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Pilot study on wastewater surveillance of dengue virus RNA: Lessons, challenges, and implications for future research

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ABSTRACT

Dengue virus (DENV) is an enveloped, single-stranded RNA virus that causes approximately 390 million infections, leading to 40,000 deaths annually. Due to the increasing trend of urbanization, water supply scarcity, and climate change, dengue is regarded as the "disease of the future," requiring robust surveillance for the early detection of DENV infection. Since the virus is shed in urine and saliva and persists in wastewater at different temperatures, our study conducted wastewater surveillance as a novel approach to monitor dengue outbreaks in the Kathmandu Valley, Nepal. The viral concentrates ($n = 34$), which were previously collected and concentrated from municipal and hospital wastewater, and river water using the electronegative membrane-vortex method, were tested for DENV using quantitative reverse transcription polymerase chain reaction (RT-qPCR) and digital PCR (RT-dPCR). Pepper mild mottle virus, a process control and endogenous biomarker, was detected in all the samples with concentrations ranging from 8.0 to 10.0 $\log_{10}$ copies/L, whereas DENV was not detected in any sample using RT-dPCR and RT-qPCR. The undetected DENV in this study could be attributed to the collection of grab wastewater samples during a low relative prevalence of dengue infection in the region, insufficient sample volume processed, probable viral nucleic acid degradation due to storage of viral concentrate at -25 °C for a long period of time, or inefficiency of the primary concentration method used. This study highlights critical gaps in knowledge and provides recommendations for future implementation of wastewater surveillance of dengue outbreaks, especially in regions where dengue was recently introduced, clinical surveillance is limited, and wastewater surveillance for polio has been adopted.

1. Introduction

Currently, during the coronavirus disease 2019 (COVID-19) pandemic, wastewater-based epidemiology (WBE) is gaining attention as an economic tool for tracing COVID-19 spread and the circulating variants of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in communities (Heijnen et al., 2021; Ahmed et al., 2020; Sherchan et al., 2020; Haramoto et al., 2020). Initially, wastewater-based surveillance was proposed to monitor illicit drug use by detecting drug metabolites (Daughton, 2001). The scope was later widened to monitor the spread of pathogenic bacteria, viruses, and protozoa (Diemert and Yan, 2019; Xagoraraki and O'Brien, 2020; Zahedi et al., 2021). Viruses cannot replicate outside host cells; therefore, their concentration in wastewater is likely to reflect the actual number of cases in a geographical area (Xagoraraki and O'Brien, 2020). Furthermore, clinical surveillance is likely to account for symptomatic patients only. However, WBE can account for asymptomatic and mild infection cases and has been effective when implemented to monitor outbreaks caused by enteric and respiratory viruses (Kishida et al., 2012; Heijnen and Medema, 2011). In addition, to enteric and respiratory viruses, arboviruses are also shed in human excrement, indicating the potential for successful application of WBE for vector-borne diseases (Xagoraraki and O'Brien, 2020; Muirhead et al., 2020).

Dengue is the most important arboviral disease among viral diseases (Yang et al., 2021) caused by the dengue virus (DENV) and transmitted by female mosquitoes of the species *Aedes aegypti* and *Aedes al-*

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bopictus (Yang et al., 2021). DENV is an enveloped, spherical in shape, single-stranded RNA virus, 10.7 kb long, 40–60 nm in size, belonging to the genus *Flavivirus* of the family *Flaviviridae*, majorly classified into four distinct serotypes (DENV 1–4) (Murugesan and Manoharan, 2020; Guzman et al., 2016). Given the potential of DENV for large-scale dissemination that can cause mass fear and casualties, it was previously listed as a Category A biological agent possessing the highest potential for adverse public health impact (Sinclair et al., 2008). At present, it is estimated that 3.9 billion people are prone to dengue and its virus, causing approximately 390 million infections, with 96 million symptomatic cases resulting in 40,000 deaths yearly (Bhatt et al., 2013). There are no specific therapeutics, but only one vaccine for dengue has been approved in approximately 20 endemic countries exclusively for people of age group 9–45 (World Health Organization 2022). DENV transmission is greatly influenced by temperature, rainfall, urbanization, and the distribution of the *Aedes* mosquito (Naish et al., 2014). Massive DENV transmission can be attributed to the capability of the vector to transmit DENV throughout its lifetime after becoming infectious and the possibility of a person getting infected with DENV four times in his/her lifetime due to the presence of four serotypes (World Health Organization 2022). *Aedes* mosquito lives in urban habitats, breeds in water-filled artificial containers and its eggs can remain viable for months and hatch once in contact with water (World Health Organization 2022). Due to the increasing trend of urbanization, scarce water supply, and climate change, dengue is now regarded as the “disease of the future” (Guzman et al., 2016), thus requiring extensive surveillance to detect DENV infection early. Interestingly, studies have been conducted to investigate the applicability of reverse transcription-quantitative polymerase chain reaction (RT–qPCR) to detect DENV in noninvasive urine and saliva samples to strengthen laboratory diagnosis of dengue in hemorrhagic patients. Such studies have confirmed the presence of DENV RNA lasting for two weeks with maximum concentrations of 5.2 $\log_{10}$ cDNA copies/mL and 6.1 $\log_{10}$ cDNA copies/mL in urine and saliva, respectively (Hirayama et al., 2012; Van den Bossche et al., 2015; Korhonen et al., 2014; Andries et al., 2015). In addition, a recent report has shown that DENV RNA persists in wastewater over a relatively large range of temperatures (Chandra et al., 2021), suggesting that wastewater surveillance deserves further investigation as a novel approach to monitor dengue outbreaks.

