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A SYSTEMATIC REVIEW AND NARRATIVE SYNTHESIS OF THE USE OF ENVIRONMENTAL SAMPLES FOR THE SURVEILLANCE OF AVIAN INFLUENZA VIRUSES IN WILD WATERBIRDS

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ABSTRACT: Wild waterbirds are reservoir hosts for avian influenza viruses (AIV), which can cause devastating outbreaks in multiple species, making them a focus for surveillance efforts. Traditional AIV surveillance involves direct sampling of live or dead birds, but environmental substrates present an alternative sample for surveillance. Environmental sampling analyzes AIV excreted by waterbirds into the environment and complements direct bird sampling by minimizing financial, logistic, permitting, and spatial-temporal constraints associated with traditional surveillance. Our objectives were to synthesize the literature on environmental AIV surveillance, to compare and contrast the different sample types, and to identify key themes and recommendations to aid in the implementation of AIV surveillance using environmental samples. The four main environmental substrates for AIV surveillance are feces, feathers, water, and sediment or soil. Feces were the most common environmental substrate collected. The laboratory analysis of water and sediment provided challenges, such as low AIV concentration, heterogenous AIV distribution, or presence of PCR inhibitors. There are a number of abiotic and biotic environmental factors, including temperature, pH, salinity, or presence of filter feeders, that can influence the presence and persistence of AIV in environmental substrates; however, the nature of this influence is poorly understood in field settings, and field data from southern, coastal, and tropical ecosystems are underrepresented. Similarly, there are few studies comparing the performance of environmental samples to each other and to samples collected in wild waterbirds, and environmental surveillance workflows have yet to be validated or optimized. Environmental samples, particularly when used in combination with new technology such as environmental DNA and next generation sequencing, provided information on trends in AIV detection rates and circulating subtypes that complemented traditional, direct waterbird sampling. The use of environmental samples for AIV surveillance also shows significant promise for programs whose goal is early warning of high-risk subtypes.

Key words: Avian influenza viruses, environmental samples, feathers, feces, sediment, surveillance, water, wild waterbirds.

INTRODUCTION

Avian influenza viruses (AIV) can cause morbidity and mortality in domestic animals, wild animals, and humans. Outbreaks of highly pathogenic avian influenza are most common in poultry and occur regularly in countries around the world (Chatziprodromidou et al. 2018). In 2014–15 an AIV outbreak in poultry in the US resulted in losses of over US$3 billion, egg shortages, and trade sanctions from 38 countries (Greene 2015). Avian influenza viruses have also caused mortality in wildlife, most notably more than 1,000 Bar-headed Geese (*Anser indicus*) found at Lake

Qinghai, China during 2005 (Chen et al. 2005). Ongoing AIV infections in people—involving multiple subtypes, including H5, H7, and H9 (Poovorawan et al. 2013)—highlight a growing public health concern due to the viruses' pandemic potential. The health effects of AIV in multiple species have highlighted the importance of surveillance to monitor and mitigate the risk of disease outbreaks.

Wild birds are reservoir hosts for all strains of AIV and play a key role in AIV ecology, particularly in waterbirds, which include species from the orders Anseriformes (i.e., ducks, geese, and swans) and Charadriiformes (i.e., gulls, terns, and waders; Olsen et al. 2006). Waterbirds are usually asymptomatic carriers of low pathogenicity avian influenza (LPAI), shedding virus in their excreta, particularly feces (Webster et al. 1978). Viral excretion by waterbirds during migration is essential to the spread AIV across geographic locations. Migration also results in the intermingling of birds from disparate locations, leading to co-infection with multiple viruses, reassortment, and the emergence of new strains (Hinshaw et al. 1980). As a result, waterbirds are a primary target for AIV surveillance.

The majority of surveillance programs are based around AIV detection (i.e., identification of AIV RNA in a sample) or isolation (i.e., growing AIV using cell or egg culture) from individual birds who are sampled through some combination of live trapping, hunting, collection of birds dead from other causes, or the use of sentinel birds. All of these methods have significant limitations that affect their utility. For example, live capture and sampling of waterbirds is often considered the gold standard for AIV surveillance because it can be used for probabilistic sampling by targeting desired species, times of year, and locations (Hoye et al. 2010). Conversely, unless they are done in conjunction with bird capture programs done for other purposes, live capture techniques are expensive, time-intensive, and require skilled personnel and specialized equipment, making them unfeasible in many jurisdictions (Whitworth et al. 2007). Partnering with hunters to test harvested waterbirds can facilitate the collection of samples, but those samples are biased by hunter locations, preferences, and restrictions (Hoye et al. 2010). Sampling of birds found dead is simple and cost-effective, but it often produces an insufficient or biased sample because it relies on opportunistic identification and submission of wild birds (Whitworth et al. 2007). Sentinel AIV surveillance involves the regular collection of serum or cloacal swabs from domestic or peridomestic waterfowl that are in contact with wild waterbirds (Globig et al. 2009). Small sample sizes and uneven geospatial distribution can limit the utility of sentinel birds by making it difficult to infer the degree to which exposure in the sentinel reflects low prevalence AIV strains (Globig et al. 2009).

Surveillance based on environmental samples has been proposed as a supplementary strategy for testing individual birds because wild waterbirds excrete the virus, and environments where waterbirds congregate (e.g., wetlands, beaches) can be heavily contaminated. Avian influenza viruses have been found in a number of different environmental substrates, including feces, feathers, water, and sediment. Given the relative novelty of this approach and the variety of sample types available, there remains uncertainty regarding how environmental sampling could or should be incorporated into AIV surveillance programs. The objective of our review was to synthesize the literature on environmental AIV surveillance, to compare and contrast the different abiotic sample types, and to identify key themes and recommendations that will aid researchers, stakeholders, and decision makers in the future implementation of AIV surveillance using environmental samples.

