Wastewater and Environmental Surveillance Summary for Influenza

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World Health Organization

This document provides information on wastewater and environmental surveillance (WES) for influenza A and B viruses. It should be used together with the accompanying *WES Guidance for one or more pathogens*, which includes general and cross-cutting information (available here).This summary is drawn from existing WHO and US CDC publicly available sources, current at the time of writing.

WES for Influenza at a glance

- Influenza is of high global public health significance both due to seasonal influenza A and B and with the pandemic potential of human and zoonotic Influenza A
- In sewered settings influenza WES has been shown to be technically feasible, and is evaluated as intermediate in terms of its actionability, operational feasibility, acceptability as well as integration as part of the flu response and as part of multitarget WES
- There is insufficient evidence to evaluate its use in non-sewered settings

Table 1 : At a glance assessment of key WES criteria for influenza for sewered settings [a,b,c,d]

Setting	Categorical Assessment (CA) / Strength of Evidence (SoE)	Public Health Significance	Actionability / Relative value	Technical Feasibility	Operational Feasibility	Acceptability	Optimisation	
							Integrated disease response	Multitarget WES
Sewered	CA							
	SoE							

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.[c]
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

- **Seasonal, pandemic and zoonotic influenza are of global human and animal health importance.** Seasonal influenza A and B viruses circulate each year in humans, with seasonality in temperate zones. Influenza A viruses also commonly circulating in avian, swine and other species. These cause spillover human infections as well as periodic zoonotic and human pandemics.
- The expanded Global Influenza Surveillance and Response System (eGISRS) provides a global platform for surveillance, preparedness, and response for influenza, SARS-CoV-2 and other respiratory pathogens.
- Case-based influenza and respiratory virus surveillance (human, avian and other animal) provide critical epidemiologic, clinical and virologic information essential to meet priority surveillance objectives of detection and assessing risk, monitoring epidemiologic and virologic characteristics of influenza, and assessing influenza prevention and control measures at an individual level along with specimens needed for vaccine strain selection.
- In some settings, WES may provide additional, relevant information on circulation of influenza viruses which is **actionable** by local authorities informing policy and public health interventions. Additional information from WES on the presence and genomic characteristics of influenza A viruses and subtypes may also be relevant and actionable for both animal and human population health.
- It **is technically feasible** to monitor influenza A and B viral levels in WES with evidence from multiple sewered settings that levels correlate with clinical cases and provide timely intelligence of seasonal influenza trends including the time of the onset, peaks and end of seasonal influenza. A few high-income countries have integrated, at scale, monitoring of influenza A together with subtypes (e.g., H5).
- Given that influenza A viruses circulate in animals and humans and there are cross-species infections, the choice of analytic methods and interpretation of influenza A results is complex. WES results for influenza must always be considered together with information from other One Health surveillance activities. One **limitation** of pooled WES samples is the inability to discern the source of the detected influenza viruses, whether a human source, a non-human animal source or both.
- The **operational feasibility** of WES programs has been shown at scale in mid to high income settings with high coverage of sewage systems, typically with the addition of influenza to existing SARS-CoV-2 WES.
- WES is moderately **acceptable,** with concern that non-human influenza A may complicate interpretation.
- If used, WES should be **integrated as part of multimodal influenza surveillance.** WES may be relatively cost-efficient and provide timely information representing more of the population than case-based surveillance that relies on individual health-care seeking behavior as well as availability of services.
- **Expanded integrated respiratory surveillance**: Multiple respiratory pathogens, including influenza, SARS-CoV-2 and RSV, have been integrated cost-efficiently as part of multitarget WES, leveraging existing WES capability and sampling, laboratory and reporting workflows, if and when they align. However program design choices are required given there are tradeoffs between sensitivity, timeliness and cost-efficiency.
- **Strengthened Pandemic Preparedness and Response**: Agile WES may be used if relevant to the context; For example , during the 2024 avian H5N1 (clade 2.3.4.4b) multispecies outbreak WES was shown to have potential as a leading indicator as part of One Health surveillance to detect infection among animals (e.g. in the US with wastewater signal from industrial dairy inputs outside the seasonal flu period).
- **Key knowledge gaps** remain which are a priority for **further applied research**, these include:
    - Context-specific value addition of routine and agile WES to current eGISRS integrated surveillance priorities considering both seasonal influenza and pandemic preparedness;
    - Feasibility and public health applications in contexts where it may have added value and provide actionable information together with current influenza surveillance;
    - Methods to characterize, quantify and interpret animal-origin and human influenza A viruses and subtypes given fragmented genome and low target numbers;
    - Costing and trade-offs for routine and agile WES influenza as part of multitarget WES

