Wastewater and Environmental Surveillance Summary for a Combined Suite of Respiratory Viruses

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World Health Organization

This document provides information on wastewater and environmental surveillance (WES) for respiratory panel viruses. It should be used together with the accompanying *WES Guidance for one or more pathogens* which includes general and cross-cutting information, and the target sheets for SARS-CoV-2 and influenza viruses (available here).

WES for a combined suite of respiratory viruses at a glance

- Respiratory viruses are of paramount global public health significance both due to their seasonal drifts and cycles of infection, and the pandemic potential of shifts from both human and some zoonotic viruses.
- In sewered settings multi-target WES for three or more respiratory viruses has been shown to be technically feasible. For the most common example, SARS-CoV-2, IVA/IVB and RSV, it is evaluated as high for actionability, operational feasibility, and integration as part of the disease response, and as part of broader multitarget WES, and moderate in terms of acceptability.
- There is insufficient evidence to evaluate its use in non-sewered settings.

Table 1: At a glance assessment of key WES criteria for combined suite of respiratory viruses (sewered and non-sewered) for the combination of SARS-CoV-2, IVA/IVB and RSV[a,b]

Setting	Categorical Assessment (CA) / Strength of Evidence (SoE)	Public Health Significance	Actionability / Relative value	Technical Feasibility	Operational Feasibility	Acceptability [c]	Optimisation	
							Integrated disease response	Multitarget WES
Sewered	CA	High	High	High	High	Intermediate	High	High
	SoE	Strong	Moderate	Strong	Strong	Moderate	Moderate	Strong
Non-sewered	CA	not separated by sewered category	Low	High	Intermediate	Intermediate	Low	Intermediate
	SoE		Inadequate evidence	Inadequate evidence	Inadequate evidence	Inadequate evidence	Inadequate evidence	Inadequate evidence

Key:

1. Categorical Assessment (CA) of criteria

Category	Code	Description
High		Criteria is evaluated as met at the highest level
Intermediate		Criteria is evaluated as met at an intermediate level (it may be that not all sub-components of the criteria are met)
Low		Criteria is evaluated as low
Not-supported		Criteria is evaluated as not supported
Not applicable		Criteria is not applicable OR cannot assessed due to inadequate evidence

2. Strength of evidence (SOE)

Evidence level	Code	Description
Strong		High quality consistent evidence, including from multiple relevant studies/settings, at scale, over a prolonged period, with evidence from program settings, not only from research studies or short projects.
Moderate		Relevant evidence is available but does not meet criteria for 'Strong' classification.[d]
Inadequate evidence		Evidence is inadequate and further study/evaluation is needed

[a] Further description of the criteria used to assess the applicability of WES for a specific pathogen, as well as the methods used to evaluate them, is included in *WES Guidance for one or more pathogens*. The assessment in Table 1 provides a snapshot at the global level, but country level assessment may differ.

[b] Sewered settings refers to closed reticulated sewage systems. Non-sewered settings refers to the diverse settings which are not 'sewered', including open drains and community sampling points. Individual small septic tanks at residential or building level are not viable to sample individually and are not considered here separately. Most WES evidence to date is reported from reticulated sewered settings, often from high-income settings. Yet much of the global population is on heterogenous non-sewered systems and this has implications for assessment of various WES categories.

[c] Experts did not achieve consensus on the assessment of these criteria. The majority view is shown here, with others evaluating both higher and lower.

[d] Evidence classified as 'Moderate' meets one or more of the following criteria: not from numerous settings, for a short period, without program-level evidence, and/or where findings are not consistent or of high quality.

