



# Wastewater surveillance for infectious agents of measles, rubella, hepatitis, influenza, mpox, and tuberculosis in South Africa, 2024

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## Summary

Wastewater and environmental surveillance (WES) has historically provided surveillance data to support monitoring of communicable diseases such as cholera and polio. More recently, WES provided surveillance data during the SARS-CoV-2 pandemic and the mpox virus (MPXV) Clade IIb multi-country outbreak. Here, we describe the application of WES for infectious disease surveillance, including measles, rubella, influenza, hepatitis A and E, mpox and tuberculosis, and describe method development and results for surveillance programmes over 2024. The National Institute for Communicable Diseases manages a national WES sentinel surveillance network comprising sampling sites at 28 wastewater treatment plants (WWTPs) across nine South African provinces and border transit points and 19 community sampling sites located within the catchment areas of three large WWTPs in Gauteng province. Grab samples of one litre were subjected to ultrafiltration followed by extraction and digital PCR for the viruses measles (MeV), rubella, hepatitis A and E, influenza A and B, SARS-CoV-2, MPXV and for *Mycobacterium tuberculosis*. The concentration of viral or bacterial genome in wastewater was determined through back-calculation and graphed and, for certain pathogens, presented together with clinical surveillance data using epidemiological curves. Sequencing of SARS-CoV-2 from wastewater demonstrated a change of variants reflecting sequences from clinical isolates from 2024. Furthermore, analysis of wastewater levels, compared with testing rates and disease incidence in two sewer sheds together with socioeconomic data, demonstrated that wastewater levels more accurately reflected the burden of disease and overcame socioeconomic factors impacting clinical test accessibility. Measles and rubella wastewater detections reflected epidemiological trends observed in clinical surveillance, including increased detections across the country during the rubella outbreak of September to December 2024. In addition, MeV RNA was detected in wastewater in certain districts across 29 epidemiological weeks even though clinical cases were not detected in those districts. Influenza virus RNA detection mirrored the season observed through the NICD's clinical syndromic sentinel surveillance programme. Hepatitis A virus DNA detection was observed in most districts through 75% of epidemiological weeks, reflecting the endemic nature of this infection. No MPXV RNA detections were observed in wastewater, nor were clinical cases reported during the period that wastewater surveillance was conducted. *Mycobacterium tuberculosis* detections in wastewater showed geographical and temporal variation, suggesting potential utility as a surveillance tool. These results to date demonstrate the potential use of wastewater and environmental surveillance for vaccine preventable diseases as a complementary surveillance tool for monitoring disease transmission, the effectiveness of vaccination programmes, and for providing early warning of seasonal or pandemic diseases.

## Introduction

Wastewater and environmental surveillance (WES) was conceived in the 1930s for the detection of *Vibrio cholerae* and poliovirus.<sup>1</sup> Progressive improvements in the detection of poliovirus from wastewater and mounting evidence of WES utility as a surveillance tool for polio culminated in the inclusion of environmental testing as part of the Global Polio Elimination Initiative's routine polio surveillance activities that started in 2005.<sup>2</sup> During the COVID-19 pandemic and following successful detection of Coronavirus-2



(SARS-CoV-2) in wastewater, multiple countries across the globe implemented WES as a complementary surveillance tool alongside clinical surveillance,<sup>3</sup> and subsequently adapted it to monitor the global multi-country and African outbreaks of mpox since 2022.<sup>4</sup>

Wastewater and environmental surveillance is utilised by the Global Polio Elimination Initiative and used to provide highly sensitive surveillance data as part of the polio elimination programme.<sup>5</sup> In 2022 and 2023, the World Health Organization (WHO) issued and subsequently updated guidance to support the implementation of WES as a complementary surveillance tool for SARS-CoV-2 outbreak preparedness and response.<sup>6</sup> In 2024, WHO released guidance on the use of WES for mpox surveillance.<sup>7</sup> Further, it has been suggested that WES be applied as a surveillance tool for other communicable disease pathogens, including those targeted for elimination and those that are seasonal or epidemic-prone.<sup>8</sup> Researchers across the world – in developed and lower-middle-income countries – are applying WES to a wide range of pathogens to establish the use-case and contexts in which this new surveillance modality offers the most useful data.<sup>9</sup>

In 2020, the National Institute for Communicable Diseases (NICD), a division of the National Health Laboratory Service, expanded polio testing at 18 WES sentinel sites to include SARS-CoV-2 across the nine provinces of South Africa (SA).<sup>10</sup> Over time, as a consequence of changes in funding, the number and location of sites changed. As of January 2025, the NICD co-ordinates a national WES sentinel surveillance network comprising sampling sites at 28 wastewater treatment plants (WWTPs) across nine South African provinces and border transit points and a network of 19 sampling sites located within the catchment areas of three large WWTPs in the densely populated Gauteng province.

In this report we describe the application of WES for infectious disease surveillance, including measles, rubella, influenza, hepatitis A and E, mpox, and tuberculosis, and describe method development and results for surveillance programmes over 2024. We also reflect on emerging interpretive strategies and on the way forward for WES in SA and beyond.

## Methods

### **Strategic partnerships and the development of the wastewater surveillance network**

In late 2020, the NICD established a strategic partnership with the Water Research Commission of SA to establish a national COVID-19 wastewater surveillance pilot programme named the 'South African Collaborative COVID-19 Surveillance System (SACCESS)' network.<sup>11</sup> Over 85 wastewater treatment sites across nine provinces in SA were tested by the SACCESS network from 2021–2022.<sup>10</sup>

In 2022, the Wastewater Genomics Syndicate, Wits Health Consortium (CVI/WGS), was established at the NICD Centre for Vaccines and Immunology (CVI) through a grant from the Gates Foundation to support SARS-CoV-2 sequencing from wastewater. The NICD CVI team, together with the NICD Sequencing Core Facility, used a modified ARTIC 1 protocol and a newly developed pipeline to identify the presence of single nucleotide polymorphisms compatible with the South African SARS-CoV-2 beta and delta



variants.<sup>12</sup> This work was expanded over 2023–2024 through a collaboration with the Andersen Laboratory (The Scripps Research Institute, La Jolla, CA, United States) and the incorporation of the Freyja bioinformatics pipeline.<sup>13</sup> Freyja uses the read-frequencies of SARS-CoV-2 mutations across the entire genome, together with a series of matrix equations and SARS-CoV-2 whole genome sequencing (WGS) results deposited in the publicly accessible database (GISAID), to determine the proportion of prevalent SARS-CoV-2 variants in a given wastewater sample.<sup>13</sup>

