ABSTRACT: Prior to the ongoing Asian H5N1 epizootic, it was believed that highly pathogenic avian influenza (HPAI) was a disease of domesticated birds and that wild birds could act only as reservoirs for the low pathogenic form (LPAI) of these viruses. The unprecedented situation in Asia during 2004 and 2005 revealed spillover of HPAI H5N1 infection to naïve populations of wild birds. To date the role wild birds may play in the ecology and epizootiology of HPAI H5N1 appears very different to that in other HPAI epizootics, and is far from being completely understood. Recent outbreaks in Russia and Central Asia, Europe, and Africa indicate transboundary spread of HPAI H5N1. Isolation of the virus from wild birds in Eurasia led to speculation that wild birds may play a role in introducing and possibly secondarily spreading the infection to disease-free areas. Several diagnostic methodologies are available to detect avian influenza infection. Generation of reliable data requires the most appropriate diagnostic approach specific to the aim of the surveillance program and ecology of the virus in both the target population and the environment. Harmonized diagnostic strategies for detection of HPAI H5N1 must be developed and followed worldwide in order to make results comparable and useful to the international scientific community. The OIE/FAO Reference Laboratory in Padova, Italy is involved in extensive surveillance of wild birds sampled in Europe, Africa, and the Middle East. Using results of this program, we developed a set of diagnostic guidelines that could be a basis for a harmonized approach by diagnostic laboratories.

Key words: Avian influenza, HPAI H5N1, laboratory diagnosis, wild birds.

According to the revised World Organization for Animal Health (OIE) definition, all avian influenza viruses belonging to H5 and H7 subtypes are defined as notifiable avian influenza (NAI) viruses, regardless of their virulence for birds (OIE, 2005). All avian influenza viruses of low pathogenicity for poultry (LPAI), including low pathogenic H5 and H7 viruses, are maintained in nature in wild birds, mainly in Anseriformes and Charadriiformes. Recent outbreaks of highly pathogenic avian influenza (HPAI) H5N1 in Russia, Central Asia, Europe, and Africa suggest the virus may have been carried by wild birds during their autumn and spring migrations. To gain more information on the presence and persistence of HPAI H5N1, several countries and international organizations developed and applied surveillance programs targeting wild bird populations. However, prior to implementing a surveillance program it is essential that the objectives are defined in order to select the most appropriate diagnostic approach.

Generally speaking, surveillance in wild birds can have two main objectives: to establish the circulation and prevalence of all avian influenza viruses (including H5 and H7 LPAI and HPAI), and to establish the presence or absence of HPAI viruses (e.g., HPAI H5N1) in the selected population at a given point in time. Assessment of NAI viruses in wild birds is relevant to identify areas at higher risk of their introduction into domestic poultry and better understand the ecology of these viruses in their natural reservoirs. The collection of viral strains and knowledge of the most prevalent hemagglutinin (H) and neuraminidase (N) subtype combinations also can be instrumental for development of vaccines for poultry. Surveillance programs focusing on detection of HPAI viruses in the wild could act as an early
warning system. This would allow rapid implementation of preventive measures in a country or in a defined area.

The two main objectives mentioned above can be achieved within the same surveillance program. However, because different diagnostic approaches may be more suitable to one or the other purpose, the main objective of a surveillance program should be identified and prioritized. We discuss the use and application of difference diagnostic approaches for wild bird surveillance. In addition, in some cases environmental sampling can be used to determine the persistence of infectious viral particles in the environment, although this type of sampling requires validation and improvement before it can be deemed a reliable means of detecting environmental contamination.

Correct selection of the target population is the first important step of any surveillance program. Although this topic is beyond the scope of our paper and will not be addressed, it is important to bear in mind that among the vast variety of wild birds, the target population might be different on the basis of the two main objectives listed above. In addition, selection of the most appropriate sample, and adequate collection, storage, and shipment is essential for the success of any surveillance program, regardless of the diagnostic approach.

Wild bird surveillance, mainly based on cloacal sampling of captured or hunted waterfowl in Europe and Africa in recent times, revealed active circulation of many LPAI subtypes. However, no HPAI H5N1 viruses were detected, even in countries in which the pathogenic virus was confirmed in ill or dead wild and domesticated birds (SANCO, 2006; Gaidet et al., 2007). This might indicate that the approach used in these surveillance programs is suitable for finding AI viruses but not HPAI viruses.