Summary

Key features of WES for multiple respiratory viruses

- Respiratory viruses are a highly significant ongoing global public health threat with very significant global pandemic potential. The viruses have the potential to rapidly evolve and spread globally, within periods of weeks. Seasonal drifts in these pathogens allows them to evade prior immunity, whether natural or vaccine-derived, with new viruses circulating annually and, in some cases, seasonally (e.g. outside of tropical areas).
- All are predominantly human-to-human respiratory pathogens, but some types of influenza and corona viruses can be transmitted to humans from animals, leading to new pandemics. Some of these pathogens can spillover from wild and domestic animals that can cause human infections and, in some cases, zoonotic and human pandemics.
- The pathogens are typically vaccine-preventable, but most vaccines are targeted to high-risk groups, or during outbreaks. Immunity once acquired is limited, often to one season, due to viral mutations, with vaccines needing to be modified seasonally in many cases.
- Global, regional and national agencies have disease monitoring and management programs, based on clinical testing and notification, which WES could support. WES for respiratory viruses should be undertaken in the context those broader surveillance efforts.
- There is good experience testing sewage for WES for multiple respiratory viruses, but not testing environmental waters. Most experience builds on that from SARS-CoV-2 as a target.
- Some high-income countries have integrated the monitoring of multiple respiratory viruses in sewered settings. This provides evidence of operational feasibility to use WES to measure changes in circulating levels and genetic lineages of the viruses.
- Studies show varying correlations between WES and clinical results, with WES signals typically leading clinical signals of annual or seasonal diseases by 1 to 3 weeks, and lagging by several months. Correlations are improved when virus prevalence and concentrations are elevated.
- Whilst no standard methods having emerged, some standardized methods have been developed, including both open source and commercial kits, for SARS-CoV-2, IAV/IBV and RSV.
- Most studies collect samples from raw or primary liquid wastewater, and some collect samples of solids, from wastewater treatment plants. Environmental waters in non-sewered areas are not well-studied for multiple target respiratory virus WES.
- There are some examples of WES being utilized to inform public health actions. No universal triggers for public health action have been developed, but studies suggest ways for establishing a baseline, and then a local threshold for public health action.
- Routine multiple respiratory virus WES can be incorporated into existing WES programs since additional viruses can be readily monitored at low marginal cost simply by testing the viral nucleic acid extracts from standard WES workflows, including for SARS-CoV-2 and poliovirus.
- Key questions to test with future research are:
    - In addition to what has emerged as the core suite (SARS-CoV-2, IAV/IBV and RSV), what are the next tier priorities among the many respiratory viruses that could be targeted?
    - What are the preferred sampling, analysis and bioinformatics workflows and how sensitive and specific are they?
    - What are the demonstrated health-impactful use cases to respond to WES evidence?

- To what extent do animal inputs hamper interpretation of IAV WES?

Key considerations relating to WES for multiple respiratory pathogens

Consideration	Suggestion
Sampling sites	*In priority order (from high to low priority):* • *Existing WES sites as used for poliovirus or SARS-CoV-2.* • *Inlets to major sentinel wastewater treatment works.* • *Possibly (but not proven) sentinel sites in non-sewered systems (i.e. gathering points).* • *Possibly (but not proven) environmental waters heavily influenced by human waste.*

Sampling approach	*Sampling should occur frequently, ideally daily, to provide early warning of the onset of seasonal diseases, since the peak can rise rapidly, rendering weekly insufficient for reliable early warning. Lower frequencies, such as weekly are sufficient for monitoring broad seasonal trends and detecting tails back towards baseline since these occur more slowly.*
	In order of preference (from high to low preference):
	• *Composite sampling of liquid wastewater or sludge is preferred:*
	  ○ *flow weighted automatic sampling of wastewater*
	  ○ *compositing serial grabs of wastewater or sludge*
	  ○ *passive 'Moore Swab' style samplers*
	• *Grab samples*

Transport and storage	*Conventional cold chain, for up to one week. Storage at warmer temperatures, and freeze-thaw, differentially degrades detectable concentrations of viruses and normalization markers so may introduce biases.*
Analytical methods	*For cost-effectiveness, broad virus groups can form a baseline, to provide early warning of rises in virus concentrations, with typing as part of an agile response. For viruses that may infect of other organ systems, respiratory types should be specifically targeted.*

Utilisation of WES evidence	*The principal use cases for WES for respiratory viruses are:*
	• *Provide early warning (1 to 3 weeks) of the onset of the rising phase of seasonal trends, and in turn:*
	  ○ *Guiding the timing of onset of seasonal vaccination programs, and their priority locations.*
	  ○ *Prepare healthcare facilities for rising cases.*
	• *Guide the choice of pathogens to test in clinical samples.*
	• *Alert the public to periods and locations of elevated viral circulation to inform decisions on protective behaviors.*
	• *Monitor trends to evaluate interventions.*

Contents

1 General information

1.1 The viruses, associated diseases, and risk factors

The most recent WES studies targeting multiple respiratory viruses, as summarized in section 2, highlighted three viruses as the most commonly tested. These are listed here in order of the number of reports of their inclusion:

- severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2);
- influenza A and B virus (IAV and IBV), (with IAV more commonly included then IBV); and
- respiratory syncytial virus (RSV).

This indicates that these three viruses have been identified in many contexts as being the highest priority for WES studies targeting respiratory viruses. However, these are not the only respiratory viruses, and one or more WES studies have targeted others, including (in no particular order):

- seasonal human coronavirus (HCoV) (not including SARS-CoV-2);
- human rhinovirus (HRV);
- human meta-pneumovirus (HMPV);
- human parainfluenza virus (HPIV);
- parvovirus (PV);
- bocavirus (BV);
- respiratory mastadenoviruses (MAV); and
- respiratory enteroviruses (EV).

