	NATIONAL CENTRE FOR ANIMAL HEALTH **LABORATORY SERVICES UNIT**	**Second edition**
	STANDARD OPERATING PROCEDURE	**Version 2018.1**
	ELISA TESTS	

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TITLE: ELISA test for *Brucella abortus*

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APPROVED BY: Head, LSU

DATE: 11.06.2018

1. INTRODUCTION

Brucella is a small Gram-negative bacterium (0.4-0.8 µm in diameter and 0.4-3.0 µm in length) which is non-flagellated, and non-spore forming. Four species are pathogenic to human: *Brucella abortus*, *Brucella melitensis*, *Brucella suis* and *Brucella canis*. All four species are exciters of Brucellosis, a disease characterized by undulating fever. Depending on exciter the disease is also called Morbus Bang (*B. abortus*) or Malta fever (*B. melitensis*). The pathogens are transmitted from animals, which are mainly affected. The infection is caused by contact with ill animals or their excrements as well as by non-pasteurized milk and milk products like fresh cheese from sheep or goat. Main entrances are skin wounds, conjunctives and digestive tract. The intact pathogens are transported by granulocytes into local lymph nodes, from where they spread haematogenous. All kind of organs can be infected.

2. PRINCIPLES

Microtiter plates are coated with inactivated antigen. Dilutions of the samples to be tested are incubated in the wells of these plates. Any antibody specific for *B. abortus* binds to the antigen in the wells and forms an antigen/antibody complex on the plate well surface. Unbound material is removed from the wells by washing. A peroxidise-labelled ani-ruminant IgG Conjugate is added, which binds to the ruminant antibody complex with the *B. abortus* antigen. Unbound conjugate is removed by washing and TMB substrate added to the wells. The degree of color that develops (Optical density) measured at 450nm) is directly proportional to the amount of antibody specific for *B. abortus* present in the sample. The result is obtained by comparing the optical density (OD) that develops in wells containing the samples with the OD from wells containing the Positive Control.

3. APPLICATION

The IDEXX Brucellosis Serum X2 test kit provides a rapid, simple, sensitive and specific method for detecting antibodies against *brucella abortus* in individual serum and in pools of up to 10 individual serum samples from ruminants.

4. OBJECTIVE

To describe the procedure for detecting antibodies against *brucella abortus* in serum samples from ruminants.

5. APPARATUS/TEST KIT/REAGENTS

IDEXX Brucellosis Serum X2

5.1 *B. abortus* antigen coated plate
5.2 Positive control
5.3 Negative control
5.4 Conjugate
5.5 TMB SubstrateN12
5.6 Stop Solution N.3
5.7 Wash Concentration (10X). **Dilute the wash Concentrate (10X) 1:10 with distilled water.**

6. TEST PROCEDURE

All the reagents must be allowed to come to 18-26 ^{o}C before use. Mix reagents by gently inverting or swirling.

6.1 Obtain coated plate and record the sample position.
6.2 Dispense 90µL wash solution into each well
6.3 Dispense 10 µL of the un diluted positive control into duplicate wells
6.4 Dispense 10 µL of the un diluted Negative control into duplicate wells
6.5 Dispense 10 µL of the un diluted serum samples or pools of up to 10 serum samples in to appropriate wells
6.6 Mix the content of the micro wells by gently tapping the plate or use micro plate shaker.
6.7 **Short Incubation**-Cover the plate and incubate for 60 minutes at 37^{o}C. The plate should be tightly sealed or incubated in a humid chamber using plate covers.
6.8 **Overnight incubation**- Cover the plate and incubate overnight for 14-18 hours at 2-8^{o}C. The plate should be tightly sealed or incubated in a humid chamber using plate covers
6.9 Remove the solution and wash each well with 300 µL of wash solution 3 times. Avoid the plate dying between plate wash. Tap each plate onto absorbent material after the final wash to remove any residual wash fluid.
6.10 Dispense 100 µL conjugate in to each well.
6.11 Incubate for 60 minutes at 37oC. The plate should be tightly sealed or incubate in a humid chamber using plate covers.
6.12 Repeat step 9
6.13 Dispense 100 µL of TMB substrate N.12 into each well.
6.14 Incubate at 18-26^{o}C for 15 minutes.
6.15 Dispense 100 µL of stop solution N.3 into each well.
6.16 Read the results at a wavelength of 450 nm.

7. RESULT INTERPRETATION

$$\textbf{SP \%} = 100 \frac{\textbf{Sample A (450) - NC}}{\textbf{PC-NC}}$$

Negative - SP % < 80

Positive - SP % ≥ 80

8. WASTE DISPOSAL

Treat it as the infectious material and dispose accordingly.

9. RISK ASSESSMENT

9.1 Handle all reagents and samples as bio-hazardous material.
9.2 Keep all reagents away from skin and eyes. If exposure should occur, immediately flush affected areas with cold water.
9.3 Wash solution, control sera, test plates, field samples and all other test kit reagents should be properly decontami-nated with bleach or other strong oxidizing agent before disposal
9.4 Take special care not to contaminate any of the test re-agents with serum or bacterial agents.
9.5 Humidity indicators are supplied with each plate. If any of the indicators exhibit a pink color, the plate may be com- promised in some way; decontaminate (i.e. wash the plate with bleach solution) and dispose of the plate.
9.6 The best results are achieved by following the protocols as they are described below, using good, safe laboratory techniques.
9.7 Do not use this kit after the expiration date.
9.8 Never pipette by mouth. Allow all reagents to come to room temperature before starting.

10. TROUBLESHOOTING

NA

11. REFERENCES

IDEXX Brucellosis Serum X 2 kit manual 06-04130-14

TITLE: **ELISA for Avian Leukosis Complex Ag**

PREPARED BY: **Serology section**

REVISED BY: **Serology section**

APPROVED BY: **Head, LSU**

DATE: **11.06.2018**

1. INTRODUCTION
Avian Leukosis viruses (ALV) produce variety of neoplastic disease including lymphoid leukosis, erythroblastosis, myelocytomatosis, and others.

2. TEST PRINCIPLES
This assay is designed to detect p27, an antigen common to all subgroup of ALV including endogenous viruses. The recommended sample types are light albumin and cloacal samples.while serum has been validated for use on the ALV-AG test, it is not a recommended sample for the detection of exogenous virus because of potential interference from endogenous sequence. A microtitration format has been developed in which anti-p27 antibody is coated onto 96-well plates.

3. OBJECTIVE
IDEXX ALV AG is an enzyme immunoassay from IDEXX for the detection of avian leukosis virus Antigen p27 in chicken serum, Cloacal or albumin samples.

4. APPARATUS/TEST KIT/REAGENTS
IDEXX ALV Ag

4.1 Anti-27 Antibody coated plate
4.2 Positive control
4.3 Negative control
4.4 Conjugate
4.5 Sample Diluent
4.6 TMB SubstrateN12
4.7 Stop Solution N.3
4.8 Albumin Wash Concentration (20X). **Dilute the wash Concentrate (10X) 1:20 with distilled water.**

5. PROCEDURE
A. Preparation of samples
- Albumin- collect light albumin and add directly to the plate without prior dilution. Freeze/thaw the sample to help reduce the viscosity.

- Cloacal swabs – place cloacal swab into 1mLculture media or sample diluents and freeze. Prior to testing, bring the sample to 18-260C and allow coarse

material to settle. Pipette 100µL of the supernatant directly onto the ELISA plate.

- Serum – for general detection of p27, the sample is added directly to the well prior dilution. Testing of serum samples for exogenous derived p27 is not recommended because of interference from from endogenous virus.

All the reagents must be allowed to come to 18-26^{o}C before use. Mix reagents by gently inverting or swirling.

5.1 Obtain coated plate and record the sample position.
5.2 Dispense 10 µL of the un diluted Positive control into duplicate wells
5.3 Dispense 10 µL of the un diluted Negative control into duplicate wells
5.4 Dispense 10 µL of samples in to appropriate wells. Samples may be tested in duplicate but a single well is acceptable. None of the samples are diluted for testing.
5.5 Cover the plate and incubate for 60 minutes at 18 - 26^{o}C.
5.6 Wash each well with 350 µL of distilled water 3-5 times. Aspirate completely.
5.7 Dispense 100 µL conjugate in to each well.
5.8 Incubate for 60 minutes at 18 - 26^{o}C.
5.9 Repeat step 6.
5.10 Dispense 100 µL of TMB substrate into each well.
5.11 Incubate at 18 - 26^{o}C for 15 minutes.
5.12 Dispense 100 µL of stop solution into each well.
5.13 Read the results at a wavelength of 650 nm.

6. RESULT INTERPRETATION and REPORTING

$$\textbf{SP ratio} = \frac{\textbf{Sample mean - NC}}{\textbf{PC-NC}}$$

Negative - SP ratio ≤0.20

Positive - SP ration ≥ 0.20

7. WASTE DISPOSAL

Treat the samples and the reagents as infectious agent and dispose properly.

8. RISK ASSESSMENT

NA

9. TROUBLESHOOTING

NA

10. REFERENCES

Avian Leukosis Complex Virus Ag test kit 06-01154-15 manual

TITLE: Ab ELISA for *Mycobacterium Paratuberculosis*

PREPARED BY: Serology section

REVISED BY: Serology section

APPROVED BY: Head, LSU

DATE: 11.06.2018

1. INTRODUCTION

Paratuberculosis, or J*ohne's* disease, is a chronic of ruminant, for which main clinical signs are abundant diarrhoea and weight loss. The infection is due to an acid fast bacillus: *Mycobacterium paratuberculosis.*

This kit is designed for the detection of antibodies directed against *Microbacterium avium ssp paratuberculosis* in individual serum plasma and milk sample from bovine and in individual serum and plasma samples from small ruminants.

2. TEST PRINCIPLES

Microplates are coated with MAP Antigen. Samples to be tested are pre-incubated with *Mycobacterium phlei* extract in order to bind unspecific Antibodies. Then samples are transferred and incubated in the wells of coated microplate. Upon incubation of the samples in the coated wells, MAP specific Antibodies form a Antibody-Antigen immune-complex.

