Monitoring exposure to avian influenza viruses in wild mammals

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ABSTRACT

1. Avian influenza (AI) viruses primarily circulate in wild waterfowl populations and are occasionally transmitted to domestic poultry flocks. However, the possible roles of other wildlife species, such as wild mammals, in AI virus ecology have not been adequately addressed.

2. Due to their habitat and behaviour, many wild mammals may be capable of transmitting pathogens among wild and domestic populations. Exposure to AI viruses has been reported in an array of wild and domestic animals. The presence of wild mammals on farms has been identified as a risk factor for at least one poultry AI outbreak in North America. These reports suggest the need for seroprevalence studies examining the exposure of wild mammals to AI viruses.

3. Serological tests are routinely used to assess domestic poultry, domestic swine and human exposure to influenza A viruses, but these tests have not been validated for use in wild mammals. As such, some of these protocols may require adjustments or may be inappropriate for use in serology testing of wild mammals. Herein, we review these serological techniques and evaluate their potential usefulness in AI surveillance of wild mammals. We call for care to be taken when applying serological tests outside their original area of validation, and for continued assay verification for multiple species and virus strains.

INTRODUCTION

Influenza viruses belong to the family Orthomyxoviridae and are lipid-enveloped viruses with a segmented, negative-sense RNA genome (Webster *et al.*, 1992). Embedded in the lipid envelope are the hemagglutinin (HA) and neuraminidase (NA) proteins responsible for virus attachment and release from host cells, respectively (Webster *et al.*, 1992). Influenza viruses are divided into three types: A, B and C. While influenza B and C viruses are primarily 'human' viruses, influenza A viruses infect a variety of species including birds, humans and other mammals (Webster *et al.*, 1992). Influenza A viruses are divided further into subtypes based on their HA and NA proteins; 16 HA (H1 – H16) subtypes and 9 NA (N1 – N9) subtypes have been isolated from avian species (Fouchier *et al.*, 2005; Olsen *et al.*, 2006). Subtypes that are not commonly isolated from humans, swine or horses are often referred to

as 'avian influenza' (AI). Most AI viruses circulating through waterfowl are classified as low pathogenic (LPAI; USGS, 2009). To date, only H5 and H7 subtypes have the potential to become highly pathogenic (HPAI) viruses, which cause high rates of poultry mortality and severe economic loss for poultry producers (Alexander, 2000; USGS, 2009). While AI has historically been considered an 'avian disease', evidence of AI infection has occasionally been reported in humans, and in a variety of domestic and wild mammalian species. In fact, in a 2002 risk assessment on LPAI H7N2 among commercial poultry farms in western Virginia, USA, the presence of mammalian wildlife was identified as a risk factor for AI infection in poultry (McQuiston *et al.*, 2005). Most recently, reports of the HPAI H5N1 Asian strain infection in mammals have sparked renewed interest in the potential roles of wild mammals in the ecology of these viruses (USGS, 2009).

The first evidence of severe disease in wild mammals attributed to AI infection was documented in harbor seals (*Phoca vitulina*) off the New England coastline during two outbreaks in 1979 and 1983, which resulted in the deaths of hundreds of these pinnipeds (Geraci *et al.*, 1982; Hinshaw *et al.*, 1984). The H7N7 and H4N5 influenza A subtypes were responsible for these outbreaks and were found to be antigenically and genetically similar to avian-derived strains although apparently adapted to these mammals (Geraci *et al.*, 1982; Hinshaw *et al.*, 1984). AI viruses have continued to be isolated from marine mammals off the New England coast representing H3, H4, H7 and H13 subtypes (Geraci *et al.*, 1982; Hinshaw *et al.*, 1984; Hinshaw *et al.*, 1986; Callan *et al.*, 1995). While the risk of marine mammals transmitting AI viruses to poultry operations or human populations is undoubtedly minimal, other wild mammals may form better links between infected wild waterfowl, poultry and/or humans. For example, evidence of AI infection has been reported in species of the mammalian families Mustelidae, Procyonidae, Sciuridae, Viverridae, Canidae, Felidae and Suidae. The home ranges of many of the species in these mammalian families may include habitats of wild birds and domestic farms or urban areas (Merrill, 1962; Dunstone & Ireland, 1989; Zeiner *et al.*, 1990; Lanski, 2003; Cleary & Craven, 2005; Wyckoff *et al.*, 2005).