Contents

1. General information

1.1. The virus and associated disease and risk factors

Influenza viruses are enveloped RNA viruses within the *Orthomyxoviridae* family. While there are four types of influenza viruses, A, B, C, and D, only influenza A and B viruses are significant human pathogens. Infection with influenza C virus generally causes mild illness and is not considered to contribute to seasonal epidemics and is not further discussed here[1]. The clinical presentation of human influenza A and B ranges from mild to severe illness and death. Symptoms begin 1–4 days after infection and usually last around a week. Common symptoms of influenza include fever, cough, sore throat, headache, body aches and fatigue. Influenza symptoms can overlap with and be indistinguishable from those of other viral respiratory illnesses, such as rhinoviruses, COVID-19 (SARS-CoV-2) and respiratory syncytial virus (RSV).

Most persons infected with seasonal influenza viruses recover without serious complications or sequelae. However, some people are at higher risk of developing severe illness, hospitalization, or death including older persons over 65 years, children under 5 years, pregnant women and persons with chronic medical conditions such as asthma, diabetes, or heart disease[2,3]. The risk of severe outcomes is greatly reduced by influenza vaccination, timely uptake of antivirals and timely access to medical and supportive care. Access to influenza-related vaccinations, antivirals and supportive health care varies widely.

1.2. Global burden, geographic distribution and seasonality

Globally, seasonal influenza viruses are widely distributed, and influenza A(H1N1)pdm09, A(H3N2) and B(Victoria) viruses were detected in all WHO regions throughout the 2023-2024 influenza season[4]. There are an estimated one billion cases of seasonal influenza occur annually, including three to five million cases with severe illness which result in 290,000-650,000 deaths from respiratory-related causes[2]. The direct and indirect economic impact is substantial due to health care costs, absenteeism and lost productivity [5,6]. While seasonal influenza viruses are detected year-round, in temperate climates they typically circulate at higher levels during autumn and winter. In tropical and subtropical climates, patterns are more complex, with circulation possible throughout the year and high variability in timing and intensity.

1.3. Routes of transmission

Seasonal influenza viruses are transmitted between humans through infectious respiratory particles either through the air, typically via direct deposition, or through contact, either direct or indirect.

1.4. Zoonotic influenza viruses and human pandemic potential

Influenza A viruses also circulate widely and routinely in many animal species, most commonly birds and swine. Influenza A viruses are further classified into subtypes based on the hemagglutinin and neuraminidase proteins on the viral surface. All known subtypes of influenza A viruses have been found

among birds, except subtypes A(H17N10) and A(H18N11) which have only been found in bats. While zoonotic transmission of influenza A viruses is uncommon, sporadic human infections and outbreaks have occurred due to influenza A viruses that usually circulate among animals. Outbreaks have been reported with high mortality, while serological studies suggest limited case ascertainment[7,8,9,10]. Since emerging in 2020, the avian influenza A H5N1 clade 2.3.4.4b has extended its host range infecting more than 200 species, including poultry, dairy cows and other farmed, companion and wild animals. As of August 2024, documented spillover human cases include seventeen cases of clade 2.3.4.4b of a total of thirty-five reported human cases of A(H5N1)[11,12].

Animal-origin influenza viruses that spillover into humans and then sustain human-to-human transmission can and have caused pandemics; with the 1919-1920 H1N1 influenza pandemic among the deadliest in human history[13].

2. Information related to influenza viruses and wastewater and environmental waters

2.1. Potential inputs to wastewater and environmental waters

Human shedding: Respiratory pathogens may contribute to wastewater and human contaminated environmental waters from fecal, urinary or other sources. Other (non-toilet) secretions also contribute to wastewater and may contain infectious virus or nucleic acid fragments; these are derived from behaviors such as brushing teeth and spitting, disposal of facial tissues, washing hands, showering and the like.

Seasonal influenza viruses specifically are shed predominantly in respiratory and nasopharyngeal secretions with viral shedding in otherwise healthy individuals typically lasting less than 6 days with an early viral peak on day 1-2 which may be reduced by use of specific antivirals[14,15]. Hospitalized, severely ill, and immunocompromised individuals have been shown to have higher viral loads and an extended duration of shedding. Seasonal influenza A and B viral RNA can be detected in the stool of both hospitalized and outpatient infected individuals including in the absence of gastro-intestinal symptoms, however there is wide variability in the frequency of fecal shedding and study methodology[16,17]. The lack of intestinal target receptors, relatively low viral load and paucity of positive cultures suggest seasonal influenza fecal RNA detections are likely due to swallowed secretions rather than direct intestinal infections[18].