3. OBJECTIVE

Paratuberculosis Screening is IDEXX enzyme immunoassay for the detection of antibodies directed against *Mycobacterium avium* spp paratuberculosis(MAP) in bovine individual serum, plasma and milk samples, and in individual serum and plasma samples from small ruminants.

4. APPARATUS/TEST KIT/REAGENTS

IDEXX Paratuberculosis Screening

- 4.1 MAP Antigen coated plate
- 4.2 Positive control
- 4.3 Negative control
- 4.4 Conjugate Concentrate
- 4.5 Dilution Buffer N.1
- 4.6 Dilution Buffer N.12
- 4.7 TBM Substrate N.9
- 4.8 Stop Solution N.3
- 4.9 Albumin Wash Concentration (20X). **Dilute the wash Concentrate (10X) 1:20 with distilled water.**

5. PROCEDURE

A. Preparation of samples

- Samples and Controls are pre-diluted and pre-incubated on an uncoated predilution microplate. Milk samples: defatted milk (obtained after centrifugation) as well as a whole milk sample can be implemented.

All the reagents must be allowed to come to 18-26oC before use. Mix reagents by gently inverting or swirling.

5.1 Obtain coated plate and record the sample position.
5.2 Dispense 10 µL of the un diluted Positive control into duplicate wells
5.3 Dispense 10 µL of the un diluted Negative control into duplicate wells
5.4 Dispense 10 µL of samples in to appropriate wells. Samples may be tested in duplicate but a single well is acceptable. None of the samples are diluted for testing.
5.5 Cover the plate and incubate for 60 minutes at 18 - 26ºC.
5.6 Wash each well with 350 µL of distilled water 3-5 times. Aspirate completely.
5.7 Dispense 100 µL conjugate in to each well.
5.8 Incubate for 60 minutes at at 18 - 26ºC.
5.9 Repeat step 6
5.10 Dispense 100 µL of TMB substrate into each well.
5.11 Incubate at 18 - 26ºC for 15 minutes.
5.12 Dispense 100 µL of stop solution N.3 into each well.
5.13 Read the results at a wavelength of 650 nm.

6. RESULT INTERPRETATION

$$\textbf{SP ratio} = \frac{\textbf{Sample mean - NC}}{\textbf{PC-NC}}$$

Negative - SP ratio ≤0.20

Positive - SP ration ≥ 0.20

7. WASTE DISPOSAL

Consider all the specimens and reagents as infectious material and dispose it properly.

8. RISK ASSESSMENT

NA

9. TROUBLESHOOTING

NA

10. REFERENCES

Mycobacterium paratuberculosis Ab test int 06-07130-23 manual

TITLE: ELISA for detection of NSP antibodies against FMD virus

PREPARED BY: Serology section

REVISED BY: Serology section

APPROVED BY: Head, LSU

DATE: 11.06.2018

1. INTRODUCTION

Foot and Mouth Disease (FMD) is the most important economic threat to the livestock industry. The highly contagious disease affects all cloven-hoofed animals and is wide-spread over the world. The FMD viruses are classified into 7 distinct serotypes which makes diagnosis using conventional serological methods complex. To control outbreaks in the future emergency vaccination will be carried out. Vaccines consist of (partly) purified structural proteins of the FMD virus and therefore vaccinated animals only elicit antibodies directed against the structural proteins of the virus. However, after infection with FMDV, antibodies directed against the structural and the non-structural proteins are produced. Therefore an ELISA detecting antibodies against non-structural proteins of FMDV detects not only infected animals but also discriminates between infected and vaccinated animals.

The PrioCHECK® FMDV NS detects antibodies directed against the non structural 3ABC protein of FMDV. The ELISA detects FMDV infected animals independent of the serotype that causes the infection and independent of the fact that the animal is vaccinated or not. The ELISA can be used to test serum samples of cattle, sheep, goats and pigs.

2. PRINCIPLES

2.1 The PrioCHECK® FMDV NS is a blocking ELISA. The Test Plates are coated with 3ABC specific monoclonal antibody (mAb), followed by incubation with antigen (3ABC protein). Consequently, Test Plates of the kit contain FMDV NS antigen captured by the coated mAb. The test is performed by dispensing the test samples to the wells of a Test Plate. After incubation the plate is washed and the Conjugate is added. FMDV NS specific antibodies, directed against the non-structural proteins, that may be present in the test sample will bind to the 3ABC protein and will block the binding of the mAb-HRPO. After incubation, the plate is washed and the Chromogen (TMB) Substrate is dispensed. After incubation at room temperature (22±3°C) the color development is stopped. Color development measured optically at a wavelength of 450 nm shows the presence of antibodies directed against Foot and Mouth Disease Virus. The PrioCHECK® FMDV NS is a single dilution test. Serum samples are tested in a 1:5 dilution.

2.2 PD-FMD DIVA Kit: Sero-conversion against NSPs (3AB3) is observed since 10-14 days after FMD virus infection. Whereas if the animal is not exposed to FMD virus infection but vaccinated with inactivated purified polyvalent FMD vaccine, no anti-NSP immune response is elicited in host's body. This differential induction of

anti-NSP antibody is exploited in DIVA ELISA to discriminate between infected and vaccinated animals. In this DIVA test reactivity of anti-3AB3 antibodies present in the serum of an infected animal (bovine species only) is assessed against purified recombinant 3AB3 (~38 kD) NSP in an indirect ELISA format. A sample producing OD value more than the fixed cut-off ratio {(test serum sample mean OD/positive control serum mean OD) x 100 i.e., percent positivity value or PP value > 40%} is qualitatively diagnosed as positive for FMD infection. The present DIVA kit as described here is a recombinant 3AB3 NSP based indirect ELISA kit designed, developed and evaluated using in-house produced and standardized reagents at the central FMD laboratory, Project Directorate on FMD, Mukteswar. This DIVA kit is very much in toe with the OIE approved test as a companion test for inactivated vaccines.

3. APPLICATION

This test is done to differentiate between vaccine antibody and infection antibody. It is also done to monitor the sero-conversion against NSPs (3ABC and 3AB3) specific monoclonal antibody.

4. OBJECTIVE

To describe the procedure for conducting the ELISA for NSP antibodies against FMD virus in serum.

5. APPARATUS/TEST KIT/REAGENTS AND BUFFER

5.1 Negative Control (Ready-to-use) - One vial contains 0.6 ml Negative Control.
5.2 Chromogen (TMB) Substrate (Ready-to-use) One vial contains 60 ml Chromogen (TMB) Substrate.
5.3 Stop Solution (Ready-to-use) - One vial contains 60 ml Stop Solution.
5.4 Package Insert, 10 plate sealers
5.5 Certificate of Analysis

Kit Components (Prionics NS ELISA)

a. Five Test Plates are delivered in bags which contain a desiccant sachet.
b. Conjugate (30x) (30x concentrated, dilute before use). One vial contains 2.5 ml Conjugate.
c. Dilution Buffer (2x) (2x concentrated, dilute before use). One vial contains 60 ml Dilution Buffer.
d. Additive (lyophilized) (Reconstitute and dilute before use). Five vials, each contains 2.5 ml lyophilized Additive. Shelf life of reconstituted additive: until expiry date at -20°C.
e. Two vials, each contains 10 ml Demineralized Water.
f. Washing Fluid (200x) (200x concentrated, dilute before use). One vial contains 60 ml washing Fluid.
g. Positive Control (Ready-to-use) - One vial contains 0.6 ml Positive Control.
h. Weak Positive Control (Ready-to-use) - One vial contains 0.6 ml Weak Positive Control.
i. Negative Control (Ready-to-use) - One vial contains 0.6 ml Negative Control.

j. Chromogen (TMB) Substrate (Ready-to-use) One vial contains 60 ml Chromogen (TMB) Substrate.
k. Stop Solution (Ready-to-use) - One vial contains 60 ml Stop Solution.
l. Package Insert, 10 plate sealers
m. Certificate of Analysis

PD-FMD DIVA Kits contents

a. 96-well Nunc Maxisorp ELISA plates (Cat. No.442404): 10 plates
b. Freeze dried recombinant 3AB3 protein (~38 kD): Ten vials (Store at -200C)
c. Each vial is to be dissolved in 1ml of Carbonate-Bicarbonate coating buffer, pH 9.5 and then added to 4.5 ml of additional coating buffer. This reconstituted vial is sufficient for coating one 96-well immunoplates. After reconstitution the vial is to be used immediately without storing further.
d. Freeze–dried positive and negative control sera: One vial each (Store at -200C)
e. Each vial is to be dissolved in 160 µl of distilled water and then distributed into single use aliquots for storage at -200C, if to be consumed in more than a month's time or else store the entire reconstituted vial at 40C. Working dilution of 1:20 in diluent buffer (as appears in the protocol) for control serum is recommended.

ii. *E. coli* lysate: One vial (Store at -200C)

a. Each vial of *E.coli* lysate is to be reconstituted with 70 µl of PBS and stored at -200C.
b. Lapin Anti Bovine-HRP conjugate (DAKO; Cat. No.P0159): 35 µl (Store at 40C)
c. Skimmed milk powder (Merck; Cat. No.1.15363.0500): 8 gram
d. Chicken serum {Sera Laboratories International Ltd. (SLI); Cat. No. S-606-HSL}: 25 mL (Store at -200C)
e. Salts for PBS, pH 7.4
f. Tween-20: 5mL
g. Carbonate-Bicarbonate coating buffer (Sigma; Cat.No.C3041): 1 capsule (for preparing 100 mL of buffer)
h. Phosphate-Citrate substrate buffer (Sigma; Cat.No.P4809): 1 tablet (for preparing 100 mL of buffer)
i. OPD (Sigma; Cat.No.P1526-100G): 50 mg (Store at -200C)
j. 30 % H_2O_2 (Merck; Cat.No.1.07210.0250): 50 µl
k. 1M H_2SO_4: 70mL

Additional Material required

i. Plate Reader e.g. Multiscan EX or equivalent. The reader has to have an appropriate filter set to read the plates at 450 nm.
ii. Plate washer e.g. Tecan EIA Tray Washer or equivalent.