Susceptibility of species within the family Mustelidae to AI viruses has been reported as a result of natural and experimental infections. For example, an outbreak of an avian-derived H10N4 within several mink (*Mustela vison*) farms in southern Sweden caused 100% mortality in these mammals, resulting in approximately 3000 deaths (Klingeborn *et al.*, 1985; Berg *et al.*, 1990). Experimentally, mink are highly susceptible to LPAI viruses by intranasal inoculation (Matsuura, Yanagawa & Noda, 1979) and by contact with other infected mink (Yagyu *et al.*, 1981; Okazaki, Yanagawa & Kida, 1983). Virus was isolated from mink nasal swabs for up to 11 days post-inoculation (dpi; Yagyu *et al.*, 1981). Striped skunks (*Mephitis mephitis*) are also susceptible to LPAI infection via intranasal inoculation or ingestion of infectious chicken eggs, and shed virus from their trachea and intestines for up to 8 dpi (Bailey, 1983). More recently, a report of stone marten (*Martes foina*) susceptibility to HPAI H5N1 infection after presumably feeding on an infected bird carcass has been published (WHO, 2006a). The combination of AI virus susceptibility and habitat, which often includes poultry farms or human settlements (Merrill, 1962; Dunstone & Ireland, 1989; Lanski, 2003), illustrates the potential risk of AI transmission by mustelids to humans and poultry.

Like the mustelids, mammals in the Procyonidae, Sciuridae and Canidae are also common on farms and in urban areas (Zeiner *et al.*, 1990; Cleary & Craven, 2005; Haemig *et al.*, 2008). Originally, the idea of raccoons (*Procyon lotor*) and 13-lined ground squirrels (*Spermophilus tridecemlineatus*) as potential transmitters of AI viruses was evaluated in experimental infections which resulted in AI positive trachea aspirates from 13-lined ground squirrels for up to 9 dpi and the seroconversion of a single raccoon (Bailey, 1983). A recent experimental

infection has shown that raccoons infected with LPAI H4N8 are capable of shedding low levels of virus for several days, and may be able to transmit the virus to nearby raccoons through aerosol transmission (Hall *et al.*, 2008a). A concurrent serological survey reported evidence of LPAI infection in raccoons trapped in three US states: Wyoming, Colorado and Maryland (Hall *et al.*, 2008a). Red foxes (*Vulpes vulpes*) frequent rural and urban habitats (Reperant *et al.*, 2008) and their proximity to humans has recently been identified as a potential risk factor for tick-borne encephalitis outbreaks in humans (Haemig *et al.*, 2008). Their proximity to humans has recently been identified as a potential risk factor for tick-borne encephalitis outbreaks in humans (Haemig *et al.*, 2008). To assess their potential roles in AI transmission, red foxes were experimentally infected with HPAI H5N1. Foxes, which were inoculated intratracheally, excreted virus for 3–7 dpi and developed severe pneumonia, myocarditis and encephalitis (Reperant *et al.*, 2008). However, foxes that were fed infected bird carcasses also shed virus for 3–5 days but developed only mild signs of disease; this may allow them to play a role in virus dispersal (Reperant *et al.*, 2008).

Civets are small omnivorous viverrids that are often hunted and trapped for their meat and medicinal properties (WAZA, 2008). In June 2005, HPAI H5N1 was isolated from three Owston's civets (*Chrotogale owstoni*) that had apparently succumbed to infection at the Owston's Civet Conservation Program in Cuc Phuong National Park, northern Vietnam (Roberton *et al.*, 2006). While the source of infection is unknown, poultry deaths were reported in villages adjacent to the park and the civet enclosures were not rodent-proof or roofed (Roberton *et al.*, 2006). Because civets are hunted and often traded at live-animal markets, they may have the potential to spread pathogens to other animals or humans (Webster, 2004). A relative of the Owston's civet, the Himalayan palm civet (*Paguna larvata*), is suspected to play a role in SARS virus transmission (Guan *et al.*, 2003).

