Methods and Applications

Wastewater-Based Monitoring of Dengue Fever at Community Level — Guangzhou City, Guangdong Province, China, May 2024

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ABSTRACT

Introduction: Traditional dengue surveillance operates reactively, frequently lagging behind viral transmission patterns and thereby impeding timely public health responses. Wastewater-based epidemiology (WBE) presents significant potential for proactive early warning systems. This study sought to implement and validate the first community-level WBE system for dengue during an active outbreak, evaluating its capacity to detect cryptic transmission and provide actionable intelligence for public health interventions.

Methods: During a dengue virus serotype 1 (DENV-1) outbreak, we collected 618 wastewater grab samples from manholes within a 200-m radius of 8 reported cases, along with matched patient serum and urine samples. We systematically compared magnetic bead and polyethylene glycol (PEG) concentration methods for viral recovery efficiency. DENV-1 ribonucleic acid (RNA) was detected and quantified using reverse transcription quantitative polymerase chain reaction (RT-qPCR). Positive samples underwent genomic sequencing and phylogenetic analysis to confirm environmental signals and determine viral lineages.

Results: The magnetic bead method demonstrated superior performance with a limit of detection of 10 copies/mL and was selected based on its higher recovery efficiency (59.7%). We successfully detected DENV-1 in 14 of 618 wastewater samples tested. Critically, a positive wastewater signal from one residential building preceded the clinical diagnosis of a new case within that same location by several hours. For a single patient, we successfully generated matched viral genomic sequences from serum, urine, and wastewater samples, providing definitive validation of the environmental signal's authenticity.

Conclusions: Community-level wastewater surveillance represents a powerful and effective tool for dengue control programs. This approach provides actionable early warnings by detecting cryptic viral transmission before cases receive clinical identification. Such capabilities enable public health authorities to deploy preemptive, geographically-targeted interventions, including vector control measures, fundamentally improving both the speed and precision of outbreak responses while helping to mitigate disease spread.

Dengue fever is an acute mosquito-borne infectious disease caused by any of the four serotypes of dengue viruses (dengue virus serotypes 1 to 4), a single-stranded ribonucleic acid (RNA) virus. The disease is endemic in over 132 countries, with an estimated 96 million symptomatic infections and 40,000 deaths annually (*1*). Traditional dengue surveillance relies primarily on passive case detection, which involves identifying dengue virus (DENV), its components, or antibodies against DENV in blood samples from symptomatic individuals. However, this approach is inherently limited by diagnostic delays, leading to a lag in outbreak detection and response. Moreover, a significant proportion of infected individuals do not seek medical attention or choose not to report their illness, contributing to the underestimation of actual case numbers. Serum epidemiological studies indicate that 4% to 92% of dengue infections are asymptomatic (*2*), however, these individuals may still contribute to viral transmission. The inability to detect asymptomatic infections not only hampers early warning and rapid intervention but also prevents accurate assessment of infection prevalence. Given these limitations, there is an urgent need for a more efficient and comprehensive surveillance strategy to enable early detection, timely outbreak control, and

effective dengue prevention.

Wastewater-based epidemiology (WBE) was initially developed to assess the prevalence of drug use in communities and has since been applied to the surveillance of various pathogens, including viruses (*3*). During the coronavirus disease 2019 (COVID-19) pandemic, WBE became widely adopted across multiple countries for monitoring severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) levels in wastewater, providing critical insights into community transmission dynamics (*4*). Compared to traditional surveillance methods, WBE offers several advantages, including near real-time monitoring of large populations at relatively low cost (*5*). Importantly, as asymptomatic individuals also excrete pathogens in urine and feces, this approach captures both symptomatic and asymptomatic infections, allowing for a more comprehensive estimation of disease prevalence. Additionally, its applicability at the community level enables early outbreak detection, rapid response, and more effective epidemic control.

Previous studies have confirmed the presence of DENV nucleic acids in the saliva and urine of infected individuals (*6*), suggesting the feasibility of wastewater-based dengue surveillance. However, only two studies to date have reported wastewater detection of DENV. Wolfe et al. (*7*) analyzed weekly wastewater solids from three wastewater treatment plants (WWTPs) in the United States and consistently detected dengue virus serotype 3 (DENV-3) RNA. Their findings suggested that wastewater-based detection of DENV RNA was possible with as few as five laboratory-confirmed dengue cases per million people. Monteiro et al. (*8*) investigated the presence of DENV RNA in wastewater samples from 11 WWTPs in Portugal, identifying two seasonal peaks in viral prevalence and load (summer and winter). While these studies demonstrated the feasibility of wastewater-based DENV surveillance, both were conducted at the WWTP level, limiting their ability to assess the sensitivity and timeliness of detection. Consequently, this approach may not be suitable for epidemic monitoring in low-prevalence areas, where viral concentrations in wastewater are likely to fall below detectable thresholds. Furthermore, neither study performed sequencing to distinguish DENV serotypes, leaving gaps in the phylogenetic characterization of circulating strains.

In this study, following the diagnosis of the first locally acquired dengue case in Guangzhou in May 2024, wastewater monitoring and epidemiological surveys were conducted in parallel. Wastewater samples were collected near the patient's residence, and two concentration methods were compared, with the magnetic bead-based method selected for routine DENV-1 monitoring. To validate the wastewater surveillance findings, serum and urine samples from patients were concurrently analyzed via polymerase chain reaction (PCR) and sequencing. To our knowledge, this represents the first study to implement wastewater-based dengue surveillance at the community level immediately following the emergence of local cases. Notably, this approach facilitated the identification of a previously undetected case based on wastewater signals. Whole-genome sequencing of DENV-1 from wastewater was successfully obtained, further demonstrating the potential of wastewater surveillance for early dengue detection and outbreak prevention.