Over 2023–2024, the CVI/WGS established a collaboration with the Gauteng City Region Observatory ([www.gcro.org](http://www.gcro.org)), a public-private social and economic observatory that conducts the annual Gauteng Quality of Life household social survey. Using prior SARS-CoV-2 sequencing results and geographic information system (GIS)-derived population data, this collaboration identified three WWTPs in Gauteng (one in each of the metropolitan areas of Tshwane, Ekurhuleni, and Johannesburg) to map sewer sheds, estimate resident population sizes, and determine associations with social determinants of health (from Quality of Life survey data), laboratory-based surveillance, and levels of SARS-CoV-2 in wastewater.<sup>14</sup> During 2023–2024, further modelling work was conducted together with the London School of Tropical Medicine and Hygiene and the South African Centre for Epidemiological Modelling and Analysis (SACEMA) to develop models that support the interpretation of SARS-CoV-2 nucleic acid concentrations in wastewater.<sup>15</sup>

In 2023, processing methods and assays to detect measles, rubella, influenza, and hepatitis A and E from wastewater were developed by the CVI/WGS team. A collaboration between the Andersen laboratory (Scripps Research), the University of Birmingham (UK), and the NICD gave rise to the Modjadji Consortium ([www.modjadji.info/](http://www.modjadji.info/)), which focused on developing amplicon sequencing approaches and Freyja-like pipelines to support WGS of infectious diseases in wastewater, including measles and tuberculosis. Most recently, and anticipating the importation and detection of mpox cases in South Africa, CVI/WGS, together with the NICD's Centre for Emerging Zoonotic and Parasitic Diseases (CEZPD), developed and validated a sample processing method and a digital PCR (dPCR) assay for the detection of mpox virus DNA. Presently, the wastewater surveillance network comprises 29 WWTP with 2–4 sites per province and 18 community sites. Sample collection is funded by the NICD through a WHO grant for polio surveillance from WWTP once a month, with the remaining weekly WWTP and all community samples funded through a Gates Foundation grant.

#### **Laboratory methods used in 2024 for detection and quantification of SARS-CoV-2, measles, rubella, influenza A and B, hepatitis A and E, and tuberculosis from wastewater samples using dPCR**

The sample processing, virus concentration, nucleic acid extraction and PCR methods that were in use in 2024 until week 41 (ending 12 October) are briefly described here. After epidemiological week 41 of 2024, and following method comparisons (results not shared here), sample concentration and nucleic acid extraction were shifted to a bead-based method<sup>16</sup> with the exception of rubella testing, which required sample processing using ultrafiltration to provide optimal results.



**Primer selection and assay validation:** Primers that amplify gene targets used by clinical molecular diagnostic assays were selected, especially where these are published by the WHO or another public health agency (Table 1). For measles detection, CVI/WGS developed their own primers.<sup>17</sup> Positive controls were identified for each target, and, using serial dilutions, the limit of detection was determined using dPCR and quantitative PCR (qPCR) in order to support interpretation of low positive results (Table 1). These assays were then applied to stored wastewater concentrates that had been made from samples collected from 2022 onwards to determine if these targets were present in wastewater. Nucleic acid specific to measles, rubella (RuV), influenza A and B (INFA and INFB), and hepatitis A and E (HAV and HEV) viruses were found to be present in retained wastewater concentrates at frequencies of 2% (47/2422), 1.6% (39/2422), 4.7% (113/2422), 3% (73/2422), 17% (349/2013) and 12% (246/2063), respectively.

**Table 1.** Target genes, reference sources, and controls used in the detection of SARS-CoV-2, measles, rubella, hepatitis A & E, mpox, and tuberculosis from wastewater samples, South Africa, 2024.

Pathogen	Target gene (reference)	Positive control	Limit of detection	
			dPCR (genome copies/uL)	qPCR (Ct threshold)
SARS-CoV-2	Nucleocapsid <sup>10</sup>	EDX SARS-CoV-2 Standard	ND	ND
Measles	Nucleocapsid <sup>18</sup>	CDC positive control	0.356	37
Rubella	5'- untranslated region <sup>19</sup>	CDC positive control	0.381	40
Hepatitis A	5'- untranslated region <sup>20</sup>	Clinical sample	0.399	38
Hepatitis E	Phosphoprotein <sup>21</sup>	Cloned plasmid	0.395	37
Influenza A	Matrix <sup>22</sup>	Inactivated culture from a clinical sample	0.573	39
Influenza B	Non-structural protein <sup>22</sup>	Inactivated culture from a clinical sample	0.637	36
Mpox	N3R & B18RPlus <sup>23</sup>	Inactivated culture from a clinical sample	ND	ND
<i>Mycobacterium tuberculosis</i>	IS6110* Glycosyl transferase <sup>24</sup>	H37Rv	ND	ND

\*in-house primers and probe; ND=not done

### Sample processing and determination of genome copies/mL of raw wastewater

One litre of raw sewage was collected using the grab method on a weekly, twice-weekly or monthly schedule from designated collection sites. Samples were transported to the NICD at 4°C within 24 hours of collection. On receipt, samples were refrigerated at 4°C and, within 24–48 hours, 200 mL of raw sewage was centrifuged at 4700 x g at 4°C for 10 minutes to clarify the sample.<sup>25</sup> Then, 70 mL of supernatant was centrifuged at 1500 x g at 4°C for 15 minutes through a Centricon® Plus-70 centrifugal ultra-filter device (Merck Millipore, Tullagreen, Ireland). The viral concentrate material retained in the ultra-filter device was



eluted to a volume of approximately 1 mL by inverting the device's components according to the manufacturer's instructions and centrifuging at 1 000 x g at 4°C for two minutes. Total nucleic acids were extracted from 200 µL of viral concentrate on the 96 KingFisher Flex Purification System (Thermo Fisher Scientific, Waltham, MA, USA) using the MagMAX™ Wastewater Ultra Nucleic Acid Isolation kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Nucleic acid extracts were subjected to absolute quantification using the QIAcuity® OneStep Advanced Probe kit and 96-well 8.5k nanoplates (Qiagen, Hilden, Germany) on QIAcuity® One, 5plex System dPCR platform (Qiagen). The assays were applied in multiplex formats with MeV and RuV, HAV and HEV, and INFA and INFB primers pooled together. An assay master mix containing 3 µL of 4× OneStep Advanced Probe Master Mix, 0.12 µL of 100× OneStep RT Mix, 0.6 µL of 20× primer-probe mix for each target and 1 µL of Enhancer GC was used. The cycling conditions were as follows: reverse transcription at 50°C for 40 minutes, reverse transcription enzyme inactivation at 95°C for two minutes, followed by 45 cycles of denaturation and combined annealing and extension at 95°C for five seconds and 60°C for one minute, respectively. The formula described in Iwu-Jaja *et al.*<sup>10</sup> was used to determine the genome copy number per microLitre of the original wastewater sample.