This study aimed to investigate the presence of DENV RNA in the wastewater of the Kathmandu Valley, Nepal, which, to our knowledge, is the first attempt in wastewater surveillance for DENV RNA. This study is significant because, unlike in endemic countries, dengue was reported for the first time in 2004 (Takasaki et al., 2008) but is still a neglected tropical disease in Nepal. Apart from vector control methods, mosquito nets are widely used to prevent mosquito-borne diseases in the country. Nevertheless, a few Nepalese are aware that dengue vectors are daytime feeders (Dhimal et al., 2014), making nets fairly ineffective in preventing dengue. Furthermore, Nepal is one of the most vulnerable countries to climate change (Tuladhar et al., 2019), and any shift toward favorable climatic conditions such as increased rainfall and temperature causes vector habitat expansion and prolongs the transmission season. The study area is of particular interest as the Kathmandu Valley is the largest urban area of Nepal with an estimated population of three million. The valley is located at 1300 m from sea level and was initially thought to be dengue-free. However, dengue vectors were identified in the valley for the first time in 2009, and subsequent studies suggest that dengue vectors are potentially established in the area (Gautam et al., 2009; Dhimal et al., 2014). Since dengue vectors have weak dispersal capacity and low average maximum flight distance (Verdonschot and Besse-Lototskaya, 2014), a closely packed population in the Kathmandu Valley is conducive to dengue outbreaks. Due to the chronic shortage of piped water supply in the valley, wells, and water storage container collections are prevalent in households, serving as potential breeding sites for mosquito vectors (Tuladhar et al., 2019). In addition, proven breeding sites, such as water-filled abandoned tires and flower pots, are common sightings (Tuladhar et al., 2019). A dengue outbreak was reported in July 2019 for the first time in the valley, and only one serotype (DENV-2) has been identified (Adhikari et al., 2020). No cases of dengue were identified in 2020, whereas data of 2021 are still lacking. As of August 2022, the Kathmandu Valley is plagued by a dengue outbreak (https://english.onlinekhabar.com/dengue-in-nepal-outbreak-and-control.html). The information collected from this study will contribute to the knowledge of the potential of WBE in monitoring dengue outbreaks in non-endemic vulnerable geographical locations.

2. Materials and methods

2.1. Sample collection

The number of dengue cases is expected to be high in the Kathmandu Valley during the monsoon period from July to September. Therefore, as shown in Table 1, previously collected 34 grab water samples during the same months in 2017–2019 from a river (n = 1), two functioning wastewater treatment plants (WWTPs) [influent and effluent (n = 13 each)], a non-functioning WWTP [influent (n = 3)], and two hospitals [untreated (n = 2), aeration tank (n = 1), and treated (n = 1)] were analyzed for DENV in this study. Wastewater samples collected in 2018 from the two functioning WWTPs in an earlier study (Tandukar et al., 2021) were also included. These WWTPs have a combined wastewater treatment capacity of only 33.5 million liters per day, resulting in the direct discharge of most sewage in rivers. Hence, a river water sample was also included. The number of confirmed dengue cases in the Kathmandu Valley in 2017–2019 (Table 1) were obtained from the annual report of the Department of Health Services, Government of Nepal.

2.2. Virus concentration and RNA extraction

Viruses in water samples were concentrated using the electronegative membrane-vortex (EMV) method previously described (Haramoto et al., 2012). The method began with addition of 500 µL of 2.5 M $MgCl_2$ to 50 mL of water samples. The water samples were then passed through an electronegative membrane filter (0.8 µm pore size and 47 mm diameter; Merck Millipore, Bellerica, MA, USA). Next, viruses were eluted by vortexing with 10 mL of an elution buffer containing 0.2 g/L $Na_4P_2O_7{\cdot}10H_2O$, 0.3 g/L $C_{10}H_{13}N_2O_8Na_3{\cdot}3H_2O$, and 0.1 mL/L Tween 80. This step was repeated with 5 mL of elution buffer, and approximately 15 mL of the eluate was recovered. The eluate was then centrifuged at 2000 × g for 10 min at 4 °C. Finally, only the supernatant was filtered through a filter unit (0.45 µm pore size, 25 mm diameter; Advantec, Tokyo, Japan), and viral concentrate was obtained using a Centriprep YM-50 ultrafiltration device (Merck Millipore). The obtained viral concentrate was then stored at −25 °C until RNA extraction in September 2021. Viral RNA was extracted from the concentrate using QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) in a QIAcube automated platform (QIAGEN) and eluted in a volume of 60 µL according to the manufacturer’s guidelines.

2.3. RT–qPCR inhibition and extraction efficiency

Pseudomonas bacteriophage Φ6 (NBRC 105,899, National Institute of Technology and Evaluation, Tokyo, Japan), a surrogate for enveloped viruses (Torii et al., 2022), was used as a molecular process control to calculate the efficiency of RNA extraction-RT–qPCR as well as to determine the presence of inhibitors. Shortly, 1 µL of Φ6 (4.2 $\log_{10}$ copies/µL) was seeded into 140 µL of concentrated samples and PCR-grade water. The seeded PCR-grade water acted as a non-inhibitory control (NIC). The extraction efficiency was calculated as the ratio of the concentration of Φ6 in samples to that in NIC.

Table 1
Sampling details and the number of confirmed dengue cases.

Year	No. of confirmed dengue cases in the Kathmandu Valley*	Month of sampling	Sample type	No. of water samples collected
2017	17	August, September	WWTP influent	4
			WWTP effluent	4
2018	11	July, August	WWTP influent	8
			WWTP effluent	5
2019	1991	August	River water	1
			WWTP influent	4
			WWTP effluent	4
			Untreated hospital wastewater	2
			Hospital wastewater (aeration tank)	1
			Treated hospital wastewater	1
Total				34

* Annual Report of the Department of Health Services, Government of Nepal (Department of Health Services, 2022).

Table 2
Primers and probes used in this study.

Assay	Function	Sequence (5′–3′)	Size (bp)	Reference
DENV	Forward primer	GARAGACCAGAGATCCTGCTGTCT	68	Gurukumar et al. (2009)
	Reverse primer	ACCATTCCATTTTCTGGCGTT		
	TaqMan MGB probe	FAM-AGCATCATTCCAGGCAC-NFQ-MGB		
PMMoV	Forward primer	GAGTGGTTTGACCTTAACGTTTGA	68	Zhang et al. (2006); Haramoto et al. (2013)
	Reverse primer	TTGTCGGTTGCAATGCAAGT		
	TaqMan MGB probe	FAM-CCTACCGAAGCAAATG-NFQ-MGB		
Φ6	Forward primer	TGGCGGCGGTCAAGAGC	101	Gendron et al. (2010)
	Reverse primer	GGATGATTCTCCAGAAGCTGCTG		
	TaqMan probe	FAM-CGGTCGTCG/ZEN/CAGGTCTGACACTCGC-IBFQ		

R denotes A or G; FAM, 6-carboxyfluorescein; NFQ, nonfluorescent quencher; MGB, minor groove binder; ZEN, ZEN internal quencher; IBFQ, Iowa Black fluorescent quencher.

