Conventional Diagnosis of Avian Influenza

7

Calogero Terregino and Ilaria Capua

7.1 Isolation of Avian Influenza Virus

7.1.1 Sample Management and Preparation

Assign an identification number to the sample as soon as it arrives at the laboratory.

Fill out a work sheet indicating the identification number of the sample, kind of sample, species, test to perform and starting date of analysis.

This work sheet should be prepared and maintained by the technician who performs the tests.

7.1.1.1 Swabs

- Under sterile conditions and using a laminar flow cabinet, transfer 2 ml of PBS solution containing antibiotics from the tube in which the swabs have been soaked into another tube.
- In case of highly contaminated swabs add 2 ml of antibiotics to obtain a final dilution of 1:2.
- Centrifuge the sample at 1,000 × g for 10 min to remove coarse particulate matter.
- Use the supernatant as an inoculum for the specific-pathogen-free (SPF) embryonated eggs.
- Keep the sample at 4°C overnight, or store it at –80°C if there are longer delays in processing until eggs can be inoculated.

7.1.1.2 Organs

- Thaw organs at room temperature or at 4°C.
- Working under a laminar flow cabinet and using sterile forceps and scissors, collect an amount of sample corresponding to 1 cm^3. Organs can be pooled according to the apparatus they belong to.
- Homogenise the sample in a mortar, adding sterile quartz sand.
- Add 9 ml PBS with antibiotics.
- Decant the homogenate into a 15-ml tube. Leave the sample at 4°C for 1 night. If the eggs will not be inoculated the next day, store the sample at –80°C until the date of the test.
- Centrifuge the sample at 1,000 × g for 10 min.
- Use the supernatant to infect embryonated eggs.

7.2 Virus Isolation

Virus is isolated following the protocol of the OIE and according to European standards (OIE Manual 2008, EC 94/2005).

7.2.1 Methods

1. Candle 9- to 11-day-old embryonated SPF fowl's eggs to check embryo viability. Mark the shell of the egg with a pen to delimit the air sac. With a manual or electric device, drill a small hole just above the air sac (Fig. 7.1).
2. Record the identification number of the sample, the passage number (1° or 2°), the kind of sample (lung, cloacal swabs, etc.) and the date of inoculation on five eggs.
3. Inoculate 0.1–0.2 ml of clarified supernatant obtained from the tracheal and cloacal swabs or from the organ homogenate into the allantoic cavity of each of the five 9- to 11-day-old embryonated SPF fowl's eggs (Fig. 7.2 a-d).
4. Seal the eggs with glue or wax (Fig. 7.3).
5. Incubate the inoculated eggs at 37°C for 7 days.

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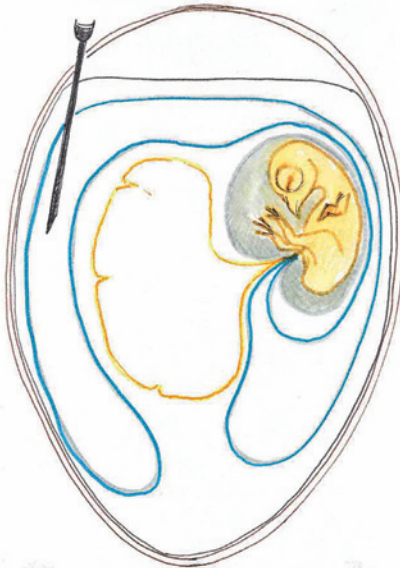
6. Candle the inoculated eggs daily to check embryo vitality.
7. The number of dead eggs for each sample should be recorded daily in the lab's working handbook.
8. Test the allantoic fluid of eggs containing dead embryos for haemagglutinating (HA) activity as indicated below (Fig. 7.4).
9. If HA activity is detected, identify the HA agents by means of the haemagglutination inhibition (HI) test as described below.
10. After 7 days, chill the remaining eggs in a refrigerator (4°C) to end the first passage.
11. The following day, open the eggs using sterile techniques under a laminar flow cabinet.

Fig. 7.1 Perforation of specific-pathogen-free (SPF) eggs for virus isolation attempts



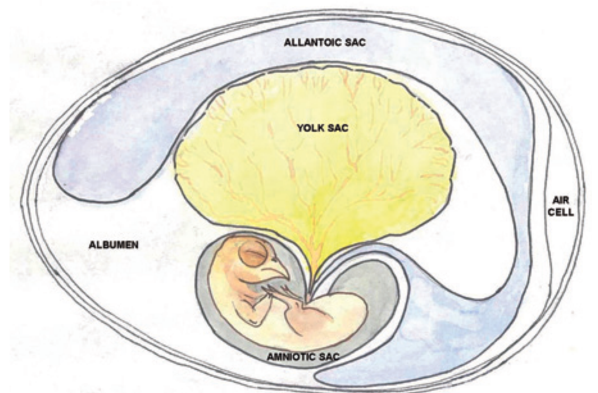


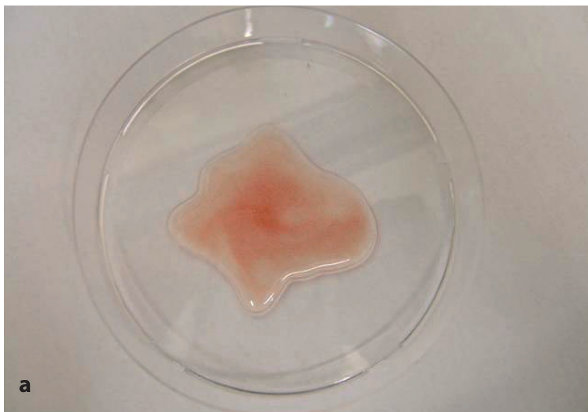
Fig. 7.2 a–d (**a**) Inoculation of SPF eggs for virus isolation attempts. (**b**) Needle introduced into the allantoic cavity, above the border of the air cell (*black horizontal line* on the egg shell). (**c**) Embryonated chicken egg: inoculation via the allantoic route. (Courtesy of Amelio Meini). (**d**) Anatomy of an embryonated chicken egg at 9–10 days of incubation: schematic view. (Courtesy of Amelio Meini)