#### **Current laboratory methods for detection and quantification of mpox virus DNA using dPCR from wastewater samples**

The mpox assay was prepared and performed using the same equipment, reagents, and consumables outlined above with minor modifications to account for the fact that MPXV is a DNA virus and reverse transcription is not required for PCR testing. The assay master mix included 0.12 µL of nuclease-free water in place of the OneStep RT Mix. The cycling conditions were as follows: an initial denaturation at 95°C for two minutes, followed by 45 cycles of denaturation at 95°C for five seconds and combined annealing and extension at 60°C for one minute.

#### **Current methods for SARS-CoV-2 and measles sequencing from wastewater samples**

SARS-CoV-2 and measles-positive samples on RT-dPCR were subjected to amplicon-based WGS using primers developed in collaboration with Quick Lab using the PrimalScheme application.<sup>26</sup> Extracted RNA was subjected to cDNA synthesis using the SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific). The resulting DNA was amplified in two pools of 400 bp using the Q5 High-Fidelity 2x Master Mix with some modifications. Finally, paired-end libraries were prepared using the Illumina COVIDSeq Kit as previously described<sup>27</sup> followed by sequencing (2 × 150 bp) on the NextSeq 2000 platform (Illumina Inc., USA). SARS-CoV-2 fastq files underwent pre-processing using an in-house pipeline, followed by analysis with the Freyja tool. The resulting Freyja output files were subsequently integrated for visualisation of trends via a publicly accessible GitHub workflow.

Measles Fastq files were submitted to an in-house bioinformatics pipeline, and consensus sequences were created and included in phylogenetic trees using publicly available clinical measles sequences.



### **Current laboratory methods for detection and quantification of *Mycobacterium tuberculosis* using dPCR from wastewater samples**

Preliminary spiking experiments and testing of routine samples (results not shown) demonstrated that a greater yield of *Mycobacterium tuberculosis* (*Mtb*) was detected in suspended wastewater solids rather than the liquid fraction. We therefore amended the processing workflows as follows: grab samples were centrifuged at 4 650g at 4°C for 30 minutes to clarify the sample by settling the sediment. Sediment was subjected to DNA extraction using the DNeasy® Powersoil® Pro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA was then subject to dPCR using the QIAcuity® One system, QIAcuity One-Step Advanced Probe RT-dPCR kit, and 96-well 8.5k nanoplates (Qiagen, Hilden, Germany) to detect *Mtb*. Two PCR targets, IS6110 and glycosyltransferase, were run as singleplex reactions using a PCR master mix containing 3 µL of 4× QIAcuity One-Step Advanced Probe Master Mix, 0.12 µL of nuclease-free water, 0.6 µL of 20× primer-probe mix for each target, and 1 µL of GC enhancer. The cycling conditions included initial denaturation at 95°C for two minutes and 45 cycles of annealing at 95°C for five seconds and extension at 60°C for one minute.

### **Current approach to epidemiological analyses and reporting of results**

For all pathogens excluding *Mtb*, the number and proportion of wastewater samples testing positive at national and sub-catchment sites and by epidemiological week were determined. The interquartile range in genome copies per millilitre was calculated. In addition, the change in proportion testing positive over time was analysed using simple regression. For SARS-CoV-2, the cumulative wastewater load by epidemiological week across the surveillance network was determined to establish national trends. For SARS-CoV-2, measles, rubella, and hepatitis A and E, epidemiological curves were created reflecting the number of positive clinical cases identified using routine surveillance data and positive wastewater results by epidemiological week. For SARS-CoV-2, epidemiological curves were provided for both the quantitative levels of SARS-CoV-2 and the proportion of variants at each week identified through the Frejya pipeline.

For measles analysis, we grouped and tallied the number of IgM-positive clinical specimens submitted for testing by epidemiological week of sample collection and district of health facility where the case was identified. Wastewater samples by epidemiological week and district were also grouped and tallied. These clinical and wastewater results were merged by epidemiological week and district, and week-district pairs where no wastewater samples were tested were eliminated. A 'positive concordant wastewater-clinical pair' was defined as any instance in a given epidemiological week in a specified district where at least one wastewater sample tested positive for MeV and one case was identified. Conversely, a 'negative concordant pair' was defined as one where all wastewater and clinical samples tested negative or no clinical samples were submitted. The remaining 'discordant' pairs were those where at least one case was detected but all wastewater samples were negative, or vice versa.



The proportion of concordant and discordant week-district pairs was determined, described, and presented in two-by-two tables.

The results were interpreted by NICD epidemiologists and shared internally within the NICD and contributed to recommendations that the NICD provides to national and provincial health departments and other key stakeholders. Results for mpox WES surveillance were also shared with the Mpox Incident Management Team and the Mpox Vaccine Technical Working Group of the National Group on Immunisation, the ministerial body advising the National Department of Health on matters pertaining to vaccination.

## Results

### Current surveillance network attributes and results summary

National and community wastewater surveillance networks are currently maintained by the NICD through grant funds. National networks comprise wastewater treatment plants in large metros and towns at land borders in all nine South African provinces. Data from the national network are used for determination of trends in infectious disease epidemiology, early warning, determination of seasonal trends, and to provide samples for sequencing of pathogens. Community wastewater surveillance networks are localised to the Gauteng province and include sampling points from sewer lines where the catchments have been carefully delineated through GIS mapping of sewer networks. Community sampling sites have been selected to identify potential utility of WES data at different population levels and to provide data for modelling to answer important questions pertaining to signal determination, estimation of population burden of disease, minimum threshold of detection and other questions (Table 2). Testing of wastewater for SARS-CoV-2, measles, rubella, hepatitis A and E, and influenza A and B was conducted for 2024, while testing for mpox and *Mtb* commenced in October and November 2024, respectively.

