# ISTITUTO ZOOPROFILATTICO SPERIMENTALE DELLE VENEZIE

# EUROPEAN UNION REFERENCE LABORATORY FOR AVIAN INFLUENZA AND NEWCASTLE DISEASE

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# INTERPRETATION OF RESULTS OF DIAGNOSTIC TESTS FOR AVIAN INFLUENZA AND NEWCASTLE DISEASE

This document provides an explanation of potential limitations of a positive or negative result to one diagnostic method, considering the provisions of Article 6 of Regulation (EU) 2020/689 on case definitions and Annex I of the same Regulation for specific case definitions for highly pathogenic avian influenza, infection with low pathogenic avian influenza viruses and infection with Newcastle disease virus.

## Explanation of the potential limitations of a positive or negative result to virus isolation

Related to Regulation (EU) 2020/689 on case definitions and Annex I of the same Regulation

- ***Annex I, Section 1, Highly pathogenic avian influenza (HPAI)***
  ***1. An animal or a group of animals must be considered, by the competent authority, as a suspected case of HPAI when it meets the criteria laid down in Article 9(1).***
  ***2. An animal or a group of animals must be considered, by the competent authority, as a confirmed case of HPAI when:***
  ***(a) The disease agent responsible for HPAI, excluding vaccine strains, has been isolated in a sample from an animal or from a group of animals;***

- ***Annex I, Section 2, Infection with low pathogenic avian influenza viruses (LPAIV)***
  ***1. An animal or a group of animals must be considered, by the competent authority, as a suspected case of infection with LPAIV when it meets the criteria laid down in Article 9(1).***
  ***2. An animal or a group of animals must be considered, by the competent authority, as a confirmed case of infection with LPAIV when:***

***(a) The disease agent responsible for infection with LPAIV, excluding vaccine strains, has been isolated in a sample from an animal or from a group of animals;***

- ***Annex I, Section 3, Infection with Newcastle disease virus (NDV)***
  ***1. An animal or a group of animals must be considered, by the competent authority, as a suspected case of infection with NDV when it meets the criteria laid down in Article 9(1).***
  ***2. An animal or a group of animals must be considered, by the competent authority, as a confirmed case of infection with NDV when:***
  ***(a) The disease agent responsible for infection with NDV, excluding vaccine strains, has been isolated in a sample from an animal or from a group of animals;***

A suspect positive result is obtained through the observation of virus-induced death/lesions of embryo in bird eggs or of characteristic morphology changes in inoculated cells. Confirmation that the virus sought has grown, also in apparently normal embryonated eggs or in cells, is obtained through the detection of a hemagglutinating activity and through the typing of the virus with reference antisera. HA activity of sterile fluids harvested from the inoculated eggs is most likely caused by an influenza A virus or an avian orthoavulavirus; however, some avian reovirus or adenovirus strains, as well as non-sterile fluid containing haemagglutinins of bacterial origin, may also cause the agglutination of RBCs. There are several recognised serotypes of avian avulaviruses and considering almost universal use of live vaccines in poultry for NDV, it is recommended to test for their presence by the haemagglutination inhibition (HI) test.
In the HI test, some level of cross-reactivity may be observed among the various avian paramyxovirus serotypes. Cross-reactivity can be observed between APMV-1 and APMV-3 viruses (particularly with the psittacine variant of APMV-3, commonly isolated from pet or exotic birds), APMV-7 or APMV 12. The risk of mistyping an isolate can be greatly reduced by using a panel of reference sera or monoclonal antibodies (MAbs) specific for APMV-1, APMV-3, APMV-7 and APMV 12. Alternatively, the presence of the virus sought can be confirmed by the use of specific conventional RT-PCR or real-time RT-PCR.
A false positive result may be due to incorrect handling during sample collection (for example during the simultaneous autopsy of birds of different origins) or sample preparation and egg inoculation in the laboratory.
As well as in embryonated eggs, avian influenza and Newcastle disease viruses can be propagated in cell cultures. Although these viruses can infect a variety of primary and continuous cell lines, many of these do not support an efficacious productive viral replication. For this reason, it is important to evaluate the susceptibility of the cell line before proceeding with the viral isolation test.
If the virus isolation test is performed correctly, a negative result is mainly ascribable to the absence of viable virus in the specimen. However, samples obtained from infected animals can still yield negative results by virus isolation due to both technical and biological factors. For instance, in the presence of low loads of viable virus, bad sample quality, incorrect handling, transportation and storing conditions may limit the viability of the virus. In mild or subclinical infections, as well as in early or delayed infections, viral loads in samples are often low and require several passages in embryonated eggs before a sufficient amount of progeny virus is produced, allowing detection by conventional methods.
False negative results may also depend on the suboptimal sensitivity of the selected diagnostic method. For example, the use of the AGID test to demonstrate the presence of AIV nucleoprotein or of matrix antigens lacks sensitivity if compared to the majority of molecular methods.