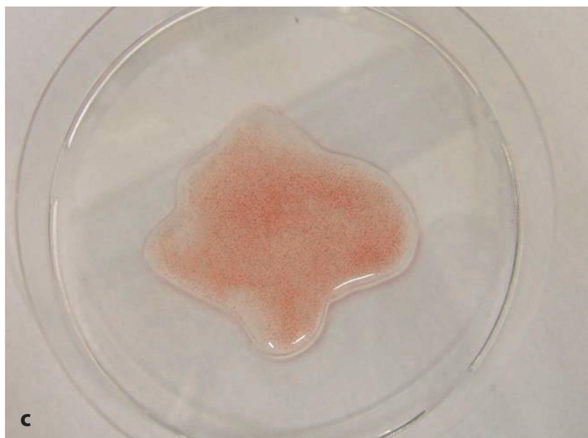
Collect approximately 10 ml of the allantoic fluid.

12. Test the allantoic fluid for the presence of HA activity by the "rapid HA test" as described below.

Fig. 7.3 Sealing of SPF eggs for virus isolation attempts

13. If at the end of the first blind passage, no HA activity is detected, use the undiluted allantoic fluid, collected from this passage, to perform a second passage in embryonated eggs as described above.
14. The mortality of inoculated eggs 24-h post-inoculation is generally considered non-specific, although some HPAI viruses, may cause embryo mortality as early as 18 h post-infection.
15. If no HA activity is detected following two passages in eggs, the sample may be considered negative.
16. When HA activity is detected, the presence of bacteria must be excluded by culture.
17. If bacteria are present, the fluids must be passed through a 450-nm membrane filter, with further addition of antibiotics, and inoculated into embryonated eggs as described above.
18. A sample giving a positive result in the rapid HA test must be tested further. These tests in-





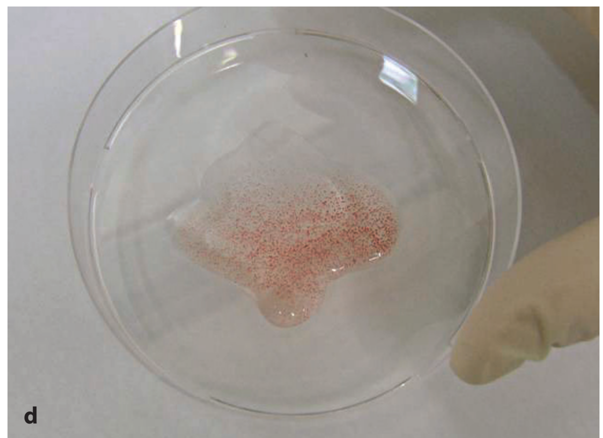


Fig. 7.4 a-d Rapid haemagglutination reaction. Incubation time: 5 s (**a**), 10 s (**b**) 20 s (**c**) and 30 s (**d**)

clude the titration of HA activity. This procedure enables the confirmation and quantification of HA, which are prerequisites to the identification of the HA agent by means of the HI test.

If a laboratory does not have the capacity to perform the HI test, the HA allantoic fluid should be sent to a National Reference Laboratory or to an International Reference Laboratory to confirm diagnosis.

7.2.2 Haemagglutination Test in Petri Dishes (Rapid HA Test)

This method is based on the reaction between the HA activity of the virus and red blood cells (RBCs). If viral replication has occurred in the embryonated SPF egg, the allantoic fluid will contain virus particles with HA activity. The latter can be visualised by adding a drop of allantoic fluid to a drop of a RBC suspension; the resulting reaction is macroscopically visible.

1. Place in a Petri dish a drop of the allantoic fluid with the same amount of 1% RBC suspension.
2. Allow the two fluids to mix.
3. Wait 30–60 s and observe whether there are any RBC aggregates (Fig. 7.4 a-d).

7.2.3 Characterisation of Avian Influenza Viruses

7.2.3.1. Haemagglutination Test in Microtitre Plates (Micro HA Test)

1. Dispense 0.025 ml PBS into each well of a plastic microtitre plate (V-bottomed wells).
2. Place 0.025 ml of virus suspension (i.e. allantoic fluid) in the first well.
3. Use a multi-channel micropipette to make twofold dilutions (from 1:2 to 1:4096) of virus suspension across the plate. Discard the last 0.025 ml.
4. Dispense 0.025 ml of PBS into each well.
5. Add 0.025 ml of 1% RBC to each well.
6. Mix by tapping gently and place at 4°C or at room temperature (20–24°C).
7. Plates are read after 30 min (at room temperature) or after 40 min (at 4°C) when the RBC controls have settled and assumed a button shape. If HA activity is present, it will appear as a fine layer of RBCs lining the entire bottom of the well. If HA activity is absent, RBCs will settle at the centre of the well in the shape of a button. The samples are read by holding the plate perpendicular to the bench, in other words by holding it vertically, against a white background and observing the presence or absence of tear-shaped streaming of the RBCs (Fig. 7.5). In wells