Avian-derived influenza virus infection in wild felids was first documented following deaths of tigers (*Panthera tigris*) and leopards (*P. pardus*) from HPAI H5N1 infection after they were fed infected poultry carcasses at zoos in Thailand in 2003 and 2004 (Keawcharoen *et al.*, 2004; Thanawongnuwech *et al.*, 2005). The first incidence of domestic cats (*Felis catus*) becoming infected with avian derived HPAI H5N1 also occurred in Thailand in 2004; 14 of 15 cats from one household died after one cat had consumed a chicken carcass on a farm where a HPAI H5N1 outbreak occurred (Kuiken *et al.*, 2006). Contact transmission of HPAI H5N1 from infected swans, ducks and chickens to domestic cats has also been documented (Leschnik *et al.*, 2007). Considering the common occurrence of domestic and feral cats on farms, the increased encroachment of humans and livestock on large wild cat territories (Sekhar, 1998), and the susceptibility of felids to HPAI H5N1 infection, it is possible that these animals could play a role in AI virus transmission to farms or other human settlements.

Many studies have illustrated the susceptibility of domestic swine (*Sus scrofa*) to AI viruses (Karasin *et al.*, 2000; Ninomiya *et al.*, 2002; Choi *et al.*, 2004). Experimental inoculation of domestic swine with 38 unique AI strains resulted in successful replication of 29 of the isolates, many with high titers of nasal shedding ($>10^4$ EID$_{50}$/mL; Kida *et al.*, 1994). As with domestic cats, domestic swine can escape from farms and give rise to feral populations in many parts of the world. Feral swine often share habitats with waterfowl and frequently come into contact with domestic swine and humans (Wyckoff *et al.*, 2005). Although experimental inoculations and AI exposure have yet to be reported in feral swine (Hall *et al.*, 2008b), it seems probable that feral swine are just as susceptible to a wide variety of AI viruses as their domestic counterparts; they may therefore play a role in AI virus transmission from wild populations to domestic farms.

To understand better the roles of wild or feral mammals in the transmission of AI viruses, a logical first step is to conduct surveillance studies for evidence of AI virus exposure in a diversity of species. In some studies, animals are surveyed for current AI infection by testing tissues, swabs or environmental samples for the presence of AI viruses; common tests include virus isolation in chicken embryos and/or reverse transcription polymerase chain reaction (USGS, 2008). While surveying for active infections only provides current information, serological surveillance, in which blood serum is tested for the presence of antibodies to a past infection, can be used to identify any past exposure to influenza viruses. These data can then be used to estimate the proportion of individual animals or populations that have ever been exposed. Certain serological tests are used to screen serum samples and identify exposure to any influenza A virus; these include agar gel immunodiffusion (AGID), and enzyme-linked immunosorbent assays (ELISA). However, researchers often want to know the exact influenza subtype to which an animal was exposed. Common subtype-specific assays include hemagglutination-inhibition (HI), virus neutralization (VN) and microneutralization (MN) tests, and single radial haemolysis (SRH) assays. Because so many serological assays exist for influenza testing in humans and domestic animals, wildlife researchers may be quick to use them for wild mammal testing as well. However, immune responses are often pathogen-dependent and can vary between species (Davis & Hamilton, 1998); a protocol that works for mammal-derived influenza A viruses may not work with avian-derived viruses, and an assay that has high sensitivity and specificity when testing for antibodies from one species may not be reliable when testing for antibodies from other species. Here, we review these traditional assays and evaluate their potential usefulness in AI surveillance of wild and feral mammals by addressing the question: do assays developed for detecting influenza A-specific antibodies in humans and/or domestic fowl adequately detect AI-specific antibodies in wild mammals?