There are a range of definitions currently used for respiratory illness. For instance, the US CDC National Syndromic Surveillance Program (NSSP)[i] uses an acute respiratory illness (ARI) metric that does not necessarily include symptoms of fever, i.e. a broad definition capturing symptomatic infections of the lower or upper respiratory tract, noting that these can also result in systemic symptoms and secondary infections. This captures a broader range of diagnoses than the previous influenza-like illness (ILI) definition that included fever. As a result, this includes influenza, RSV and COVID-19, as well as non-pyrogenic diseases, such as the 'common cold'. Risk factors centre around airborne person-to-person spread. The similar and overlapping symptoms and risk factors for these diseases means that from a public health perspective they are often grouped, and hence it is logical to group them for WES programs.

1.2 Global burden and geographic distribution

Collectively, acute respiratory illnesses are the leading cause of morbidity and mortality globally in children between their neonatal and under five years.[1] They are distributed globally and typically occur in seasonal patterns.

[i] https://www.cdc.gov/nssp/index.html

1.3 Routes of transmission

Respiratory viruses are shed in high concentrations in nasopharyngeal secreta discharged via the nose and mouth through exhalation, exacerbated by the sneezing and coughing reflexes triggered during symptomatic infection. The viruses are primarily spread via airborne pathways, person-to-person.

1.4 Zoonotic hosts and potential reservoirs

Most of the respiratory viruses infecting humans are not typically zoonotic. However, zoonoses can arise through mutations occurring resulting in viruses circulating in animals becoming infectious to and circulating between humans (as reported for some coronaviruses and influenza viruses). Some zoonotic respiratory viruses (such as some influenza virus strains) can infect humans, albeit typically only sporadically. Of more importance from a WES perspective, the specificity of WES targets needs to consider that some respiratory viruses, particularly influenza viruses, are common in animals, and may enter wastewater and environmental waters from animal sources, including direct contributions from animals and their waste, as well as animal products such as meat or milk. This needs to be considered in designing WES analytical and bioinformatic methods. Further testing can help to identify the mix of viruses present, which may help determine whether they are more commonly associated with animal or human sources.

1.5 Human pandemic potential

Due to their rapid person-to-person transmission, and high frequency of mutations leading to the ability to evade immunity from previous vaccination, acute respiratory viruses have high pandemic potential, with seasonal cycles of global pandemic spread being observed annually for multiple respiratory viruses.

2 Respiratory viruses and wastewater and environmental waters

2.1 Potential inputs to wastewater and environmental waters

Respiratory viruses are shed primarily via nasopharyngeal secreta, with the extent of shedding in faeces varying between viruses, and during infections. Shedding patterns vary between viruses, but typically the viruses are shed for one to some days prior to symptom onset, and for one to some weeks following symptom cessation. Virus concentrations reported in wastewater are typically lower than those for faecal-oral pathogens, with results sometimes being below limits of detection (approximately 10 gc/L of wastewater or 1,000 gc/g of solids) until the start of the rising phase of seasonal disease trends, and peaking at up to 1 million gc/L or gc/g of wastewater and solids, respectively, for most viruses responsible for ARI, albeit lower peaks typically reported for viruses other than SARS-CoV-2.[2,3] The higher typical concentrations of SARS-CoV-2 is important as it may mean that experience with WES for SARS-CoV-2 is more favorable than experience with other less numerous ARI viruses.

Some respiratory viruses, such as IAV/IBV, may be present in wastewater and environmental waters from animal waste, including wild and domesticated animals. This needs to be considered when assessing the specificity of assays.

2.2 Target persistence, degradation and risk of infectious virus

Multiple viruses associated with ARI have been routinely simultaneously detectable in wastewater samples collected in many countries, including high and low income countries in Asia,[4,5] Europe,[2,6–8] United States,[3,9,10] and Canada.[11] Based on these and their cited studies, the viral genetic targets, and in some cases proteins, are sufficiently robust when processed under common workflows that WES is technically achievable under the conditions and transit time periods associated with diverse sanitation systems across different climate zones. This, combined with the elevated levels of viral targets during seasonal disease peaks, and available analytic methods, make these viruses technically highly feasible targets for WES.

In some studies the persistence of respiratory viral RNA and DNA in wastewater and solids has been systematically assessed, with results supporting its persistence as being long enough for WES to be technically achievable, even at ambient temperatures. For instance:

- In wastewater, the concentration of RNA from indigenous SARS-CoV-2, RSV, IAV/IBV and PMMoV, was monitored upon receipt and then after three and six days at two temperatures (4°C and ambient temperature) and following freeze-thaw at -20˚C in sewage samples.[12] At both 4°C and ambient temperature the concentration of extractable, detectable, and quantifiable SARS-CoV-2 RNA concentrations were stable, and RSV RNA did not drop significantly. However, at -20˚C the concentration of SARS-CoV-2 RNA was statistically significantly reduced (by 1-2 $\log_{10}$), and there was evidence of reduced RSV RNA concentrations. This was possibly linked to the freeze-thaw effect. The normalisation marker PMMoV decay was negligible under all conditions, which may lead to an underestimation of pathogenic virus shedding by the population when evaluating sewage subjected to freeze-thaw. The IAV/IBV levels were too low

to provide reliable data on stability. Although included as part of the assessment of enteric and not respiratory viruses, it was noted that AdV DNA was stable under all conditions.