MATERIALS AND METHODS

Peer-reviewed and grey literature were selected for review using a systematic search procedure (Fig. 1). Relevant peer-reviewed literature on the use of abiotic environmental samples for AIV surveillance in wild waterbirds was found by searching PubMed, Science Direct, EBSCO Host, and Ovid (Embase/Medline/Biological ab-

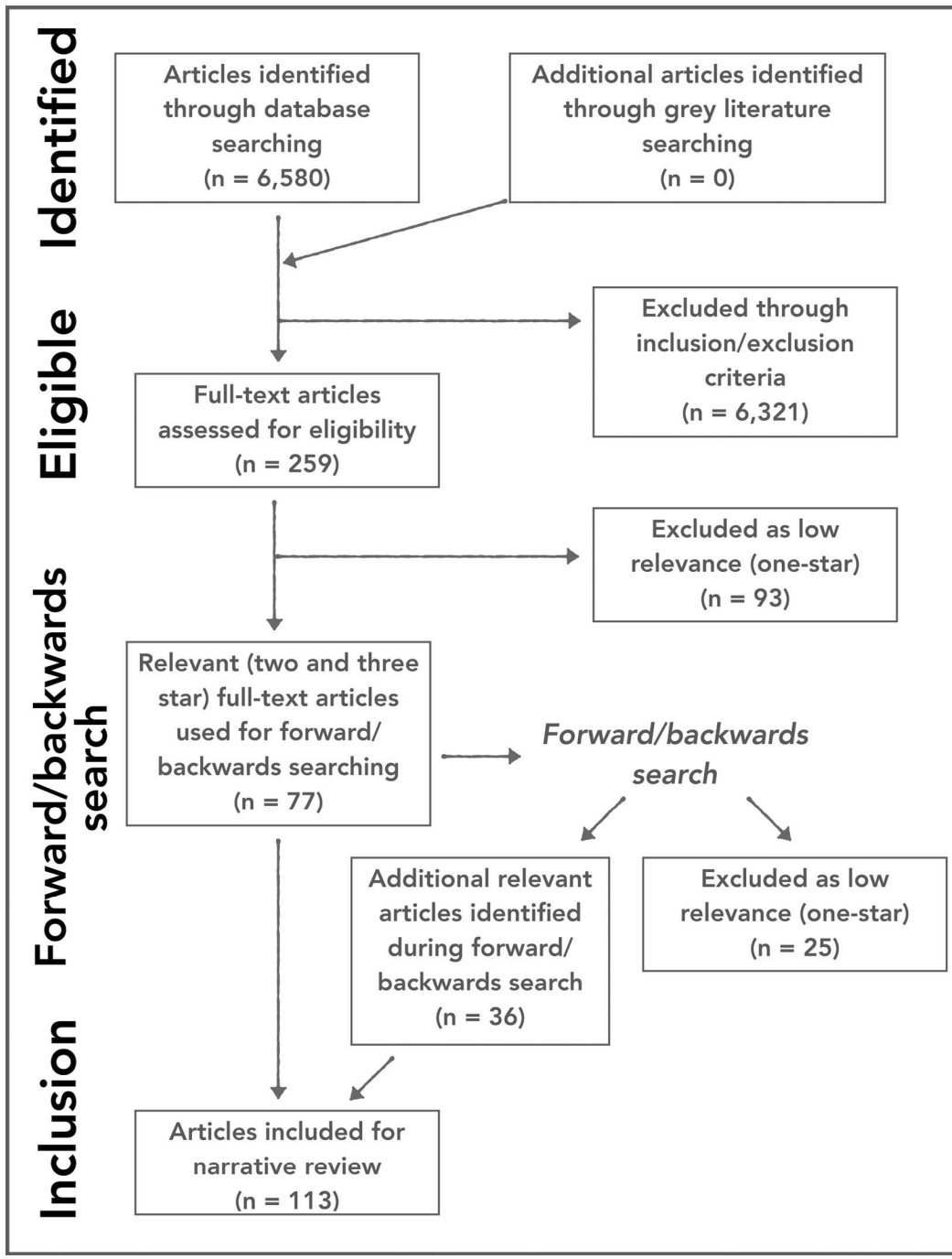

FIGURE 1. Flow chart of the systematic literature review procedures, relating to the use of environmental samples for avian influenza virus surveillance in wild waterbirds. Reviewed literature was found by searching PubMed, Science Direct, EBSCO Host, and Ovid (Embase/Medline/Biological abstracts) databases for publications with dates between 1 January 2005 and 30 January 2019, and containing terms belonging to five major groups (animal, diagnostic, disease, environment, and surveillance).

stracts) databases. Search criteria included publication dates between 1 January 2005 and 30 January 2019 and using terms belonging to five major groups (animal, diagnostic, disease, environment, and surveillance; Supplementary Material Table 1). Titles, abstracts, and keywords of retrieved articles were screened based on inclusion and exclusion criteria (Supplementary Table 2). The search terms were also entered into internet search engines to cover any recent news not yet published by peer review (Supplementary Table 3); however, no relevant grey literature results were found. A screening procedure ranked peer-reviewed articles into categories of three stars (most relevant) to one star (least relevant; Supplementary Table 3). A forward and backwards search of literature cited in the two- and three-star papers was performed to find relevant articles that might have been missed. Narrative review and synthesis were based on the full body text of the final two- and three-star peer-reviewed articles (n=113). Narrative synthesis is a nonstatistical review method used for identifying themes across diverse bodies of literature (Arai et al. 2007). Briefly, included articles were analyzed by tabulating study methodology and results according to both a priori and emergent themes and then summarizing, comparing, and contrasting the data within each theme (Rodgers et al. 2009).

Ecological drivers and patterns of AIV transmission rates in waterbirds are known to differ by habitat (e.g., tropical vs. temperate, northern vs. southern; Gaidet et al. 2012; Ferenczi et al. 2016). To determine the diversity of waterbird habitats represented in the reviewed literature, we plotted the approximate locations of sampling sites from the 21 unique field studies that collected either water, sediment, feathers, or a combination thereof on maps of the world's terrestrial biomes plus the "rock and ice" ecoregion. The terrestrial biomes represent the 14 major global habitat types that have similar climates, vegetation structure, and biodiversity features (Dinerstein et al. 2017). The shapefile data were downloaded from the Ecoregions2017 interactive map (Resolve 2020). One of the 21 field studies (Numberger et al. 2019) was excluded because no location for field sampling sites was provided, and sampling sites from three studies (n=2 sediment, n=1 multiple) could only be approximated by a random point within Cambodia due to the lack of a more precise site description (Horm et al. 2011, 2012a; Deboosere et al. 2012). The map was replicated by sample type, whereby the multiple category contained sampling sites where either water and feathers (n=1 sampling site; Gaidet et al. 2018) or water and sediment were collected (n=6 sampling sites; Vong et al. 2008; Stallknecht et al. 2010; Horm et al. 2012a; Vogel et al. 2013). Data manipulation and plotting were performed in QGIS version 3.2.1-Bonn (QGIS 2018).

RESULTS

Sample types used for AIV testing

There were four major environmental sample types used for AIV testing in the reviewed literature: feces (n=55 studies), feathers (n=5 studies), water or ice (n=46 studies), and sediment or other soil-based substrates (n=15 studies). Eighteen studies used multiple environmental sample types. Table 1 provides an overview of the major advantages and disadvantages of samples collected directly from waterbirds vs. environmental samples.

