Methods and Applications

Early Detection of the Emerging SARS-CoV-2 BA.2.86 Lineage Through Wastewater Surveillance Using a Mediator Probe PCR Assay — Shenzhen City, Guangdong Province, China, 2023

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ABSTRACT

Introduction: The emergence of the new severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Omicron sublineage, BA.2.86, has sparked global public health concerns for its potential heightened transmissibility and immune evasion. Utilizing data from Shenzhen's city-wide wastewater surveillance system, we highlight the presence of the BA.2.86 lineage in Shenzhen.

Methods: A mediator probe polymerase chain reaction (PCR) assay was developed to detect the BA.2.86 lineage in wastewater by targeting a specific mutation (Spike: A264D). Between September 19 and December 10, 2023, 781 wastewater samples from 38 wastewater treatment plants (WWTPs) and 9 pump stations in ten districts of Shenzhen were examined. Through multiple short-amplicon sequencing, three positive samples were identified.

Results: The BA.2.86 lineage was identified in the wastewater of Futian and Nanshan districts in Shenzhen on December 2, 2023. From December 2 to 10, a total of 21 BA.2.86-positive wastewater samples were found across 6 districts (Futian, Nanshan, Longhua, Baoan, Longgang, and Luohu) in Shenzhen. The weighted average viral load of the BA.2.86 lineage in Shenzhen's wastewater was 43.5 copies/L on December 2, increased to 219.8 copies/L on December 4, and then decreased to approximately 100 copies/L on December 6, 8, and 10.

Conclusions: The mediator probe PCR assay, designed for swift detection of low viral concentrations of the BA.2.86 lineage in wastewater samples, shows promise for detecting different SARS-CoV-2 variants. Wastewater surveillance could serve as an early detection system for promptly identifying specific SARS-CoV-2 variants as they emerge.

The detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) BA.2.86 was first reported in Israel and Denmark in late July 2023 (*1*). On August 17, 2023, the World Health Organization designated BA.2.86 as a variant under surveillance due to its global spread and potential increased transmissibility and immune evasion (*2*), possibly attributed to its 34 spike protein mutations in addition to those of the BA.2 lineage (*3*–*4*). Therefore, evaluating the prevalence and dynamics of the BA.2.86 lineage is crucial. Clinical diagnoses post-pandemic are limited, causing delays in surveillance. Hence, wastewater-based epidemiological surveillance can complement clinical methods, offering a cost-effective and unbiased approach for monitoring SARS-CoV-2 variants (*5*).

We established a citywide wastewater surveillance system in Shenzhen City, Guangdong Province, to monitor the presence of SARS-CoV-2. The system covers a population of over 18 million across the sewersheds of 38 wastewater treatment plants (WWTPs) and 9 pump stations. In this study, we conducted real-time monitoring of the BA.2.86 lineage using a mediator probe polymerase chain reaction (PCR) assay and identified its emergence in Shenzhen through wastewater surveillance.

METHODS

To assess the trend of SARS-CoV-2 infection, a wastewater surveillance system was established in December 2022 in Shenzhen using 6 WWTPs and 9 pump stations (*6*). This system expanded on March 21, 2023, to include 47 sampling sites covering 38 WWTPs and 9 pump stations across ten districts in Shenzhen. Wastewater samples of 125 mL each were collected at hourly intervals throughout the day and concentrated using a modified polyethylene glycol (PEG) precipitation method (*7*). Nucleic acids were

then extracted using an automatic extraction platform (HBNP-9601A, Hybribio, China). Subsequent quantitative reverse transcription PCR (qRT−PCR) testing for SARS-CoV-2 RNA was conducted using the Hybribio 2019-nCoV nucleic acid detection kit (Hybribio, China). Samples with cycle threshold (Ct) values ≤32 were subjected to multiple short-amplicon sequencing on the DNBSEQ-G99 sequencing platform (MGI, China) (*6*). The relative abundance of SARS-CoV-2 variants in wastewater samples was estimated using the Freyja deconvolution tool (*8*).

