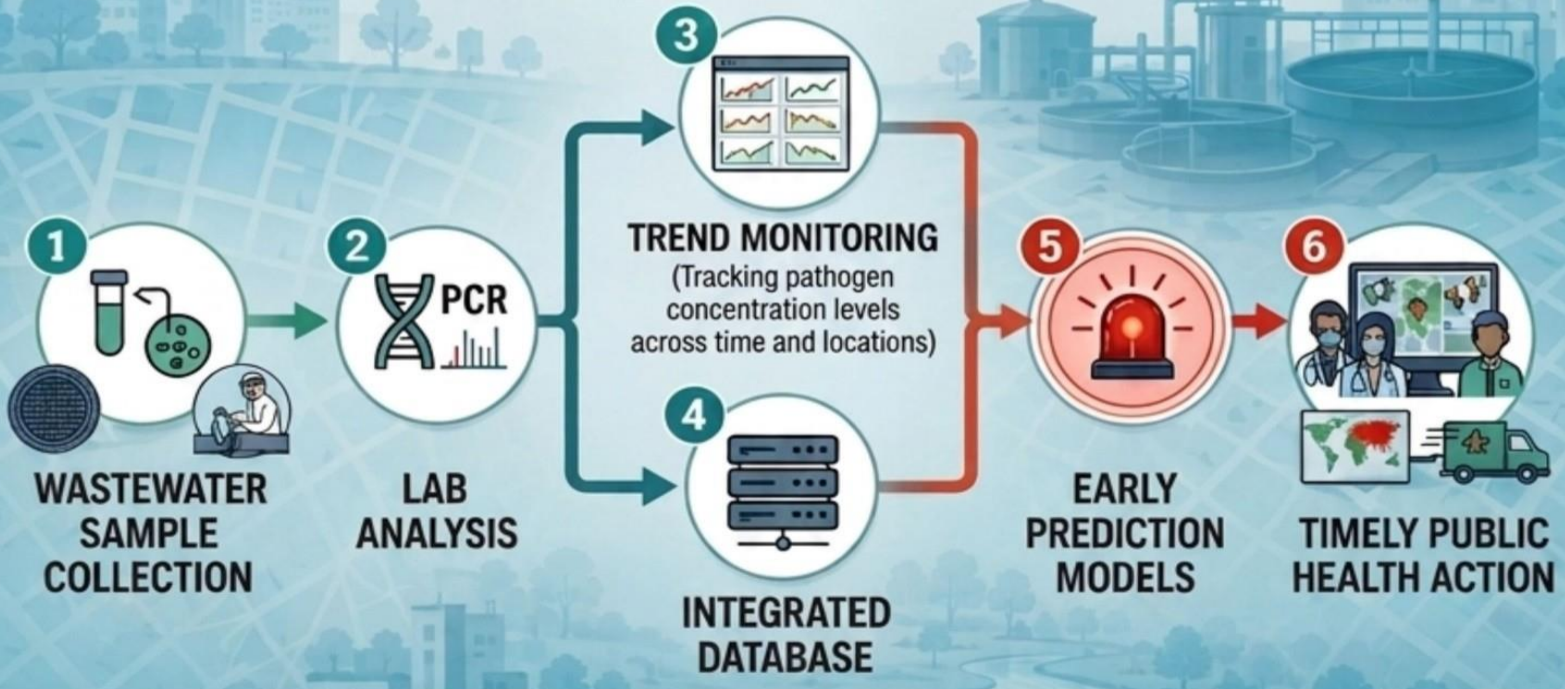
स्वास्थ्य एवं
परिवार कल्याण मंत्रालय
MINISTRY OF
HEALTH AND
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राष्ट्रीय रोग नियंत्रण केन्द्र
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DELHI
दिल्ली-११००५४

APSi

NATIONAL GUIDELINES ON WASTEWATER SURVEILLANCE FOR PATHOGENS OF PUBLIC HEALTH IMPORTANCE



National Centre for Disease Control (NCDC)
Directorate General of Health Services (DGHS)
Ministry of Health and Family Welfare
Government of India
New Delhi

स्वास्थ्य एवं
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NATIONAL GUIDELINES ON WASTEWATER SURVEILLANCE FOR PATHOGENS OF PUBLIC HEALTH IMPORTANCE

National Centre for Disease Control (NCDC)
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2026

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Government of India
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Ministry of Health and Family Welfare



MESSAGE

Globally, wastewater surveillance has become a crucial addition to traditional disease monitoring. It provides real-time, community-level data on infectious diseases that is independent of health-seeking behaviour of a person, accessibility to health care facilities and availability of laboratory tests. The experiences gained during the COVID-19 pandemic have clearly demonstrated the utility of this approach in monitoring disease trends, supporting timely public health action, and strengthening preparedness for upcoming surge in clinical cases.

The development of these guidelines reflect the commitment to building an integrated and resilient surveillance ecosystem under the One Health framework. By standardizing methodologies for sample collection, laboratory testing, quality assurance, data interpretation, and reporting, these guidelines aim to ensure consistency, reliability, and comparability of wastewater surveillance activities across the country. They will serve as a valuable resource for public health authorities, laboratories, academic institutions, and implementing agencies engaged in surveillance and outbreak preparedness.

The successful implementation of wastewater surveillance will require sustained collaboration among the health, water and sanitation, environmental, urban development, and research sectors. I am confident that these guidelines will facilitate coordinated efforts to generate actionable evidence for the decision-making and strengthen our collective capacity to prevent and respond to public health threats.

I commend the efforts of all experts, technical institutions, and partner organizations who have contributed to the preparation of these guidelines. I am confident that this publication will serve as an important reference document and contribute significantly to advancing India's public health surveillance capabilities.

I extend my best wishes to all stakeholders for the successful implementation of these guidelines and for their continued efforts towards safeguarding the health and well-being of our population.

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(Punya Salila Srivastava)

#StopObesity

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Message

Effective disease surveillance is the cornerstone of any robust public health system. It provides the critical epidemiological insights needed to understand the true extent of disease burden, target interventions, and protect our most vulnerable populations.

Traditionally, our defence systems have relied heavily on clinical surveillance—detecting infected individuals after they seek medical care. While indispensable, this approach has inherent limitations. Clinical data can be skewed by underreporting, individual health-seeking behaviours, and unequal access to healthcare facilities.

To bridge these gaps, Wastewater Surveillance (WWS) is emerging globally as a transformative, cost-effective tool to complement traditional clinical systems. By systematically collecting and analysing nucleic acids from sewage samples at strategic locations, WWS acts as an early warning system for an entire community without relying on individual healthcare access.

India has already witnessed the value of wastewater surveillance. Environmental surveillance has played a critical role in supporting the country's polio eradication efforts for over two decades. More recently, during the COVID-19 pandemic, wastewater surveillance demonstrated its potential to detect changes in virus circulation, complement clinical surveillance, and support timely preparedness and response measures. These experiences have reaffirmed the importance of incorporating innovative surveillance approaches into our public health systems.

As we move forward, it is imperative to expand the application of wastewater surveillance to priority pathogens of public health importance. The National Guidelines on Wastewater Surveillance for Priority Pathogens provide a comprehensive framework for the planning, implementation, quality assurance, data interpretation, and use of wastewater surveillance across the country. The guidelines aim to promote harmonized practices, strengthen laboratory and surveillance capacities, and facilitate the generation of reliable evidence to support public health decision-making.

The successful implementation of these guidelines will require sustained and collaborative efforts among the health, water and sanitation, urban development, environmental, and research sectors stakeholders. Such multi-sectoral partnerships, guided by the principles of the One Health approach, will be instrumental in strengthening our preparedness against existing and emerging public health threats.

I congratulate all the experts, institutions, and partner organizations whose dedication and technical expertise have contributed to the development of these guidelines. I am confident that this document will serve as a valuable resource for public health professionals, laboratories, policymakers, and implementing agencies, and will significantly strengthen India's disease surveillance ecosystem.

I extend my best wishes to all stakeholders for the successful implementation of these guidelines and for their continued commitment to safeguarding the health of our nation.

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Message

Data-driven public health actions rely on timely and comprehensive disease intelligence. While clinical surveillance remains our primary line of defense, it captures only a fraction of the population- those who actively seek medical care. The **'National Guidelines on Wastewater Surveillance for Pathogens of Public-Health Importance'** address this critical gap by establishing a passive, cost-effective, and population-wide environmental screening mechanism.

I am pleased to share that, in line with global initiatives to secure early warnings of priority pathogens circulating in the community, NCDC has developed a document on **'National Guidelines on Wastewater Surveillance for Pathogens of Public- Health Importance'**. This document is a result of extensive consultations with wide range of experts who have experience in Wastewater Surveillance (WWS). Critically, it recognizes and addresses the unique practical challenges and feasibility of implementing WWS within the Indian scenario- taking into account diverse sewage systems, variable laboratory infrastructure, and stringent biosafety requirements.

The guidelines have been developed by the **Ministry of Health and Family Welfare, Government of India** in collaboration with **Alliance for Pathogen Surveillance Innovations (APSI) consortium,** that includes leading scientific institutions. These guidelines provide a standardized blueprint for institutionalizing Wastewater-Based Epidemiology (WBE) across India.

I congratulate the National Centre for Disease Control and commend the dedicated efforts of the **Enterovirus Division of NCDC** for bringing out this important guidance document. I am entirely confident that this framework will significantly strengthen the Waste water Surveillance capabilities of our country.

(Loveneesh G. Krishna)

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Message

In the wake of complex global health challenges, the Government of India remains steadfast in its commitment to strengthen the nation's disease surveillance architecture. The integration of environmental surveillance into our public health system represents a paradigm shift- moving from reactive clinical reporting to proactive action to prevent the upsurge in clinical cases.

The *"National Guidelines for Wastewater Surveillance for Pathogens of Public Health Importance"* marks a significant milestone in this journey. By leveraging wastewater as a sensitive indicator of pathogen burden in the community, we create a powerful tool that complements our traditional clinical surveillance systems. This document provides a standardized, scalable, and scientific approach that will enable state and district-level authorities to detect, track, and respond to infectious disease threats.

For the Ministry, this initiative is not merely a technical upgrade; it is a critical component of the broader mandate to ensure health security for all. Wastewater surveillance provides us with an objective data stream that is free from the biases of healthcare-seeking behavior, allowing for more precise resource allocation and data-driven policymaking.

I commend the efforts of all experts, technical institutions, and partner organizations who have contributed to the preparation of these guidelines. I am confident that this publication will serve as an important reference document and contribute significantly to advancing India's public health surveillance capabilities.

The success of these guidelines rests on the synergy between public health officials, municipal authorities, and laboratory networks. I urge all states and union territories to adopt these protocols diligently as we work towards building a more resilient, responsive, and robust health system for the nation.

(**Harsh Mangla**)

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Government of India
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MINISTRY OF HEALTH & FAMILY WELFARE



MESSAGE

Wastewater surveillance has emerged as an important public health tool for early detection and monitoring of infectious diseases at the community level. By identifying pathogens and their genetic material in wastewater, this approach can provide timely information on disease circulation, including infections that may remain undiagnosed or unreported through routine clinical surveillance.

The publication of the **'National Guidelines on Wastewater Surveillance for Pathogens of Public-Health Importance'** represents an important step towards strengthening India's integrated disease surveillance and public health preparedness. The document provides a coordinated framework for the planning, implementation, laboratory testing, interpretation and reporting of wastewater surveillance activities across the country.

A standardized national approach is essential to ensure that surveillance data generated by different institutions and jurisdictions are reliable, comparable and useful for public health action. This document outlines the principles for selecting surveillance sites, collecting and transporting samples, applying appropriate laboratory methods, ensuring quality assurance, analyzing trends and submission of wastewater surveillance data to the Integrated Health Information Platform (IHIP). This data can be merged with clinical and demographic data sets to generate early-warning signals with the help of prediction models. This will ultimately help in timely public-health action.

I appreciate the contributions of the subject experts, technical working groups, laboratory professionals, public health specialists and partner institutions involved in the development of this national document. Their collective expertise and commitment have helped create a practical and scientifically sound framework suited to India's public health needs.

I am confident that this document will support the systematic expansion of wastewater surveillance for priority pathogens and strengthen the country's ability to detect, assess and respond to emerging public health threats.

I encourage all stakeholders to adopt the guidance provided in this document and contribute towards building a resilient, coordinated and evidence-based surveillance system.

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Acknowledgement

The ***National Guidelines on Wastewater Surveillance for Pathogens of Public Health Importance*** represent the collective endeavor and profound contributions of numerous experts, institutions, and stakeholders dedicated to fortifying India's public health surveillance architecture.

The National Centre for Disease Control (NCDC) extends its deepest gratitude to the Ministry of Health and Family Welfare (MoHFW), Government of India, for facilitating this initiative. We are particularly grateful to Ms. Punya Salila Srivastava, Secretary; Dr. Rakesh Gupta, Additional Secretary; and Mr. Harsh Mangla, Joint Secretary, Ministry of Health and Family Welfare, Government of India for their visionary leadership and steadfast support throughout this process.

We also extend our heartfelt thanks to Dr. Loveneesh G Krishna, Director General of Health Services (DGHS), for his guidance and continuous support. We are profoundly grateful to Dr. Sandhya Kabra, Director, NCDC, for her pivotal insight, unwavering advocacy, and the critical support during the publication of these guidelines. Furthermore, we highly value the foundational expertise and sustained encouragement provided by Prof. (Dr.) Ranjan Das, former Director, NCDC, throughout this endeavor.

We offer our profound gratitude to Prof. (Dr.) Atul Goel, former Director General of Health Services (DGHS), whose foresight and initiative catalyzed the development of this national document. We would like to sincerely acknowledge the members of APSI who initiated the formulation of this document. Special recognition is due to the Expert Committee, whose rigorous review considerably elevated its scientific rigor.

The foundation of this document rests on the pioneering legacy of NCDC's Enterovirus Division, which has spearheaded wastewater surveillance for over two decades. The vast experience garnered from Polio Surveillance and COVID-19 standardizations forms the bedrock of these guidelines. We commend the dedicated officers and staff of this Division for their relentless

commitment. Furthermore, we recognize the vital technical acumen provided by subject matter experts at NCDC and the Integrated Disease Surveillance Programme (IDSP), whose practical insights on targeted public health actions have been instrumental.

It is our earnest hope that this document serves as a cornerstone resource to strengthen Integrated Disease Surveillance, accelerate the early detection of priority pathogens, and elevates public health preparedness and response capabilities nationwide.

Enterovirus Division
National Centre for Disease Control
Directorate General of Health Services
Ministry of Health and Family Welfare
Government of India

Preface

For over two decades, Wastewater Surveillance (WWS) has served as a highly effective global complement to Acute Flaccid Paralysis (AFP) surveillance for the poliovirus. This utility was further demonstrated during the COVID-19 pandemic, when researchers worldwide leveraged WWS to monitor infection trends. In India, multiple institutions adopted this approach, yielding critical insights that helped assess the national epidemiological landscape. Consequently, wastewater testing protocols for COVID-19 were successfully standardized across key organizations, including the NCDC, DBT, DST, CSIR, ICMR, and various state government institutions.

The Alliance for Pathogen Surveillance Innovations (APSI)-India, established in 2021 at the Centre for Scientific and Industrial Research (CSIR)-Centre for Cellular and Molecular Biology (CCMB), Hyderabad, with support from the Rockefeller Foundation. It is a pan-India consortium of premier academic, research, and medical institutions - including NCBS, TIGS, Ashoka University, CSIR-IGIB, CSIR-NCL, IISER, PKC, and the Pasteur Institute. APSI advances national pathogen tracking through genome sequencing, bioinformatics, and wastewater-based epidemiology. By developing affordable, indigenous detection tools and embedding surveillance technologies into public health systems, APSI strengthens India's preparedness under the One Health framework. Further details can be found at https://data.ccmb.res.in/apsi/. The APSI Consortium approached Ministry of Health & Family Welfare (MoHFW) to scale these efforts nationally. The Directorate General of Health Services, Delhi (DGHS) constituted an expert committee to draft the National Guidelines on WWS in coordination with APSI.

The Integrated Disease Surveillance Programme (IDSP), operating under the National Centre for Disease Control (NCDC), is India's primary framework for tracking epidemic-prone diseases. By pairing wastewater surveillance with the clinical data available on the Integrated Health Information Platform (IHIP) portal, we can significantly enhance the nation's early warning systems, expedite outbreak detection, and fortify overall public health preparedness.

India's vast population and rapidly expanding sanitation infrastructure present a unique opportunity to establish a standardized national framework for WWS. The National Centre for

Disease Control (NCDC) and the Alliance for Pathogen Surveillance Innovations (APSI)-India consortium have jointly developed the *National Guidelines on Wastewater Surveillance for Pathogens of Public Health Importance*. These guidelines provide a comprehensive framework covering laboratory testing protocols, data interpretation and submission, and the translation of WWS data into actionable public health measures.

This document serves as a practical reference for institutions establishing new WWS systems, as well as those seeking to strengthen or standardize existing programs. It is designed for a diverse audience of stakeholders involved in planning, implementing, and managing WWS initiatives, including public health and environmental professionals, laboratory personnel, researchers, program managers, and policymakers.

Structured across thirteen interconnected sections, the guidelines guide the reader through the complete WWS workflow from initial planning to public health action. Designed as a modular reference rather than a sequential text, users can consult specific chapters based on their role and stage of program implementation. For example, program planners might focus on surveillance objectives, site selection, and governance, while laboratory personnel can refer directly to the testing, sequencing, biosafety, and data reporting sections. Extensive cross-referencing is included to ensure seamless navigation across related topics.

This document underwent an extensive consultation process, having been shared with all Indian States as well as the Indian Council of Medical Research (ICMR) for their critical review and constructive inputs.

Given the dynamic nature of wastewater surveillance, this guidance should be utilized alongside prevailing national policies and contemporary scientific insights. To ensure its continued relevance, the document will undergo periodic updates to incorporate new technological and scientific developments.

Table of Contents

Chapter 1 Introduction	**1**
Chapter 2 Scope of the Document	**3**
Chapter 3 Priority Pathogens for Wastewater Surveillance	**5**
Chapter 4 Selection of Sites for Wastewater Surveillance	**19**
Chapter 5 Collection of Wastewater Samples & Transport to the Laboratory	**23**
Chapter 6 Laboratory Testing Procedure for Viral Pathogens in Wastewater	**28**
Chapter 7 Laboratory Testing Procedure for Antimicrobial Resistance (AMR) Genes in Wastewater	**45**
Chapter 8 Interpretation of Laboratory Data and Surveillance Analytics	**50**
Chapter 9 Sequencing Approaches for Wastewater Surveillance	**55**
Chapter 10 Biosafety and Risk Assessment	**64**
Chapter 11 Submission of Wastewater Surveillance Data to the Integrated Health Information Platform (IHIP)	**66**
Chapter 12 Using the Wastewater Surveillance Data for Public Health Action	**71**
Chapter 13 Ethical and Legal Considerations for Wastewater Surveillance	**73**
References	
Annexures	
List of Acronyms	
List of Contributors	

1. Introduction

Disease surveillance is essential to provide inputs for necessary actions to be taken by a public health system. Surveillance is central to infectious disease epidemiology helping to understand the extent of disease burden and identifying vulnerable and high-risk populations. Traditionally, disease surveillance has relied on detecting infected individuals within a population. However, direct estimates from such data can be biased due to underreporting and are largely dependent on the health-seeking behavior of individuals and access to health care facilities.

Wastewater Surveillance (WWS) is emerging as a powerful tool to complement clinical surveillance for various pathogens. WWS is characterized by the systematic collection of wastewater samples from selected locations, analysis of nucleic acid in samples to detect, quantify and characterize infectious disease pathogens. While WWS does not provide individual data, it is cost-effective and is particularly useful in low-resource settings where obtaining representative data on the infectious disease burden is especially challenging. WWS reflects contributions from the entire community, including individuals who are non-infected, asymptomatic, symptomatic, or severely ill individuals, thereby providing a population-level signal of pathogen circulation. In contrast, clinical surveillance primarily captures individuals who seek testing (symptomatic) or treatment (ill/severely ill)[1] **(Figure 1)**.