Feces: Feces were the most commonly used environmental sample for AIV surveillance. Feces can be considered an intermediate sample type between samples obtained directly from wild waterbirds (e.g., cloacal swabs) and environmental substrates (e.g., water). For example, cloacal swabs and feces can be handled and analyzed the same way in the laboratory (Spackman and Lee 2014) but, similar to other environmental samples, fecal samples are often dissociated from the source animal, resulting in a loss of ecological data. Many studies using both cloacal swabs and fecal samples did not separate out testing results between sample types (Kou et al. 2009; Breed et al. 2010; Muzyka et al. 2012), and some studies that involved capture of waterbirds collected fecal samples from those birds instead of cloacal swabs (Latorre-Margalef et al. 2016). However, the majority of the reviewed studies collected feces from the environment without the direct handling of waterbirds. Fecal samples can be collected by flushing flocks and collecting the recently deposited feces on the ground (Gaidet et al. 2018), by gathering feces deposited on vinyl sheets placed under species roosting in trees (Kishida et al. 2008), or through more opportunistic methods. Our review will primarily focus on environmental samples other than feces due to the similarities between traditional direct waterbird samples and fecal samples.

TABLE 1. Comparison of the principal advantages and disadvantages for samples collected directly from wild waterbirds vs. indirectly from the environment. The comparison synthesizes systematically reviewed literature on the use of environmental samples for avian influenza virus (AIV) surveillance in wild waterbirds. Reviewed literature was found by searching PubMed, Science Direct, EBSCO Host, and Ovid (Embase/Medline/Biological abstracts) databases for publications with dates between 1 January 2005 and 30 January 2019 and containing terms belonging to five major groups (animal, diagnostic, disease, environment, and surveillance).

Method	Main advantages	Main disadvantages
Samples collected through live capture, hunting, or direct handling of wild waterbirds.	Provides exact ecological context (e.g., host species and sex) and primary location of viral shedding (e.g., oropharyngeal, cloacal). Can be used to determine AIV prevalence rate. Can readily be compared to historical studies due to similarity of methods.	Difficult or expensive to collect sufficient sample size to be representative of viruses found in all local waterfowl populations. Negative effect on waterbirds (e.g., potential for increased stress, injury, or mortality). Sampling restricted to certain locations or times of year (e.g., due to hunting restrictions, urban environments).
Samples collected from the environment.	May provide information on viruses from multiple birds, thereby increasing efficiency of AIV detection. Sample collection typically does not require specialized equipment or highly trained personnel. Can provide information on AIV transmission through the environment.	The number of birds contributing virus to one sample is unknown. Most sample types require processing steps prior to testing (e.g., removal of inorganic material, concentration). Unknown, and likely variable, time of persistence between viral deposition by birds and detection in sample.

Water: Laboratory studies that use AIV-inoculated samples have demonstrated it is possible to detect or isolate AIV from a variety of water sources, including distilled water, filtered water, and natural river, lake, brackish, and sea water (Brown et al. 2009; Shoham et al. 2012; Zhang et al. 2014). In contrast, field studies have only successfully detected or isolated AIV from fresh lake water or ice sources (Zhang et al. 2006). This discrepancy might be explained by sampling bias, because only one of the reviewed manuscripts reported AIV testing results from brackish or seawater collected from the field (Pérez-Ramírez et al. 2012). Alternatively, it could reflect AIV prevalence in bird species using those environments, because fresh fecal samples collected around freshwater lakes also had higher detection rates than those collected around saline or brackish lakes (Ofula et al. 2013). Avian influenza viruses in water are present in lower concentrations than in fecal samples (VanDalen et al. 2010); thus large volumes (i.e., 1 L or more) or a concentration step are often used prior to detection or isolation (Bridle et al. 2013). The detection rate of AIV in water collected from field studies ranged from 0% to 6% (Pérez-Ramírez et al. 2012; Okuya et al. 2015), and AIV were successfully identified using study methods with and without a concentration step. Methods used in the reviewed field studies varied widely. For example, the volume of water collected varied from about 1 mL to 50 L per sample (Deboosere et al. 2011; Ornelas-Eusebio et al. 2015). The degree of methodological variation in sample volume, concentration methods, and locations and times of sample collection make it extremely difficult to identify which protocols are associated with higher detection rates.

Soil-based substrates: Avian influenza viruses have been identified in a number of soil-based wetland substrates including soil, mud, sand, and sediment (Lang et al. 2008; Horm et al. 2012a; Poulson et al. 2017). Sediment is the organic and inorganic material that collects at the bottom of a water body and was the most common soil-based substrate investigated (n=7 studies). Avian influenza viruses are

expected to concentrate in this layer as they settle out of the water column (Stallknecht and Brown 2009). Although the depth of sediment sample collection is typically not reported, sediment in shallow water is thought to be an important medium for fecal-oral transmission among dabbling ducks (Franklin et al. 2011). Superficial sediment can also contain the most recently deposited viral particles. Avian influenza virus detection and isolation in sediment can be hampered by low viral concentrations, viral inactivation, RNA degradation, and the presence of inhibitory substances (Lang et al. 2008; Horm et al. 2012b); however, in some scenarios, AIV RNA can also be preserved by being bound within the substrate matrix (Poulson et al. 2017). The detection rate of AIV in sediment and other soil-based substrates collected from field studies ranged from 0% to 56% (Lang et al. 2008; Horm et al. 2012a) and, similar to water studies, sediment studies used a wide range of field and laboratory methodologies.

Feathers: Avian influenza viruses can be introduced onto feathers through contact with water contaminated by feces (VanDalen et al. 2010) or through contact with respiratory secretions from preening or allogrooming (Delogu et al. 2010). Avian influenza viruses have been detected by PCR from feathers from multiple locations on the bird such as wing, breast, or tail (Aiello et al. 2013). Uropygial secretions present on feathers can also act to concentrate AIV (Delogu et al. 2010), and feathers often remain longer in the environment than feces (Aiello et al. 2013). The detection rate of AIV in feathers collected from field studies ranged from 0% to 39% (Lebarbenchon et al. 2013; Gaidet et al. 2018). To date, feathers have been the least investigated environmental sample source, and the advantages and disadvantages of using feathers to identify AIV are not well understood.