Summary results for samples collected from 01 January to 30 November 2024 are presented in Table 3. Notable findings include a 56%, 34%, and 16% positivity rate for SARS-CoV-2, HAV, and HEV, respectively. Influenza A virus was detected marginally more often than INFB (10% vs 6%), whilst the MeV and RuV were least frequently detected at 7% and 6%, respectively.



**Table 2.** Attributes of national sentinel and sub-catchment (case study) wastewater surveillance networks co-ordinated by the NICD, South Africa, 2024.

	<b>National wastewater sentinel surveillance network</b>	<b>Community wastewater surveillance network</b>
<b>Purpose of surveillance</b>	To support national communicable disease risk assessment and to provide insight into infectious disease epidemiology	To demonstrate the utility of WES data at different population levels and to provide data for modelling
<b>Number of sites (number of districts covered)</b>	27 (19)	22 (3)
<b>Where samples are drawn from</b>	Influent entering wastewater treatment plants (WWTPs) in nine provinces located in larger metropolitan areas and in towns located at major land borders	Inspection holes in sewage lines supplying communities of different sizes and socioeconomic features in Gauteng only.* Hospital effluent from three tertiary hospitals in Gauteng districts only.
<b>Size of population contributing to samples</b>	5 000 to 900 000 (WWTPs serving smaller towns to vast metros)	1 000 to 40 000 (hospital manholes to sewer lines draining larger communities)
<b>Sampling frequency</b>	Weekly (one sample per month for polio)	Weekly or twice weekly**
<b>Pathogens detected</b>	Polio (monthly, by culture), SARS-CoV-2, measles, rubella, hepatitis A & E, influenza, mpox	SARS-CoV-2, measles, rubella, hepatitis A & E, influenza, mpox
<b>Nucleic acid sequencing</b>	Polio, SARS-CoV-2, measles	SARS-CoV-2, measles. (methods in development for all other pathogens)

\*One sub-catchment site in each of the City of Tshwane, the City of Ekurhuleni, and the City of Johannesburg.

\*\* Twice weekly for Ekurhuleni and Tshwane sites, weekly for Johannesburg sites.

WES=wastewater & environmental sampling, BMGF=Bill & Melinda Gates Foundation.



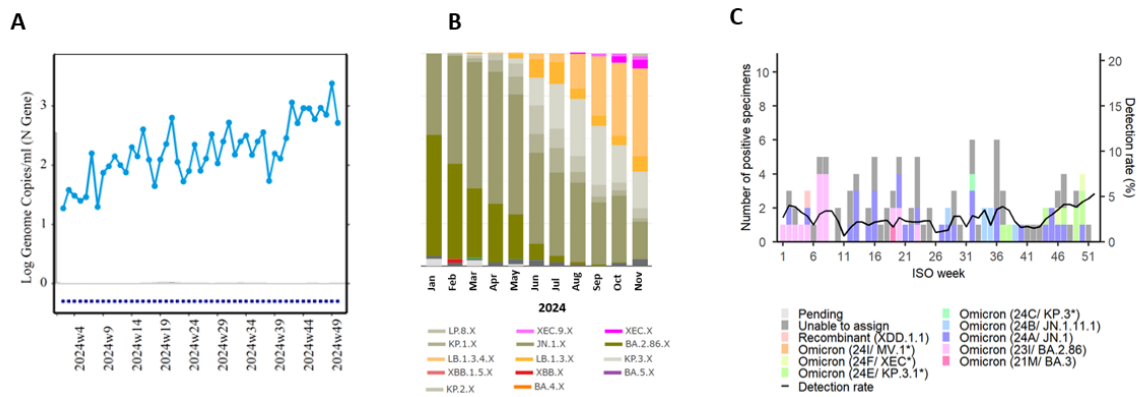
**Table 3.** Results from testing of wastewater samples for infectious diseases by surveillance site category from epidemiological weeks 1–48 (01 January to 30 November 2024), South Africa.

	National sentinel sites		Community sites		All sites	
	#positive/#tested (n/N, %)	Concentration per reaction (IQR in gc/uL)	#positive/#tested (n/N, %)	Concentration per reaction (IQR in gc/uL)	#positive/#tested (n/N, %)	Concentration per reaction (IQR in gc/uL)
<b>SARS-CoV-2</b>	653/1 041 (63)	11,4–25,4	770/1 516 (51)	7,9–22,4	1 426/2 561 (56)	7,9–25,4
<b>Measles</b>	68/1 041 (7)	0,41–0,81	109/1 516 (7)	0,42–1,19	177/2 561 (7)	0,41–1,19
<b>Rubella</b>	60/1 041 (6)	0,39–0,40	90/1 516 (6)	0,39–0,41	150/2 561 (6)	0,39–0,41
<b>Hepatitis A</b>	397/1 041 (38)	0,79–2,21	468/1 516 (31)	0,75–1,85	865/2 561 (34)	0,75–2,21
<b>Hepatitis E</b>	234/1 041 (22)	0,76–1,90	164/1 516 (11)	0,40–0,79	398/2 561 (16)	0,40–1,90
<b>Influenza A</b>	115/1 041 (11)	0,40–0,78	148/1 516 (10)	0,39–0,75	256/2 561 (10)	0,39–0,78
<b>Influenza B</b>	142/1 041 (14)	0,39–0,77	119/1 516 (8)	0,40–0,80	148/2 561 (6)	0,39–0,80
<b>Mpox</b>	0/97	0	0/154	0	0/251	0
<b>Mtb IS6110*</b>	92/97 (95)	0,40–1 118	132/154 (86)	0,39–627	224/251 (89)	0,39–1 118
<b>Mtb GTF**</b>	70/97 (72)	0,38–59,0 (GTF)	70/154 (45)	0,39–42,90	140/251 (56)	0,38–59

Gc/uL = genome copies per microliter; \*IS6110 = multicopy IS6110 sequence detection assay for *Mycobacterium tuberculosis* (Mtb); \*\*GTF = glycosyl transferase gene detection assay for Mtb.

