Developing Measurements of Health Markers in Wastewater

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When someone uses the bathroom, they excrete bacteria, viruses, and chemical metabolite markers that reflect the state of their health. These markers end up in wastewater, which represents a pooled sample from everyone that contributed to it and can provide a way to monitor the health of that population; this practice is called wastewater-based epidemiology (WBE). Researchers and public health officials have developed different assays, which are tests to determine the quantity of health markers in wastewater such as pathogenic viruses, bacteria, and chemical metabolites (See Table 1). Assays can also be developed for specific hormones or metabolic indicators of a particular disease. In this brief, we focus on the development and validation of assays designed to detect viral and bacterial pathogens.

What is an assay, and what are the differences between a clinical and wastewater assay?

An assay is a test that is used by researchers and clinicians to detect and quantify how much of a specific component is present in a sample. Many assays for pathogen detection in clinical and wastewater samples work by recognizing and amplifying genomic signatures (e.g., DNA and RNA) unique to those target pathogens so that researchers can detect and distinguish them from the vast background of other microbes in a sample. The assays designed for clinical use might only work for a particular type of sample (that is, urine, feces, sputum, or saliva) and not be able to tolerate the wide variety of compounds that end up in wastewater. Assays developed for wastewater must be sufficiently robust to resist potentially inhibitory substances that may interfere with the detection of a given target in the complex wastewater matrix. Clinical samples also often have relatively few types of organisms present compared with wastewater, so assays suitable for wastewater must be able to detect and distinguish pathogens among a more diverse background of molecules. Clinical assays frequently can be applied to wastewater samples, but this desirable outcome is not a given and may require assay modification followed by empirical testing and validation. Challenges in validating assays for wastewater arise from the need for having access to the pathogen of interest (as a positive control) and a target-free wastewater sample (negative control) that then can be fortified with the pathogen to evaluate assay detection limits. Absent of a standard of the pathogen of concern, experimentalists may resort to collecting wastewater from a location (for example, daycare, school, or a long-term care facility) with an active, known outbreak.

What makes for a good target (or biomarker) when monitoring diseases in wastewater?

- It's excreted in urine or stool in concentrations high enough to enable detection
- It's stable in wastewater (i.e., it's not appreciably degraded during transport in the sewer)
- It's directly related to infection, disease, or the human behavior monitored

How do you develop successful assays?

A reliable assay is **specific** (few false positives), is **sensitive** (few false negatives), and yields **timely results**. Assays based on amplifying targeted genetic markers (for example, PCR-based assays such as RT- qPCR/ddPCR) align well with those criteria. Yet these molecular assays require prior knowledge about the genomes of targeted pathogens, their variants, and all close relatives. The assays being developed must also be tested against as many known closely related organisms or viruses that are non-pathogenic, non-targeted, or both, to determine specificity. This way, the assay will reliably be interpreted as detecting and quantifying the targeted pathogen and not a similar but non-pathogenic cousin. Another important practice to implement when developing an assay is to use internal

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controls. These controls can be either cultivated target organisms or an analogous organism with distinct genetic targets but similar properties that are added to wastewater samples in a known concentration. When added just prior to the detection step, they can provide assurance that the assay is providing positive results when expected. When added before sample manipulation (e.g., filtration of samples to concentrate a target), they can provide important information on the recovery efficiency, consistency, and sensitivity of the assay.

Repurposing existing assays

There are many assays designed to detect known pathogens in individual clinical patients or other environmental settings (e.g., in contaminated food or processing facilities). The development and validation of these assays can be leveraged by repurposing them for WBE. This was almost universally done with WBE for SARS CoV-2 by using the existing assays approved by the Centers for Disease Control and Prevention and World Health Organization. The genomes of many SARS CoV-2 viruses from patient samples were sequenced and multiple unique regions were identified, and the resulting assays were tested for specificity and sensitivity. Building on previously developed clinical assays thus can accelerate the successful deployment of WBE assays for new threats: for example, clinical test kits applied to pre-processed (i.e., concentrated/purified) wastewater can increase assay efficacy, sample throughput, assay availability and scale-up.

Experience gathered during the COVID-19 pandemic can serve as a WBE road map for developing and validating novel assays

The goal of developing or adopting an assay for WBE is to collect actionable information where it does not otherwise exist. An ideal assay is sensitive to a low number of targets, specific for the pathogen and its variants, and provides a quantifiable signal that corresponds proportionally to target concentration (e.g., SARS-

