



SADC Harmonized SOP for Avian Influenza HA and HI Serological Tests

Version 2.0

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This Standard Operating Procedure (SOP) is available at:

<http://www.fao-ectad-gaborone.org>

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Text affected In this edition the following items have been either added or removed: (1) addition of the internet link address, (2) addition of a new section on Content, (3) addition of a new section on Introduction (Point 1) where Step 1 and Step 2 tests for the diagnosis of AI are described, (4) addition of a new section on Distribution list, (5) additional notes under the section on Criteria for acceptance and rejection of samples, (6) addition of a new section on the usage of 2 subtype antigens with different neuraminidase in Materials (Point 7), (7) addition of a new section on External quality control (Point 10.2), (8) additional notes under the section on the Interpretation of results (Point 11), (9) addition of a new section on Operational statistics (Point 12.2), (10) addition of a new section on Reference (Point 15), (11) Removal of Point 16 (Amendment history/list) at the back of the document, (12) addition and documentation of a new section on Amendment issued in front of the document, and (13) substitution of word "button" by "streaming" throughout the test.

DISTRIBUTION LIST

This SOP has been prepared for use by National Veterinary Laboratories of the following SADC member states:

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- Botswana
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- Malawi
- Mauritius
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AND

The following Veterinary Laboratories:

- Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI), South Africa
- Allerton Provincial Veterinary Laboratory, South Africa
- Potchefstroom Provincial Veterinary Laboratory, South Africa
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1. INTRODUCTION

According to OIE/FAO guidelines, serological diagnosis of Avian Influenza (AI) should be performed in 2 steps, unless the subtype circulating in a given country is already known. The first step aims at the detection of antibodies to any AI virus. The second step, to be performed on samples that are positive to step 1, identifies the viral subtype causing infection.

The tests prescribed for step 1 are Agar gel immunodiffusion (AGID) (indicated for chicken and turkey but completely unreliable in waterfowl and yet to be fully validated in other avian species) and Enzyme linked immunosorbent assay (ELISA) (highly sensitive test, but it lacks in specificity). These tests are able to **detect antibodies to the group antigen of influenza A viruses** that are present in all influenza A viruses regardless of the H or N subtype. Positive reactions to these tests indicate that the birds have encountered an influenza A virus but no information on the AI subtype which has caused seroconversion can be deduced.

The haemagglutination inhibition (HI) is ideal for step 2 which involves the **detection of subtype-specific antibodies (H subtype)**. This test is to be used in birds that are known to be infected with AI, either following a positive serologic test against the group (type A) antigen, or as a result of clinical history. The test is used to identify the haemagglutinin subtype of the virus causing the seropositivity.

2. PURPOSE, SCOPE AND PRINCIPLE OF THE TEST

To detect subtype-specific antibodies (H subtype) to AI.

Note:

It is highly **recommended** that Step 1 tests (group antigen) are performed before HA/HI is carried out.

In this case (i.e. where the Laboratories start with AGID or ELISA, as Step 1 test) the Purpose of the test will read as follows:

To identify the haemagglutinin subtype of the virus (i.e. AI subtype) that has caused seropositivity of serum samples on Step 1-tests (AGID or ELISA).

The AI virus is capable of haemagglutinating cockerel/chicken red blood cells (CRBC) that are used in the test as the indicator. In this test, sera from the suspected cases are mixed with the AI Virus strain antigens. In positive cases there will be binding of the antibodies to a specific antigen. This binding inhibits the haemagglutination of the indicator cells by the antigen, causing the formation of sediments at the bottom of the wells, appearing like **tear-shaped streaming** (herein referred to as streaming). The result of a positive case is detected as a 'streaming' and the negative result shows haemagglutination of the cells due to the presence of the antigen.

3. SAFETY CONSIDERATION AND PRECAUTIONS

All blood samples should be regarded as potentially capable of transmitting diseases. Handling of such blood samples (e.g. decanting, aliquoting, etc) should therefore always take place in the safety cabinet.

All safety measures as applicable to the laboratory must be followed at all times. These measures include:

- Wearing of protective clothing at all times. These include coat, gloves and, if the need arises, face mask during all manipulations.

- disinfecting of the working bench before and after completing the test
- All spills or bench contaminations of any sort during testing process should be decontaminated using 70% alcohol or any other recommended disinfectant (according to manufacturer's instruction or as documented in SOP)
- Disposal of used and contaminated materials in accordance with applicable procedure

4. PERSONNEL

- Technicians/ technologists/veterinarian
- All personnel performing the test should be declared competent and authorised by management
- Criteria for declaring personnel competent should be clearly defined by management and training records should be available in personnel file.