6. PROCEDURE
Prio CHECK FMDV NS ELISA

SOLUTIONS TO BE MADE IN ADVANCE Dilution buffer working solution

- Dilute Dilution Buffer (2x) (Component 3) 1/2 in demineralized water; e.g. for one Test Plate prepare 24 ml (add 12 ml Dilution Buffer (2x) to 12 ml demineralized water). Can be stored at 5±3°C for up to 24 hours.
- Equilibrate the vial to 22±3°C and reconstitute 1 the Additive (Component 4) with 2.5 ml Demineralized Water (Component 5). Can be stored at -20oC until expiry date.
- Dilute reconstituted additive 1/10 in dilution buffer working solution; e.g. for one Test Plate prepare 24 ml (add 2.4 ml reconstituted additive to 21.6 ml dilution buffer working solution). Unused ELISA buffer can be stored at 5±3°C for up to 24 hours.
- Conjugate dilution
- Dilute Conjugate (30x) (Component 2) 1/30 in ELISA buffer; e.g. for one plate prepare 12 ml (add 400 µl Conjugate (30x) to 11.6 ml ELISA buffer). The diluted conjugate must be prepared just before use.
- Dilute Washing Fluid (200x) (Component 6) 1/200 in demineralized water. The amount of Washing Fluid is sufficient to prepare a final volume of 12 liters washing solution. Stability of washing solution: 1 week stored at 22±3°C.
- Reconstitution of the lyophilized Additive should be performed as follows:
- Equilibrate the vial to 22±3°C.
  - ✓ With the vial in an upright position, tap the vial gently against the worktop to ensure that the content is on the bottom of the vial.
  - ✓ Carefully open the vial.
  - ✓ Add the specified amount of Demineralized Water (Component 5).
  - ✓ Replace the stopper on the vial and allow the lyophilized material to dissolve.
  - ✓ Gently agitate the vial so that any remaining dry material will be dissolved.
  - ✓ Allow the material to stand at least for 15 minutes at 22±3°C before use.
  - ✓ Mix gently and intermittently (formation of foam should be avoided).

b. DAY 1 (**incubation with test serum)**

- Dispense 80 µl ELISA buffer to all wells of the Test Plate (Component 1).
- Dispense 20 µl of Negative Control (Compo- nent 9) to wells A1 and B1.
- Dispense 20 µl of Weak Positive Control (Com- ponent 8) to wells C1 and D1.
- Dispense 20 µl of Positive Control (Compo- nent 7) to wells E1 and F1.
- Dispense 20 µl of test samples to the remaining wells.
- Seal the Test Plate using the enclosed plate sealers.
- Shake the Test Plate gently.
- Incubate overnight (16–18 hours) at 22±3℃.

c. DAY 2 incubation with conjugate

- Empty the Test Plate after the incubation period and wash the plate 6 times with 200 to 300 µl washing solution. Tap the plate firmly after the last washing step.
- Dispense 100 µl of diluted conjugate to all wells.
- Seal the Test Plate using the enclosed plate sealers.
- Incubate 60±5 minutes at 22±3^{O}C.

d. Incubation with chromogen (TMB) substrate

- Empty the Test Plate after the incubation period and wash the plate 6 times with 200 to 300 µl washing solution. Tap the plate firmly after the last washing step.
- Dispense 100 µl of Chromogen (TMB) Substrate (Component 10) to all wells.
- Incubate 20 minutes at 22±3oC.
- Add 100 µl of Stop Solution (Component 11) to all wells.
- Mix the content of the wells of the Test Plate prior to measuring.
- Note: Start the addition of Stop Solution 20 minutes after the first well was filled with Chromogen (TMB) Substrate. Add the Stop Solution in the same order and at the same pace as the Chromogen (TMB) Substrate was dispensed

e. PD-FMD DIVA ELISA

I. Dissolve the freeze dried recombinant protein in the vial with 1 ml of coating buffer and add another 4.5 ml of coating buffer to it (One vial is sufficient for coating one 96-well ELISA plates). The reconstituted protein vial should be used for coating immediately without storing. Reconstitute the freeze dried vials of positive (one month postinfection serum) and negative (one month post vaccination serum) control serum with distilled water as appears on the sticker and store at – 20^0C after reconstitution in single use aliquots. Dissolve the content of freeze dried vial carrying *E. coli* lysate in 70 µl of PBS and store at – 20^0C after use.

II. Coat 96-well polystyrene (Nunc Maxisorp) Immuno plates with the diluted recombinant protein @ 50 µl per well (~50 ng of purified recombinant protein per well). Tap the plate gently from all sides and incubate (refrigerate) the plate at 40C for overnight.

III. Remove the plates from the refrigerator and thaw them at 370C for 15 minutes.

IV. In a low protein binding Perspex plate dilute the test and the supplied negative and positive control sera @ 1:20 in diluent buffer. Only serum from bovine host is compatible with this test. Prepare a total volume of 220 µl of diluted serum so that 100 µl of the mixture can be transferred to the coated ELISA plates in duplicate. On a coated ELISA plate a total of 45 test sera samples, one positive and one negative control serum and two background controls can be accommodated. For background controls only 100 µl of diluent buffer is dispensed without any serum.

V. Give three continuous wash (no hold time) with wash buffer.

VI. Transfer 100 µl of the serum and diluent buffer mixture from Perspex plate to the ELISA plate in duplicate wells. Incubate for 1 hour at 370C and tap the plate gently from all sides at every 15 minute intervals or incubate for 30 minutes in a plate shaker at 370C with 20-30 rpm.

VII. Give three washes of 3 minute soak period each.

VIII. Dispense anti-bovine-HRP conjugate diluted in the diluent buffer (1:2000) @ 50 µl per well. Incubate for 1 hour at 370C and tap the plate gently from all sides at every 15 minute intervals or incubate for 30 minutes in a plate shaker at 370C with 20-30 rpm. 6ml of diluted conjugate solution is sufficient for an ELISA plate.

IX. Give three washes of 5 minute soak period each.

X. Add freshly prepared substrate solution @ 50 µl per well and incubate for 15 minutes at 37^0C without shaking. Then stop the colour reaction by adding 1M

H2SO4 @ 50 µl per well.

XI. Measure the optical density values at wave length of 492 nm (Reference 620 nm).

7. RESULT INTERPRETATION

a. The PrioCHECK FMDV NS ELISA

- Measure the optical density (OD) of the wells 450 nm within 15 minutes after color development has been stopped.
- Calculate the mean OD_{450} value of wells A1 and B1 (Negative Control = OD_{450} max).
- The percentage inhibition (PI) of the Controls and the test sera are calculated according to the formula below.
- The OD_{450} values of all samples are expressed as

Percentage Inhibition (PI) relative to the OD_{450} max.

$$PI = 100 - \left| \frac{OD_{450}\ \text{test sample}}{OD_{450}\ \text{max}} \right| \times 100$$

If the OD450 of a test sample is higher than the OD450 max, the Percent Inhibition can be interpreted as 0%. **Interpretation of the percent inhibition**

b. PD-FMD DIVA

- To reduce inter-run variation due to differences in absolute absorbance between runs, final results for each test serum needs to be expressed as the PP value {(test serum sample mean OD/positive control serum mean OD) x 100 i.e., percent positivity value or PP value $\geq$ 40%}, calculated by dividing the reaction of the test serum by that of the positive control serum and then multiplying with 100. The results should be interpreted based on the following cut-off zones.

1. Precaution /Limitation of Procedure

If the mean OD450 of the Negative Control is below 1.000 possibly the Chromogen (TMB) Substrate is too cold.
In that case warm the solution to 22±3°C or incubate up to 30 minutes. If the mean OD450 of the Negative Control is above 2.000 a shorter incubation period with
Chromogen (TMB) Substrate is recommended.

2. Reporting

2.1. The PrioCHECK FMDV NS ELISA
PI = <50% (negative)
Antibodies against the NS protein of FMDV are absent in the test sample.
PI = 50% (positive)
Antibodies against the NS protein of FMDV are pre- sent in the test sample.

Quality Control

2.2. PrioCHECK FMDV NS ELISA
- The OD450 max (mean OD450 of the Negative Control) must be >1.000.
- The mean percentage inhibition of the Weak Positive Control must be >50%.
- The mean percentage inhibition of the Positive Control must be >70%.
- Not meeting any of these criteria is reason to discard the results of that specific Test Plate.

2.3. PD-FMD DIVA - the test is to be considered valid provided the mean absorbance of the positive control wells is not less than 0.8. Likewise the plate has to be rejected if the mean absorbance of the supplied negative control serum is > 0.3. The O.D. in back ground control wells should be less than 0.1.

2.4. PD-FMD ELISA

- 3AB3 NSP reactivity positive: If PP value is more than 40%
- 3AB3 NSP reactivity negative: If PP value is less than 40%

8. WASTE DISPOSAL

All sample leftover materials are disinfected with 2% Virkon solution and other infectious materials are autoclaved**.**

9. RISK ASSESSMENT

9.1 Handle all reagents and samples as bio-hazardous material.
9.2 Keep all reagents away from skin and eyes. If exposure should occur, immediately flush affected areas with cold water.
9.3 Wash solution, control sera, test plates, field samples and all other test kit reagents should be properly decontaminated with bleach or other strong oxidizing agent before disposal
9.4 Take special care not to contaminate any of the test re-agents with serum or bacterial agents.
9.5 Humidity indicators are supplied with each plate. If any of the indicators exhibit a pink color, the plate may be com- promised in some way; decontaminate (i.e. wash the plate with bleach solution) and dispose of the plate.
9.6 The best results are achieved by following the protocols as they are described below, using good, safe laboratory techniques.
9.7 Do not use this kit after the expiration date.
9.8 Never pipette by mouth. Allow all reagents to come to room temperature before starting!

