Istituto Zooprofilattico Sperimentale delle Venezie

GUIDELINES AND MINIMUM REQUIREMENTS FOR DIAGNOSIS OF H5Nx HPAI INFECTION IN MAMMALS

1. Clinical signs

Clinical signs are highly variable and influenced by factors such as the virulence of the infecting virus, route and dose of infection, the species affected, age, sex, concurrent diseases, environment and the immune status of the host. Signs described so far in the order *Carnivora*, in particular in domestic carnivores, include loss of appetite, apathy, hypersalivation, fever, dyspnea (shallow and accelerated breathing), nasal discharge and nervous signs such as circling, lack of coordination, epileptic seizures, increased muscle tension and lethargy. Animals may also die without premonitory signs and sudden increased mortality may be the only evidence in farmed carnivores.

Both for domestic and wild mammals of the order *Carnivora*, asymptomatic infections have been reported.

In countries where mass mortality of pinnipeds was observed, this was preceded by an extensive mortality of seabirds. The mass mortality event described is consistent with systemic infection with highly pathogenic influenza virus resulting in acute encephalitis and pneumonia.The clinical signs presented by sick animals while still alive were mainly neurological (lethargy, inability to stand or walk, tremors, convulsions, paralysis) and respiratory (breathing difficulty, dyspnea, tachypnea, eye discharge, nasal and mouth secretions), in addition to miscarriages. Many HPAI H5N1 infected South American sea lions were pregnant and several abortions were observed [1].

The signs reported in cetaceans (e.g. *Phocoena phocoena*), after they had been infected with the HPAI H5N1 virus, were swimming in circles, inability to right themselves, and drowning.

2. Gross lesions

On post-mortem examination, multiple internal organs are often congested and hemorrhagic, but in some cases minimal lesions may be detected at macroscopic level due to the peracute nature of the disease. The most common pathological findings include bronchointerstitial pneumonia, edematous or hemorrhagic lungs, meningoencephalitis, brain congestion and hepatitis. Necrosis and inflammation can also be identified in the heart, kidney, spleen, intestine and pancreas.

3. Differential diagnosis

Clinical signs associated with an H5 HPAI infection are similar to the ones caused by other infectious and non-infectious agents. Infections in mammals of the order *Carnivora* with H5N1 HPAI viruses of the 2.3.4.4b clade have been commonly associated with the involvement of the respiratory and nervous apparatus. Pathologies that affect these organs in the species of interest (e.g. rabies, intoxicants, Canine Distemper disease, tetanus, morbillivirus, brucella, toxoplasma, herpesvirus infections, etc.) should also be included in the diagnostic algorithm, to ascertain the cause of disease.

4. Suspected case definition

Whenever the official veterinarian has a suspicion of an outbreak of H5 HPAI in mammals, the competent authority must ensure that an investigation is performed and satisfactorily completed before the presence of the infection can be is excluded. A suspected case is defined as such when a mammal:

- shows clinical signs which could be indicative of HPAI infection (see paragraph 1);
- has a confirmed history of exposure to, or highly probable contact with an HPAI positive dead or sick domestic or wild animals;
- is found dead in areas/holdings characterized by the active circulation of HPAI viruses in wild and/or domestic birds, irrespective of the occurrence of mass mortality events.

5. Samples collection and transport

It is imperative to take into account the risk to public and animal health when handling or sampling suspect cases. For the transport of samples, please refer to the guidelines for the diagnosis of AI in birds.

Sample size for both virological and serological sample collection varies on expected prevalence, population size and desired confidence level, as described in the table reported below. In the lower part of the table, the number of samples required per shed in domestic mammal farms; in case of five or more sheds, sampling must in any case take place uniformly in the different breeding structures.

Expected prevalence		5%		10%		20%	
		*95% LC**	*99% LC*	*95% LC*	*99% LC*	*95% LC*	*99% LC*
Population size (wild or domestic)	50	34	41	22	29	12	17
	100	44	59	25	35	13	19
	500	55	82	28	42	13	20
	1000	57	86	28	43	13	20
	3000	58	88	28	43	13	21
	5000	58	89	28	44	13	21
	7000	58	89	28	44	13	21
	10000	58	89	28	44	13	21
Number of samples to be taken in each breeding shed	1 shed	60	90	30	45	20	30
	2 sheds	120	150	60	90	40	60
	3 sheds	180	210	90	135	60	90
	4 sheds	240	280	120	180	80	120
	5+ sheds	250	300	150	200	100	150