5. SAMPLE REGISTRATION

Use details on your Serology submission form

Important details should include but are not limited to:

- Name, physical address and geographical coordinates of owner, with telephone and fax numbers (where applicable)
- Name and postal address of sender, with telephone and fax number, and date of submission
- Disease suspected
- Samples submitted and tests required
- Different species on the farm and number
- Length of time on the farm; if recent arrival, where from
- Date of first cases and of subsequent cases or losses
- Description of the spread of infection in the flock
- Number of animals dead, the number showing clinical signs (and their age if possible)
- The clinical signs and their duration
- Vaccination history

6. CRITERIA FOR ACCEPTANCE/REJECTION OF SAMPLES

The criteria for acceptance and/or rejection of samples should be documented. The personnel receiving samples should be trained to recognise abnormal samples. When samples are rejected owner or sender should be advised immediately not to expect any result (or part of the result) from the laboratory

Only whole clotted blood or separated serum is acceptable

Haemolysed/autolyzed specimens are not acceptable and should not be tested

7. MATERIALS AND EQUIPMENT

7.1. Materials

Reagents/ Consumables

- Antigens H1 to H16 (*specify your antigen source*)
Initial testing should be performed using H5 and H7 subtype antigens.

At least two antigens of the same H subtype but with different neuraminidase subtypes (e.g. H5N1 and H5N9 and H7N1 and H7N3) should be used in the initial approach to diagnosis (See Point 11 below).

- Positive and Negative controls (*Specify your source*)
- Distilled water
- Alsever's solution
- Glucose
- Sodium chloride
- Tri-sodium citrate (2H₂O (2-hydrate)
- Citric acid
- PBS
- Sodium Chloride (NaCl)
- Potassium Chloride (KCL)
- Disodium Hydrogen orthophosphate Na₂HPO₄
- Potassium di-hydrogen orthophosphate KH₂PO₄
- Red blood cells

7.2. Equipment

- Autoclave
- Bench top centrifuge
- Heparinised capillary tubes, plasticine
- Microhaematocrit centrifuge
- Microhaematocrit reader
- Microtitre agglutination plates
- Multichannel pipette
- 1000µL sample tips
- 300 µL sample tips
- 1000µL measuring cylinder
- 1000ml storage bottles
- 500 ml bottles
- Magnetic stirrer + magnet
- Paper towel
- Permanent marker
- pH meter
- Refrigerator / cold room
- Refrigerated incubator
- Single channel pipette
- Syringes
- Timer
- Water bath

8. SAMPLE HANDLING AND PREPARATION

- Keep samples at +2 to +8⁰C before testing
- Separate serum from the whole blood sample by centrifugation
- *Optional:* Inactivate sera in water bath at 56⁰C for 30 minutes before use

Note:

When the Laboratory opts not to inactivate the sera, the Quality Manager should cross out the sentence referring to inactivation (bullet number 3 above) and initialise.

9. TEST PROCEDURE

9.1 Pre-test

9.1.1 Preparation of Phosphate buffer saline - PBS

- Sodium Chloride (NaCl) 8.00g
- Potassium Chloride (KCL) 0.20g
- Disodium Hydrogen orthophosphate Na_2HPO_4 1.14g
- Potassium di-hydrogen orthophosphate KH_2PO_4 0.20g
- Distilled Water up to 1000 ml
- Adjust pH to 7.2-7.4
- Aliquot into 500 ml bottles.
- Autoclave at 121°C for 15 minutes

ALTERNATIVELY

Reconstitute PBS powder according to manufacturer's instructions OR use ready to use liquid PBS

9.1.2 Preparation of Alsever's solution

- Glucose 20.5g
- Sodium chloride 4.2g
- Tri-sodium citrate ($2\text{H}_2\text{O}$ (2-hydrate)) 8.0g
- Citric acid 0.55g
- Distilled water 1000 ml

Dispense into 10 ml or 50 ml amounts

Autoclave at 121°C for 15 min

9.1.3 Preparation of erythrocytes (RBC)

1. Prepare the RBC stock solution as follows:
 - (a) Place 5 ml of Alsever's solution into each of two syringes.
 - (b) Use the 2 syringes with Alsever's solution from (a) to draw 5 ml whole blood from each of 2 birds free of AI to give a total mixture volume of 10 ml in each of the 2 syringes.
 - (c) Pool the 2 mixtures of blood and Alsever's in the 2 syringes into a universal bottle to give a total volume of 20ml. Mix gently. This will be your stock solution.
 - (d) Store the stock solution at $4^\circ\text{C} \pm 2^\circ\text{C}$. (The stock solution can be stored for up to 5 days provided there is no haemolysis). Cells for immediate use (working solution) are prepared freshly each day from the stock solution.
2. Prepare the working solution from the stock solution as follows:
 - (a) Mix the stock solution cells gently, but ensuring that it is well mixed and aliquot 1 ml per test plate.
 - (b) Centrifuge the aliquoted cells at 2200 RPM for five minutes and discard the supernatant to remove the Alsever's solution.
 - (c) Add PBS and centrifuge at 2200 rpm for 5 min and remove supernatant. Repeat this step twice more.
3. Determine the packed cell volume (PCV) of the prepared working solution as follows:
 - (a) Fill two capillary tubes with the blood from step 2(c) above and centrifuge in a

