ISTITUTO ZOOPROFILATTICO SPERIMENTALE DELLE VENEZIE

EUROPEAN UNION REFERENCE LABORATORY FOR AVIAN INFLUENZA AND NEWCASTLE DISEASE

SOP VIR 005

ISOLATION AND CHARACTERIZATION OF AVIAN INFLUENZA VIRUSES USING SPF EMBRYONATED CHICKEN EGGS AND HAEMAGGLUTINATION INHIBITION TEST

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 12/10/21.

The information in this document is subject to change without notice.

Revision history

Revision	Date	Description
ed. 01	October 2021	• Updated references • Specification on the number of swabs to pool by species
ed. 00	December 2020	Initial release

Contents

1. Purpose and field of application

This protocol describes the procedure to isolate avian influenza viruses (AIV) in biological samples, and to preliminary characterize the HA subtype-specific of AIVs based on the haemagglutination inhibition test using specific polyclonal sera. The procedure can be successfully applied to organ homogenates, faeces and tracheal and cloacal swabs appropriately collected and preserved.

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, Manual of Diagnostic Tests and Vaccine for Terrestrial Animals, Chapter 3.3.4. Avian influenza (including infection with high pathogenicity avian influenza viruses) (Version adopted in May 2021);
- IZSVe PDP VIR 005.

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. Materials

- 1% chicken red blood cells (RBCs) suspension derived from specific pathogen free (SPF) chickens (**):
- 6-week-old SPF or specific antibodies negative (SAN) chickens;
- 9-11 day-old SPF embryonated chicken eggs;
- AI polyclonal chicken antisera (H1-H16) (^);
- Antibiotic PBS pH 7.4 ± 0.2 (*);
- Negative control serum (^);
- PBS pH 7.2;
- Sterile quartz powder;

(*) Very high levels of antibiotics may be necessary for faecal samples; appropriate levels are: 10,000 IU/ml penicillin, 10 mg/ml streptomycin, 0.25 mg/ml gentamycin, and 5,000 IU/ml nystatin. These levels may be reduced by up to five-fold for tissues and tracheal swabs. A 10% of glycerol may be added to the solution to better preserve sample stability and integrity, particularly during prolonged storage at a low temperature.

(**) Refer to the manufacturer's instructions for storage conditions. However, it is recommended to check RBCs daily before use to ensure they have not haemolysed. In case the suspension appears pink, the cells have started to lyse and a fresh suspension should be prepared.

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

5. Procedure

The properly prepared suspected pathological material is inoculated in the allantoic cavity of SPF embryonated chicken eggs. The presence of haemagglutinating activity is detected by haemagglutination (HA) testing of harvested allantoic liquid and subtype characterization is achieved by means of haemagglutination inhibition (HI) test. Overall, the procedure includes the following steps:

1. Viral isolation and rapid HA test;
2. Titration of the HA agent;
3. Typing by HI test with polyclonal antisera.

In specific cases and only in laboratories with appropriate facilities, the procedure may also include the assessment of the pathogenicity level of the isolates by means of the intravenous pathogenicity test (IVPI). This *in vivo* test is not routinely performed on avian influenza isolates and usually carried out on notifiable H5 and H7 isolates which are determined as LPAI by conventional sequencing and on the index case of an epidemic. In such cases, the inoculation of birds is required to confirm LPAI as the possibility of virus cultures containing mixed populations of viruses of high and low pathogenicity cannot be ruled out. Inoculation of birds may also be required to assess virulence of HA subtypes other than H5 and H7 (e.g. H3, H9 and H10 subtypes) when associated with the occurrence in the field of severe clinical signs and high economic losses.

5.1. Preparation of samples

5.1.1. Swabs

If samples are submitted as dried swabs, they should be placed into a tube containing a sufficient amount of antibiotic PBS to ensure full immersion (about 1 ml). In case the samples are conferred in antibiotic PBS solution lower than 1 ml, addition of refrigerated antibiotic PBS is required. Allow the samples to stand at room temperature for a minimum of 1/2 hours or at 4°C overnight. Then, centrifuge the samples at 1,000 x g for 10 minutes and use the supernatant for egg inoculation. Store the samples between +2 and +8°C if the inoculation is carried out within four days, otherwise store at ≤ -70°C until use.