Concurrent with the decline in SARS-CoV-2 RNA concentrations in wastewater throughout September 2023, nearly all samples failed to satisfy the criteria for sequencing (Ct value ≤32). To circumvent this issue and effectively track the emergence of the BA.2.86 lineage, we implemented the mediator probe PCR assay (MPro assay). Between September 19 and December 2, 2023, we collected wastewater samples weekly from the 47 sampling sites to detect the BA.2.86 lineage. Commencing from December 4, 2023, the sampling and testing regimen was intensified to once every two days. A cumulative total of 781 samples were gathered specifically for BA.2.86 lineage analysis (Supplementary Table S1, available at https://weekly.chinacdc.cn/). We extracted RNA from the concentrated wastewater samples and synthesized cDNA using the HyperScript III 1st Strand cDNA Synthesis Kit (EnzyArtisan, China). The MPro assay (MPro BA.2.86) targeting the BA.2.86-specific mutation (Spike: A264D) was then employed to examine the cDNA of wastewater samples. This assay combines mediator probe PCR (*9*) and a molecular beacon reporter with minimal background fluorescence (*10*), as depicted in Figure 1A. The mediator probes, upon binding to mutant DNA templates (BA.2.86 lineage), release correct mediator primers due to *Taq* DNA polymerase's 5'-flap endonuclease activity. These primers then initiate the extension on the reporter, producing a fluorescent signal during the PCR extension phase. In the case of wild-type DNA templates (variants other than BA.2.86 lineage), incorrect mediator primers are released, leading to a negative test result as they are unable to extend on the reporter.

The MPro BA.2.86 assay was carried out in a reaction mixture of 25 μL, comprising 10 μL of DNA template and 15 μL of PCR master mix. The master mix included 10 mmol/L Tris-HCl (pH 8.0), 50 mM KCl, 7 mmol/L MgCl2, 0.2 mmol/L deoxynucleoside triphosphates (dNTPs), 1 U of Taq HS DNA polymerase (Takara, Japan), 600 nmol/L each of forward and reverse primers with sequences 5'-TGAC TCCTGGTGATTCTTCT-3' and 5'-GCACAGTCT ACAGCATCTG-3', respectively, 600 nmol/L of a mediator probe (5'-GCCGTGTCCCTCTGCAGC ACCAGCTGTCCA-NH2C7-3'), and 60 nmol/L of a universal molecular beacon reporter (5'-HEX-CGCG CGACTGGGCAGGGACACGGCTCGTCTCGGA CGGCTGCGCGCG-BHQ1-3') (*10*). The PCR and melting curve analysis (MCA) were performed on a SLAN 96S real-time PCR platform (Hongshi Medical Technology Co. Ltd., China). The thermal cycling program was as follows: 95 °C for 30 seconds; 45 cycles of 10 seconds at 95 °C, and 1 minute at 60 °C; a final stabilization at 35 °C for 40 minutes, 95 °C for 2 minutes, and 45 °C for 2 minutes. For MCA, the temperature was incrementally increased from 45 °C to 95 °C at a rate of 0.04 °C per step. Fluorescence intensities were recorded on the HEX channel during the PCR extension phase. The MCA step verified the presence of the specific mutation (Spike: A264D) by its unique melting temperature (Tm=76.5±1 °C). Samples exhibiting both a real-time PCR amplification curve below the threshold Ct value of 40 and a melting curve with a Tm of 76.5±1 °C were deemed positive.

To determine the limit of detection (LOD) of the MPro BA.2.86 assay, a plasmid containing an insertion sequence of the BA.2.86 lineage (accession number on GISAID: EPI_ISL_18096761) was serially diluted from 10^6 to 5 copies/reaction (Figure 1B). Each dilution (10^6 to 10^2 copies/reaction) was tested in triplicate to generate a standard curve (Figure 1C). To assess the analytical specificity of the MPro BA.2.86 assay, we analyzed 178 oropharyngeal swab samples collected from CDCs in various districts and sentinel hospitals. These samples were previously confirmed by sequencing to contain the BA.2.86 variant and 16 other prevalent variants (Table 1). Further details regarding these methodologies are furnished in the Supplementary Material (available at https://weekly.chinacdc.cn/).