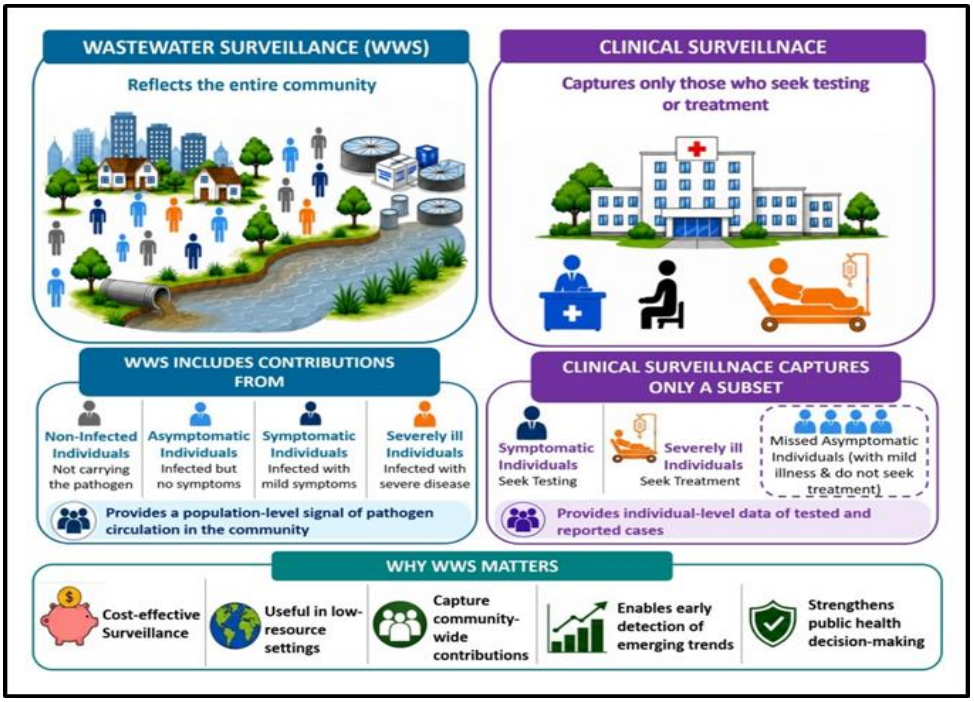


Figure 1. Broader Scope of Wastewater Surveillance

Globally, WWS has gained significant attention over the past five years. It has been extensively used for poliovirus surveillance and has played a critical role in eradication efforts. Its utility was further demonstrated during the COVID-19 pandemic, when several countries leveraged WWS to detect early signals of transmission and identify "silent waves" ahead of clinical case surges. More recently, WWS has also been explored for monitoring antimicrobial resistance (AMR), enabling the tracking of resistance genes in communities and hospitals.[2-5]

The relevance of WWS is further strengthened within the One Health framework, which recognizes the interconnectedness of human, animal, and environmental health. Wastewater systems act as convergence points for these domains, making WWS a valuable tool for integrated surveillance of pathogens, including emerging and re-emerging infections[6]. In the context of "Disease X" a term used to denote unknown pathogens with pandemic potential, WWS offers a proactive approach for early detection and preparedness, enhancing the ability to identify novel threats before they manifest widely in clinical settings[7].

Various pilot studies and initiatives undertaken across Indian cities have demonstrated the feasibility of WWS in urban and peri-urban settings, including the early detection of pathogens such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) prior to their appearance in clinical data[8-11].

This document seeks to lay the foundation for an integrated, scalable, and cost-effective national WWS system aligned with India's public health priorities. Recognizing the evolving nature of pathogens, technologies, and surveillance needs, these guidelines are intended to be dynamic and will require periodic review and updating to remain relevant and effective.

2. Scope of the Document

This document provides a comprehensive and standardized framework for implementing wastewater surveillance. It is intended to serve as a reckoner for national and state-level health authorities, IDSP units, municipal bodies, testing laboratories and other relevant stakeholders involved in disease surveillance and public health response.

The scope of the guidance includes the technical, operational, and institutional components necessary to establish and sustain WWS systems, with adaptation based on local contexts. Whereas this document provides summaries for detection of specific pathogens such as SARS-CoV-2, influenza, respiratory syncytial virus, the procedures can be expanded to other pathogens of public health concern in India. Procedures include a description of the sampling, processing and detection of selected pathogens in wastewater utilizing reverse transcription-quantitative polymerase chain reaction (RT-qPCR) methods. Sequencing platforms are also described, which help in detecting the circulating variants and further understanding the evolution and epidemiology of these pathogens.

Specifically, the document covers:

- Priority pathogens for wastewater surveillance, with provisions for expanding the list based on emerging public health threats.
- Site selection strategies for sewage collection, considering infrastructure availability, population representativeness, and public health risk.
- Sampling methodologies, including grab, composite, and passive sampling approaches and transport of wastewater samples to designated laboratories.
- Standardized laboratory methods, including:
    - ❖ Concentration of pathogens from wastewater samples
    - ❖ Nucleic acid extraction
    - ❖ Real-time PCR testing and estimation of viral titres
    - ❖ Genomic sequencing of samples to detect pathogens or resistance genes
    - ❖ Biosafety and risk assessment
    - ❖ Ethical and legal considerations for WWS

- Data interpretation frameworks to support early warning, trend analysis, and integration with clinical surveillance systems.
- Use of WWS data for Public Health Action.
- Data reporting and submission protocols, including linkage with the Integrated Health Information Platform (IHIP) under the IDSP.
- Guidance on translating WWS data into public health action, including triggers for alerts, targeted interventions, and communication with local authorities.

While the document is technical in nature, it is designed to be actionable by a wide range of stakeholders and to promote inter-sectoral collaboration and create a uniform national approach while allowing for local innovation and adaptation.

3. Priority Pathogens for Wastewater Surveillance

Wastewater surveillance (WWS) provides population-level information on infectious disease circulation by capturing biological signals from both symptomatic and asymptomatic individuals, including those who do not access healthcare services. As wastewater may contain a wide range of human pathogens and environmental organisms, systematic prioritization of target pathogens is a critical foundational step in the planning, design, and implementation of WWS programmes.

Not all pathogens are equally suitable for wastewater-based monitoring, nor do all generate information that supports timely public health decision-making. National and subnational authorities are therefore encouraged to focus surveillance efforts on pathogens of high public health importance for which wastewater data can meaningfully strengthen existing surveillance systems and inform response actions.

3.1 PRINCIPLES FOR PRIORITIZING PATHOGENS

The selection of priority pathogens for wastewater surveillance should follow a structured and context-specific process. Particular consideration should be given to pathogens that cause substantial morbidity or mortality, have epidemic or pandemic potential, or are characterized by silent transmission due to asymptomatic or mild infection. In such contexts, wastewater surveillance may detect community transmission that is not readily captured through clinical surveillance alone. Priority setting should also account for pathogens that disproportionately affect vulnerable or underserved populations with limited access to healthcare and diagnostic services.

The principal criteria for prioritization include[12]:

A. Relevance for Public Health Decision-Making

A primary consideration in selecting pathogens for wastewater surveillance is whether the generated data can be translated into practical public health action. Priority should be given to pathogens for which wastewater trends can meaningfully support interventions, including:

i. Detecting increase in transmission before clinical cases rise

ii. Supporting focused diagnostic testing or case-finding activities

iii. Informing immunization planning or booster strategies

iv. Guiding water, sanitation, and hygiene (WASH) risk mitigation measures

v. Strengthening outbreak preparedness and response at local or national levels

Surveillance activities should not be undertaken in isolation. In the absence of a clearly defined mechanism linking WWS outputs to public health decisions, the value of implementing such surveillance is limited.

B. Scientific, Technical, and Operational Suitability

Not all pathogens are equally amenable to wastewater surveillance. Selection should be guided by biological and technical characteristics that support reliable detection and interpretation, such as:

i. Regular and sufficient excretion of the pathogen or its genetic material into wastewater

ii. Persistence in environmental matrices long enough to allow sampling and analysis

iii. Availability of well-established and reproducible laboratory assays

Operational considerations are equally important for sustained surveillance and include:

i. Sampling approaches that are logistically feasible and representative

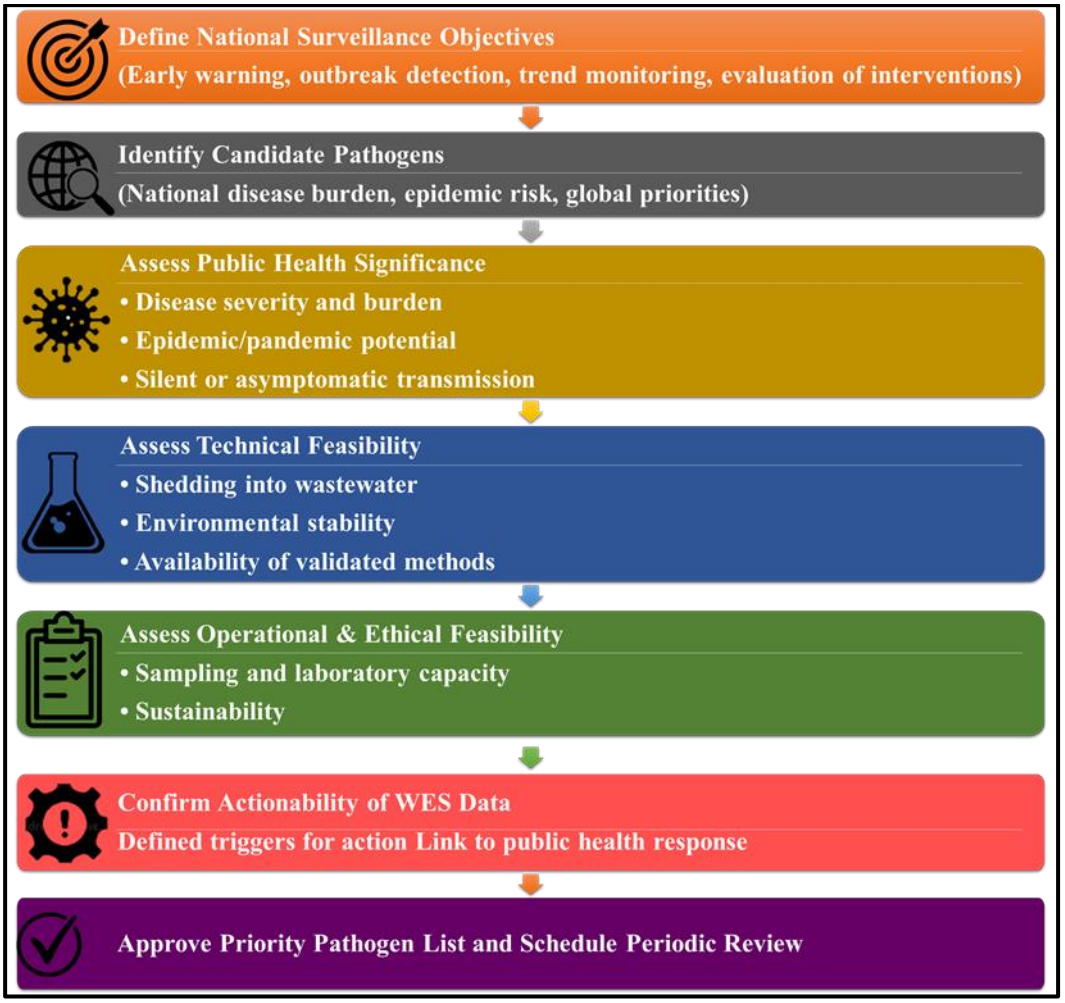
ii. Access to adequately equipped laboratories and trained personnel

iii. Implementation of quality management systems to ensure consistency and data comparability over time

Together, these factors determine whether WWS can be maintained as a dependable, long-term public health surveillance tool.

3.2 PATHOGEN PRIORITIZATION PROCESS

The prioritization of pathogens for wastewater surveillance should follow a stepwise decision-making process, as illustrated in **Flowchart 1**, incorporating public health relevance, technical feasibility, and actionability (adapted from WHO, 2024)[12].



Flowchart 1: Stepwise Decision-Making Process for Prioritization of Pathogens for Wastewater Surveillance

3.3 CURRENT PRIORITY PATHOGENS FOR NATIONAL WWS PROGRAMMES

The prioritization of pathogens for wastewater surveillance is guided by global experience with emphasis on pathogens that are consistently shed in human excreta, technically detectable in wastewater matrices, epidemiologically significant, and capable of informing timely public health action. The following pathogens are currently recommended as potential priority targets for WWS.

3.3.1 Poliovirus

Poliovirus, a small non-enveloped enterovirus is the most established pathogen for wastewater and environmental surveillance globally[2]. In the current pre-eradication phase, wild poliovirus types 2 and 3 have been globally eradicated, while transmission of wild poliovirus type 1 (WPV1) persists in a small number of geographic settings. At the same time, circulating vaccine-derived polioviruses (cVDPVs), particularly type 2, have emerged

as the predominant cause of paralytic polio worldwide. This evolving epidemiological landscape has increased the importance of WWS, as poliovirus transmission frequently occurs in the absence of reported clinical cases. The virus is shed at high concentrations in human feces, both by recently oral polio vaccine (OPV) vaccinated individuals and for prolonged periods, by persons with primary immunodeficiency. The absence of a lipid envelope confers high environmental stability and resistance to common disinfectants, enabling poliovirus to persist for weeks in wastewater and remains detectable across diverse sanitation systems[13].

The Global Polio Eradication Initiative (GPEI) has integrated wastewater surveillance with acute flaccid paralysis (AFP) surveillance in more than 80 countries, expanding and strengthening surveillance networks[14-15]. Recent studies in 2024–2025 reported genetically linked cVDPV2 in wastewater from multiple European countries, illustrating silent, geographically widespread virus circulation absent clinical cases and underscoring the sensitivity of environmental surveillance in polio-free regions. Genomic sequencing has clarified cross-border transmission pathways and lineage linkages, while isolated detections of wild poliovirus type 1 in sewage further highlight the ongoing risk of importation even without paralytic disease. Looking ahead, research priorities emphasize integration of wastewater and clinical surveillance, advanced molecular and sequencing tools for enhanced sensitivity, and sustainable, standardized surveillance networks to support eradication. India, having eliminated wild polio through rigorous AFP and environmental surveillance, continues to leverage wastewater sampling to confirm the absence of poliovirus and maintain high confidence in its polio-free status, illustrating the value of sustained wastewater and environmental monitoring even after eradication[16].

Although, WWS for poliovirus is highly informative, its implementation must be accompanied by strict biosafety and biosecurity measures**,** including full compliance with global poliovirus containment requirements. Effective surveillance further depends on careful sentinel site selection, standardized sampling and laboratory protocols, and systematic integration of wastewater findings with AFP surveillance and immunization data.

3.3.2 SARS-CoV-2

SARS-CoV-2 is an enveloped RNA virus that remains a significant public health threat due to periodic surges and continuous viral evolution. Infected individuals, including those who

are asymptomatic, shed viral RNA in feces and respiratory secretions, which enter wastewater systems and enable the use of wastewater monitoring. Although infectious virus does not persist in wastewater, viral RNA is sufficiently stable to enable robust detection, quantification, and genomic characterization across a wide range of environmental conditions.

Following the onset of the COVID-19 pandemic, wastewater and environmental surveillance has rapidly emerged as a valuable approach for targeting SARS-CoV-2 as an early warning indicator of community transmission. Since 2020, more than a thousand studies have been conducted worldwide, spanning diverse geographical settings and wastewater systems, to assess the feasibility, sensitivity, and public health utility of this approach. Despite this extensive body of work, significant heterogeneity remains in sampling strategies, virus concentration methods, nucleic acid extraction, molecular targets, and data normalization, underscoring the ongoing need for standardized and harmonized protocols. Collectively, these studies have demonstrated a range of outcomes, including the ability of wastewater signals to precede clinical case trends, reflects infection dynamics, identify resurgences and variant circulation, and complement traditional surveillance systems, while also highlighting methodological limitations and context-specific challenges that influence data interpretation[17-22].

Several countries have developed dedicated dashboards to support standardized data entry, visualization, and interpretation of SARS-CoV-2 quantification from wastewater surveillance (**Box 1**). These dashboards are typically managed by public health agencies or research consortia and allow laboratories to upload viral concentration data, sampling metadata, and quality indicators in near real time. The platforms integrate geospatial mapping, enabling timely situational awareness at local, national, and sometimes regional levels. In India, the Integrated Health Information Platform (IHIP) has been developed for the Ministry of Health and Family Welfare (MoHFW) under the Integrated Disease Surveillance Programme (IDSP) as a central system where SARS-CoV-2 wastewater surveillance data can be entered and linked with other public health information.

As SARS-CoV-2 is primarily a respiratory virus with high transmissibility, strict biosafety and biosecurity measures must be ensured when initiating wastewater surveillance to protect personnel involved in sample collection, handling, and laboratory analysis.



Box 1: Global Wastewater Surveillance Dashboards

3.3.3 Influenza A and B

Influenza viruses are RNA viruses with a segmented genome and a lipid envelope. Of the known influenza virus types, types A and B are responsible for recurrent outbreaks in human populations and are therefore most relevant for routine public health surveillance. Influenza A and B viruses, despite being primarily respiratory pathogens, are increasingly recognized as detectable targets in wastewater surveillance, as infected individuals can shed viral RNA in faeces and through activities such as hand washing, bathing, laundering, and disposal of respiratory secretions. Recent global studies have shown that influenza RNA can be consistently detected in municipal wastewater and that temporal trends often align with, or precede, clinical influenza activity, emphasizing its value for seasonal outbreak monitoring[23]. Evidence from pilot and research studies in India similarly indicates the feasibility of detecting influenza viral RNA in sewage, supporting its potential integration into existing environmental surveillance platforms developed for polio and SARS-CoV-2. Although environmental conditions limit viral infectivity, RNA detection provides meaningful insights into community transmission dynamics rather than risk of exposure. Importantly, wastewater surveillance may also capture signals from zoonotic influenza viruses shed by animals, particularly in settings with close human–animal interfaces or mixed waste streams, offering

an opportunity for early detection of novel or genetically re-arranged strains with pandemic potential[24-27].

3.3.4 Monkeypox Virus

Monkeypox virus is an enveloped DNA virus. Monkeypox virus is closely related to the smallpox virus, as both are orthopoxviruses**,** sharing similar genomic structure and replication strategies, although Mpox generally causes a less severe and less transmissible disease than smallpox. Infected individuals can shed viral DNA through multiple routes, including skin and mucosal lesions, respiratory secretions, saliva, urine, faeces, semen, contaminated grey water from bathing and laundering. Although DNA viruses are generally more environmentally stable than RNA viruses, evidence on Monkeypox virus persistence in wastewater remains limited. Few available studies suggest that infectious virus can persist for some time in untreated wastewater, with degradation influenced by temperature and chemical conditions, while the public health risk from wastewater exposure has not been documented[28-32]. Overall, WWS is considered a case-based surveillance tool for Mpox, particularly in sewered settings, while further research is needed to optimize methods, understand viral stability, and expand applicability to unsewered contexts.

3.3.5 Respiratory Syncytial Virus

Respiratory syncytial virus (RSV) is an enveloped RNA virus, a leading cause of lower respiratory tract infections, particularly among infants, young children, older adults, and immunocompromised populations. RSV is primarily transmitted via respiratory droplets and direct contact however, infected individuals can shed viral RNA through faeces, likely due to the swallowing of respiratory secretions and gastrointestinal passage of the virus. Multiple studies have confirmed the presence of RSV RNA in both raw wastewater and wastewater solids, demonstrating that it can be monitored through wastewater surveillance. As an enveloped virus, RSV is less environmentally stable, its RNA persistence in wastewater is affected by temperature, storage conditions, and freeze–thaw cycles, with studies indicating better short-term stability at ambient temperature or 4°C compared to freezing. Experience from WWS in several countries has shown that RSV wastewater signals display clear seasonality and correlate well with clinical indicators such as test positivity and hospitalizations, although in some settings wastewater trends may lag behind clinical case data rather than provide early warning[24-25,33-36].

Major public health agencies are increasingly considering RSV as a target within integrated wastewater surveillance programmes, primarily to complement clinical surveillance of respiratory infections. In the United States, the CDC has incorporated RSV into the National Wastewater Surveillance System (NWSS) as part of multipathogen monitoring alongside SARS-CoV-2 and influenza, with a focus on tracking seasonal trends and supporting situational awareness. Similarly, health agencies in Canada, Europe, and other regions are targeting RSV in wastewater to monitor seasonal onset and intensity, inform the timing of immunoprophylaxis strategies, and complement clinical surveillance, particularly where testing coverage is limited.

3.3.6 Measles, Mumps and Rubella Virus

Measles, mumps and rubella (MMR) are enveloped RNA viruses that infect only humans. Global strategies target elimination of measles and rubella by 2030, while mumps control relies on sustained high MMR coverage rather than a formal elimination goal[37]. Although transmission is primarily respiratory, all three viruses can be shed during acute infection, mainly in urine and upper respiratory secretions**,** resulting in low-level inputs to wastewater. Vaccine-derived RNA shedding can also occur at low concentrations. As enveloped viruses, they are less environmentally stable than non-enveloped enteric viruses, but viral RNA can persist long enough (hours to days in sewer systems) to allow detection by molecular methods. Wastewater surveillance experience is strongest for measles**,** with multiple pilot studies and emerging national multipathogen programmes demonstrating technical and operational feasibility, early-warning potential, and the ability to distinguish wild-type from vaccine strains using targeted assays and sequencing. In contrast, evidence for rubella and mumps in wastewater remains very limited, with only sporadic detections reported, indicating a need for further research before routine WWS application for these pathogens[38-42].