In contrast to seasonal influenza A, not much is known about whether and how frequent zoonotic influenza viruses are shed in feces and urine of infected humans. Highly pathogenic avian influenza (HPAI) H5N1 can result in higher rates of gastrointestinal symptoms in humans infected through spillover infections and has been demonstrated ex-vivo to bind to human gut receptors with local replication[19,20]. Likewise ex-vivo experiments have shown various cell lines including human intestinal epithelial cells to be susceptible to novel swine origin H1N1 as well as avian H5N1 and H9N2 influenza[21,22]. Combined, these suggest that fecal source shedding, including the potential for infectious virions, may be higher in pandemic human influenza relative to seasonal flu should pandemic strains show similar intestinal organ tropism[21,19,23,20,24].

2.2. Non-human source and zoonotic shedding

Influenza A viruses also circulate widely in avian, swine and other animal hosts and may contribute to wastewater and other human waste impacted waters[25,26]. During the ongoing 2024 highly pathogenic avian influenza (HPAI) H5N1 clade 2.3.4.4b outbreak there has been an unprecedented number of new animal species infected. Among them, was the first reported HPAI H5N1 outbreak among dairy cattle in the United States of America. In this outbreak, surveillance of wastewater, that likely included industrial dairy discharges including milk, detected influenza A viruses and confirmed the viruses as A(H5N1) with additional genomic characterization[27,28]. This information provided retrospective information on the temporal and geographic spread of the virus in the US among cattle within the areas sampled.

Human and animal waste can end up in WES samples. These depend on many factors including the type of sewage system, the land use and wildlife habitats in the catchment, the extent and type of industrial contributions, storm water, and other infiltration. This context is important for interpreting the meaning of WES influenza virus detections, especially animal source or spillover human zoonotic viruses.

2.3. Influenza-related target persistence, degradation and risk of infectious virus

To detect influenza-related targets in wastewater and interpret quantitative RNA levels and trends it is important to consider: the amount of virus shed into the system at various points, its relative partitioning within sewage components; the transit time within the wastewater system; the degradation rate to the sampling point; any inhibitory factors present; as well as the methodological approaches and limitations to adjust for these variables.

The previous section noted the current evidence on shedding from human and non-human sources and knowledge gaps which are relevant to the amount of virus shed into the system at various points. There is limited data on the degradation rates in different conditions of influenza viral RNA in wastewater. Increased temperature is associated with more rapid degradation; in one study, influenza A viruses were more susceptible to rapid decay relative to other pathogens tested including SARS-CoV-2, norovirus and salmonella[29], while a second multi-pathogen study showed that degradation was modest (<20%) across a range of temperatures and duration related to typical sewage transit times[30].

There is evidence that influenza partitions with the solid rather than aqueous component with much higher concentrations in suspended solids and sludge fractions than in supernatant[31,32,33]. There is currently no evidence that influenza viruses grow or replicate outside of a host.

It is also important to consider whether there might be infectious virus which poses a risk to sanitary workers and others in contact with wastewater and human contaminated environmental waters. Studies that have collected fecal specimens from patients infected with seasonal influenza viruses, conducted among individuals with severe influenza virus infection, show a low rate of culture positivity suggesting infectious virus in stool may be rare[16,17]. However, standard protections for infectious hazards are recommended for those with occupational exposure.

2.4. Seasonal Influenza WES experience

There is growing at-scale, multi-year experience of use of WES for seasonal influenza A and B in a variety of high and middle-income global settings including in Europe, Northern America, Asia, Oceania and Africa[30]. Empirical data from multiple settings show that it is analytically feasible to detect and interpret influenza A and influenza B PCR result. Further, there have been consistent and high correlations between public health surveillance metrics for influenza over time (such as outpatient influenza-like cases, laboratory confirmed influenza hospitalizations, and school absenteeism) and quantitative wastewater results in various settings and scales[31,35,36,37,38,39,40,41,42,43,30,44,45,33,46]. Influenza WES has typically been integrated with SARS-CoV-2 as part of multi-target WES drawing on existing sample collection and workflows where these are synergistic and with public-facing national WES dashboards showing influenza results together with SARS-CoV-2, Respiratory Syncytial Virus (RSV) or other pathogens. However, recovery from the solid phase is much higher than the liquid phase, and multiplex

approaches need optimization and adjustment when different targets are present at markedly different levels. For these reasons, some groups have adopted different sampling or analytic workflows including parallel single-plex to optimize sensitivity. There are trade-offs and choices made between optimizing sensitivity for influenza +/- other pathogens and cost-efficient work flows which optimize resource use.