Biotic environmental samples: It is of note that AIV has also been found in aquatic plants and animals. Aquatic invertebrate filter feeders such as bivalves and *Daphnia* are of particular interest because they can accumulate AIV through their normal feeding behavior (Faust et al. 2009; Marschang et al. 2009). For example, AIV has been found to naturally concentrate in zebra mussel tissues and can remain infective from days to weeks (Marschang et al. 2009; Stumpf et al. 2010). By harboring infective AIV, aquatic invertebrates have the potential to infect waterbirds through shedding active AIV back into the water or by being consumed (Stumpf et al. 2010). Other studies have found that the presence of filter feeders alone (Faust et al. 2009) or in combination with sediment and plants (Horm et al. 2012b) decreases AIV in the water column. Thus, the dynamics of AIV in aquatic organisms is likely dependent on the complex interactions among waterbirds, AIV, aquatic organisms, and the environment, and was determined to be beyond the scope of our review, which instead focused on abiotic substrates.

Collection of environmental samples

Diversity of study settings: The reviewed literature included 21 unique studies where samples were collected in the field (vs. laboratory experiments). Field studies composed 13 of the 46 studies involving water, 10 of 15 involving sediment, and three of five involving feathers. Five studies used more than one sample type. The level of detail regarding the field settings varied widely between articles, with some providing a thorough description of the local ecology (Hénaux et al. 2012) and others providing only high-level details such as country of origin (Horm et al. 2012a). Despite the global distribution of waterbirds (Olsen et al. 2006), inland temperate biomes from the northern hemisphere are over-represented among field studies of AIV using environmental samples (Fig. 2). The represented tropical biomes are all sites within one country (Cambodia) and there are no sampling sites from the southern hemisphere. Unequal representation of ecosystems is problematic as the ecology of waterbirds and AIV can vary among biomes and between hemispheres (Gaidet et al. 2012; Ferenczi et al. 2016). Similarly, only two of 21 studies included ecosystems with brackish or saline water (Pérez-Ramírez et al. 2012;

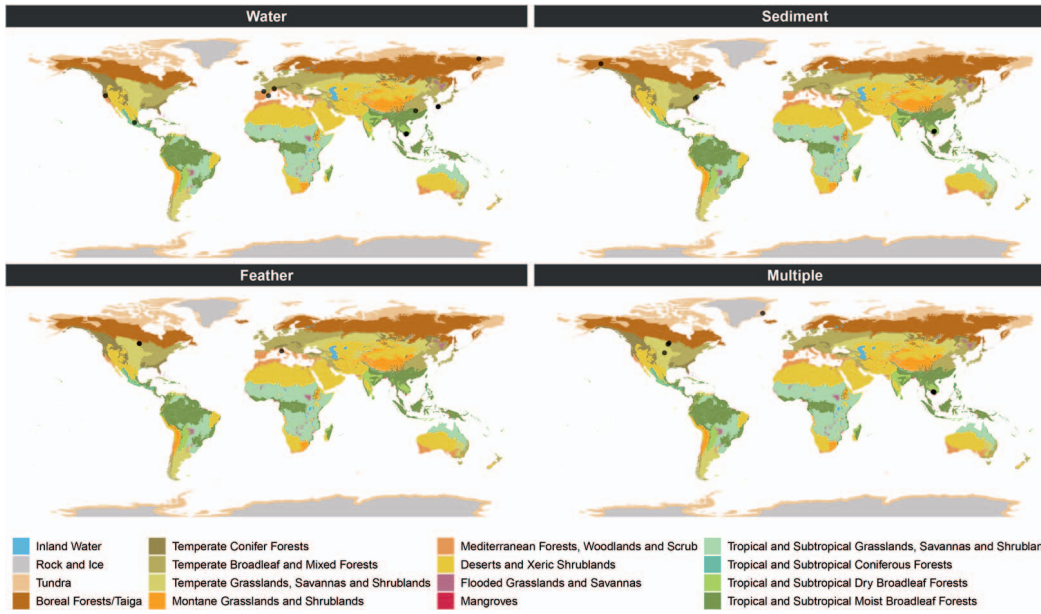

FIGURE 2. Map of the 14 terrestrial biomes and the rock and ice ecoregion (Dinerstein et al. 2017) represented in sampling sites from 21 peer-reviewed field studies that used environmental samples for the surveillance of avian influenza virus in wild waterbirds. Reviewed literature was found by searching PubMed, Science Direct, EBSCO Host, and Ovid (Embase/Medline/Biological abstracts) databases for publications with dates between 1 January 2005 and 30 January 2019, and containing terms belonging to five major groups (animal, diagnostic, disease, environment, and surveillance). The 21 field studies used either water, sediment, feather, or multiple (a combination of either water+sediment or water+feathers) sample types. Black dots are the approximate location of sampling sites from the 21 field studies, excluding one study that did not provide a location for any sampling sites. Three sampling sites (n=2 sediment, n=1 multiple) could only be approximated by a random point within Cambodia due to the lack of a more precise sampling site description. Terrestrial biome data is courtesy of Resolve (2020), accessed under Creative Commons 4.0 license.

Poulson et al. 2017). The timing of field sampling is spread more evenly across the year. Six of 21 studies collected samples from most or all biologically relevant seasons (i.e., from three or four seasons in temperate regions or from dry and wet seasons in tropical regions), although the majority of samples were still collected in the autumn or winter (in 13 of 21 studies), presumably to reflect the typical peak in LPAI prevalence in waterbirds in temperate northern regions (Olsen et al. 2006).

Anthropogenic habitat disturbance can also affect AIV in waterbirds and the environment. For example, the provision of resources through activities such as baiting traps can increase waterbird concentration, thereby increasing AIV transmission and shedding (Soos et al. 2012). Avian influenza viruses are more likely to be detected in sediment samples taken from field sites with high levels of anthropogenic modification (Himsworth et al. 2020), and locations that were baited, managed, or urban were over-represented among the reviewed field studies with higher detection rates. For the most part, the degree of anthropogenic habitat modification was not described or investigated in the included studies.

Diversity of waterbird species: Eight of the 21 unique field studies targeted the order Anseriformes or their habitats. The remaining studies indicated they were targeting species from the order Charadriiformes (one of 21), both Anseriformes and Charadriiformes (one of 21), the order Gruiformes (two of 21), migratory species generally (four of 21), or did not identify a target species or group (five of

21). Given the indirect nature of environmental sampling, it was nevertheless often uncertain which species were actually contributing to the sample results. The exception is from two studies using feathers where samples were clearly collected in direct association with handling live birds and the exact species of origin could be recorded. The species sampled in these studies were either Mallards (*Anas platyrhynchos*; Delogu et al. 2010) or duck species generally (Lebarbenchon et al. 2013). Other solutions used to provide additional ecological context regarding the species of origin included collecting samples from single-species flocks (Gaidet et al. 2018) and recording observed waterbird species abundance at the field site (Pérez-Ramírez et al. 2012). For studies collecting observational data, the majority reported on the most common waterbird species observed (Vogel et al. 2013), whereas fewer performed systematic surveys of all waterbirds present (Zhang et al. 2006; Hénaux et al. 2012; Pérez-Ramírez et al. 2012; Densmore et al. 2017).