*LC: level of confidence

Virological testing

In the majority of H5 HPAI positive live domestic/farmed carnivores (e.g. cats, minks) and wild mammals (e.g. foxes, sea lions), the virus has been commonly detected in nasal or tracheal/oropharyngeal swabs and, to a lesser extent, in rectal swabs. In consideration of this, *in vivo* non-harmful sampling should include samples from the upper respiratory tract (URT) (e.g. nasal or tracheal/oropharyngeal swabs) and/or samples from the lower respiratory tract (LRT) (e.g. bronchoalveolar lavage). Caution is advised when performing *in vivo* sampling of the LRT, due to the risks associated with increased aerosolization of the virus during this procedure.

Nonetheless, for moribund or neurological animals, as well as in case of carcasses, H5 HPAI virus has mainly been found in the lower respiratory tract and at the level of the central nervous system, hence both these anatomical sites should be examined. To target the LRT, we recommend the collection of the bronchoalveolar

lavage and/or sampling of multiple parts of the lung (e.g. different lobes), favoring specimen that include not only the alveolar tissue but also part of the bronchial tree. Cerebrum, cerebellum, Ammon's corn and medulla oblongata were generally found all positive in foxes, although the highest viral loads were mostly detected in the cranial/frontal part of the cerebrum and the cerebellum. Furthermore, it is likely that the first sites involved in the infection are the olfactory bulbs. In case the number of carcasses to be examined is very high, a simple access to the brain can be achieved by reaching the foramen magnum and coring the caudal portions of the brain. To this end, expose the neck upwards, make an initial cut of the skin and then free the atlanto-occipital joint from muscles and tendons, using a knife. Perform a core sample using a sterile disposable spoon, forceps, scissors and knife (see pictures below).

However, with this method less materials of CNS (above all of cerebrum) is generally obtained, so deep coring is recommended to sample as frontally/cranially as possible.
In suids, nasal swabs often tested negative in HPAI H5NX infections; it is therefore recommended to carry out a deep pharyngeal swab or, better still, a lung sampling from dead animals.
In marine mammals, HPAI H5NX viruses have mainly been detected in oropharyngeal/oral swabs (live animals) and in the spleen, brain and lungs (dead animals). Rectal swabs were positive less frequently.

Serological sampling
Blood sampling should be carried out approximately 10-14 days after the assumed onset of infection and repeated after two-three weeks, to compare the antibody titers in acute-phase sera to those detected in convalescent-phase sera

6. Procedures for molecular and serological laboratory testing

Molecular testing
For virus detection, AI molecular methods applied for testing birds can also be used for mammalian samples processing. In case of positive results to AIV screening (e.g. SOP VIR 018), downstream H5 subtyping has to be performed. The EURL AI/ND has validated some procedures for Eurasian H5 detection (e.g. 143, 1004) and pathotype definition based on the sequence of the haemagglutinin cleavage site (e.g. SOP VIR 125). Detailed protocols are available on the EURL AI/ND website (https://www.izsvenezie.com/reference-laboratories/avian-influenza-newcastle-disease/diagnostic-protocols/).

Antibody detection
The serological assays that have been repeatedly used by the EURL for AI/ND in ferrets, minks, dogs, cats and pigs naturally or experimentally infected with HPAI H5N1 virus of clade 2.3.4.4b are here below reported. If required, the EURL can provide assistance for testing or confirming HPAI doubt/positive serum samples obtained from mammals.

ELISA Test
A preliminary screening can be performed with a commercially available multi-species competitive ELISA Kit targeting antibodies specific for Nucleoprotein (NP) of type A influenza viruses. Prior to routine testing, it is recommended to carefully evaluate the validity of ELISA tests in the species under examination due to the unknown diagnostic performance of these commercial assays with mammalian species other than swine and equine. We therefore recommend carrying out an additional confirmatory test (e.g. MN).