### SARS-CoV-2 quantitative sequencing results

From epidemiological weeks 1–48 (01 January to 30 November) 2024, 56% (1426/2561) of wastewater samples tested positive for SARS-CoV-2 at median concentrations of 2.37 (IQR 7.9–25.4) genome copies/mL (Table 3). Cumulative weekly levels between 1–2x10<sup>3</sup> log copies/mL were observed across the year (Figure 1A), with intermittent increases. A sustained increase was observed from week 41 (6–12 October 2024) as laboratory concentration methodology changed from ultrafiltration to bead-based extraction. A peak in week 47 (17–23 November 2024) was observed, which was supported by sentinel site data (Figure 1B). A breakdown of levels at individual sites over the time period is available at <https://wastewater.nicd.ac.za/>. Both wastewater and clinical surveillance detected a dominance of Omicron lineages throughout the year (Figure 1C), although the maximum number of clinical samples successfully sequenced each week across all the respiratory surveillance programmes was not more than eight and usually less than four. Initially, BA.2.86 was most frequently detected, but from March until May 2024, JN.1 and its sub-lineages accounted for more than 50% of detected strains in both wastewater and sentinel surveillance. LB.1.3 and its sub-lineages emerged towards the end of the year.



**Figure 1.** A) Cumulative levels of SARS-CoV-2 RNA in wastewater samples across national and community sentinel sites; B) the relative frequency of SARS-CoV-2 lineages in wastewater across national and community sentinel sites as determined by the Frejya tool; and C) the number (left vertical axis) and proportion testing positive (right vertical axis) of SARS-CoV-2-positive samples differentiated by variant from severe respiratory illness clinical sites across South Africa; all by epidemiological weeks 1-49 (A,B) or 1-52 (C), January to December, 2024.

### Measles detection and sequencing results

Over epidemiological weeks 1–48 of 2024, 177/2 561 (6.9%) wastewater samples tested positive for MeV (Table 3). The Northern Cape and Gauteng provinces had the highest detection rates (13% [3/23] and 8% [144/1 793], respectively), while only one district had no positive detections. There was a trend towards increasing wastewater positivity over the course of the year, likely due to improved laboratory processing (Figure 2A & B). However, during the rubella outbreak (September to December 2024), particularly in Gauteng, many clinically suspected rubella cases tested positive for both measles and rubella IgM. Wastewater findings suggest that true measles cases were present, as sustained positive wastewater results were found during week 26 and onwards, particularly in Gauteng.





**Table 4.** The number and percentage of district-time pairs (comparing wastewater PCR and clinical serology surveillance results for wastewater and patient (blood) samples collected from the same district during the same epidemiological week) with concordant and discordant measles results observed from weeks 1–48 (01 January to 30 November inclusive), 2024, South Africa.

		District-time pairs: wastewater measles detection		Total week-district pairs
		At least 1 wastewater sample positive (n, %)	No wastewater samples positive (n, %)	
<b>District time-pairs: clinical measles case detection</b>	At least one case positive	48 (14)	64 (18)	112 (32)
	No positive cases	29 (8)	213 (60)	242 (68)
	Total	77 (22)	277 (78)	354

Measles virus was successfully sequenced from seven wastewater samples, with genome coverage ranging from 13% to 92% (Table 5). Phylogenetic analysis (not shown) showed that all but one sequence aligned with genotype B3.

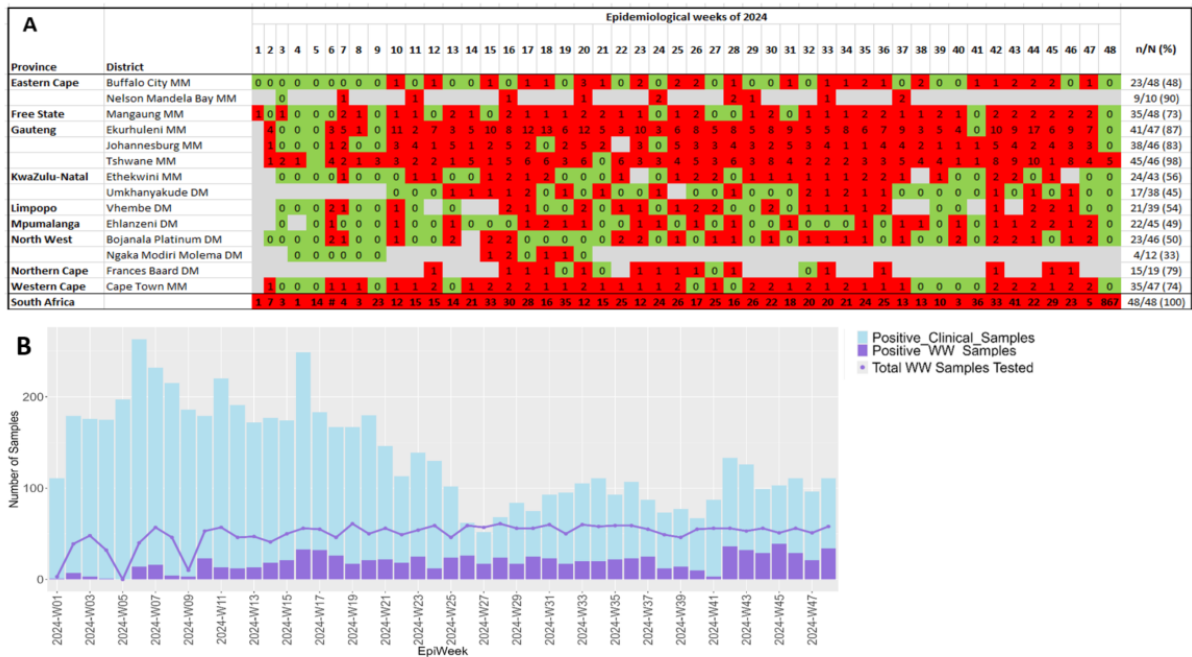
**Table 5.** Results from amplicon sequencing of measles from wastewater for seven specimens that tested positive for measles since 01 October 2024, South Africa.