METHODS

Serum and urine samples from confirmed cases (Supplementary Material, available at https://weekly.chinacdc.cn/) were obtained from hospitals or district CDCs in Guangzhou. On the day of diagnosis, 1 mL of serum was collected from each case. Subsequently, a total of 36 midstream urine samples (50 mL each) were collected from three cases. Given the urgent need for epidemic response, grab wastewater samples were collected as a practical alternative to 24-hour composite sampling. In total, 618 grab samples were obtained from wastewater manholes within 200 m of all confirmed case residences — a distance corresponding to the typical activity range of *Aedes* mosquitoes (Figure 1). Additionally, 24-hour composite influent samples were collected from WWTPs to enable a comprehensive assessment of detection sensitivity. Each wastewater sample volume was 500 mL. Wastewater surveillance encompassed all of Guangzhou during the initial wave of local dengue fever outbreaks, with sampling conducted at 115 distinct sites. All samples were transported at 4 °C to the laboratory for analysis within 24 hours.

Serum RNA was extracted using the magnetic bead virus nucleic acid extraction kit (Jiangsu Bioperfectus Technologies Co., Ltd, Taizhou, Jiangsu, China). A 2 mL-aliquot of the urine sample was used for one-step virus concentration and RNA extraction using a magnetic bead-based biological sample virus

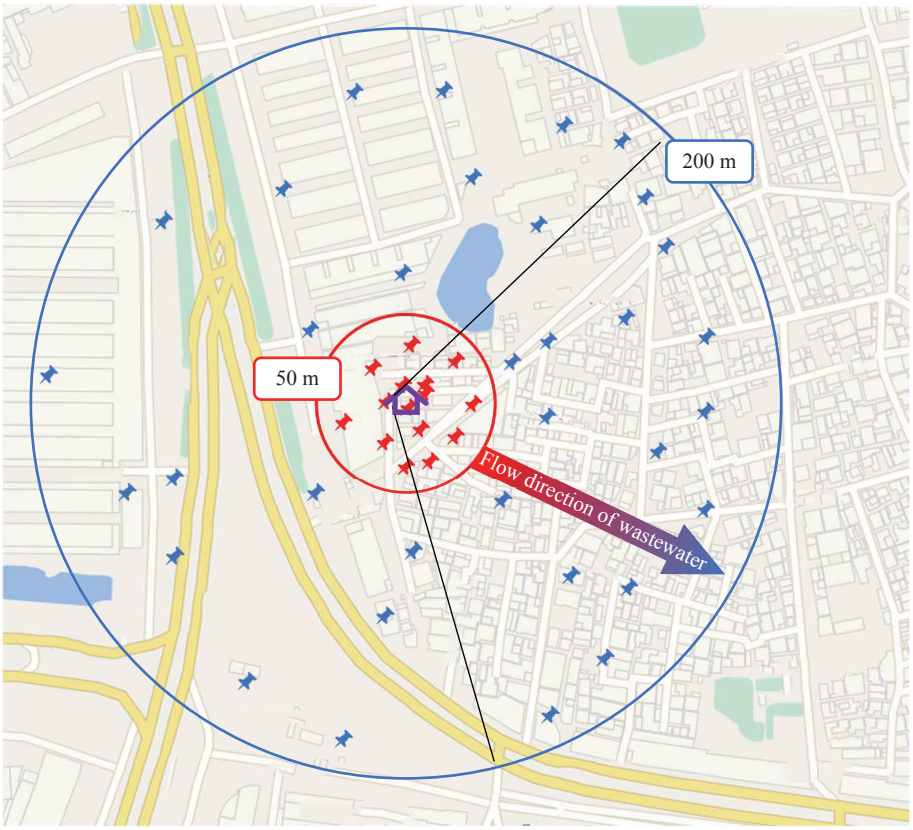

FIGURE 1. A schematic layout of sampling sites from wastewater manholes within 200 m of the residence of the first locally acquired dengue case (Case 1).

concentration kit (Suzhou Advanced Molecular Diagnostics Co., Ltd, Suzhou, Jiangsu, China).

Two concentration methods, polyethylene glycol precipitation and the magnetic bead-based method, were systematically compared in terms of limit of detection (LOD), PCR inhibition, and recovery efficiency (Supplementary Material). Primers and probes used are shown in Supplementary Table S1 (available at https://weekly.chinacdc.cn/). The method demonstrating superior performance was selected for wastewater sample concentration.

To further confirm the type of DENV infection in patients, reverse transcription PCR (RT-PCR) of serum RNA was conducted on four serotypes. The RNA concentration of DENV-1 in wastewater and urine samples was obtained by RT-qPCR. Sanger and whole-genome sequencing of DENV-1 was performed in positive serum, urine, and wastewater samples. Sequence analysis and visualization were carried out. The detailed descriptions are shown in the Supplementary Material.

For DENV-1 detection in wastewater samples, spiked murine hepatitis virus (MHV) served as the sample processing control (*9*). Following RNA extraction, the OneStep™ PCR Inhibitor Removal Kit (Zymo Research, CA, USA) was employed to eliminate potential inhibitors.

RESULTS

Genotype Branches of DENV-1 in Serum Samples

DENV-1 target sequences were successfully obtained from serum samples of 6 out of 8 cases, with GenBank (National Center for Biotechnology Information genetic sequence database) accession numbers PQ326420-PQ326425. Phylogenetic tree analysis revealed that the DENV infections in 2 cases belonged to DENV-1 genotype I (branch 1 and branch 2), while the DENV infections in 4 cases belonged to DENV-1 genotype III (branch 3) (Figure 2). Through online Nucleotide Basic Local Alignment Search Tool (BLAST) analysis, branches 1, 2, and 3 showed the closest similarity to KY057370 (isolated in 2012, Indonesia), OQ678061 (isolated in 2019, Cambodia), and MZ312929 (isolated in 2018, India), respectively.