2.4. RT–qPCR analysis

RT was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). A previously developed assay that represents all serotypes of DENV was used in this study (Gurukumar et al., 2009). The complete list of primers and probes used is given in Table 2. All the RT–qPCR assays were performed using a Thermal Cycler Dice Real-Time System III (Takara Bio, Kusatsu, Japan). Reaction mixtures (25 μL) contained 12.5 μL of Probe qPCR Mix with UNG (Takara Bio), 0.1 μL each of forward and reverse primers (100 pmol/μL), 0.05 μL of a probe (100 pmol/μL), and 2.5 μL of template cDNA. Thermal conditions for qPCR included incubation at 25 °C for 10 min, followed by 95 °C for 30 s, and 45 cycles of 95 °C for 5 s and 60 °C for 30 s (for DENV and Φ6) or at 60 °C for 60 s (for PMMoV). Tenfold serial dilutions (5.0×10^0–5.0×10^5 copies/reaction) of gBlocks gene fragment (Integrated DNA Technologies, Coralville, LA, USA) were used to draw a standard curve and negative control comprised of PCR-grade water. All samples were tested in duplicate, and the cutoff cycle threshold value was 40.

2.5. RT–dPCR analysis

RT–dPCR analysis was performed in singlicate using the QIAcuity Digital PCR system (QIAGEN) to analyze the presence of DENV only. The same DENV assay for RT–qPCR analysis was used. Optimized dPCR reaction mixture comprised of reagents from QIAcuity One-Step Viral RT–PCR Kit (QIAGEN): 11 μL of RT One-Step Viral RT–PCR Master Mix, 0.44 μL of Multiplex Reverse Transcription Mix, 0.18 μL each of forward and reverse primers (100 pmol/μL), 0.09 μL probe (100 pmol/μL), 25.51 μL of RNase-free water, and 6.6 μL of template RNA. A total of 40 μL of the reaction mixture was transferred to a 24-well QIAcuity Nanoplate 26 K (QIAGEN) and sealed. The temperature profile used for RT–dPCR was as follows: 50 °C for 40 min, 95 °C for 2 min, followed by 45 cycles of 95 °C for 5 s and 60 °C for 30 s. Negative control comprised of RNase-free water, whereas four tenfold serial dilutions (8.0×10^1–8.0×10^4 copies/μL) of gBlocks gene fragment (Integrated DNA Technologies) were also included as positive controls and to evaluate the performance of DENV assay. The number of valid partitions ranged from 23,199 to 25,469 (89.2–98.0%). Based on the partition volume of 0.82 nL, dead volume ranged 19.1–21.0 μL.

3. Results and discussion

3.1. RNA extraction efficiency and performance of RT–qPCR assay

The average RNA extraction-RT–qPCR efficiency obtained in this study was 95.0% ± 50.9% (n = 34). Two samples demonstrated <10% (poor) extraction-RT–qPCR efficiency (6.7% and 0%) due to poor extraction and/or presence of PCR inhibitors and as recommended (Haramoto et al., 2018), were not included for further qPCR analysis. The slope of the standards for Φ6, PMMoV, and DENV assays was −3.21, −3.23, and −3.30, respectively. The amplification efficiencies for three assays were 104.9% (Φ6), 103.9% (PMMoV), and 101.1% (DENV). Y-intercept values were 37.89 (Φ6), 40.28 (PMMoV), and 39.27 (DENV). The correlation coefficient (R^2) values for the assays were 0.998 (Φ6), 0.999 (PMMoV), and 0.993 (DENV).

3.2. Performance of DENV assay using dPCR

The expected and measured concentration of gBlocks gene fragment and the number of valid partitions in the dPCR run is given in Table 3. The average difference between expected and measured concentrations was 0.81 ± 0.01 $\log_{10}$ (n = 4). Fig. 1 shows the regression plot drawn using $\log_{10}$ transformed expected and measured concentrations of gBlocks gene fragment.

3.3. Detection of DENV and PMMoV in water samples

PMMoV was quantified as a process control to assess viral recovery in this study. PMMoV was detected in all tested samples with concentra-

Table 3
Expected and measured concentrations of gBlocks gene fragment using dPCR.

Expected concentration of gBlocks gene fragment (copies/µL)	dPCR performance			
	No. of partitions			Measured concentration of gBlocks gene fragment (copies/µL)
	Positive	Negative	Total	
8.0×10^4	18,121	5397	23,518	1.3×10^4
8.0×10^3	3292	22,160	25,452	1.2×10^3
8.0×10^2	368	25,077	25,445	1.3×10^2
8.0×10^1	35	24,225	24,260	1.2×10^1

Table 4
Detection of viruses in water samples using qPCR.

Year	No. of samples tested	DENV		PMMoV	
		No. of positive samples (%)	Concentration ($\log_{10}$ copies/L) (Min–Max)	No. of positive samples (%)	Concentration ($\log_{10}$ copies/L) (Min–Max)
2017	8	0 (0)	N/A	8 (100)	8.7–9.6
2018	11	0 (0)	N/A	11 (100)	8.0–10.0
2019	13	0 (0)	N/A	13 (100)	8.1–9.7
Total	32	0 (0)	N/A	32 (100)	8.0–10.0

N/A, not applicable.

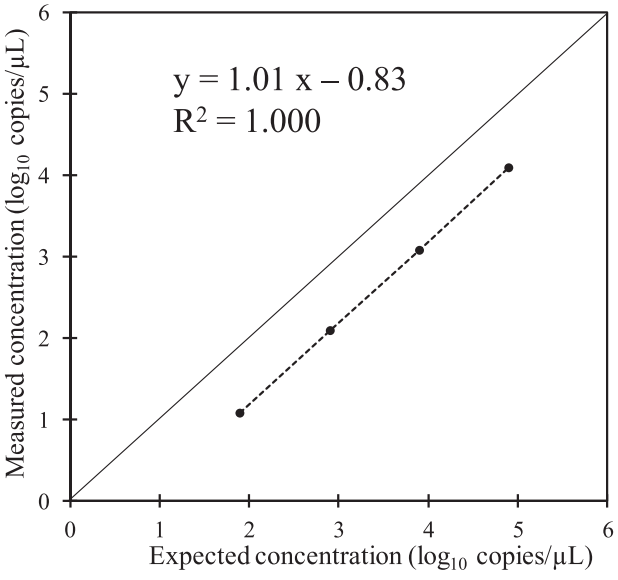

Fig. 1. Regression plot demonstrating the accuracy of DENV assay using dPCR.

tions ranging from 8.0 to 10.0 $\log_{10}$ copies/L. A recent review on enteric viruses in water (Haramoto et al., 2018) has reported PMMoV concentrations in raw wastewater worldwide ranged from approximately 6.8 to 10.3 $\log_{10}$ copies/L, which suggests that significant virus recovery was achieved (Table 4). However, it should be noted that recovery of an enveloped virus including DENV is likely to differ from a non-enveloped virus such as PMMoV. In contrast, qPCR did not detect DENV in any tested samples ($n = 32$). Since PCR inhibition was suspected in two samples, all the samples ($n = 34$) were subsequently tested for the presence of DENV using dPCR. The dPCR technology used in this study divides the samples into approximately 26,000 partitions, providing higher tolerance to inhibitors. Regardless, none of the samples tested positive for DENV using RT–dPCR. The limit of detection (LOD) for DENV using both qPCR and dPCR was 4.2 $\log_{10}$ copies/L.