Influenza WES results are typically shown as quantitative or semi-quantitative results which may be normalized by a fecal or other biomarker (such as PMMoV) or flow which adjust for factors such as dilution and/or population. Experience from multiple sewered settings suggests that, depending on the surveillance systems being compared, WES may be used as a leading (or near real-time) indicator to reliably detect the onset and timing of peaks of seasonal influenza in communities at various scales[31,36,39,41,45]). There have also been proof of concept use applications in highly localized settings such as schools and universities and other facilities[44,4733].

Most examples of WES for influenza in the literature use laboratory methods that can distinguish influenza viruses by type (either A or B), but not by subtype (e.g., distinguishing A(H3N2) from A(H1N1)pdm09 or A(H5N1)). Numerous research and surveillance groups have now reported on their methods to subtype influenza viruses from WES with use of targeted primers and probes and including sequence confirmation[31,36,37,48,49]. Investigators from Texas have reported on a hybrid-capture sequencing approach of 450 distinct pathogenic viruses including influenza as well as identification of Influenza A virus subtypes H1N1, H3N2 and H5N1[42,28]. Given viral fragmentation and multiple individual sources in the pooled sample preclude long read sequencing, all these approaches must address methodological challenges including with coupling H and N gene sequences from wastewater pooled sequences.

There is ongoing research and development to further innovate and validate these and other WES applications for influenza; these include monitoring serologic immune markers and evaluating their relationship to population level immunity[18].

2.5. One Health WES experience encompassing avian, animal and spillover influenza A

There is limited One Health influenza WES experience; this requires consideration of all influenza viruses which may contribute to wastewater and environmental waters, the interpretation of these results and consideration of any proportionate actions. For influenza A, these include influenza A human seasonal viruses as well as influenza A viruses which may come from avian, non-human animal, spillover human and pandemic human sources. As noted previously, there are already multimodal influenza surveillance systems including avian, other animal and human surveillance and it is not yet clear if WES meets a specific surveillance priority where there is a gap.

Nevertheless, there is one recent example to consider in the 2024 outbreak of influenza A(H5N1) among dairy cattle in the United States where there were discordant signals between WES and clinical surveillance; with high levels of influenza A viruses in wastewater that were not consistent with human influenza cases from clinical surveillance. Additional retrospective analysis of wastewater samples have shed light on the timing and extent of the 2024 dairy cattle H5N1 outbreak in the US given wastewater could contain industrial waste from milk processing or affected farms[50,28].

Preliminary research suggests that sequencing the influenza A virus nucleic acids detected in wastewater specimens can further distinguish the influenza A virus subtypes, therefore providing an opportunity to prospectively monitor influenza from human and animal sources in the same water body[26]. Development and application of targeted primers and probes relevant to the emerging epidemic/pandemic contexts and rapid turn-around time is reported to be technically feasible for applied research laboratories[37]. The US National Wastewater Surveillance System has integrated at scale surveillance for subtype H5 using published methods[50] at more than 150 sites across 41 states since May 2024 in addition to ongoing surveillance for seasonal influenza A viruses, SARS-CoV-2 and other pathogens[51].

3. Influenza surveillance

3.1. Overall influenza surveillance and response

The global influenza strategy spans the period 2019 – 2030 and comprehensively outlines a holistic approach to influenza from surveillance to disease prevention and control inclusive of strengthening seasonal prevention and control as well as preparedness for future pandemics[33]. The WHO-led expanded Global Influenza Surveillance and Response System (eGISRS) is made up of laboratory, epidemiologic, and surveillance teams that routinely conduct facility-based integrated, surveillance for influenza, SARS-CoV-2 and RSV[53,54]. eGISRS provides a global mechanism of surveillance, preparedness and response for seasonal, pandemic and zoonotic influenza; a platform for monitoring influenza epidemiology and disease; and alert for novel influenza viruses and other respiratory pathogens.

Multiple surveillance approaches can be used to detect influenza viruses and monitor the circulation and severity of seasonal and non-seasonal influenza viruses[55]. Integrated sentinel surveillance for human influenza is commonly conducted globally and includes syndromic surveillance of influenza like illness (ILI) and severe acute respiratory infection (SARI) with linked clinical, demographic and laboratory testing. Mortality and virologic surveillance are also important to provide information on severity of illness from and circulation of influenza viruses Resources constraints may limit influenza surveillance to the months when seasonal viruses typically circulate at higher levels. . Case-based surveillance provides samples for genomic characterization of circulating influenza strains and is essential to develop and optimize influenza vaccines. eGISRS priorities are to strengthen case-based surveillance with integration within country diagnostic and surveillance systems.