Swabs from the same anatomical site and originating from the same epidemiological unit can also be pooled into groups up to 5 samples. In particular conditions, as during an epidemic emergency, for gallinaceous species pooling up to 10 swabs into an appropriate antibiotic PBS volume may be required to increase testing capacity and shorten turnaround times. For other species (e.g., ducks and geese), it is recommended to pool maximum 5 swabs.

For pooling swab samples, which are submitted individually in tube still containing 1 ml of antibiotic PBS, mix the tubes by vortexing for 2 minutes and transfer 0.1 or 0.2 ml (for groups of respectively 10 or 5 samples) from each swab suspension into a new tube to give the 1 ml volume for the inoculum.

5.1.2. Organs

Thaw the organs at room temperature or between +2 and +8°C. Cut the tissue material into small pieces using sterile forceps and scissors and transfer about 1 cm^3 of tested material into a sterile mortar. Homogenize the sample with a pestle by adding an appropriate amount of sterile quartz powder and antibiotic PBS. Generally, an approximate 10% (w/v) suspension in antibiotic PBS is used (about 9 ml). Transfer the homogenate in a 15 ml sterile test tube and incubate at room temperature for 1/2 hours or at 4°C overnight. Centrifuge the sample at 1,000 x g for 10 minutes and collect the supernatant to infect the embryonated eggs. In case inoculation is not carried out on the same day, store the samples between +2 and +8°C up to four days or at ≤ -70°C for longer periods.

Organs can also be pooled according to the anatomical site they are sampled from. They can be grouped as follows: 1. Brain; 2. Lungs and trachea; 3. Liver, heart, spleen and kidneys; 4. Intestines and caecal tonsils.

5.1.3. Faeces

Place about 1 g of tested material into a sterile mortar and homogenize the sample with a pestle by adding antibiotic PBS. Faeces samples are processed individually and generally, an approximate 20% (w/v) suspension in antibiotic PBS is used. Transfer the homogenate in a 15 ml sterile test tube and incubate at room temperature for 1/2 hours or at 4°C overnight. Centrifuge the sample at 1,000 x g for 10 minutes and collect the supernatant to infect the embryonated eggs. In case inoculation is not carried out on the same day, store the samples between +2 and +8°C up to four days or at ≤ -70°C for longer periods.

5.2. Virus isolation

For each batch of SPF embryonated chicken eggs, two to five eggs should be incubated to verify whether they are free of bacterial contamination or viral infection.

- Place at least four 9 to 11 day-old SPF embryonated eggs for each sample on a tray and candle the eggs to check embryo vitality before inoculation;
- Drill or punch a small hole through the shell near the edge of the air sac and label all eggs with a unique sample identification number below the inoculation hole;
- Inoculate 0.1-0.2 ml of clarified supernatant into the allantoic cavity of each egg with a 1 ml syringe;
- Seal the hole with non-toxic nail varnish and incubate the inoculated eggs at 37°C in a thermostat for 6-7 days;
- Candle the eggs daily to check embryo vitality and daily record the number of dead eggs for each sample. Those containing dead or dying embryos must be first chilled between +2 and +8°C for a minimum of 4 hours and then opened by cutting the shell above the air space with sterile scissors. Collect up to 5 ml per egg of allantoic liquid with a sterile pipette and transfer into a sterile test tube labelled with the corresponding unique sample identification number;
- Perform the rapid HA test as follows:
    1. Place a small aliquot of allantoic liquid on a Petri dish;
    2. Add a drop of 1% RBCs suspension;

3. Mix the two components by gently rotating the plate;
4. After 2 minutes, check for the presence of haemagglutinating activity. If it is detected, identify the HA agents by means of the HI test (paragraph 5.4.);

If haemagglutination occurs, RBCs agglutinate with formation of macroscopically visible granules. On the contrary, RBCs remain in suspension falling to the bottom of the plate after about 3-4 minutes

- After a 6/7-day incubation, chill the remaining eggs between +2 and +8°C for a minimum of 4 hours or overnight to terminate the first passage (EP1). Open the eggs, collect the allantoic liquid and perform the rapid HA test as described above. If at the end of the first passage no HA activity is detected, use the undiluted allantoic fluid as inoculum to perform a second blind passage (EP2) in embryonated eggs following the steps as above.