Several limitations can explain the failure to detect DENV in this study. First, this is a retrospective study, and the previously stored viral concentrates were analyzed. Storage of viral concentrate at −25 °C for a long period of time might have also resulted in the decay of DENV RNA. The concentration volume of water can also affect the detection of viruses, and 50 mL might have been inadequate. The EMV method, designed to prioritize the concentration of non-enveloped viruses (Haramoto et al., 2012), was used to concentrate viruses from water samples. In the EMV method, viruses adsorbed to the electronegative membrane are concentrated from the supernatant, resulting in probable loss of virus present in the pellet. A lipid bilayer covers enveloped viruses, such as DENV, which are naturally hydrophobic, and tend to attach to solid particles, including pellets (Ye et al., 2016). Even the data available for the reported number of confirmed dengue cases are summarized for the whole year only without further granularity. Number of confirmed cases on a weekly or monthly basis are still unavailable. There is a high possibility that grab samples might have been collected during the low prevalence of dengue infection as peak months of dengue cases can vary, as previously seen in 2010 (August), 2013 (October), and 2016 (September) (Acharya et al., 2016; Khetan et al., 2018). Furthermore, the confirmation of dengue cases in Nepal is often delayed or questionable due to the lack of molecular diagnostic facilities and the use of rapid diagnostic kits with low specificity (Adhikari et al., 2020; Gupta et al., 2018).

3.4. Scope, challenges, and future direction of wastewater surveillance of dengue outbreaks

DENV can either cause mild disease or severe dengue, with the former occurring when any serotype causes a primary infection in a non-immune population. In contrast, severe dengue occurs when a secondary infection with another serotype occurs in the same population (Adhikari et al., 2020; Wilder-Smith et al., 2019). In the recent outbreak in 2019, mild disease was generally observed in the Kathmandu Valley (Adhikari et al., 2020). Such pauci-symptomatic cases can make determining the actual degree of viral circulation difficult, given the low molecular diagnostic testing capability. In addition, the determination of co-circulating serotypes is critical to conclude the possibility of severe dengue and in that regard, WBE can be a valuable tool.

Despite the potential, the accuracy of wastewater surveillance can be affected by different variables associated with sampling, sample processing, molecular detection, and data analysis (Ahmed et al., 2020). Among all variables, selecting a highly efficient method for the primary concentration of DENV is critical due to the probable low concentration of DENV in wastewater. Urine constitutes only 1% of the total vol-

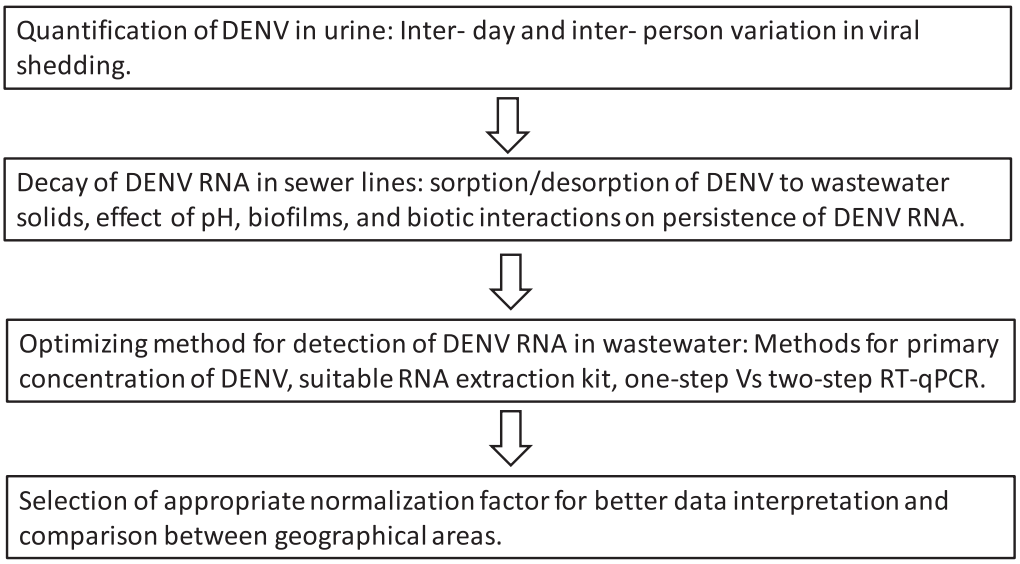

Fig. 2. Flowchart highlighting key research gaps in wastewater surveillance of dengue outbreaks.

umetric flow in municipal wastewater (Landry and Boyer, 2016). The form of DENV, whether it exists as the genome, (non)-infectious virions, or virus-antibody complex, remains unclear. Inter-day and inter-person variation of DENV excretion in urine and saliva has been observed in previous studies (Van den Bossche et al., 2015; Andries et al., 2015), and a clear picture of such variations is necessary as it adds value in determining the probable number of dengue cases in a catchment area. In addition, increase in catchment runoffs due to rainfall events during dengue outbreaks are likely to further dilute viruses in wastewater. WBE studies on viruses shed in feces are usually conducted based on 24 h composite or morning grab (considering early morning defecation habit) samplings once a week, which facilitates selection of sampling day when rainfall is least likely to affect wastewater flow. However, researchers are probably deprived of such facilities when conducting wastewater surveillance for dengue.