10. TROUBLESHOOTING

NA

11. REFERENCES

- Sørensen K.J., Madsen K.G., Madsen E.S., Salt J.S. Nqindi J. Mackay D.K.J. Differentiation of infection from vaccination in foot-and-mouth disease by the detection of antibodies to the non-structural proteins 3D, 3AB and 3ABC in ELISA using antigens expressed in baculovirus. Arch Virol (1998) 143: 1461-1476

- Differentiation of Infected from Vaccinated Animals (DIVA) ELISA (r3AB3 NSP based), central FMD laboratory, Project Directorate on FMD, Mukteswar

TITLE: ELISA test for Mycoplasma

PREPARED BY: Serology section

REVISED BY: Serology section

APPROVED BY: Head, LSU

DATE: 11.06.2018

1. INTRODUCTION

Mycoplasma galliseptum/synoviae infections in chickens and turkeys are characterized by respiratory rales, coughing, and nasal discharge and clinical signs are usually slow to develop in chickens. *Mycoplasma synoviae* (MS) and *Mycoplasma gallisepticum* (MG) infection of chickens is associated with acute to chronic infectious synovitis, air sacculitis and increased condemnation losses. MS may be vertically transmitted from infected hens to progeny or horizontally transmitted via the respiratory tract. MS serologic testing serum plate agglutination (SPA) and hemagglutination-inhibition (HI) test} and culture techniques are needed to confirm MS-negative and MS-infected chicken flocks.

2. PRINCIPLES

The assay is designed to measure antibody bonded to antigen coated plates. The principle of the test is as follows: Serum obtained from chickens or turkeys exposed to MG or MS antigens contain specific MG or MS antibodies. Serum, diluted in Dilution Buffer, is added to an antigen coated plate. Specific antibody in the serum forms an antibody-antigen complex with the antigen bound to the plate. After washing the plate, an affinity purified goat anti-chicken IgG (H+L) peroxidase conjugate is added to each well. The antibody-antigen complex remaining from the previous step binds with the conjugate. After a brief incubation period, the unbound conjugate is removed by a second wash step. Substrate, which contains a chromagen (ABTS), is added to each well. Chromagen color change (from clear to green- blue) occurs in the presence of the peroxidase enzyme. The relative intensity of color developed in 15 minutes (compared to controls) is directly proportional to the level of antibody in the serum. After the substrate has incubated, Stop Solution is added to each well to terminate the reaction and the plate is read using an ELISA plate reader at 405-410 nm.

3. APPLICATION

The kit is used to diagnose Mycoplasma infection in chicken and turkey.

4. OBJECTIVE

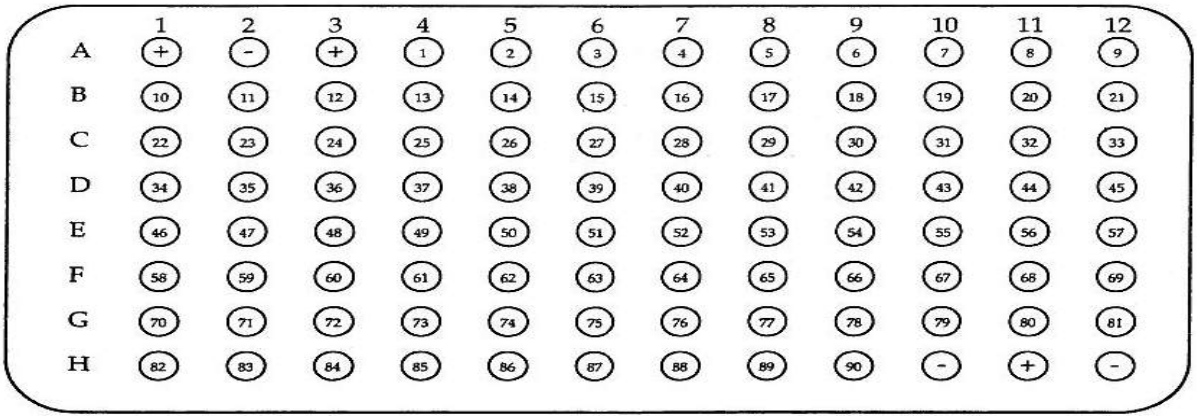
The Synbiotics' ProFLOK® MG/MS Combo ELISA kit is a presumptive *Mycoplasma gallisepticum (MG) and Mycoplasma synoviae (MS)* screening test for the detection of MG and MS antibodies in chicken and turkey serum.

5. APPARATUS/TEST KIT/REAGENTS

5.1 1 antigen coated plate (MGIMS)
5.2 10 µl Positive Control Serum (MGIMS)
5.3 10 µl Normal Control Serum (MGIMS)
5.4 100 µl Goat anti-Chicken 19G (H+L) Peroxidase Conjugate Solution (MGIMS)
5.5 40 mL Dilution Buffer Plus
5.6 10 mL ABTS-Hydrogen Peroxide Substrate Solution
5.7 2.5 mL 5X Stop Solution, 5% SDS (dilute [1:5] with laboratory grade water)
5.8 20 mL 20X Wash Solution (dilute [1:20) with laboratory grade water)
5.9 High precision pipette (i.e. 1-20 microliter pipette)
5.10 0.2 ml., 1.0 mL, and 5.0 mL pipettes
5.11 8 or 12 channel pipette (or transplating device) and pipette tips
5.12 2 graduated cylinders (50 mL)
5.13 1 mL or 5 mL borosilicate glass test tubes
5.14 Uncoated low binding 96 well test plates (i.e. Nunc catalog #269620)
5.15 Laboratory grade (distilled or R.O.) water
5.16 96 well plate reading spectrophotometer with 405-410 nm filter
5.17 Plate washing apparatus
5.18 Waste container with bleach or other oxidizing agent

6. PREPARATION OF REAGENTS AND SOLUTIONS

Dilute serum samples using Dilution Buffer in a clean, uncoated 96 well microtiter plate. Frozen serum samples should be completely thawed and thoroughly mixed before diluting. Set up samples and controls as shown in Figure 1.



6.1 Add 300 µl, Dilution Buffer to each well of an uncoated 96 well microtiter plate. This plate is referred to as the serum dilution plate.
6.2 Add 6 µl unknown serum per well as per Figure 1 (producing a 1:50 dilution). Start with well A4 and end with well H9 (moving left to right, row by row of wells). For example, wells 1 through 30 contain the diluted sera of flock 1 and wells 31-60 contain the diluted sera of flock 2, etc.
6.3 Add 6 µl of Normal Control Serum (producing a 1:50 dilution) to wells A2, H10, and H12.

6.4 Aspirate and remove any liquid in dilution plate wells AI, A3, and H11.
6.5 Allow all diluted serums to equilibrate in Dilution Buffer for 5 minutes before transferring to an antigen coated ELISA plate.
6.6 Diluted serum should be tested within 24 hours. This dilution format provides adequate quantities of diluted serum samples to conduct three additional ProFLOK® ELISA tests using the same serum dilution plate.

A. Preparation of Positive Control
A Positive Control Serum for each agent has been provided with this kit. Dilute the appropriate volume of the appropriate Positive Control Serum with Dilution Buffer (1:50) in a clean, glass test tube. For example, dilute 6 μl. of positive control serum in 300 μl. Dilution Buffer. Mix well. 150 μl, of diluted Positive Control is needed per ELISA plate.

B. Preparation of Conjugate Solution
The horseradish peroxidase conjugated anti-chicken IgG (H+L) is supplied in HRP stabilizer. Dilute 100 μl, stock conjugate in 10 mL Dilution Buffer (1:100 dilutions). Mix well. This 10 mL preparation will supply sufficient conju- gate for one 96 well ELISA plate.

C. Preparation of 1X Wash Solution
Dilute 20 mL concentrated Wash Solution in 380 mL laboratory grade (distilled or R.O.) water (1:20). Mix well. Approximately 400 mL Wash Solution is needed for each 96 well ELISA plate.

D. Preparation of the Substrate Solution
The Substrate Solution is ready to use. Each plate will re- quire approximately 10 mL substrate solution. For best results, the substrate solution must be equilibrated to room temperature before use.

E. Preparation of 1X Stop Solution
Dilute 2.5 mL concentrated Stop Solution in 10 mL laboratory grade (distilled or R.O.) water (1:5). Mix well. Approximately 12.5 mL Stop Solution is needed for each 96 well ELISA plate.

F. TEST PROCEDURE
- Remove an antigen coated test plate from the protective bag and label according to the dilution plate identification.
- Add 50 μl, Dilution Buffer to all wells on the test plate.
- Add 50 μl, diluted Positive Control Serum to wells A1, A3, and H11. Discard pipette tip.
- Using an 8 or 12 channel pipette transfer 50 μ*l/well* of each of the diluted serum samples and Normal Control Serum samples from the dilution plate to the corresponding wells of the coated test plate (yields a 1: 100 dilution. Discard pipette tips after each row of sample is transferred of samples to the

ELISA plate should be done as quickly as possible.

- Incubate plate for 30 minutes at room temperature.
- Tap out liquid from each well into an appropriate vessel containing bleach or other decontamination agent.
- Using an 8 or 12 channel pipette (or comparable automatic washing device), fill each well with approximately 300 µl, Wash Solution. Allow to soak in wells for 3 minutes; then discard contents into an appropriate waste container (waste container should contain bleach solution). Tap in- verted plate to ensure that all residual liquid is removed.
- Repeat wash procedure 2 more times.
- Using an 8 or 12 channel pipette (or transplating device) dispense 100 µl, diluted conjugate (prepared as described above) into each assay well. Discard pipette tips.
- Incubate for 30 minutes at room temperature. Wash as in steps above.
- Using an 8 or 12 channel pipette (or transplating device) dispense 100 µl, diluted Substrate Solution (prepared as described above) into each test well. Discard pipette tips.
- Incubate 15 minutes at room temperature.
- Using an 8 or 12 channel pipette (or transplating device) dispense 100 µl, diluted Stop Solution (prepared as de- scribed above) into each test well.
- Allow bubbles to dissipate before reading plate.
- Read the plate using an ELISA plate reader set at 405-410 run. Be sure to blank the reader as directed.