- haematocrit for 3 minutes.
- (b) Read off the PCV from the haematocrit reader. The PCV should be in the range of 55%- 70%. If the PCV is less than 55%, the cells must be re-centrifuged and excess PBS removed and if its more than 70% dilute with more PBS.
- (c) Prepare **1% cells** from the PCV determined working solution from 3 according to the following formula:

$$C1 \times V1 = C2 \times V2$$

Where:

C1 is the original PCV

C2 is the concentration required (i.e. 1%)

V1 is volume of Packed Cells

V2 is the Volume of the 1% cells to be prepared

OR

Mix **1 part RBC** with **99 parts PBS**

9.2 Methodology

9.2.1 Haemagglutination (HA) test

- (1) Dispense 0.025 ml of PBS into each well of a plastic V - bottomed microtitre plate
- (2) Place 0.025 ml of antigen (virus suspension) to the first well in rows A and B and make twofold dilutions of 0.025 ml volumes of the virus suspension across the plate, discard 0.025 ml from the last well

Note: read package insert carefully to familiarise with manufacturer's instruction on the reconstitution of the antigen. Dilute only if so directed or if reading overshoots the plate

- (3) Dispense a further 0.025 ml of PBS to each well
- (4) Dispense 0.025 ml of 1% (v/v) chicken RBCs to each well
- (5) Make cell control wells, rows C and D, by placing 0.05 ml PBS followed by 0.025ml of 1% cockerel red blood cells.
- (6) Mix by tapping the plate gently and then allow the RBCs to settle for 45 minutes at room temperature ($23^{\circ}\text{C} \pm 2^{\circ}\text{C}$), if ambient temperatures are high/ low place in a refrigerated incubator set at the appropriate temperature or for 60 minutes at 4°C by which time control RBCs should be settled to a distinct button
- (7) HA is determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The titration should be read to the highest dilution giving complete HA (no streaming); this represents 1 HA Unit (HAU) and can be calculated accurately from the initial range of dilutions
- (8) Calculate the amount of antigen required for the number of plates. Make a 4 HAU of the antigen as follows:

e.g. Antigen haemagglutination titre	1 HA Unit	= 640
	4 HA Units	= $640 \div 4$
		= 160

Therefore the antigen is used at a dilution of 1:160 in HI

- (9) Carry out a back titration as follows:
 - a) Place 0.025 ml of PBS in two rows to the wells 1-6.
 - b) Add 0.025 ml of 4 HAU antigen to the first well. Make two fold dilutions to the 6th

well and discard 0.025 ml.

- c) Add a further 0.025 ml of PBS to the wells.
- d) Prepare the cell control wells by placing 0.025 ml of PBS to wells 7 – 12.
- e) Add 0.025 ml of 1% (v/v) chicken RBCs to each well, mix gently and incubate for 45 minutes at room temperature
- f) The antigen with correct haemagglutinating titre (i.e. 4 HAU) will be expected to yield complete HA at 2 HAU and 1 HAU in the back titration. If this is not the case, the antigen titre can be corrected by either concentrating or further diluting it.
- g) Only when satisfactory antigen titre is confirmed by back titration that the next step (i.e. Haemagglutination inhibition – Point 9.2.2) should be carried out.

9.2.2 Haemagglutination inhibition (HI) test

Time should not be wasted before serological positivity to Notifiable AI (NAI) viruses as detected by Step 1 test (for Laboratories starting with Step 1 tests: AGID or ELISA) is ruled out or confirmed.

- (1) Mark the HI test plates according to the plate layout.
- (2) Dispense 0.025 ml of PBS into each well of a plastic V-bottomed microtitre plate
- (3) Using two rows per sample, Place 0.025 ml of test serum into the first and second well of the plate. Include one positive and negative control (prediluted 1 in 4) per batch of test plates and treat them the same way as the test sera.
- (4) Make twofold dilutions of 0.025 ml volumes of the serum from the second well across the plate. Discard 0.025 ml from the last well.

Note: For Laboratories testing large number of samples whereby only H5 and H7 are targeted, it is acceptable to stop the serial dilution at 1:32, then positive samples be further titrated to determine the exact end point.