- In solids, the concentration of RNA from spiked SARS-CoV-2, RSV, HCoV, HRV, and IAV, (along with indigenous PMMoV), was monitored for 50 days at three temperatures (4°C, 22°C, and 37°C) in sewage sludge.[13] At all temperatures the reduction in extractable, detectable, and quantifiable RNA concentrations was 0 to 20% per day, (with one day being approximately the typical residence time of sewage in small to mid-sized piped sewerage systems). Inactivation followed classical first-order kinetics with daily decay rate constants (k) varying from 0 at 4˚C, to 0.2 at 37˚C, and one $\log_{10}$ reduction occurring after approximately > 50, 30, and 10 days at 4, 22, and 37˚C, respectively. The normalisation marker PMMoV decay was negligible even after 50 days, which may lead to an underestimation of pathogenic virus shedding by the population when evaluating aged sewage.

Whilst all targets are stable enough for WES to be technically feasible for all respiratory viral pathogens evaluated, the differences in persistence between the RNA and DNA of various pathogenic viruses, between pathogens and normalization markers, and under different storage and freeze-thaw conditions, have implications for selecting methods for sample storage and transport. These differences mean that workflows validated for more robust viruses may not be as sensitive for less robust pathogens. These differences can also bias comparisons made between:

- studies not using the same end-to-end workflows and methods;
- different target viruses within the same studies; and
- pathogens compared to normalization markers.

2.3 Respiratory virus WES experience

Several multi-target WES studies have been reported, undertaken as pilot or experimental WES studies, although not as part of routine, broader, integrated public health surveillance programs. For instance:

- Frozen samples of primary settled sludge collected as 24-hour composites thrice-weekly from a large (1.5 m people) WWTP in California, United States, were retrospectively tested for IAV/IBV, RSV A/B, HPIV 1–4, HRV, seasonal HCoV, and HMPV, over a 17-month period, and tested using probe-based RT-dPCR.[3] PMMoV was used as a normalization marker. For the viruses that were commonly detected in wastewater, statistically significant positive correlations were found between one another, and between the reported clinical tests and the concentrations of viral RNA in wastewater. The correlation between viruses may be related to the common general determinants of respiratory transmission. For instance, there was evidence of reduced concentrations of all the commonly detected viruses in wastewater following the SARS-CoV-2 omicron BA.1 transmission surge which was related to changes in human behavior.
- Raw sewage samples collected as 24-hour composites weekly to monthly over two years from four WWTPs in Northern Tuscany, Italy, were tested for SARS-CoV-2, HAdV, RSV A/B, and IAV/IBV, to evaluate whether WES provided value to complement clinical surveillance.[6] The workflow included centrifugation, PEG extraction, kit-based nucleic acid extraction, PCR inhibitor removal, and PCR, followed by typing. For SARS-CoV-2, WES correlated poorly with clinical and hospitalization data due to WES detecting circulation during periods of minimal

clinical presentation, possibly due to Omicron having less severity and/or a reduction in clinical testing. In contrast, IAV was not detected despite clinical presentation, which was attributed to poor method sensitivity. Most HAdVs were type F41, which was not respiratory, highlighting the need to improve specificity by monitoring specific types and not just total viruses to understand respiratory threats. Poor correlation between WES and clinical RSV data was possibly due to the low quantity and relevance of the clinical data.