3.3.7 Hepatitis A Virus (HAV) and Hepatitis E Virus (HEV)

Hepatitis A virus (HAV) and Hepatitis E virus (HEV) are distinct RNA viruses that share several epidemiological and environmental characteristics. Both are non-enveloped RNA viruses with multiple genotypes that exhibit strong geographic variation. Six genotypes of HAV are recognized, of which genotypes I, II and III account for most human infections. Genotype IV, V, and VI infect simians (monkeys and apes). HEV comprises eight (1-8) genotypes, with genotypes 1-4 responsible for the majority of human disease. Genotypes 1

and 2 predominantly circulate in Asia and Africa, whereas genotypes 3 and 4 are more common in Europe, the Americas, and parts of Asia. HAV and HEV viruses are shed in high concentrations in human faeces, often before the onset of symptoms, making wastewater a reliable aggregate sample for monitoring community transmission. Their non-enveloped structure confers substantial environmental stability, allowing viral RNA to persist in wastewater and environmental waters with limited degradation under typical sanitation and climatic conditions. WWS studies across diverse settings have consistently demonstrated the detectability of HAV and HEV in untreated sewage, surface waters. For HAV, multiple investigations have shown good temporal correlation between wastewater signals and reported cases, with evidence of early warning up to several weeks before clinical detection during outbreaks. WWS experience for HEV also confirms frequent detection, though interpretation can be more complex due to zoonotic genotypes and contributions from animal reservoirs, particularly pigs and other animals like wild boars, deer, rabbits, sheep, goats, foxes, and rats. Overall, accumulated evidence supports the technical feasibility and public health value of including HAV and HEV as priority pathogens in WWS to complement clinical surveillance and inform timely preventive interventions[43-47].

3.3.8 Rotavirus

Rotavirus is a non-enveloped RNA virus and a leading cause of acute gastroenteritis. Several studies have shown that people infected with rotavirus, particularly infants and young children**,** may shed the virus in their stool without showing clinical symptoms**.** Viral shedding can occur before symptom onset**,** during mild or subclinical infections, and even after recovery**,** sometimes for several days to weeks. Although viral loads in asymptomatic individuals are generally lower than in symptomatic cases, they are still sufficient to contribute to community-level transmission and to detectable signals in wastewater. This asymptomatic and prolonged shedding is one of the key reasons rotavirus is consistently detected in wastewater and why WWS is useful for capturing the true circulation of rotavirus beyond clinically reported cases. Owing to its non-enveloped structure, rotavirus is highly stable in wastewater and environmental waters, showing resistance to temperature fluctuations, pH variation, and conventional wastewater treatment processes, with infectious particles and viral RNA frequently persisting in sewage and irrigation waters. Wastewater based studies across multiple regions, including Europe, the Middle East, Asia, and the Americas, have consistently demonstrated widespread circulation of rotavirus in community

sewage, and clear seasonal patterns corresponding to clinical gastroenteritis trends. Recent wastewater based surveillance experience further shows that rotavirus RNA concentrations in wastewater correlate with reported infection rates and vaccination coverage, highlighting the value of WWS as a complementary tool for monitoring disease burden, evaluating vaccine impact, and identifying transmission hotspots, especially in settings with limited clinical reporting[48-52].

3.3.9 Adenovirus

Human adenoviruses are non-enveloped DNA viruses which are associated with a range of clinical syndrome including respiratory infections, gastroenteritis, conjunctivitis, and febrile illness. Adenoviruses are commonly and persistently shed in faeces**,** often at high concentrations and for prolonged periods, including by asymptomatic individuals, making them frequent constituents of domestic wastewater. Due to their non-enveloped structure, adenoviruses exhibit high environmental stability and resistance to physical and chemical stress. This allows Viral DNA to persist allowing through wastewater collection systems and in some cases, treatment processes. Adenovirus genomes have been repeatedly detected in raw sewage, treated effluents, and environmental waters, demonstrating technical detectability in wastewater matrices. However, despite these favourable characteristics, wastewater surveillance for adenovirus remains relatively limited, with most studies focusing on presence and persistence rather than systematic trend analysis or linkage to population-level disease burden. Consequently, wastewater surveillance for adenoviruses is a promising yet evolving approach. Additional research is required to improve epidemiological interpretation and support the use of wastewater data for public health action[53-55].

3.3.10 Arboviruses

Arboviruses comprise a heterogeneous group of viruses transmitted mainly by mosquitoes and other vectors. They are responsible for a substantial global burden of febrile and neuroinvasive diseases. Most arboviruses of public health importance are enveloped RNA Viruses, including dengue, chikungunya, zika, west nile, japanese encephalitis and yellow fever viruses with oropouche an important emerging arbovirus. The infected individuals release viral RNA to wastewater systems, predominantly through urinary shedding and occasionally via other secretions. Arboviruses are less stable than non-enveloped viruses, yet their RNA can persist in wastewater for several days. However, because shedding is

generally low-level and short-lived for most arboviruses, wastewater surveillance remains technically demanding.

Experience with wastewater surveillance for arboviruses remains an emerging field, with most evidence derived from pilot studies, proof-of-concept investigations, or outbreak-related applications. Among the major arboviruses, dengue virus and zika virus currently have the strongest evidence for wastewater detection. In contrast, wastewater surveillance of chikungunya virus, West Nile virus, and Japanese encephalitis virus has been evaluated in only a limited number of studies. In contrast, published data are currently lacking for yellow fever and oropouche viruses. These gaps highlight that, although arboviral WWS has clear theoretical and practical potential, its application remains uneven across pathogens and requires further research, particularly for the less-studied zoonotic arboviruses[56-58].

3.3.11 *Vibrio cholerae*

Detection of *V. cholerae* in wastewater and surface water can provide early warning of outbreaks, identify high-risk areas, and guide WASH interventions. Infected individuals shed large quantities of the organism in faeces. The symptomatic cases excrete extremely high concentrations for approximately 1–2 weeks, while asymptomatic carriers shed at much lower levels and typically for a very short duration. The bacterium shows moderate persistence in wastewater and can survive for weeks in fresh and brackish waters. It also has a natural environmental reservoir, which complicates interpretation of wastewater signals.

World Health Organization (WHO) notes that WWS for *V. cholerae* is technically feasible using culture and molecular methods, but its routine public health application remains limited. Most experience to date comes from pilot and research studies rather than operational programs. Documented studies from India, Haiti, Uganda, and the African Great Lakes region demonstrate the feasibility of wastewater and environmental surveillance for monitoring *Vibrio cholerae*, while also highlighting important knowledge gaps that require further investigation before routine implementation[59-65].

3.3.12 *Salmonella typhi* and *paratyphi*

Salmonella enterica serovars typhi and *paratyphi* are Gram-negative, facultative intracellular bacteria that cause enteric fever in humans and are transmitted predominantly through fecally contaminated food and water. Following symptomatic or asymptomatic infection, these pathogens are shed mainly in faeces and, less frequently, in urine, with shedding beginning

within days of infection and usually lasting several weeks. However, a clinically important proportion of infected individuals (approximately 2–5%) develop chronic gallbladder carriage, resulting in intermittent, long-term release of bacteria into wastewater systems. In the environment, *S. typhi* and *S. paratyphi* are relatively fastidious organisms with limited persistence. They are highly sensitive to chlorine although they may remain detectable for days to weeks in untreated wastewater and surface waters. Their survival is influenced by temperature, disinfectants, and biological interactions. Wastewater surveillance has therefore been explored as a complementary population-level tool to address major gaps in clinical surveillance, particularly in endemic settings where blood-culture–based reporting is incomplete. At present, there is no established at-scale implementation of WWS for enteric fever, and the strength of evidence remains moderate to inadequate, with methodological standardization and interpretation frameworks still under development. Nonetheless, multiple applied research studies have demonstrated the feasibility of detecting *S. typhi* (and to a lesser extent *S. paratyphi*) in wastewater, and coordinated investigations are ongoing in India to evaluate its utility for estimating disease burden, monitoring antimicrobial resistance, and supporting vaccine and WASH prioritization[66-72].

3.4 DISEASE X

Disease X is a placeholder term introduced by the WHO to represent a future epidemic or pandemic caused by a currently unknown pathogen capable of infecting humans. The concept acknowledges that emerging infectious diseases particularly those of zoonotic origin continue to pose significant global health threats due to ecological disruption, increased human animal interface, urbanization, climate change, and rapid international travel. Most candidate pathogens for Disease X have been expected to be RNA viruses because of their high mutation rates, genetic recombination potential, and ability to adapt quickly to new hosts. Disease X therefore represents not a specific illness, but a strategic preparedness framework emphasizing anticipatory surveillance, One Health integration, and resilient public health systems to mitigate the impact of the next unknown pandemic threat.

Many public health agencies, scientists and researchers are exploring several methods to detect unknown or high-risk pathogens in sewage through targeted and untargeted approaches such as[73-76]:

a. Using metagenomic sequencing and next-generation genomic methods to screen wastewater for diverse viral and microbial genetic material, allowing detection of unusual or previously undetected pathogens that could represent Disease X–like threats. This involves casting a wide net beyond known viruses and tracking patterns of genetic divergence compared with baseline wastewater signals.

b. National and regional surveillance systems, such as sample networks in the United States (through the CDC's National Wastewater Surveillance System) and pilot programs in China, are incorporating "Disease X" as a conceptual priority target. These systems combine 24-hour composite sampling with genomic analysis to capture broad pathogen landscapes in urban and high-risk settings.

c. Research is focusing on strategic sensor placement and advanced sampling strategies so that signals of emerging pathogens can be detected earlier and localized more precisely, which is crucial for early intervention in a potential Disease X event.

d. International collaborative efforts aim to standardize protocols and develop tools for detecting virus families with pandemic potential spanning coronaviruses, filoviruses, arenaviruses, and other zoonotic threats thus preparing wastewater systems to flag unexpected signals.

e. Scientific reviews and global surveillance analyses emphasize that wastewater systems can be adapted for emerging pathogen detection by integrating sentinel sites (such as airport wastewater), genomic analysis, and real-time data sharing to provide early warning of unusual pathogen circulation.

3.5 MULTIPATHOGEN SURVEILLANCE APPROACHES

Where capacity permits, it is encouraged to adopt multipathogen WWS approaches using shared sampling and laboratory workflows. This improves efficiency, reduces costs, and strengthens preparedness for emerging threats. However, surveillance objectives, analytical methods, and interpretation frameworks must be clearly defined for each pathogen to ensure meaningful outputs[12].

3.6 REVIEW AND UPDATING OF PRIORITY PATHOGENS

Priority pathogen lists should be reviewed periodically to reflect emerging and re-emerging threats, changes in disease epidemiology, advances in laboratory methods, and evolving national priorities.

Table 1: Priority Pathogens and Surveillance Objectives

Pathogen	Primary Objective	Key Public Health Use
Poliovirus	Detection of silent and low-level transmission	Verification of eradication and outbreak response
SARS-CoV-2	Trend monitoring and early warning	Preparedness, response, and healthcare system planning
Influenza A and B	Seasonal trend monitoring	Pandemic preparedness and situational awareness
Monkeypox virus	Situational awareness of community transmission	Support for outbreak monitoring and response
Respiratory syncytial virus (RSV)	Monitoring seasonal circulation patterns	Health system preparedness, particularly for paediatric care
Measles virus	Detection of cryptic or undetected circulation	Support for elimination verification and outbreak risk assessment
Mumps virus	Exploratory detection of community circulation	Supplementary surveillance in elimination or low-incidence settings
Rubella virus	Detection of potential silent transmission	Support for elimination goals and congenital rubella syndrome prevention
Hepatitis A virus (HAV)	Detection of community-level transmission	Early warning for outbreaks and support for vaccination strategies
Hepatitis E virus (HEV)	Identification of endemic or outbreak-related circulation	Risk assessment and water safety management
Rotavirus	Monitoring population-level circulation	Assessment of vaccination impact and seasonal dynamics
Adenovirus	Exploratory assessment of population circulation	Supplementary epidemiological insight and method development
Arboviruses	Exploratory detection of community-level circulation	Supplementary situational awareness and outbreak risk assessment
Vibrio cholerae	Early outbreak signal detection	Targeted WASH interventions and rapid public health response
Salmonella typhi	Population-level trend estimation	Monitoring disease burden and vaccine impact
Disease X	Early detection and monitoring of unknown or emerging pathogens	Timely signals for outbreak preparedness, risk assessment, and informed public health response planning.

4. Selection of Sites for Wastewater Surveillance

Selection of sites for wastewater surveillance (WWS) is a critical step and should be aligned with clearly defined surveillance objectives. The sites should be chosen based on the epidemiology of the disease under surveillance, population dynamics, site accessibility and safety to allow regular sampling by field personnel without operational constraints.

4.1 TYPES OF SITES FOR WASTEWATER SURVEILLANCE

The sampling sites may include convergent sewer networks that feed into treatment facilities, storm drains, septic tanks or rivers and lakes where untreated sewage/wastewater is disposed of into surface waters. Additionally, surveillance can be focused on high footfall sites such as airports, flights, hospitals, schools, and prisons by monitoring outflowing sewage at a building or compound level.

4.1.1 Converging Sewer Networks

Sewer networks that collect wastewater from households are optimal locations for systematic wastewater surveillance. Sampling is preferably carried out at points where wastewater (containing human faecal material) from a well-defined catchment converges, such as the influent of a wastewater or sewage treatment plant (WWTP/STP), providing a representative sample of the contributing population.

In large urban sewer systems, the catchment may be divided into smaller surveillance units by selecting upstream sampling locations, such as pumping stations or major collector pipes. This approach allows each sampling site to represent a defined population (approximately 100,000–300,000 people). Epidemiological assessment of each catchment can then guide the selection of surveillance sites that best represent populations at increased risk of disease transmission[78-79]. A detailed sewage network map and clearly defined micro sewersheds will help in the selection of sub-sites that can be scaled up during peak outbreak periods to identify disease hotspots and scaled down during lean periods (**Figure 2**).

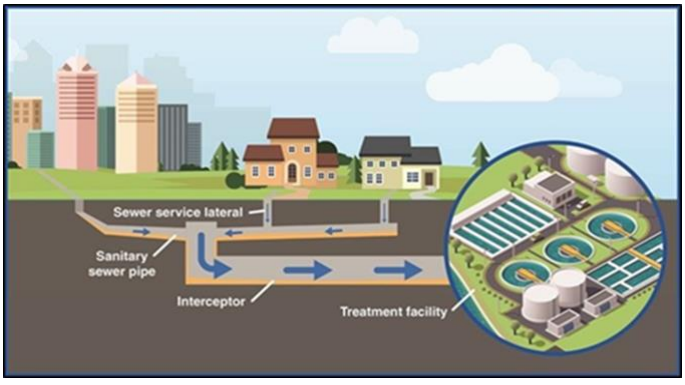


Figure 2: Example of a Converging Sewer Network

When identifying a sampling site, the surveillance programme should consult with both local sanitary engineers and epidemiological experts who can assist in evaluating sewer and wastewater systems in the area and provide information about the catchment populations.

4.1.2 Storm Drains and Open Canals

In settings where closed sewer networks are absent or limited, open canals or drainage channels may provide the only feasible option for wastewater surveillance. Prior to site selection, the drainage system should be thoroughly assessed and mapped with support from local sewer engineers to identify representative and operationally suitable sampling locations. A field assessment of the drainage network allows identifying any blockages or flowing obstructions that may prevent wastewater from parts of the catchment from reaching the selected sampling location. It also helps to detect potential inputs of toxic wastes that may affect wastewater quality and surveillance results.

When detailed sewer network maps are lacking, wastewater drainage routes can be mapped using Global Positioning System (GPS) coordinates. These data can be used to prepare "blue line maps" and, through suitable modelling approaches, can help estimate the population contributing to a given sampling location[79].

4.1.3 Open fields, latrines and septic tanks

Wastewater sampling is generally not recommended in areas where human waste is managed through individual latrines, septic tanks, or open fields without a connected sewer network, as these systems do not represent a well-defined catchment population. Similarly, sewage treatment facilities receiving septage from septic tanks or pit latrines are not preferred sampling locations because the wastewater originates from multiple, often unrelated, sources rather than a defined geographic catchment. Sites with low or intermittent wastewater flow should also be avoided, as sensitivity of the surveillance may be reduced owing to dilution and environmental degradation or inactivation of microbial targets[79].

4.1.4 STPs associated with Hospitals

Hospital on-site sewage treatment plants (STPs) are important sites for hospital-based wastewater surveillance due to the aggregation of patients, antimicrobial resistance, and emerging pathogens. Sampling wastewater from hospital STPs and proximate open drains

enhances the capacity of surveillance systems to detect and monitor pathogens of public health concern. The influent of the hospital STP is the optimal sampling location because it receives raw, untreated wastewater. Notably, appropriate biosafety precautions are essential for personnel involved during sampling owing to potentially high pathogen loads.

4.2 ACCESSIBILITY

Before establishing wastewater surveillance locations, practical factors such as site accessibility and transport requirements should be evaluated to ensure safe and uninterrupted sampling. Field teams may need to access the site on foot and remain there for a period of time to complete collection activities. Difficult-to-access locations can lead to inconsistent sampling, operational delays, or safety risks, ultimately affecting the quality of wastewater surveillance data. Sampling sites should ideally be selected away from heavy traffic, pedestrian disturbance, and industrial activities, while also providing suitable conditions for the deployment and operation of equipment such as autosamplers.

As sampling is typically conducted during early morning hours to coincide with peak wastewater flow (peak toilet usage), site access during this period should be verified. Locations that become inaccessible because of seasonal flooding or other environmental conditions are generally unsuitable for routine surveillance but may be used temporarily during outbreak[79].

After identifying areas of epidemiological interest (such as monitoring for SARS-CoV-2, poliovirus, or hepatitis viruses etc.), workshops and field assessments should be conducted to determine feasible and cost-effective sewage sampling points. Final site selection should be guided by a thorough risk assessment, epidemiological context, and the anticipated role of environmental surveillance within the broader disease monitoring system. Ongoing coordination with local authorities and facility operators is essential to ensure uninterrupted access, regulatory compliance, and smooth operational functioning.

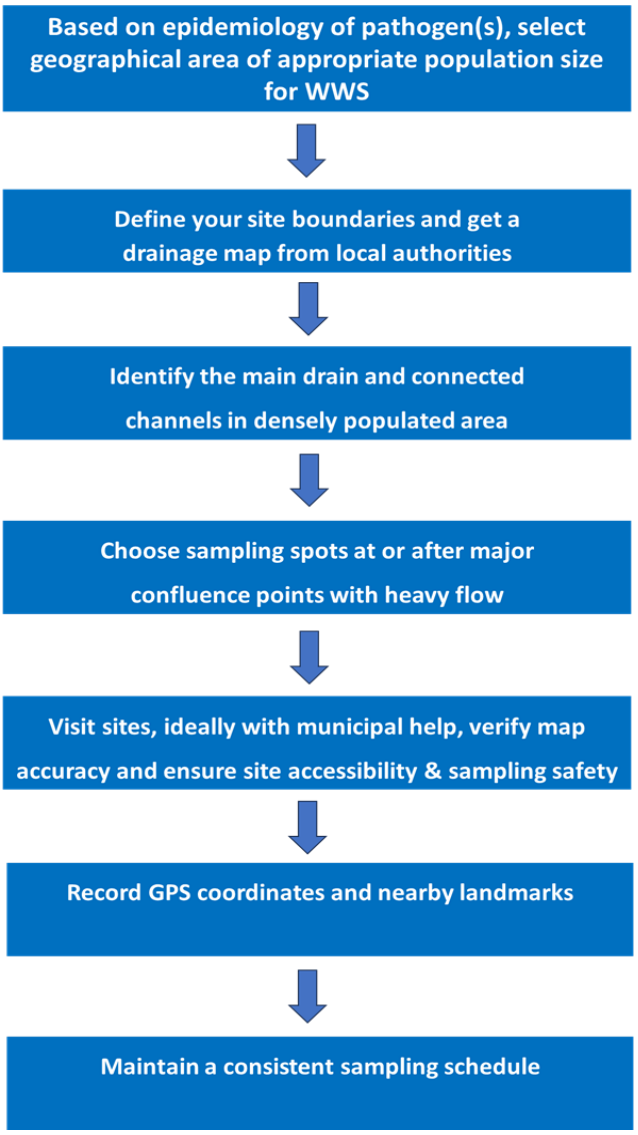
The total number of sampling sites may be adjusted (increased or decreased) in response to program requirements. Certain sites can be designated for long-term monitoring, allowing samples to be collected at regular intervals (e.g., weekly to once in every two months), in line with defined surveillance goals. In contrast, additional sites may be chosen for short periods to strengthen monitoring in areas experiencing elevated risk, including active outbreaks. Sampling at these temporary locations should be discontinued once the situation stabilizes, typically within a limited timeframe. Furthermore, some locations may

operate only during specific seasons, with the duration and timing of sample collection varying according to local conditions.

