ISTITUTO ZOOPROFILATTICO SPERIMENTALE DELLE VENEZIE

EUROPEAN UNION REFERENCE LABORATORY FOR AVIAN INFLUENZA AND NEWCASTLE DISEASE

SOP IMM 063

DETECTION OF ANTIBODIES TO TYPE A INFLUENZA VIRUS BY AGAR GEL IMMUNODIFFUSION ASSAY (AGID)

This protocol is a copy of the standard operating procedure used by the EURL for AI and ND at the Istituto Zooprofilattico Sperimentale delle Venezie. Released on 30/12/20.

Contents

1. Purpose and field of application

This protocol describes the procedure to detect antibodies induced by any influenza A virus in blood sera of birds. The presence of influenza A virus can be confirmed in AGID test by demonstrating the presence of the nucleocapsid or matrix virus proteins, both of which are common to all influenza A viruses.

As not all avian species may produce precipitating antibodies following infection with influenza viruses, this protocol is not recommended to test sera of anseriformes, which have a low production of precipitating antibodies.

2. References

- Commission Decision 2006/437/EC approving a Diagnostic Manual for avian influenza as provided for in Council Directive 2005/94/EC;
- OIE - World Organization for Animal Health, Terrestrial Manual Chapter 3.3.4. Avian influenza (Version adopted in May 2015);
- C.W. Beard. Demonstration of type specific antibody in mammalian and avian sera by immunodiffusion. Bull World Health Organ. 42(5): 779–785, 1970;
- IZSVe PDP IMM 063.

3. Safety

Individual laboratories are responsible for ensuring that all the procedures described in this document are conducted under high safety standards, including awareness on chemical and biological risks. For this latter, BSL2 or BSL3 facilities must be used, depending on the risk hazard. Safety rules at individual laboratories must be agreed with the biosecurity and biosafety officer and acknowledged by all the staff members involved.

4. Reagents

- AI positive control serum (^);
- AI reference antigen specific for AGID (^);
- Distilled water;
- Noble Agar;
- Sodium Chloride (NaCl);

(^) To be used according to the manufacturer's instructions.

5. Procedure

5.1. Preparation of samples

If samples are submitted as whole blood, centrifuge the blood at 2,500 rpm for 10 minutes to separate the serum. Carefully pipette or decant serum off red blood cells pellet into another labelled 50 ml centrifuge tube and refrigerate at 4°C.

5.2. Reference antigen and positive control serum preparation

If AGID antigen and/or serum are freeze-dried, they must be reconstituted with appropriate amount of distilled water according to the manufacturer's instructions. If not completely used, the antigen can be stored between +2°C and +8°C up to seven days or at ≥ -70 °C if occasionally used. To avoid frequent freezing and thawing, control serum can be prepared, labeled and stored at -20°C in multiple aliquots. When more than one aliquot is used, pool before starting the test.

5.3. Agar dishes preparation

Tests are usually carried out using gel of Noble agar and NaCl dissolved in 100 ml of distilled water to a thickness of 2–3 mm in Petri dishes, which are incubated in a humidified chamber. The preparation of the agar dishes is carried out following the steps reported below:

- Dissolve 8 g of NaCl in 100 ml of distilled water in a volumetric flask;
- Add 1.25 g of Noble agar and shake gently;
- Immerse the flask in a boiling water bath until the agar is completely dissolved, or place it in a microwave until the agar is completely dissolved (about 5 minutes at approximately 800 Watt for 100 ml of agar);
- Gently shake the flask and verify that the agar is completely dissolved, otherwise repeat the previous step;
- Place the 90 mm Petri dishes on a perfectly horizontal surface and distribute to each 15 ml of the agar solution with a graduated pipette;
- Leave the dishes uncovered and allow the agar to cool at room temperature;
- Close the dishes and label each plate of the batch with the production date. Then, seal the dishes in an air-tight plastic bag labelling the expiry date;
- Store the agar dishes upside down (to prevent the development of condensation) at +4°C for no longer than 15 days;

5.4. AGID

- Bring the agar dish to room temperature and record the identification number of each test serum on the dishes
- Drill 5 mm-diameter holes on the agar layer using a template cutter. Remove the agar plugs with a steel tip or a Pasteur pipette connected to a vacuum pump;
- Distribute 25 µl of AI reference AGID antigen (Ag) in the central well, 25 µl of AI positive control serum (S+) into the two peripheral wells, which are located in the right and left side of the central well and add 25 µl of each test serum (Se) to the remaining wells, as shown in Figure 1. This layout ensures that each serum is adjacent to the positive serum and the reference antigen;
- Incubate the dishes in a humid chamber at room temperature for 48 hours;

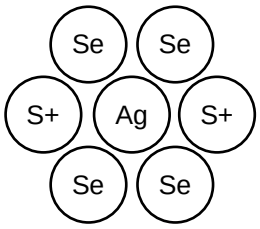

Figure 1. AGID well pattern. Ag = Antigen; Se = Test serum; S+ = Positive control serum.

6. Interpretation of results

Wells should be examined for precipitin lines by reading the plate 48 h post-incubation. The precipitin lines are best observed against a black background that is illuminated from behind.

6.1. Reliability of the test

For a test to be considered valid, a continuous precipitin line must be clearly identifiable between the positive serum and the reference antigen well. If the expected result is not observed, the test is considered not valid and must be repeated.

6.2. Clinical specimens

Interpret reference lines at the sample wells according to the following table:

Output	Result
The precipitin line between the test serum and the reference antigen well is continuous with the line between the reference antigen and the positive serum well	Positive
(i) No precipitin line between the reference antigen and the test serum; (ii) crossed lines between the test serum and the positive serum well	Negative

The AGID is a serological screening test for detection of generic influenza A infections in serum of birds and it must be followed by HI tests for subtyping influenza A positives as to H5 and H7, as recommended by the OIE guidelines.

7. Characteristics of the method

This standard operating procedure is a validated and accredited procedure according to the ISO/IEC 17025.