Logistics of sample collection: Relative to the live capture of wild waterbirds specifically for the purposes of AIV surveillance, the collection of all environmental sample types is less technical, requires less equipment, and can be undertaken in areas where capture or hunting is prohibited (e.g., urban areas, wildlife reserves; Deliberto et al. 2009; Pannwitz et al. 2009; VanDalen et al. 2010; Ofula et al. 2013). Most studies do not specifically discuss barriers and opportunities for environmental sample collection. Nevertheless, based on the distribution of the sample types in the environment and comments regarding impediments encountered in the field, some generalizations regarding sample collection can be made. Water and sediment, in particular, are easy to find and access in the majority of water bodies (Okuya et al. 2015; Densmore et al. 2017). Collecting water and sediment can be difficult in certain circumstances—such as in freezing temperatures or locations with large amounts of rocks or roots. The relative ease of obtaining feathers and feces can also vary by waterbird species and habitat. For example, the collection of feathers and feces can be challenging for bird species that spend the majority of their time in the water (Deliberto et al. 2009) or in wetlands surrounded by heavy undergrowth. Collection of feather and fecal samples is most efficient when done in conjunction with existing wild bird handling programs or circumstances that allow for high concentrations of easily accessible birds (e.g., bird banding stations, baited traps, colony nesting sites).

Analysis of environmental samples

Sample processing: Environmental samples can require additional preanalysis processing compared to feces and cloacal swabs. Substrates that contain rocks and plant material might need to be filtered. Additionally, homogenization or concentration might be required because AIV is often present in low concentrations and is unevenly distributed in environmental substrates (Bridle et al. 2013). Concentration is particularly important for water samples (Poulson et al. 2017) and can be achieved using a filtration and protein-based elution step (e.g., glass wool filter and beef extract eluent; Deboosere et al. 2011) and followed by a secondary concentration step (e.g., precipitation with PEG6000 and centrifugation; Deboosere et al. 2011; Bridle et al. 2013). Avian influenza viruses in water have also been concentrated using chicken erythrocytes, which can subsequently be used for virus isolation in embryonated chicken eggs (Khalenkov et al. 2008). Elution and concentration methods can also facilitate AIV detection sensitivity in mud samples (Horm et al. 2011; Deboosere et al. 2012); however, these methods can also result in the accumulation of PCR inhibitors (e.g., humic acids; Deboosere et al. 2012). Feathers do not require additional processing steps (Lebarbenchon et al. 2013; Gaidet et al. 2018).

Avian influenza virus presence: The presence of AIV in a sample is usually identified through virus isolation (VI), reverse transcription PCR, or both (Cattoli and Capua 2007). In general, PCR is more sensitive than VI (Munster et al. 2009; Stallknecht et al. 2012),

because VI only detects infectious virus, whereas PCR can detect both infectious and noninfectious viruses, as well as free viral RNA (Cattoli and Capua 2007). As such, AIV RNA remains detectable by PCR in the environment for longer than detection of the virus through VI (Stallknecht et al. 2010), suggesting that PCR is preferable over VI when there is a prolonged or unknown time period between AIV deposition in the environment and sample collection or where rapid viral degradation is expected due to hostile environmental conditions (e.g., times of year or locations with high ambient heat). The PCR might also be the most appropriate detection method in environmental samples that typically have low viral concentrations—such as water samples— because VI performs best with high concentrations of active virus (VanDalen et al. 2010). Detection by PCR can be inhibited in sediment samples due to the presence of compounds such as heme and humic acids (Lang et al. 2008). The optimal detection method in relation to timing of viral deposition, in different environments, and in different sample types remains to be determined.

Avian influenza virus characterization: Avian influenza viruses are mainly subtyped based on the hemagglutinin (HA) and neuraminidase (NA) surface proteins or the genes encoding them. Serological assays (i.e., hemagglutination inhibition and neuraminidase inhibition), subtype-specific PCR, or sequencing can be used to characterize AIV (Spackman 2014). Each method has its own strengths and advantages; for instance, serological assays are relatively inexpensive and quick (Pedersen 2014), whereas PCR is highly sensitive and specific (Cattoli and Capua 2007). The ideal characterization method thus varied according to specific surveillance needs and resources and was reflected by a relatively even distribution across the reviewed field literature that collected water, sediment, or feathers (serological assays in two of 21 studies, PCR in five of 21 studies, sequencing in six of 21 studies, and multiple methods in four of 21 studies). Sequencing has particular potential for use with environmental substrates that are disassociated from a source animal. Unlike characterization by PCR or serological methods, AIV sequences found in environmental samples can be placed in context through comparison to sequences of influenza viruses from birds or other animals. For example, sequencing AIV from environmental substrates has determined relationships to outbreak viruses (Fujimoto et al. 2010), probable geographic regions and times of viral origin (Zhang et al. 2006; Cheon et al. 2018), and the occurrence of reassortment events (Nakagawa et al. 2018).

Sequencing of AIV can be done either on isolates obtained through VI (Ornelas-Eusebio et al. 2015) or directly on the environmental sample (Lang et al. 2008). Sequencing of AIV through VI was the most common method used with water samples (five of six water studies using sequencing), and has the advantage of being able to identify specific viral strains because the HA and NA sequences originate from the same viral particle (such as H5N1). Traditional characterization techniques might not work in sediment (Tindale et al. 2020), and for these samples next generation sequencing (NGS) technologies that can identify low concentrations and short strands of AIV RNA in complex substrates are particularly promising (Tindale et al. 2020). The interpretation of sequence data obtained directly from environmental samples (without VI) can be challenging (Lang et al. 2008) because these samples can contain multiple strains of AIV. Therefore, it might not be possible to determine which HA and NA sequences came from which virus. In these scenarios the HA and NA subtypes can only be reported separately (e.g., H5 and N1) and it is not usually possible to identify specific AIV strains (Lang et al. 2008; Latorre-Margalef et al. 2016).