For samples with positive HA, the presence of bacteria must be excluded by means of *in vitro* culture. Should bacteria contamination be observed, allantoic liquids must be filtered through a 450 nm-membrane filter, with further addition of antibiotics and again inoculated into embryonated eggs as described above to ascertain that HA activity is due to the virus presence.

Particular conditions, such as epidemic emergencies, may require a rapid egg passage approach consisting in two rapid passages in SPF eggs lasting three days. However, any variation in the standardized procedure has to be carefully evaluated and authorized by the Head of the Laboratory.

5.3. Haemagglutination (HA) test

The allantoic liquid pool of each sample positive to the rapid HA test must be titrated by HA test before performing the HI test (paragraph 5.4.). It is recommended to test the sample in duplicate to avoid errors in the HA titre determination. The test is performed using V-bottomed microwell plastic plates with a final volume of 75 µl.

- Dispense 25 µl of PBS into the wells of the first two rows (“A” and “B”) of a V-bottomed 96-microwell plate (Figure 1) to test the sample in duplicate;
- Add 25 µl of sample to the first well of rows “A” and “B” (A1 and B1);
- Make serial two-fold dilutions across the plate by transferring 25 µl from the first well to the following ones (from A1 to A12 and from B1 to B12);
- Discard the remaining 25 µl;
- Add 25 µl of PBS to each well of rows “A” and “B”;
- Add 50 µl of PBS to each well of row “C” to arrange the RBCs control line;
- Add 25 µl of 1% chicken RBCs suspension to each well of rows “A”, “B” and “C”;
- Gently shake the plate and incubate at room temperature for 30 minutes or at +4°C for 1 hour to allow the RBCs in the control wells to settle and assume a button shape;
- Read the plate by holding it vertically and observing the absence or presence of a tear-shaped streaming of erythrocytes. In case of complete agglutination of the RBCs no streaming is observed. On the contrary, in wells without haemagglutination activity the RBCs flow at the same rate as the RBCs in the control wells (Figure 1, row “C”);

- The HA titre of the virus isolate is given by the highest dilution inducing complete agglutination of the RBCs. This represents 1 haemagglutinating unit (HAU).

		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
		1	2	3	4	5	6	7	8	9	10	11	12
Test sample	A	O	O	O	O	O	O	O	O	O	O	O	O
Test sample (rep.)	B	O	O	O	O	O	O	O	O	O	O	O	O
RBCs control	C	O	O	O	O	O	O	O	O	O	O	O	O
	D	O	O	O	O	O	O	O	O	O	O	O	O
	E	O	O	O	O	O	O	O	O	O	O	O	O
	F	O	O	O	O	O	O	O	O	O	O	O	O
	G	O	O	O	O	O	O	O	O	O	O	O	O
	H	O	O	O	O	O	O	O	O	O	O	O	O

Figure 1. Set-up of a V-bottomed 96-microwell plate to perform the HA test sample titration. Rep. = replicate.

5.4. Haemagglutination Inhibition test (HI)

The HI is performed by testing the diluted allantoic fluid with reference polyclonal antisera H1-H16 to determine the presence of any AIV and characterize the virus subtype. Control of the 4 virus HAU, negative control serum and RBCs control should be included in each HI test session to assess test reliability.

5.4.1. Reference antisera and negative control serum preparation

Prepare a panel including sera to all AI HA subtypes (H1-H16) and a serum to be used as negative control. A limited panel may be used to screen the unknown isolates for the most probable strains and subtypes (as a minimum H5 and H7 subtypes) based on the epidemiological situation of the area where the samples are collected from.

If reference antisera and/or negative control serum are freeze-dried, they must be reconstituted with an appropriate amount of sterile distilled water according to the manufacturer's instructions and stored at ≤ -18°C. To avoid frequent freezing and thawing, sera can be prepared, labeled and appropriately stored in multiple aliquots.

5.4.2. Procedure

The following procedure refers to the assessment of the test sample against the whole HA (H1 to H16) sera panel in order to allow a full subtype identification of influenza isolate.