The exclusion criteria for wastewater sampling are:

i. Areas near heavy industries (industrial effluents may dilute or degrade viral RNA, affecting reliability)
ii. Any sites not practical for field sampling, such as the area around a manhole that does not offer enough space or safety for field personnel

Briefly, a site that is physically accessible and safe ensures consistent, reliable sampling, reduces risks to personnel, and improves the overall efficiency of viral wastewater surveillance programs. An overview of the key considerations and decision-making steps involved in selecting appropriate wastewater sampling sites is presented in **Flowchart 2.**



Flowchart 2: Overview of the Selection Process for Wastewater Sampling Sites

5. Collection of Wastewater Samples & Transport to the Laboratory

5.1 SAMPLE TYPE

Effective wastewater surveillance relies on the sample type as well as systematic and standardized sampling approaches to obtain representative samples that accurately reflect the catchment population. Ideally, samples should be collected at points upstream of any chemical addition to ensure that they accurately represent untreated influent. Microbes may be present in the dissolved phase, associated with suspended solids, or distributed between both fractions, depending on their biological characteristics. For wastewater surveillance, two common sample types are influent wastewater (untreated) and primary sludge.

5.1.1 Untreated wastewater influent: It is raw municipal sewage collected before treatment and is the commonly used sample matrix for wastewater surveillance. It primarily comprises domestic wastewater containing human fecal and urinary waste, along with contributions from commercial and institutional establishments. Depending on the characteristics of the sewerage network and the catchment area, untreated influent may also receive industrial effluents and varying amounts of storm water or groundwater through infiltration and inflow. Consequently, the physical and chemical characteristics of untreated influent may vary across locations and seasons, particularly during periods of heavy rainfall, and these factors should be considered when designing sampling strategies[80-81].

5.1.2 Primary sludge (primary settled solids): In a sewage treatment plant (STP), primary sludge is generated when suspended solids in raw sewage settle in the primary clarifier[82]. As many viruses and bacteria preferentially adsorb to solid particles, primary sludge generally contains higher concentrations of microbial pathogens than untreated wastewater influent, making it a highly sensitive matrix for wastewater surveillance [83-85]. For example, SARS-CoV-2 RNA has consistently been detected at higher concentrations in primary sludge than in untreated influent, thereby improving analytical sensitivity[84,86]. However, the complex sludge matrix contains substances that may inhibit nucleic acid extraction and PCR-based detection, requiring appropriate sample processing and inhibitor removal[86]. Sampling may also be influenced by plant design, accessibility of sludge collection points, and operational practices[83].

5.2 SAMPLE VOLUME

Wastewater samples are typically collected in volumes of 1 liter or 100 milliliter grab sludge sample (that will yield approximately 1-2 grams of sludge solid). Large sample volumes offer limited advantage due to the presence of higher concentrations of wastewater constituents and suspended solids, which may introduce PCR inhibitors and reduce the efficiency of nucleic acid detection[87-88].

5.3 SAMPLING FREQUENCY

The frequency of wastewater sampling should be guided by the purpose of surveillance and the pathogen under monitoring/of interest. For routine long-term monitoring of pathogen circulation, weekly to fortnightly sampling frequency is sufficient. In contrast, surveillance aimed at early detection or early warning may require more frequent sampling. Based on the epidemiological/surveillance signals, sampling frequency may be adjusted or intensified at specific sites.

5.4 SAMPLING METHODS

Selection of an appropriate sampling method should align with the surveillance objectives, target pathogens, available resources, and site-specific considerations, such as wastewater flow characteristics, operational feasibility, and accessibility. The most commonly used wastewater sampling methods include grab sampling, composite sampling, passive and robotic sampling, (**Figure 3**) which are described in the following sections.

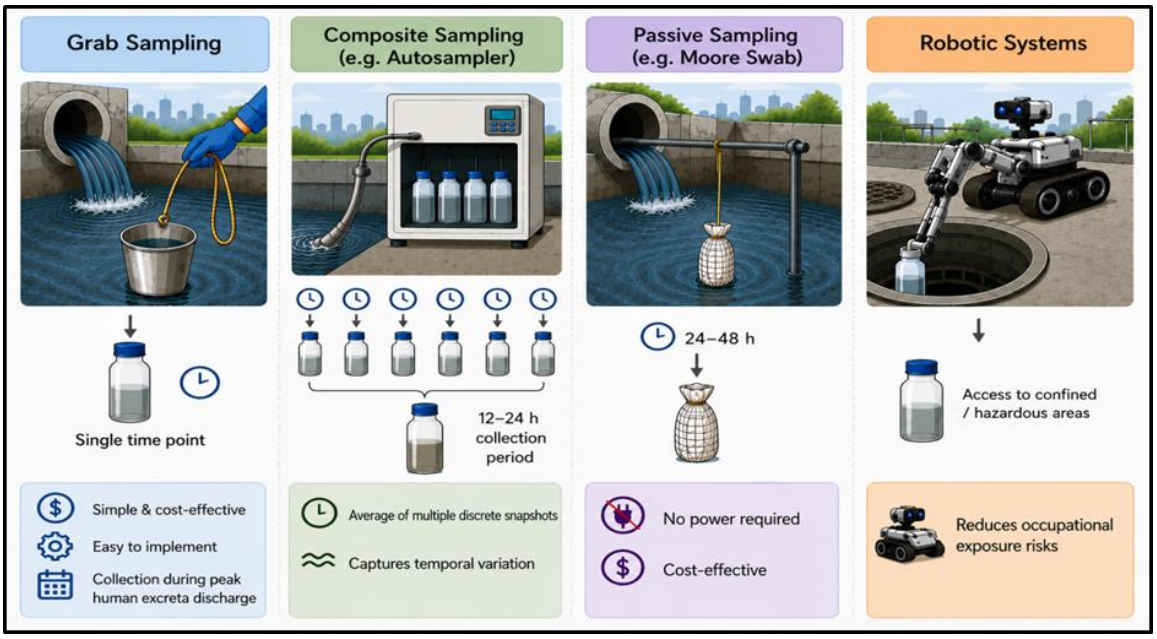


Figure 3: Different Types of Wastewater Sampling Methods

5.4.1 Grab Sampling

Grab sampling involves the collection of a single discrete wastewater sample at a specific time and location[83] (**Figure 4**). It is typically obtained during early morning hours (8 a.m. – 10 a.m.) to coincide with peak human excreta discharge, thereby increasing the likelihood of pathogen detection. Grab sample provides a "snapshot" for the catchment population. While grab sampling is simple, cost-effective and easy to implement logistically, it may be subject to temporal variability. Detailed procedure for the grab sampling and template for metadata collection can be found in **annexure I and IIC** respectively. For smaller catchment areas, where adequate wastewater mixing is unlikely, composite or passive sampling may be preferred over grab sampling, which is discussed in the later sections.



Figure 4: Pictorial Representation of Wastewater Sample Collection

5.4.2 Composite Sampling

Composite sampling, performed using conventional refrigerated autosamplers provides a representative sample over the collection period while preserving biological targets (such as viral nucleic acids and antibiotic resistance genes). It involves collecting multiple wastewater subsamples at predetermined intervals over a defined duration (typically 12-24 hours) and combining them into a single composite sample, thus avoiding the impact of diurnal wastewater fluctuations. In the absence of automated equipment, composite samples can also be prepared manually by collecting and pooling discrete samples at regular intervals, thus represent the average characteristics of multiple discrete "snapshots" collected over a defined period. Therefore, they provide a more representative measure of community-wide fecal inputs than grab samples because they account for temporal variations in wastewater composition[83,87-88].

Autosampling can be performed using time-weighted, flow-weighted, or continuous composite sampling strategies. In time-weighted sampling, equal volumes of wastewater are collected at predetermined intervals, whereas flow-weighted sampling adjusts the sample volume in proportion to wastewater flow rate, resulting in a more representative estimate of pathogen concentrations over the sampling period. Although flow-weighted sampling generally provides better representation of wastewater characteristics, it requires more sophisticated equipment and reliable flow measurements, making its implementation more technically demanding[89].

However, autosamplers are relatively expensive and require considerable logistical support, including installation, power supply, sample cooling, routine maintenance, and appropriate transport and site security (**annexure IIA**: Checklist for Deployment of Autosamplers and Robotic Systems; **annexure IIB**: Operation and Maintenance of Refrigerated Autosamplers and Robotic Systems; and **annexure IIC:** Template for Metadata Collection). Consequently, their use is generally limited to locations with adequate infrastructure and operational resources [83,87,90].

5.4.3 Passive Sampling

Passive sampling is an adsorption-based approach that uses adsorbent materials, such as electronegative membranes or Moore swabs, which are placed in wastewater streams for extended periods (typically 24–48 hours) to obtain time-integrated signals or composite samples[88,89]. A Moore swab consists of cotton gauze (approximately 120 cm × 15 cm) folded multiple times into a thick 8-ply pad and securely tied with nylon fishing line or string[91]. This method has gained attention as a sensitive and a cost-effective alternative, particularly in resource-constrained or hard-to-access settings, or where installation of autosamplers is not feasible. Passive samplers do not require power or complex equipment. Additionally, passive sampling can improve pathogen detection sensitivity while reducing the need for frequent site visits.

Three types of passive samplers were evaluated including electronegative membrane filters, cotton buds, and medical gauze for SARS-CoV-2 wastewater surveillance as alternatives to conventional autosamplers and grab sampling[92]. Among these, electronegative membrane filters were identified as one of the most effective and practical options.

Passive samplers generally provide qualitative or semi-quantitative data. Interpretation of the material accumulated on these samplers is influenced by several variables, including wastewater flow conditions, adsorption characteristics, deployment duration, and analytical recovery[89].

5.4.4 Robotic Sampling

Robotic systems technologies have the potential to address limitations of manual grab sampling by facilitating access to confined or hazardous sewer environments and reduce occupational exposure risks (**annexure IIA**: Checklist for deployment of autosamplers and robotic systems; **annexure IIB**: Operation and Maintenance of Refrigerated Autosamplers and Robotic Systems; and **annexure IIC:** Template for metadata collection). In India, robotic sewer inspection platforms developed by Fluid Robotics have been piloted in cities such as Mumbai and Pune for in-pipe sewer sampling applications, pollution mapping and COVID-19 RNA detection (Pune Knowledge Cluster[93] (https://www.pkc.org.in/explore-projects/health/waste-water-surveillance/), Fluid Robotics Pvt. Ltd.). In addition, Genrobotics (Bandicoot) and Solinas systems are used for inspection and site preparation in open drains and manholes (https://www.genrobotics.com/ and https://solinas.in/; **annexure IID:** Case Studies of Indian Robotic Pilots).

5.5 TRANSPORT TO THE LABORATORY

Transport the wastewater samples to the laboratory as soon as possible to minimize degradation of the samples (particularly nucleic acids), while maintaining the cold chain (2-8˚C) throughout transport.

6. Laboratory Testing Procedure for Viral Pathogens in Wastewater

Wastewater surveillance (WWS) for viral pathogens has emerged as a valuable public health tool and is now being implemented across diverse geographical and epidemiological settings at national and sub-national levels. While the underlying principles of WWS are broadly consistent, laboratory methodologies vary depending on surveillance objectives, available infrastructure, technical capacity, and local disease dynamics[94]. The selection and design of laboratory workflows are influenced by factors such as resource availability, biosafety considerations, analytical throughput, target pathogen characteristics, and the intended use of surveillance data (e.g., early warning, trend monitoring, or outbreak response).

Despite this methodological diversity, laboratory testing for viral pathogens in wastewater generally follows a common analytical framework. No single wastewater molecular testing workflow is universally optimal for all pathogens or surveillance objectives, as methods often need to be adapted based on the target organism and monitoring purpose. This typically includes standardized procedures for sample storage and transport, sample inactivation and homogenization, concentration of viral particles or nucleic acids, extraction of viral RNA or DNA, and molecular detection using validated assays. Each step plays a critical role in ensuring data quality, sensitivity, and comparability across time and locations. The recommended laboratory procedures for wastewater-based detection of pathogens generally comprise the following key components: sample storage, inactivation, homogenization, concentration, nucleic acid extraction, and molecular detection (**Figure 5**).

Wastewater is a potentially infectious material, and its concentration procedures may generate bioaerosols. Appropriate risk assessment and mitigation measures should be applied prior to laboratory work. Detailed biosafety precautions to be taken during the entire process of wastewater collection, transport and testing are described in Chapter 10.

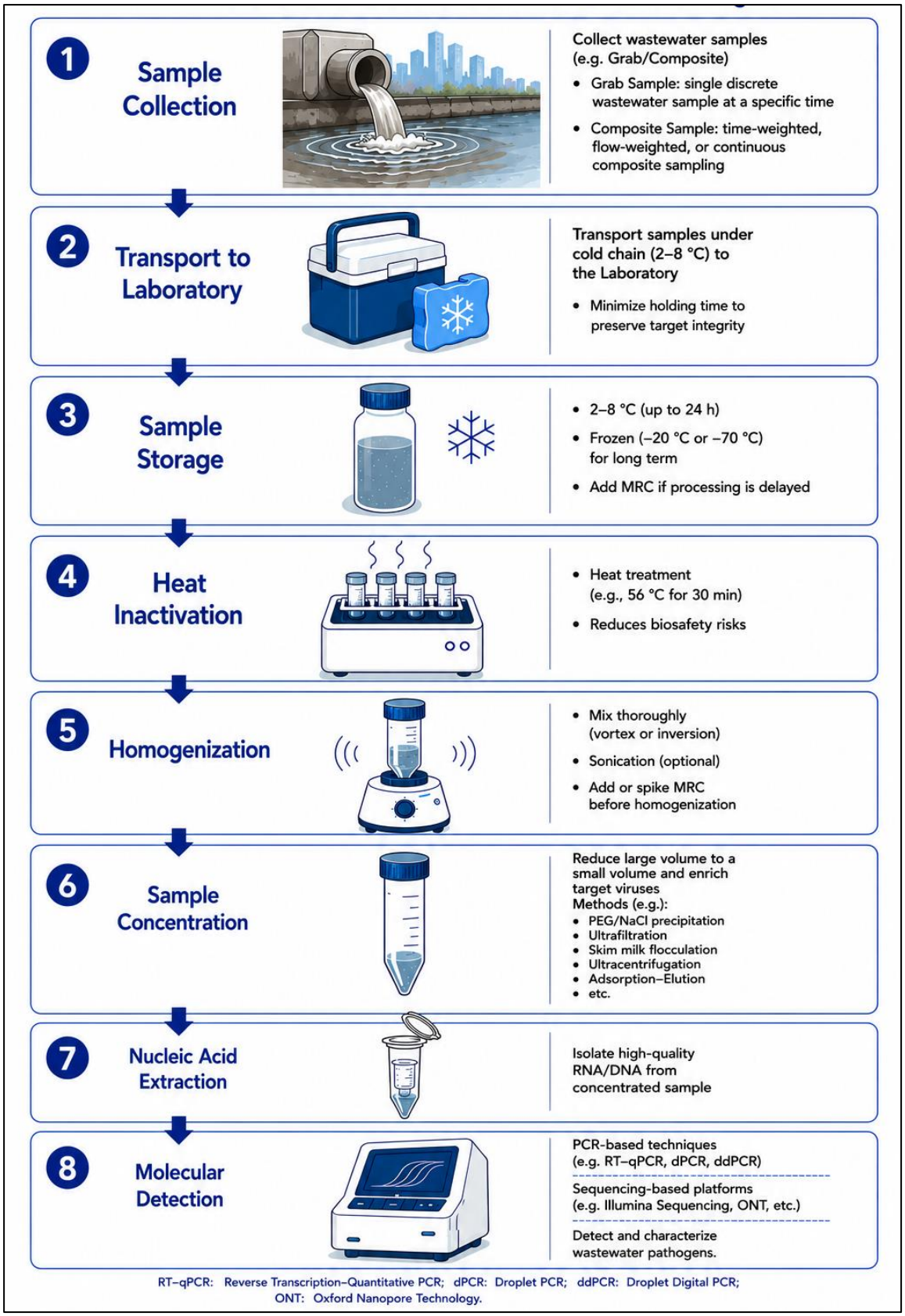


Figure 5: Workflow for Wastewater-Based Detection of Pathogens

6.1 SAMPLE STORAGE

Upon receipt in the laboratory, samples should be processed as soon as possible, till then samples can be stored at 2-8 °C (up to 24 hours) or frozen at -20°C or -70°C for long term use to minimize viral nucleic acid degradation. If sample processing is not possible on the same day, a matrix recovery control (MRC) should be added to the sample [87].

6.2 HEAT INACTIVATION

To reduce biosafety risks to laboratory personnel and the environment during wastewater processing, heat inactivation may be performed. Inactivation at 56°C for 30 minutes has been shown to result in minimal impact on RNA quantification[95].

6.3 HOMOGENIZATION

Wastewater samples should be thoroughly mixed prior to further processing. Mixing may be achieved mechanically or by repeated inversion in the case of liquid samples. Additional homogenization methods, such as sonication, may be employed to disrupt wastewater solids and facilitate the release of viral particles. A matrix recovery control (MRC) should be added or spiked prior to the homogenization step. The role and application of MRC are described in Section 7.9 of this chapter.

6.4 SAMPLE CONCENTRATION

Concentration refers to the process of reducing a large volume of wastewater to a much smaller volume while retaining and enriching the target viruses. In wastewater surveillance, pathogens are typically present at low concentrations and are diluted within complex matrices containing organic matter, chemicals, and inhibitory substances. Concentration is therefore a critical pre-analytical step that increases the likelihood of detecting viral RNA or DNA and improves the sensitivity, reliability, and reproducibility of downstream molecular assays such as qPCR, RT-qPCR or sequencing.

6.4.1 Importance of Concentration in Wastewater Surveillance

Wastewater samples often contain pathogens at concentrations below the direct detection limits of molecular methods. By concentrating samples, the effective target copy number per reaction is increased, enabling:

a. Improved detection of low-prevalence infections in the community
b. Earlier identification of increasing transmission trends
c. More consistent quantification across sampling locations and time points

Without an efficient concentration step, false negatives may occur, particularly during periods of low disease incidence.

6.4.2 Virus Concentration Methods

6.4.2.1 Ultrafiltration (UF)

Ultrafiltration is a size-based, pressure-driven separation technique that employs semi-permeable membranes, typically characterized by molecular weight cut-offs (MWCOs) ranging from approximately 10 to 150 kDa. The pressure allows passage of water and smaller molecules through the membrane, while retaining the larger particles such as bacteria, viruses, viral particles, etc.

Ultrafiltration may experience pore clogging when treating turbid samples; however, tangential flow ultrafiltration (TFUF) reduces fouling by directing water parallel to the membrane surface, making it suitable for high-turbidity samples. In wastewater surveillance, ultrafiltration is an important concentration technique used to recover or enrich low-abundance pathogens, including SARS-CoV-2 from raw sewage. Some commercially and commonly used centrifugal ultrafilters (e.g., Amicon and Centricon filters) are available for the concentration of wastewater samples. Ultrafiltration has been reported for concentrating wastewater-associated viruses, including SARS-CoV-2 and enteric viruses such as adenovirus, norovirus, rotavirus, and poliovirus[96-97].

6.4.2.2 Polyethylene glycol (PEG) precipitation

Polyethylene glycol (PEG) precipitation is a well-established conventional method for the concentration of viruses. Originally developed for the purification of arboviruses and bacteriophages, it has since been adapted for the concentration of a broad range of RNA

viruses, including Hepatitis E virus, influenza virus, respiratory syncytial virus and coronaviruses[98].

PEG is a non-ionic, water-soluble polymer that facilitates virus concentration through a volume exclusion mechanism, typically in the presence of sodium chloride. In this process, PEG sequesters water molecules from the surrounding environment, thus reducing the hydration layer around viral particles and promotes their aggregation and subsequent precipitation. This method preserves viral envelopes under low-temperature and high-salt conditions. The concentrated viral particles are recovered by centrifugation and resuspended in a smaller volume for downstream analysis. The PEG method has been widely used due to its simplicity, rapid processing, cost-effectiveness, preservation of viral integrity, and particularly suitable for large-volume samples. Its efficiency may vary depending on virus type and environmental conditions, and therefore it is often selected based on surveillance objectives and sample characteristics.

This method has been widely used for concentration of wastewater-associated viral pathogens, particularly SARS-CoV-2 and enteric viruses such as poliovirus, rotavirus and hepatitis A/E viruses. Its application for other viruses such as influenza virus, respiratory syncytial virus, etc. is emerging and requires further method validation and standardization[99-102].

