies on STEC are still limited in the Kathmandu Valley—particularly in wastewater—despite being a common cause of outbreaks worldwide. This study successfully determined the occurrence and reduction in STEC in two WWTPs in the Kathmandu Valley and provided data on STEC in the wastewaters. These results showed the ineffective treatment of the STEC genes from the influent to the discharged effluent due to the high concentrations in both and a low LRV between the two. The inability to decrease the pathogen demands the installation of a disinfection unit that uses chlorination, which is cost-effective and energy-efficient, and better maintenance of the treatment techniques presently implemented to reduce the concentrations and infectivity of the pathogens. To further decrease the pathogen load in the WWTPs, hospitals must be required to build a treatment system that performs disinfection of both the influent and the effluent, such as also using chlorination from the HWW before being discharged into the sewers that lead to the municipal WWTP; these may assist in lowering the WWTP effluent pathogen concentrations in the Kathmandu Valley.

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