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EUROPEAN UNION REFERENCE LABORATORY FOR AVIAN INFLUENZA AND NEWCASTLE DISEASE

SOP VIR 007

ISOLATION AND CHARACTERIZATION OF NEWCASTLE DISEASE 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 30/12/20.

Contents

1. Purpose and field of application 3
2. References 3
3. Safety 3
4. Materials 3
5. Procedure 4
5.1. Preparation of samples 4
5.1.1. Swabs 4
5.1.2. Organs 4
5.1.3. Faeces 5
5.2. Virus isolation 5
5.3. Haemagglutination (HA) test 6
5.4. Haemagglutination Inhibition test (HI) 7
5.4.1. Reference antiserum and negative control serum preparation 7
5.4.2. Procedure 7
5.5. Intracerebral pathogenicity test (ICPI) 9
6. Interpretation of results 9
6.1. Virus isolation 9
6.2. HI test 9
6.2.1. Reliability of controls 10
6.2.2. Clinical specimens 10
6.3. Intracerebral pathogenicity index test 10
7. Characteristics of the method 10

1. Purpose and field of application

This protocol describes the procedure to isolate avian paramyxovirus type-1 (APMV-1), the causative agent of Newcastle disease (ND), in biological samples. The identification of APMV-1 is achieved by means of the haemagglutination inhibition (HI) test using the specific polyclonal antiserum. The procedure can be successfully applied to organ homogenates, faeces and tracheal and cloacal swabs appropriately collected and preserved.

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 VIR 007.

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 (**);
- 9-11 day-old SPF embryonated chicken eggs;
- Antibiotic PBS pH 7.4 ± 0.2 (*);
- APMV-1 polyclonal chicken antiserum (^);
- Negative control serum (^);
- One-day-old SPF or specific antibody negative (SAN) chickens;
- 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 antiserum.

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 intracerebral pathogenicity test (ICPI). This *in vivo* test is usually carried out on the index case of an epidemic but not routinely performed on APMV-1 isolates. In case of an outbreak, the inoculation of birds is required to assess the strain virulence.

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, pooling up to 10 swabs may be required to increase testing capacity. In this regard, Spackman and colleagues (BMC Vet Res. 2013 Feb 22;9:35. doi: 10.1186/1746-6148-9-35) proved that no differences in ND virus detection could be observed regardless of the number of pooled specimens, may this be of 1, 5 and/or 11 samples. However, any variation in the standardized procedure has to be carefully assessed and authorized by the Head of the Laboratory.
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 between +2 and +8°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 for 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
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

Test sample

Test sample (rep.)

RBCs control

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 the reference polyclonal antiserum to determine the presence of APMV-1. In order to assess test reliability, control of the 4 virus HAU, negative control serum and RBCs control should be included in each HI test session.

5.4.1. Reference antiserum and negative control serum preparation

If reference antiserum and negative control serum are freeze-dried, they must be reconstituted whit 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

- 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 and +8°C until use
- Prepare the 4 HAU and RBCs controls in row "D" (six wells per control) of a V-bottomed 96-microwell plate (Figure 2) by adding 25 µl of PBS to the wells from D2 to D12;

- Add 25 µl of PBS to all wells of the first three rows of the plate;
- Add 25 µl of reference chicken antiserum to the first two wells of the first column (A1 and B1) to test sample in duplicate;
- Use row “C” 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 of the first three rows and to the 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. Discard the remaining 25 µl;
- Add 25 µl of PBS to all wells of row “D”;
- 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
APMV-1 reference antiserum	A	O	O	O	O	O	O	O	O	O	O	O	O
APMV-1 reference antiserum (rep.)	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. Example of HI test set-up of 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. Rep. = replicate.

5.5. Intracerebral pathogenicity test (ICPI)

The intracerebral pathogenicity index (ICPI) is used to assess the virulence of APMV-1 isolates for chickens by observing clinical sign in SPF or SAN infected birds over a 8-day period.

- Prepare 10 one-day-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 0.05 ml of the diluted virus intracerebrally into each of the 10 one-day-old chicks (chicks must be over 24-hours and under 40-hours old at the time of inoculation);
- Check the birds at 24-hour intervals for 8 days. At each observation, chicks are assigned a score rating 0 = normal; 1 = sick; 2 = dead. Dead birds should be assigned a 2-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 ICPI:

Clinical sign	**Days after inoculation**								**Score**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	
Normal	10	4	0	0	0	0	0	0	14 X 0 = 0
Sick	0	6	10	4	0	0	0	0	20 X 1 = 20
Dead	0	0	0	6	10	10	10	10	46 X 2 = 92
									Total score = 112/80*
									ICPI = 1.4

(*) Number of observations given by 10 birds monitored for 8 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 Newcastle disease virus 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
(i) No inhibition of haemagglutination by the polyclonal reference antiserum and/or (ii) The titre of the reference antiserum to the isolate is more than 3-fold lower than the expected titre	Negative

In case an isolated virus showing HA activity turns out to be negative for ND to HI test, further investigations will be required to identify the haemagglutinating agent.

6.3. Intracerebral pathogenicity index test

The ICPI is the average score per bird and per observation over a 8-day period. The most virulent viruses will give indexes that approach the maximum score of 2.0, whereas lentogenic and asymptomatic enteric strains will give values close to 0.0. Any avian paramyxovirus type 1 strain yielding a value > 0.7 in an ICPI test is considered a Newcastle disease virus, as outlined in the Council Directive 92/66/EEC.

7. Characteristics of the method

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