Data analysis and interpretation

Prevalence estimation: In general, environmental samples do not provide accurate measures of AIV prevalence in birds. This is because water and sediment samples can contain excretions from any number of individuals, or none at all. Even for fecal

and feather samples, it is difficult or impossible to determine if each sample represents one unique individual or if multiple samples originated from the same bird (Lee et al. 2010). Environmental samples can still provide important epidemiological information by mirroring the trends in AIV detection rates found in waterbirds. For example, although the apparent prevalence rate in eastern US shorebird fecal samples did not exactly match those from cloacal samples, the same interannual trends (i.e., increasing or decreasing, compared to the prior year) were found in both sample types (Stallknecht et al. 2012). The fact that a single environmental sample might contain AIV from a number of different birds could be viewed as an advantage, because it means that these samples might be able to provide more information on a per-sample basis compared to samples obtained directly from individual animals. Indeed, Lang et al. (2008) found that detection rates in sediment samples was higher than that in contemporary wild waterbird swabs (Runstadler et al. 2007). The increased detection efficiency of environmental samples might be particularly beneficial where the prevalence of AIV in waterbirds is low (Hoye et al. 2010).

Trends in AIV subtypes: Environmental samples appear to be valuable for identifying trends in circulating AIV subtypes. Studies have shown that subtypes found in sediment can reflect the predominant strains circulating in birds (Lang et al. 2008) and might be more sensitive than direct waterbird sampling for identifying low-prevalence subtypes. For instance, Lebarbenchon et al. (2011) found that AIV subtypes isolated from Minnesota lake water did not become the predominant strain found in waterbirds until the following year. This finding is likely because viruses being carried by a small proportion of the waterbird population have a lower probability of being identified using direct waterbird sampling, particularly if the host is a species that is rare or difficult to capture (Lang et al. 2008; Lee et al. 2010). Environmental samples might also contain more subtypes on a per-sample basis compared to bird samples, because Himsworth et al. (2020) found that approximately 25% of sediment samples contained more than one subtype with up to eight NA and eight HA subtypes in a single sample.

Effect of abiotic factors: Results of environmentally based AIV surveillance must also be interpreted in light of a variety of abiotic factors that can influence viral persistence and detection. These include physiochemical factors such as temperature, pH, and salinity, which can affect the survival of AIV in water (Stallknecht and Brown 2009). Laboratory studies using distilled water demonstrate that the ideal conditions for AIV persistence are neutral to slightly basic pH, low temperatures, and freshwater (Brown et al. 2009). The effect of temperature, pH, and salinity has been confirmed with AIV inoculation in natural surface waters (Keeler et al. 2013; Zhang et al. 2014) but remains to be fully investigated under field conditions. Fluctuations in environmental conditions can also influence detection rates; for instance, exposure to freeze-thaw cycles can degrade AIV in water (Stallknecht et al. 2010). Environmental persistence can also vary by viral strain (Brown et al. 2009; Horm et al. 2012b).

Adding ecological context: Unlike traditional AIV surveillance, AIV identified in environmental samples cannot be associated with epidemiologic data, such as the species of the host bird (Stallknecht et al. 2012), unless these samples are augmented by data collected from other sources. Collecting environmental samples deposited by only one species is a technique that can be used to connect data obtained from environmental samples with data on the host population (i.e., the typical migratory, behavioral, and physiologic patterns of the targeted species). However, this approach is only feasible for feces and feathers deposited on land-based locations where single-species flocks are resting or foraging (Gilbert et al. 2012; Gaidet et al. 2018).

Bird observations taken at or prior to the time of environmental sample collection can suggest the waterbird species that might be contributing to AIV found in the environment (Pannwitz et al. 2009; Densmore et al. 2017).

Bird observations can be biased because they can overlook cryptic species or those present only in low abundances (Lee et al. 2010). More importantly, there is no direct link between the observation and the sample. This limitation can be overcome by environmental DNA—or DNA barcoding—by using wild bird DNA found within the environmental sample itself to infer the host species (Lee et al. 2010). Theoretically, environmental DNA could be used with all sample types, but has only been used with fecal and water samples in association with AIV surveillance (Lee et al. 2010; Vogel et al. 2013).

DISCUSSION

Our results suggest that environmental samples can be a valuable adjunct to direct sampling of wild waterbirds for the purposes of AIV surveillance. Indeed, environmental sample types could be highly complementary for programs with multiple surveillance aims (Table 1). The sample type selected will also depend on the context. For instance, it might be more efficient to use direct waterbird samples when surveillance programs can be piggybacked onto existing bird capture programs, whereas environmental samples might be more efficient for jurisdictions without the expertise or resources to collect waterbird samples. For this reason, samples must be selected that are ideal in light of the unique goals and constraints of each surveillance program.

Surveillance goals

Clarifying the epidemiology of AIV: Samples collected directly from waterbirds are most appropriate where the goal is to understand the epidemiology of AIV, provided those samples adequately represent the larger waterbird population. Waterbird samples can provide both the source attribution and ecological metadata that give a specific time, location, and host context to the virus. This information is essential for calculating epidemiological parameters such as viral or subtype prevalence rates and for understanding the dynamics of AIV in waterbird populations. Environmental samples can be useful where the goal is to understand the role of the environment itself in the ecology of AIV (Lebarbenchon et al. 2011). For example, PCR could be used to screen potential environmental reservoirs for AIV (e.g., lake water, beach sand) and then VI and sequencing AIV from both the environmental and local waterbirds would provide support for AIV transmission between the environment and waterbirds if infective, genetically similar AIV was found. Superficial sediment might be of particular interest for evaluating fecal-environment-oral transmission of AIV in waterbirds, because superficial sediment is expected to contain a higher concentration of AIV than the surrounding water (Horm et al. 2012b), and is also consumed by dabbling waterbirds as they feed (Franklin et al. 2011).

Early detection: Providing an early warning for possible AIV spillover to domestic species or humans is the most commonly cited reason for the surveillance of AIV in wild waterbirds, and environmental samples are particularly well-suited to this goal. Surveillance for early detection differs from epidemiological goals in that it is principally concerned with the presence and spread of high-risk AIV segment subtypes (e.g., LPAI H5, LPAI H7, any highly pathogenic avian influenza). Therefore, the early detection goal does not require the source attribution and ecological metadata needed for epidemiological assessments, nor does it require linking HA and NA subtypes because risk assessment is primarily based on HA subtype detection. Environmental samples can also be used to monitor geographic and temporal trends in circulating high-risk HA subtypes over time. For example, sediment has been found to contain the predominant viral subtypes concurrently circulating in waterbirds (Lang et al. 2008). Certain environmental sample types (e.g., sediment) can even have higher AIV detection rates and contain a greater number of HA and NA subtypes compared to direct waterbird sampling (Lang et al. 2008; Himsworth et al. 2020), thereby further increasing the probability that high-risk strains will be identified.