The viral concentrations in positive samples were quantified using a standard curve. We combined viral concentrations in wastewater from WWTPs in 10 districts to determine the weighted average viral load of the BA.2.86 lineage in wastewater in Shenzhen. The formula used for calculation was adapted from a previous study (*11*).

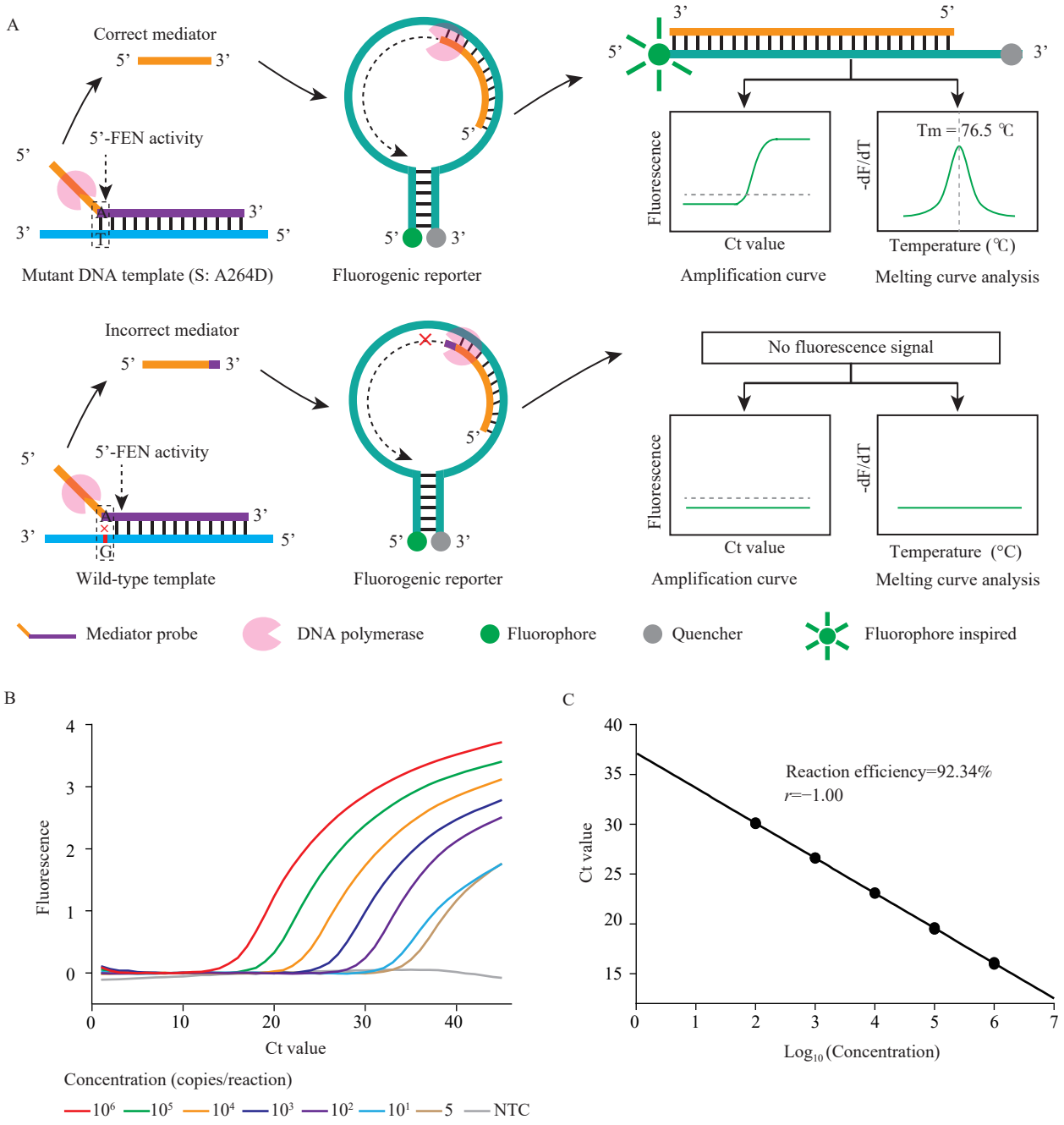

FIGURE 1. Development and analytical evaluation of the MPro BA.2.86 assay. (A) Detection of the specific mutation (Spike: A264D) of BA.2.86 through mediator probe PCR. (B) Analytical sensitivity of the MPro BA.2.86 assay. (C) Construction of the standard curve for the MPro BA.2.86 assay.
Note: Seven plasmid concentrations ranging from 10^6 to 5 copies per reaction are displayed alongside a no-template control (NTC).

$$\text{Weighted average viral load}_{city} = \frac{\sum_{n=1}^{N} C_n \times Q_n}{\sum_{n=1}^{N} Q_n}$$

Where C_n represents the concentration of the BA.2.86 lineage in wastewater at WWTP n (copies/L), and Q_n represents the daily 24-hour volume of treated wastewater at WWTP n (L/day).

RESULTS

We developed the MPro BA.2.86 assay to detect a specific mutation (Spike: A264D) in the BA.2.86 lineage, with a LOD of 5 copies/reaction (Figure 1B). This indicates its ability to detect low-concentration samples. The assay's standard curve exhibited a

TABLE 1. Detection results of 178 oropharyngeal swab samples previously confirmed to contain the SARS-CoV-2 variant by whole genome sequencing (column 1) using the MPro BA.2.86 assay.

SARS-CoV-2 variant	Total number	MPro BA.2.86 assay	
		Spike: A264D	Wild-type
BA.2.86	4	4	0
BA.5.2.48	10	0	10