# Explanation of the potential limitations of a positive or negative result to molecular tests

Related to Regulation (EU) 2020/689 on case definitions and Annex I of the same Regulation

- ***Annex I, Section 1, Highly pathogenic avian influenza (HPAI)***
  ***1. An animal or a group of animals must be considered, by the competent authority, as a suspected case of HPAI when it meets the criteria laid down in Article 9(1).***
  ***2. An animal or a group of animals must be considered, by the competent authority, as a confirmed case of HPAI when:***
  ***(b) Nucleic acid specific to the disease agent for HPAI, that is not a consequence of vaccination, has been identified in a sample from an animal or from a group of animals;***

- ***Annex I, Section 2, Infection with low pathogenic avian influenza viruses (LPAIV)***
  ***1. An animal or a group of animals must be considered, by the competent authority, as a suspected case of infection with LPAIV when it meets the criteria laid down in Article 9(1).***
  ***2. An animal or a group of animals must be considered, by the competent authority, as a confirmed case of infection with LPAIV when:***
  ***(b) Nucleic acid specific to the disease agent for infection with LPAIV, that is not a consequence of vaccination, has been identified in a sample from an animal or from a group of animals;***

- ***Annex I, Section 3, Infection with Newcastle disease virus (NDV)***
  ***1. An animal or a group of animals must be considered, by the competent authority, as a suspected case of infection with NDV when it meets the criteria laid down in Article 9(1).***
  ***2. An animal or a group of animals must be considered, by the competent authority, as a confirmed case of infection with NDV when:***
  ***(b) Nucleic acid specific to the disease agent for infection with NDV, that is not a consequence of vaccination, has been identified in a sample from an animal or from a group of animals;***

The nucleic acid of the specific AIV and NDV responsible of the disease generally comply with a more or less recent infection and may be indicative of an active outbreak.
The increased sensitivity of the molecular methods leads to the detection of target RNA also in samples with a low viral load and/or in the absence of viable virus. Thus, care should be taken when interpreting laboratory results of molecular tests. This issue may be overcome by testing multiple samples from the same cohort of infected birds, especially relevant when analysing samples from domestic poultry for disease investigation.
In case genome fragments from the lab environment, such as carry-over from previous PCR experiments or contaminant nucleic acids from positive samples, are accidentally incorporated into the PCR reaction, they can be amplified during the reaction yielding false positives. Nucleic acids contamination, particularly from PCR products, can be hardly reduced or removed once it has occurred. In order to prevent such setback, best laboratory practices should be implemented when performing PCR tests, including the adoption of dedicated laboratory equipment and unidirectional rooms for different steps of the workflow, the use of filter tips and proper chemical-physical decontamination techniques.

Lacking to detect the nucleic acid of the specific agent responsible for the disease is generally indicative of a non-infectious status of the tested animals. However, failures in molecular tests for the detection of AIV or NDV may occur in complex sample matrices, such as cloacal swabs, faeces and tissues (e.g.