6.4.2.3 Ultracentrifugation (Solid/Pellet-based concentration)

Ultracentrifugation concentrates viruses based on differences in size and density by applying centrifugal forces (typically up to or exceeding 100,000 × g) to wastewater samples. Viral particles are sedimented into a pellet at the bottom of the centrifuge tube, while lighter components and suspended debris remain in the supernatant. The resulting pellet with enriched viral particles is resuspended in a smaller volume for downstream analyses. This method enables recovery of relatively intact viral particles without the use of chemical precipitants. Although ultrafiltration is an effective virus concentration method, its performance can be limited by clogging of the membrane pores when processing highly turbid samples. Tangential flow ultrafiltration (TFUF) overcomes this issue by directing the sample flow parallel to the membrane surface, thereby reducing membrane fouling and improving filtration efficiency for high-turbidity wastewater [97].

Ultracentrifugation is particularly useful for recovering relatively intact viral particles for downstream analyses requiring preservation of viral structure including enveloped viruses

(e.g., SARS-CoV-2 and influenza viruses) and non-enveloped viruses (e.g., hepatitis E virus)[101,103]. Although it provides high purification efficiency, its application in routine wastewater surveillance remains limited because of specialized equipment requirements and lower scalability compared with methods such as PEG precipitation, ultrafiltration, and adsorption–elution approaches.

6.4.2.4 Electronegative Membrane Filtration (Adsorption–Elution)

Electronegative membrane filtration, also known as the virus adsorption–elution (VIRADEL) method, is a virus concentration technique based on the adsorption of viral particles onto negatively charged membrane filters during filtration. Prior to filtration, wastewater samples are typically conditioned by adjusting pH and adding multivalent cations (such as magnesium or aluminium ions). These ions facilitate virus attachment to the membrane by reducing surface charge repulsion and enhancing binding between viral particles and the filter[98].
After filtration, retained viral particles are recovered either by elution or by direct processing of the membrane for nucleic acid extraction.

This method is commonly used for non-enveloped viruses, such as poliovirus and hepatitis A virus, as they are more stable in wastewater and exhibit physicochemical properties that favor adsorption to membrane surfaces. Electronegative membrane filtration can also be applied to enveloped viruses, including SARS-CoV-2, although recovery efficiency may vary depending on virus characteristics, wastewater composition, and sample conditioning conditions.

6.4.2.5 Skim milk flocculation (SMF)

The skim milk flocculation (SMF) method concentrates viruses through the formation of protein flocs under acidic conditions. Acidification induces precipitation of skim milk proteins, which adsorb viral particles present in wastewater. The resulting flocs, containing virus–protein complexes, are recovered by sedimentation or low-speed centrifugation. This method is suitable for resource-limited settings due to its low cost and minimal equipment requirements.

SMF is used for concentrating non-enveloped enteric viruses, including poliovirus, norovirus, etc. from environmental and wastewater. Although its application has been explored for enveloped viruses, such as SARS-CoV-2, recovery efficiency may vary depending on viral properties and wastewater matrix characteristics[104-105].

6.4.2.6 Aluminum Flocculation Method

Aluminum flocculation is a chemical precipitation method used for concentrating viruses from large volumes of water or wastewater. The technique involves adding an aluminum salt (commonly aluminum chloride ($AlCl_3$)), which undergoes hydrolysis under appropriate pH conditions to form aluminum hydroxide [$Al(OH)_3$] flocs. Viral particles are concentrated through adsorption onto these flocs. The flocs are then recovered by sedimentation or low-speed centrifugation, and the associated viruses are subsequently eluted for downstream processing. The recovered viral material is resuspended and processed for further analysis, including nucleic acid extraction and RT-qPCR. This method has been applied for rotavirus, adenovirus and has also been evaluated for SARS-CoV-2, respiratory syncytial virus from wastewater matrices[106-108].

6.4.2.7 Affinity-Capture Magnetic Hydrogel Particle Method

Affinity-capture magnetic hydrogel particles are magnetic beads embedded within a hydrophilic hydrogel matrix functionalized with ligands such as dyes or peptides that selectively bind viral particles with high affinity. When introduced into wastewater samples, viral particles attach to these particles and are subsequently separated and concentrated using an external magnetic field from complex environmental matrices. This approach improves assay sensitivity by enriching viral targets and reducing inhibitors, thereby enhancing downstream detection methods such as RT-qPCR and sequencing[109-110].

It is also compatible with high-throughput and automated processing workflows. However, capture efficiency may vary depending on viral characteristics, including presence of envelope and surface proteins, as well as ligand specificity. This method has been applied for SARS-CoV-2 and has potential as an emerging concentration approach for other viruses, including influenza virus and RSV.

6.4.2.8 Two-Phase Separation (PEG-dextran) Method

The PEG–dextran two-phase method was adapted for the concentration of poliovirus from wastewater and was subsequently adopted by the WHO as the standard method for poliovirus environmental surveillance under the GPEI[111].

This method is based on the formation of an aqueous biphasic system using two polymers, i.e. polyethylene glycol (PEG) and dextran to concentrate viruses from wastewater. The wastewater sample is firstly clarified by centrifugation to remove large suspended solids and the pellet is retained as viruses may adsorb to particulate matter. The clarified wastewater

is then mixed with PEG and dextran, left in a separation funnel and incubated typically at 4°C to allow phase separation. This forms two immiscible distinct phases: an upper PEG-rich phase and a denser lower dextran-rich phase. Under appropriate salt and pH conditions, viral particles preferentially partition into the lower dextran-rich phase and the interphase owing to differences in their physicochemical properties, including size, surface charge, and hydrophobicity. Following phase separation, the virus-rich lower phase and interphase are collected and combined with the initial retained pellet for further use.

The procedure is relatively labor-intensive, requires overnight phase separation, and involves multiple processing steps. Consequently, its selection depends on the surveillance objectives, laboratory capacity, and target pathogens. The PEG–dextran two-phase method has been widely applied for the concentration of non-enveloped poliovirus and has also been used for other viruses such as SARS-CoV-2 (enveloped virus) in wastewater. Nevertheless, evidence indicates that its recovery efficiency for enveloped viruses such as SARS-CoV-2 may be lower than that achieved by alternative concentration method such as PEG-NaCl[112].

6.4.3 Factors Influencing the Selection of a Virus Concentration Method

The selection of an appropriate wastewater concentration method should be guided by technical, operational, and epidemiological considerations including[95]:

i. **Target Pathogen Characteristics:** Viral structure (enveloped vs. non-enveloped), genome type (RNA or DNA), size, and surface charge influence recovery efficiency. Methods optimized for one virus may perform poorly for others.

ii. **Wastewater Matrix and Sample Type:** The composition of wastewater (raw sewage, influent, open drains, or treated effluent) affects method performance. High solids content, organic load and the presence of PCR inhibitors must be considered.

iii. **Recovery Efficiency and Sensitivity:** The method should consistently achieve high and reproducible recovery of viral nucleic acids, particularly at low concentrations, to support trend analysis and early warning.

iv. **Compatibility with Downstream Analysis:** Concentration techniques should not introduce substances that inhibit nucleic acid extraction, PCR amplification, or sequencing.

v. **Sample Volume and Throughput:** Methods should be scalable to process large volumes where required and suitable for routine surveillance with reasonable turnaround time (TAT).

vi. **Operational Feasibility:** Considerations include cost, availability of reagents, equipment requirements, biosafety, technical expertise, and suitability for implementation in resource-limited settings.

vii. **Standardization and Comparability:** Preference should be given to methods that are well-documented, widely used, and amenable to harmonization across laboratories to allow inter-site and temporal comparisons.

6.5 NUCLEIC ACID EXTRACTION

Viral nucleic acid (RNA/DNA) needs to be extracted from the wastewater sample/concentrate before it is subjected to the molecular detection, quantification and characterization. Wastewater is a complex environmental matrix that may contain substances known to interfere or inhibit viral detection and quantification. Selection of an extraction method may depend on factors such as wastewater matrix characteristics, cost considerations, availability, and compatibility with existing laboratory equipment or required laboratory instrumentation. Extraction procedures may be conducted using automated platforms (e.g., robotic extraction systems) or manual methods, depending on the laboratory capacity.

Nucleic acid (RNA or DNA) can be extracted using commercially available kits specifically designed for environmental or wastewater samples that minimize the effect of PCR inhibitors and help to maintain nucleic acid integrity. The extracted RNA or DNA needs to be aliquoted into separate vials prior to storage at -70°C or lower to limit repeated freeze–thaw events and reduce the risk of nucleic acid degradation.

Examples of commercially available extraction kits suitable for wastewater or environmental samples analysis are provided in **Table 2**.

Table 2: Commercially Available Extraction Kits Suitable for Wastewater or Environmental Samples Testing

S. No.	Kit Name	Extraction Basis	Manufacturer
1	Wizard® Enviro TNA Kit	Vacuum capture / spin	Promega
2	DNeasy PowerSoil Pro Kit	Mechanical & chemical lysis followed by silica membrane–based nucleic acid purification.	Qiagen
3	AllPrepPowerViralDNA/RNAKit	Spin-column	Qiagen
4	MagMAX™ Wastewater Ultra Nucleic Acid Isolation Kit	Magnetic bead	Thermo Fisher Scientific
5	MagMAX™ Wastewater Ultra Nucleic Acid Isolation Kit with Virus Enrichment	Viral pre-enrichment + extraction	Thermo Fisher Scientific

This list is not exhaustive and does not constitute endorsement of any specific product.

6.6 DETECTION AND QUANTIFICATION OF VIRAL NUCLEIC ACID

Quantification of viral genetic material in wastewater enables monitoring trend of a pathogen over time in a specific geographical location. Detection and quantification of viral nucleic acid is commonly performed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR), digital PCR (dPCR) or Droplet Digital PCR (ddPCR). Selection of PCR method is based on surveillance objectives, target concentration levels, available equipment, and required sensitivity.

Selection of gene targets and assay kits may vary depending on the pathogens of interest (here viruses), availability of real time PCR machine compatibility with the selected assay, and prior laboratory experience. In addition to standardized target gene assays, user-designed primer and probe sets may be employed to detect specific genomic regions of interest.

6.6.1 Considerations for Planning Nucleic Acid Detection and Quantification Assays[113-115]

i. Select highly conserved genomic regions to minimize false negative results caused by viral mutations.

ii. Choose short amplicons (typically 70–150 base pairs) and avoid genomic regions with extensive secondary structure.

iii. Select target regions that are relatively resistant to degradation during transport, storage, and processing.

iv. Prefer genomic targets with high copy numbers (e.g., *N* gene, *M* gene, or 5′UTR, where applicable) that are suitable for multiplex assays.

v. Optimize primers/probe to maximize amplification efficiency while minimizing secondary structure formation and primer-dimer interactions.

vi. Design assays according to the viral genome type (RNA or DNA) and its replication strategy.

vii. For RNA viruses, ensure prompt sample stabilization (e.g., RNA preservatives or maintenance of the cold chain).

viii. Consider that wastewater contains organic matter, chemical contaminants, and microbial communities that may inhibit nucleic acid amplification.

ix. Include internal amplification controls and incorporate strategies to assess and mitigate PCR inhibition.

x. Define limit of detection (LOD) and limit of quantification (LOQ) based on the expected viral concentrations in wastewater and assay performance.

xi. Consider digital PCR for low-abundance targets or where inhibition is likely.

xii. Ensure that assays are highly specific to the target virus and minimize cross-reactivity with related viruses or non-target environmental nucleic acids.

xiii. Include appropriate quality control measures (e.g., extraction controls, recovery controls, and inhibition controls) to enable accurate interpretation of quantitative results (refer to Section 6.7 of this chapter). Apply standardized reporting metrics to facilitate comparisons across sampling sites and over time.

6.6.2 PCR-Based Detection Platforms for Wastewater Surveillance

a) RT-qPCR

RT-qPCR continues to be regarded as the benchmark molecular technique for simple, fast, and cost-effective measurement of viral RNA/DNA. It is the most common and recommended method for target-specific quantification in wastewater due to its sensitivity and scalability. It requires standard curves (based on serially diluted positive control) for absolute quantification. Representative potential RT-qPCR targets used for the detection and quantification of viral pathogens in wastewater surveillance are summarized in **Table 3**.

In wastewater-based monitoring, RT-qPCR performance can be compromised by inhibitory substances in the sample (wastewater) matrix, potentially reducing detection sensitivity or lead to false-negative results. Therefore, inhibition assessments and appropriate controls are critical to ensure accurate detection and quantification of viral targets (refer Section 6.7 of this chapter).

b) Digital PCR (dPCR) and Droplet Digital PCR (ddPCR)

Digital PCR (dPCR) and droplet digital PCR (ddPCR) are increasingly used as alternative molecular platforms for the detection of viral variants and the absolute quantification of viral nucleic acids in complex environmental matrices such as wastewater. Unlike conventional real-time quantitative PCR these techniques determine target copy numbers using Poisson statistics and do not require standard curves for quantification. They are particularly suitable for low-abundance targets and inhibitor-rich samples[116-117]. Consequently, dPCR-based methods are valuable for early outbreak detection, monitoring changes in viral load, and supporting wastewater-based public health surveillance. However, despite these advantages, their routine implementation is often constrained by higher instrumentation and operational costs[89].

Droplet Digital PCR (ddPCR) is a subset of dPCR and is increasingly applied in wastewater-based epidemiology due to its high analytical sensitivity and ability to provide accurate absolute quantification of viral nucleic acids. In ddPCR, the PCR reaction mixture is partitioned into thousands of nanoliter-sized water-in-oil droplets, with each droplet functioning as an individual amplification compartment. Following PCR amplification, droplets are analyzed for fluorescence and classified as either target-positive or negative. Poisson statistics allow the absolute quantification of target molecules by determining their

concentration from the distribution of positive and negative droplets. The partitioning process also minimizes the influence of PCR inhibitors commonly present in wastewater and other environmental samples, thereby improving detection sensitivity and quantification reliability compared with conventional RT-qPCR[116].

Table 3: Potential Viral RT-qPCR Targets for Wastewater Surveillance

Virus	RT-qPCR Gene Targets[#] (WWS-adapted assays/ Clinical Studies)
Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)[118-120]	*N* gene (*N1*/*N2*), *E* gene
Poliovirus (PV)[121-123]	*VP1* (viral protein) gene, 5′UTR (5' untranslated region)
Influenza A virus (IAV)[124-126]	Matrix (*M*) gene, *HA* gene (subtype-specific)
Influenza B virus (IBV)[124-126]	Matrix (*M*) gene or *NP* gene or *NS* gene, *HA* gene (specific for influenza B lineages)
Respiratory Syncytial Virus (RSV)[108,126-127]	*N* gene, *M* gene
Hepatitis A virus (HAV)[101,128-130]	5′UTR (5′-untranslated region)
Hepatitis E virus (HEV)[101,128-130]	ORF (ORF2/ORF3 overlapping region)
Measles virus (MeV)[38,131-133]	*N* gene
Rubella virus (RuV)[134]	*E1* gene region (envelope glycoprotein - based on RT-PCR assay)
Rotavirus (RV)[135-136]	NSP3 (non-structural protein 3) [VP7 & VP4: RT-PCR genotyping assays]
Human Adenovirus (HAdV)[137]	Hexon gene
Monkeypox virus (MPXV)[28,138-139]	G2R, F3L

#The listed targets represent commonly reported and are not intended to be an exhaustive compilation of all available molecular targets. Some of the targets are from clinical molecular diagnostic assays and have been applied or adapted for wastewater-based surveillance (WWS). Additional reported targets may require further analytical validation, inter-laboratory evaluation, and standardization before routine application in wastewater surveillance.

6.7 STANDARDS AND CONTROLS

Standards are used to generate calibration curves that allow accurate quantification of viral nucleic acid and ensure assay consistency across runs. Controls (such as positive, negative, extraction, and inhibition controls) verify that sample processing, amplification, and detection are functioning correctly and help identify contamination, assay failure, or PCR inhibition. Together, standards and controls ensure accuracy, reliability, and comparability of viral wastewater surveillance results which are explained below.

a) **No template control (NTC):** No template control (molecular grade water) should be incorporated in each run to monitor contamination of the PCR reagents. An NTC reaction contains all real-time PCR components except the template. Detection of a positive signal in an NTC reaction indicates the presence of contaminating nucleic acids.

b) **Extraction Control:** It monitors the effectiveness of the extraction process and serves as an indicator of the co-purification of nucleic acid, thus validating the accuracy of the overall test.

c) **Positive Control (PC)**: It must be run during each assay to confirm reaction efficiency. For quantification, the gene copies per µl (g.c./µl) of the tested sample can be calculated using the standard curve obtained by testing positive controls of known concentration (or known copy number). For quantitative real time PCR, the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines should be followed[113,140].

d) **Calibrators:** Appropriate DNA or RNA calibrators can be included alongside each batch of samples for every PCR run to ensure assay performance and consistency. A calibrator control is a reference standard with a known target concentration used to normalize and compare results across different runs or batches. The calibrator helps convert Ct values into quantitative data, corrects for run-to-run variability, and ensures consistency in viral load estimation over time. This is especially important in wastewater surveillance, where trends and comparisons between samples are critical.

e) **Technical Replicates of Samples**: Wastewater samples should be analyzed in technical replicates (preferably in triplicates) to account for variability inherent to environmental matrices. Where variation is observed among replicates, the lowest

cycle threshold (Ct) value, corresponding to the highest estimated concentration, may be used for analysis.

f) **Inhibition Assessment:** Wastewater matrices contain a wide range of substances, including organic matter, chemicals, and microbial by-products that can interfere with nucleic acid extraction, reverse transcription, and amplification reactions. Systematic assessment of inhibition can be done using internal amplification controls or exogenous nucleic acid spikes and is essential to ensure reliability of viral quantification data. Inhibition may be identified through delayed or suppressed amplification signals relative to control reactions.

g) **Normalization Controls:** Normalization is the process of adjusting measured viral concentrations to account for technical and sample-related variability, including differences in wastewater dilution, flow rate, sample processing efficiency, nucleic acid recovery, and fluctuations in the population contributing to the sewer network. Appropriate normalization improves data quality, enables meaningful comparisons across sampling sites and time points, and strengthens confidence in trend interpretation[95].

The importance of normalization includes:

i. **Reducing technical variabilit**y: Normalize using endogenous or exogenous reference controls to account for differences in viral recovery, nucleic acid extraction efficiency, and PCR performance.

ii. **Improving quantitative accuracy:** Normalization minimizes technical variation and provides more reliable estimates of viral RNA or DNA concentrations.

iii. **Enhancing data comparability:** Normalized data allow comparisons across wastewater samples, surveillance locations, and sampling periods by reducing the influence of non-biological variability (or technical noise).

Common normalization approaches include:

i. **Flow-based normalization:** Adjust viral concentrations using wastewater flow rates to compensate for dilution caused by rainfall, groundwater infiltration, or fluctuations in sewage volume

ii. **Fecal biomarker normalization**: Use human fecal indicators such as Pepper Mild Mottle Virus (PMMoV) or crAssphage to normalize viral concentrations to the human fecal load, thereby improving comparisons between samples and correlation with infection trends[95,141].

iii. **Exogenous spike-in controls:** Add a known quantity of a non-target virus before nucleic acid extraction to evaluate viral recovery and monitor sample processing efficiency. Murine Hepatitis Virus (MHV) is commonly used as a process control in SARS-CoV-2 wastewater surveillance because it has physicochemical characteristics similar to those of SARS-CoV-2[142].

h) Matrix Recovery Control (MRC) in Wastewater Viral Surveillance

Wastewater is a complex and highly variable matrix, and significant losses of viral particles or nucleic acids may occur during sample concentration, extraction, and purification steps. A matrix recovery control (MRC) is therefore essential for evaluating the efficiency and reliability of wastewater-based viral surveillance workflows[95].

Matrix recovery controls consist of a known quantity of an exogenous surrogate virus or nucleic acid that is added or spiked into the wastewater sample prior to processing. This control is used to assess losses occurring across concentration, extraction, and detection steps. The recovery of the control is measured alongside the target virus to estimate overall process efficiency. This approach helps identify samples with low recovery that may affect data quality and interpretation.

Inclusion of MRCs improves data comparability across samples, sites, and time points by accounting for variability in sample processing efficiency. Recovery efficiency thresholds should be predefined, and samples that do not meet acceptance criteria should be flagged, reprocessed where possible, or interpreted with caution. For

minimum reporting requirements, the identity, concentration, and point of addition of the recovery control should be documented, along with calculated recovery efficiency. Common matrix recovery controls used for wastewater studies for enveloped viruses (used for targets such as SARS-CoV-2 and influenza viruses) include Bovine coronavirus (BCoV), Murine hepatitis virus (MHV), Bacteriophage Φ6 (enveloped RNA phage) and Bovine respiratory syncytial virus and for non-enveloped viruses (used for targets such as norovirus, adenovirus, or enteroviruses) include Mengovirus, Murine norovirus (MNV) and MS2 bacteriophage[95-96].