- Raw sewage samples were collected over six months from WWTPs in Xi'an city, China, and RT-qPCR was used to determine concentrations of SARS-CoV-2, IAV, IBV, RSV, hantavirus, and norovirus.[4] The trends in concentrations in sewage for four targets (SARS-CoV-2, IAV, RSV, and hantavirus) aligned with sentinel hospital percent positivity data. Biweekly sequencing of SARS-CoV-2, norovirus and hantavirus identified circulating genotypes. Given the limitations in clinical surveillance, the wastewater surveillance was considered potentially more informative of seasonal trends and genotypes.
- Untreated raw sewage samples from two WWTPs in the Kathmandu Valley, Nepal, were tested over seven months for SARS-CoV-2, IAV, RSV and tested using eight workflows.[5] The preferred workflow involved simple centrifugation, target nucleic acid protection, and kit-based extraction, followed by RT-qPCR. All three viruses were detected and quantified, with some variants being characterized.
- Untreated raw sewage from 10 WWTPs in Finland was tested over a two-year period for multiple pathogens and their variants.[7] Respiratory pathogen targets included SARS-CoV-2, IAV, RSV, HCoV, HMPV, MAV, and HRV, with PMMoV and CrAssphage included as normalization markers. There was no actionable public health objective for the project, but rather broader objectives were identified. These included conducting a trial of the multi-pathogen WES approach, supporting pandemic preparedness, raising awareness among the public, engaging with stakeholders, and providing experience in multi-pathogen infectious disease surveillance.
- Untreated 24 h composite raw sewage samples were collected twice-weekly from eight WWTPs in Germany over 12 months and tested for IAV, IBV, and RSV.[2] The WES trends were statistically significantly correlated with those of clinical cases. However, the sensitivity of the WES methods were too low to provide a reliable means of early warning ahead of clinical surveillance when cases were rising from low levels. The implications are that to provide value as an early warning indicator during periods of low virus circulation in areas that already have sensitive clinical surveillance, WES sensitivity for the viruses evaluated needs to be improved.
- Untreated raw sewage 24 h composite samples were collected up to five times per week from up to ten WWTPs in Switzerland over 3.5 years and tested for SARS-CoV-2 (throughout the study period) and RSV and IAV/IBV (for just under half of the study period).[8] Pharmaceuticals commonly used for symptom relief were monitored from some of the same sample points, albeit at a lower frequency. The concentrations of the targeted viruses in wastewater correlated well with both clinical case reports and with levels of relevant pharmaceuticals during most of the study period. However, during some periods increases in pharmaceuticals in wastewater were not correlated with increases in the target viruses, which was attributed to infections with pathogens that were reported at elevated levels from clinical testing but that were not targeted by WES. One implication is that due to the diversity of common respiratory pathogens, integrated surveillance of multiple pathogens, more than just SARS-CoV-2, IAV/IBV and RSV, is

necessary for WES to cover all significant causes of respiratory symptoms and associated pharmaceutical use.

- Untreated raw sewage samples were collected from three WWTPs in Saskatchewan, Canada, over six months and tested using an end-to-end portable RT-qPCR detection kit for SARS-CoV-2, IAV, IBV, and RSV.[11] The concentrations of viruses from the WES study were statistically significantly correlated with clinical cases for SARS-CoV-2 with a lag time of 4 days, and for IAV with a lead time of 10 days. There were insufficient detections of IBV and RSV during this study to assess relationships. The implications are that portable kit-form end-to-end WES methods have the potential to provide WES evidence in locations without access to laboratories.
- Untreated raw sewage samples were collected weekly from a WWTP in Valencia, Spain, over 2.5 years and tested using RT-qPCR for SARS-CoV-2, RSV, and IAV, along with normalization markers PMMoV, crAssphage, and somatic coliphages.[14] The clinical data for the respiratory viruses strongly correlated with their concentrations in wastewater after normalization based on WWTP inflow or physico-chemical markers, less well when using PMMoV, and showed weak correlations when normalizing using crAssphage or somatic coliphages. The inflow-normalized correlations were sufficient for WES to demonstrate potential for early warning of each of the three pathogenic viruses, with one week being the optimal lead time. The differential correlations achieved using different normalization markers demonstrate the importance of evaluating and selecting a suitable normalization approach.
- Untreated raw sewage samples were collected weekly from WWTPs in three cities (one WWTP in two cities, and two in a third) in Wisconson, United States, over nine months and tested using RT-qPCR for RSV and IAV.[9] The testing was undertaken as part of the US CDC National Wastewater Surveillance System (NWSS) (https://www.cdc.gov/nwss/index.html), that also included SARS-CoV-2 and the normalization marker PMMoV. The clinical data from emergency department visits for IAV and RSV statistically significantly correlated with their concentrations in wastewater, being stronger for IAV than RSV. The WES signal showed both a lead (variable) and lag (up to three months) relative to the clinical data. The authors recommended more frequent than weekly testing in future studies seeking to provide a reliable lead indicator, with daily testing recommended to detect the rising phase of seasonal disease trends early. No actionable public health decisions were directly related to the WES data, but it was considered complementary, and useful to inform disease patterns, such as the start of seasonal diseases.
- Primary settled sludge collected as 24-hour composites 2-7 times per week from 175 WWTPs from 36 states across the United States were tested for SARS-CoV02, IAV/IBV, RSV, and HMPV, over a 12-month period, and tested using probe-based RT-dPCR.[10] PMMoV was used as a normalization marker. Based on an analysis of their data, the authors proposed state-based trigger levels that indicated the seasonal onset for the rising, peak, and falling phases of each disease. For instance, a rising phase trigger level was the consistent detection over 14 days across the state of ≥ 2,000 gc/g solids (double the assay sensitivity). The authors demonstrated significant differences between the timing of seasonal trends for different states and diseases. Correlations with clinical evidence were not provided, but the authors noted that clinical data was reporting the more severe symptomatic infections in humans, whereas WES measured viral shedding from the whole human population, and for IAV, animals whose waste goes to sewer.