In the following sections, we describe assays that are currently available to detect antibodies to influenza A viruses. While none of the following serological tests has been validated for use with wild mammals, some tests may perform better than others. Wildlife researchers should take into consideration the advantages, disadvantages and potential pitfalls of each test when surveying for AI exposure in wild mammals (Table 1) and keep in mind that more than one test may be necessary for confirmation.

AGID

The AGID test has been a standard screening tool for poultry antibodies to influenza A viruses. All influenza A viruses have antigenically similar nucleocapsid and matrix proteins. Concentrated virus preparations containing either or both of these antigens are used in the AGID test. The antigen preparation is added to a centre well in an agar-gel matrix and the positive control sera and poultry test sera are added to surrounding wells. The agar plates are incubated overnight and if a serum sample is positive for antibodies to influenza A viruses, a precipitation line will generally form where the antigen and antibodies meet. However, not all species produce precipitating antibodies. For example, some avian species do not form precipitins to various antigens (Toth & Norcross, 1981; Higgins, 1989). While the AGID is a standard test in the poultry industry, it has not been validated for the use with any species other than domestic chickens and turkeys (OIE, 2008).

HI

The HI test is useful in detecting HA subtype-specific antibodies (e.g. H1, H2) and is based on the ability of the HA protein to agglutinate (bind together) red blood cells (RBCs). Known

Table 1. Advantages, disadvantages and potential pitfalls in the use of traditional serological assays to detect avian influenza antibodies in wild mammals

Assay	Advantages	Disadvantages	Potential pitfalls
AGID	• Simple and inexpensive • Few reagents • Can be performed without infectious virus • Useful for general influenza A screening • 'Next-day' results	• Subjective interpretation of results • Does not distinguish between subtypes	• Depends on the formation of precipitating antibodies. Not all species produce precipitating antibodies
HI	• 'Same-day' results • Subtype specific • Can be performed without infectious virus • Established protocols for domestic animals and humans	• Not practical for general influenza A screening • Reagents are costly and difficult to acquire • Cross-reactions can occur between subtypes • Requires large amount of sera	• The use of horse RBCs has greatly improved sensitivity in humans and domestic animals but still does not reliably detect H7 exposure in some mammals
VN and MN	• Considered 'gold standard' • Subtype specific • Established protocols for domestic animals and humans • Sensitive and specific when testing humans and domestic animals • 'Next-day' results with MN	• Requires infectious virus • Requires tissue culture • Some influenza A viruses can be difficult to culture • 3–4 days for standard VN results • Not practical for general influenza A screening • Requires large amount of sera	• Unless a standardized protocol is used, caution must be taken when comparing titers between laboratories
SRH	• Can be performed without infectious virus • Similar sensitivity as VN and HI • Subtype specific • 'Next-day' results	• Subjective interpretation of results • Cross-reaction and non-specific binding can occur • Not practical for general influenza A screening	• Non-specific antibodies should be removed by adsorption with other influenza A viruses • No standard OIE or WHO protocol for inter-laboratory comparison
cELISA bELISA	• Sensitive and specific • 'Same-day' results • Can be performed without infectious virus • Can be designed for screening or subtyping • Requires small amount of sera	• Published protocols require reagent production (not commercially available) • Require frequent optimization	• May be useful for multiple species and multiple subtypes but not currently validated • No standard OIE or WHO protocol for inter-laboratory comparison

AGID, agar gel immunodiffusion; bELISA, blocking ELISA; cELISA, competitive ELISA; HI, hemagglutination-inhibition; MN, microneutralization; OIE, The World Organisation for Animal Health; RBCs, red blood cells; SRH, single radial haemolysis; VN, virus neutralization; WHO, World Health Organization.

subtypes of influenza viruses are mixed with serum samples and then added to chicken RBCs (OIE, 2008). If the serum in question contains antibodies specific to that influenza subtype, then the antibodies will bind to the virus and prevent the virus from agglutinating the RBCs. An HI titer is assigned based on the last dilution of sera that prevents hemagglutination. With the HI test, the HA subtype and antibody titer can be determined, although titers may differ slightly between laboratories.