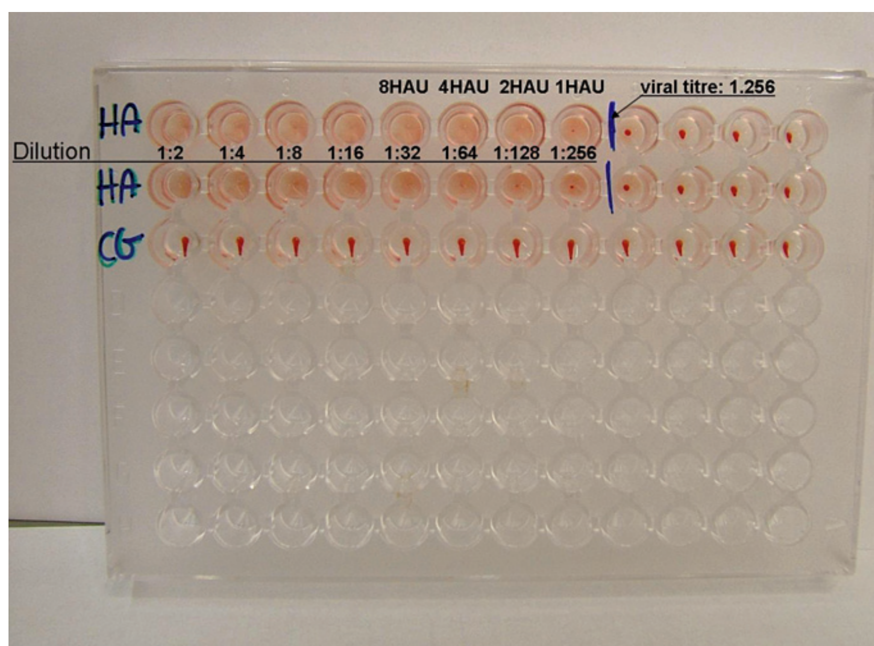


Fig. 7.5 Haemagglutination reaction in microplates

with HA activity, there will be no streaming. In wells without HA activity, the RBCs should flow at the same speed as the RBCs in the control wells in which only PBS and RBCs have been dispensed.

The HA titre of the viral suspension under examination is the highest viral dilution that causes agglutination of the RBCs (no streaming). This dilution is defined as containing one haemagglutinating unit (HAU). To perform the HI test and characterise the virus subtype, a 4-HAU antigen solution is conventionally used, i.e. containing four times that viral concentration. For example: if the HA titre obtained is 1:512 (1 HAU), 4 HAU will be obtained by dividing that titre by 4 (512:4=128). A dilution of 1:128 of the haemagglutinating allantoic fluid will be used to prepare the antigen solution for the HI test. For accurate determination of the HA content, this should be done from a close range of an initial series of dilutions, i.e. 1/3, 1/5, 1/7 etc.

7.2.3.2 Haemagglutination Inhibition Test (HI Test)

This method is based on a reaction between the virus and the specific antiserum. When the antiserum reacts with the virus, it binds to the epitopes that are responsible for haemagglutination; thus, these epitopes will not be available to bind to RBC. If the antiserum is not specific for the virus, haemagglutination will occur, indicating non-identity between the two reagents.

Reagent Preparation

- *Reference antiserum*: Reconstitute one vial of freeze-dried serum with 1 ml of sterile distilled water or according to the manufacturer's instructions. Prepare 16 antisera against all the different known AI subtypes and include one against Newcastle disease (ND) virus. Unused reconstituted antisera may be aliquoted and stored at –20°C.
- *Virus to be characterised*: Prepare the viral suspension with 4 HAU as described above.

Procedure

1. Dispense 0.025 ml PBS into all wells of a plastic microtitre plate with V-bottomed wells, except the first well of the 4-HAU control row, generally H1–H6.
2. Place 0.025 ml of each reference antiserum in the first wells of the first column of the plate (A1–G1) and use the last row (H) to titrate the 4 HAU and for the RBC control (Fig. 7.6).
3. Use a multichannel micropipette to obtain two-fold dilutions of all sera across the plate and discard the last 0.025 ml.
4. Add 0.025 ml of diluted allantoic fluid containing 4 HAU in each well from row A to row G.
5. In the first two wells of the last row (4-HAU virus titration control: H1–H6) of each plate, dispense 0.025 ml of diluted allantoic fluid containing 4 HAU and make two-fold dilutions from the second well to the sixth well (H2-H6). Discard the last 0.025 ml.
6. Add 0.025 ml of PBS in all wells of the virus control and 0.050 ml of PBS in the RBC control wells (H7–H12).
7. Mix by tapping gently and place the plate at +4°C for 40 min or at room temperature for 30 min.
8. Add 0.025 ml 1% RBCs to all wells.
9. Mix by gentle tapping and place at 4°C or at room temperature. Plates are read after 30–40 min, when the RBCs control has settled. This is done by holding the plate in a perpendicular position to the bench, in other words by holding it vertically, against a white background and observing the presence of tear-shaped streaming at the same speed as that occurring in the RBCs control wells.

Results

In the first three wells (H1–H3) of the 4-HAU control, haemagglutination must be observed. In well H4, a partial haemagglutination (half of a tear-shaped drop) and in wells H5 and H6 no haemagglutination should be seen. Wells H1–H6 correspond to 4 HAU, 2 HAU, 1 HAU, 0.5 HAU, 0.25 HAU and 0.125 HAU respectively.

The virus is identified on the basis of the correspondence with the reference antiserum, which inhibits its haemagglutinating activity. In case of identity, the titre of the reference antiserum with the virus under examination should be equal to or ± 1 dilution of its titre with a homologous antigen (Ag) (see Fig. 7.6).

The 4HAU control ensures that the correct amount of Ag has been used in the test.

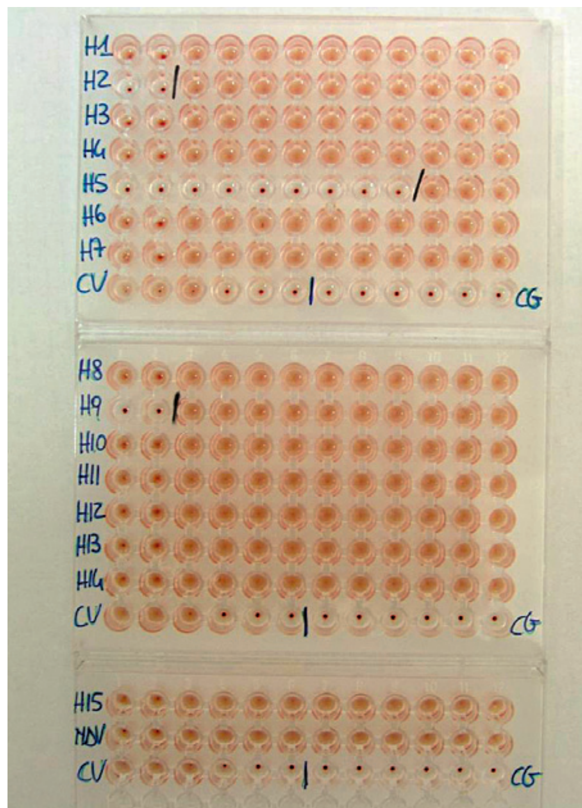


Fig. 7.6 Typing of avian influenza virus by the haemagglutination inhibition test. The strain was identified as H5 subtype. Weak cross-reactions with H2 and H9 antisera were observed. *CV* 4 HA unit control; *CG* red blood cell control