9 RESULT INTERPRETATION

9.1 Calculate the average Positive Control Serum absorbance (Optical Density [O.D.]) using the absorbance values of wells AI, A3, and HI1. Calculate the Normal Control Serum absorbance using values obtained from wells A2, H10, and H12. Record both averages.

9.2 Subtract the average normal control absorbance from the average positive absorbance. The difference is the Corrected Positive Control

9.3 Calculate a sample to positive (Sp) ratio by subtracting the average normal control absorbance from each sample absorbance. The difference is divided by the corrected positive control Use the following equation format:

$$SP = \frac{(\text{SAMPLE ABSORBANCE} - \text{AVERAGE NORMAL CONTROL ABSORBANCE})}{\text{CORRECTE POSITIVE CONTROL ABSORBANCE}}$$

An ELISA titer for MGIMS can be calculated by the following suggested equation:

LOG10 TITER = (1.464 x LOGIO Sp) + 3.197
TITER = ANTILOG of LOGlo TITER

Example:

1. Example Positive Control Absorbance: 0.585, 0.610, 0.590
Average = (0.585 + 0.610 + 0.590) 13 = 0.595
2. Example Normal Controls: 0.078, 0.067, 0.057
Average = (0.078 + 0.067 + 0.057) 13 = 0.067 (0.595) - (0.067) = 0.528
Example Sp value calculation: Absorbance of sample = 0.560
(0.560) - (0.067) / 0.528 = 0.934
Example of Calculation of titer using the Sp from above:
LogTiter = 1.464 x (Log10 0.934) + 3.197
Titer = ANTILOG 3.15
Titer = 1413

10 WASTE DISPOSAL

Consider as infectious materials and dispose the samples and kits properly after the test.

11 RISK ASSESSMENT

11.1 Handle all reagents and samples as bio-hazardous material.
11.2 Keep all reagents away from skin and eyes. If exposure should occur, immediately flush affected areas with cold water.
11.3 Wash solution, control sera, test plates, field samples and all other test kit reagents should be properly decontami-nated with bleach or other strong oxidizing agent before disposal
11.4 Take special care not to contaminate any of the test re-agents with serum or bacterial agents.
11.5 Humidity indicators are supplied with each plate. If any of the indicators exhibit a pink color, the plate may be com- promised in some way; decontaminate (i.e. wash the plate with bleach solution) and dispose of the plate.
11.6 The best results are achieved by following the protocols as they are described below, using good, safe laboratory techniques.
11.7 Do not use this kit after the expiration date.
11.8 Never pipette by mouth. Allow all reagents to come to room temperature before starting.

12 TROUBLESHOOTING

NA

13 REFERENCES

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Kleven, S.H. and H.W. Yoder, Jr. Mycoplasmosis, In: *A Laboratory Manual for the Isolation and Identification of Avian Pathogens,* 3rd ed., American Association for Avian Pathologists. Kendall/Hunt Publishing Company, Dubuque, Iowa. pp.57-62. 1989.

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Schwartz, L.D., *Mycoplasma synoviae.* In: *Poultry Health Handbook.* 2nd ed.,The Pennsylvania State University. University Park, Pennsylvania. pp. 64-65. 1977.

TITLE: LPB-ELISA for FMD

PREPARED BY: Serology section

REVISED BY: Serology section

APPROVED BY: Head, LSU

DATE: 11.06.2018

1. INTRODUCTION

Foot and mouth disease (FMD) is caused by a virus of the genus Aphthovirus, family Picornaviridae. There are seven serotypes of FMD virus (FMDV), namely O, A, C, SAT 1, SAT 2, SAT 3, and Asia 1, that infect cloven- hoofed animals. Infection with any one serotype does not confer immunity against another. Of the domesticated species, cattle, pigs, sheep, goats and water buffalo are susceptible to FMD. Many species of cloven-hoofed wildlife may become infected, and the virus has occasionally been recovered from other species as well. Infection of susceptible animals with FMDV can lead to the appearance of vesicles on the feet, in and around the oral cavity, and on the mammary glands of females. The vesicles rupture and then heal whilst coronary band lesions may give rise to growth arrest lines that grow down the side of the hoof. The age of lesions can be estimated from these changes as they provide an indicator of the time since infection

Within serotypes, many strains can be identified by biochemical and immunological tests. Virus neutralization tests (VNTs) and ELISAs for antibodies to structural proteins are used as serotype-specific serological tests. VNTs depend on tissue cultures and are therefore more prone to variability than ELISAs; they are also slower and subject to contamination. ELISAs for detection of antibodies have the advantage of being faster, and are not dependent on cell cultures. The ELISA can be performed with inactivated antigens, thus requiring less restrictive bio-containment facilities. The liquid phase blocking ELISA is a technique for the detection of FMDV antibodies in serum. The assay can be used as a single dilution screening assay, and also as a quantitative titration assay, resulting in an end-point titre determination for each serum

2. TEST PRINCIPLES

2.1 PD-FMD

A serial two fold dilutions of test serum are mixed with equal volume of a constant dose of viral inactivated antigens in a liquid medium and allowed to react overnight at 4°C. The antigen-antibody reaction is carried out in a suspension (or liquid medium), and the antigen is blocked by the homologous antibodies, if present, in the test serum for subsequent detection by guinea pig serum. Next day, the antigens which are not completely blocked by the antibodies in the test serum are trapped to the wells of the ELISA plates by the pre-coated type-specific rabbit antibodies. Antigen and background controls are added containing antigen without serum and blocking buffer respectively as described in plate

layout. Subsequently, the presence of antigen is traced by adding pre-titrated guinea pig (type-specific) serum and anti-guinea pig-HRPO conjugate and substrate reaction is followed in a standard ELISA procedure. Color reaction is stopped and measured in terms of optical density (OD) at 492 nm wavelength with reference to 620nm. OD of reference antigen controls are used for the calculation of 50% inhibition in OD for calculation of the titers.

2.2 BDSL, WRL

The test is based upon specific blocking of the FMDV antigen in liquid phase by antibodies in the test serum sample. Rabbit antisera specific for the different serotypes of FMDV are passively adsorbed to polystyrene microwells. After the test serum is allowed to react with the specific FMDV antigen, the test serum/antigen mixture is then transferred to an ELISA plate coated with FMDV trapping antibodies. The presence of antibodies to FMDV in the serum sample will result in the formation of immune complexes and consequently reduce the amount of free antigen trapped by the immobilized rabbit antisera. In turn, fewer guinea pig anti-FMDV detecting antibodies will react in the next incubation step. After the addition of enzyme-labelled (HRP) anti-guinea pig Ig conjugate and substrate/chromogen solution, a reduction in colour development will be observed when compared to controls containing free antigen only.

3. APPLICATION

The kit is used for serotyping of FMD in animals.

4. OBJECTIVE

To describe the procedures for serotyping of FMD in animals.

5. APPARATUS/TEST KIT/REAGENTS

5.1 96-well Nunc Maxisorp ELISA plates (cat. No. 442404)
5.2 Freeze dried Anti-FMDV coating serum: Type specific anti-146S FMDV serum, raised in rabbits.
5.3 Freeze dried Anti-FMDV tracing serum: type specific anti-146S FMV serum, raised in guinea pig.
5.4 Freeze dried inactivated Type O, A, and Asia1 antigens.
5.5 Anti-guinea pig conjugate: Rabbit/ goat anti-guinea pig immunoglobulin HRPO conjugate (DAKO)
5.6 Chromogen: OPD (Orthophenylenediamine dihydrochloride, Sigma)
5.7 Hydrogen peroxide (H_2O_2)
5.8 Phosphate buffer saline vials (Sigma)
5.9 Substrate buffer capsules
5.10 Coating buffer tablets
5.11 Tween-20

Materials needed but not provided:

- Precision pipettes: multi channel pipettes variable range from 50 to 200µl & Single channel pipettes variable range from 1 to 20µl, 20 to 100µl, 50 to 200µl and 200 to 1000µl along with disposable tips.
- Distilled water

- H_2SO_4
- Wash bottle fitted with Immunowasher
- 1 container: 1 to 2 liters
- ELISA plate reader, 492 nm filter.
- Photometer: Multiscan type Micro pate ELISA reader with an interference filter of 492nm and reference filter of 620nm.
- Refrigerator: range of +2ºC to +6ºC
- Freezer: range of -15 to -20ºC.
- Incubator: warm wall incubator maintained at +35ºC to 37ºC.
- pH meter: with accuracy of 0.01pH units or good quality pH strips with varying range from 2 to 10 can be used.
- Glassware/ plastic ware: flasks (50-5000ml), graduated cylinders (10-2000ml), graduated pipettes (1-100ml).
- Weighing balance

6. PROCEDURE

6.1 PD-FMD

Coating: Coat the individual plates with type-specific (O, A, Asia1) rabbit (dilute the serum as specified in the serum vial) serum in coating buffer (Carbonate-Bicarbonate buffer, pH 9.6). Dispense 50µl of diluted serum to each wells of ELISA plate. Tap gently for even distribution of coating solution to wells and cover with lid. Incubate at 37°C for 1 hr followed by incubation at 4ºC overnight.

Preparation of Ag-Ab mixture: Prepare two-fold dilutions (4 dilutions starting from 1:16) of serum samples in a low binding Perspex plate with PBS containing 0.05% Tween-20. Distribute 75 µl each dilutions of the serum sample to four separate (labeled O, A, and Asia1) Perspex plates. Dilute the antigen as specified in the antigen batch provided. Add equal volume of diluted antigen (75µl) to all the distributed serum samples. For antigen control, add equal volume of PBS-Tween 20 (0.05%) to the already diluted antigen. The dilution of each antigen and antibody in this step will be strictly made with PBS containing tween-20 (not the PBS containing 0.1% tween-20 as used for washing of the plate). Incubate the diluted antigen and antibody mixture at 4ºC overnight without disturbance.