Originally, it was hypothesized that AI viruses do not induce a humoral (antibody) response in mammals, because antibody detection in AI-infected ferrets (*Mustela putorius furo*) was found to be lower than that in ferrets infected with mammalian-derived strains of influenza A virus (Hinshaw *et al.*, 1981). However, subsequent AI studies conducted on ferrets, ducks, pigs and mice suggested that the failure to detect mammalian antibodies specific to AI viruses was actually due to problems with the HI test (Lu, Webster & Hinshaw, 1982; Kida *et al.*, 1994; Ninomiya *et al.*, 2002). While mammalian antibodies to AI viruses frequently did not inhibit hemagglutination of homologous avian-derived virions, these antibodies did inhibit hemagglutination of viral subunit antigens and/or mammalian-derived viruses of the same subtype (Lu, Webster & Hinshaw, 1982). More recently, the HI test has been improved for detecting antibodies against AI viruses in humans by using horse RBCs in the assay (Stephenson *et al.*, 2003, 2004; Puzelli *et al.*, 2005; Gill *et al.*, 2006; Meijer *et al.*, 2006; Traenor *et al.*, 2006; Jia *et al.*, 2008). Horse erythrocytes contain almost exclusively SAα2,3-Gal linkages (the cellular binding sites of AI viruses), whereas the traditionally used chicken or turkey RBCs contain a mixture of SAα2,6-Gal (the cellular binding sites for human-derived influenza viruses) and SAα2,3-Gal linkages (Stephenson *et al.*, 2003). While the horse-RBC HI test has worked well for testing selected mammalian sera for most AI subtypes, problems in identifying exposure to the H7 subtype still have not been resolved (WHO, 2006b; Kayali *et al.*, 2008).

Often, the neuraminidase inhibition (NAI) assay is performed in conjunction with the HI assay, which results in the identification of both the HA and NA subtypes. The World Health Organization (WHO) suggests the use of both assays when subtyping antibodies acquired through animal surveillance (WHO, 2002). An NAI protocol is detailed in the WHO manual (WHO, 2002) and to date, no concerns about the NAI test have been published.

Virus neutralizing and microneutralization tests

VN tests are often considered the 'gold standards' of serology. In influenza testing, the VN test detects subtype specific circulating antibodies directed against the HA antigen (Stephenson *et al.*, 2007). Briefly, infectious virus of a known subtype is combined with serum samples, allowed to incubate and added to a cell monolayer (usually MDCK cells) for 3–4 days. Results are often expressed as a titer based on cytopathic effect in the monolayer caused by viral infection. The MN test is a variation of the VN assay performed in microtiter plates with 'next day' results expressed as a titer based on an enzymatic reaction. The MN assay can therefore accommodate more samples at once and produce more timely results (Okuno *et al.*, 1990; Rowe *et al.*, 1999). Although considered equally (or more) sensitive and strain-specific as the horse RBC HI tests, VN and MN titers can also differ slightly between laboratories (Stephenson *et al.*, 2007; Kayali *et al.*, 2008; Wood, 2008). Inter-laboratory differences in sera preparation, quantity of virus added, neutralization time and dilution may all contribute to variability (Stephenson *et al.*, 2007). To compare results between laboratories, suggested VN and MN protocols have been published by WHO for influenza surveillance in lower mammals and birds (WHO, 2002).

SRH assays

SRH assays were developed for the detection of HA- and NA-specific antibodies in humans. These assays use antibody diffusion in agar gel and the hemolyzing property of influenza antigen-antibody complexes to measure the antibody content of test sera (Morley *et al.*, 1995). Influenza virus particles are attached to the surface of RBCs and combined with a source of complement (serum proteins that aid in immune response) and agar in a Petri dish. After the gel has cooled, heat inactivated serum samples are added to wells. As plates are incubated, antibody from serum samples diffuses from the wells into the gel at a rate proportional to the concentration of antibodies. HA-specific antibodies attach to a homologous virus-RBC complex causing complement mediated hemolysis as it diffuses through the gel. The size of the resulting zone of hemolysis is proportional to the amount of HA-specific antibody contained in the test samples. The SRH test has sensitivity equivalent to the VN and horse RBC HI tests for detecting H5-specific antibodies in human sera (Stephenson *et al.*, 2003). Although the clarity of zones is sometimes variable, the test is rapid, convenient and can be performed with inactivated viruses (Wood *et al.*, 2001).