- (5) Add 4 HAU of virus/antigen in 0.025 ml to each of the wells except the first column (serum control) well. Carry out another back titration and cell controls (see sections 9.2.1 9, a to f) and leave for 30 minutes at room temperature or 60 minutes at 4°C
- (6) Add 0.025 ml of 1% (v/v) chicken RBCs to each well and after gentle mixing, allow the RBCs to settle for 45 minutes at room temperature, i.e. about 20°C, or for 60 minutes at 4°C if ambient temperatures are high, by which time control RBCs should be settled to a distinct button
- (7) The HI titre is the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The agglutination is assessed by tilting the plates. Only those wells in which the RBCs stream at the same rate as the serum control wells (containing test serum and RBCs only) should be considered to show inhibition.

9.3 Reference to worksheets and Forms

- (1) Technical records for HA are recorded on Poultry haemagglutination worksheet – Give Sheet code e.g.WS/VS/001
- (2) Test sample results are recorded on HI result sheet - Give Sheet code e.g.WS/VS/005

10. CRITERIA FOR TEST ACCEPTANCE/QUALITY CONTROL (QC)

Results are accepted on the basis of:

- (1) Antigen back titration to give complete haemagglutination up to 1 HAU

- (2) Cell control and serum control should show complete streaming
- (3) Positive control should fall within one log difference of the supplier's predetermined titre.
- (4) In the negative control wells, only the serum control well should show a streaming

10.1 Internal QC

- (i) On each day the test is carried out; plot the titre of the positive control on the trend analysis record form, either FM/VS/017 or FM/VS/018 for H5 and H7 antigens respectively. Include the date of testing and the initials of the operator
- (ii) Raise a non conformance whenever the control falls outside the limits and withhold the results until corrective action is complete. If a positive trend is noted; titrate the positive control until a dilution which is within limit is established. If a negative trend is noted, use a new vial of positive control
- (iii) From the graph, make concluding statements quarterly. Investigate shifts in trends and make corrective action as stated above

10.2 External QC

- (i) Participation in a proficiency testing (PT) scheme (or ring trial) is a must for assuring the validity of results produced by the laboratory

Alternatively,

- (ii) Inter-laboratory comparison (ILC) with other laboratories doing similar work can be carried out. In this case, please ensure that the laboratory is accredited (ISO/IEC 17025) or has effectively implemented a QMS

Note: results of PT and ILC must be analysed and interpreted. Where outliers or discrepancies occur, an appropriate root cause analysis must be conducted and corrective actions taken.

11. READING AND INTERPRETATION OF RESULTS

The observation of inhibition, i.e. a streaming that flows at the same rate as the cell control streaming (tear drop) indicates the presence of antibodies to AI virus.

	1	2	3	4	5	6	7	8	9	10	11	12
A	SC	4	8	16	32	64	128	256	512	1024	2048	4096

- A sample is considered positive if it causes inhibition of the haemagglutinating activity of 4 HAU at a titre of at least 1:16 (2⁴)
- Serum with no streaming in the serum control well should be reported as inconclusive and a rebleed of the bird requested.
- Sera from species other than chicken may cause non specific haemagglutination. This can be overcome as follows: add 0.025 ml of packed chicken RBC's to each 0.5 ml of test sera, shake the mixture gently, allow it to stand at room temperature for 30 minutes, centrifuge it at 2200 rpm, then decant and use the supernatant for testing.
- Low degree cross-reactivity with other H subtypes may be observed due to homology with the neuraminidase antigen
- This cross-reactivity is generally not higher than 1:16 (2⁴) and disappears with another antigen with different neuraminidase.

For example:

A serum sample is positive to H9N2 at a titre of 1:256 (2^8). If tested with H5N2 antigen a positive inhibition result may be observed at 1:8 (2^3). When tested with an H5N9 antigen the sample will be negative

12. REPORTS AND RECORDS

12.1 Laboratory reports and records

- (1) Follow reporting procedure – Give reference to the procedure e.g. GP/VS/018
- (2) Fill notifiable disease form if any samples test positive according to the respective procedure in the Quality Procedures Manual

12.2. Operational statistics

Quarterly operational statistics must be compiled and reported for consolidation into the SADC Diagnostic Subcommittee's report that will be available online at <http://www.fao-ectad-gaborone.org>

13. RETENTION OF SAMPLES

- (1) Short term storage is up to 7 days at +2 to +8°C
- (2) Long term up to 2 years at -20°C

14. WASTE DISPOSAL

Waste is disposed of according to your general disposal procedure – Give a cross-reference to your SOP (or instruction) e.g. GP/VS/006

15. REFERENCE

- (1) *OIE Manual: Manual of Diagnostic, Tests and Vaccines for Terrestrial Animals, 2008*
- (2) Official Journal of the European Union: Commission Decision 8 June 2009: *amending Decision 2007/268/EC on the implementation of surveillance progress for avian influenza in poultry and world birds to be carried out in the Member States*
- (3) OIE/FAO guidelines for correct application and interpretation of diagnostic results for the diagnosis of AI on serum samples

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