7.2.3.3 Neuraminidase Inhibition Test

The neuraminidase inhibition (NI) test is a laboratory procedure aimed at characterising the neuraminidase subtype of influenza viruses. The assay is based on the reaction between the unknown neuraminidase viral subtype and monovalent antisera able to inhibit the enzymatic activity of neuraminidase. Nine different neuraminidase subtypes (N1–N9) of influenza type A are known and all of them have been associated with isolates obtained from birds.

This complex method is based on inhibition of neuraminidase protein activity with monovalent antisera raised against each of the neuraminidase subtypes. The enzymatic activity of neuraminidase is detected through the release of N-acetyl neuraminic acid from the fetuin substrate. Subsequently, the addition of periodate oxidises the N-acetyl neuraminic acid into β-formyl pyruvic acid, while the addition of thiobarbituric acid (TBA) develops a chromophore that can be extracted with acid butanol. The results may be read colorimetrically using a spectrophotometer, with the optical reading proportional to the neuraminidase activity in the original preparation. The enzymatic activity will be inhibited only by the specific antiserum against the neuraminidase subtype of the virus that is being characterised.

In practical terms, if the antiserum is not specific for the neuraminidase, enzymatic activity is retained, resulting in the production of a pink-coloured solution. If the specific antiserum binds to the neuraminidase protein, then enzyme activity is inhibited and the chromophore remains white (colourless).

This test should be performed only after the HA subtype of the virus has been characterised.

Safety

The NI test releases toxic compounds during the immersion of tubes in boiling water. Thus, this phase should be carried out under a fume hood and operators should wear a protective mask.

Equipment

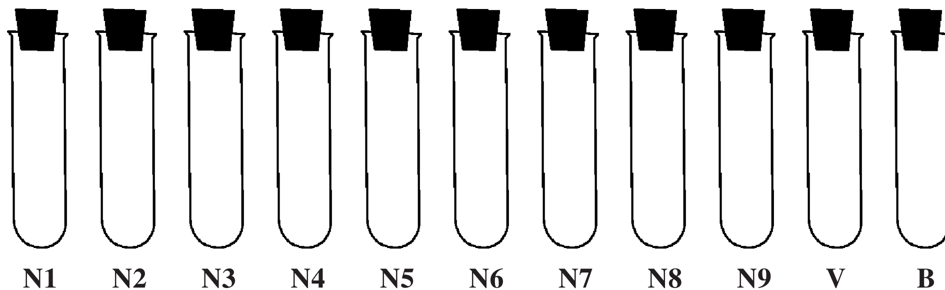
- *100- to 300-μl pipettes*
- *Laminar-flow cabinet*
- *37 ± 2°C incubator*
- *56–100°C water bath*
- *Spectrophotometer (549 nm) (optional)*
- *Plastic test tubes 12×75 mm*
- *Glass test tubes 16×100 mm*
- *Syringes*
- *Rubber plugs*
- *Rack for pipettes*
- *10-ml pipettes*

Working Reagents for Neuraminidase Inhibition Assay

- *Antisera against each of the nine different neuraminidase subtypes. The antisera should be stored at –20°C*
- *Standard fetuin substrate*
- *0.1M PBS (pH 5.9)*
- *0.025M Sodium periodate in 0.125 N* H_2SO_4
- *2% Sodium arsenite in 0.5N HCl* ($NaAsO_2$)
- *0.1M TBA (pH 9.0)*
- *5% 10N HCl butanol acid (optional)*
- Instructions for the preparation of these solutions are provided in Annex 4.

Procedure

1. Source antisera for each different subtype of neuraminidase (9 in total). The haemagglutinin of the sera should be different from that of the virus being tested. Example: if the virus under examination is H7, then antisera H1N1, H1N2, H5N3, H8N4, etc. should be chosen. Do not select antisera raised against H7 viruses.
2. Dilute the antisera 1:5 in PBS (pH 5.9)
3. Dilute the virus 1:15 in PBS (pH 5.9) if the HA titre is > 1:64. Dilute the virus 1:13 if the HA titre is ≤ 1: 64.
4. Set up the antisera and the sample as shown in the scheme below:



V Virus control, *N* neuraminidase subtype, *B* blank (reaction control)

5. Dispense 100 µl of the diluted antiserum into each glass test tube (serum anti-N1 into tube N1, serum anti-N2 into tube N2, etc.).
6. Add 100 µl of diluted virus to all tubes except tube B (blank).
7. Add 100 µl of PBS (pH 5.9) into tube V (virus control) and 200 µl into tube B (reaction control).
8. Seal each tube with its plug and incubate the closed tubes at room temperature for 30 min.
9. Add 300 µl of standard fetuin to each tube. Carefully shake the tubes for 15 s to mix the reagents.
10. Seal the tube with its plug and incubate at 37°C for 16–20 h.
11. Add 200 µl of sodium periodate to each tube. Carefully shake the tubes for 15 s in order to allow the reagents to mix.
12. Seal each tube with its plug and incubate at 37°C for 30 min.
13. Add 200 µl of sodium arsenite to each tube. Carefully shake the tubes to mix the reagents. A brown colour will develop and then disappear. It is possible at this stage to stop the assay by storing the tubes at +4°C.
14. Add 2 ml of TBA to each tube. Carefully shake the tubes for 20 s to mix the reagents.
15. Remove the plug and partially immerse the tubes in boiling (100°C) water for 7.5 min.