Washing: Wash the plate by adding washing buffer using immune-wash and discard the contents of wells by abrupt downward hand motion. Repeat the washing 3 times with 5 minutes of hold period between each wash. Slap the inverted microplate 3-4 times onto a dust free absorbent pad to remove all residual contents in the wells.

Dispensing of antigen-antibody (Ag-Ab) mixture: Dispense 50µl of the overnight incubated Ag-Ab mixture in duplicate to the ELISA plates as instructed in the plate lay out. Incubate the plates at 37°C for 1 hr.

Wash the plate as in the step 3.

Addition of tracing antibodies: Dilute the guinea pig serum, as specified in the serum vials, in blocking buffer. Add the 50µl of diluted tracing serum to each type-specific plate. Incubate at 37°C for 1 hr. Wash the plate as in the step 2.

Conjugate: Add the anti-guinea pig-HRPO conjugate diluted in blocking buffer to all the wells. Incubate at 37°C for 1 hr. Wash the plate as in step 2.

Substrate reaction: Add freshly prepared substrate solution to the plate and keep in the incubator at 37°C for 15 minutes.
Stop the color reaction by adding 50µl of stopping solution. Read the plate at 492 nm.

6.2 **BDSL, WRL**
Coating of Microplates

- Gently agitate the contents of vials of trapping rabbit antibody stock (FMDV serotypes O, A, C, SAT 1, SAT 2, SAT 3 and/or Asia 1).
- Prepare a 1:1000 working dilution of trapping antibody stock in coating buffer in a volume sufficient for the number of plates required (5 ml per plate plus an additional 1 ml, e.g. 6 µl of rabbit antibody stock in 6 ml of coating buffer) and agitate gently to ensure uniform dispersion.
- For the serotype(s) being tested, immediately dispense 50 µl volumes of the working dilution of the respective trapping antibody into all 96 wells of appropriately labelled polystyrene microplate(s) (**NUNC Maxisorp**). Make sure that all the plates are aligned correctly (with the letters on the left hand side), as the ELISA reader will only accept the plates in one position. Tap the sides of the microplates to ensure that the trapping antibody is evenly distributed over the bottom of each well.
- Cover or seal the microplates and incubate at +1 to 8^{o}C.
- Return the remainder of the aliquot of the FMDV serotype O, A, C, SAT 1, SAT 2, and SAT 3 and/or Asia 1 trapping rabbit antibody stock to 1 to 8^{o}C.

Test and Control Serum Incubation (Liquid phase)
SCREENING ASSAY

- Agitate the test and control sera gently to ensure homogeneity.
- Test and control sera will be added to the wells of the **polypropylene** U-bottom microplate at a dilution of 1/16.
- First, prepare a 1/16 dilution of each control and test sera in suitable dilution tubes (e.g. for each control serum (C++, C+ and C-, add 15 µl of an undiluted control bovine serum to 225 µl of Diluent Buffer **A**; for each test serum, add 10 µl of undiluted serum to 150 µl of Diluent Buffer **A**). Again, gently agitate these dilutions to ensure homogeneity. **These volumes are sufficient for 1 microplate; volumes will need to be increased according to the number of microplates being run in the assay**.
- Return the undiluted test sera and the remainder of the undiluted control sera stocks to +1 to 8^{o}C for future use.
- According to the **Plate Layout #1** (**Appendix 1**) add 50 µl volumes of prediluted test and control sera to the wells of a **polypropylene** U-bottom microplate. Add 50 µl of Diluent Buffer **A** to the antigen control (Ca) wells.
- Record details and position of test sera on the **ELISA Data Sheet** (**Appendix 3**).

TITRATION ASSAY

Two-fold dilution range (for evaluation of positive sera)

- First prepare a 1/16 dilution of the control sera as in **5.2.1**. Next, prepare a 1/8 dilution of test sera in suitable dilution tubes or microplates (e.g. for each test serum add 20 µl of undiluted serum to 140 µl of Diluent Buffer **A**). **These volumes are sufficient for 1 microplate; volumes will need to be increased according to the number of microplates being run in the assay**. For each run and for each FMDV serotype the C++ control should be additionally titrated in the same manner as a test serum.
- Dispense 50 µl of Diluent Buffer **A** to all columns 3-12 for each **polypropylene** U-bottom microplate. According to **Plate Layout #2** (**Appendix 2**), add 50 µl volumes of the prediluted control sera to the appropriate wells. Add 50 µl of Diluent Buffer **A** to the antigen control (Ca) wells.
- According to the **Plate Layout #2**, add 50 µl volumes of prediluted test serum and the C++ control to the appropriate wells of row A or E, columns 3-12. Carefully mix the 100 µl contents in the well by filling and emptying the pipette tip several times, taking care not to introduce air bubbles. This will result in a 100 µl volume of a 1/16 test serum dilution. Transfer 50 µl of this dilution to the next row (A to B) and carefully mix the contents of row B. This will result in a doubling dilution. Transfer 50 µl from row B into the next row (B to C) and repeat the mixing procedure. Transfer 50 µl from row C to row D, mix carefully and finally discard 50 µl of this dilution. This will result in a test sample dilution series from 1/16 to 1/128 in 50 µl volumes. For the next test sample, repeat the above procedure for rows E through H using new pipette tips.
- Record details and position of test sera on the **ELISA Data Sheet** (**Appendix 4**).

Five-fold dilution range (for evaluation of post-vaccinal sera)

- Firstly, prepare a 1/16 dilution of the control sera as in 5.2.1 but for each control serum, add
- 20 µl of an undiluted control serum to 300 µl of Diluent Buffer **A**. According to **Plate Layout #2** (**Appendix 2**), add 60 µl volumes of the prediluted control sera to the appropriate wells of the **polypropylene** microplates. Add 60 µl of Diluent Buffer **A** to the antigen control (Ca) wells.
- Secondly, prepare a 1/5 dilution of each test serum (and the C++ control) by adding 20 µl of undiluted serum to 80 µl of Diluent Buffer **A**.
- Next, add 60 µl of Diluent Buffer **A** to all columns 3-12 in each **polypropylene** microplate. According to **Plate Layout #2** (**Appendix 2**), add 15 µl volumes of 1/5 diluted test serum (and the C++ control) to the appropriate wells of rows A or E, columns 3-12. This will result in a five-fold dilution. Carefully mix the 75 µl contents in the well by filling and emptying the pipette tip several times, taking care not to introduce air bubbles. This will result in a 75 µl volume of a 1/25 test serum dilution. Transfer 15 µl of this dilution from row A to B and carefully mix the contents of row B. Transfer 15 µl of this dilution from row B to C and repeat the mixing procedure. Transfer 15 µl of this dilution from row C to D, mix carefully and finally discard 15 µl of the dilution from row D. This will result in a test sample dilution series from 1/25 to 1/3125 in 60 µl volumes. For the next test sample, repeat the above procedure for rows E through H using new pipette tips.
- Record details and position of test sera on the **ELISA Data Sheet** (**Appendix 4**).

Addition of FMDV Antigen

- Prepare a working dilution of the FMDV antigen (FMDV serotype O, A, C, SAT 1, SAT 2, SAT 3 and/or Asia 1) in Diluent Buffer **A** in a volume sufficient for the microplates used (5 ml per plate plus an additional 1 ml for two-fold dilution range plates or 6 ml per plate plus an additional 1 ml for five-fold dilution range plates).

Suggested working dilutions:

O_1 Manisa	n	1:x
A_{22} IRQ 24/64	n	1:x
C PHI 7/84	n	1:x
SAT 1 (105)	n	1:x
SAT 2 Eritrea	n	1:x
SAT 3 (309)	n	1:x
Asia 1 Shamir	n	1:x

- For evaluation of positive sera, add 50 µl of the antigen working dilution to all 96 wells of the respective **polypropylene** U-bottom microplate. All wells should now contain 100 µl total of a serum dilution and antigen. This will result in a final serum dilution of 1/32 to 1/256 in the titration assay.
- For evaluation of post-vaccinal sera, add 60 µl of the antigen working dilution to all 96 wells of the respective **polypropylene** U-bottom microplate. All wells should now contain 120 µl total of a serum dilution and antigen. This will result in a final serum dilution of 1/50 to 1/6250 in the titration assay.
- Briefly place the microplates on an orbital shaker (or tap the sides of the microplates) to ensure thorough mixing.
- Incubate the **polypropylene** microplates at +1 to 8^{o}C overnight. Do not stack any more than four test plates in order to prevent thermal gradient formation. Seal the top plate or cover with an empty plate.
- **Return the remainder of the master stock antigens to -90 to -50^{o}C for further use.**

Transfer of Serum/Antigen Mixture to the ELISA Plate

- By inverting the microplate and using an abrupt downward hand motion, discharge the contents of all antibody coated microplates (**NUNC Maxisorp**) into a sink or other reservoir and slap the inverted microplates onto a lint-free absorbent towel to remove all residual contents. Using the Handiwash or equivalent, fill all 96 wells of all microplates with wash buffer. After filling, again discharge the contents of the microplates and slap the inverted microplates onto a lint-free absorbent towel to remove all residual contents. Repeat with two more wash cycles of filling and emptying.
- After three complete wash cycles and ensuring that no residual contents are left in the microplates, immediately transfer 50 µl volumes of the serum/antigen mixture from the **polypropylene** U-bottom microplates to the appropriate wells of the

different polystyrene microplates (**Nunc Maxisorp**) according to the same plate layout as being used for the "liquid phase".

- Cover or seal the microplates and place on an orbital shaker housed in a '+37°C' warm incubator or hot room and incubate at 35 to 39°C for 1 hour with continuous shaking.

Preparation of Diluent Buffer B

- M Phosphate Buffered Saline, pH 7.4.+/- 0.20 plus 0.05% Tween 20 plus 5% (w/v) Skimmed Milk Powder
- On the day of testing, calculate the amount of Diluent Buffer **B** required and prepare by adding skimmed milk powder to a final concentration of 5% (w/v) to the required volume of Diluent Buffer **A**.
- For example: to prepare 100 ml Diluent Buffer **B** add 5 gm of skimmed milk powder to
- 100 ml of Diluent Buffer **A**.
- Adjust the pH to pH 7.4 +/- 0.20 using 0.1 M NaOH.