Normally, concentrating <100 mL of untreated wastewater is deemed sufficient for WBE (Kitajima et al., 2020); however, volume greater than >200 mL may need to be processed for adequate quantification of DENV, depending on the number of dengue cases and the effect of rainfall. No studies have reported the efficiency of DENV recovery using different available virus concentration methods. However, a study (Ahmed et al., 2020) using a surrogate for enveloped viruses has shown that pretreatment of a sample with $MgCl_2$ followed by adsorption-extraction method to be highly efficient in concentrating enveloped viruses. The mechanism of adsorption is based on the electrostatic attraction between viruses and electronegative membrane filter in the presence of $MgCl_2$. The adsorption-extraction method is advantageous as it facilitates the concentration of a higher volume of wastewater compared with other methods, covers both solid and liquid fractions of wastewater, and does not require additional concentration steps that can also result in loss of viruses. Altogether, the adsorption-extraction method has potential but further investigations on the performance of other existing methods, such as polyethylene glycol precipitation, ultrafiltration, concentrating pipette, and centrifugation, are also required. The centrifugation method involves settling down of solid fraction of wastewater and RNA extraction directly from the pellet. The added benefit of the centrifugation method is that it is economical and can be easily implemented in low- and middle-income countries. Recently, a concentration method involving the combination of dead-end hollow-fiber ultrafiltration and concentrating pipette has been developed to concentrate a large volume of wastewater (2 L) with whole process recovery efficiency of > 20% for an enveloped virus (McMinn et al., 2021). The combination method can concentrate 8-times volumes of existing methods and lower the effect of rainwater dilution during wastewater surveillance.

Post concentration of viruses, RT–qPCR results can also be affected by RNA extraction and RT steps. Only one study has compared five commercially available RNA extraction kits and reported comparable recovery for a surrogate of enveloped viruses to date (Qiu et al., 2021). Nevertheless, the use of kits with fewer steps that facilitate the removal of PCR inhibitors is recommended (Ahmed et al., 2020). RT is also performed separately (two-step) or combined with qPCR in a single tube (one-step). One-step RT–qPCR has shown higher sensitivity in detecting genes in low abundance as it allows higher volume of nucleic acid analysis (Qiu et al., 2021) and also lowers the probability of contamination than two-step RT–qPCR. When repeated freeze-thaw cycles of RNA samples can be avoided, one-step RT–qPCR is advised for future studies focusing on environmental surveillance of dengue. Given that wastewater samples are prone to nonspecific amplification and the likelihood of DENV concentration to be near the lower limit of detection, we recommend sequencing for confirmation when DENV is detected. A whole process control (WPC) and molecular process control (MPC) must be included before the concentration of water samples and RNA extraction, respectively, for better data interpretation (Haramoto et al., 2018).

Data generated from wastewater surveillance of viruses are interpreted to estimate disease incidence or even predict outbreaks (Ahmed et al., 2020; Xagoraraki, 2020). Such interpretation requires information regarding the population covered by the WWTP, disease incubation period, DENV shedding rate, and the average flow of wastewater in WWTP. Critical research gaps, such as the decay of DENV in sewer lines due to pH, biofilms, and biotic interactions, still exist (Fig. 2). Quantifying the decay rate of DENV reduces bias in estimating disease incidence or predicting viral disease fluctuation in a community. WBE studies involve quantification of indicator non-pathogenic viruses, such as PMMoV and crAssphage along with the target virus as an internal control to assess viral recovery or as a population normalizing factor (Haramoto et al., 2020; Wilder et al., 2021). Any change in the concentration of PMMoV and crAssphage can indicate a change in fecal matter concentration in raw wastewater. Normalization of target virus concentration to an indicator virus enables data comparison between cities, cancels the effect of population change or any storm events affecting target virus detection, and helps deal with non-detects (left-censored data) to observe the trend of diseases in long-term epidemiological studies. Like indicator viruses, a consensus on a suitable urine biomarker for

normalization of viruses found in wastewater shed primarily via urine is required. Twelve urinary biomarkers have been previously proposed (Rico et al., 2017; Daughton, 2012; Chiaia et al., 2008; Chen et al., 2014); of which, cotinine and caffeine are most beneficial (Rico et al., 2017) due to the high usage of nicotine and caffeine products, stability in wastewater under different temperatures, and low intra- and inter-day variation in concentrations. A threshold value for urinary biomarker concentration should be established to confirm the acceptable urine composition in wastewater. Quantification of indigenous viruses is still advised whenever surrogate viruses as a WPC are not spiked into water samples to assess viral recovery.

In regions without extensive sewer lines connected to a centralized WWTP, urine-diverting dry toilets (UDDTs) are heavily promoted (Etter et al., 2011). UDDTs facilitate source separation of urine and feces and allow phosphorous recovery from urine for agricultural purposes. UDDTs are installed as mobile public toilets or in agricultural areas of the Kathmandu Valley. In Australia, wastewater samples collected from aircraft and cruise ships have been analyzed, showing that wastewater testing and clinical testing can be employed together to detect SARS-CoV-2 infections among travelers (Ahmed et al., 2020). Similarly, mobile public UDDTs in the Kathmandu Valley can also be sampled for epidemiological studies.

A limitation of this study is that it solely focuses on qPCR and dPCR as the core analytical technologies for detecting DENV despite the recently increased usage of metagenomics approach to detect known and new viruses in untreated wastewater (Ng et al., 2012). Primers and probes to detect all strains of DENV-1–4 worldwide have been developed, enabling rapid detection of DENV economically by qPCR and dPCR (Santiago et al., 2013). We also acknowledge that wastewater surveillance for dengue is a relatively new approach and challenging with little significance in hyperendemic regions where all four serotypes exist. However, in regions, such as the Kathmandu Valley, where dengue was only recently introduced, clinical surveillance is weak, and wastewater surveillance for polio is carried out (Garg et al., 2018), additional surveillance of DENV can be beneficial.

4. Conclusions

Our study is the first to investigate DENV in wastewater; however, DENV RNA was not detected in any sample tested. Regardless, the presence of DENV RNA in urine and saliva and the nature of DENV RNA to persist in wastewater over varying temperatures suggest that wastewater surveillance deserves further investigation as a novel approach to monitor dengue outbreaks. The absence of a well-established method for concentrating DENV in wastewater means existing methods that can concentrate large volumes of wastewater and incorporate both solid and liquid fractions of wastewater for viral recovery should be evaluated in future research. Appropriate WPC and MPC should be added, and nucleic acid should be extracted using kits with fewer steps that also facilitates removal of PCR inhibitors. With these guidelines, a reliable protocol can be developed to detect DENV and its serotypes in wastewater which will contribute significantly in regions where dengue was recently introduced, clinical surveillance is weak, and wastewater surveillance for polio is carried out.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Ocean Thakali: Visualization, Investigation, Formal analysis, Conceptualization, Methodology, Writing – original draft. **Sunayana Raya:** Investigation. **Bikash Malla:** Investigation. **Sarmila Tandukar:** Investigation. **Ananda Tiwari:** Investigation, Formal analysis, Writing – review & editing. **Samendra P. Sherchan:** Writing – review & editing. **Jeevan B. Sherchand:** Writing – review & editing. **Eiji Haramoto:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing.