7. Laboratory Testing Procedure for Antimicrobial Resistance (AMR) Genes in Wastewater

7.1 IMPORTANCE OF AMR SURVEILLANCE IN WASTEWATER

Antimicrobial resistance (AMR) is the ability of microorganisms to become less susceptible to antimicrobial agents, making infections difficult to treat. It is recognized as one of the top ten significant global public health threats worldwide.

Wastewater surveillance (WWS) has emerged as a technically and operationally feasible approach for monitoring AMR, with some studies showing good agreement between the prevalence and proportions of AMR pathogens detected in wastewater and those observed through clinical surveillance. However, most available evidence comes from pilot and proof-of-concept studies, and standardized methodologies have not yet been established[143-150]. At present, there are no documented examples of routine wastewater surveillance for AMR that have consistently informed public health actions or providing sufficient evidence to support programme implementation or cost-benefit analyses. As a result, the application of WWS for AMR pathogens and antimicrobial resistance genes (ARGs) in public health practice remains largely conceptual rather than operationally established. Nevertheless, based on emerging evidence, considering primary research and systematic reviews, WWS for the detection of AMR has the potential to provide information on the following aspects[151-153]:

i. Monitoring long-term population level AMR dynamics
ii. Demonstrating an estimate of overall resistance burden
iii. Estimating the baseline ARG concentrations at hospital and community levels
iv. May help in identifying geographic AMR hotspots to complement existing surveillance

This information may be potentially useful for implementation of following public health actions:

a. Infection control: Support targeted public health interventions, including improvements in water, sanitation and hygiene (WASH), infection prevention and control (IPC) practices, and facility maintenance in areas where elevated levels of AMR are detected.

b. Trending: Monitor the prevalence and distribution of AMR pathogens and ARGs over time and across locations to identify trends and support long-term surveillance. Where human-derived signals can be distinguished from animal and environmental sources, these data may also help inform antimicrobial policies.

c. Diagnostic testing: Help prioritize antimicrobial susceptibility testing and other diagnostic investigations for the pathogen-AMR combinations.

d. Selecting preferred antimicrobials: Support antimicrobial stewardship by informing the selection of effective antimicrobial agents based on local resistance patterns and discouraging the use of agents with high levels of resistance.

e. Development of new antimicrobials: Identify priority areas for research and development of new antimicrobial agents where resistance to existing treatments is increasing.

f. Vaccination programmes: Inform the prioritization of vaccine development and targeted vaccination programmes for vaccine-preventable AMR pathogens in populations with a high burden of disease.

Moreover, wastewater surveillance for AMR is more challenging than surveillance for many infectious diseases because AMR bacteria and ARGs originate from multiple human, animal and environmental sources[154]. Consequently, the detection of AMR bacteria or ARGs in wastewater does not necessarily reflect the burden of human infection alone. Animal waste, environmental waters, wastewater treatment systems, sewer biofilms and environmental microflora can all contribute to the AMR signal. These non-human sources may be particularly important in non-sewered settings or sewer systems receiving storm water, environmental water or livestock waste. In addition, the survival of AMR bacteria in wastewater varies between species. Hence, there is a need to define benchmarks and thresholds for WWS of ARGs to determine when increases in pathogen diversity and abundance should trigger a ‘red flag’ for public health response. Some organisms persist longer than others, while some may proliferate within sewer biofilms. Horizontal gene transfer among environmental bacteria may also influence the abundance and distribution of ARGs independently of human infections. Therefore, AMR wastewater surveillance data should be interpreted cautiously, taking into account local environmental conditions, sewer infrastructure, sampling strategy and other potential sources of AMR to distinguish true population-level trends from environmental variability[143] (**Figure 6**).

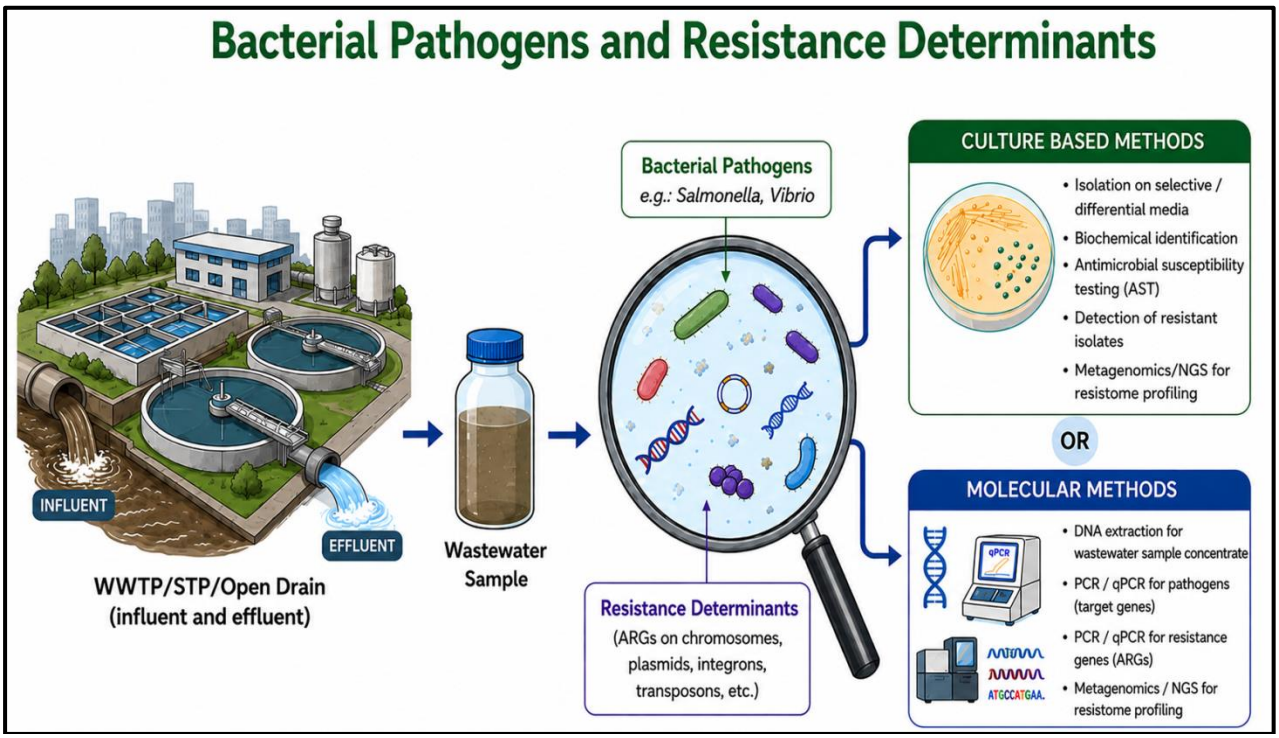


Figure 6: Overview of Wastewater Surveillance for the Detection of Bacterial Pathogens and AMR Determinants

7.2 PRIORITY ARGs FOR INDIA

Selecting targets for routine AMR wastewater surveillance requires identifying priority pathogen-antimicrobial resistance combinations. However, the large diversity of pathogens and resistance profiles makes prioritization a complex task. Therefore, the initial selection can focus on a limited number of pathogen-ARG combinations that are most relevant to local public health needs and feasible to monitor. Target selection also needs to reflect the intended objectives of the surveillance programme. In India, the AMR surveillance strategy can be aligned with the priority resistance genes identified under the National Action Plan on Antimicrobial Resistance (NAP-AMR) and the Indian Council of Medical Research (ICMR) AMR surveillance priorities. These include clinically significant genes associated with resistance to critically important antimicrobial classes and those with a high potential for rapid dissemination. Key target genes include[155-156]:

a. **Carbapenem resistance genes:** *blaNDM*, *blaKPC*, and *blaCTX-M*, which are commonly associated with multidrug-resistant *Enterobacterales* and other Gram-negative pathogens. These genes confer resistance to β-lactam antibiotics, including extended-spectrum cephalosporins and carbapenems.

b. **Colistin resistance gene:** *mcr-1*, an important plasmid-mediated resistance determinant linked to resistance against colistin, another last-line antimicrobial used for severe multidrug-resistant infections.
c. **Tetracycline resistance genes:** *tet* gene groups, which are widely distributed among environmental and clinical bacterial populations and serve as indicators of antimicrobial selection pressure.
d. **Sulfonamide resistance genes:** *sul* gene groups, commonly detected in wastewater environments and associated with resistance to sulfonamide antibiotics.
e. **Quinolone resistance genes:** *qnr* gene groups, which mediate plasmid-associated resistance to fluoroquinolones and contribute to reduced susceptibility in clinically relevant pathogens.
f. **Macrolide resistance genes:** *erm* gene groups, responsible for resistance to macrolides through ribosomal target modification and frequently associated with mobile genetic elements.

7.3 SELECTION OF SAMPLING SITES BASED ON SURVEILLANCE OBJECTIVES

The selection of sampling locations for wastewater AMR surveillance should be guided by the surveillance objectives and the population of interest. For community-level surveillance, influent from municipal wastewater treatment plants (WWTPs) is the preferred sampling location, as it provides a representative composite of wastewater from the contributing population and enables longitudinal monitoring of AMR trends over time. Where surveillance of defined catchment populations or identification of geographic hotspots is required, upstream sewer network locations (e.g. pumping stations or open drains) may be selected. For healthcare-associated AMR surveillance, hospital wastewater (untreated influent or effluent prior to treatment) is the preferred sampling matrix, as it reflects the burden of AMR organisms and ARGs associated with healthcare settings and complements clinical surveillance, infection prevention, and control programmes[143,157].

7.4 LABORATORY TESTING APPROACHES

For wastewater sample concentration and nucleic acid extraction, the methods outlined in Chapter 6 should be followed. In addition to the procedures described therein, a critical mechanical lysis step (e.g. bead-beating) must be included to ensure efficient disruption of

bacterial cells and optimal recovery of bacterial DNA. Wastewater contains a diverse microbial community, including Gram-positive bacteria with thick cell walls, biofilm-associated microorganisms, and bacteria attached to suspended particulate matter, which may not be efficiently lysed by chemical methods alone. Incorporation of mechanical disruption enhances cell lysis, improves bacterial DNA yield, thereby providing a more representative profile of the microbial community for downstream molecular analyses[158-159].

Following the sample concentration and nucleic acid extraction procedures described in Chapter 6, detection of AMR pathogens or ARGs in wastewater may be undertaken using culture-based and/or culture-independent molecular approaches[145,160-162], depending on the surveillance objectives, laboratory capacity, and intended public health use of the data.

a. Culture-based detection involves the isolation of indicator organisms, such as *Escherichia coli* (including extended-spectrum β-lactamase (ESBL)-producing *E. coli*), *Enterococcus spp.*, or other priority bacterial pathogens, using selective and differential culture media. Isolates should undergo antimicrobial susceptibility testing (AST) using standardized methods in accordance with recognized international standards (e.g. CLSI or EUCAST). Where appropriate, phenotypic confirmation of specific resistance mechanisms may be performed, followed by whole-genome sequencing (WGS) of selected isolates to characterize antimicrobial resistance genes (ARGs), sequence types, virulence factors, plasmids, and other mobile genetic elements. Culture-based surveillance provides phenotypic evidence of antimicrobial resistance and facilitates direct comparison with clinical AMR surveillance programmes, including WHO GLASS.

b. For culture-independent molecular detection, the nucleic acids may be used for targeted quantitative polymerase chain reaction (qPCR), digital polymerase chain reaction (dPCR) or droplet digital polymerase chain reaction (ddPCR) assays for the detection and quantification of priority ARGs and AMR pathogens. In addition, shotgun metagenomic sequencing may be employed for comprehensive characterization of the wastewater resistome and mobilome, enabling the detection of a broad spectrum of known and emerging resistance determinants. The same nucleic acid extracts may also be used for parallel viral sequencing, thereby supporting an integrated, resource-efficient, and multi-target wastewater surveillance strategy[143,146-148,163].

8. Interpretation of Laboratory Data and Surveillance Analytics

Interpretation of wastewater surveillance (WWS) data is a critical step in transforming laboratory findings into meaningful public health information. The concentrations of genetic material or biomarkers detected in wastewater provide an indirect estimate of disease circulation within a community. However, generated data do not directly correspond to the exact number of infected or colonized individuals. Therefore, wastewater data should always be interpreted cautiously, considering test sensitivity, quality control performance, environmental and population-related factors that may influence the results. Furthermore, single measurements should generally not be interpreted in isolation. Repeated sampling over time is necessary to identify meaningful trends and fluctuations[164].

8.1 QUALITY ASSURANCE IN INTERPRETATION

Reliable interpretation of WWS data requires necessary quality control measures that should be an integral part of WWS testing. Proper use of positive controls, negative controls, and internal amplification controls during molecular testing should be ensured. These measures help to ensure that the results are accurate and reliable. Use of normalization control helps to distinguish true epidemiological changes from variations attributable to wastewater characteristics. If possible, assessment of recovery efficiency should be performed during wastewater concentration and nucleic acid extraction procedures. The heterogeneous nature of wastewater requires internal amplification control to mitigate the effect of PCR inhibitors. Replicate testing and the use of standardized protocols are important for maintaining consistency, reproducibility, and reliability of the results.

8.2 INTERPRETATION OF QUANTITATIVE DATA

Quantitative wastewater data is commonly expressed as copies per liter (copies/L), gene copies per liter, genome equivalents per liter, relative abundance, or normalized concentration values. These measurements are used to estimate the amount of a target pathogen, antimicrobial resistance gene, or biomarker present in wastewater samples. Therefore, to report viral nucleic acid or biomarker concentrations accurately, laboratory personnel should convert PCR-derived concentration estimates to concentrations per unit volume of the

original (unconcentrated) wastewater sample. This conversion can be done by considering the template volume used in the PCR assay (and reverse transcription reaction, if conducted separately), the concentration factor resulting from nucleic acid extraction, and the concentration factor introduced during wastewater sample processing.

8.3 INTERPRETATION OF DETECTION RESULTS

A. Positive Detection (Positive Result)

A positive result, or the detection of viral nucleic acid or a biomarker in wastewater, indicates active circulation of the pathogen or ongoing transmission within the catchment population. The presence of viral RNA in wastewater is confirmed when RT-qPCR amplification crosses the threshold before cycle 40 (Ct < 40) during the exponential phase, or when RT-dPCR/RT-ddPCR detects at least three positive droplets. If multiple assays or PCR replicates are performed, detection in any one assay or replicate is sufficient to classify the sample as positive. The positive results may also occur due to the presence of asymptomatic or undiagnosed cases in the community. Therefore, positive results should be interpreted cautiously.

B. Non-Detection (Negative Result)

Interpretation of negative results should always be performed by considering analytical sensitivity, assay performance, and quality control outcomes. A non-detection or no amplification result does not necessarily confirm the complete absence of the pathogen or biomarker in the community. Such results might be occurred due to very low prevalence within the population or because the concentration of the target organism is below the assay detection limit. No amplification may also result from sample degradation during transport or storage, inefficient sample concentration, or poor nucleic acid extraction efficiency. In addition, the presence of PCR inhibitors in wastewater can significantly affect amplification and should always be considered during result interpretation. Furthermore, data generated from samples with failed quality control parameters should be interpreted cautiously or repeated or excluded from public health decision-making.

8.4 OTHER FACTORS AFFECTING INTERPRETATION

Interpretation of WWS data may be influenced by several environmental and population-related factors. Environmental conditions such as rainfall, temperature, pH, sunlight exposure, and wastewater travel time can affect the stability and concentration of target analytes in wastewater. Population dynamics including population movement, tourism, sewer connectivity, and industrial discharge may also contribute to variations in wastewater composition. In addition, laboratory factors such as sampling methodology, concentration efficiency, nucleic acid extraction recovery, PCR inhibition, assay sensitivity and specificity can influence analytical results. Therefore, these factors should always be documented and carefully considered during data interpretation.

8.5 TREND ANALYSIS

Trend analysis of WWS data provides a quantitative approach to evaluate temporal changes in nucleic acid or biomarker concentration within a community. Rather than relying on visual interpretation, statistical methods should be applied to pathogens concentration to identify meaningful changes in infection dynamics, including both reported and unreported cases.

Trend estimation requires a minimum of three sampling time points; therefore, with a weekly sampling schedule, at least three weeks of data is generally needed to generate reliable trend estimates. The identified trends can be categorized according to the following to support public health decision-making.

a) Direction (increasing, decreasing, or stable)
b) Duration (short-term or sustained)
c) Magnitude of the peak (the rise in viral titres)

As pathogen nucleic acid or biomarker concentrations in wastewater typically follow a log-normal distribution, they should be log10-transformed prior to statistical analysis. Linear regression using sampling date as the independent variable is recommended for estimating temporal trends, when log-transformed data are used, the regression slope may be converted into **Percent Daily Change (PDC)** using the equation:

$$\mathbf{PDC} = (10^{\text{slope}} - 1) \times 100$$

To avoid bias in trend estimation, samples with pathogen nucleic acid or biomarker concentrations below the assay limit of detection should be retained in the analysis by assigning a value equal to one-half of the detection limit.

Trend direction should be determined by evaluating the statistical significance of the regression slope and may be further supported by applying a predefined minimum Percent Daily Change threshold. Based on these criteria, trends can be classified as increasing, decreasing, or plateau (stable) trend.

A. Increasing Trend

A sustained increase in concentration over multiple sampling events may indicate:

- **i.** Increasing transmission within the community,
- **ii.** Expansion of infection clusters,
- **iii.** Rising viral or antimicrobial resistance burden,
- **iv.** Emergence of an outbreak.

Note: Early increases in wastewater signals may precede clinical case reporting because infected individuals can shed pathogens before symptom onset or testing.

B. Decreasing Trend

A decline in wastewater concentrations over time may indicate:

- **i.** Reduction in disease transmission,
- **ii.** Successful public health interventions,
- **iii.** Reduced shedding within the population,
- **iv.** Declining outbreak intensity.

Note: Temporary decreases may also occur due to dilution effects, rainfall events, industrial discharge, or variations in wastewater flow.

C. Stable Trend

Relatively stable concentrations over time may suggest:

- **i.** Endemic circulation at a constant level,
- **ii.** Persistent background presence of ARGs or pathogens,
- **iii.** Stable community transmission dynamics.

Note: Stable trends should still be monitored continuously for sudden deviations.

8.6 IMPORTANCE OF TREND-BASED INTERPRETATION

Trend analysis is generally more informative than interpreting absolute numerical values. Variability in wastewater characteristics may influence measured concentrations. Therefore:

i. Consecutive increases are often more meaningful than a single high value,
ii. Longitudinal monitoring improves reliability,
iii. Weekly or biweekly sampling provides better interpretation than sporadic sampling.

Note: Graphical visualization of trends over time is strongly recommended.

8.7 LIMITATION OF WASTEWATER SURVEILLANCE

Wastewater surveillance has several inherent limitations:

i. Inability to identify infected individuals
ii. The severity of disease cannot be predicted, though it gives an estimate of rise in number of cases
iii. Lack of direct prevalence estimation
iv. Influence of environmental degradation
v. Differences in sewer infrastructure
vi. Methodological variability between laboratories.

Therefore, wastewater data should be interpreted as a population-level indicator rather than an exact measure of disease burden. A single site that is consistently monitored at fixed intervals using the same laboratory procedure will give a good idea about circulation of pathogen in the population.

9. Sequencing Approaches for Wastewater Surveillance

9.1 IMPORTANCE OF SEQUENCING IN WWS



Genomic sequencing is a key component of wastewater surveillance that complements molecular detection by providing detailed genetic information on pathogens and antimicrobial resistance determinants present in wastewater. Unlike targeted nucleic acid detection methods, which primarily indicate the presence and quantity of a specific target, sequencing enables comprehensive characterization of genetic diversity within a community. For viral pathogens, sequencing facilitates the identification and monitoring of circulating lineages, detection of mutations and variants of public health significance, assessment of transmission dynamics, and discrimination between vaccine-derived and wild-type strains, particularly of the polio virus. These data strengthen outbreak investigations, support risk assessment, and inform public health responses[3,165].

In addition to viral surveillance, sequencing has become an important tool for population-level monitoring of antimicrobial resistance (AMR). Metagenomic and targeted sequencing approaches enable the characterization of antimicrobial resistance genes (ARGs), identification of clinically important resistance determinants, and evaluation of temporal and spatial trends in the wastewater resistome, thereby supporting early warning of emerging AMR threats and complementing conventional clinical surveillance[166].