Addition of Detecting Antibody

- Immediately before the end of the serum/antigen mixture incubation for the first plate, prepare a 1:100 working dilution from the homologous detecting antibody stock (anti-FMDV serotype O, A, C, SAT 1, SAT 2, SAT 3 and/or Asia 1) in Diluent Buffer **B** in a volume sufficient for the plates being used (5 ml of working dilution per plate plus an additional 1 ml, e.g. 60 µl of guinea pig antiserum stock in 6 ml Diluent Buffer **B**).
- Return the remainder of the detecting guinea pig antibody stocks to +1 to 8°C.
- After 1 hour of incubation, remove the microplates from the incubator and wash the microplates with wash buffer as described previously.
- For each serotype, immediately after washing, add 50 µl volumes of the working dilution of the detecting antibody (FMDV serotype O, A, C, SAT 1, SAT 2, SAT 3 and/or Asia 1) into all 96 wells of the respective microplates.
- Tap the sides of the microplates to ensure that the working dilution is evenly distributed over the bottom of each well.
- Cover or seal the microplates and place on an orbital plate shaker housed in a '+37°C' warm incubator or hot room at 35 to 39°C for 1 hour with continuous shaking.

Addition of Conjugate

- Immediately before the end of the detecting antibody incubation, prepare a 1:200 working dilution of the conjugate in Diluent Buffer **B** in a volume sufficient for all microplates (5 ml of working dilution per plate plus an additional 1 ml, e.g. 30 µl of conjugate stock in 6 ml of Diluent Buffer **B**). Both the **conjugate stock** and its working dilution should be handled with care; agitation should be gentle but thorough.
- Return the remainder of the **conjugate stock** to -30 to -5°C.
- After 1 hour of incubation, remove microplates from the incubator and wash with wash buffer as described previously. Immediately after washing, add 50 µl volumes of the working dilution of conjugate into all 96 wells of each microplate. Tap the sides of the microplates to ensure that the conjugate working dilution is evenly distributed over the bottom of each well.

- Cover or seal the microplates and incubate for 1 hour at +35 to 39°C with continuous shaking.

Addition of Substrate/Chromogen and Stopping Solutions

- Immediately before the end of the conjugate incubation, prepare the substrate/chromogen solution in a volume sufficient for the number of plates being run (e.g. for 1 plate, dilute 30 µl of substrate stock (H_2O_2) in 6 ml of chromogen stock solution (OPD)). This represents
- 3.3 mM OPD and 4.4 mM H_2O_2. This final substrate/chromogen solution should be colourless and stored in the dark. If coloured, it should be discarded.
- A clean microplate (not coated with trapping antibody) will be used as the "blanking plate" for the photometric reading (this plate can be washed later and reused for blanking procedures in future tests).
- After 1 hour of conjugate incubation, wash the microplates as described previously. Ensure that all 96 wells of each microplate have been completely flooded with wash buffer to eliminate unreacted conjugate.
- Immediately after washing, add 50 µl volumes of the substrate/chromogen solution to the wells of the microplates, starting with the first column of the "blanking plate" followed by all 96 wells of the microplates in the test run. Immediately begin timing after filling the first wells and incubate at ambient temperature for 15 minutes without plate shaking.
- After 15 minutes of substrate/chromogen incubation, immediately add 50 µl volumes of the stopping solution (1.25 M sulphuric acid) starting with the first column of the "blanking plate" followed by all 96 wells of the microplates in the test run. Briefly place the microplates on the shaker (or tap the sides of the microplates) to ensure even mixing. All wells should now contain 50 µl of substrate/chromogen solution plus 50 µl of stopping solution.

Measurement of Substrate Development

- Place the "blanking plate" in the carriage of the photometer and initiate the blanking sequence.
- Place the first microplate of the test run in the carriage of the photometer and initiate the reading sequence. Repeat for each microplate.

7. RESULT INTERPRETATION and REPORTING

7.1 PD-FMD

- Performance of a test can be determined from positive reaction of the known antigens (approximately 1.0) employed in the test and a clear background reaction. The interpretation of the result should be done on the basis of the corrected OD value (OD of test well OD of background well).
- Percent reactivity against each serum dilution is calculated as follows:

$$\% \text{ Reactivity} = \frac{\text{Mean OD of test wells}}{\text{Mean OD of antigen control wells}} \times 100$$

- Titer of serum sample is expressed as the reciprocal of the serum dilution giving 50% optical density (OD) as compared to the antigen control or in other words, reciprocal of the serum dilution which inhibits 50% of the guinea pig serum binding to the homologous virus.

7.2 BDSL, WRL

Data Expression

Microplate readings will be used in two types of data analysis:

A) Percent Inhibition (PI) values which are used for Quality Assurance (QA) acceptance. These PI values are calculated as follows:

$$\textbf{PI} = 100 - \left(\frac{\text{Replicate OD of control}}{\text{Median OD of Ca}} \times 100\right)$$

B) Percentage Inhibition (PI) values which are used for acceptance of replicate values for test sera and diagnostic interpretation. These PI values are calculated as follows:

$$\textbf{PI} = 100 - \left(\frac{\text{Replicate OD of test serum}}{\text{Median OD of Ca}} \times 100\right)$$

Calculation and Acceptance of Control Data

The data expressed in OD values and PI values for the antigen control (Ca) and the data expressed in PI values for the three other controls (C++, C+ and C-) are used to determine whether or not the test has (been) performed within acceptable limits of variability and therefore, whether or not the test sera data may be accepted for any given microplate.

Given that the variation in OD values and PI values of the controls included with the kit should be normally distributed, Upper (UCL) and Lower Control Limits (LCL) for these controls have been established.

Precaution /Limitation of Procedure

- All the glass wares to be used should be clean and sterile.
- Slight drop in pH (less than 7.2) in washing buffer can adversely affect antigen antibody reaction. Ensure correct pH everday before use.
- Microbial contamination/ precipitation formation of any degree in any of the reagents/ buffer will very much reduce the specificity of the test.
- Mark the plate orientation properly with water proof marker before starting the test to avoid any confusion which may arise later.
- Do not stack plates one over other in incubator.
- Prepare fresh substrate solution every time.
- Do not store prepared buffers more than 1 month.
- Check pH of every buffer every day before starting the test.
- The chromogen (OPD) stock solution and H_2O_2 / OPD solution should be stored in the dark at all times.

Reporting

Diagnostic Interpretation of Test Sera Data

The diagnostic threshold for this assay is set at 50% inhibition (50 PI).

Screening assay

- If both replicate PI values of a test serum fall below 50 PI then that test serum is considered to be negative.
- If either or both replicate PI values of a test serum fall above 50 PI, then that test serum is **tentatively** considered to be positive and should be retested according to the titration assay procedure for both confirmation and estimation of antibody titre.

Two-fold dilution range

- If all replicate PI values of a test serum fall below 50 PI, then that test serum is considered to be negative.
- If one of the two replicate PI values of a test serum dilution of 1/32 fall above 50 PI but all replicate PI values at subsequent dilutions fall below 50 PI, then the titre of the test serum is 32 but is considered to be negative.
- If both of the replicate PI values of a test serum dilution of 1/32 fall above 50 PI and all replicate PI values at subsequent dilutions fall below 50 PI, then the titre of the test serum is 45 and is considered to be positive. This is the threshold titre that constitutes positivity.

- The titre of a test serum demonstrating PI values above 50 PI at dilutions greater than 1/32 can be assessed by reference to Table 2.
- An antibody titre of >90 indicates that the animal, at the time of bleeding, was protected against infection from the homologous antigen of the particular FMDV serotype.

Five-fold dilution range

- The titre of the test serum demonstrating replicate PI values above 50 PI can be assessed by reference to Table 3**.** An antibody titre of = or >112 indicates that the animal, at the time of bleeding, was protected against infection from the homologous antigen of the particular FMDV serotype. The higher the antibody titre, the greater is the confidence of protection.

Quality Control

1. PD-FMD

Performance of a test can be determined from positive reaction of the known antigens (approximately 1.0) employed in the test and a clear background reaction.

2. BDSL, WRL

Calculation and Acceptance of Control Data

- The data expressed in OD values and PI values for the antigen control (Ca) and the data expressed in PI values for the three other controls (C++, C+ and C-) are used to determine whether or not the test has (been) performed within acceptable limits of

variability and therefore, whether or not the test sera data may be accepted for any given microplate.

- Given that the variation in OD values and PI values of the controls included with the kit should be normally distributed, Upper (UCL) and Lower Control Limits (LCL) for these controls have been established.
- The controls should be examined in the following order:

First Level of Microplate Acceptance - Antigen Control (Ca)

- Before calculating PI values, compare the replicate OD values of the Ca control to the UCL and LCL. Both intermediate OD values (i.e. the two values remaining after discarding the lowest and highest values) must fall within these limits. If not, the plate should be rejected.
- Only the two intermediate OD values are used for the calculation of the median Ca OD value and used in subsequent PI calculations.

Second Level of Microplate Acceptance - Antigen Control (Ca), Strong Positive (C++), Moderate Positive (C+) and Negative (C-) Control Sera

- For the antigen control (Ca) and for each control serum (C++, C+ and C-), the replicate PI values should be calculated and recorded on the **ELISA DATA SHEET** (**Appendix 3 or 4**).

Antigen control (Ca)

- Compare the replicate PI values of the Ca controls to the UCL and LCL for the Ca and use the criteria in Table 1 to accept or reject each **individual** microplate.

Strong Positive, Moderate Positive and Negative Control Sera (C++, C+ and C-)

- Compare the corresponding replicate PI values to the UCL and LCL for the strong positive, moderate positive and negative control sera (C++, C+ and C-) and use the criteria in Table 1 to accept or reject each **individual** microplate.