Hemagglutination Inhibition Tests
ELISA-positive sera should be further analysed by the hemagglutination inhibition (HI) test performed according to the WOAH Terrestrial manual [2] using the homologous antigen. A contemporary H5 HPAI strain belonging to the circulating clade can be used as an antigenic surrogate of the virus responsible for the outbreak. Briefly, three volumes of receptor-destroying enzyme (RDE Seiken, Japan) are added to one volume of serum. The mixture is incubated overnight at 37 °C, and subsequently inactivated at 56 °C for 30 min and brought to a final dilution of 1:10 by adding six volumes of phosphate buffered saline (PBS). To remove non-specific hemagglutinating factors, if present, one volume of packed chicken erythrocytes can be added to 20 volumes of serum and incubated at 4 °C under gentle shaking for 1h, before removing erythrocytes by centrifugation at 300 x g for 5 min. HI tests are performed using four hemagglutinating units of virus with 0.5% chicken erythrocytes according to standard procedures. Two-fold dilutions are tested starting from 1:10 dilution.

Neuraminidase Inhibition Assay
The neuroaminidase inhibition (NI) test is performed using inactivated antigens (as the ones used for HI) selected on the basis of the targeted N. In brief, sera are incubated for 20 min at room temperature (RT) in a

1:1 ratio with the test antigen previously diluted 1:20 in a PBS solution. Fetuin is added and gently mixed before an overnight incubation at 37 °C. To convert free N-acetyl neuraminic acid into β-formyl pyruvic acid, sodium periodate is added and the solution is incubated for 30 min at 37 °C. The revelation of free β-formyl pyruvic is achieved upon addition of 2% sodium arsenite and thiobarbituric acid. In the absence of antibodies inhibiting neuraminidase enzymatic activity, a pink color is observed upon incubation at a boiling temperature in a water bath.

Microneutralization Assay

For a subset of HI and ELISA positive sera, the microneutralization (MN) assay should be carried out as a confirmatory test. The MN assay is performed according to the standard procedure described by the Center for Disease Control (CDC) [3]. Briefly, sera should be used after the same RDE-based pretreatment adopted for HI testing. Briefly, two-fold dilutions of the treated sera are prepared in Dulbecco modified Eagle medium (D-MEM). The dilutions are then mixed to a 1:1 ratio with a virus solution containing 100 TCID 50% of the H5 HPAI virus and incubated for 1 h at 37 °C. To each well, 1.5×10^4 MDCK cells are added and incubated with the virus-serum mixture for 18–20 hs, at 37 °C with 5% CO_2.

After incubation, cells are fixed with a cold solution of 80% acetone and incubated for 1h at RT with a 1:1 mixture of primary anti-influenza A nucleoprotein monoclonal antibodies at a dilution of 1:4000 in blocking buffer. After washing, cells are incubated for 1h with a secondary goat anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP) at a dilution of 1:6000. After washing, a substrate based on o-phenylenediamine dihydrochloride (OPD) and citrate buffer is added to each well for 15 min at RT and then stopped with a 0.5 N sulfuric acid solution.

The optical absorbance of wells should be read at 490 nm to identify the reciprocal of the highest serum dilution resulting in 50% virus infection of cells.

Interpretation of results

For a better interpretation of results generated through the HI and MN assays, sera of mammals potentially exposed to both the Goose/Guangdong (Gs/GD) lineage HPAI viruses and to the non-Gs/GD lineage H5Nx viruses, should be tested with more than one virus/antigen. In particular, a second antigen/virus representative of the non-Gs/GD H5Nx viruses circulating in Europe should be included. Given the considerable antigenic distance between these viruses, a differential in the inhibiting/neutralizing titers is expected. For example, the EURL has observed that positive sera from mammals exposed to or infected with 2.3.4.4b H5N1 viruses have a 8-16 fold lower reactivity with non-Gs/GD European H5Nx viruses than with 2.3.4.4b clade H5N1 viruses.

Moreover, the MN assay has proven more sensitive than the HI assay in the detection of subtype specific antibodies in mammals exposed to or infected with 2.3.4.4b H5N1 viruses.

Reference

1. Gamarra-Toledo, V., Plaza, P. I., Gutierrez, R., Inga-Diaz, G., Saravia-Guevara, P., Pereyra-Meza, O., ... Lambertucci, S. A. (2023). Mass Mortality of Marine Mammals Associated to Highly Pathogenic Influen- za Virus (H5N1) in South America. BioRxiv, 2023.02.08.527769. Available at: <https://doi.org/10.1101/2023.02.08.527769>
2. Chapter 3.3.4., Avian influenza (including infection with high pathogenicity avian influenza viruses.
3. Kitikoon, P.; Vincent, A.L. Microneutralization assay for swine influenza virus in swine serum. *Methods Mol. Biol.* **2014**, *1161*, 325–335.