Data Availability

Data will be made available on request.

References

Heijnen, L., Elsinga, G., de Graaf, M., Molenkamp, R., Koopmans, M., Medema, G., 2021. Droplet digital RT-PCR to detect SARS-CoV-2 signature mutations of variants of concern in wastewater. Sci. Total Environ. 799, 149456. doi:10.1016/j.scitotenv.2021.149456.

Ahmed, W., Angel, N., Edson, J., Bibby, K., Bivins, A., O'Brien, J.W., Choi, P.M., Kitajima, M., Simpson, S.L., Li, J., Tscharke, B., Verhagen, R., Smith, W.J.M., Zaugg, J., Dierens, L., Hugenholtz, P., Thomas, K.V., Mueller, J.F., 2020a. First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: a proof of concept for the wastewater surveillance of COVID-19 in the community. Sci. Total Environ. 728, 138764. doi:10.1016/j.scitotenv.2020.138764.

Sherchan, S.P., Shahin, S., Ward, L.M., Tandukar, S., Aw, T.G., Schmitz, B., Ahmed, W., Kitajima, M., 2020. First detection of SARS-CoV-2 RNA in wastewater in North America: a study in Louisiana, USA. Sci. Total Environ. 743, 140621. doi:10.1016/j.scitotenv.2020.140621.

Haramoto, E., Malla, B., Thakali, O., Kitajima, M., 2020. First environmental surveillance for the presence of SARS-CoV-2 RNA in wastewater and river water in Japan. Sci. Total Environ. 737, 140405. doi:10.1016/j.scitotenv.2020.140405.

Daughton, C.G., Daughton, C.G., Jones-Lepp, T.L., 2001. Illicit drugs in municipal sewage. In: Pharmaceutical and Care Products in the Environment, 791. American Chemical Society, Washington D.C., pp. 348–364. doi:10.1021/bk-2001-0791.ch020 Symposium Series.

Diemert, S., Yan, T., 2019. Clinically unreported salmonellosis outbreak detected via comparative genomic analysis of municipal wastewater Salmonella isolates. Appl. Environ. Microbiol. 85. doi:10.1128/AEM.00139-19, ee00139–19.

Xagoraraki, I., O'Brien, E., O'Bannon, D., 2020. Wastewater-based epidemiology for early detection of viral outbreaks. In: Women in Water Quality. Springer, Cham, pp. 75–97 Women in Engineering and Science Series.

Zahedi, A., Monis, P., Deere, D., Ryan, U., 2021. Wastewater-based epidemiology-surveillance and early detection of waterborne pathogens with a focus on SARS-CoV-2, *Cryptosporidium* and *Giardia*. Parasitol. Res. 120, 4167–4188. doi:10.1007/s00436-020-07023-5.

Kishida, N., Morita, H., Haramoto, E., Asami, M., Akiba, M., 2012. One-year weekly survey of noroviruses and enteric adenoviruses in the Tone River water in Tokyo metropolitan area, Japan. Water Res. 46, 2905–2910. doi:10.1016/j.watres.2012.03.010.

Heijnen, L., Medema, G., 2011. Surveillance of influenza A and the pandemic influenza A (H1N1) 2009 in sewage and surface water in the Netherlands. J. Water Health 9, 434–442. doi:10.2166/wh.2011.019.

Muirhead, A., Zhu, K., Brown, J., Basu, M., Brinton, M.A., Costa, F., Hayat, M.J., Stauber, C.E., 2020. Zika virus RNA persistence in sewage. Environ. Sci. Technol. Lett. 7, 659–664. doi:10.1021/acs.estlett.0c00535.

Yang, X., Quam, M.B.M., Zhang, T., Sang, S., 2021. Global burden for dengue and the evolving pattern in the past 30 years. J. Travel Med. 28, taab146. doi:10.1093/jtm/taab146.

Murugesan, A., Manoharan, M., 2020. Dengue virus. In: Emerging and Reemerging Viral Pathogens, 2020. Elsevier, Amsterdam; The Netherlands, pp. 281–359. doi:10.1016/B978-0-12-819400-3.00016-8.

Guzman, M.G., Gubler, D.J., Izquierdo, A., Martinez, E., Halstead, S.B., 2016. Dengue infection. Nat. Rev. Dis. Prim. 2, 16055. doi:10.1038/nrdp.2016.55.

Sinclair, R.G., Choi, C.Y., Riley, M.R., Gerba, C.P., 2008. Pathogen surveillance through monitoring of sewer systems. Adv. Appl. Microbiol. 65, 249–269. doi:10.1016/S0065-2164(08)00609-6.

Bhatt, S., Gething, P.W., Brady, O.J., Messina, J.P., Farlow, A.W., Moyes, C.L., Drake, J.M., Brownstein, J.S., Hoen, A.G., Sankoh, O., Myers, M.F., George, D.B., Jaenisch, Thomas, Wint, G.R.W., Simmons, C.P., Scott, T.W., Farrar, J.J., Hay, S.I., 2013. The global distribution and burden of dengue. Nature 496, 504–507. doi:10.1038/nature12060.

World Health Organization, Vaccines and immunizations: dengue. https://www.who.int/news-room/questions-and-answers/item/dengue-vaccines (Last accessed on June 15, 2022)

Naish, S., Dale, P., Mackenzie, J.S., McBride, J., Mengersen, K., Tong, S., 2014. Climate change and dengue: a critical and systematic review of quantitative modelling approaches. BMC Infect. Dis. 14, 167. doi:10.1186/1471-2334-14-167.

World Health Organization, Dengue and severe dengue. https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue (Last accessed on June 15, 2022)

Hirayama, T., Mizuno, Y., Takeshita, N., Kotaki, A., Tajima, S., Omatsu, T., Sano, K., Kurane, I., Takasaki, T., 2012. Detection of dengue virus genome in urine by real-time reverse transcriptase PCR: a laboratory diagnostic method useful after disappearance of the genome in serum. J. Clin. Microbiol. 50, 2047–2052. doi:10.1128/JCM.06557-11.

Van den Bossche, D., Cnops, L., Van Esbroeck, M., 2015. Recovery of dengue virus from urine samples by real-time RT-PCR. Eur. J. Clin. Microbiol. Infect. Dis. 34, 1361–1367. doi:10.1007/s10096-015-2359-0.