Surveillance constraints

Resources: The availability of resources for sample collection and analysis are primary considerations when selecting a sample type for AIV surveillance. Direct sampling of wild waterbirds requires considerable resources for field work; for instance, live capture involves special equipment (e.g., funnel traps, net guns) and considerable time spent in the field by well-trained biologists to safely and successfully trap waterbirds. This investment can be minimized when collecting waterbird samples in conjunction with established bird banding programs. By comparison, environmental samples are much faster to collect and do not require special skills or equipment but do require considerable resources for laboratory work. Water samples often need special equipment to filter and concentrate the diluted AIV therein (Deboosere et al. 2011), whereas sediment samples can require advanced molecular techniques (e.g., NGS) for AIV detection and characterization (Himsworth et al. 2020).

Restrictions: Direct sampling of waterbirds is subject to a number of legal restrictions. Hunting is often restricted to particular seasons, locations, and species (Deliberto et al. 2009) and usually requires licenses, thereby depending on good partnerships with local hunters to provide a sufficient sample size. Similarly, live capture of waterbirds usually requires permits or ethics board approvals and is not allowed for certain species, seasons, and locations.

Operational requirements: Timeliness, meaning the reporting of surveillance results soon enough to allow implementation of risk-mitigation strategies (Hoinville et al. 2013), is an operational requirement for early detection surveillance programs. Waterbird samples typically take longer to collect, whereas most environmental samples take longer to analyze. For this reason, it is critical for each AIV surveillance program to review the workflow from sampling through reporting to determine whether turnaround targets can be met. Large regional or national surveillance programs can collect and analyze hundreds of thousands of samples (Deliberto et al. 2009), and the ability to scale up operations to accommodate this is critical to the success of these programs. The scalability of certain environmental samples is primarily limited by the lack of automated laboratory protocols. For instance, the extraction of AIV RNA from sediment currently requires a manual protocol (Lang et al. 2008; Himsworth et al. 2020), which poses a significant barrier to operationalizing programs using these substrates. Possible solutions include further development of alternative protocols for time-consuming steps; for example, use of formalin-fixed erythrocytes for concentration of water samples (Dovas et al. 2010), or development of partnerships between multiple laboratories to analyze large numbers of environmental samples that require labor-intensive protocols (Pérez-Ramírez et al. 2012).

Knowledge gaps and priorities for future study

Although the literature that we reviewed provided abundant information to suggest the utility of environmental samples for AIV surveillance, there remain a number of significant knowledge gaps (Fig. 3).

Consistency among sample types: In a surveillance context, environmental samples are only useful if AIV found in the environment reflects those circulating in wild birds; however, the degree of consistency among AIV found in birds and the environment over time and space is not well understood. Inconsistency could lead to problems in interpreting data generated from environmental samples. A particular concern is if AIV persists in environmental substrates for prolonged periods of time—as has been documented in laboratory settings (Stallknecht and Brown 2009)—because it might then be possible to identify viruses or RNA in environmental samples that are no longer circulating in waterbird populations. Conversely, viruses that are currently circulating in wild waterbird populations might not be found in environmental samples that do not contain waterbird secretions or where AIV have degraded. Longitudinal studies that directly compare waterbird to environmental

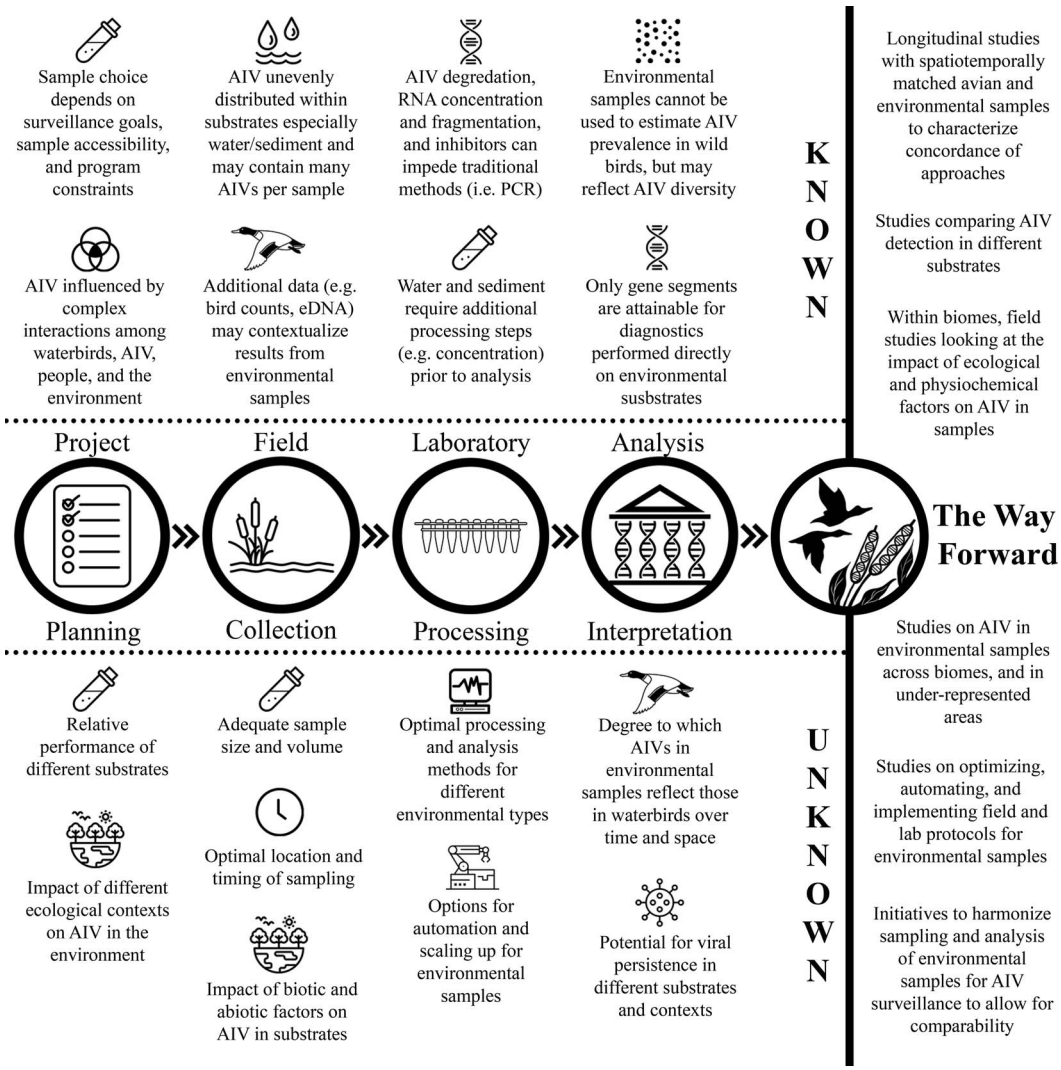

FIGURE 3. Overview of key knowledge, knowledge gaps, and future directions in the use of environmental samples for avian influenza virus (AIV) surveillance in wild waterbirds. The overview synthesizes peer-reviewed literature systematically retrieved from PubMed, Science Direct, EBSCO Host, and Ovid (Embase/Medline/Biological abstracts) databases, with publication dates between 1 January 2005 and 30 January 2019, and which contained terms belonging to five major groups (animal, diagnostic, disease, environment, and surveillance).

samples from the same locations and same sampling times are needed to understand how AIV in the environment change in relation to natural fluctuations in AIV abundance and diversity in waterbirds.