Sample ID	Epidemiological week of sample collection	Measles genotype detected	RT-dPCR concentration (copies/µL)	No. of dPCR positive partitions	400 bp Amplicon concentration (ng/µL)	% genome covered
ART-MEV-24-2250_D	43	B3	2.484	6	1.89	71.4
ART-MEV-24-2254_D	43	B3	0.826	2	5.7	45.3
ART-MEV-24-2257_D	43	Unassigned	0.941	2	3.37	-
ART-MEV-24-2262_D	43	B3	2.082	5	5.56	39
ART-MEV-24-2266_D	43	B3	5.356	13	2.43	75.8
ART-MEV-24-2268_D	43	B3-like	1.216	3	7.01	12.8
ART-MEV-24-2283_D	47	B3	3.485	9	1.85	92.1
Positive control		Vaccine strain	-	-	54.1	99.7

### Rubella

From epidemiological weeks 1–48 (01 January to 30 November), 150/2 561 (5.8%) wastewater samples tested positive for rubella (Table 3). No seasonality in positivity rate was detected, but there was a trend towards increasing positivity over the course of the year, likely due to the outbreak of rubella across South Africa from September to December 2024 and improved laboratory processing (Figures 3A & B). The Northern Cape and Gauteng provinces had the highest number and proportion of positive detections (3/23 (13%) and 110/1 793 (6%), respectively), whilst KwaZulu-Natal and Free State had the lowest detection rates at 6/150 (4%) and 4/95 (4%), respectively.





**Figure 4.** A) Number of wastewater samples testing positive for hepatitis A in South African provinces by epidemiological weeks 1–48 (01 January to 30 November) of 2024.

Red=positive, green=negative, grey=sample not taken. The number in each cell reflects the number of samples testing positive. B) The number of clinical samples testing positive for hepatitis A (light blue) and wastewater samples testing positive for hepatitis A (purple) by epidemiological weeks 1–48 (01 January to 30 November) 2024, South Africa. Figure B: 'EpiWeek' is the epidemiological week during which the clinical sample for laboratory testing was collected by the healthcare provider.

### Hepatitis E

From epidemiological weeks 1–48 (01 January to 30 November 2024), 398/2 561 (16%) wastewater samples tested positive for HEV (Table 3). No seasonality in positivity rate was detected, but there was a trend towards increasing positivity over the course of the year, likely due to improved laboratory processing (Figure 5). Among districts with more than 20 samples, there was also marked geographical variation in positivity rates, with rates exceeding 50% in Northern Cape (Frances Baard District) and as low as 4% in Limpopo (Vhembe district). A median of nine samples were positive every epidemiological week across the country. The cities of Cape Town, Ekurhuleni, and Johannesburg had consistently high results, with a third to a quarter of results testing positive.