Korhonen, E.M., Huhtamo, E., Virtala, A.M., Kantele, A., Vapalahti, O., 2014. Approach to non-invasive sampling in dengue diagnostics: exploring virus and NS1 antigen detection in saliva and urine of travelers with dengue. J. Clin. Virol. 61, 353–358. doi:10.1016/j.jcv.2014.08.021.

Andries, A.C., Duong, V., Ly, S., Cappelle, J., Kim, K.S., Lorn Try, P., Ros, S., Ong, S., Huy, R., 2015. Value of routine dengue diagnostic tests in urine and saliva specimens. PLoS Negl. Trop. Dis. 9, e0004100. doi:10.1371/journal.pntd.0004100.

Chandra, F., Lee, W.L., Armas, F., Leifels, M., Gu, X., Chen, H., Wuertz, S., Alm, E.J., Thompson, J., 2021. Persistence of dengue (serotypes 2 and 3), Zika, yellow fever, and murine hepatitis virus R.N.A. in untreated wastewater. Environ. Sci. Tech. Lett. 8, 785–791. doi:10.1021/acs.estlett.1c00517.

Takasaki, T., Kotaki, A., Nishimura, K., Sato, Y., Tokuda, A., Lim, C.K., Ito, M., Tajima, S., Nerome, R., Kurane, I., 2008. Dengue virus type 2 isolated from an imported dengue patient in Japan: first isolation of dengue virus from Nepal. J. Travel Med. 15, 46–49. doi:10.1111/j.1708-8305.2007.00165.x.

Dhimal, M., Aryal, K.K., Dhimal, M.L., Gautam, I., Singh, S.P., Bhusal, C.L., Kuch, U., 2014a. Knowledge, attitude and practice regarding dengue fever among the healthy population of highland and lowland communities in central Nepal. PLoS One 9, e102028. doi:10.1371/journal.pone.0102028.

Tuladhar, R., Singh, A., Varma, A., Choudhary, D.K., 2019. Climatic factors influencing dengue incidence in an epidemic area of Nepal. BMC Res. Notes 12, 131. doi:10.1186/s13104-019-4185-4.

Gautam, I., Dhimal, M., Shrestha, S., Tamrakar, A., 2009. First record of *Aedes aegypti* (L.) vector of dengue virus from Kathmandu, Nepal. J. Nat. Hist. Mus. 24, 156–164. doi:10.3126/jnhm.v24i1.2298.

Dhimal, M., Gautam, I., Kreß, A., Müller, R., Kuch, U., 2014b. Spatio-temporal distribution of dengue and lymphatic filariasis vectors along an altitudinal transect in central Nepal. PLoS Negl. Trop. Dis. 8, e3035. doi:10.1371/journal.pntd.0003035.

Verdonschot, P.F.M., Besse-Lototskaya, A.A., 2014. Flight distance of mosquitoes (Culicidae): a metadata analysis to support the management of barrier zones around rewetted and newly constructed wetlands. Limnologica 45, 69–79. doi:10.1016/j.limno.2013.11.002.

Adhikari, S., Basnyat, B., Maharjan, K., 2020. Preparing for the dengue explosion in Kathmandu, Nepal. Lancet Glob. Health 8, e331–e332. doi:10.1016/S2214-109X(20)30007-3.

Tandukar, S., Ghaju Shrestha, R., Malla, B., Sthapit, N., Sherchand, J.B., Sherchan, S.P., Haramoto, E., 2021. Virus reduction at wastewater treatment plants in Nepal. Environ. Chall. 5, 100281. doi:10.1016/j.envc.2021.100281.

Department of Health Services, Nepal, annual report. https://dohs.gov.np/wp-content/uploads/2021/07/DoHS-Annual-Report-FY-2076-77-for-website.pdf (Last accessed on June 17, 2022)

Haramoto, E., Katayama, H., Asami, M., Akiba, M., 2012. Development of a novel method for simultaneous concentration of viruses and protozoa from a single water sample. J. Virol. Methods 182, 62–69. doi:10.1016/j.jviromet.2012.03.011.

Torii, S., Wakana, O., Zhu, Y., Thakali, O., Malla, B., Yu, Z., Zhao, B., Arakawa, C., Kitajima, M., Hata, A., Ihara, M., Kyuwa, S., Sano, D., Haramoto, E., Katayama, H., 2022. Comparison of five polyethylene glycol precipitation procedures for the RT-qPCR based recovery of murine hepatitis virus, bacteriophage phi6, and pepper mild mottle virus as a surrogate for SARS-CoV-2 from wastewater. Sci. Total Environ. 807, 150722. doi:10.1016/j.scitotenv.2021.150722.

Gurukumar, K.R., Priyadarshini, D., Patil, J.A., Bhagat, A., Singh, A., Shar, P.S., Cecilia, D., 2009. Development of real time PCR for detection and quantitation of dengue viruses. Virol. J. 6, 10. doi:10.1186/1743-422X-6-10.

Zhang, T., Breitbart, M., Lee, W.H., Run, J.Q., Wei, C.L., Soh, S.W.L., Hibberd, M.L., Liu, E.T., Rohwer, F., Ruan, Y., 2006. RNA viral community in human feces: prevalence of plant pathogenic viruses. PLoS Biol. 4, e3. doi:10.1371/journal.pbio.0040003.

Haramoto, E., Kitajima, M., Kishida, N., Konno, Y., Katayama, H., Asami, M., 2013. Occurrence of pepper mild mottle virus in drinking water sources in Japan. Appl. Environ. Microbiol. 79, 7413–7418. doi:10.1128/AEM.02354-13.

Gendron, L., Verreault, D., Veillette, M., Moineau, S., Duchaine, C., 2010. Evaluation of filters for the sampling and quantification of RNA phage aerosols. Aerosol Sci. Technol. 44, 893–901. doi:10.1080/02786826.2010.501351.

Haramoto, E., Kitajima, M., Hata, A., Torrey, J.R., Masago, Y., Sano, D., Katayama, H., 2018. A review on recent progress in the detection methods and prevalence of human enteric viruses in water. Water Res. 135, 168–186. doi:10.1016/j.watres.2018.02.004.

Ye, Y., Ellenberg, R.M., Graham, K.E., Wigginton, K.R., 2016. Survivability, partitioning, and recovery of enveloped viruses in untreated municipal wastewater. Environ. Sci. Technol. 50, 5077–5085. doi:10.1021/acs.est.6b00876.

Acharya, B.K., Cao, C., Lakes, T., Chen, W., Naeem, S., 2016. Spatiotemporal analysis of dengue fever in Nepal from 2010 to 2014. BMC Public Health 16, 849. doi:10.1186/s12889-016-3432-z.