BA.5.2.49	10	0	10
BF.7.14	10	0	10
XBB.1.5	23	0	23
XBB.1.16	42	0	42
XBB.1.9.1	8	0	8
FL.2	8	0	8
FL.4	3	0	3
EG.1	3	0	3
EG.5.1	10	0	10
HK.3	10	0	10
XBB.1.22	8	0	8
XBB.1.22.1	11	0	11
FY.3	13	0	13
XBB.1.22.2	2	0	2
XBB.2.3	3	0	3

reaction efficiency of 92.34% and a Pearson correlation coefficient (r) of −1.00 (Figure 1C), showcasing its quantitative detection potential. Analytical specificity was evaluated by testing 178 oropharyngeal swab samples (Table 1), previously confirmed to contain 17 common variants, including BA.2.86. The assay demonstrated 100% sensitivity [4/4, 95% confidence interval (*CI*): 51.01%, 100%] in identifying the BA.2.86 lineage and 100% specificity (174/174, 95% *CI*: 97.84%, 100%) when testing these samples.

Between September 19 and November 26, 2023, we collected wastewater samples weekly from 47 sampling sites, analyzing each using the MPro BA.2.86 assay. Out of 564 samples, none tested positive. However, on December 2, two samples from Liantangwei pump station (FT04) and Shekou WWTP (NS01) tested positive for the BA.2.86 lineage (Figure 2). In response, we doubled the sampling frequency to bi-daily. By December 4, two additional samples, one from Longhua and the other from Nanshan, were found to be positive for the BA.2.86 lineage, along with repeated positive results from FT04 and NS01. To verify the MPro BA.2.86 assay outcomes, we conducted multiple short-amplicon sequencing on three samples: the initial two positives and the one with the lowest Ct value. The sequencing indicated that the prevalence of BA.2.86 in these samples was 100%, 65.51%, and 100%, respectively (Figure 2C).

