ISTITUTO ZOOPROFILATTICO SPERIMENTALE DELLE VENEZIE

EUROPEAN UNION REFERENCE LABORATORY FOR AVIAN INFLUENZA AND NEWCASTLE DISEASE

SOP IMM 065

DETECTION OF ANTIBODIES TO NEWCASTLE DISEASE VIRUS BY 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 30/12/20.

Contents

1. Purpose and field of application

This protocol describes the procedure to detect antibodies induced by avian paramyxovirus type 1 (APMV-1) in blood sera of chickens (*Gallus gallus*) and other avian species. Chicken red blood cells (RBCs) are mixed with a standard concentration of the APMV-1 antigen and with the serum to be tested to determine whether agglutination occurs. If no or insufficient antibodies to APMV-1 are in the test serum, the reference antigen will bind to RBCs inducing haemagglutination. Otherwise, when antibodies to APMV-1 are present, these will bind to the antigen inhibiting the agglutination of the RBCs, which will settle and assume a bottom shape at the bottom of the well.

2. References

- Council Directive 92/66/EEC introducing Community measures for the control of Newcastle disease;
- OIE - World Organization for Animal Health, Terrestrial Manual, Chapter 3.3.14. Newcastle disease (Version adopted in May 2012);
- IZSVe PDP IMM 065.

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

- 0.01 M sterile PBS pH 7.0-7.2;
- 1% and 10%(*) chicken red blood cells (RBCs) suspension derived from specific pathogen free (SPF) chickens (**);
- Negative control serum (^);
- Reference APMV-1 antigen (^);
- Reference positive control antiserum (^);

(*) Use the 10% chicken RBCs suspension in case the HI test is performed on sera from species other than chicken (see paragraph 5.5.)

(**) 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 first phase of the procedure consists of the HA titration of the APMV-1 reference antigen, while the second phase involves the detection of antibodies in chicken sera and/or in sera from species other than chickens by means of haemagglutination inhibition (HI) test. Dilutions and distribution of reagents and samples in the V-bottomed microwell plates can be performed manually or by using a liquid handling system.

5.1. Sample preparation

If samples are submitted as whole blood samples, they should be centrifuged at 1,000 x g for 10 minutes to separate the serum. The supernatant should then be collected avoiding aspiration of red blood cells and placed between +2°C and +8°C until use. Sera from avian species other than chickens may cause non-specific agglutination of RBCs. In order to perform a valid HAI test, the inactivation of any factor responsible for non-specific agglutination must be ensured by pre-treating the sera with a 10% RBCs suspension following the procedure outlined in paragraph 5.5. Sera collected from species bred for hunting purposes may undergo an additional heat-treatment at 56°C for 30 minutes prior to HI testing.

5.2. Reference antigen and control sera preparation

If reference antigen and/or control sera are freeze-dried, they must be reconstituted with an appropriate amount of distilled water according to the manufacturer's instructions. If not completely used, the reconstituted sera can be stored at ≤ -18°C and the antigen between +2°C and +8°C up to seven days, or at ≤ -70 °C for occasional use. To avoid frequent freezing and thawing, sera can be prepared, labeled and appropriately stored in multiple aliquots.

Reference antigens deriving from the same virus strain and from more than one vial should be pooled before starting the test to reduce tube-based variation in titre. However, reference antigen should be thawed and back titrated each test day (see paragraph 5.3.) to confirm that there is no loss of titre in the storage process and to ensure the correct dilution of the antigen.