Results

Tubes containing a pink coloured solution = no inhibition has occurred. The antiserum used is not specific for the neuraminidase.

Tubes containing colourless or a white-coloured solution compared to virus control tube = total inhibition. The antiserum used is specific to the neuraminidase.

For example, if a white/colourless solution has been obtained using antiserum against N2 then the neuraminidase is type 2 (Fig. 7.7).

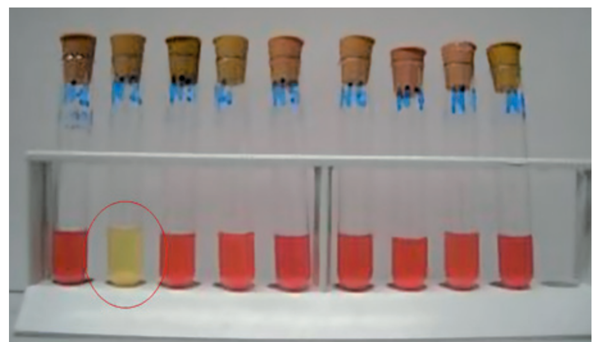


Fig. 7.7 Typing of avian influenza virus by the neuraminidase inhibition test. The strain was identified as N2 subtype (*red circle*)

7.2.3.4 In vivo Pathogenicity Test

The virulence for chickens of influenza A viruses isolated from birds must be assessed using the intravenous pathogenicity index (IVPI) test.

Reagents and Equipment

- *Allantoic fluid containing virus to be assessed*
- *Sterile isotonic saline*
- *10 SPF or specific-antibody-negative (SAN) 6-week-old chickens*
- *1-ml syringes*
- *Test tubes, cotton wool*
- *Negative-pressure isolators*
- *Feed, litter and drinking water*
- *Disposable bags*

Procedure

1. Dilute fresh infective allantoic fluid with a HA titre > 1:16 1:10 in sterile isotonic saline. The fluid should be from the lowest passage available, preferably from the initial isolation and without prior selection.
2. Inject 0.1 ml of the diluted virus intravenously into each of ten 6-week-old SPF or SAN chickens.
3. The birds are examined at 24-h intervals for 10 days. At each observation, each bird is assigned a score of 0 if normal, 1 if sick, 2 if severely sick and 3 if dead.
   The judgement of sick vs severely sick is a subjective clinical assessment. Normally, 'sick' birds show one of the following signs and 'severely sick' more than one of the following signs:
   - *Respiratory involvement*
   - *Depression*
   - *Diarrhoea*
   - *Cyanosis of the exposed skin or wattles*
   - *Oedema of the face and/or head*
   - *Nervous signs*

   Dead birds must be scored as 3 at each of the remaining daily observations after death. On welfare grounds, when birds are too sick to eat or drink, they must be killed humanely and scored as dead at the next observation since they are expected to die within 24 hours without intervention. This approach is acceptable to accreditation authorities.

The IVPI is the mean score per bird per observation over the 10-day period. An index of 3.00 means that all birds died within 24 h while an index of 0.00 means that no bird showed any clinical signs during the 10-day observation period.

A simple method for recording results and calculating indices is shown in the following example:

Clinical signs	Days after the inoculation Number of chickens with specific signs										Total score
	1	2	3	4	5	6	7	8	9	10	
Normal	10	0	0	0	0	0	0	0	0	0	10 × 0 = 0
Sick	0	6	0	0	0	0	0	0	0	0	6 × 1 = 6
Severely sick	0	2	4	2	0	0	0	0	0	0	8 × 2 = 16
Dead	0	2	6	8	10	10	10	10	10	10	76 × 3 = 228
											TOTAL = 250/100 IVPI = 2.50

10 birds observed for 10 days = 100 observations
Index = mean score per bird per observation = 250/100 = 2.50.
Any influenza A virus, regardless of subtype, yielding a value > 1.2 in an IVPI test is considered to be a highly pathogenic avian influenza (HPAI) virus.
Data from Commission Decision of 4 August 2006 approving a Diagnostic manual for avian influenza as provided for in Council Directive 2005/94/EC (2006). Official Journal of the European Union L 237/1-27.

7.3 Serology

7.3.1 Introduction

Domestic and wild birds infected with AI viruses through natural exposure or vaccinated against AI viruses develop antibodies that can be detected by means of serological tests. The antibody response to AI viruses in birds is directed against the immunogenic proteins of the virus. Natural infection with AI viruses elicits the production of both type-specific (against type A matrix (M) and nucleopro-

tein Ag) and subtype-specific (against the HA and N proteins) antibodies. It is therefore essential to implement a diagnostic protocol while bearing in mind the diagnostic significance of the result. Tests that are able to detect antibodies against type A Ag are group-specific, i.e. detection of these antibodies indicates that the bird has been infected (or vaccinated) with an influenza A virus of any subtype. These tests are the agar gel immunodiffusion (AGID) assay and the enzyme-linked immunosorbent assay (ELISA), both of which are directed against the NP and/or M proteins.

Subtype-specific antibodies are detected by means of the HI test and indicate that infection (or vaccination) has occurred with a virus of a specific H subtype. The HI test gives no indication of the N subtype of the virus that has elicited the immune response, as the antibodies measured by this test are only those targeting the haemagglutinin.

Generally speaking, serological diagnosis of AI is performed in two steps, unless the subtype circulating in a given area is already known. The first step aims at the detection of antibodies to any AI virus and is implemented by applying the AGID assay or the ELISA. The second step, performed on samples determined to be positive in step 1, identifies the viral subtype causing infection.

7.3.1.1 Step 1: Detection of Group Antigen (Type A) Antibodies

These tests are able to detect antibodies to the group Ag of influenza A viruses. The antigen-antibody reaction is against the NP or M proteins of AI viruses. These Ags are present in all influenza A viruses regardless of the H or N subtype. Serological positivity to these tests indicates that the birds have encountered an influenza A virus, but no information on the AI subtype that has caused seroconversion can be deduced.

Note: Serological positivity to type A in waterfowl (wild and domestic) is a very common finding.