From December 2 to December 10, 2023, we collected 217 wastewater samples, of which 21 samples across six districts — Futian, Nanshan, Longhua, Baoan, Longgang, and Luohu — tested positive for the BA.2.86 lineage (Figure 2A). The weighted average viral load of BA.2.86 in Shenzhen's wastewater was 43.5 copies/L on December 2, which increased to 219.8 copies/L by December 4, before declining and stabilizing at approximately 100 copies/L on December 6, 8, and 10 (Figure 2B). Spatial analysis revealed that the initial positive PCR signals for the BA.2.86 lineage were detected in samples from Futian and Nanshan districts, which serve as the central port areas of Shenzhen. Subsequently, the lineage was identified in four other neighboring districts: Longhua, Baoan, Longgang, and Luohu (Figure 2D). No positive signals were found in samples from the four districts of Guangming, Yantian, Pingshan, and Dapeng, which do not have direct connections to Futian and Nanshan.

DISCUSSION

This study documented the appearance of the BA.2.86 lineage in Shenzhen through wastewater monitoring. Despite detecting the BA.2.86 lineage in the wastewater of six districts in Shenzhen until December 10, 2023, it showed a declining viral load, and there was no widespread outbreak at a regional level.

Early detection and monitoring of new variants of SARS-CoV-2 are essential to inform public health strategies (*12*). Scaling up clinical surveillance by testing individual patient samples for specific variants of the virus is impractical, particularly in regions with limited resources (*8*). Recent studies have indicated that wastewater sequencing is an effective and supplementary method for tracking SARS-CoV-2 variants (*13*). Yet, full genome sequencing remains costly and inefficient, especially for underprivileged and remote areas (*14*). While qRT-PCR is a well-established technique for detecting single nucleotide mutations in SARS-CoV-2 variants and is broadly used (*15*), the mediator probe PCR method outperforms qRT-PCR in its ability to minimize the detection of non-specific amplification products (*9*). Additionally, qRT-PCR encounters challenges when targeting more than ten mutations. In contrast, combining mediator probe PCR with MCA analysis, utilizing a dual-