ELISA

ELISAs can be designed to detect antibodies to all influenza A viruses or only subtype-specific antibodies. Many variations of ELISAs are used in serology but all follow the same basic principles. Antigen-antibody complexes are allowed to form, and an enzyme-labelled secondary antibody is added. This conjugated-secondary antibody is specific for the antigen or antibody, depending on the ELISA platform used. Similarly to the MN test, a substrate is added and the subsequent enzymatic reaction is measured by a spectrophotometer.

Indirect ELISAs (iELISAs) were the first to be developed for detection of antibodies to influenza A viruses in exposed or 'at risk' poultry flocks (Snyder *et al.*, 1985; Abraham *et al.*, 1988; Adair *et al.*, 1989). The iELISA was found to perform as well, or better, than the AGID and HI tests in detecting antibodies to AI in chickens and turkeys (Snyder *et al.*, 1985; Abraham *et al.*, 1988; Adair *et al.*, 1989). However, the iELISA requires species-specific conjugated antibodies, so a different conjugated antibody is needed for each species tested.

Unlike the iELISA, blocking ELISAs (bELISAs) and competitive ELISAs (cELISAs) are not species-specific because the secondary antibody used in these tests is specific for the monoclonal antibody used, regardless of the species being tested. The cELISA has been evaluated for use with chickens, turkeys, game birds, ratitites and penguins (Shafer, Katz & Eernisse, 1998). In these avian species, the cELISA was more sensitive and more specific than the AGID test (Shafer, Katz & Eernisse, 1998), as sensitive and specific as the HI test (Shafer, Katz & Eernisse, 1998), and able to detect some antibodies earlier in infection than the AGID and HI tests (Zhou *et al.*, 1998). While the cELISA has only been evaluated for use with avian sera, the bELISA has been used to assess exposure of some mammals to human and swine influenza viruses. The bELISA detected antibodies to influenza A viruses in naturally infected marine mammals, in ferrets experimentally infected with human influenza isolates and in swine experimentally infected with swine influenza isolates (De Boer, Back & Osterhaus, 1990).

CONCLUSIONS

Exposure to AI viruses has been documented in several mammalian species. Because of their susceptibility to AI infection and their potential contact with domestic animals and/or humans (e.g. through overlapping habitats or feeding behaviours, or via 'wet-markets' where livestock are sold alive), certain wild mammal species may be capable of transmitting AI

viruses between wild waterfowl and domestic animals or humans (Clark & Hall, 2006). Increased interest in the role of wild mammals in influenza ecology has led to wildlife biologists entering the field once occupied only by poultry producers, human and veterinary diagnosticians, and primary influenza research laboratories. Studies are already underway to characterize seroprevalences in some wild mammalian species (Hall *et al.*, 2008a,b; Marschall *et al.*, 2008). While there is a need for these studies, researchers must choose diagnostic tests carefully to avoid underreporting influenza virus exposure. Table 1 summarizes the advantages and disadvantages, potential pitfalls and necessary protocol adjustments when using traditional serological tests for wild mammal seroprevalence studies. However, even with protocol adjustments, a WHO working group on influenza research has expressed concern that some human seroprevalence studies may still be reporting false-negative H5 and H7 results due to possible limitations and variability of serological tests (WHO, 2006b).

When using a serological test outside of its original validation, it is difficult to predict if the test will perform correctly (Gardner, Hietala & Boyce, 1996). Differences in pathogens, host immune responses and various environmental exposures may have serious effects on the outcome of a test. It is also important that validation is not a 'one-time' experiment, but that assays are continually reassessed for different species, pathogens and other conditions (Jacobson, 1998). AI viruses are continually changing and adapting to new hosts, which emphasizes the continued need for assay validation for multiple species and multiple virus strains, to understand better the incidence of false-negative results that may affect prevalence estimates in wild mammals.

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