Agar Gel Immunodiffusion (AGID) Assay

This is a simple and reliable test in chicken and turkey sera. It is very specific but is of limited sensitivity; thus, it must be used as a diagnostic tool on a flock basis. It can be performed in any laboratory with basic equipment. It is completely unreliable in waterfowl, as these birds do not produce precipitating antibodies. It has not been validated in other avian species.

Enzyme-Linked Immunosorbent Assay (ELISA)

This is a test that requires more advanced laboratory equipment, including a spectrophotometer. It is highly sensitive but lacks specificity. In the case of indirect ELISA tests, care must be taken to ensure that the secondary antibody in the test (anti-species) is directed against the species under examination. Competitive ELISA tests have the advantage that serum of any species can be examined. The instructions of the assay's manufacturer should be followed and assumptions on test reactivity for species other than those mentioned in the kit's specifications should be avoided.

7.3.1.2 Step 2: Detection of Subtype-Specific (H Subtype) Antibodies

This test is used in birds known to be infected with AI virus – either following a positive serological test against the group (type A Ag) or as a result of clinical history. The test identifies the haemagglutinin subtype of the virus causing the seropositivity. The HI test is used for this purpose. Low-level cross-reactivity with other H subtypes may be observed due to homology with the neuraminidase Ag. This cross-reactivity is generally not higher than 1:16 (2^4) and disappears with another Ag containing a different neuraminidase subtype. For example: A serum sample is positive to H9N2 at a titre of 1:256 (2^8). If tested with H5N2 Ag, a positive inhibition result is observed at 1:8 (2^3). When tested with an H5N9 antigen the sample will be negative.

7.3.2 Agar Gel Immunodiffusion (AGID) Assay

This test is widely and routinely used to detect the presence of antibodies against influenza A virus in the serum of birds. Since it is very specific but is of limited sensitivity, it is used as a diagnostic tool on a flock basis. Antibodies against influenza A viruses are detected by the lines formed following precipitation of the immune complex established between antibody in the test sera and the reference Ag.

7.3.2.1 Preparation of Agar Dishes

1. Dissolve 8 g NaCl in 100 ml distilled water in a volumetric flask.
2. Add 1.25 g Noble agar and mix gently.
3. Dissolve the agar by immersing the flask in a boiling-water bath until the agar is completely dissolved.
4. Transfer 15 ml of the agar solution to each 90 mm Petri dish.
5. Leave the dishes uncovered and allow the agar to cool at room temperature.
6. Label the batch of agar dishes with the production date on each lid, then seal the dishes in an air-tight plastic bag. The agar dishes can be stored up-side-down (to avoid the condensation of water droplets on the lids) for up to 15 days at +4°C.

7.3.2.2 Test Procedure

1. Record the identification number of the samples on the dishes.
2. Punch wells with the agar punch as shown in the Figures 7.8, 7.9. Remove the agar plugs with a steel tip or a Pasteur pipette attached to a vacuum pump.
3. Place 30 µl Ag into the central well.
4. Add 30 µl positive antiserum (S+) into two wells that are directly opposite from each other (Figs. 7.8, 7.9).
5. Place 30 µl of the serum under examination (SE) in each of the remaining wells. The layout of the reagents ensures that each SE is adjacent to a well containing a positive serum S+ and to one containing Ag.
6. Incubate the dishes in a humid chamber at room temperature for 48 h.

7.3.2.3 Interpretation of the Results

1. Read the plates 48 h post-incubation using a diffuse light source, with illumination from below the plate.
2. The test is valid when a precipitation band is seen between the wells containing the positive serum and the central well containing the Ag (Figs. 7.8, 7.9).
3. The sample is positive when a precipitation band is observed between the well containing the test serum and the central well containing the Ag. The band must be continuous (i.e. show identity) with the precipitation band formed between the positive serum and the Ag.
4. The sample is negative when no precipitation band is seen between the well containing the SE and the central well containing the Ag.

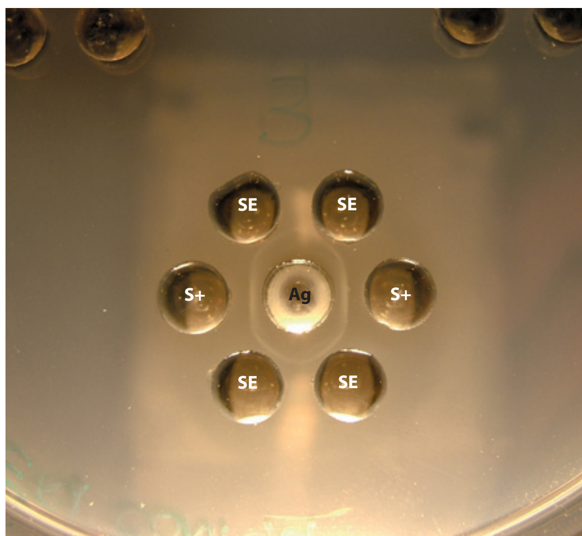


Fig. 7.8 Agar gel precipitation assay. *Ag* Reference antigen, *S+* positive serum, *SE* serum under examination

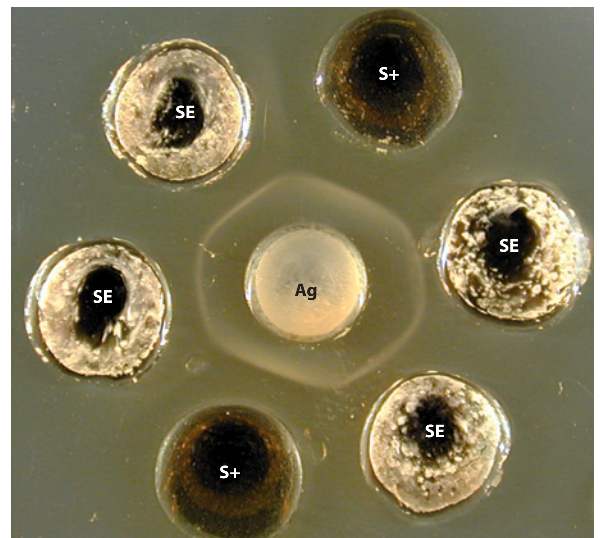


Fig. 7.9 Agar gel precipitation assay: *Ag* Antigen, *S+* positive serum, *SE* serum under examination

7.3.3 Enzyme-Linked Immunosorbent Assay (ELISA) To Detect Antibodies Against Avian Influenza Virus Type A

An ELISA is a useful and sensitive test that can be applied for serological screening. A variety of different kits are now available on the market, allowing detection of the presence of specific antibodies against the NP and the M of proteins AI viruses. The instructions of the assay kit's manufacturer must be followed carefully when performing the test.