Khetan, R.P., Stein, D.A., Chaudhary, S.K., Rauniyar, R., 2018. Profile of the 2016 dengue outbreak in Nepal. BMC Res. Notes 11, 423. doi:10.1186/s13104-018-3514-3.

Gupta, B.P., Haselbeck, A., Kim, J.H., Marks, F., Saluja, T., 2018. The dengue virus in Nepal: gaps in diagnosis and surveillance. Ann. Clin. Microbiol. Antimicrob. 17, 1–5. doi:10.1186/s12941-018-0284-7.

Wilder-Smith, A., Ooi, E.E., Horstick, O., Willis, B., 2019. Dengue. Lancet 394, 350–363. doi:10.1016/S0140-6736(18)32560-1.

Ahmed, W., Bivins, A., Bertsch, P.M., Bibby, K., Choi, P.M., Farkas, K., Gyawali, P., Hamilton, K.A., Haramoto, E., Kitajima, M., Simpson, S.L., Tandukar, S., Thomas, K.V., Mueller, J.F., 2020b. Surveillance of SARS-CoV-2 RNA in wastewater: methods optimization and quality control are crucial for generating reliable public health information. Curr. Opin. Environ. Sci. Health 17, 82–93. doi:10.1016/j.coesh.2020.09.003.

Landry, K.A., Boyer, T.H., 2016. Life cycle assessment and costing of urine source separation: focus on nonsteroidal anti-inflammatory drug removal. Water Res. 105, 487–495. doi:10.1016/j.watres.2016.09.024.

Kitajima, M., Ahmed, W., Bibby, K., Carducci, A., Gerba, C.P., Hamilton, K.A., Haramoto, E., Rose, J.B., 2020. SARS-CoV-2 in wastewater: state of the knowledge and research needs. Sci. Total Environ. 739, 139076. doi:10.1016/j.scitotenv.2020.139076.

Ahmed, W., Bertsch, P.M., Bivins, A., Bibby, K., Farkas, K., Gathercole, A., Haramoto, E., Gyawali, P., Korajkic, A., McMinn, B.R., Mueller, J.F., 2020c. Comparison of virus concentration methods for the RT-qPCR based recovery of murine hepatitis virus, a surrogate for SARS-CoV-2 from untreated wastewater. Sci. Total Environ. 739, 139960. doi:10.1016/j.scitotenv.2020.139960.

McMinn, B.R., Korajkic, A., Kelleher, J., Herrmann, M.P., Pemberton, A.C., Ahmed, W., Villegas, E.N., Oshima, K., 2021. Development of a large volume concentration method for recovery of coronavirus from wastewater. Sci. Total Environ. 774, 145727. doi:10.1016/j.scitotenv.2021.145727.

Qiu, Y., Yu, J., Pabbaraju, K., Lee, B.E., Gao, T., Ashbolt, N.J., Hrudey, S.E., Diggle, M., Tipples, G., Maal-Bared, R., Pang, X., 2021. Validating and optimizing the method for molecular detection and quantification of SARS-CoV-2 in wastewater. Sci. Total Environ. 812, 151434. doi:10.1016/j.scitotenv.2021.151434.

Xagoraraki, I., 2020. Can we oredict viral outbreaks using wastewater surveillance? J. Environ. Eng. 146, 01820003. doi:10.1061/(ASCE)EE.1943-7870.0001831.

Wilder, M.L., Middleton, F., Larsen, D.A., Du, Q., Fenty, A., Zeng, T., Insaf, T., Kilaru, P., Collins, M., Kmush, B., Green, H.C., 2021. Co-quantification of crAssphage increases confidence in wastewater-based epidemiology for SARS-CoV-2 in low prevalence areas. Water Res. X 11, 100100. doi:10.1016/j.wroa.2021.100100.

Rico, M., Andrés-costa, M.J., Picó, Y., 2017. Estimating population size in wastewater-based epidemiology. Valencia metropolitan area as a case study. J. Hazard. Mater. 323, 156–165. doi:10.1016/j.jhazmat.2016.05.079.

Daughton, C.G., 2012. Real-time estimation of small-area populations with human biomarkers in sewage. Sci. Total Environ. 414, 6–21. doi:10.1016/j.scitotenv.2011.11.015.

Chiaia, A., Banta-Green, C., Field, J., 2008. Eliminating solid phase extraction with large volume injection LC/MSMS: analysis of illicit and legal drugs and human urine indicators in US wastewaters. Environ. Sci. Technol. 42, 8841–8848. doi:10.1021/es802309v.

Chen, C., Kostakis, C., Gerber, J.P., Tscharke, B.J., Irvine, R.J., White, J.M., 2014. Towards finding a population biomarker for wastewater epidemiology studies. Sci. Total Environ. 487, 621–628. doi:10.1016/j.scitotenv.2013.11.075.

Etter, B., Tilley, E., Khadka, R., Udert, K.M., 2011. Low-cost struvite production using source-separated urine in Nepal. Water Res. 45, 852–862. doi:10.1016/j.watres.2010.10.007.

Ahmed, W., Bertsch, P.M., Angel, N., Bibby, K., Bivins, A., Dierens, L., Edson, J., Ehret, J., Gyawali, P., Hamilton, K., Hosegood, I., 2020d. Detection of SARS-CoV-2 RNA in commercial passenger aircraft and cruise ship wastewater: a surveillance tool for assessing the presence of COVID-19 infected travelers. J. Travel Med. 27, taaa116. doi:10.1093/jtm/taaa116.

Ng, T.F.F., Marine, R., Wang, C., Simmonds, P., Kapusinszky, B., Bodhidatta, L., Oderinde, B.S., Wommack, K.E., Delwart, E., 2012. High variety of known and new RNA and DNA viruses of diverse origins in untreated sewage. J. Virol. 86, 12161–12175. doi:10.1128/JVI.00869-12.

Santiago, G.A., Vergne, E., Quiles, Y., Cosme, J., Vazquez, J., Medina, J.F., Medina, F., Colón, C., Margolis, H., Muñoz-Jordán, J.L., 2013. Analytical and clinical performance of the CDC real time RT-PCR assay for detection and typing of dengue virus. PLoS Negl. Trop. Dis. 7, e2311. doi:10.1371/journal.pntd.0002311.

Garg, A., Pattamadilok, S., Bahl, S., 2018. Successes and challenges of expansion of environmental poliovirus surveillance in the WHO south-east Asia region. WHO S. East Asia J. Public Health 7, 122–128. doi:10.4103/2224-3151.239424.