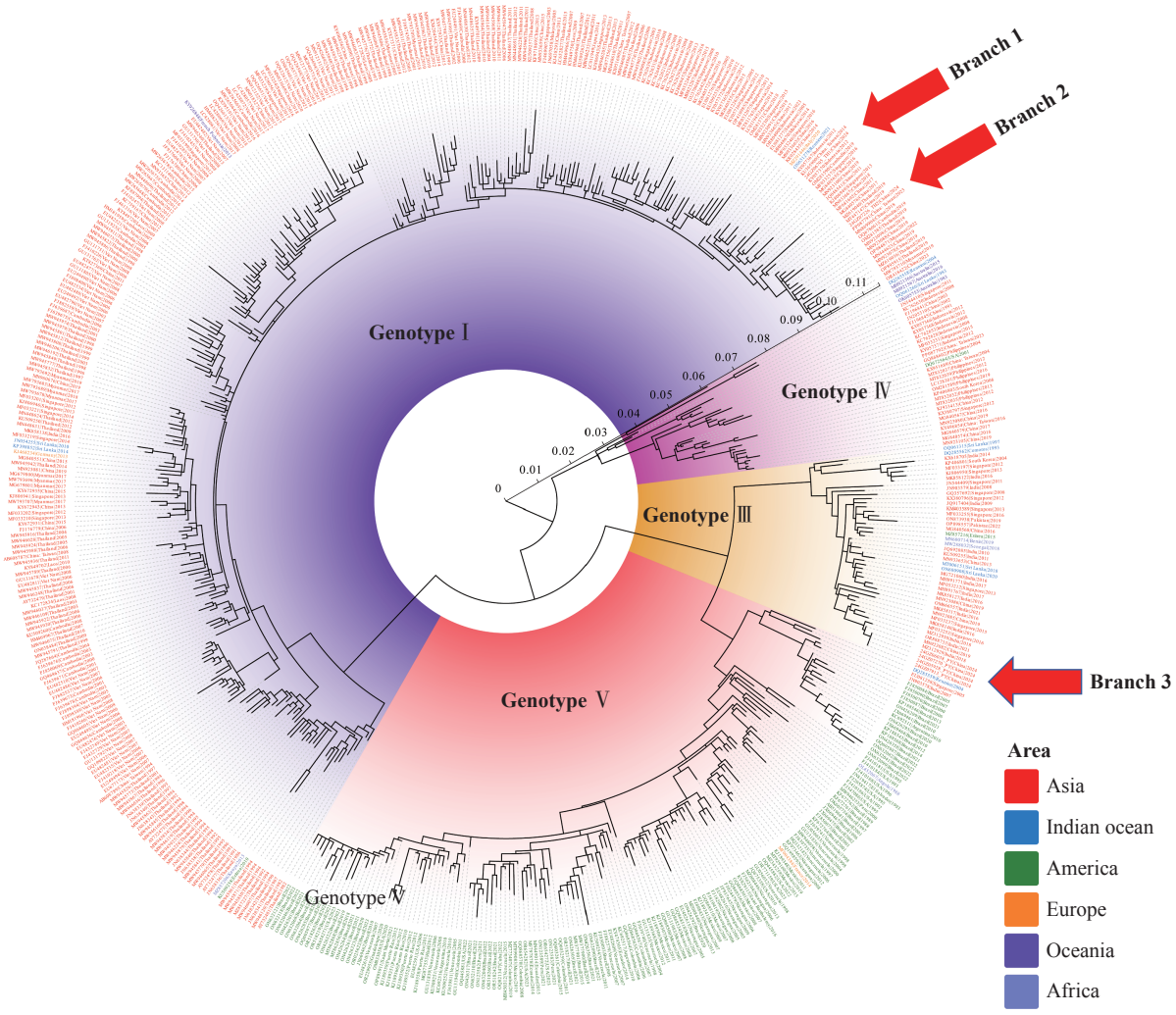

FIGURE 2. The phylogenetic tree of dengue virus serotype 1 (DENV-1) branches in serum samples.

These sequences demonstrated close phylogenetic relationships with Southeast Asian strains, consistent with genomic analysis results from previous dengue cases in Guangzhou (*10*).

RNA Concentration of DENV-1 in Urine Samples

Urine samples demonstrated a 100% positivity rate for DENV-1, with RNA concentrations ranging from 7.0 to 50,065.5 copies/mL, consistent with previously reported values (10^2 to 10^4 copies/mL) (*6*). Prior studies (*6*,*11*–*12*) indicate that the primary source of detectable DENV RNA in wastewater originates from patient urine, whereas SARS-CoV-2 RNA predominantly derives from fecal shedding, with reported concentrations ranging from 10^2 to 10^8 copies/mL (*13*). Consequently, the substantially lower concentration of DENV in wastewater compared to SARS-CoV-2 presents a significant analytical challenge for wastewater-based surveillance of dengue fever.