Consistency among different environmental sample types is also unknown. For example, AIV can persist longer in water than in feces in the laboratory (Franklin et al. 2011). These results need to be confirmed in field settings, because there are additional factors (e.g., degree of exposure to ultraviolet light, desiccation) that could affect the speed of AIV degradation outside the laboratory (Karmacharya et al. 2015). Although detection rates seem to vary between sample types (i.e., studies using sediment generally have a higher AIV detection rate than studies using water), the differences between field settings (e.g., terrestrial biome, season), field methods (e.g.,

volume of sample collected, number of samples collected), laboratory methods (e.g., concentrated or unconcentrated sample) provide a significant barrier for comparing sample types in a meaningful way. Future studies should therefore aim to collect multiple environmental sample types under the same field and laboratory conditions whenever possible to make direct comparisons.

Effect of micro- and macroenvironmental factors on environmental samples: Microenvironmental factors such as temperature, pH, and salinity are recognized to affect viral longevity in the laboratory (Stallknecht and Brown 2009), yet the manner in which abiotic and biotic substrate characteristics interact to affect viral persistence in complex natural settings is not understood. The effects of ecosystem-level factors on AIV in the environment also have yet to be fully described, which is a problem because these factors can influence the persistence and distribution of AIV in environmental samples. For example, AIV can persist in the environment for extended periods of time in certain settings—such as cold, freshwater lakes in northern latitudes (Shoham et al. 2012). Ecosystem factors can also influence how the waterbird hosts use their habitat and thus where AIV are deposited in the environment. Future studies of AIV using environmental samples should seek to include and compare a variety of ecological settings, particularly those that are currently underrepresented in the literature (e.g., southern hemisphere, saline or brackish environments, coastal regions, tropical regions). These studies should also aim to assess the effect of microenvironmental factors, waterbird species diversity, and study site characteristics (e.g., anthropogenic modification) on AIV in environmental samples.

Optimizing protocols for environmental samples: Regardless of the environmental sample type, there are no standard sample collection or analysis protocols evident in the reviewed literature. Using optimized, validated protocols is important for designing a surveillance program and for the interpretation of the program's results. How to approach sample size calculations is a dilemma as most require a priori knowledge or estimates regarding sampling frame, an estimate of the expected parameter (e.g., number of subtypes identified), and anticipated range of variation (Dohoo et al. 2012), none of which are established for environmental samples. These calculations are particularly problematic for water and sediment where there is no clear definition of what consists as an appropriate sampling unit and where the denominator for a sample size calculation has the potential to be infinite. A solution to the problem might be empirical or simulation studies that compare the number of sampling units to the surveillance result (e.g., number of subtypes identified) and visualize the point of diminishing returns at different spatial scales (e.g., within a beach vs. across beaches).

Sample processing methodologies also require further refinement. For instance, when an automated ultrafiltration method developed in the laboratory setting was used for the collection and concentration of natural lake water, it was found that the filter tended to clog with high-turbidity water (Francy et al. 2013). With regard to identifying the presence of AIV in environmental samples, low AIV concentration in these substrates often necessitated a more liberal PCR cycle threshold (Ct) cut-off than is typically used for samples obtained directly from waterbirds. Although the PCR Ct cut-off in waterbirds typically is $\leq$35 (Spackman and Suarez 2008), there is no consensus on what threshold should be for environmental samples; Ct$\leq$40 (Hénaux et al. 2012), $\leq$42 (Deboosere et al. 2011), and $\leq$45 (Stallknecht et al. 2010) have also been used for water samples. Similarly, it can be difficult to know how to interpret the results of NGS performed directly on environmental samples because it can be difficult to confirm results using other diagnostic methods. Traditional metagenomic thresholds (i.e., the number of reads needed to establish that an AIV subtype is present) are often unhelpful because of an overall low concentration of target RNA in these substrates compared to biological samples. Future studies should aim to refine and optimize the environmental surveillance workflow from sampling to data interpreta-

tion, while holding ecosystems and sample types constant to ensure interpretable comparisons can be made.

Study limitations and conclusions

Our study has several important limitations. First, we excluded biotic samples from our review due to the additional layer of complexity imparted by the ecology and distribution of the sample itself. Aquatic plants and animals, however, have significant promise for AIV surveillance and warrant further investigation and review. Second, although fecal samples were over-represented as a sample type in the reviewed literature, it is concurrently also possible that our search strategy missed surveillance results that considered fecal samples equivalent to cloacal samples and did not expressly mention feces in the study's title, abstract, or keywords. Finally, although the narrative synthesis approach is helpful for analyzing literature with significant methodological heterogeneity (Arai et al. 2007), it prohibits the numerical synthesis and comparison enabled by other review methodologies (e.g., Cochrane-style systematic reviews). Once the body of literature regarding AIV surveillance using environmental samples has been expanded and harmonized, it might be prudent to again review the field with even more rigorous approaches that can yield definitive conclusions and recommendations.

Environmental samples might have a role to play in AIV surveillance. Environmental samples might be particularly beneficial in the early detection of high-risk AIV subtypes, because they provide high information density per sample, might represent multiple birds or species within one sample, and a large number of samples can be collected easily and quickly, making them attractive for large-scale (e.g., national) surveillance programs; however, their utility in this context remains to be confirmed. Given that environmental samples and those obtained from waterbirds have different strengths and limitations, these two approaches must be used in a complementary manner to address the range of surveillance goals and needs for AIV risk assessment and mitigation. There are also still many questions that need to be answered in order to determine how best to design, implement, and interpret AIV surveillance systems based on environmental samples (Fig. 3). Despite these gaps in our knowledge, environmental samples currently show enough promise that they warrant concerted, ongoing investment as a tool to help us better predict and prevent future outbreaks of this devastating virus.

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SUPPLEMENTARY MATERIAL

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