These in turn complement One Health surveillance including avian, other animal and environmental surveillance of influenza A viruses, as well as other intersectoral pandemic preparedness activities given influenza A remains a pandemic threat, likely with a zoonotic source.

4. WES objectives, approaches and related public health actions

WES is always considered in the context of local, multi-modal surveillance. Surveillance from multiple sources should be integrated and complement each other to provide actionable intelligence, not stand alone. WES must have potential to provide additional value in the local context to be considered for implementation. This also includes doing no harm and not undermining event-based influenza surveillance.

WES (at scale), principally for seasonal influenza together with other respiratory pathogens have been implemented in some high and middle-income countries at national or subnational levels since 2022. At the current time, WHO does not request that countries report WES as part of eGISRS.

WES may contribute to the 2nd strategic objective of the Global Influenza Strategy 2019-2030 to strengthen global influenza surveillance. This may specifically include to strengthen integrated surveillance and laboratory systems, expand capability for intersectoral investigation and response to human seasonal influenza as well as to zoonotic influenza outbreaks and other respiratory disease outbreaks and characterise disease burden [56].

Specific approaches for WES of influenza may include one or more of the following:

4.1. Routine WES for Influenza

Routine influenza WES at relevant geographic scales and periods of interest providing

- quantitative seasonal influenza trends with identification of season or outbreak onset, peak, fluctuations and end/offset as well as out-of-season influenza virus circulation (with consistency over time not influenced by changes in health seeking behaviors or availability of services);
- identification of circulating influenza virus subtypes +/- targeted mutations of interest

Routine WES for influenza involves consistent sampling and analytic methods and for consistent periods (such as prior to and during the expected flu season)

4.2. Agile WES for Influenza

Agile influenza WES when, and if, triggered by specific local risk assessment or a heightened spillover or pandemic influenza risk providing

- Detection of influenza viruses, with targeted sampling as well as appropriate laboratory assays, and may assist for:
  - early warning
  - characterization of spatial and temporal contours as part of outbreak response or to better define epidemiology of seasonal influenza (e.g., in non-temperate climates)

Agile WES means that it is time-limited surveillance with a specific trigger to initiate and is differentiated from routine surveillance; agile WES involves establishing new time-limited activities or purposive changes in the existing WES program, e.g. sampling more frequently or in different locations, reducing the turn-around time to results, and/or performing new of different analyses.

- genomic characterization (subtypes and mutations of interest)

Countries may add specific WES approaches and objectives relevant to their context and needs.

Note these all require **specific preparedness activitie**s to establish local capability and capacity to implement, as well as **continuous quality improvement** to strengthen cost-effective seasonal influenza and integrated respiratory surveillance and for multitarget WES. Contextual methodological development, validation and optimization are required for application at scale, including for normalisation to adjust for dilution and population as well as for genomic characterization or subtyping.

In relation to animal and spillover influenza A viruses and pandemic preparedness, protocols for identification of circulating subtypes as part of surveillance have been developed for influenza A/H5, A/H7 and A/H9. In the context of the novel multistate outbreak of A/H5N1 clade 2.3.4.4b in dairy cattle in the US, H5 subtype surveillance together with Influenza A virus surveillance has been implemented at scale in the US CDC NWSS program, including more than 340 sites since May 2024, however it appears that subtype monitoring has not yet been implemented at scale elsewhere[51]. Many laboratories performing wastewater testing have established some capability to evaluate influenza A virus including subtyping and/or sequencing as influenza A and H5N1 were suggested targets as part of the GLOWACON global synchronised sampling exercise with engagement of more than 30 countries with wide global distribution.

An additional benefit is that methodologic advances for WES are likely to have direct applications for other forms of One Health influenza surveillance such as for avian surveillance and in high-risk human: animal interfaces such as wet markets, farms, and abattoirs.

4.3. Potential public health actions arising from the addition of WES for seasonal influenza

Likely public health uses and related actions arising from routine WES for influenza (over other influenza surveillance alone) may include:

- timely identification of out of season circulation as well as local season or outbreak onset, intensity, peaks and offset/end - (not otherwise possible), which in turn lead to specific and impactful public health decisions which optimise resource allocation and health outcomes. These may include one or more of:
  - administrative decisions for vaccine campaign timing, health system logistics and staffing etc
  - patient care with advice on heightened risk and use of antivirals etc
  - public communication to inform protective (use of mask, social distancing etc) and health seeking behaviours (accessing vaccines, antivirals and/or care etc)
- cost-effective integrated respiratory surveillance providing timely infection activity trends for influenza as well as SARS-CoV-2, RSV and/or other respiratory pathogens to inform pathogen specific and pathogen-agnostic actions including broader health system, clinical care and public communications complementing symptom-based surveillance
- multi-year, multi-season comparisons with stable WES methods, given uptake and access to clinical testing are influenced by multiple factors and only involve symptomatic patients.