pancreas, liver) due to the presence of PCR inhibitors that may be responsible for false-negative results. Incorporation of an internal control will prevent such events and help to ensure test reliability. Improved nucleic acids extraction methods have been developed to eliminate most PCR inhibitors from test samples. Pre-analytical procedures for sample preparation might also help to prevent issues caused by PCR inhibition. As the system used affects the success of downstream molecular tests on clinical samples, care should be taken when selecting the most appropriate reagents, commercial products and procedures for the molecular diagnosis of AIV and NDV; at the same time, the method should be standardized and validated on the platforms available at each laboratory.
Furthermore, it has to be kept in mind that assay inclusivity, and thus the rate of false negatives, can be dependent from oligonucleotides development that might be designed for the detection of specific targets. For example, H5 and H7 primer/probe sequences designed for the detection of Eurasian H5 and H7 isolates might not be suitable for H5 and H7 from other parts of the world. Each set of primers and probes needs to be validated against a wide panel of viruses from a broad range of geographic areas and time periods in order to assure test applicability to numerous avian species.
Due to the large genetic diversity among AOAV-1 (NDV) viruses a laboratory testing algorithm should ideally be developed taking into account the purpose for which the test is being applied. Furthermore, this should consider the relevance of the test for the detection of local co-circulating strains (but also consider the risk of introduction of new viruses) and be appropriately validated for the purpose for which it is used.

## Explanation of the potential limitations of a positive or negative result to serological tests

Related to Regulation (EU) 2020/689 on case definitions and Annex I of the same Regulation

- ***Annex I, Section 1, Highly pathogenic avian influenza (HPAI)***
  ***1. An animal or a group of animals must be considered, by the competent authority, as a suspected case of HPAI when it meets the criteria laid down in Article 9(1).***
  ***2. An animal or a group of animals must be considered, by the competent authority, as a confirmed case of HPAI when:***
  ***(c) Positive result to an indirect diagnostic method, that is not a consequence of vaccination, has been obtained in a sample from a kept animal or from a group of kept animals showing clinical signs consistent with the disease or epidemiologically linked to a suspected or confirmed case;***

- ***Annex I, Section 2, Infection with low pathogenic avian influenza viruses (LPAIV)***
  ***1. An animal or a group of animals must be considered, by the competent authority, as a suspected case of infection with LPAIV when it meets the criteria laid down in Article 9(1).***
  ***2. An animal or a group of animals must be considered, by the competent authority, as a confirmed case of infection with LPAIV when:***
  ***(c) Positive result to an indirect diagnostic method, that is not a consequence of vaccination, has been obtained in a sample from a kept animal or from a group of kept animals showing clinical signs consistent with the disease or epidemiologically linked to a suspected or confirmed case;***

- ***Annex I, Section 3, Infection with Newcastle disease virus (NDV)***
  ***1. An animal or a group of animals must be considered, by the competent authority, as a suspected case of infection with NDV when it meets the criteria laid down in Article 9(1).***

***2. An animal or a group of animals must be considered, by the competent authority, as a confirmed case of infection with NDV when:***

***(c) Positive result to an indirect diagnostic method, that is not a consequence of vaccination, has been obtained in a sample from a kept animal or from a group of kept animals showing clinical signs consistent with the disease or epidemiologically linked to a suspected or confirmed case;***

Serological diagnosis of viral infections is widely used in routine laboratory work. Besides bringing many advantages, such as rapidity and possibility of testing many samples at the same time, several routinely used serological tests have great limitations in the determination of classes and types of antibodies as well as a precise protective titre.

Serological tests are not able to identify the presence of antibodies against the virus sooner than 7-10 days from infection. For this reason, it is always useful to combine serological and virological testing should a recent infection be suspected.

In case a commercial ELISA kit is used, it is important to verify that the kits are validated for the specific species of interest and for the specific purpose(s) for which they are going to be used. Several different tests and antigen preparation methods previously evaluated and validated by the manufacturer are applied. For this reason, it is of utmost importance to follow the instructions for use in order to avoid unreliable results.

If the AGID test is used, a false negative result may be due to the fact that not all avian species produce precipitating antibodies following infection with influenza viruses, for example ducks. Furthermore AGID has generally a lower sensitivity for detecting generic influenza A infections compared to ELISA and it should be taken into account if a low titre in the sample is expected.

As for the HI test, chicken sera rarely give nonspecific positive agglutination reactions and any pre-treatment of the sera is unnecessary. Sera from species other than chickens may sometimes cause agglutination of chicken RBCs resulting in nonspecific agglutination. Therefore, each serum should first be tested for this idiosyncrasy and, if present, inhibited by adsorption of the serum with chicken RBCs.