5.3. Haemagglutination test (HA)

The procedure allows to determine the HA titre of the antigen in order to calculate the 4 haemagglutinating units (HAUs), which are conventionally used in the HI test. It is recommended to test the antigen 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 antigen in duplicate;
- Add 25 µl of reference antigen 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 antigen titre is given by the highest dilution causing complete agglutination of the RBCs. This represents 1 haemagglutinating unit (HAU);
- Standardize the reference antigen in order to obtain a concentration of 4 HAU per 25 µl. Use the dilution that contains four times the viral concentration of the highest dilution causing complete haemagglutination. For example, if 1 HAU is at a dilution of 1:512, 4 HAU are obtained by diluting the antigen in PBS 1:128 (512: 4 = 128). Place the diluted antigen at +4°C until use;
- Perform a back titration to verify HA units (4 HAU/25 µl) by performing a second HA test prior to the HI test as outlined in paragraph 5.4. If the working solution does not have the expected HA titre, it must be adjusted accordingly by adding more antigen to increase units or by diluting to decrease units until 4 HAU/25 µl is obtained.

5.4. Haemagglutination Inhibition test (HI) on chicken sera

The following steps refer to the assessment of a test serum against the APMV-1 reference antigen with the aim of determining the presence of specific antibodies. In case more than one sample is analyzed, arrange the V-bottomed microwell plastic plates by assigning a separate row to each tested sample. However, set the test to allow incubation times to be strictly observed and make sure plates are read promptly when the RBCs control has completely settled. Include the appropriate controls with each batch of HI tests.

A virus back-titration of the working solution of the virus (4 HAU antigen) and RBCs control should be arranged on each plate to assess test reliability; negative serum and reference antiserum must be arranged in two rows of the plate or of each 10-plate batch and used respectively as negative and positive control sera to assess results obtained with unknown tested samples.

- Arrange a V-bottomed 96-microwell plate by setting up the test serum in row “A”, the positive and negative control sera respectively in rows “B” and “C” and the control of the 4 antigen HAU and RBCs in row “D” (six wells per control) (Figure 2). Number the plate and record the position of the test serum;
- Add 25 µl of PBS to all wells;
- Add 25 µl of test serum, positive and negative control sera to the appropriate wells in column 1 (respectively to A1, B1 and C1);

- Make serial two-fold dilutions across the plate by transferring 25 µl of both test serum and positive/negative control sera from the first well to the following ones (from A-C1 to A-C12);
- Discard the remaining 25 µl;
- Add 25 µl of standardized antigen dilution to all wells containing test serum, positive and negative control sera and to D1 and D2 wells;
- Make serial two-fold dilutions from D2 to D6 to obtain 4, 2, 1, 0.5, 0.25 and 0.125 HAU and discard the remaining 25 µl;
- Add 25 µl of PBS to all wells of row "D", with the exception of D1;
- Gently shake the plate and incubate at room temperature for 30 minutes or at +4°C for 1 hour;
- 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.

When a large number of samples is tested, the V-bottomed plate can also be vertically arranged by inverting the columns with the rows, as in Figure 3. In this case, a maximum serum dilution of 1:256 is obtained by making serial two-fold dilutions across the plate from H1 to A1, and six and two wells of the same row are respectively assigned to the 4 HAU and the RBCs controls (i.e. row "4" in Figure 3).

5.5. Haemagglutination Inhibition test (HI) on sera of avian species other than chicken

To perform a valid HI test, sera from species other than chicken must be pre-treated with a 10% RBCs suspension following the procedure below in order to remove factors that may cause non-specific agglutination. For some species such as guinea fowls, quails, ostriches and species bred for hunting purposes, an additional inactivation pre-treatment in a water bath at 56°C for 30 minutes should be carried out prior performing the procedure as described hereafter.

- Arrange a V-bottomed 96-microwell plate by setting up the test serum in row "A", the positive and negative control sera respectively in rows "B" and "C" and the control of the 4 antigen HAU and RBCs in row "D" (six wells per control) (Figure 2). Number the plate and record the position of the test serum;
- Add 50 µl of PBS to the first well of the test serum row (A1);
- Add further 25 µl of PBS to all test wells excluding the well of column 2 (A2);
- Add 25 µl of PBS to all wells of rows "B", "C" and "D";
- Add 25 µl of test serum, positive and negative control sera to the appropriate wells in column 1 (respectively to A1, B1 and C1);
- Make serial two-fold dilutions across the plate by transferring 25 µl of positive and negative control sera from the first well to the following ones (from B-C1 to B-C12);
- Discard the remaining 25 µl;
- Add 25 µl of 10% chicken RBCs suspension to the test well of column 1 (A1);