Table 1. Characteristics of assays used for different wastewater targets

Target	Type	Assays	Maturity
Respiratory disease targets			
SARS-CoV-2 and variants	RNA virus	RT-qPCR, RT-ddPCR, targeted amplicon sequencing	High
Influenza	RNA virus	RT-qPCR, RT-ddPCR	Low/Mid
Respiratory syncytial virus	RNA virus	RT-qPCR, RT-ddPCR	Low
Enteric disease targets			
Norovirus	RNA virus	RT-qPCR, RT-ddPCR	High
Rotavirus	RNA virus	RT-qPCR, RT-ddPCR	Low
Salmonella	Bacteria	qPCR, ddPCR, culture	Mid
Campylobacter	Bacteria	qPCR, ddPCR, culture	Mid
Shigella toxin-producing E. coli	Bacteria	qPCR, ddPCR, culture	Mid
Vector-borne disease targets			
West Nile virus	RNA virus	RT-qPCR, RT-ddPCR	Low
Other emerging disease targets			
mpox virus	DNA virus	qPCR, ddPCR	Low/Mid
Poliovirus	RNA virus	RT-qPCR, RT-ddPCR, targeted amplicon sequencing	High
Pharmaceuticals and drugs			
Prescription drugs	Metabolite	Mass spectrometry	High
Illicit drugs	Metabolite	Mass spectrometry	High
Ingested chemicals			
Caffeine	Metabolite	Mass spectrometry	High
Nicotine	Metabolite	Mass spectrometry	High
Human biomarkers			
Creatinine	Metabolite	Mass spectrometry	Mid
crAssphage	DNA virus	qPCR, ddPCR	Mid
Stress hormones	Metabolite	Mass spectrometry	Mid

CoV-2 virus counts indicative of infected people). The COVID-19 pandemic was unique in that individual testing was, at least for a period, widespread for symptomatic and non-symptomatic people. The value of WBE for COVID-19 was evident early in the pandemic, when it was possible to compare the concentration of SARS-CoV-2 in wastewater and the number of recorded infections within sample sizes ranging from individual buildings to wastewater treatment facilities serving hundreds of thousands of people. The pandemic provided the unusual duality of testing approaches, allowing the opportunity for WBE assays to be optimized for representative sampling, efficient extraction of genetic material, and the comparison of viral concentrations in wastewater to numbers of cases.

The connection between WBE and individual testing data has continued to shift throughout the pandemic. Currently, fewer tests are being administered, with a bias toward confirming likely infections. At home testing now increasingly supplants clinical testing efforts. As such, WBE for COVID-19 has become a primary source of disease surveillance at the population level.

It is important to align wastewater pathogen assay data with more traditional surveillance data, which include individual testing, syndromic monitoring from clinics, hospital admissions, and deaths. Integrating wastewater data with other traditional metrics can improve predictions of disease prevalence, rapidly identify communities experiencing active outbreaks and those coming out of infection surges, and improve the equitable and timely allocation of public health resources.

Potential roles for pathogen assays applicable to wastewater

Beyond the current pandemic, additional pathogen assays are beginning to become more prevalent (see Table 1). Without widespread individual testing, wastewater monitoring can provide valuable early warnings for new outbreaks of pathogens, such as mpox and poliovirus. Endemic or more common or seasonal diseases can also be monitored, thereby allowing public health entities to forewarn local hospitals and clinics about their prevalence and projected hospital bed capacity needs. More explorative, less directed approaches that involve high throughput sequencing of wastewater can provide research scientists and public health officials with a new lens to monitor emerging pathogens, variants, and novel threats (new genetic targets) that may be observed in wastewater before infected individuals present in healthcare settings for treatment.

Conclusions

The widespread adoption of WBE worldwide in response to the ongoing COVID-19 pandemic has accelerated the development of additional assays that can monitor human health at the population level. Table 1 lists the assays for various targets of human health and their characteristics. For a more extensive list, see Adhikari and Halden (2022). Aside from the ongoing pandemic and successive outbreaks caused by new variants, researchers can monitor other pathogens where only syndromic or limited data are available. Normally, people would have to seek health care and be tested to identify an incidence or outbreak of disease. By applying several assays for specific pathogens, public health entities can monitor the presence and spread of specific pathogens across geographic space and time.

Assays developed or adopted for use with wastewater can also help address an outbreak known only through syndromic data. For example, gastrointestinal distress could be linked to bacterial pathogens (such as *Campylobacter, Escherichia,* or *Salmonella*) or instead by viral pathogens (such as Norovirus). Infection dynamics and treatment options differ between pathogens (e.g., bacteria, viruses, fungi), as do the sources and transmission routes for the associated infections, which can inform strategies for testing for contaminated food or environments. For example, an increase in respiratory illnesses in a school or school district may be quickly identified as, for example, another outbreak of SARS-CoV-2, seasonal influenza, or RSV. Broadening the repertoire of assays suitable for WBE and refining the case for their uses will provide public health officials with better, more informative data on which to base best mitigation decisions.

References

Adhikari, S., and R.U. Halden. "Opportunities and limits of wastewater-based epidemiology for tracking global health and attainment of UN sustainable development goals." *Environment International,* vol. 163, 2022.

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