ATTACHMENT 7

Environmental Sampling (Feces/Water)

Introduction

Avian Influenza viruses (AI) are released by waterfowl through the intestinal tract and viable virus can be detected in both feces and the water in which the birds swim, defecate and feed. This is the principle means of virus spread to new avian hosts and potentially to poultry and other susceptible livestock. Analysis of both water and fecal material from waterfowl habitat can provide evidence of AI virus circulating in wild bird populations, the specific AI subtypes, levels of pathogenicity, and possible risks to livestock.

Technical Aspects of Sampling Water

AI is relatively stable in water, especially at colder temperatures. The longevity of viable virus (weeks to months) allows for an integration of activity on a site basis. However, in the absence of established serial sampling, pinpointing the time at which a site becomes contaminated would be difficult. The advantages of including waterfowl sites as a point of sampling lie in the ease of collecting samples and the potential to sample the potential contaminating influence many birds at once. This method would provide a cost-effective, geographically explicit methodology. Moreover, given the ease of sampling, more sites could be sampled, providing for a higher resolution surveillance network.

Technical aspects to monitor water samples for AI involve collecting specified volumes of water (usually 50-500ml), transporting the samples on ice or frozen, concentrating the virus present either by filtration or precipitation/centrifugation, and inoculating the virus onto chicken eggs or cell culture for virus growth. The virus replicates in the cultures and is characterized by serological or molecular methods to determine specific subtypes. Alternative methods of analysis involve extracting viral genetic material from the sample with detection using molecular techniques such as reverse transcription-polymerase chain reaction (RT-PCR) and subsequent sequencing to determine subtype. Refinement of these methods still needs to be done. However, these techniques have the advantage of rapid results. All of the procedures for monitoring AI in water samples are generally established, and with proper expertise and equipment can be easily adapted to most laboratory settings.

Technical Aspects of Sampling Feces

Fecal sampling is used extensively in monitoring studies for AI in wild bird populations. The principal advantages of this method are that the costs and effort of capturing birds are avoided and large sample numbers can be quickly and easily obtained. It also is a

good method to determine the presence or absence of virus in bird populations present at a specific location. The disadvantage is that species identification is not always possible, determination of prevalence is complicated by the possibility of repeated sampling of individuals, and the sensitivity of the method is lower than for oral swabs. However, the increased sampling effort occurring because of reduced sampling costs are anticipated to offset any short comings owing to decreased sensitivity. Information from the field could be used to generate an environmental risk map related to specific areas (habitats) associated with potential AIV transmission.

Infectivity of the virus is maintained up to 4 days in wet feces at 25^{o}C. Best analytical results come from fresh fecal samples that are either processed quickly or frozen until processing. Thus this method of sampling, while providing good information, is best applied while birds are present at a location such that the samples are as fresh as possible. By restricting fecal collection to fresh samples, it allows for population census data to be collected, and by inference estimates of the species sources of the contamination. Species and individual identification through genetic typing of feces would allow estimates of prevalence.

Accredited laboratories have the capacity and infrastructure to analyze a limited number of samples for AI. The anticipated sampling effort for this surveillance study will require an investment in equipment and staff to provide results in a timely fashion. Equipment needs include real-time PCR thermalcyclers, RNA extraction capabilities, DNA sequencing capabilities, tissue culture and egg culture facilities, ultracold freezers, centrifuges and vacuum pumps.

Methodology

Sampling for highly pathogenic avian influenza (HPAI), such as highly pathogenic H5N1 avian influenza, from environmental deposition of virus by waterfowl should be accomplished by collecting and analyzing feces (Attachment 10) and water from areas of known use by high risk species (e.g., transcontinental migrants). The general challenges faced include; 1) Determining locations used by high risk species, 2) Refinement of existing methods for detecting the virus in water and fecal samples and developing the analytical infrastructure and capacity, 3) Design of a sampling system using composite samples for analysis.

Table 7-1. Qualitative comparison of environmental sampling methods.