RNA Concentration and Sequence Distribution of DENV-1 in Wastewater Samples

Both concentration methods demonstrated a LOD of 10 copies/mL (Supplementary Table S2, available at https://weekly.chinacdc.cn/), with no evidence of PCR inhibition (Supplementary Table S3, available at https://weekly.chinacdc.cn/). However, the magnetic bead-based method exhibited superior recovery efficiency (59.7%) compared to polyethylene glycol precipitation (50.3%) (Supplementary Table S4, available at https://weekly.chinacdc.cn/). Given its enhanced recovery rate and automation potential, the magnetic bead-based method was selected for routine wastewater monitoring. In total, 14 wastewater samples

tested positive for DENV-1, yielding a positivity rate of 2.3%. All positive samples were collected from wastewater manholes within 50 m (straight-line distance) downstream of patients' residences during the initial 5 days of sampling. Sanger sequencing results of the RT-qPCR products from positive wastewater samples further confirmed the detection of DENV-1 sequences. The sequence alignment coverage reached 100.0%, with similarity ranging from 98.2% to 100.0%. RNA concentrations ranged from 0.7 to 97.5 copies/mL. These values were slightly lower than those reported in WWTPs (31–450 copies/mL) (*8*), highlighting potential differences in sampling environments and viral persistence.

Whole-genome sequencing revealed that the read counts of the obtained DENV-1 sequences from wastewater, urine, and serum samples from the same patient were 6,311/559,029 (1.1%), 206,362/341,880 (60.4%), and 1,573,040/1,590,306 (98.9%), respectively (Figure 3). These results indicate that the degradation of DENV-1 sequences in wastewater is substantially greater than in serum and urine samples. This finding suggests that wastewater-based monitoring and whole-genome sequencing of dengue fever present greater technical challenges compared to clinical specimens.

DISCUSSION

Ongoing dengue control efforts have successfully maintained low case numbers in Guangzhou. Between 2010 and 2019, annual dengue incidence fluctuated from 58 to 1,925 cases, with a significant outbreak in 2014 resulting in 38,036 reported cases (*10*). In recent

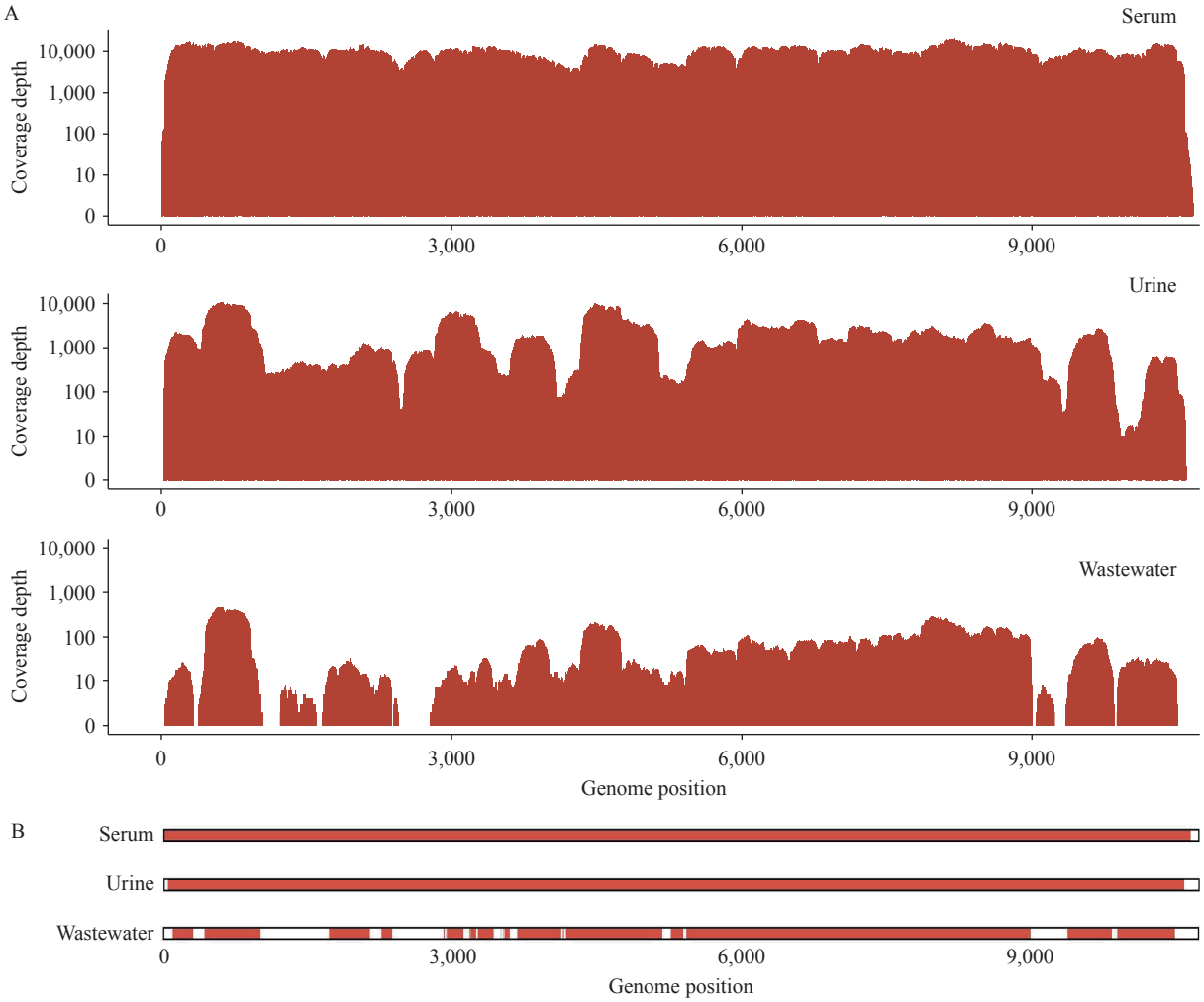

FIGURE 3. Distribution of dengue virus serotype 1 (DENV-1) sequences in serum, urine, and wastewater samples based on whole-genome sequencing. (A) The distribution and coverage depth of all sequences; (B) The distribution of sequences with a coverage depth of no less than 10.