7.3.4 Detection of Specific Antibody Subtypes by the Haemagglutination Inhibition (HI) Test

This method is based on a reaction between a HA virus and an antiserum containing specific antibodies to that virus. When the HA virus is incubated with its specific antibodies, the natural HA activity of the virus is inhibited. This is visualised by adding to the well a suspension of RBCs, which will sediment in the shape of a button.

Since the HI test is both qualitative and a quantitative, a known amount of antigen, in most cases 4 HA units, must be used.

7.3.4.1 Preparation of Red Blood Cell Suspensions for HA and HI Tests

A 1% RBC suspensions is used in the HA and HI assays of chicken sera, while a 10% RBC suspension is used in the pre-treatment of sera originating from species other than chickens. Nonspecific reactions, due to the presence of nonspecific HA in the sera of species other than chickens, may occur during the HI assay. To avoid this, non-chicken sera are pre-treated with chicken RBCs to remove non-specific HA.

Procedure

1. Collect 5 ml of blood from at least three SPF chickens with a syringe containing sufficient Alsever's solution to yield a ratio of 1:1.
2. Pool the contents of the syringes and centrifuge the blood suspension at 1,000 g × 10 minutes. Discard Alsever's solution or supernatant.
3. Wash the RBCs twice in PBS solution, centrifuging at 1,000 g × 10 minutes after each washing.
4. The supernatant is removed with a pipette, leaving the packed RBCs which are then used to prepare a RBC suspension of appropriate concentration for a given test. The suspension can be stored at +4°C for 7 days.

10% RBC suspension: Prepare 9 ml of 0.05% bovine albumin PBS solution and add 1 ml of packed RBCs.

1% RBC suspension: Prepare 99 ml of 0.05% bovine albumin PBS solution and add 1 ml of packed RBCs. The correct percentage should be checked by one of the following methods.

7.3.4.2 Measurement of the RBC Concentration

Spectrophotometric Method

This system measures the RBC concentration indirectly, by quantifying the haemoglobin content of the solution.

1. Set up two cuvettes each with 3.6 ml of distilled water and 0.4 ml of the suspension to be read in the spectrophotometer at 545 nm wavelength.
2. Refer to the PBS 0.05% albumin solution as the blank.
3. A 1% RBC suspension should result in an optical density (O.D.) of 0.250 nm.

Microhematocrit Tube Method

The haematocrit may be measured manually by centrifugation.

1. Fill a standard capillary microhaematocrit tube with blood and seal the tube at the bottom.
2. Centrifuge the tube at 10,000 rpm for 5 min. The RBCs have the greatest mass and are forced to the bottom of the tube.
3. Measure the height of the red cell column as a percent of the total blood column. The higher the column of red cells, the higher the hematocrit and the percentage.

Cell Counting Chamber

In this procedure, either the Thoma, Burker, or Malassez chamber can be used. In each of these chambers, 75 million to 80 million RBCs/1 ml correspond to a concentration of 1%.

7.3.4.3 Haemagglutination Inhibition Test for Chicken Sera

Reagents

- *PBS*
- *PBS and albumin (PBS/albumin 0.05%)*
- *Freeze-dried reference antigen diluted with PBS to obtain 4 UHA per 0.025 ml*
- *Chicken RBCs suspension (1%)*
- *Negative control chicken serum*
- *Positive control chicken serum*

Note: Reconstituted reference sera must be store at –20°C and reconstituted reference antigens at –80°C.

Procedure

1. Dispense 0.025 ml PBS into all the wells of a microtitre plate, with the exception of the H1 well.
2. Dispense 0.025 ml of serum into the first wells of the microtitre plate (column 1). Add 0.025 ml of the positive control serum (with known HI titre) to the F1 well and 0.025ml of negative control serum to the G1 well. Control sera should be ideally included in each plate or batch of 10 plates. The 4-HAU control and RBCs control should be included in each plate.
3. Using a multi-channel micropipette, make two-fold dilutions of the sera (A1–A12) across the plate. Discard the last 0.025 ml.
4. Add 0.025 ml of antigen suspension containing 4 HAU across the plate, with the exception of row H.
5. Add 0.025 ml of antigen suspension containing 4 HAU to the first two wells of row H, then make two-fold dilutions from H2 to H6 (discard the last 0.025 ml) in order to obtain 4, 2, 1, 0.5, 0.25, 0.125 HAU. Wells H1–H6 contain the 4-HAU control titration.
6. Add 0.025 ml PBS + albumin 0.05% to all wells of the H row.
7. Mix by gentle tapping. Place the plate at +4°C for 40 min or at room temperature for 30 min.
8. Add 0.025 ml of 1% RBCs suspension to all wells.
9. Mix by gentle tapping. Incubate at +4°C for 40 min or at room temperature for 30 min.
10. Read the plates after 30–40 min, when the RBC control has settled. This is done by holding the plate in a vertical position and recording the occurrence or absence of tear-shaped streaming of the RBCs at the same speed as in control wells containing RBCs (0.025 ml) and PBS (0.05 ml) only (Figs. 7.10, 7.11). The HI titre is the highest dilution of serum causing complete haemagglutination inhibition.