9.2 OVERVIEW OF SEQUENCING APPROACHES

Sequencing wastewater samples is technically demanding. Wastewater is a highly heterogeneous matrix containing nucleic acids from humans, animals, industrial, plants waste and diverse microbial communities, together with organic matter, chemicals and other substances that may inhibit downstream molecular analyses. In contrast to clinical specimens, wastewater represents a composite sample derived from numerous individuals, animals and

various microbial communities and therefore contains fragmented and mixed genetic material from multiple sources. Consequently, successful sequencing depends on the quality, quantity and integrity of the target nucleic acid, as well as effective removal of inhibitory substances during sample processing. Samples selected for sequencing should satisfy predefined laboratory quality criteria, such as acceptable nucleic acid recovery, inhibition assessment and target concentration (e.g., Ct value, where applicable), to maximize the likelihood of generating high-quality sequencing data (**annexure IIIA)**. The choice of sequencing approach should be guided by the surveillance objectives, target organism, laboratory capacity, turnaround time and available resources. The principal sequencing approaches used in wastewater surveillance include Targeted (amplicon-based) sequencing, Shotgun metagenomic sequencing, and Hybrid capture (probe-based enrichment) sequencing. These sequencing technologies have been widely evaluated for wastewater-based surveillance and have been shown to provide a scalable and cost-effective means of monitoring pathogens circulating within populations[165-167].

9.2.1 Targeted (Amplicon-Based) Sequencing: Targeted sequencing relies on PCR amplification of predefined genomic regions using pathogen-specific primers prior to sequencing. Depending on the surveillance objective and assay design, the amplified regions may represent a single genomic target, multiple targets, or overlapping amplicons spanning the entire genome (whole genome amplicon sequencing). Briefly, whole-genome amplicon sequencing is a targeted sequencing approach in which multiple PCR primer pairs are designed to amplify overlapping fragments that collectively cover the entire genome before sequencing. This approach provides high sequencing depth across the amplified regions while reducing background nucleic acids from non-target organisms.

Key Characteristics

a. High analytical sensitivity for pathogens presents at low abundance.

b. Provides high sequencing depth across targeted genomic regions.

c. Enables efficient monitoring of known pathogens, mutations, lineages and variants of public health importance.

d. Cost-effective for routine surveillance of known threats (e.g., SARS-CoV-2) with relatively simple downstream bioinformatics analysis.

Limitations:

a. Limited ability to discover novel or highly divergent pathogens outside the targeted regions.

b. Primer mismatches caused by genomic evolution may reduce amplification efficiency and genome coverage.

9.2.2 Shotgun Metagenomic Sequencing: Shotgun metagenomics sequences all genetic material (nucleic acid) present in wastewater sample without prior enrichment or targeting. Sequences are identified computationally after sequencing.

Key Characteristics

a. Provides an unbiased overview of microbial diversity.

b. Allows analysis of microbial communities and co-circulating agents

c. Enables detection of unexpected, novel, or emerging pathogens

d. Metagenomics is most valuable for sentinel surveillance and outbreak investigation, particularly when the causative pathogen is unknown.

Challenges/Limitations:

a. Requires deeper sequencing depth due to low nucleic acid abundance

b. Higher cost and analytical complexity (need advanced bioinformatics expertise)

9.2.3 Hybrid Capture/ Probe-Based Sequencing (e.g. Viral Surveillance Panels): Hybrid capture sequencing uses labeled probes that bind to genomes of interest. Nucleic acids are selectively enriched from complex wastewater samples before sequencing, allowing simultaneous detection of multiple pathogens.

Key Characteristics

a. Enables multi-pathogen surveillance in a single assay.

b. Improves detection sensitivity compared to untargeted metagenomics.

c. Supports surveillance of both RNA and DNA viruses/microbes.

d. For instance, this approach may allow monitoring of respiratory, enteric and emerging pathogens together.

9.3 SEQUENCING PLATFORMS AND WORKFLOWS

Sequencing methodology plays a pivotal role in the detection and characterization of pathogens in wastewater surveillance. Each sequencing platform offers distinct strengths and limitations[168]. Current sequencing technologies encompass short-read, long-read, and length-agnostic approaches, enabling researchers to balance genome coverage, sequencing depth, throughput, and error profiles according to specific surveillance objectives.

Among these, long-read sequencing platforms, particularly Oxford Nanopore Technologies (ONT), are well suited for resolving structural variants, atypical nucleotide compositions, and long or complex genomic regions that are often difficult to characterize using short-read sequencing methods. This section provides an overview of the major sequencing platforms and their workflows employed in wastewater surveillance.

9.3.1 Short-Read Sequencing Platforms

9.3.1.1 Illumina Sequencing: Illumina employs a sequencing-by-synthesis (SBS) approach that uses fluorescently labeled, reversible terminator nucleotides in combination with bridge amplification. Here, DNA fragments (typically 75–300 base pairs) are immobilized on a solid surface through complementary primers and undergo bridge amplification, resulting in dense clusters composed of many identical copies of each original fragment.

Incorporation of each nucleotide is optically detected through fluorescent signals emitted by reversible terminator-labeled bases, producing highly accurate reads. This chemistry delivers a very low overall error rate (approximately 99.9% accuracy), with errors predominantly occurring as base substitutions rather than insertions or deletions (indels), allowing reliable sequencing.

Illumina platforms typically generate paired-end reads, providing sequence information from both ends of each DNA fragment, which substantially improves read alignment, genome assembly, and taxonomic resolution in complex samples. Bioinformatics tools then align the reads to reference target (e.g., viral) genomes to reconstruct consensus sequences and identify mutations. Illumina platform is widely adopted for its reliable read accuracy and sensitivity, which is particularly advantageous for detecting rare or low

abundance pathogens in wastewater, and high-throughput routine surveillance. It enables reliable variant calling, lineage assignment, and mutation frequency analysis.

Limitations:

a. Short reads make reconstruction of full viral genomes and complex variants more challenging.

b. Structural variations may be difficult to resolve.

For Illumina sequencing, the workflow generally includes:

i. cDNA synthesis from extracted RNA

ii. Targeted amplification using multiplex PCR with specific primer pools

iii. Pooling of amplified products

iv. End repair and A-tailing of amplicons

v. Adapter ligation with unique indices/barcodes

vi. Library purification and amplification

vii. Quantification and quality assessment of the final library

viii. Loading of libraries onto the Illumina sequencing flow cell

ix. Sequencing run on an Illumina platform (e.g., MiSeq)

9.3.1.2 Ion Torrent Sequencing: Ion Torrent sequencing employs semiconductor technology to detect hydrogen ions (H^+) released during DNA synthesis, i.e. during the incorporation of deoxynucleotide triphosphates (dNTPs) into the growing DNA strand[169]. DNA fragments ligated with sequencing adapters are clonally amplified on beads using emulsion PCR and subsequently loaded into proton-sensitive semiconductor wells. During sequencing, nucleotides are introduced sequentially, and each incorporation event releases H^+ ions, resulting in a local pH change[168]. This pH change is detected by the semiconductor sensor and converted into an electrical signal. The magnitude of the signal is proportional to the number of nucleotides incorporated in a single cycle.

Ion Torrent sequencing enables rapid sequencing and is particularly well suited for targeted sequencing applications. However, homopolymeric regions are prone to higher error rates because accurately estimating the number of consecutively incorporated identical nucleotides from signal intensity can be challenging, thus require careful interpretation[169-170].

Steps in Ion Torrent Sequencing:

i. cDNA preparation from extracted RNA
ii. Targeted Multiplex PCR using Primer Pools for Second strand synthesis
iii. Combining target amplification reactions
iv. Partial digestion of amplicons
v. Ligation of adapters and barcodes
vi. Library purification and amplification
vii. Purification and quantification of amplified library
viii. Loading of the Ion Chef instrument with sequencing chips, reagents and sample libraries
ix. Loading the prepared sequencing chips on a ION GeneStudio S5 plus sequencer

9.3.2 Long-Read Sequencing Platforms

9.3.2.1 Oxford Nanopore Technology (ONT): Nanopore sequencing determines nucleotide sequences by measuring changes in electrical current as single DNA or RNA molecules pass through a protein nanopore. This approach generates long continuous reads, sometimes spanning entire viral genomes, and produces data in real time, thus enabling faster data turnaround time.

ONT offers real-time sequencing and portability (e.g., MinION), preferred for rapid, decentralized, and field-deployable applications which is valuable for outbreak monitoring and early warning systems. Long reads improve genome assembly, genome resolution, handling mixed or fragmented viral genetic material in wastewater samples, detection of recombination events, identification of complex mutations and haplotype resolution. However, ONT has slightly higher error rates (like indels), requiring advanced error correction methods to ensure accurate base calling[171-172]. The workflow for amplicon-based ONT sequencing (adapted for wastewater) is outlined in **annexure IIIB**.

Steps in Oxford Nanopore Sequencing:

i. cDNA preparation from extracted RNA
ii. Targeted Multiplex PCR using Primer Pools for Second strand synthesis
iii. QC check on 2% agarose gel
iv. Addition of Rapid Barcode for Nanopore Library preparation

v. Pooling samples and clean-up

vi. Library quantification using Qubit fluorometer

vii. Loading library preparation

9.3.2.2 Pacific Biosciences (PacBio): PacBio sequencing utilizes single-molecule real-time (SMRT) technology, in which individual DNA molecules are read multiple times and sequenced in real time. By employing circular consensus sequencing (CCS), it generates highly accurate long consensus sequences, commonly referred to as HiFi reads[173-174]. The long-read capability of SMRT technology enables sequencing of complete or near-complete viral genomes. In wastewater surveillance, it facilitates the characterization of fragmented or genetically diverse viral populations and improves the detection of co-occurring mutations, insertions, deletions, and structural variants[174]. However, slightly higher error rates require bioinformatic correction for accurate interpretation.

9.4 VIRUS-SPECIFIC SEQUENCING CONSIDERATIONS

The sequencing protocols are tailored to genome type, shedding patterns and public health relevance in India. The summary of virus-specific sequencing parameters is given in **Table 5**.

9.5 METAGENOMIC SEQUENCING FOR COMPREHENSIVE PROFILING FOR AMR GENES/PATHOGEN

While targeted assays focus on predefined genes, shotgun metagenomic sequencing provides a culture-independent approach for characterizing the wastewater resistome (total ARG content – both known and emerging ARGs) and mobilome, including mobile genetic elements such as plasmids, integrons, and transposons. This approach is particularly important in wastewater environments, where high microbial diversity and continuous mixing of human, industrial, and animal waste facilitate horizontal gene transfer. Metagenomic analyses are typically performed using high-throughput sequencing platforms. Illumina sequencing is used for high-depth, high-accuracy short read data suitable for quantitative resistome profiling, whereas Oxford Nanopore Technologies (ONT) supports long-read sequencing for improved reconstruction of ARG–mobile element linkages and plasmid structures. Overall, metagenomics is essential for understanding the ecological and evolutionary context of AMR dissemination in wastewater environments[146,148,175]. **Table 6** provides a summary of the major parameters for AMR testing in wastewater samples.

Table 5: Virus-Specific Sequencing Parameters

Virus	Genome Type	Recommended Sequencing Approach	Preferred Platform	Key Genomic Regions	Minimum Depth	India-specific Considerations derations
SARS-CoV-2	Enveloped ssRNA (+)	Targeted Amplicon / Hybrid Capture	ONT / Illumina	Full genome (ARTIC regions)	>1000×	Variant tracking; multi-respiratory panels
Poliovirus	Non-enveloped ssRNA (+)	Targeted Amplicon	ONT / Illumina	VP1 + full genome	>500×	Strict containment; cVDPV differentiation
Influenza A/B	Enveloped segmented ssRNA (-)	Amplicon / Metagenomic	Illumina / ONT	HA, NA segments	>500×	Seasonal & zoonotic surveillance
RSV	Enveloped ssRNA (-)	Hybrid Capture / Amplicon	Illumina / ONT	*G*, *F* genes	>300×	Paediatric burden
HAV / HEV	Non-enveloped ssRNA (+)	Nested / Targeted Amplicon	Illumina / ONT	VP1-2A / ORF2	>300×	Waterborne outbreaks in unsewered areas
Measles / Rubella	Enveloped ssRNA (-/+)	Targeted Amplicon	Illumina	N / H / E1	>200×	Elimination verification
Arboviruses	Enveloped ssRNA (+)	Metagenomic / Hybrid Capture	Illumina / ONT	*NS5*, *E* genes	>500×	Urban seasonal co-circulation
Monkeypox	Enveloped dsDNA	Targeted Amplicon / Capture	ONT / Illumina	Clade-specific markers	>300×	Emerging clade differentiation

Table 6: Summary of AMR Testing Parameters

ARG / Gene Group	Resistance Class	Preferred Method	Platform	Normalisation Marker	India-specific Considerations
blaNDM, blaKPC, blaOXA-48	Carbapenemases	qPCR + Metagenomics	Illumina/ ONT	16S rRNA	Hospital & community dissemination
blaCTX-M	ESBL	qPCR + Sequencing	Illumina	Faecal markers	Widespread in urban sewage
mcr-1 / mcr-2	Colistin	Targeted qPCR + Metagenomics	ONT	16S rRNA	Animal-agriculture interface
tet, sul, qnr, erm	Multiple classes	qPCR panel + Metagenomics	Illumina/ ONT	PMMoV / Flow	High antibiotic usage in human & veterinary sectors

9.6 BIOINFORMATICS ANALYSIS

Standardized bioinformatics pipelines shall be used (**annexure IIIC & IIID**). The results should be interpreted together with quantification and normalization data. Consensus sequences and metadata are uploaded to national repositories following ethical and data privacy guidelines.

10. Biosafety and Risk Assessment

10.1 BIOSAFETY CONSIDERATIONS FOR HANDLING WASTEWATER SAMPLES

Wastewater may contain infectious agents, including enteric pathogens and respiratory viruses shed in feces, along with a complex mixture of chemical contaminants. Consequently, all wastewater samples should be considered potentially infectious and handled accordingly. Laboratory personnel should adhere to appropriate biosafety practices during sample collection, transportation, processing, testing, and disposal. Furthermore, all procedures should be performed following a comprehensive risk assessment.

10.2 COLLECTION

Sample collection should preferably be carried out by two trained personnel. Before initiating the sample collection, laboratory personnel must don appropriate personal protective equipment (PPE), including double gloves, shoe covers, mask, head cover, gown, and eye protection goggles. Perform hand hygiene immediately after glove removal.

10.3 TRANSPORT

Transport the samples to the laboratory as soon as possible to minimize degradation of the samples (particularly nucleic acids), while maintaining the cold chain (2-8˚C, avoid freezing) throughout transport. Samples should preferably be transported in a triple-packaging system consisting of a primary leak-proof sample container, a secondary sealed leak-proof container with absorbent material, and a rigid outer transport container to ensure safe transport and maintain sample integrity

10.4 PROCESSING

According to WHO, at a minimum, all laboratory facilities processing environmental samples should meet basic WHO Biosafety Level 2 (BSL-2) laboratory standards. In addition appropriate Biosafety Level 3 (BSL-3) precautions including respiratory protection and designated areas for donning and doffing personal protective equipment (PPE) should be implemented. All procedures generating aerosols should be carried out in a certified biosafety cabinet (BSL- class 2). Laboratory workflow should be unidirectional to minimize the risk of contamination[176-177].

10.5 HEAT INACTIVATION

To reduce biosafety risks to laboratory personnel and the environment during wastewater processing, heat inactivation may be performed. Inactivation at 56°C for 30 minutes has been shown to result in minimal impact on RNA quantification.

10.6 RISK ASSESSMENT

Prior risk assessment should be done for all the procedures and necessary mitigation measures should be taken to reduce the risk to acceptable levels. A laboratory-specific risk assessment should be performed before handling wastewater samples. It should consider the sample type, target pathogen(s), laboratory procedures, aerosol generation potential, biosafety level, facility design, staff competency, and available biosafety controls. The findings should be used to implement appropriate risk mitigation measures and ensure the safe handling, processing, and disposal of wastewater samples. The chapter on risk assessment in the WHO Laboratory Biosafety Manual may be referred for risk assessment in wastewater testing procedures and implementing mitigation measures[177].

10.7 BIOMEDICAL WASTE MANAGEMENT

Laboratory waste generated during processing of wastewater samples should be autoclaved and managed before disposal in accordance with Bio-medical Waste Management Rules 2016 (BMWM), Ministry of Environment, Forest and Climate Change (MoEFCC)[178].

11. Submission of Wastewater Surveillance Data to the Integrated Health Information Platform (IHIP)

Institutional Framework and Protocols for the Integration of Wastewater Surveillance (WWS) Data into the Integrated Health Information Platform (IHIP)

11.1 BACKGROUND

The Integrated Disease Surveillance Programme (IDSP) is a decentralized, technology-driven initiative by the Ministry of Health and Family Welfare designed for real-time epidemic intelligence. It coordinates a nationwide network across district, state, and national units to detect, track, and respond to early warning signals of public health outbreaks.

The Integrated Health Information Platform (IHIP) is a state-of-the-art, web-enabled platform developed under IDSP to provide near-real-time, case-based disease surveillance. It uses advanced GIS capabilities for spatial heat-mapping and features interoperable modules to break data silos across various national health programmes. It visualizes clinical, genomic, and environmental datasets simultaneously, allowing health officials to deploy rapid, localized outbreak responses.

The Existing Surveillance Architecture of IHIP is built on three core reporting streams, traditionally called Form S (Syndromic), Form P (Presumptive/Clinical), and Form L (Laboratory confirmed). However, to capture environmental vectors and non-clinical data streams, the ecosystem has expanded to encompass advanced dedicated modules:

1. IDSP Core Disease Surveillance Module: Captures data on 33+ priority health conditions using standardized electronic forms filled by frontline health workers, medical officers, and network laboratories.
2. INSACOG Whole Genome Sequencing (WGS) Module: Integrates line lists and genomic variant analyses directly from Indian SARS-CoV-2 Genomics Consortium (INSACOG) labs.
3. Wastewater Tracking (WWT/WWS) Surveillance Summary Report: An expanding system designed to handle multi-centric sewage data. This framework complements clinical

reporting by serving as an early-warning signal, identifying community transmission up to 1 to 2 weeks before it manifests in clinical diagnostic spikes.

11.2 STANDARDIZED DATA VARIABLES FOR WWS INTEGRATION

To achieve seamless data harmonization across various sentinel collection nodes, data uploaded to the IHIP-WWS sub-module must follow a highly structured metadata format. Submissions are divided into four essential variable categories:

1. Epidemiological & Pathogen Targets

- **a.** Target Organisms: Categorized by public health priority (e.g., SARS-CoV-2, *Vibrio cholerae*, *Salmonella typhi*, Poliovirus, Influenza A/B).
- **b.** Quantitative Assay Metrics: Specific assay parameters including target genes (e.g., *N1*, *N2*, *ORF1ab*), raw and adjusted Cycle Threshold (Ct) values, and copy numbers per milliliter (copies/mL) of wastewater.
- **c.** Genomic Lineage Signatures: Next-Generation Sequencing (NGS) metrics, including Pango lineages, mutations of interest, and relative variant abundance percentages within the community composite sample.
- **d.** Anti-Microbial Resistance (AMR) Markers: Abundance profiles of specific Antibiotic Resistance Genes (ARGs) alongside phenotypic signatures of resistant bacterial isolates.

2. Spatial & Catchment Metadata

- **a.** Unique Node ID: System-generated unique alphanumeric identifier for every approved, designated wastewater sampling site.
- **b.** Geospatial Coordinates: High-precision latitude and longitude coordinates logged via the IHIP geo-tagging module.
- **c.** Site Typology: Classification of the sampling node (e.g., Sewage Treatment Plant (STP) Inflow, Open Drain, Hospital Wet-Well, Community Wet-Well).
- **d.** Estimated Population Catchment: Approximate headcount of the population draining into the specific sewershed.

3. Temporal & Sample-Handling Chronology

a. Sampling Typology: Method used for collection (e.g., 24-hour time-weighted composite, flow-proportional composite, or instantaneous grab sampling).

b. Chronological Logging: Exact timestamps detailing sample collection, laboratory arrival, extraction initiation, and final digital validation.

4. Environmental & Contextual Confounders

a. Physicochemical Properties: On-site measurement parameters including sample temperature, pH levels, total suspended solids (TSS), and electrical conductivity.

b. Hydrological Inflow Data: Total daily sewage flow rate at the plant or estimated flow velocities in open drains.

c. Meteorological Interferences: Categorical indicators for recent precipitation events (e.g., No Rain, Light Rain, Heavy Monsoonal Deluge) to correct for dilution errors.

11.3 FREQUENCY OF DATA SUBMISSION AND REPORTING TIMELINES

To preserve the predictive value of Wastewater Surveillance, strict reporting cadences must be maintained by all participating sentinel entities:

a. Routine Surveillance Cadence: Data logging must occur on a consistent weekly or bi-weekly cycle during periods of standard baseline activity.

b. System Timeliness Threshold: To maintain surveillance validity, data must be uploaded within 48 hours of laboratory assay completion. All designated sentinel sites are expected to maintain an 80% or greater timeliness consistency rating over a rolling 52-week year.

c. Trigger-Based Accelerated Reporting: Upon detection of specific epidemiological triggers, the reporting frequency automatically shifts to daily or real-time event-based notification. These triggers include:

i. Detection of a highly critical pathogen in a previously uninfected catchment (e.g., Wild Poliovirus or Novel Influenza strain).

ii. A sustained, statistically significant increase in pathogen copy numbers over two consecutive sampling cycles.

iii. Detection of novel variants or dangerous mutation profiles via the genomic sequencing pipeline.