Environmental detection of AI viruses may be a possible indicator to establish virus persistence following cleaning and disinfection procedures during and after an AI outbreak (Suarez et al., 2003). However, sampling to determine the presence and persistence of AI viruses in the natural environment has not been extensively applied. Although not specifically recommended, collection of fresh faecal dropping can substitute for cloacal swabbing in wild birds (Munster et al., 2005).

Serology offers a relatively inexpensive and practical way to determine circulation and prevalence of influenza viruses in the avian population. In the framework of a surveillance effort in domesticated birds, serological diagnosis is considered a suitable approach to monitor the AI-free status of farms, including backyard and industrial birds. On the contrary, the application of current serological methods in wild bird surveillance will generate information of very limited use. Antibodies to the group antigen of influenza A viruses may be detected by agar gel immunodiffusion (AGID) and immunoenzymatic tests. These tests are unable to give any indication of the virus subtype. Because several species of wild birds are the natural reservoirs of AI viruses, the detection of group-specific rather than subtype-specific antibodies is a common finding of no diagnostic relevance.

The above-mentioned tests have additional limitations for use in wild fowl. Waterfowl usually do not produce serum precipitins (Higgins, 1989) and, therefore in spite of exposure, they rarely yield positive results in the AGID test. Furthermore, most of the current commercial immunoenzymatic tests are based on indirect detection of specific antibodies through antispecies conjugates. These tests were developed for detecting infection in intensively reared poultry, namely chickens and turkeys, and therefore employ antichicken IgG secondary antibodies. These kits have not been validated for wildfowl, and their reliability in these species remains to be established.

Hemagglutination inhibition assay (HI) provides information on the presence of
antibodies directed against a specific subtype and can be applied on a variety of bird species. The sera of certain avian species contain nonspecific hemagglutinins that interfere with the HI test. Such sera are therefore treated prior to the test. In any case, given that wild birds are the reservoir of AI viruses, detection of antibodies against NAI only represents evidence of an exposure to H5 or H7 antigens and no information on whether exposure was recent or not, or on the H and N combination of the viruses or the pathotype.

Traditionally, laboratory protocols for detection and identification of avian influenza viruses were based on virus isolation (VI) in specific-pathogen-free eggs or in cell cultures. This methodology still represents the gold standard and the official method for detection of AI viruses (OIE, 2005). It can be used on all types of clinical, laboratory, and environmental specimens including swabs, organs, faeces, and other organic and inorganic materials. Virus isolation is the only method that can determine the persistence of viable virus in the environment. In extensive surveillance efforts in wild birds, however, its use is limited mainly because it is laborious, time consuming, and cannot be performed on large numbers of samples. In particular, limiting factors appear to be the availability of staff, equipment (e.g., biosafety cabinets, incubators), eggs, and reagents. Virus isolation can yield positive results only if the samples contain viable virus particles. In some instances, wildlife sample collection occurs in remote areas, where it is not always possible to ensure proper storage and/or prompt shipment of samples to the laboratory. In these cases, VI attempts might lead to false-negative results.

The available immunoenzymatic (antigen capture) methods for influenza virus antigen detection generally are easy to perform and can be applied in the laboratory and directly in the field. They are directed to detection of one of the major viral proteins (nucleoprotein or hemagglutinin) and do not require viable virus to yield a positive result. The methodology is now easily accessible from many companies on the international market. Some products developed for influenza virus detection in humans can be applied to other animal species, such as poultry (Cattoli et al., 2004; Bai et al., 2005; Woolcock and Cardona, 2005). Recently, several kits for the rapid detection of AI antigens in poultry also were developed and marketed. Most of these kits can detect influenza type A antigens (i.e., viral matrix protein or nucleoprotein); thus they cannot provide any information about the H or N subtype or the pathotype. It is not possible to express an overall opinion on the performances of these kits, as full field validation currently is unavailable. However, generally speaking, published data (Cattoli et al., 2004; Woolcock and Cardona, 2005) and laboratory experience in poultry testing indicate that antigen capture methods are useful in some situations but have limited sensitivity and specificity compared to other methods. Analytical sensitivity is generally in a threshold range between $10^4$ and $10^3$ EID$_{50}$, and this should be kept in mind when issuing a diagnosis based on these methods. Certain types of specimens, such as faeces or internal organs, sometimes lead to false positive results, thus limiting the specificity of the test.