There was one report of routine WES providing actionable data when undertaken as part of integrated surveillance for RSV. Whilst not integrated with other respiratory WES targets, it is mentioned here as it

was not covered in the related SARS-CoV-2 and IAV/IBV summaries. Untreated raw 24 h composite sludge samples were collected from 5 to 7 times per week from one WWTP in each of the cities Ottawa and Hamilton, Canada, over nine months and tested using RT-dPCR for RSV, along with normalization marker PMMoV.[15] The relationship between the WES and clinical trends were different in the two cities, both in the rising and falling phase of the season. The WES data provided a lead indicator of the start of the RSV season of 36 and 12 days for the two cities, respectively, when compared with data from clinical test results from the community and hospitalization cases. The trends also differed in the falling phase. These differences were attributed to variations between the cities in how the seasonal RSV infections spread and the age and immunological status of the populations affected. The public health actions triggered by the WES data included initiating seasonal immunoprophylaxis programs, and preparing hospitals for increased presentations.

Key observations from these studies that are of importance when designing WES for ARI including the following:

- The WES analytical approach should have sufficient specificity to target the virus genotypes that typically present as 'respiratory' if the objective is to provide information on respiratory infections since there may be similar viruses that infect other body systems and create other symptoms. Examples include EV and MAV.[6]
- The WES analytical approach should target the virus genotypes that typically infect humans if the objective is to provide information on human infections since there may be if similar viruses that infect animals. Examples include zoonotic IAV strains.[10]
- Normalisation needs to be undertaken intelligently and is more challenging when targeting multiple viruses. For instance, PMMoV is either as stable, or more stable, than most respiratory viruses, when exposed to freeze-thaw or warm storage, with the relative stabilities differing between viral targets.[12] The choice of normalisation approach can lead to biases that differ between viral targets and these need to be understood.
- For WES to provide an actionable lead indicator for ARI pathogens, the rising phase of seasonal cycles can be too fast for weekly or monthly sampling frequencies to be optimal, and daily frequencies are preferred to provide early warning of seasonal onset.[9]
- There is a high degree of confidence from multiple studies (cited above) that data from WES trends *typically* both lead and lag clinical data. However, the specific relationships between WES and clinical data can be markedly different between locations, pathogens, and seasons. Differences in relationships include whether and to what extent the WES data leads and/or lags clinical data, and how the WES and clinical datasets correlate during the rise, peak, and tail of those seasonal trends. As a broad observation, the WES signal leads the clinical by one to two weeks, and WES lags clinical by months. However, no one generalisable pattern can be consistently described to set WES early warning lead times, WES actionable trigger levels, or other information on trends. Data from routine WES for ARI pathogens needs to be interpreted intelligently, as part of integrated surveillance, for the specific populations covered by the sample locations, specific to the methods used, and based on historical data. Nonetheless, trigger levels can be established based on analysis of sufficiently large datasets,[15] and public health actions can be triggered by WES data.[15]

3 Respiratory virus surveillance

3.1 Overall respiratory virus surveillance and response

Syndromic public health surveillance for respiratory illness is passive, as it is informed using a body of data obtained from clinical presentations. This includes presentations at general healthcare facilities and hospital emergency wards for respiratory illnesses.

The expanded Global Influenza Surveillance and Response System (eGISRS) provides a global platform for surveillance, preparedness, and response for influenza, SARS-CoV-2 and RSV. The WHO Coronavirus Laboratory Network (CoViNet) provides similar support dedicated to SARS-CoV-2.

Case-based respiratory virus surveillance (human, and for some viruses animal) provide critical epidemiologic, clinical and virologic information essential to meet priority surveillance objectives of detection and assessing risk, monitoring epidemiologic and virologic characteristics, and assessing prevention and control measures at an individual level along with specimens needed for vaccine strain selection.

There are a range of definitions currently used for respiratory illness. For instance, the US CDC National Syndromic Surveillance Program (NSSP)[ii] uses an ARI metric that does not necessarily include symptoms of fever, i.e. a broad definition capturing symptomatic infections of the lower or upper respiratory tract, noting that these can also result in systemic symptoms and secondary infections. This captures a broader range of diagnoses than the previous influenza-like illness (ILI) definition that included fever. As a result, this includes influenza, RSV and COVID-19, as well as non-pyrogenic diseases, such as the 'common cold'.