If whole virus antigen in HI test for subtyping is used, it is important to ensure that two antigens for each haemagglutinin subtype are used with heterologous neuraminidase i.e. H5N1 and H5N3, to eliminate the possibility of interference in the assay with anti N antibodies that can lead to false typing results. To prevent steric nonspecific inhibition, the H antigen used to test unknown serum must be of a different N subtype than the unknown sera, or the H antigen used can be recombinant or purified H protein that lacks N protein.

Furthermore, reference antigens produced with historic strains may reduce the sensitivity of the HI assay when used for the detection of antibodies against currently circulating ND viruses. For this reason, it is important to investigate the antigenic relationships between the antigen used in the laboratory and the current circulating viruses.

Keep in mind that also HI test and ELISA may measure antibodies to different antigens; depending on the system used. ELISAs may detect antibodies to more than one antigen whereas the HI test is most probably restricted to those directed against the HN protein.

# Explanation of the potential limitations of result to pathotyping diagnostic methods

Related to Regulation (EU) 2020/689 on case definitions and Annex I of the same Regulation

- ***Annex I, Section 1, Highly pathogenic avian influenza (HPAI)***
  ***3 For the purposes of this case definition, the disease agent responsible for HPAI must be either***
  ***(a) An influenza A virus of H5 and H7 subtypes or any influenza A virus with an intravenous pathogenicity index (IVPI) greater than 1,2; or***
  ***(b) An influenza A virus of H5 and H7 subtypes with a sequence of multiple basic amino acids present at the cleavage site of the haemagglutinin molecule (HA0) that is similar to that observed for other HPAI isolates...***

- ***Annex I, Section 2, Infection with low pathogenic avian influenza viruses (LPAIV)***
  ***3. For the purposes of this case definition, the disease agent of infection with LPAIV must be any influenza A virus of H5 and H7 subtypes that are not HPAI viruses.***

- ***Annex I, Section 3, Infection with Newcastle disease virus (NDV)***
  ***3. For the purposes of this case definition, the disease agent responsible for infection with NDV must be any avian paramyxovirus type 1 (APMV-1) (avian Avulavirus type 1) that either:***
  ***(a) Has an intracerebral pathogenicity index (ICPI) of 0.7 or greater; or***
  ***(b) Presents multiple basic amino acids at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein. The term 'multiple basic amino acids' refers to at least three arginine or lysine residues between residues 113 and 116. Failure to demonstrate the characteristic pattern of amino acid residues as described above would require characterisation of the isolated virus by an ICPI test. In this definition, amino acid residues are numbered from the N-terminus of the amino acid sequence deduced from the nucleotide sequence of the F0 gene (113–116 corresponds to residues –4 to –1 from the cleavage site).***

For all the H5 and H7 viruses the amino acid sequence of the connecting peptide of the haemagglutinin must be determined. If the sequence is similar to the one observed for other HPAI isolates, the isolate being tested will be considered as HPAI (refer to the table that lists all the reported haemagglutinin proteolytic cleavage sites of HA0 protein for H5 and H7 LPAI and HPAI viruses, based on the deduced amino acid sequence that can be found on the OFFLU website (http://www.offlu.net/fileadmin/home/en/resource-centre/pdf/Influenza_A_Cleavage_Sites.pdf).
We must take into account the fact that we may come across viruses with cleavage sites with a low degree of similarity with respect to the ones previously identified. The presence of a polybasic site indicates a high pathogenic potential. In case of a first identification of peculiar polybasic cleavage sites, it is always useful to carry out a biological test of pathogenicity (IVPI).
In case of Newcastle disease suspect case, failure to detect viruses with multiple basic amino acids at the F0 cleavage site using molecular techniques does not necessarily confirm the absence of a virulent virus. In case a primer mismatch for a virulent virus detection is assumed, and/or in the event that a mixed population of virulent and a-virulent viruses is suspected on the basis of clinical disease indicators or of the diagnostic tests performed, further samplings and analyses should be required. The employment of approved methods which might include virus isolation followed by in-vivo assessment of virulence (ICPI) and the use of specific PCRs that prove to reliably detect virulent virus or a combination of methods are recommended. Considering that classic viruses of the ND pigeon variant, despite having multiple basic amino acids at the F0 cleavage site, show a very different behaviour in poultry, the intracerebral pathogenicity index (ICPI) often remains the only way to understand the real pathogenic power of these strains in avian species other than Columbiformes.