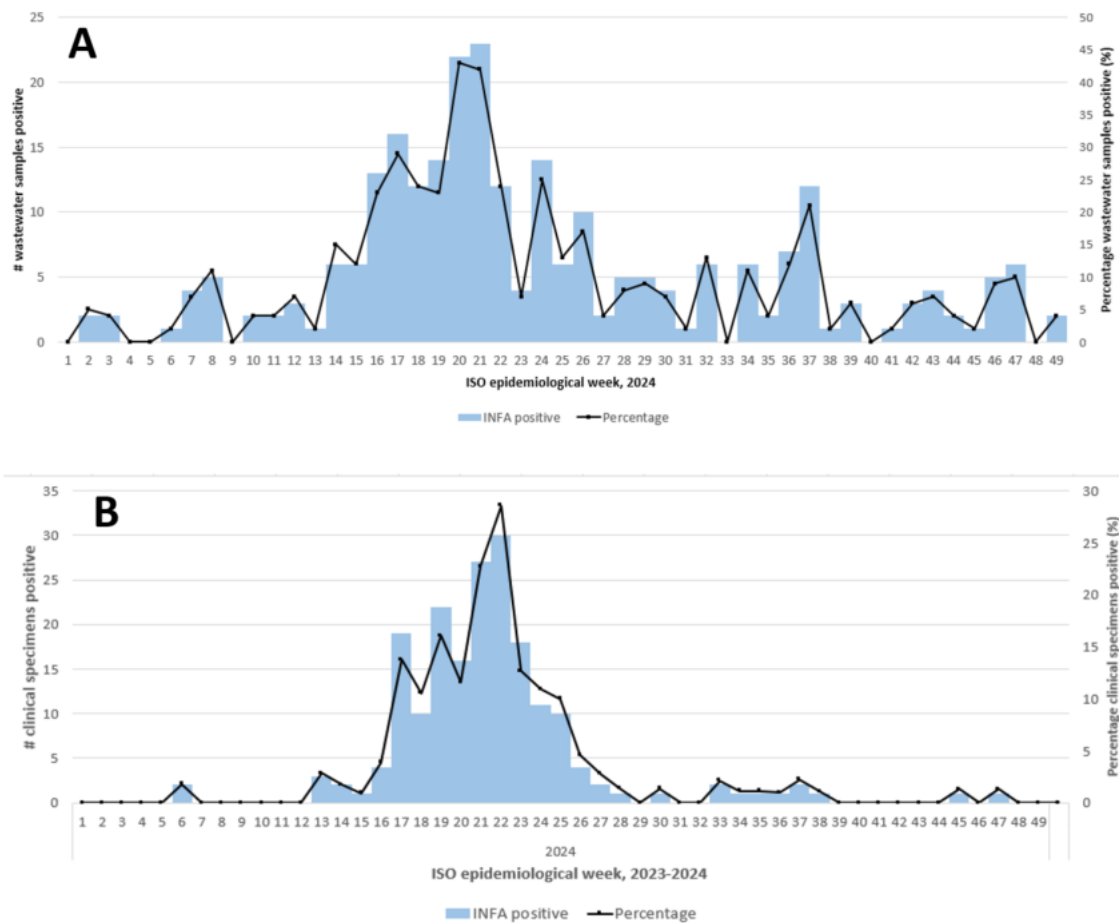


		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	Grand Total	
Eastern Cape	Buffalo City	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	1	1	0	0	0	0	0	0	0	1	1	0	1	0	0	2	0	2	1	2	3	2	3	0	1	0	2	2	2	2	1	2	0	35/129 (27)	
	Nelson Mandela Bay	1	0	0	0	0	0	0	0	1	1	0	0	1	2	1	1	0	0	1	0	0	0	0	0	2	0	0	1	0	0	1	1	0	1	2	1	1	2	1	0	2	1	1	2	1	0	0	6/13 (46)		
Free state	Mangaung	0	0	0	0	0	0	0	0	1	1	0	0	1	2	1	1	0	0	1	0	0	0	0	0	0	2	0	0	1	0	0	1	1	0	1	2	1	1	2	1	0	2	1	1	2	1	0	29/95 (31)		
Gauteng	Ekurhuleni	1	0	0	0	0	4	0	0	1	2	1	0	1	3	5	2	2	0	1	3	0	4	0	1	8	1	4	3	0	8	1	1	3	4	5	4	3	1	2	0	2	7	6	1	3	2	0	100/821 (12)		
	Johannesburg	0	1	0	0	1	2	0	0	2	3	0	2	3	0	4	3	1	1	4	0	2	0	3	2	1	3	3	0	5	0	2	1	2	2	3	3	2	5	0	2	3	2	3	1	0	0	77/320 (24)			
	Tshwane	0	0	0	0	1	0	0	1	2	2	1	0	2	0	1	1	1	3	0	5	0	0	0	2	1	6	0	2	2	0	1	3	4	4	2	1	3	0	1	0	1	0	1	2	0	0	60/648 (9)			
KwaZulu-Natal	Ethekwini	0	0	0	0	0	1	0	1	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	1	0	1	1	1	1	1	1	0	0	0	1	0	0	0	0	14/81 (17)			
	Umkhanyakude	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	9/69 (13)		
Limpopo	Vhembe	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3/76 (4)		
Mpumalanga	Ehlanzeni	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	9/79 (11)	
North West	Bojanala Platinum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	10/86 (12)		
	Ngaka Modiri Molema	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3/24 (13)		
Northern Cape	Frances Baard	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	0	1	1	1	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	13/23 (57)		
Western Cape	Cape Town	0	0	0	0	0	1	0	0	1	1	0	0	0	1	1	1	1	1	2	0	0	0	1	1	1	0	1	2	0	1	0	1	0	1	1	0	0	0	0	0	0	0	0	1	1	1	1	2	0	30/93 (32)
South Africa		1	1	1		3	8	1	1	9	11	3	2	9	8	16	10	5	11	4	6	9	2	9	19	4	18	10	3	19	5	12	8	15	21	16	13	11	9	1	10	17	14	11	11	8	3	398/2557 (16)			

**Figure 5.** The number of wastewater samples testing positive for hepatitis E in South African provinces by epidemiological weeks 1–48 (01 January to 30 November 2024) of 2024. Red=positive, green=negative, and grey=sample not taken. The number in each cell reflects the number of samples testing positive.

### Influenza A

From epidemiological weeks 1–48 (01 January to 30 November 2024), 256/2 561 (10%) wastewater samples tested positive for INFA (Table 3). The Northern Cape, Western Cape, and Free State provinces had the highest detection rates at 15/23 (65%), 50/93 (54%), and 91/95 (54%), respectively, while the lowest overall detection rate was observed in Gauteng at 561/1 795 (31%). A distinct seasonality was observed (Figure 6A), with over 25% of wastewater samples testing positive between epidemiological weeks 16–23 (14 April to 08 June). Two smaller peaks were noted at epidemiological weeks 36–46 (01 September to 16 November). The peak in the proportion of wastewater samples testing positive corresponded with the influenza A season in 2024 as determined through sentinel site syndromic surveillance (Figure 6B), which was determined from clinical surveillance by the NICD to occur between weeks 17–29 (21 April to 20 July).<sup>28</sup>



**Figure 6.** Number and proportion testing positive for influenza A of A) wastewater samples from national and community wastewater surveillance sites and B) clinical samples from persons enrolled at sentinel surveillance sites for severe respiratory illness in South African provinces by epidemiological weeks 1–49 of 2024 (01 January to 07 December 2024).

ISO=International Standards Organisation 8601 standard for naming epidemiological weeks.

### Mpox

Mpox was not detected in routine surveillance samples collected from national and sub-catchment sites during the period of implementation from epidemiological weeks 40–48 (01 October to 30 November).

### Tuberculosis

Evidence of *Mtb* bacteria was detected in 56% (140/251) of wastewater samples collected across national and sub-catchment sites from epidemiological weeks 40–48 (01 October to 30 November) using the *Mtb*-specific glycosyl transferase target (Table 3). There was a trend towards agreement in levels (high, medium, low, negative) between both DNA targets (Figure 7) from weeks 44–45 (24 October to 09 November) and also evidence of variation in quantitative levels across different sites, namely community, hospital, and wastewater treatment sites, with the highest levels observed in samples collected from community sites.



District	Site type	Epidemiological week			Epidemiological week		
		44A	44B	45A	44A	44B	45A
		IS6110 sequence			GTF gene		
COJ	WWTP	3,91		17,71	0,39		0,82
	WWTP	57,69		55,24	4,13		2,92
	WWTP	6,59		26,41	0,43		2,47
	WWTP	8,90		29,07	0,00		2,39
	Comm	10,05		38,92	1,97		5,14
	Comm	4,03		6,50	0,00		0,81
	Comm	0,41		6,99	0,41		0,41
COE	WWTP	53,95		23,74	1,62		0,83
	WWTP	4,41		4,91	0,00		0,82
	Comm	22,85		1,63	2,82		0,41
	Hospital	72,77		20,90	4,33		2,38
	Comm	526,90		18,89	42,87		0,84
	Comm	528,30	9,13	221,30	30,23	1,68	36,40
	Comm	61,70	20,37	7,39	4,61	0,00	0,84
	Comm	1,55		0,79	0,00		0,00
	Comm	1,57	1,28	2,86	0,00	0,00	0,00
	WWTP	36,55	7,46	158,80	2,68	0,00	18,83
	Airport	0,00		0,00	0,00		0,00
COT	WWTP	47,73		25,24	4,28		2,10
	WWTP	6,58		3,24	1,23		0,00
	Comm	11,85	20,37	5,09	0,41	2,48	0,84
	Hospital	12,51	0,00	0,45	0,00	0,00	0,41
	Comm	0,39	4,24	0,40	0,00	0,00	0,40
	Comm	5,69	0,00	0,00	0,86	0,00	0,00
	Comm	5,14	12,44		0,40	0,00	
Comm	2,37	0,79	0,00	0,00	0,00	0,00	



**Figure 7.** High (red), medium (orange), low (yellow) and negative (green) levels of *Mycobacterium tuberculosis* IS6110 and glycosyl-transferase genome copies/uL of wastewater from wastewater treatment plants (WWTP), community (Comm) and hospital wastewater sampling sites in Gauteng Province for weeks 44 and 45, 2024, South Africa.