years, dengue cases have remained consistently low, with only 9–37 cases reported annually from 2020 to 2022. Dengue virus RNA concentrations in urine, as documented in this and previous studies, range from 10^1 to 10^4 copies/mL (*6*). Assuming an average daily urine output of 2 L per person, daily dengue virus shedding is estimated at 2×10^4 to 2×10^7 copies per individual, approximately 100 times lower than SARS-CoV-2 shedding [1.27×10^6 to 1.04×10^8 copies/(day·person)] (*12*). The combination of low dengue prevalence and substantially reduced viral shedding creates considerable challenges for wastewater-based surveillance. Unlike SARS-CoV-2, which remains detectable in wastewater from large catchments even with minimal case numbers, dengue virus was only detectable within a 50 m radius of infected residences, with WWTP samples falling below detection limits. Under these conditions of low case numbers and limited viral shedding, wastewater-based surveillance at WWTPs provides insufficient sensitivity for effective outbreak detection. Instead, robust dengue surveillance requires targeted sampling at the community level, with strategic focus on locations proximal to identified cases.

Despite these challenges, our findings demonstrate the significant potential of wastewater-based surveillance as a complementary tool for community-level dengue monitoring. Notably, all positive wastewater samples in this study correlated precisely with epidemiological survey results, with no false-positive detections observed. More importantly, wastewater surveillance demonstrates promise for early detection of asymptomatic or pre-symptomatic infections, enabling timely interventions. The first

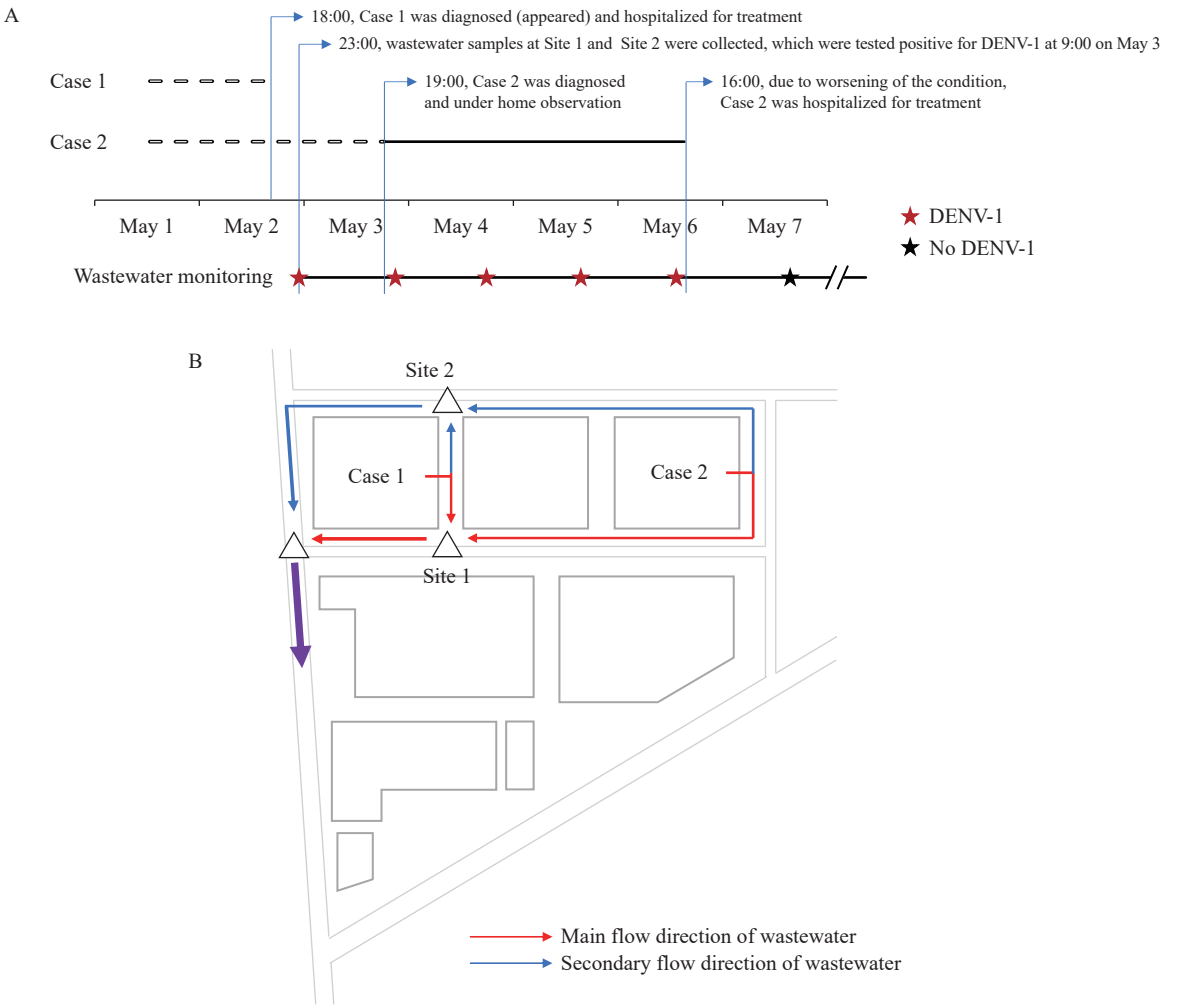

FIGURE 4. Schematic diagram of wastewater surveillance identifying Case 2 prior to clinical diagnosis. (A) Timeline of clinical confirmation/hospitalization for Case 1 and Case 2, and corresponding positive wastewater signals. (B) Relative geographical locations of Case 1 and Case 2 residences, and case-origin attribution of positive wastewater samples.
Abbreviation: DENV-1=dengue virus serotype 1.