Fecal Sampling	
Pros	**Cons**
Technically easy sample acquisition. Sampling represents non technical approach and would not require extensive training or experience by field personnel.	Viable virus restricted to fresh samples (1-4 days)
Generate large sample numbers quickly.	Large sample numbers can swamp lab systems (applies to all methods)
Does not require handling or capturing animals	
Low cost, well established technique amenable for high through-put screening (modified APHIS RT-PCR method). Sample analysis is transferable across labs.	
Capable of identifying HPAI contaminated sites/locations/regions. Prevalence would be estimated on a site basis. Information from the field could be used to generate an environmental risk map related to specific areas (habitats) associated with potential AIV transmission.	Identity of species and individuals unlikely, estimates of prevalence not possible. Species identification possible through molecular fingerprinting, but at additional cost.
BSL-2 laboratory conditions sufficient for initial diagnostic screening.	Requires Biosafety level 3 capabilities for virus isolation
Summary: An approach based on fecal sampling could be immediately implemented and may represent the only reasonable approach in areas where bird capture is not practical.	
Water Sampling	
Pros	**Cons**
Low cost	Biosafety level 3 capabilities for virus isolation
Effectively sample all or most birds present on the body of water	Analyses potentially complicated if multiple strains of AI present in water samples.
Samples easily, quickly obtained	Large volumes of water needed to concentrate virus for analysis, transportation and logistical issue
Virus stable, especially at moderate pH and low temperature	Longevity complicates interpretation on initial timing of contamination.
Does not require handling or capturing animals	Identity of species and individuals not possible/difficult. Prevalence calculation restricted to a site basis system.
Generate large sample numbers	Large sample numbers can swamp lab systems - need analysis infrastructure
Can provide large scale spatial risk assessment of HPAI contamination.	May need to validate technique

Sampling strategies to detect highly pathogenic H5N1 avian influenza virus in waterfowl populations will change depending upon the risk assessment and management goals and prevailing status of the pathogen in North America. For first detection of highly pathogenic H5N1 avian influenza virus in migratory birds efforts should focus on likely cross-over routes of birds from Asia to North America (e.g., Alaska and North Slope). Efforts should focus on areas of high aggregations of waterfowl intersecting with logistical sampling support, e.g., National Wildlife Refuge (NWR) system and state waterfowl management areas. While highly pathogenic H5N1 avian influenza virus may cross from Asia to North America at any point the surveillance network needs to be

tactically practical without compromising its ability for detection. Once highly pathogenic H5N1 avian influenza virus gains a foothold in North America the surveillance network should be placed along known waterfowl movement paths from the point of origin (i.e., point of detection). These paths can be inferred from known migration routes based on waterfowl telemetry data. However, practically, and given the patterns emerging in Eurasia, once highly pathogenic H5N1 avian influenza virus gains a foothold in North America the likelihood of rapid and diffusive spread across the continent is high. At this point local waterfowl and environmental sampling should target areas of strategic value, e.g., human population centers and areas of high density of poultry production. In the former case, such areas would be represented by urban zoo-parks and lakes. These areas would represent the highest level of risk of human contact with contaminated water and/or waterfowl. In the latter case, ponds, lakes and waterfowl management areas around high density poultry production areas would provide the best ability to assess risk of transmission to humans and poultry. Surveillance efforts patterned on these areas are best amenable to local and state efforts for first detection and subsequent risk assessment once the highly pathogenic H5N1 avian influenza virus achieves enzootic status in North America.

There is an inherent conflict between the need for high resolution surveillance, the number of samples generated, the time to analyze those samples, and the cost of analysis. If the goal is first detection, methods that integrate across many individuals and species at a particular site without loss of sensitivity should be preferred. Currently analysis of fresh fecal samples is the best method to achieve these goals. For logistically practical and economic reasons sample analysis should focus on composite samples on a per site basis; this bulk sample minimizes effort in both data collection and analysis, while greatly increasing the probability of detection. Given the expected rarity of highly pathogenic H5N1 avian influenza virus in current migratory bird populations, this approach will allow for a substantially reduced number of samples to be analyzed. Table 7-2 provides a hypothetical, but plausible, example of the expected number of tests per composite fecal sample necessary to detect Highly pathogenic H5N1 avian influenza virus. When prevalence is very low (e.g., 10^{-7}) almost all composites will test negative and on average only a single test will be needed to determine the absence of highly pathogenic H5N1 avian influenza virus in that composite sample.

