Istituto Zooprofilattico
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GUIDELINES AND MINIMUM REQUIREMENTS FOR DIAGNOSIS OF H5N1 HPAIV INFECTION IN CATTLE

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This situation is evolving rapidly. Check back frequently the EURL website for updated versions.

These recommendations are based on the findings in the dairy cows in the USA and on the information provided by the US representatives

1. Premise

The H5 HPAI situation in dairy cows in the USA is evolving rapidly, and important features of disease ecology such as the source of the infection, transmission dynamics and infection duration are still under investigation and may vary from a context to another.

2. Clinical signs in suspected cases

- Sudden drop in feed intake with concurrent decreased rumination and rumen motility;
- Subsequent marked drop in individual/herd milk production. More severely affected cows may have thickened, discolored milk that almost appears like colostrum or may have essentially no milk; increased somatic cell count has been observed as well;
- Lethargy, dehydration and fever;
- Changes in manure - most reporting indicates tacky or loose faeces in affected cattle.

3. Samples collection and transport

- Lactating cows

In case of suspect, samples from dairy cattle (milk/udder secretions) should be collected from clinically affected animals (up to 20 individuals). It is of utmost importance that each quarter of an individual is sampled and pooled together, as there have been reports of only one quarter having virus. Submit to the laboratory between 3-10 ml of milk per animal under refrigeration. For submission of milk in transport medium, refer to paragraph 5 “Procedures for molecular laboratory testing”.
Based on the currently available information, it appears that the virus replicates particularly in the mammary tissue, as Ct values in milk samples can be as low. In the USA, other tissues and stool have been rarely found to be positive (at high Ct) in animals with confirmed virus detection in mammary tissue and milk. So far, whole blood tested negative. No information on meat or other edible organs are available to date.
It is highly recommended to test any other sick and dead wild/domestic mammals (in particular barn cats) or birds in close proximity to the premises where suspected dairy cows are located.

Recommendations for other types of cattle production are given below:

- Calves and dairy beef: testing is currently not recommended in the USA.
- Pregnant, springing heifers and breeding bulls: nasal swabs should be collected. A single sterile swab can be used to sample both nostrils of individual animals, ensuring that the mucosa is brushed firmly. Place the swab

in the transport medium (e.g. PrimeStore MTM - Longhorn, BHI, or PBS with antibiotics/antimycotics), vigorously swirl and press the head against the wall of the tube to release the liquid. Do not retain the swab into the medium. PrimeStore MTM (Longhorn) is the only medium that can be submitted without refrigeration. Do not submit dry swabs. Do not pool nasal swabs.

4. Procedures for molecular testing

Milk samples

Process samples within 24-48 hours. Because of milk composition and thickness in diseased animals, the NVSL, USDA and APHIS recommend diluting one part of milk with three parts of Primestore MTM (i.e. transferring 0.5 mL milk into a vial containing 1.5 mL medium) to improve nucleic acids extraction. It is important not to exceed the recommended ratio. Concentrating the milk by centrifugation is not helpful. To increase testing capacity, laboratories may pool up to 5 milk samples from different individuals (200 μl each) of the same epidemiological unit.

Alternatively, if staff collecting milk at farms is provided with 5 mL tubes pre-filled with 1.5 mL Primestore MTM, it is possible to use a standard dry swab for milk collection and dilution, as follows: dip the swab into the milk to adsorb fully, and then transfer swab head into the tube containing Primestore MTM. Swirl the swab into the medium, press the head against the wall of the tube to release the liquid and finally discard the swab.

It is recommended to submit to the laboratory both diluted and undiluted milk.

The EURL for AI/ND has determined analytical sensitivity of a number of testing procedures (i.e. SOPs VIR 018, 143, 1004 for the H5 target, 1005) employing H5 HPAI spike-in of bulk milk and of individual mastitic milk diluted in Primestore MTM. The following extraction kits were evaluated as being fit for purpose:

- MagMAX Pathogen RNA/DNA Kit (Applied Biosystems) on a KingFisher Flex Purification System (Thermofisher Scientific) according the procedure reported in SOP VIR 1000 ed. 02.). Two hundred microliters (200 μL) sample (milk diluted in Primestore MTM) were processed;
- MagMAX Core Nucleic Acid Purification Kit (Applied Biosystems) on a KingFisher Flex Purification System (Thermofisher Scientific). For each sample, the lysis/binding solution was prepared pipetting 350 μl MagMAX CORE Lysis Solution, 350 μl MagMAX CORE Binding Solution and 9 μl intype IC-RNA (Indical Bioscience). Two hundred microliters (200 μL) sample (milk diluted in Primestore MTM) were processed with the workflow "Simple" following the instructions reported in the user manual.

Please note that the above recommendations on sample preparation and nucleic acids extraction refer to avian influenza testing only and their fitness for the detection of dairy cow pathogens considered in the differential diagnosis from the same sample aliquot need to be assessed. The use of different transport media for milk samples (e.g. bacteriostatic preservatives, guanidine-based media) has not been tested at the current stage and therefore their use should follow assessment.

Swab samples

Refer to the procedures reported in SOP VIR 1000 ed. 02. The MagMAX Core Nucleic Acid Purification Kit (Applied Biosystems) has yet to be validated on swabs, but based on the data on milk samples it should be considered equivalent to the MagMAX Pathogen RNA/DNA Kit (Applied Biosystems).

5. Procedures for serological testing

To better characterize the extent of past exposure events, animals can also be tested serologically by hemagglutinin inhibition (HI) test using A(H5N1) HPAI strain belonging to 2.3.4.4b clade as HA antigen. The HI assay for bovines and small ruminants should be conducted according to the guidelines for the diagnosis of influenza A in mammals that were previously shared on Mattermost in October 2023 (GUIDELINES AND MINIMUM REQUIREMENTS FOR DIAGNOSIS OF INFLUENZA A IN MAMMALS - AI-ND EURL.docx). As little or partial information is available regarding the profile of seroconversion of HPAI-infected cattle and the diagnostic performance of the available serological assays, we recommend coupling the HI assay with at least one

complementary screening serological test (e.g. multi-species competitive ELISA kit targeting the NP protein). Performance testing of several ELISA kits and other serological assays is currently ongoing at the EURL and results will be shared as soon as possible.

References

- USDA, Animal and Plant Health Inspection Service. Testing recommendations for Influenza A in cattle. Version.2024.04.01. Available at: https://www.aphis.usda.gov/sites/default/files/nvsl-hpai-dairy-testing-recommendations.pdf
- IZSVe-EURL AI/ND. Diagnostic protocols. Available at: https://www.izsvenezie.com/reference-laboratories/avian-influenza-newcastle-disease/diagnostic-protocols/