locally acquired dengue case was clinically diagnosed and hospitalized at 18:00 on May 2. Later that evening at 23:00, wastewater monitoring and epidemiological surveys were initiated (Figure 4). Remarkably, despite the hospitalization of this initial case, positive wastewater signals persisted at Site 1 and Site 2 from May 2 to May 6. This unexpected persistence prompted further investigation. Since grab wastewater samples were collected from manholes beneath the patient's building rather than from wastewater storage tanks, we excluded the possibility that the detected viral signals originated from Case 1. Subsequent epidemiological investigations identified a second infected individual residing in the same building at 19:00 on May 3. This patient remained at home under observation for three days before hospitalization on May 6. The spatiotemporal correlation between wastewater viral concentrations and confirmed cases strongly indicates that the wastewater signals at Site 1 and Site 2 were attributable to Case 2. These findings underscore the utility of wastewater surveillance in identifying undetected transmission chains. Furthermore, trends in wastewater viral levels provide valuable insights into the temporal dynamics of outbreaks, offering a complementary tool for real-time epidemiological monitoring and early intervention strategies.

Although wastewater monitoring for dengue frequently yields negative results, these findings provide valuable insights for tracking epidemic trends. For instance, wastewater surveillance continued for nine days following the hospitalization of Case 2, during which all wastewater samples collected near the residence tested negative — consistent with the absence of further infections. A similar pattern was observed for other patients, as no additional cases emerged during their hospitalization, aligning with the lack of detectable DENV-1 in wastewater samples collected around their residences. These results highlight the potential of wastewater surveillance as a valuable tool for assessing the ongoing risk of dengue transmission and determining whether continued prevention and control measures are necessary in a given area.

While this study successfully demonstrated the potential of wastewater-based surveillance for early detection and outbreak monitoring, several limitations warrant consideration in future research. First, the study relied on grab sampling, which may have reduced detection sensitivity. Given the generally lower concentrations of DENV compared to SARS-CoV-2, employing larger-volume samples, 24-hours composite sampling, or solid-phase extraction methods (which enhance concentration factors) could improve detection sensitivity (*3*,*8*,*14*–*15*). Enhanced sensitivity would extend the effective monitoring range, increasing the likelihood of detecting infections at a given density of sampling sites. Second, sequence analysis of DENV-1 ribonucleic acid (RNA) from urine and wastewater samples revealed significant viral degradation in wastewater. As samples were collected in close proximity to infected residences, degradation is unlikely to have occurred within the sewer system. Instead, it is more plausible that RNA degradation resulted from suboptimal conditions during sample transport and storage prior to analysis. Future studies should focus on optimizing these processes to minimize degradation, potentially through the use of stabilizing agents or rapid processing techniques to preserve viral RNA integrity. Third, this study was conducted during a period of sporadic dengue cases in Guangzhou, whereas dengue outbreaks typically peak in October and November. Ongoing surveillance efforts aim to assess the correlation between wastewater detection rates at WWTP inlets and the number of clinically diagnosed cases within corresponding catchment areas. This extended monitoring will provide critical insights into the feasibility of wastewater-based surveillance for outbreak prediction at a larger scale.

Conflicts of interest: No conflicts of interest.

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SUPPLEMENTARY MATERIAL

Case Information

In May 2024, through systematic epidemic surveillance and comprehensive record analysis, a total of 8 cases from 5 distinct epidemic locations were identified during the initial wave of local dengue fever outbreaks in Guangzhou. All cases were confirmed to be infected with DENV-1 through blood sample analysis conducted by hospitals or district CDCs. The diagnostic methodology adhered to the latest dengue fever diagnostic standard (*1*). All diagnostic results included positive detection based on DENV-1 nucleic acid identification. Case numbers were assigned chronologically according to the date of discovery. All 8 patients represented locally acquired dengue fever cases.

Concentration Methods

PEG precipitation method. A 50 mL wastewater sample was centrifuged at 4 °C and 2,000 *g* for 2 minutes. From the resulting supernatant, 40 mL was mixed with 4.0±0.1 g of polyethylene glycol (PEG) and 0.80±0.01 g of sodium chloride, then shaken at 4 °C and 150 r/min for 120 minutes before centrifugation at 4 °C and 4,800 x *g* for 45 minutes. After removing the supernatant, the remaining 1 mL was transferred to a 1.5 mL tube and centrifuged at 4 °C and 20,000 x *g* for 8 minutes. The supernatant was discarded, leaving 500 μL of concentrate, of which 200 μL was used for nucleic acid extraction using the Tianlong method. Specifically, the concentrated virus solution underwent extraction using Nucleic acid extraction or purification reagents T183 with the Nucleic Acid Extractor GeneRotex 96 (Xi'an Tianlong Technology Co., Ltd, Xian, Shaanxi, China). This process yielded 80 μL of nucleic acid solution for PCR analysis.

Magnetic bead-based method. One-step virus concentration and nucleic acid extraction for wastewater samples were performed using a magnetic bead wastewater virus concentration kit combined with a wastewater molecular concentrater (WMC-24C, Suzhou Advanced Molecular Diagnostics Co., Ltd, Suzhou, Jiangsu, China). A 15 mL wastewater sample was centrifuged at 4 ℃ and 2,500 *g* for 10 minutes. The supernatant, enhancer, and eluent were then added according to the protocol described by Xu et al. (*2*). The instrument automatically executed sequential steps including nucleic acid fragment release from wastewater using lysis buffer, nucleic acid adsorption by magnetic beads, nucleic acid washing, and final elution. This automated process yielded 80 μL of nucleic acid solution suitable for PCR analysis.