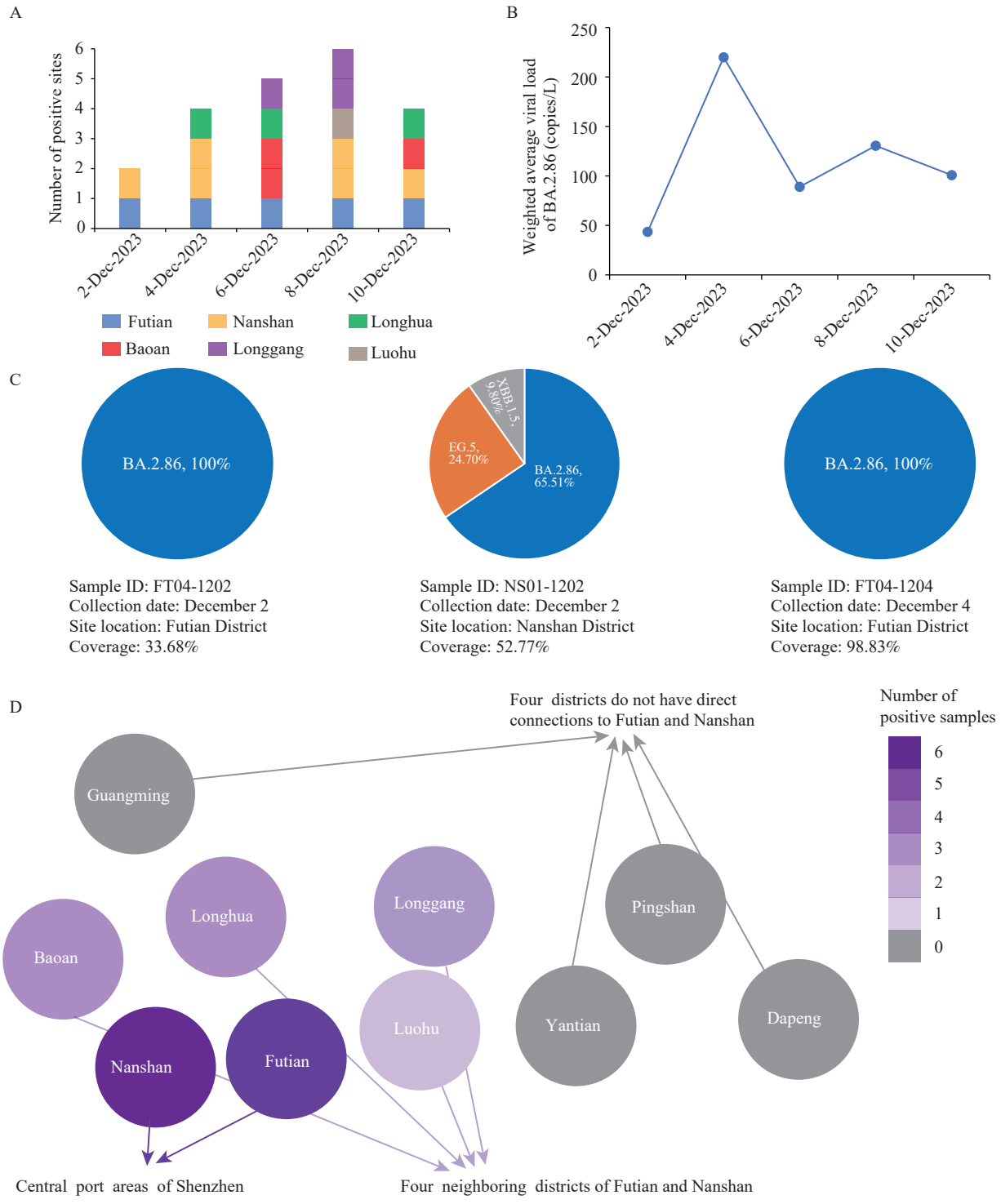

FIGURE 2. The emergence of BA.2.86 lineage derived from monitoring wastewater in ten districts of Shenzhen city in Guangdong Province, China, 2023. (A) The number of sampling sites per day with wastewater samples tested positive for BA.2.86 lineage. (B) Viral loads of BA.2.86 lineage in wastewater in Shenzhen (a weighted average of WWTPs in ten districts). (C) Variant prevalence of three wastewater samples tested positive by the MPro BA.2.86 assay. (D) Distribution of wastewater samples tested positive for BA2.86 lineage in ten districts of Shenzhen from December 2 to December 10, 2023.

labeling approach, enables the detection of over 30 mutations in the Omicron variant of SARS-CoV-2 within a single PCR reaction (*16*). This breakthrough suggests a method for comprehensive and adaptable screening for mutations across various SARS-CoV-2 variants in wastewater. In this study, we have

developed an MPro assay specifically for detecting a mutation associated with the BA.2.86 lineage. This assay is designed for execution on widely available real-time PCR thermal cyclers, which are predominantly used for SARS-CoV-2 detection. With the capability to analyze up to 96 samples simultaneously in a 96-well plate format, the assay can deliver results within three hours. This rapid and cost-effective method is ideally suited for the real-time surveillance of the BA.2.86 lineage, and its scalable nature holds promise for future adaptation to detect a broad array of SARS-CoV-2 variants.

Until December 10, 2023, no indigenous BA.2.86 case had been reported in Shenzhen. The detection of the BA.2.86 lineage in Shenzhen was identified through wastewater samples taken on December 2 using the MPro BA.2.86 assay and confirmed through wastewater sequencing. This indicates the effectiveness of wastewater surveillance in offering early detection capabilities before clinical surveillance.

Through wastewater surveillance, the presence of the BA.2.86 lineage was initially detected in samples from the key port areas of Futian and Nanshan. Subsequently, it appeared in four neighboring districts while it remained undetected in the four non-adjacent districts. These findings suggest that the BA.2.86 cases may have been introduced into the Futian and Nanshan districts from international sources, highlighting the need for enhanced COVID-19 monitoring in port areas.