Aetiological surveillance for ARI is even less sensitive than syndromic surveillance. Clinical surveillance only diagnoses the specific aetiology of ARI in the most severe of cases, if there is perceived to be a medical reason to conduct such a test, along with the opportunity. Therefore, clinical data on the pathogens in circulation is biased towards severe cases occurring in settings with readily available diagnostic services. That leaves most infections and illness cases either undiagnosed or only identified syndromically and without an identified aetiology.

3.2 Acute respiratory illness surveillance systems and data sources

In most settings, positive clinical samples are notified to the regional and national surveillance systems. However, there is a lack of standardization in analytical laboratory methods for detecting and enumerating viruses, whether in clinical or veterinary samples. In addition, in areas where the infections are less common, tests for the viruses are less likely to be requested. This leads to challenges in comparing results where different analytical laboratory methods have been used, and likely significant under-ascertainment and reporting.

[ii] https://www.cdc.gov/nssp/index.html

4 WES objectives and related public health actions

4.1 Routine WES for respiratory viruses

At the time of writing there are a small but growing number of examples of routine WES for respiratory viruses. The largest is the US CDC National Wastewater Surveillance System (NWSS) (https://www.cdc.gov/nwss/index.html) which expanded beyond SARS-CoV-2 to cover IAV/IBV and RSV. Concentrations of all three groups of viruses in wastewater are measured and are expressed on a five-point scale from 'very low' to 'very high'. The program helps to provide early warning of periods of increased infection risk due to elevated virus circulating in the community by yielding results before increases in clinical cases are identified by capturing viruses shed from infected persons yet to present for clinical testing.

4.2 Agile (or responsive) WES for respiratory viruses

Agile surveillance has been demonstrated for respiratory pathogens. For instance, the Danish Statens Serum Institut (SSI) (https://en.ssi.dk) maintains a routine WES program that covers SARS-CoV-2, IAV/IBV and RSV, similar to the NWSS. An agile response was initiated during the 2023/24 respiratory disease peak season to include the bacterial pathogens *Bordetella pertussis* and *Mycoplasma pneumoniae* in response to epidemic levels of infection observed from clinical samples during the autumn of 2023. Increased WES for both bacterial pathogens is intended to continue until their levels reduce low enough to cease the program.

4.3 Potential public health actions arising from the addition of WES for respiratory viruses

Based on the current state of the science, WES has a proven role in identifying the onset of seasonal rises in diseases caused by respiratory viruses, which could inform public health actions, such as encouraging:

- vaccination targeting and promotion; and
- hospital preparedness.

Additional roles proposed include public health actions, such as encouraging:

- raising awareness to allow the community to allow them to reduce their risk of exposure; and
- clinical testing for specific diseases.

5 WES additional methodological considerations for respiratory viruses

This section should be read in conjunction with general methodological consideration in Section 5 of *Wastewater and environmental surveillance for one or more pathogens: Guidance on prioritization, implementation and integration* (available here).

Importantly, most of the respiratory viruses have singled-stranded RNA genomes, with most of these RNA genomes being positive-sense, and RSV and IAV/IBV being negative-sense. Atypically among the common human pathogenic viruses, MAV has a double-stranded DNA genome. Most of these viruses are enveloped, except HRV, EV, and MAV.

The commonality of structure and genome, with most respiratory viruses being enveloped RNA viruses, may be useful when adopting combined sampling and analytical workflows to simultaneously test for multiple viruses. In practice, however, as reported in section 2.2, there are some differences in both the persistence of detectable viral targets in wastewater[12] and solids[13] and of the concentration and extraction efficiency of viruses.[2]

5.1 Sampling methods

As reported in section 2.3, a variety of sample types and sampling methods have been used in WES studies targeting multiple target respiratory viruses, with most studies using either simple grab samples or 24-hour composite samples, with both wastewater and solids having been targeted. Experience with multiple target respiratory viruses is largely limited samples from WWTPs influents, and in some cases sewers, rather than unsewered contexts, such as onsite sewage management systems or open water.

5.2 Laboratory methods and interpretation

A wide range of methods have been successfully used for detecting and quantifying respiratory viruses in sewage, but in studies that compared methods, their performance was different, highlighting the value in careful method selection and optimization. For instance, using indigenous viruses in sewage samples, seven virus concentration methods (including chemical precipitation, filtration, and centrifugation) and nine RNA extraction kits (both with and without inhibitor removal) were compared for IAV/IBV and RSV A and B. For IAV and IBV five and three, respectively, probe-primer combinations were compared.[2] Large (up to 2 $\log_{10}$) and statistically significant differences were found between the various virus concentration and RNA extraction methods used. Performance was reasonably comparable between viruses for any one method. The choice of primer and probe had either no or only a small effect in this study. The study highlighted the value of comparing methods to select the most sensitive, and identified that for the viruses studied, the performance was similar such that one preferred workflow could be effectively used to test both viruses, avoiding the need to set up separate workflows for different targets.