Analytical Methods

Limit of detection (LOD) test. Different volumes of inactivated DENV-1 were introduced into DENV-1-free wastewater samples to achieve final concentrations of 0 copies/mL (negative control), 5 copies/mL, 10 copies/mL, 50 copies/mL, 100 copies/mL, and 250 copies/mL. Three parallel samples were prepared for each concentration level. Wastewater samples underwent concentration and extraction using both methods described above, followed by RT-qPCR detection for DENV-1. Each sample was amplified in triplicate. The concentration producing at least 95% positive replicates was designated as the LOD for the RT-qPCR assay (*3*).

PCR inhibition test. DENV-1-free wastewater samples underwent concentration and extraction using both methods described above. Subsequently, 40 μL of nucleic acid solution was spiked with 10 μL of 2.6×10^6 copies/mL DENV-1 plasmid for RT-qPCR analysis. Parallel testing was conducted using nuclease-free water instead of nucleic acid solution as a control. Each sample was amplified in triplicate. Samples were considered to contain PCR inhibitors if the mean quantification cycle (Cq) value exceeded the reference Cq value for nuclease-free water by >2 cycles (*4*).

Recovery efficiency test. Various ribonucleic acid (RNA) concentrations of pepper mild mottle virus (PMMoV) were employed to calculate recovery efficiency. DENV-1-free wastewater samples underwent concentration and extraction using both methods described above. Additionally, 200 μL of wastewater samples were directly extracted for nucleic acid using the Tianlong method as the reference standard. RT-qPCR detection of PMMoV was performed on nucleic acid extracts using the Hieff Unicon® V Universal Multiplex One Step RT-qPCR Probe Kit (Yisheng Biotechnology (Shanghai) Co., Ltd, Shanghai, China). Primer and probe sequences are detailed in Supplementary Table S1. Each sample was amplified in triplicate. PMMoV recovery efficiency was calculated based

SUPPLEMENTARY TABLE S1. Primer and probe sequences for polymerase chain reaction (PCR) amplification.

Amplification method	Virus	Primer or probe	Sequence (5'–3')	Genomic target	Amplicon size (bp)	References
RT-PCR	DENV-1	Forward primer	TGAGACACCCAGGATTCACGG	membrane glycoprotein M (partial) + envelope protein E (complete) + nonstructural protein NS1 (partial)	1,782	this study
		Reverse primer	TRGCTGATCGAATTCCACACAC			
	DENV-2	Forward primer	GACACGAACYGAAACATGGATGTC		1,696	
		Reverse primer	CCAGCTCACAACRCAACCAC			
	DENV-3	Forward primer	CAAGTCGAGAAGGTAGAGACATGG		1,847	
		Reverse primer	AGYTCATTGGCTATTTGYTTCCAC			
	DENV-4	Forward primer	CCCATCYTACGGAATGCGATG		1,592	
		Reverse primer	ACTGTTCTGTCCAAGTGTGCAC			
RT-qPCR	DENV-1	Forward primer	CAAAAGGAAGTCGYGCAATA	RNA-dependent RNA polymerase NS5 (partial)	112	(*7*-*8*)
		Reverse primer	CTGAGTGAATTCTCTCTGCTRAAC			
		Probe	6-FAM-CATGTGGYTGGGAGCRCGC-BHQ1			
	PMMoV	Forward primer	GAGTGGTTTGACCTTAACGTTTGA	replicase protein (partial)	68	(*9*-*10*)
		Reverse primer	TTGTCGGTTGCAATGCAAGT			
		Probe	CY5-CCTACCGAAGCAAATG-MGB			
	MHV	Forward primer	GGAACTTCTCGTTGGGCATTATACT	membrane protein (partial)	108	(*11*)
		Reverse primer	ACCACAAGATTATCATTTTCACAACATA			
		Probe	CY5-ACATGCTACGGCTCGTGTAACCGAACTGT-BHQ3			

Abbreviation: DENV-1=dengue virus serotype 1; DENV-2=dengue virus serotype 2; DENV-3=dengue virus serotype 3; DENV-4=dengue virus serotype 4; PMMoV=pepper mild mottle virus; MHV=murine hepatitis virus; Y=C/T; R=A/G.

SUPPLEMENTARY TABLE S2. Detection sensitivity of two concentration methods for dengue virus serotype 1 (DENV-1) in wastewater samples by reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Concentration method	5 copies/mL	10 copies/mL	50 copies/mL	100 copies/mL	250 copies/mL
Magnetic bead-based method	6/9 (66.7)	9/9 (100.0)	9/9 (100.0)	9/9 (100.0)	9/9 (100.0)
Polyethylene glycol precipitation method	8/9 (88.9)	9/9 (100.0)	9/9 (100.0)	9/9 (100.0)	9/9 (100.0)

Note: Data are presented as positive/total replicates (positivity rate, %). Example: 6/9 (66.7)=6 positives out of 9 replicates (66.7%).

SUPPLEMENTARY TABLE S3. The quantification cycle (Cq) value of two concentration methods for dengue virus serotype 1 (DENV-1) in polymerase chain reaction (PCR) inhibition test.

Concentration method	Cq value		
	Range	Mean	ΔCq
Magnetic bead-based method	30.03–30.15	30.09	0.62
PEG precipitation method	29.82–30.90	30.34	0.37
Reference	29.82–31.30	30.71	–

Note: "–" means that the data is not applicable.

SUPPLEMENTARY TABLE S4. The mean ribonucleic acid (RNA) concentration and recovery efficiency of two concentration methods for pepper mild mottle virus (PMMoV) in wastewater samples by reverse transcription polymerase chain reaction (RT-qPCR).