A key limitation of this study, however, was the lack of validation using a substantial set of clinical samples of the BA.2.86 lineage. Additionally, not all infected individuals shed the SARS-CoV-2 virus in their feces or urine discharged into the wastewater network, and some infected individuals face dilution effects in wastewater, leading to the missing detection in the corresponding sewershed.

In conclusion, wastewater surveillance has revealed the presence of the BA.2.86 lineage in Shenzhen, serving as an efficient method for early detection of emerging variants. The newly devised MPro assay for the BA.2.86 lineage can be expanded to promptly identify different SARS-CoV-2 variants in wastewater moving forward.

Conflicts of interest: No conflicts of interest.

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SUPPLEMENTARY MATERIAL

Detailed Methods of Analytical Evaluation of the MPro BA.2.86 Assay

To determine the limit of detection (LOD) of the MPro BA.2.86 assay, a plasmid containing an insertion sequence of BA.2.86 lineage (accession number on GISAID: EPI_ISL_18096761) was prepared in a serial dilution (10^6, 10^5, 10^4, 10^3, 10^2, 10^1, 5 copies/reaction) and each of the five dilutions (10^6, 10^5, 10^4, 10^3, 10^2 copies/reaction) was used in triplicate to construct a standard curve using the SLAN 96S real-time PCR detection system software (version 8.2.2; Hongshi Medical Technology Co. Ltd., Shanghai, China). The LOD was determined as the lowest concentration that yielded no more than one negative result in 20 replicates (positive rate ≥ 95%).

To assess the analytical specificity of the MPro BA.2.86 assay, we analyzed 178 oropharyngeal swab samples collected by district Centers for Disease Control and Prevention and sentinel hospitals in Shenzhen. These samples were previously confirmed by sequencing to contain the BA.2.86 variant and 16 other prevalent variants: BA.2.86 (*n*=4), BA.5.2.48 (*n*=10), BA.5.2.49 (*n*=10), BF.7.14 (*n*=10), XBB.1.5 (*n*=23), XBB.1.16 (*n*=42), XBB.1.9.1 (*n*=8), FL.2 (*n*=8), FL.4 (*n*=3), EG.1 (*n*=3), EG.5.1 (*n*=10), HK.3 (*n*=10), XBB.1.22 (*n*=8), XBB.1.22.1 (*n*=11), FY.3 (*n*=13), XBB.1.22.2 (*n*=2), and XBB.2.3 (*n*=3). Nucleic acids were extracted from the swab samples using the GeneRotex96 automatic extraction platform (TIANLONG, China). Subsequent cDNA synthesis was performed using the HyperScript III 1st Strand cDNA Synthesis Kit (EnzyArtisan, China). The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) genomes from these samples were sequenced on an Illumina MiniSeq platform according to the manufacturer's instructions.

SUPPLEMENTARY TABLE S1. Serial number, sampling site, location (area + longitude and latitude), and number of wastewater samples collected from 38 wastewater treatment plants and 9 pump stations in Shenzhen from September 19 to December 10, 2023.