The approximate sample sizes necessary for assuring a high probability of detecting highly pathogenic H5N1 avian influenza virus depends on its prevalence in the population, which is currently unknown. However, a preliminary estimator is:

$$p^* = 1 - (1 - r/m)^{1/n} \qquad \text{(eq. 1)}$$

where p^* is the proportion of infected individual samples across all composite samples, r is the number of composite samples that test positive for the presence of highly pathogenic H5N1 avian influenza virus, m is the total number of composite samples tested, and n is the number of individual samples in each composite sample (e.g., fecal count or volume). Rearranging eq. 1 provides an estimate of the number of individual

fecal samples needed to detect highly pathogenic H5N1 avian influenza virus, for a given population level prevalence;

$$n = \ln(1-r/m) / \ln(1-p^*) \qquad \text{(eq. 2)}$$

Table 7-2. Expected number of tests needed for a single positive reaction for each composite sample containing 100 individual fecal samples, *n*, as a function of expected prevalence of HPAI, *p*. Calculation is based on the binomial probability model describing the average number of tests needed as $(n+1) - n(1-p)^n$

Prevalence in Waterfowl (p)	Individual fecal samples/composite (n)	Mean # composite samples to test
10^{-3}	100	10.5
10^{-4}	100	2.0
10^{-5}	100	1.1
10^{-6}	100	1.0
10^{-7}	100	1.0

The results for various hypothetical values of r, m, n, and p^* are shown in Table 7-2. Thus, if highly pathogenic H5N1 avian influenza virus prevalence is 10^{-6} and 10,000 independent fecal samples are collected, analysis of 100 composite samples would result in detecting the presence of highly pathogenic H5N1 avian influenza virus in one composite. These two equations allow us to initially estimate the number of fecal samples to be collected and to estimate prevalence of highly pathogenic H5N1 avian influenza virus in the population.

Table 7-3. Number of individual fecal samples *n*, for a fixed prevalence p^*, needed to detect the presence of HPAI in 1 out of 100 composite samples. Calculation is based on the probability model given by eq. 2.

Prevalence in Waterfowl (p^*)	Number of positive composites (r)	Number of composites (m)	Number of individual samples (n)
10^{-3}	1	100	10
10^{-4}	1	100	100
10^{-5}	1	100	1005
10^{-6}	1	100	10050
10^{-7}	1	100	10050

Safety

Given the concern of introduction of highly pathogenic H5N1 avian influenza virus into North America, and the potential for human infection, significant precautions should be taken by workers conducting the environmental sampling and those handling the samples in the laboratory. In the lab, standard BSL-3 precautions are required for virus isolation, and BSL-2 precautions for molecular diagnostics. In the field, workers should wear disposable gloves and garments. Gloves should be decontaminated with 70% ethanol frequently, or changed often as necessary. Mucous membranes (eyes, nose, throat) should be protected from splashes and aerosols. This may require covering with protective equipment such as goggles and hepafiltered masks in some cases. Field workers should avoid direct contact with animals after handling environmental samples until decontamination procedures are completed (e.g. changing garments and gloves). Untrained workers (such as the general public) should be discouraged from collecting and submitting environmental samples for testing.

Summary

Monitoring of water and/or fecal samples gathered from waterfowl habitat is a reasonably cost effective, technologically achievable means to assess risks to poultry in the western hemisphere to new, potentially highly pathogenic subtypes of AI. A surveillance system based on water sampling is not ready to implement. However, the validation of this method could come on-line in a short period of time and would represent considerable cost savings without loss of sensitivity. Fecal sampling is an established technique and is ready for use in surveillance with the establishment of sampling guidelines. Both approaches yield advantages where individual bird sampling is too costly or logistically impractical. Either approach could yield a spatial and habitat risk assessment for site contamination with highly pathogenic H5N1 avian influenza virus. The main considerations are where and when to get the samples, ensuring proper storage and

transport, and the capacities and capabilities of the laboratories doing the analyses. Real-time reporting and the infrastructure to support such reporting is a serious constraint on any surveillance system. The ability to integrate, analyze, and responsibly disseminate these data is critical and needs to be addressed.