Other potential public health uses (which require additional research and validation) which could add value over current surveillance are:

- timely identification of unusual signals and initiation of further investigation, e.g.
  - identification of subtype of concern or rapid increase in population viral levels
  - discordant wastewater and clinical surveillance results which may suggest a zoonotic source (or a change in tissue tropism and shedding)

The potential agile surveillance applications described below first require additional research as well as local validation, optimization and specific preparedness activities.

Potential public health uses and related actions arising from agile WES for influenza include:

- characterizing the geographic and temporal detections in response to an outbreak or particularly severe epidemic (with a high burden of illness) to assist in the geographic targeting and evaluation of the response (similar to that used during the emergency phase of the SARS-CoV-2 pandemic).
- as part of a One Health surveillance response to an animal outbreak with spillover to humans, agile WES could be used to understand geographic and temporal trends in detection of a specific influenza A subtype. However, there would need to be careful consideration of the potential human and other animal source contributions to wastewater and environmental waters.

- The targeted One Health sampling strategy will follow from the specific surveillance goals which may be human and/or animal focused.
- Human-to human transmission (rather than spill-over) with its associated pandemic threat could trigger an escalated WES response similar to the COVID pandemic
- Targeted primers and probes are required to subtype and/or sequence the pathogenic threat of contextual interest (e.g., seasonal flu strain, HPAI H5N1, other HPAI, novel swine H1N1 or other) against the background of circulating seasonal and animal influenza viruses. These also require local validation and optimization and contextual interpretation.

Other potential use cases for hyper-localized settings also exist, such as for residential aged care facilities, hospitals, or correctional facilities or other populations both at heightened risk and to whom benefits can accrue through targeted interventions.

5. WES additional methodological considerations for influenza

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).

5.1. Sampling methods

Routine WES surveillance requires stable sampling; with stability of sampling location, sampling type and frequency to monitor quantitative trends of influenza population viral levels during the period of interest. For seasonal influenza in temperate zones, the influenza season period is targeted; including just prior to the beginning of the influenza season, in order to identify the start, and periodically through to the season's end. However off-season sampling may be considered to characterize out-of-season influenza circulation and for situational awareness.

Sampling site selection aims to identify informative sentinel sites and generally prioritizes high population coverage with geographic representation of large population centers. For non-reticulated and reticulated sewage systems alike, sentinel locations are sought where the catchment population is representative of the community of interest and it is reasonable to then generalize results to the broader at-risk population. These are aligned to the current sampling priorities for both SARS-CoV-2 and polio WES in most settings but may differ for mpox or other pathogens with different epidemiology.

The choice of sampling locations requires local expertise with knowledge of the sanitation system and the dynamic human inputs from persons residing, working or visiting locations which may be sampled. As human and animal-origin influenza A viruses, co-circulate and their RNA can be detected from human and animal by-products, there should be efforts to understand the sources of waste that deposit into a site, including from humans, animals, or industrial, commercial, or manufacturing processes. A further consideration is the dilution and inhibition which can arise from chemical pollutants. All these inputs may be dynamic.

As highlighted in the latter knowledge gaps section, there is a relative paucity of evidence related to optimal sampling (e.g. type of sample, matrix (solids or liquids), frequency, site) for the diverse global settings without improved sewage which requires further applied research[32,34].

Cost-efficiency is optimized when sampling work-flows for existing WES are utilized, when and where this is possible, with alignment to the specific surveillance objective/s; ie using the same sampling and transport processes as are already in place for SARS-CoV-2, polio or other WES targets. Intermittent cross-sectional sampling at season onset, mid-season and late in the season in temperate zones may provide adequate data if the influenza (or combined respiratory) surveillance objective is primarily to detect the start and end of the season and/or to characterize the relative prevalence of subtypes.

Frequency, end-to-end turn-around time and, if needed, location of prospective sampling can also be modified to meet specific, time-limited, agile surveillance objectives with consideration of feasibility and costs to minimize additional resource requirements. Storage of samples collected within the multi-target WES program also allows additional testing of retrospective samples if this is indicated.

Other considerations for sampling, such as the choice of sampling type (liquid grab, composite, passive, settled solids/activated sludge), frequency of sampling, transport and turn-around time, and triggers for new or changed sampling, are covered in the WES consideration for one or more pathogens document.