11.4 USER WORKFLOW AND ACCESS PROTOCOLS ON THE PORTAL

The workflow within the IDSP-IHIP platform uses role-based, secure credentials to ensure data integrity and prevent unauthorized adjustments:

1. Authentication: Personnel access the system via the official IHIP login Page using unique assigned credentials. Profile fields marked with a red asterisk (*) must be updated annually.
2. Data Ingestion: Trained laboratory data operators enter parameters using dedicated digital web forms or standard, schema-validated bulk CSV uploads.
3. Analytical Quality Control: The system parses the raw input metrics, automatically converting spatial coordinates into localized map layers for visualization. Operators can review generated tables to correct manual entry errors.
4. Server Commit: Clicking the "Submit" button pushes the finalized data record to the central IDSP servers. Once submitted, data records are locked permanently and cannot be modified or deleted.

11.5 ANALYTICS AND VISUALIZATIONS GENERATED FROM WWS ON IHIP

Integrating Wastewater Surveillance (WWS) data into the IHIP enables four key categories of real-time public health analytics:

a. Temporal Trend & Early Warning Analytics: Interlinked timelines plotting normalized pathogen copy loads (copies/mL) and average PCR Cycle Threshold (Ct) values. This visualizes community infection waves and tracks the 7-to-14-day lead-time before these cases show up in clinical facilities.

b. Geospatial Hotspot Analytics: Built-in GIS maps that color-coded sewer sheds and open drains based on viral or bacterial intensity. Overlaying these with clinical data (Forms P and L) helps identify "silent" transmission zones where community shedding is high but clinical diagnostic testing is low.

c. Genomic Variant Dashboards: Stacked bar charts and variant tracking matrices that show the relative abundance percentage of emerging mutations or lineages (e.g., specific sub-variants) detected via Next-Generation Sequencing (NGS).

d. AMR Stewardship Metrics: Radar and longitudinal trend charts tracking the presence, distribution, and concentrations of high-priority Antimicrobial Resistance Genes (ARGs) across different geographic zones to guide localized antibiotic stewardship.

12. Using the Wastewater Surveillance Data for Public Health Action

The ultimate objective of integrating Wastewater Surveillance (WWS) data into the IHIP ecosystem is to translate environmental signals into decisive, preventive public health interventions. By capturing pathogen shedding from both asymptomatic and pre-symptomatic individuals, WWS overcomes the limitations of healthcare-seeking behaviour. It provides an unbiased, population-level overview of disease transmission that serves as a powerful instrument for early warning and timely containment.

To operationalize these data insights, public health authorities at the District, State, and National levels must execute structured actions across three core operational pillars:

12.1 STRATEGIC RESOURCE ALLOCATION FOR CLINICAL READINESS

i. **Strengthening targeted surveillance and diagnostic capacity** in affected sewersheds when an unusual spike or a critical Ct-value drop is observed, through deploying diagnostic kits, mobile testing vans, and fever clinics to that catchment area.

ii. **Enhanced Healthcare preparedness through** utilizing the lead time provided by WWS trends, hospital networks can optimize bed availability, secure oxygen supplies, and manage stockpiles of specific therapeutics before clinical admissions surge.

iii. **Implement timely public health interventions such as** localized pulse vaccination drives or mop-up immunization campaigns within the affected demographic zones when increasing level of pathogen loads (such as Poliovirus, *Vibrio cholerae*, or Influenza) are detected.

12.2 LOCALIZED CONTAINMENT AND ENVIRONMENTAL INTERVENTIONS

i. **Enabling Spatial Vigilance through** GIS hotspot mapping on IHIP to implement micro-containment protocols and localized public health interventions rather than applying district-wide lockdowns.

ii. **Guiding Environmental interventions by triggering** water-quality testing, localized chlorination verification, leak repairs in overlapping water-sewer lines, and targeted

cleaning of open drainage networks following identification of enteric pathogens e.g., *Salmonella typhi*).

12.3 RISK COMMUNICATION AND EVIDENCE-BASED POLICY DECISIONS

i. **Risk communication through development of location specific** IEC materials depicting messages such as high pathogen loads, promoting hand hygiene, water boiling protocols, or early clinic reporting.

ii. **Evidence-Based Policy Decisions through** longitudinal data trends to issue precise health advisories, travel updates, or therapeutic guidelines tailored to the shifting genomic and volume profiles of circulating strains.

13. Ethical and Legal Considerations for Wastewater Surveillance

Wastewater surveillance is a non-invasive public health tool, as it does not require the collection of samples from individuals or direct interaction with populations[87,94,179]. From an ethical and legal perspective, it is generally considered a less than minimal risk approach because it does not involve direct human participation, linked identifiers, or individual-level data. Nevertheless, the implementation of wastewater surveillance must be guided by essential ethical safeguards to ensure responsible use and timely action. As emphasized by organizations such as the World Health Organization (WHO), the National Academies of Sciences, Engineering, and Medicine, and the Association of State and Territorial Health Officials (ASTHO), the following remains crucial for the effective and ethical application of wastewater surveillance[87,89,180-183].

13.1 ETHICAL CONSIDERATIONS

i. **Privacy and confidentiality**

   a. Wastewater surveillance data must not be used to identify individuals, households, or specific persons and strict anonymity must be maintained.

   b. Combining wastewater data with clinical or demographic datasets that may enable individual/household identification should be avoided.

   c. Sampling strategies should ensure representation at the population level only, not individual level.

ii. **Avoid Unnecessary Granularity**

   Sampling should preferably be conducted at large-scale points, such as wastewater treatment plants, sewer networks, or defined zones.

iii. **Non-Stigmatizing Use of Data**

   a. Data should not be presented in ways that label or stigmatize communities, such as calling areas as “infected” or “hotspots” without context.

   b. Findings should be communicated as trends**,** signals**,** or indicators, rather than definitive conclusions.

   c. Misuse leading to discrimination, panic, or misinformation must be strictly avoided.

iv. Transparency

a. Relevant stakeholders, including concerned government bodies, public health authorities, and municipal bodies **s**hould be informed.

b. Documentation covering purpose, sampling locations (generalized), and data use should be maintained.

c. Findings should be shared through reports, dashboards, or briefings.

d. Data should not be publicly displayed if it has been collected from sewersheds that cover less than 3000 people, or from facility/institute-specific sampling locations.

v. Communication

a. Clearly communicate that wastewater data reflects community-level trends, not individual infections**.**

b. Explain limitations, including variability in viral shedding and environmental influences.

c. Ensure messaging is accurate, simple, and non-alarming, ideally coordinated with public health authorities.

13.2 LEGAL CONSIDERATIONS

i. Ethical Approval Requirements

Wastewater surveillance generally qualifies for exemption from review by an Institutional Ethics Committee (IEC), as it does not involve direct interaction with human participants or the collection of identifiable personal data.

However, formal ethics review or approval may be required under the following circumstances:

a. When surveillance targets small or identifiable populations, such as residents of prisons, hostels, schools, or other closed settings, where findings could be attributed to a specific group.

b. When the sampling scale is sufficiently small to increase the risk of indirect identification of individuals or groups, thereby raising concerns regarding privacy and confidentiality.

c. When surveillance findings could have direct social, legal, economic, or public health implications for identifiable individuals or groups, including the potential for stigmatization, discrimination, or other adverse consequences.

d. When the institute or funding agency requires review for all human-related research, including wastewater-based epidemiology.

ii. Data Protection

a. Since no personal data is involved, wastewater surveillance generally falls outside strict data protection regulations.

b. However, best practices include secure storage, restricted access, and maintaining anonymization.

iii. Inter-Agency Coordination

a. Coordination should be established with local government, health departments, and concerned authorities with wastewater management.

b. Information should be provided to local government health departments who are responsible for necessary public health action.

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ANNEXURE I

Annexure IA: Grab Sample Collection

1. Sample collection should preferably be carried out by two trained personnel.
2. Personnel must don appropriate personal protective equipment (PPE), including double gloves, shoe covers, mask, head cover, gown, and eye protection goggles, before initiating sample collection.
3. Sterile sample collection bottles should be labeled with relevant metadata, including site name, collection date, and time of collection.
4. Wastewater samples should be collected from the midstream by lowering a sterile steel bucket attached to a rope.
5. The collected sample should be filtered through a muslin cloth placed over a funnel, positioned on a sterile collection bottle. Ensure the bottle is securely closed after filling.
6. Use disinfectant (1% sodium hypochlorite) to wipe any spillage on the bottle surface.
7. Secure each sample bottle in an individual secondary container, such as a zip-lock bag.
8. Samples must be stored in a sample carrier box with frozen ice packs and transported to the laboratory within 2–4 hours of collection.
9. Dispose-off PPE and any contaminated materials in appropriate biohazard waste disposal bags.

Annexure IB: List of items required for wastewater sample collection

i. Sterile high-density polyethylene (HDPE) or polypropylene bottles with a capacity of 500 mL to 1 L (autoclavable)
ii. Plastic funnel (autoclavable)
iii. Insulated sample transport/ carrier box with ice packs (frozen)
iv. Freshly prepared 1% Na Hypochlorite solution
v. Laboratory coats
vi. Disposable hand gloves
vii. Surgical masks
viii. Eye protection goggles
ix. Muslin cloth
x. Rope
xi. Sterile Bucket (stainless steel)
xii. Labels
xiii. Laboratory Request Form (for sample details)
xiv. Tissue Paper
xv. Permanent marker
xvi. Biohazard bags for waste disposal

ANNEXURE II

Annexure IIA Deployment Checklist for Autosamplers and Robots

1. Site assessed for power supply, security, flow stability, and accessibility

2. Equipment (autosampler or robot) calibrated and all tubing/filters clean and functional.

3. Refrigeration unit (if applicable) tested and set to maintain 2–8 °C.

4. Robotic system (e.g., Fluid Robotics) pre-tested for navigation and sampling module operation.

5. Battery or solar power backup confirmed operational.

6. Safety protocols and PPE requirements for retrieval team documented.

7. Metadata logging system (digital or manual) prepared.

8. Emergency maintenance contact details established with urban local body.

Annexure IIB Operation and Maintenance of Refrigerated Autosamplers and Robotic Systems

1. Install sampler or robot at approved site inside a secure, weather-proof enclosure.

2. Program the device for 24-hour time-weighted or flow-weighted composite collection.

3. Verify initial collection volume and temperature maintenance.

4. Retrieve composite sample daily or as scheduled while maintaining cold chain (2-8 °C).

5. Clean tubing, replace filters, and inspect for blockages or sediment build-up.

6. Robotic systems: Download mapping and sampling data, recharge batteries, and schedule next deployment.

7. Record all maintenance activities, performance metrics, and any deviations in the logbook.

Annexure IIC Template for Metadata Collection

- Sample ID and Site name
- GPS coordinates and Catchment description
- Sampler type (autosampler / robotic system / manual)
- Deployment start and end date/time
- Sampling mode (time-weighted / flow-weighted)
- Total volume collected and average temperature during collection
- Robotic system model (e.g., Fluid Robotics) and any mapping data generated
- Notes on operational issues (blockages, monsoon impact, maintenance performed)

Annexure IID Case Studies of Indian Robotic Pilots

- **Fluid Robotics (Mumbai and Pune)**: AI-powered in-pipe and floating robots deployed for sewer network mapping, pollution load quantification (>650 MLD in early pilots), and automated sewage sample collection. Successfully adapted during the COVID-19 pandemic for safe collection of samples for SARS-CoV-2 RNA detection in complex urban drains and rivers. Demonstrates strong potential for integration with viral and AMR WWS in both sewered and open-drain settings.

- **Genrobotics (Bandicoot series) and Solinas Systems**: Deployed in Ahmedabad, Bengaluru, and other cities for sewer inspection and cleaning. These systems improve site accessibility and safety for autosampler deployment in manholes and open drains, supporting elimination of manual scavenging while enabling consistent WWS sampling.

ANNEXURE III

Annexure IIIA Checklist for Sequencing Readiness

1. Sample should meet the concentration and extraction criteria (recovery efficiency ≥ 50% using matrix recovery control[1]).

2. Inhibition assessment completed and acceptable (internal control Ct shift ≤ 3 cycles[2]).

3. The RT-qPCR quantification is performed (Ct value < 35 or quantifiable viral load documented).

4. Nucleic acid stored at −70 °C or lower with minimal freeze-thaw cycles.

5. Appropriate positive, negative, extraction, and no-template controls should be included.

6. Sample metadata (site name, GPS coordinates, sampling date/time, flow/volume, and normalization factor) recorded.

8. Library preparation reagents, sequencing platform, and consumables confirmed available.

9. Bioinformatics pipeline and reference databases verified as current.

[1] It means that after processing a complex wastewater sample, at least 50% of the target analyte (virus, bacteria, etc.) is successfully retained and detected compared to the original amount, with that percentage verified by a control substance added to the matrix.

[2] An internal control Ct shift (or ΔCt) of cycles in a real-time PCR (qPCR) assay typically indicates that the sample quality is acceptable and the results are valid. The ΔCt method is used to normalize data.

Annexure IIIB Amplicon-based ONT Sequencing (Adapted for Wastewater)

1. Start with DNA/RNA extracted as per standard procedures.
2. Perform cDNA (if RNA virus) synthesis using random hexamers[3] or virus-specific primers.
3. Amplify target genomic regions using appropriate multiplex primer pools (e.g., ARTIC V4.1 or updated schemes for SARS-CoV-2).
4. Purify amplicons and add Oxford Nanopore barcodes using Rapid Barcoding Kit or Native Barcoding Kit.
5. Quantify the final library using Qubit fluorometer.
6. Prime the flow cell and load the prepared library onto MinION or GridION sequencer.
7. Initiate sequencing with real-time base-calling.
8. Monitor run quality and stop when sufficient read depth is achieved.
9. Transfer Fast5/Fastq files for downstream bioinformatics analysis.

[3] Random hexamers are short, random-sequence oligodeoxyribonucleotides ($d(N)_6$) used to prime reverse transcription for cDNA synthesis. They bind randomly along RNA templates, enabling the amplification of all RNA types.

Annexure IIIC Recommended Bioinformatics Pipelines and Quality Metrics

1. Quality control and trimming[4]: FastQC[5] / fastp[6] (Phred score[7] > Q30, adapter removal).
2. Alignment and assembly: Minimap2 or nf-core/viralrecon pipeline[8].
3. Variant calling and lineage assignment: Pangolin and Nextclade (for SARS-CoV-2); equivalent validated tools for other viruses.
4. Minimum quality thresholds: ≥ 90% genome coverage, mean read depth.
5. Final outputs: Consensus sequence in FASTA format, variant report, and phylogenetic tree (if applicable).

[4] It is a critical preprocessing step in bioinformatics, where low-quality bases, adapter sequences, and contamination are removed from raw sequencing reads (FASTQ files) before downstream analysis.
[5] FastQC is a bioinformatics tool used for quality control (QC) of high-throughput sequencing data, primarily in FASTQ format.
[6] Fastp is a highly efficient, all-in-one preprocessing tool for FASTQ files generated.
[7] A Phred quality score (Q-score) is a logarithmic metric used in DNA sequencing to measure the probability (P) that a specific base call is incorrect: $\mathbf{Q} = -10 \log_{10}(\boldsymbol{P})$. Higher scores indicate higher confidence, with Q30 (1 in 1,000 error rate) being a standard benchmark for high-quality data. Scores are commonly encoded in FASTQ files using ASCII characters.
[8] nf-core/viralrecon is a Nextflow-based pipeline designed for viral assembly and intra-host variant calling from Illumina/Nanopore data, often using Minimap2 to map reads.

Annexure IIID Recommended Bioinformatics Tools for Resistome Analysis

1. Quality filtering and trimming: fastp or Trimmomatic.
2. Assembly: MEGAHIT or SPAdes[9].
3. ARG annotation: Comprehensive Antibiotic Resistance Database (CARD), ResFinder, ARG-ANNOT.
4. Taxonomic profiling: Kraken2 or MetaPhlAn.
5. Mobilome (MGE) analysis: PlasFlow or MobileElementFinder.
6. Visualisation: Heatmaps of ARG abundance and co-occurrence networks with mobile genetic elements.

[9] MEGAHIT (Megagenome Assembler) and SPAdes (specifically metaSPAdes) are two of the most widely used *de novo* metagenomic assemblers for sequencing data.

List of Acronyms

AFP	Acute flaccid paralysis
$Al(OH)_3$	Aluminum hydroxide
$AlCl_3$	Aluminum chloride
AMR	Antimicrobial resistance
APHL	Association of Public Health Laboratories
ARG	Antimicrobial resistance gene
AST	Antimicrobial susceptibility testing
ASTHO	Association of State and Territorial Health Officials
BCoV	Bovine coronavirus
BSC	Biosafety cabinet
BSL	Biosafety level
BMWM	Bio-medical Waste Management Rules
CDC	United States Centers for Disease Control and Prevention
CCS	Circular consensus sequencing
cDNA	Complementary deoxyribonucleic acid
CHIKV	Chikungunya virus
CLSI	Clinical and Laboratory Standards Institute
COVID-19	Coronavirus disease 2019
CPHEEO	Central Public Health and Environmental Engineering Organisation
CT	Cycle threshold
cVDPV	Circulating vaccine-derived poliovirus
ddPCR	Digital droplet polymerase chain reaction
DENV	Dengue virus
dMIQE	Digital MIQE guidelines
dNTPs	Deoxynucleotide triphosphates
DNA	Deoxyribonucleic acid
dPCR	Digital polymerase chain reaction
E. coli	*Escherichia coli*
ESBL	Extended-spectrum beta-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing

FAO	Food and Agriculture Organization
GC	Genome copies
GEPI	Global Polio Eradication Initiative
GI	Gastrointestinal / Genogroup I (for norovirus)
GII	Genogroup II (for norovirus)
HAdV-40/41	Human adenovirus type 40/41
HAV	Hepatitis A virus
HEV	Hepatitis E virus
HiFi reads	High-fidelity sequencing reads
ICMR	Indian Council of Medical Research
IDSP	Integrated Disease Surveillance Programme
IEC	Institutional Ethics Committee
INSACOG	Indian SARS-CoV-2 Genomics Consortium
IPC	Infection prevention and control
IPV	Inactivated poliovirus vaccine
IVA/IVB	Influenza A virus / Influenza B virus
LOD	Limit of detection
LOQ	Limit of quantification
MHV	Murine hepatitis virus
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
MMR	Measles, mumps and rubella
MNV	Murine norovirus
MoEFCC	Ministry of Environment, Forest and Climate Change
MoHFW	Ministry of Health and Family Welfare
MPXV	Mpox virus (formerly monkeypox virus)
MRC	Matrix recovery control
MS2	Bacteriophage MS2
MWCO	Molecular weight cut-off
NAP-AMR	National Action Plan on Antimicrobial Resistance
NASEM	National Academies of Sciences, Engineering, and Medicine
NCDC	National Centre for Disease Control
NGS	Next-generation sequencing

NMCG	National Mission for Clean Ganga
NTC	No template control
NWSS	National Wastewater Surveillance System (CDC)
ONT	Oxford Nanopore Technologies
OPV	Oral poliovirus vaccine
ORF	Open reading frame
PacBio	Pacific Biosciences
PCR	Polymerase chain reaction
PC	Positive control
PEG	Polyethylene glycol
PDC	Percent daily change
PMMoV	Pepper mild mottle virus
PPE	Personal protective equipment
PV	Poliovirus
QC	Quality control
QMS	Quality management system
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RNase P	Ribonuclease P
rDNA	Ribosomal deoxyribonucleic acid
RdRp	RNA-dependent RNA polymerase
Rotavirus (RV)	Rotavirus
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcription-polymerase chain reaction
RT-qPCR	Reverse transcription - quantitative polymerase chain reaction
RuV	Rubella virus
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SBS	Sequencing-by-synthesis
SMF	Skim milk flocculation
SMRT	Single-molecule real-time sequencing
STP	Sewage treatment plant
TAT	Turnaround time
TFUF	Tangential flow ultrafiltration

TSS	Total suspended solids
UF	Ultrafiltration
VIRADEL	Virus adsorption–elution method
WASH	Water, sanitation and hygiene
WBE	Wastewater-based epidemiology
WES	Wastewater and environmental surveillance
WGS	Whole genome sequencing
WHO	World Health Organization
WPV1	Wild poliovirus type 1
WWS	Wastewater surveillance
WWT	Wastewater tracking
WWTP	Wastewater treatment plant
ZIKV	Zika virus

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