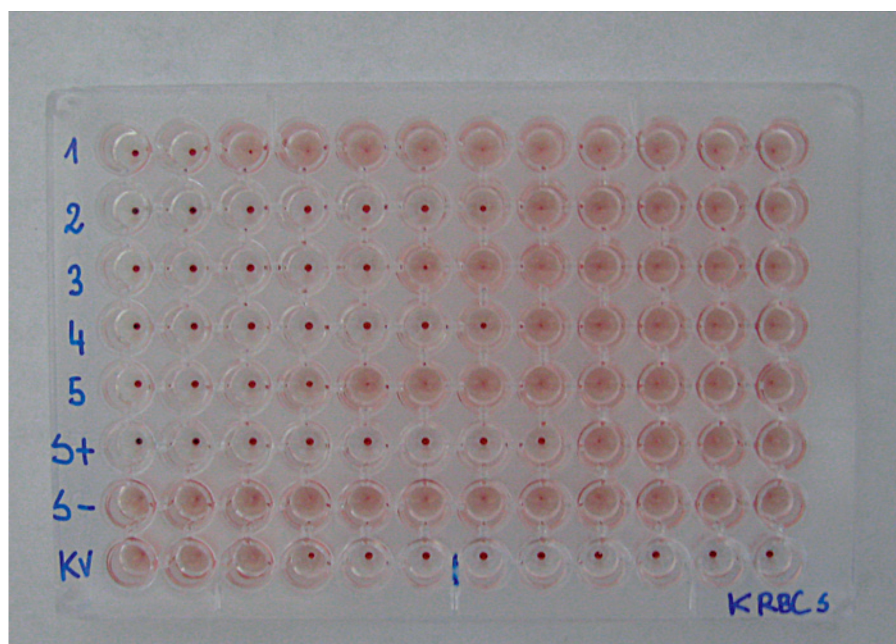


Fig. 7.10 Haemagglutination inhibition assay. *A1–A5* Test sera, *S-* negative serum, *S+* positive serum; the last row is the 4-HA unit control (*KV*) and the last six wells contain the RBCs control (*KRBCS*)

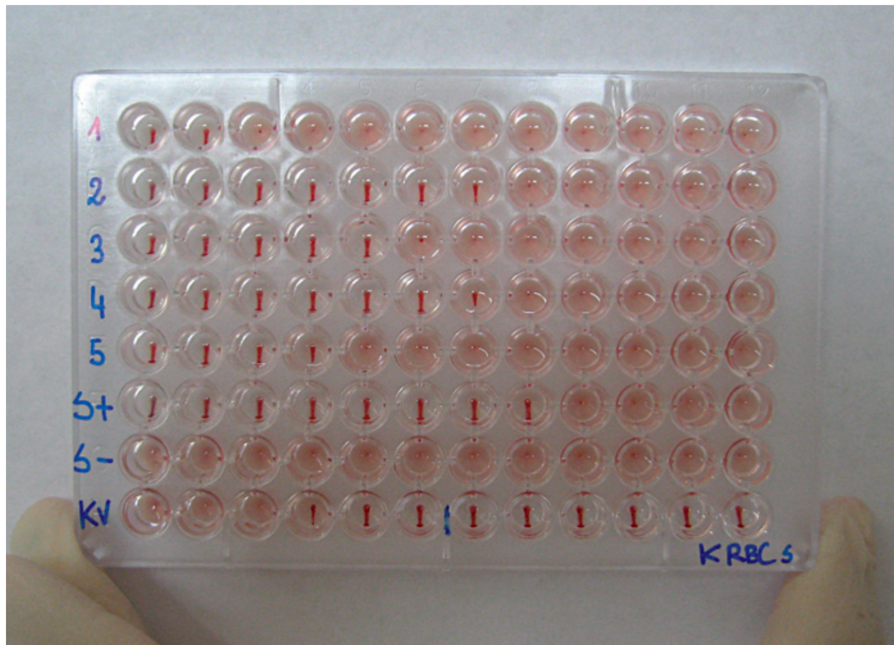


Fig. 7.11 Haemagglutination inhibition assay. *A1–A5* Test sera, *S-* negative serum, *S+* positive serum; the last row is the 4-HA unit control (*KV*) and the last six wells contain the RBCs control (*KRBCS*). Same plate as Fig. 7.10, tilted

Interpretation of the Results

1. The test is valid if:
   - The *negative control serum* has a titre of less than 2^3.
   - The *positive control serum* has a titre that coincides with its declared titre or is within one dilution of its declared titre.
   - Complete haemagglutination is observed in the first three wells (H1–H3) of the 4-HAU control row (containing respectively 4, 2, 1 HAU)
2. The sample is considered negative if the HI titre is < 1:8. This means that the serum does not contain specific antibodies to that subtype of AI virus.
3. The sample is considered positive if the HI titre is ≥ 1:16. This means that the serum contains specific antibodies to that subtype of AI virus.

7.3.4.4 Haemagglutination Inhibition Test for Species Other than Chickens

Non-specific reactions frequently occur when sera samples coming from birds other than chickens are used. Sera must be pre-treated as described below.

Pre-treatment of Sera

1. Dispense 0,050 ml PBS into wells in the first column of the microplate (wells A1–E1).
2. Leave the second row (A2–E2) empty.
3. Dispense 0.025 ml PBS into all other wells of the microtitre plate.
4. Add 0.050 ml of test sera to the first wells of the microplate (column 1).
5. Add 0.050 ml of a 10% RBCs suspension to the first wells (column 1).
6. Incubate the plate for 30-40 min at room temperature, and wait for the 10% RBCs suspension to settle.
7. Transfer 0.025 ml of the supernatant from the wells of the first column to the wells of the second column.
8. Transfer an additional 0.025 ml of the supernatant from the wells of the first column to the wells of the third column. Make two-fold serial dilutions of the sera from the third column to the last column (12). Discard the last 0.025 ml.
9. The sera are now ready to be treated as chicken sera. Column 1 should be excluded from the test.

It is also recommended to inactivate the non-specific haemagglutinating agents in the serum of game birds (pheasant, partridge, etc.) and of quails, ostriches and guinea fowl by heat treatment in a water bath at 56°C for 30 min prior to testing.