5.2. Laboratory methods, bioinformatics and interpretation

In the context of multiple-target WES building on existing SARS-CoV-2 WES, pre-analytic methods including sample collection, extraction and concentration are aligned and are synergistic, to those established for SARS-CoV-2[18]. Methods must be optimized and validated for each specific pathogen and for local conditions. Multiple laboratories have reported superior results (including recovery and sensitivity) with solid matrix over liquid samples for influenza however other sample types are commonly used for other pathogens (e.g., trap and grab for polio and liquid phase or passive samples for SARS-CoV-2). Further, influenza viruses are typically present in lower concentration than SARS-CoV-2 as well as fecal biomarkers so use of multiplex assays require consideration of the differential target numbers between the pathogens and adjustments such as relative sample volumes to minimize spurious non-detections of the rarer target. Currently, multiple research laboratories, even when the same sample is used, run these as parallel singleplex assays which entails significantly higher costs. Noting these technical considerations, trade-offs must be considered as the ability to combine workflows as far as reasonably practicable provide significant cost and resource efficiencies. Widely used and validated molecular methods, including single-plex and multiplex methods have been reported to detect the presence of influenza A and B virus RNA above detection limits and normalize quantitative results with faecal biomarkers or flow rate to adjust for population, dilution and other confounders[45,57–61].

There is growing literature on standardized, scalable methods to subtype from wastewater samples with RT-PCR and genomic sequencing[31,37,49,50]. There are various platforms and bioinformatics pipelines in use which draw on the global reference databases of influenza sequences. Reference sequences are available, CDC provides one such published sequencing reference[62].

Notably, given that influenza A viruses are also animal pathogens with a wide range of avian, swine and other hosts and can also potentially infect a wide range of species, there is a need to consider the likelihood of non-human contributions into WES in interpreting results (and value of WES for One Health surveillance including for animal surveillance). Validated, scalable methods are needed to distinguish influenza A virus subtypes as well as provide further confirmation and genomic characterization in reference laboratories as needed to inform proportionate actions. Detections of influenza A virus subtypes that are typically of animal origin are likely to come from animals and cannot be attributed to a human source (e.g., as a spillover infection) from WES alone. Depending on the context, they may require further investigation and triangulation with other clinical and zoonotic surveillance information; for example, repeated WES detection of a H5 subtype or H5N1 sequence in the absence of known local animal infections. While the inability to discriminate between source in a pooled sample represents a known limitation, it also provides an opportunity for WES to contribute to both human and zoonotic surveillance simultaneously[26,50,63].

5.3. Reporting and communications

Globally, there are a wide variety of bespoke country and location specific reports and dashboards including WES results and useful visualizations for influenza as well as other respiratory pathogens (e.g

SARS-CoV-2, RSV, human metapneumovirus) with normalization by fecal biomarkers. The European Union Wastewater Observatory for Public Health is one resource with monthly reports summarizing available global WES activity inclusive of SARSR-CoV-2, influenza, RSV and other pathogens with links to country and subnational dashboards[64].

While there is some convergence, a wide diversity of analytic and reporting approaches remains including those to normalize, adjust, aggregate and display data. Information of interest for influenza includes those pertinent to all WES as described in the guidance document. These include : the trends (change) in influenza viral activity levels geographically and over time; influenza type and subtype (if characterized), geographical context for the sampling site(s) (for example, population mobility between the site's catchment and neighboring communities, sources of waste that are captured at the site, proximity of the site to avian and animal sources). It is best practice to visualize and report on WES data alongside other surveillance data for influenza and other respiratory pathogens, which provide additional context to the WES data and improve integration of relevant influenza surveillance together for accessible combined intelligence and response, this approach is illustrated by the Swiss infectious diseases dashboard[65].

Communication to relevant stakeholders must be tailored according to the surveillance objective and context including responsible health departments, health system administrators, clinicians, the general public and, as indicated, One Health actors from government, industry and animal health practitioners.

5.4. Acceptability of WES for influenza viruses

As a large pooled population sample, individuals are not identified in WES and samples are collected for specific public health objectives with controls in place by the responsible health authority. There does not appear to be ethical concerns raised by population-level WES for respiratory pathogens in general.

However for influenza A, there are some specific concerns arising from the complexity of interpretation of PCR results for influenza A viruses. As noted previously, if influenza WES is implemented, consideration of animal source influenza viruses and clinical surveillance information is important to ensure proportionate actions are taken and resources are not diverted from higher priority influenza surveillance activities. As noted, implementation of influenza WES must not undermine or compromise higher priority influenza surveillance activities such as surveillance using approaches such as sentinel surveillance for influenza-like illness or severe acute respiratory illness and virologic surveillance. Cross-cutting ethical issues are discussed in the WES overview document.