- Prepare the viral suspension of the harvested allantoic fluid to obtain a concentration of 4 HAU per 25 µl, which are conventionally used to perform the HI test. Use the dilution that contains four times the viral concentration

of the highest dilution inducing complete agglutination of the RBCs (as outlined in paragraph 5.3.). For example, if 1 HAU is at a dilution of 1:512, 4 HAU are obtained by diluting the haemagglutinating allantoic liquid in PBS 1:128 (512:4 = 128). Place the diluted allantoic fluid between +2°C and +8°C until use;

- Arrange three V-bottomed 96-microwell plates for each sample. Number the plate and record the position of each reference antiserum;
- Prepare the 4 HAU and RBCs controls in the last row of each plate (six wells per control) (Figure 2) by adding 25 µl of PBS to the wells from H2 to H12 of the first and second plate and from D2 to D12 of the third plate;
- Add 25 µl of PBS to all wells of the first and second plate, with the exception of the 4 HAU and RBCs controls rows, and to all wells of the first three rows of the third plate;
- Add 25 µl of each reference chicken antiserum (1 well per antiserum) to the first well of the first column (A1-G1 on the first and second plate, A1 and B1 on the third plate). Using a multichannel micropipette make serial two-fold dilutions across the plate by transferring 25 µl of antisera from the first well to the following ones. Discard the remaining 25 µl;
- Use row “C” of the third plate to arrange the negative control serum. Add 25 µl of negative serum to C1 and make a two-fold dilution across the plate by transferring 25 µl of serum from the first well to the following ones. Discard the remaining 25 µl;
- Add 25 µl of standardized virus dilution to all wells, except for rows “H“ and “D” of the third plate
- Add 25 µl of standardized virus dilution to the H1 and H2 wells of the first and second plate and to the D1 and D2 wells of the third plate. Make serial two-fold dilutions from H2 to H6 and from D2 to D6 to obtain 4, 2, 1, 0.5, 0.25 and 0.125 HAU. Discard the remaining 25 µl;
- Add 25 µl of PBS to all wells of rows “H” and “D” of the third plate;
- Gently shake the plate and incubate at room temperature for 30 minutes;
- Add 25 µl of 1% chicken RBCs suspension to all wells;
- Gently shake the plate and incubate at room temperature for at least 30 minutes or at +4°C for 1 hour;
- Read the plate by holding it vertically against a white background to observe either the absence or the presence of a tear-shaped streaming of erythrocytes. Only those wells in which the RBCs stream at the same rate as the RBCs in the control wells should be considered as showing inhibition of haemagglutination;
- Record which dilution of the reference serum inhibits the haemagglutination, if it occurs.

		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
		1	2	3	4	5	6	7	8	9	10	11	12
H1 reference antiserum	A	O	O	O	O	O	O	O	O	O	O	O	O
H2 reference antiserum	B	O	O	O	O	O	O	O	O	O	O	O	O
H3 reference antiserum	C	O	O	O	O	O	O	O	O	O	O	O	O
H4 reference antiserum	D	O	O	O	O	O	O	O	O	O	O	O	O
H5 reference antiserum	E	O	O	O	O	O	O	O	O	O	O	O	O
H6 reference antiserum	F	O	O	O	O	O	O	O	O	O	O	O	O
H7 reference antiserum	G	O	O	O	O	O	O	O	O	O	O	O	O
Control of 4 HAU and RBCs	H	O	O	O	O	O	O	O	O	O	O	O	O

Figure 2. Example of HI test set-up of the first out of three V-bottomed 96-microwell plates. In row “H” six wells are assigned to the control of the 4 HAU and the remaining to the control of the RBCs.

5.5. Intravenous pathogenicity test (IVPI)

The intravenous pathogenicity index test (IVPI) is used to assess the virulence of AIVs for chickens by observing clinical sign in SPF or SAN infected birds over a 10-day period.