Concentration method	Mean ribonucleic acid (RNA) concentration (× 10^6 copies/mL)	Recovery efficiency (%)
Magnetic bead-based method	5.99±0.15	59.7
PEG precipitation method	5.05±0.07	50.3
Reference	10.04±1.85	–

Note: "–" means that the data is not applicable.

on the ratio of detected mean viral concentration in concentrated samples versus unconcentrated samples, as determined by RT-qPCR analysis.

RT-PCR for DENV Serotypes

One-step RT-PCR of serum RNA was performed using the C1000 Touch™ Thermal Cycler (Bio-Rad, CA, USA) to confirm the DENV serotype. The 50 μL RT-PCR cocktails were prepared using PrimeScript™ II High Fidelity One Step RT-PCR Kit (Takara, Shiga, Japan), with primer sequences shown in Supplementary Table S1. The RT-PCR cycling conditions were as follows: reverse transcription for 30 minutes at 45 ℃, an initial denaturation for 2 minutes at 94 ℃, followed by 40 cycles consisting of amplification for 15 s at 98 ℃, 15 s at 50 ℃ and 30 s at 68 ℃, and a final extension for 5 min at 68 ℃. The amplicon length was analyzed using QIAxcel System (QIAGEN, Hilden, Germany).

RT-qPCR for DENV-1

One-step RT-qPCR assays for DENV-1 concentration of urine and wastewater samples were performed on the Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, MA, USA). The 20 μL RT-qPCR cocktails contained 10 μL of 2× One Step RT-qPCR Probe Buffer IV and 2 μL of One Step Probe Enzyme Mix IV (ABclonal Technology, Wuhan, Hubei, China), 0.5 μL of 10 μM each primer and probe (Sangon Biotech, Shanghai, China), 5 μL of RNA, and 1.5 μL of nuclease-free water. The sequences of primers and probe are shown in Supplementary Table S1. The target fragment is the same as that used for blood sample diagnosis by RT-qPCR of nucleic acid in the above dengue fever diagnostic standard (*1*). The RT-qPCR cycling conditions were as follows: UDG reaction for 5 min at 25 ℃, reverse transcription for 5 min at 50 ℃, an initial denaturation for 3 min at 95 ℃, followed by 45 cycles consisting of amplification for 15 s at 95 ℃ and 31 s at 55 ℃. Nuclease-free water was used as the negative amplification control. For the standard curve, 4.5×10^9 copies/mL of DENV-1 RNA standard solution (BNCC, Xinyang, Henan, China) was sequentially diluted 10 fold to 4.5×10^8 to 4.5×10^1 copies/mL, and each concentration gradient were amplified in sextuplicate.

A negative wastewater and a positive sample (200 μL of 4.5×10^5 copies/mL of inactivated DENV-1) were included as the negative and positive extraction control, respectively. The two samples were simultaneously subjected to RNA extraction and RT-PCR to check for contamination during the experimental process. Each sample was amplified in duplicate. If a wastewater sample was tested positive, other commercial kits would be used for rechecking.

Sequencing

The positive RT-PCR and RT-qPCR products were submitted to Guangzhou Tianyi Huiyuan Gene Technology Co., Ltd (Guangzhou, Guangdong, China), and Sangon Biotech (Shanghai, China) Co., Ltd. for Sanger sequencing, respectively. One serum sample (from Case 4), one positive urine sample (from Case 4), and one positive wastewater sample (from the site around residence of Case 4) were selected for whole-genome sequencing, following a procedure similar to that of Su et al. (*5*). Reverse transcription was performed on their RNA solution using SuperScript™ IV VILO™ Master Mix (Invitrogen, MA, USA) to obtain complementary DNA (cDNA). A multiplex PCR method with multiple primer combinations was used to perform targeted amplification of the entire DENV genome on cDNA (*6*). After purification, quantification, and normalization of the amplification products, Nextera® XT DNA Library Preparation Kit (Illumina, CA, USA) was used to construct a sequencing library. After purification, quantification, normalization, and mixing of the library, the concentration of the mixture for sequencing was 1.2–1.5 pM. The MiniSeq™ Mid Output Kit (Illumina, CA, USA) was used on the Miniseq sequencing platform for paired-end 150 cycles of sequencing.

Sequence Analysis and Visualization

Using the online alignment software Nucleotide Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information, the sequences obtained by Sanger sequencing were aligned with all sequences from nucleotide databases to identify aligned species. Additionally, for the Sanger sequences of positive RT-PCR

products, IQtree software (v2.3.6, https://iqtree.github.io/about) was used to construct the phylogenetic tree based on the Maximum Likelihood method and calculate branch length based on Bayes method. The beautification and display of phylogenetic tree were completed through ChiPlot software.

CLC Genomics Workbench (QIAGEN, Hilden, Germany) was used for quality control, trimming, and mapping of whole-genome sequencing downstream data (*5*). The genomes submitted under GenBank (genetic sequence database) accession numbers NC_001477 (DENV-1), NC_001474 (DENV-2), NC_001475 (DENV-3), and NC_002640 (DENV-4) were used as the reference sequences for initial mapping. The consensus sequence for the initial mapping was generated with a coverage depth of no less than 10, which was then used as a reference sequence for the second mapping and correction to generate the final consensus sequence. Statistical analysis and data visualization were conducted using Hiplot (https://hiplot.org).

Research Ethics

This study received approval from the Ethics Committee of the Guangzhou Center for Disease Control and Prevention (CDC) under protocols GZCDC-ECHR-2022P0044 and GZCDC-ECHR-2023P0009. Written informed consent was obtained from all patients for surveillance activities and data collection related to disease control and subsequent analysis. All personally identifiable information in this study was pseudonymized to protect participant privacy.

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