6. Integrated surveillance and multitarget WES considerations

6.1. Integration of influenza WES into existing influenza surveillance and response

- As WES for seasonal influenza is relatively recent and its place in multimodal influenza surveillance is evolving, there has not yet been substantial integration at global or local levels (with some frontrunner exceptions such as Hungary and Switzerland[65]). There is potential for improved integration, including at the planning stage to optimize complementary multimodal surveillance, as well as at the analysis and reporting stage to better visualize and enable use of combined influenza information to inform public health policy and practice decisions.

6.2. Integration of influenza as part of multi-target WES surveillance

- Existing SARS-CoV-2 or other WES activities allows the integration of influenza WES at low marginal cost with substantial alignment with multiple work-flows although trade-offs may need to be considered between optimal methods for individual pathogen sensitivity and resource allocations
- Likewise, routine WES activities for influenza provide local capability to which agile WES can be initiated, enhancing **local epidemic/pandemic preparedness and response capability for emergent zoonotic or pandemic influenza.**
- In many high-income settings, multitarget WES surveillance already combines multiple respiratory pathogen targets from the same samples including, for example influenza, RSV and SARS-CoV-2 combined contributing to **integrated respiratory surveillance** with publicly accessible dashboards showing trends for multiple respiratory pathogens.

7. Key knowledge gaps and applied research priorities

7.1. Key strengths and limitations

Key strengths and limitations of WES are described in the overall guidance document noting the relative paucity of WES evidence from settings without improved sanitation (other than for polio).

Limitations specific to WES for influenza viruses have been highlighted in this summary and include:

- Concentrations of seasonal influenza A and B viruses are typically much lower in wastewater as compared to SARS-CoV-2 (and are predominantly seasonal in temperate climates)
- Early stage of development, harmonization and standardization of WES for influenza affecting all aspects; including optimal sampling, wet and dry analytic methods, bioinformatic pipelines, interpretation, integration and reporting (with some frontrunners)
- Current lack of scaleable robust analytic methods including subtyping and sequencing
- Inability to distinguish the source of any influenza A viruses from WES samples as from avian, other animal influenza A and/or human spillover
- Limited data on human shedding of influenza viruses, including among those infected with zoonotic-origin influenza viruses
- Paucity of pathogen specific information on stability in wastewater and environmental waters in relevant conditions including seasonal influenza and other influenza A as well as likelihood of infectious virus

7.2. Applied research priorities given their potential for improved influenza surveillance

Key recommended areas of applied research include:

- Harmonization (and standardization where feasible and appropriate) of WES methods across the entire work-flow with establishment of best practice approaches and requirements
- Evidence to define the context-specific value addition and limitations of both routine and agile WES to current influenza surveillance priorities considering seasonal influenza, animal influenza which poses a zoonotic threat to humans (such as the H5N1 clade 2.3.4.4b outbreak), as well as pandemic preparedness;
    - Including modelling of the relative value of WES together with other respiratory disease surveillance and response data in relation to different surveillance objectives for early warning, quantitative trends and genomic characterization
    - With particular emphasis on contexts not well studied where WES could add value to current influenza surveillance and result in actionable information;
- Further development, optimisation and evaluation of methods to characterize, quantify and discern animal, spillover and seasonal human influenza A viruses which are scaleable and low cost which may include hemagglutinin subtyping, multiplex, metagenomic and other approaches;

- Costing of the local and contextual resource requirements for initiation and maintenance of routine and agile WES influenza and the associated cost-benefit profile as part of multitarget testing and with relevance to different global contexts.
- Characteristics and associated genetic mutations contributing to population or disease variability in tissue tropism and shedding by influenza viral subtype (including seasonal, avian and other animal influenza viruses)

Other basic and applied research directions which may expand WES utility to assess whether and to what extent WES can provide robust, reliable information include:

- Characterization of unusual signals, e.g.
  - identification of influenza virus subtype of concern or rapid increase in viral levels
  - discordant wastewater and clinical surveillance results such as due to a non-human animal source (or a change in tissue tropism and shedding)
- representative, complete and timely monitoring of strains in circulation allowing:
  - better epidemiologic and virologic characterisation in settings where this is lacking
  - real-time assessment of current circulating strains and vaccine match/mismatch
- Molecular markers of heightened risk for spillover and pandemic potential influenza strains

This last point arises from the demonstration that specific haemagglutinin mutations in avian influenza alter affinity for human receptors with tissue specific tropism, coupled with the improved population coverage of WES vis a vis case-based clinical samples, there may be utility in monitoring such molecular markers to assess pandemic potential of field isolates [66].

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