- Prepare 10 six-week-old SPF or SAN chicks for each test sample;
- Dilute 1:10 fresh infectious allantoic liquid with an HA titre >1:16 in sterile PBS. The liquid should be from the lowest passage available, preferably from the initial isolation and without any prior selection;
- Inject 0.1 ml of the diluted virus intravenously into each of the 10 six-week-old chicks;
- Check the birds at 24-hour intervals for 10 days. At each observation, chicks are assigned a score rating 0 = normal; 1 = sick; 2 = severely sick; 3 = dead. The classification of sick and severely sick birds is based on a subjective clinical evaluation. "Sick" birds normally show one of the following symptoms, while "severely sick" birds suffer from more than one:
    - Respiratory involvement;
    - Depression;
    - Diarrhoea;
    - Cyanosis of the exposed skin surface or wattles;
    - Oedema of the face and/or head;
    - Nervous signs;

Dead birds should be assigned a 3-rating on each of the remaining daily observations after death. With respect to animal welfare, birds which are too sick to feed or drink must be euthanized and scored as dead at the next observation, since they are expected to die anyway within 24 hours.

The following example shows a simple method of recording results and calculating IVPI:

Clinical sign	**Days after inoculation**										**Score**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	
Normal	10	2	0	0	0	0	0	0	0	0	12 X 0 = 0
Sick	0	4	2	0	0	0	0	0	0	0	6 X 1 = 6
Severely sick	0	2	2	2	0	0	0	0	0	0	6 X 2 = 12
Dead	0	2	6	8	10	10	10	10	10	10	76 X 3 = 228
											Total score = 246/100* IVPI = 2.46

(*) Number of observations given by 10 birds monitored for 10 days.

6. Interpretation of results

6.1. Virus isolation

If no HA activity is detected following two passages in SPF embryonated chicken eggs, the sample is considered as “negative” for avian influenza and for any haemagglutinating agent.

6.2. HI test

The haemagglutination and the inhibition of haemagglutination are assessed by reading the plate. If an antigen/antibody reaction occurs, agglutination of the RBCs is inhibited and the RBCs flow at the same rate as the RBCs control wells. This is recorded using a “+” symbol. On the contrary, a “-” symbol is used in the presence of haemagglutination.

6.2.1. Reliability of controls

The HI test is conforming only if the controls generate the following expected results:

Control	**Expected Result**
4 HAU	Complete haemagglutination in the first 3 wells, partial haemagglutination in the fourth well, and no visible haemagglutination in the last two wells
RBCs	No evidence of haemagglutination
Negative serum	Titre $\leq$ 1:8

The test is non-conforming if the controls do not provide the expected results. In this case, the test must be repeated.

6.2.2. Clinical specimens

The HI titre of a virus isolate is given by the highest serum dilution inducing complete inhibition of the haemagglutination (formation of the tear-shaped streaming of RBCs).

The result of the analysis of clinical samples, as described in this procedure, is expressed as follows:

Output	Result
The titre of the reference antiserum to the isolate is equal to the expected one or, at the most, not less than 2 or 3 folds of its expected titre	Positive for AI and HA subtype determined
(i) No inhibition of haemagglutination by any polyclonal reference antisera and/or (ii) The titre of the reference antiserum to the isolate is more than 3-fold lower than the expected titre	Negative

Note that cross-reactions between HA subtypes may occur during the typing test of the isolates and should be assessed when interpreting the results. In case an isolated virus showing HA activity turns out to be negative for AI to HI test, further investigations will be required to identify the haemagglutinating agent.

6.3. Intravenous pathogenicity test

The IVPI is the average score per bird and per observation over a 10-day period. An index of 3.00 means that all birds have died within 24 hours, while an index of 0.00 indicates that none of the birds have shown clinical signs during the 10-day observation period. Any influenza A type yielding a value >1.2 in an IVPI test is considered to be a high pathogenic virus (HPAI). Note that up to date, all the notified HPAI viruses belong to the H5 and H7 subtypes. However, some non-H5/H7 AI viruses (e.g. some H10 or H9 strains) may cause high lethality when inoculated intravenously in chickens even if these are not so highly lethal via the natural route of administration and lack the hemagglutinin cleavage site sequence compatible with HPAI viruses. The resulting high IVPI value of these strains should be interpreted as a laboratory artefact and therefore they should not be considered as real HPAI viruses.

7. Characteristics of the method

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