Several protocols based on different molecular methodologies, such as RT-PCR, real-time RT-PCR (rRT-PCR) and NASBA (nucleic acid sequence-based amplification), were developed in the last decade for detection of influenza viral RNA in clinical and laboratory specimens. They are generally very sensitive, specific, and adaptable to high throughputs. PCR-based and sequencing protocols are available to detect subtype and pathotype of the virus directly on clinical materials, thus allowing a rapid turnaround time and faster characterization. Careful evaluation of the method employed to extract the
RNA is necessary to avoid false-negative results due to inefficient extraction or presence of contaminants that can inhibit the molecular reactions. On the other hand, molecular methods based on enzymatic amplification of nucleic acid are at risk of generating false-positive results due to cross-contamination of samples, particularly when high numbers of samples are processed at the same time, as might happen during extensive surveillance programs.

Unpublished observations on the performance of rRT-PCR for detection of HPAI H5N1 in carcasses of wild swans (Cygnus olor) at the Italian Reference Laboratory showed that a higher amplification signal and lower cycle threshold (Ct) values could be obtained in tracheal and lung tissues of infected birds. Comparisons of samples from the intestinal contents and pooled lungs and trachea showed differences ranging from 3 to 9 Ct. Whether this was due to a different viral load in the tissues or to the presence of PCR inhibitors in those particular specimens is not known, but sampling the respiratory tract of wild birds may be more suitable for molecular detection of HPAI H5N1 viruses.

At the time of writing, rRT-PCR can probably be considered the preferred molecular method for wild bird surveillance, as it allows high throughput in combination with high sensitivity and specificity. However, no official methods exist for the molecular detection of AI viruses in domestic and wild birds. This results in different protocols used among diagnostic laboratories and can make the comparison and interpretation of results difficult. Recently, a selection of molecular protocols, including conventional RT-PCR and rRT-PCR, were evaluated in inter-laboratory trials (Slomka et al., 2007) and will be included among the recommended protocols in the diagnostic manual for AI published by the European Union.

Based on the characteristics of the different methods and taking into account the main objectives of a surveillance program, guidelines for an appropriate diagnostic approach can be summarized as follows:

To determine circulation and prevalence of AI viruses (including H5 and H7 LPAI and HPAI viruses):

1. Active surveillance in captured or hunted wild birds.
2. Cloacal swabs and, when possible, tracheal or oropharyngeal swabs. Environmental sampling of fresh faecal droppings also can be performed.
3. Rapid molecular screening tests specific for type A influenza viruses are recommended. Test positive and uncertain samples by VI. Rapid molecular subtyping also can be performed on positive samples.
4. Serology and rapid diagnostic immunoenzymatic methods are not recommended. Current serological tests used on wild birds probably will lead to results that can be difficult to interpret. The cost and limited sensitivity of antigen capture immunoenzymatic methods suggest their use in extensive surveillance programs is questionable.

To determine presence or absence of HPAI viruses in the population of a certain area:

1. Collect samples in any reported cases of abnormal mortality or abnormal behavior in wild birds.
2. Active surveillance on species at high risk. Obtain respiratory and digestive tract samples by swabbing or by collection of internal organs.
3. Screen suspected cases by molecular target-specific techniques (e.g., for H5 or H7 subtype, in addition to type A specific tests) or directly by VI. Rapid immunoenzymatic tests also can be applied in suspect cases, paying attention to the quality and the type of material processed and following manufacturer’s instructions to avoid specific-
ity problems. HPAI viruses are present in significant amounts in selected samples collected from moribund birds or from birds that died during the acute phase of the disease. High titers reached in internal organs under these circumstances may overcome the sensitivity limits of the immunoenzymatic methods. A positive test therefore can provide a preliminary indication concerning the etiology of the investigated mortality. However, samples should be tested by another independent rapid test (e.g., molecular test) regardless of the antigen capture immunoenzymatic results. All samples tested positive by immunoenzymatic or molecular tests should be confirmed by VI, and full characterization of the isolate should follow, particularly for samples submitted from supposed HPAI-free areas. Serology is not recommended.

To make results comparable and useful to the international scientific community, it is essential that harmonized diagnostic strategies aiming at the detection of influenza viruses, and specifically to HPAI and HPAI H5N1 viruses, are developed and followed worldwide. Regardless of approach used, virus isolation techniques can be delayed but never abandoned, as they still remain the only diagnostic tools that enable complete and genetic characterization of the virus. Collecting virus isolates is of fundamental importance to increase our knowledge of AI, including selection of candidate vaccine strains, as well as pathogenesis and evolutionary studies on influenza viruses.

LITERATURE CITED


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