Serial number	Sampling site	Area	Longitude and latitude	Number of samples
FT07	Futian-1 WWTP	Futian District	114°0′58″E, 22°31′42″N	17
FT08	Futian-2 WWTP	Futian District	114°1′13″E, 22°31′40″N	17
FT09	Binhe WWTP	Futian District	114°5′47″E, 22°32′8″N	17
FT01	Fuxing PS	Futian District	114°5′13″E, 22°32′7″N	17
FT02	Tianmian PS	Futian District	114°4′35″E, 22°32′27″N	17
FT03	Huanggang village PS	Futian District	114°3′52″E, 22°31′28″N	17
FT04	Liantangwei PS	Futian District	114°0′51″E, 22°33′37″N	17
FT05	Shixia PS	Futian District	114°3′1″E, 22°31′28″N	17
FT06	Futian free zone PS	Futian District	114°3′11″E, 22°30′6″N	17
N1	Shekou WWTP	Nanshan District	113°53′38″E, 22°27′52″N	17
N2	Nanshan WWTP	Nanshan District	113°53′45″E, 22°30′54″N	17
N3	Xili WWTP	Nanshan District	113°57′20″E, 22°35′25″N	17
N4	Chuangye Road PS	Nanshan District	113°55′50″E, 22°31′4″N	17
N5	Dengliang Road PS	Nanshan District	113°54′48″E, 22°31′6″N	17
N6	Qianhai PS	Nanshan District	113°54′32″E, 22°31′31″N	17
BA-09	Xixiang Guwu-1 WWTP	Baoan District	113°50′41″E, 22°35′2″N	17
BA-10	Xixiang Guwu-2 WWTP	Baoan District	113°50′39″E, 22°34′58″N	17
BA-08	Airport south WWTP	Baoan District	113°49′36″E, 22°35′20″N	17
BA-06	Fuyong-1 WWTP	Baoan District	113°46′50″E, 22°40′33″N	17
BA-07	Fuyong-2 WWTP	Baoan District	113°46′49″E, 22°40′28″N	14
BA-03	Shajing-1 WWTP	Baoan District	113°46′45″E, 22°44′53″N	17
BA-04	Shajing-2 WWTP	Baoan District	113°46′48″E, 22°44′43″N	17

Continued

Serial number	Sampling site	Area	Longitude and latitude	Number of samples
BA-05	Shajing-3 WWTP	Baoan District	113°46′55″E, 22°44′51″N	17
BA-01	Songgang-1 WWTP	Baoan District	113°50′45″E, 22°47′39″N	17
BA-02	Songgang-2 WWTP	Baoan District	113°50′43″E, 22°47′48″N	17
YT-01	Yantian WWTP	Yantian District	114°15′3″E, 22°33′47″N	17
LF	Luofang WWTP	Luohu District	114°8′44″E, 22°32′35″N	17
HH	Honghu WWTP	Luohu District	114°6′56″E, 22°34′31″N	17
L5	Hengling WWTP	Longgang District	114°20′21″E, 22°45′49″N	17
L7	Banxuegang WWTP	Longgang District	114°3′9″E, 22°40′3″N	17
L6	Buji WWTP	Longgang District	114°6′46″E, 22°35′26″N	17
L1	Pinghu WWTP	Longgang District	114°8′3″E, 22°42′38″N	17
L2	Egongling WWTP	Longgang District	114°9′45″E, 22°40′36″N	17
L3	Pudixia WWTP	Longgang District	114°8′18″E, 22°38′26″N	17
L4	Henggang WWTP	Longgang District	114°14′0″E, 22°41′8″N	17
GM-1	Guangming WWTP	Guangming District	113°54′38″E, 22°46′26″N	17
GM-2	Gongming WWTP	Guangming District	113°52′12″E, 22°42′35″N	17
LHMZ	Minzhi WWTP	Longhua District	114°2′20″E, 22°37′0″N	17
LHYQ	Longhua-1 WWTP	Longhua District	114°2′22″E, 22°40′45″N	17
LHEQ	Longhua-2 WWTP	Longhua District	114°2′30″E, 22°40′54″N	17
LHGL	Guanlan WWTP	Longhua District	114°3′37″E, 22°44′3″N	17
PSSY	Shangyang WWTP	Pingshan District	114°24′21″E, 22°42′37″N	12
PSST	Shangyang WWTP	Pingshan District	114°24′21″E, 22°46′20″N	12
PSLT	Longtian WWTP	Pingshan District	114°21′27″E, 22°45′56″N	12
DPST	Shuitou WWTP	Dapeng District	114°29′31″E, 22°34′18″N	17
DPDC	Dongchong WWTP	Dapeng District	114°34′6″E, 22°29′29″N	17
DPKY	Kuiyong WWTP	Dapeng District	114°25′31″E, 22°36′59″N	17