- Gently shake the plate and incubate at room temperature for at least 30 minutes;
- Dilute the test serum by transferring 25 µl from A1 to A2 (dilution 1:4) and further 25 µl from A1 to A3 (dilution 1:8);
- Make serial two-fold dilutions across the plate by transferring 25 µl of the test serum from A3 to A12 and discard the remaining 25 µl
- The test serum is now ready to be treated as chicken serum by following the next steps. Column 1 should be excluded from the test;
- Add 25 µl of standardized antigen dilution to all wells containing test serum, positive and negative control sera and to D1 and D2 wells;
- Make serial two-fold dilutions from D2 to D6 to obtain 4, 2, 1, 0.5, 0.25 and 0.125 HAU and discard the remaining 25 µl;
- Add 25 µl of PBS to all wells of row D, with the exception of D1;
- Gently shake the plate and incubate at room temperature for 30 minutes or at +4°C for 1 hour;
- 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.

Reference antigen

Reference antigen (rep.)

RBCs control

	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
A	O	O	O	O	O	O	O	O	O	O	O	O
B	O	O	O	O	O	O	O	O	O	O	O	O
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 reference antigen titration. Rep. = replicate.

	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 serum	A	O	O	O	O	O	O	O	O	O	O	O	O
Positive control antiserum	B	O	O	O	O	O	O	O	O	O	O	O	O
Negative control serum	C	O	O	O	O	O	O	O	O	O	O	O	O
Control of 4 HAU and RBCs	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 2. HI test set-up on a V-bottomed 96-microwell plate. In row “D” six wells are assigned to the control of the 4 HAU and the remaining to the control of the RBCs.

	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	
	H	G	F	E	D	C	B	A	
Test serum	O	O	O	O	O	O	O	O	1
Positive control antiserum	O	O	O	O	O	O	O	O	2
Negative control serum	O	O	O	O	O	O	O	O	3
Control of 4 HAU and RBCs	O	O	O	O	O	O	O	O	4
	O	O	O	O	O	O	O	O	5
	O	O	O	O	O	O	O	O	6
	O	O	O	O	O	O	O	O	7
	O	O	O	O	O	O	O	O	8
	O	O	O	O	O	O	O	O	9
	O	O	O	O	O	O	O	O	10
	O	O	O	O	O	O	O	O	11
	O	O	O	O	O	O	O	O	12

Figure 3. HI test set-up on a vertically-arranged V-bottomed 96-microwell plate. In row "4" six wells are assigned to the control of the 4 HAU and the remaining to the control of the RBCs.

6. Interpretation of results

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.

6.1. Reliability of controls

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

Control	Expected Result
4 antigen HAU	Complete haemagglutination in the first 3 wells, partial haemagglutination in the fourth well ("half drop"), and no visible haemagglutination in the last two wells
RBCs	No evidence of haemagglutination
Positive antiserum	Titre either equal or higher/lower of one log base 2 to its expected titre
Negative serum	Presence of haemagglutination

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

6.2. Clinical specimens

The antibody titre of a test serum is given by the highest serum dilution inducing complete inhibition of the haemagglutination (formation of the tear-shaped streaming of RBCs). In case of HI test on serum from species other than chicken, the first dilution of the serum is excluded from the reading.

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

Output	Result
Serum titre $\geq$ 1:16	Positive
Serum titre $\leq$ 1:8	Negative

7. Characteristics of the method

This standard operating procedure is a validated and accredited procedure according to the ISO/IEC 17025. To ensure optimal HI results when determining APMV-1 serologically, it is essential to carry out the test procedure in exactly the same way. Any modification of this standard operating procedure could invalidate the test results.