COJ=City of Johannesburg; COE=City of Ekurhuleni; and COT=City of Tshwane.

## Discussion

In this collation of NICD surveillance results from national and community wastewater sentinel sampling sites, we demonstrate the feasibility and complementary nature of WES when combined with clinical surveillance data for infectious diseases.

Whilst clinical surveillance for infectious diseases is an essential component of disease control programmes to detect cases and outbreaks, conduct case monitoring and contact tracing, provide post-exposure prophylaxis, implement quarantine, and monitor routine vaccination programmes, clinical surveillance is limited by patient health-seeking behaviour and clinician diagnostic testing practices. Health-seeking behaviour is influenced by symptom severity, and persons with mild infections or who are asymptomatic may not make contact with the healthcare system. Additionally, health system functioning, budgetary constraints and clinician awareness may limit submission of good-quality specimens for laboratory detection. Wastewater and environmental surveillance overcome these inherent limitations by providing samples representative of populations and/or communities for pathogen detection. Furthermore, WES provides an alternative and independent data source to corroborate or



complement clinical surveillance data, especially where clinical testing may be limited through accessibility or health system issues.

In summary, for the diseases under wastewater surveillance, our findings demonstrated the complementarity of WES in the following ways:

- Regarding SARS-CoV-2, WES continues to provide quantitative and sequencing data that supports interpretation of clinical syndromic surveillance programmes, especially when clinical sequence data are based on few samples.
- Regarding measles and rubella, WES data identify districts with undetected circulation of MeV, whilst RuV WES data complemented clinical surveillance during a nationwide rubella outbreak. Although measles has been detected in wastewater by other groups,<sup>29</sup> the NICD is the first to demonstrate the potential utility of WES as a surveillance tool during outbreaks.<sup>17</sup>
- Hepatitis A WES data indicate the degree of endemicity of HAV in the absence of a systematic surveillance programme for acute jaundice syndrome.
- Hepatitis E WES data demonstrate that this virus is more prevalent than previously thought and open the way for further investigations to determine exposure risks and disease transmission pathways.
- We demonstrated that influenza WES data are able to identify the timing of the onset of the influenza season, confirming global observations of the same.<sup>30</sup>
- Regarding mpox, in a nationwide analysis of MPXV WES data, Adams *et al.* demonstrated that the negative predictive value of mpox wastewater surveillance is greater than 80%.<sup>31</sup> Against this background, our negative WES MPXV findings during the October–November 2024 period, together with the absence of clinical cases during this period, are likely due to the absence of widespread mpox transmission.
- Lastly, WES surveillance for *Mtb* holds promise, and further studies comparing clinical case load with quantitative wastewater data will identify if these data may be used to guide public health interventions.

We have recognised a number of limitations, some of which are intrinsic to WES and others that are amenable to intervention, as follows:

1. Whilst our PCR assays are always controlled by the addition of positive, negative and reaction controls, there are no external quality assurance or accreditation programmes for WES. As the quantities of genomic material are small, there may be extensive, statistically significant within- and between-sample variations in PCR detection results for WES. Our laboratory is collaborating with global initiatives to strengthen WES laboratory testing quality.
2. For all of the infectious diseases under surveillance in our programme, appropriate interpretative thresholds are still unclear. Modelling of WES data together with clinical surveillance data and social determinants of health is underway and will support guideline development.
3. Public health initiatives, in response to positive WES findings, are evolving. These are likely to be disease-specific – for example, detection of measles in wastewater may trigger enhanced case finding, whilst sustained detection of influenza in wastewater may hasten health promotion regarding vaccine uptake amongst vulnerable persons.



These findings are preliminary and, with the exception of SARS-CoV-2 results, were not available in the public domain in 2024. A number of developments are in progress to support utilisation for public health decision-making across the diseases under surveillance. These include:

- Ongoing refinement in wastewater processing methodology to ensure removal of PCR inhibitors, increased sensitivity and greater reproducibility of results.
- Collaborative modelling efforts with SACEMA and the Modelling and Analytics Hub, Africa (MASHA) to examine the relationships between wastewater levels of VPDs, clinical surveillance data and social determinants of health. This will allow for clearer insight into the significance of positive findings, the development of thresholds for public health action, and disease-specific applications, such as monitoring of elimination (measles) or motivation for inclusion of newer vaccines into health programmes.
- Field application of data to determine district-level applications of WES. Two surveillance officers have been employed to support the cities of Tshwane and Ekurhuleni by providing clinical and wastewater data for VPDs.
- Improved communication of VPD WES results through website and dashboard communication tools.

## Conclusion and recommendation

Through these data, the NICD has demonstrated the complementarity of WES with clinical surveillance data across a spectrum of epidemic-prone, endemic or seasonal, faeco-oral or respiratory-transmitted VPDs, even after as short a period as a single year of routine implementation. We recommend ongoing work, including field integration of WES data, modelling to support interpretation, refinement of laboratory methods, and development of enhanced communication tools to support identification of and application to areas of greatest benefit. As the public health benefit of WES becomes clearer, it may be appropriate to reduce programme dependence on grant funding.

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## Ethical considerations

Measles, rubella, hepatitis A and E, and mpox are notifiable medical conditions (National Health Act, 2003 (Act no. 61 of 2003)), and clinical surveillance for diseases caused by these pathogens is conducted by the NICD under the approval of the ethics protocol reviewed by the University of the Witwatersrand Human Research Ethics Committee (M210752). Wastewater samples are collected from national sentinel sites by wastewater treatment plant staff at these facilities and through arrangements with the National Department of Health. Wastewater samples are collected from community sampling sites with permission from the Departments of Water and Sanitation of the cities of Johannesburg, Ekurhuleni, and Tshwane metros.

## Conflict of interest

None of the authors have declared conflicts of interest.



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