Most studies used PCR-based methods. More recently a proof-of-concept study used biosensors detecting viral proteins to provide more rapid tests of wastewater for SARS-CoV-2, IAV, and RSV, along with caffeine for normalization[16]. The principal challenge with such rapid tests relates to their limited

sensitivity and specificity. However, in some situations, the ability to deploy such assays, at remote and even in-field locations, the simplicity of the assays, and rapid reporting of results, may make them more useful than PCR and metagenomic methods, such as in remote or resource-constrained contexts.

5.3 Reporting and communication

The principal user of WES programs that target multiple respiratory viruses is the public health and hospital sector, to help provide information on targeting the timing and location of vaccines, and preparing healthcare facilities for elevated presentations. Communication to the public can include general advice on the presence of an elevated risk of transmission, which can permit the public to take increased precautions against infection, such as mask-wearing and distancing.

In practical terms, reporting results for WES for respiratory viruses, the approach adopted is consistent with that used for SARS-CoV-2 programs. That is, for academic studies, the results are typically reported in terms of genome copies of specified targets per unit volume or mass of sample, and the information is targeted to a scientific audience. For reporting to the public and public health decision-makes, tables and timeseries plots are typically used. For instance, the United States CDC NWSS program reports results in categories, e.g. on a five-point scale from ‘very low’ to ‘very high’.

5.4 Acceptability of WES for respiratory viruses

ARIs are diseases that effect everyone, are all circulating at some level globally, and all countries that test reliably are finding cases. As such they do not carry any particular stigma. However, periods of elevated ARIs may have sociopolitical impacts. For instance, if there is publicly reported evidence of a significantly elevated level of respiratory viruses in circulation within a population, that may have implications on people’s willingness to participate in activities where they may become infected. This may deter people from travelling to particular locations, or from attending crowded places such as public transport, meeting venues, and major events. They are diseases that have high pandemic potential that may lead to impacts on business or tourism if found. Therefore, the acceptability of WES for respiratory viruses is rated as moderate.

6 Integrated surveillance and multitarget WES considerations

6.1 Integration of respiratory virus WES into existing surveillance and response

Routine, operational use of WES for multiple respiratory viruses as part of integrated public health surveillance is being demonstrated in an increasing number of contexts, including the United States NWSS and Danish SSI programs as described in section 4. These programs illustrate that WES can provide useful information as a complement to clinical surveillance, primarily for early identification of the onset of seasonal diseases. WES provides information for monitoring trends in prevalence and circulating variants, which can be integrated with, be complementary to, and add value to, public health surveillance.

6.2 Integration of multi-target WES together with respiratory viruses

Several studies and some routine operational programs have combined WES for multiple respiratory viruses with other pathogens, including non-viral respiratory pathogens, and gastrointestinal pathogens, including poliovirus. This proves that multiple respiratory viruses can be tested alongside other pathogens as part of multi-target WES, and within existing multi-modal public health surveillance programs linked to public health action.

7 Key knowledge gaps and applied research priorities

Both clinical and WES analytical methods have their own biases. This creates challenges in comparing within each discipline (i.e. comparing clinical studies with one another; or WES studies with one another) and even more so when undertaking integrated WES across disciplines (i.e. comparing clinical with WES studies).

There are no agreed standard methods for any components of the WES workflows, and the most appropriate methods to use may differ between contexts. Variables include which respiratory viruses are to be tested, what, if any, other targets are to be tested alongside the respiratory viruses, the sensitivity and specificity required for the particular use case, and the organizational, technical and financial capacity of the analytical parties involved in delivering the WES program.

In the short term the priority is to develop good practice guidance on selecting the most suitable workflows and methods, and these should be evaluated prior to committing to any largescale study. Setting trigger levels and relating those to public health actions likely requires local evidence from ongoing programs.

In the longer term, standardization of WES methods will improve comparability between WES and clinical results within, and between, studies.

Further studies, particularly over longer timeframes, and in multiple contexts, are necessary to provide more quantitative evidence to inform benefit-cost analysis. Whilst only one of the studies cited in this document was routine and coupled to public health action, collectively the studies published to date have demonstrated the technical feasibility of WES for multiple respiratory viruses, in some cases along with other targets (as discussed in section 6.2). The next step is to implement more routine programs to demonstrate the delivery of actionable results that inform beneficial public health interventions and to refine methods and set triggers for action.

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