Table 1. Ca, C++, C+ and C- Control Data

Replicate PI Value		**Status**
In*	**Out****	
4	0	Accept
3	1	Accept
2	2	Reject
1	3	Reject
0	4	Reject

*In Within UCL and LCL
*Out Outside UCL and LCL

- Microplate rejected if any one of the Ca, C++, C+ or C- controls fails to meet PI acceptance criteria.

8. WASTE DISPOSAL

Consider it as infectious agent and dispose properly following proper SOP.

9. RISK ASSESSMENT

9.1 Handle all reagents and samples as bio-hazardous material.
9.2 Keep all reagents away from skin and eyes. If exposure should occur, immediately flush affected areas with cold water.
9.3 Wash solution, control sera, test plates, field samples and all other test kit reagents should be properly decontami-nated with bleach or other strong oxidizing agent before disposal
9.4 Take special care not to contaminate any of the test re-agents with serum or bacterial agents.
9.5 Humidity indicators are supplied with each plate. If any of the indicators exhibit a pink color, the plate may be com- promised in some way; decontaminate (i.e. wash the plate with bleach solution) and dispose of the plate.
9.6 The best results are achieved by following the protocols as they are described below, using good, safe laboratory techniques.
9.7 Do not use this kit after the expiration date.
9.8 Never pipette by mouth. Allow all reagents to come to room temperature before starting!

10. TROUBLESHOOTING

NA

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Liquid Phase Blocking ELISA, central FMD laboratory, Project Directorate on FMD, Mukteswar

TITLE: ELISA for New Castle Disease

PREPARED BY: Serology section

REVISED BY: Serology section

APPROVED BY: Head, LSU

DATE: 11.06.2018

1. INTRODUCTION

Newcastle disease is an infection of domestic poultry and many other bird species with virulent Newcastle disease virus. NDV synonymous with avian paramyxovirus serotype 1 (PMV-1), is an RNA virus and the most important of the 9 known PMV serotypes as a pathogen for poultry. Newcastle Disease Virus (NDV) causes a range of disease states from mild respiratory disease to severe diarrhea and death. The severity of the disease is determined by the infecting strain of NDV. Highly pathogenic strains (Velogenic NDV) can cause swelling of the tissues around the eyes, diarrhoea and death within 8 days after exposure. Moderately pathogenic strains (Mesogenic NDV) produce acute respiratory tract infections and reductions in egg production. Milder strains (Lentogenic NDV) produce an in apparent respiratory infection.

NDV can be isolated from oropharyngeal or cloacal swabs or tissues from infected birds by inoculation of the allantoic cavity of 9 to 11 day old embryonated chicken eggs. Infection is confirmed by recovery of a hemagglutinating virus that is inhibited with NDV antiserum or by detection of NDV RNA by reverse transcriptase PCR. A rise in NDV antibody titer by hemagglutination-inhibition or ELISA of paired serum samples indicates NDV infection. Newcastle disease viruses, whether virulent field viruses or live vaccine, can produce a transitory conjunctivitis in humans, but the condition has been limited primarily to laboratory workers and vaccination teams exposed to large quantities of virus.

2. PRINCIPLES

Serum obtained from chickens or turkeys exposed to MG or MS antigens contain specific MG or MS antibodies. Serum, diluted in Dilution Buffer, is added to an antigen coated plate. Specific antibody in the serum forms an antibody- antigen complex with the antigen bound to the plate. After washing the plate, an affinity purified goat anti-chicken IgG (H+L) peroxidase conjugate is added to each well. The antibody-antigen complex remaining from the previous step binds with the conjugate. After a brief incubation period, the unbound conjugate is removed by a second wash step. Substrate, which contains a chromagen (ABTS), is added to each well. Chromogen color change (from clear to green- blue) occurs in the presence of the peroxidase enzyme. The relative intensity of color developed in 15 minutes (compared to controls) is directly proportional to the level of antibody in the serum. After the substrate has incubated, Stop Solution is added to each well to terminate the reaction and the plate is read using an ELISA plate reader at 405-410 nm

3. OBJECTIVE

3.1 Assessment of antibody levels in the bird to the NDV group antigen
3.2 To aid in the detection of pre and post-vaccination NDV antibody levels in chickens.

4. APPARATUS/TEST KIT

4.1 High precision pipette (i.e. 1-20 microliter pipette)
4.2 0.2 ml., 1.0 mL, and 5.0 mL pipettes
4.3 8 or 12 channel pipette (or transplating device) and pipette tips
4.4 2 graduated cylinders (50 mL)
4.5 1 mL or 5 mL borosilicate glass test tubes
4.6 Uncoated low binding 96 well test plates (i.e. Nunc catalog #269620)
4.7 Laboratory grade (distilled or R.O.) water
4.8 96 well plate reading spectrophotometer with 405-410 nm filter
4.9 Plate washing apparatus
4.10 Waste container with bleach or other oxidizing agent

5. REAGENTS, SOLUTION AND BUFFER

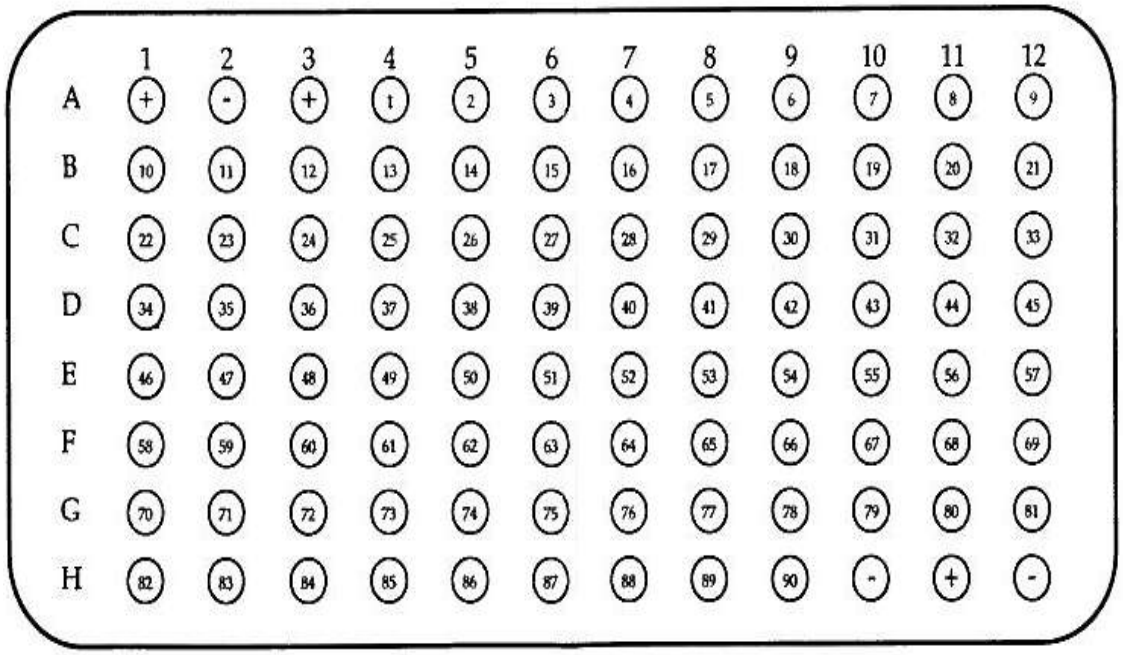
Reagents Required

5.1 1 antigen coated plate *(MGIMS)*
5.2 10 µl Positive Control Serum *(MGIMS)*
5.3 10 µl Normal Control Serum *(MGIMS)*
5.4 100 µl. Goat anti-Chicken 19G (H+L) Peroxidase Conjugate Solution *(MGIMS)*
5.5 40 mL Dilution Buffer Plus
5.6 10 mL ABTS-Hydrogen Peroxide Substrate Solution
5.7 2.5 mL 5X Stop Solution, 5% SDS (dilute [1:5] with laboratory grade water)
5.8 20 mL 20X Wash Solution (dilute [1:20) with laboratory grade water)

6. PROCEDURE

Sample Dilution Procedure

- Dilute serum samples using Dilution Buffer in a clean, uncoated 96 well microtiter plate. Frozen serum samples should be completely thawed and thoroughly mixed before diluting. Set up samples and controls as shown in Figure 1.



Preparation of the serum dilution plate

- Add 300 µl, Dilution Buffer to each well of an uncoated 96 well microtiter plate. This plate is referred to as the serum dilution plate.
- Add 6 µl unknown serum per well as per Figure 1 (producing a 1:50 dilution). Start with well A4 and end with well H9 (moving left to right, row by row of wells). For example, wells 1 through 30 contain the diluted sera of flock 1 and wells 31-60 contain the diluted sera of flock 2, etc.
- Add 6 µl of Normal Control Serum (producing a 1:50 dilution) to wells A2, H10, and H12.
- Aspirate and remove any liquid in dilution plate wells A1, A3, and H11.
- Allow all diluted serums to equilibrate in Dilution Buffer for 5 minutes before transferring to an antigen coated ELISA plate.
- Diluted serum should be tested within 24 hours. This dilution format provides adequate quantities of diluted serum samples to conduct three additional ProFLOK® ELISA tests using the same serum dilution plate.

Preparation of Positive Control

- A Positive Control Serum for each agent has been provided with this kit. Dilute the appropriate volume of the appropriate Positive Control Serum with Dilution Buffer (1:50) in a clean, glass test tube. For example, dilute 6 µl of positive control serum in 300 µl. Dilution Buffer. Mix well. 150 µl, of

diluted Positive Control is needed per ELISA plate.

Preparation of Conjugate Solution

- The horseradish peroxidase conjugated anti-chicken IgG (H+L) is supplied in HRP stabilizer. Dilute 100 µl, stock conjugate in 10 mL Dilution Buffer (1:100 dilutions). Mix well. This 10 mL preparation will supply sufficient conjugate for one 96 well ELISA plate.

Preparation of IX Wash Solution

- Dilute 20 mL concentrated Wash Solution in 380 mL laboratory grade (distilled or R.O.) water (1:20). Mix well. Approximately 400 mL Wash Solution is needed for each 96 well ELISA plate.

Preparation of the Substrate Solution

- The Substrate Solution is ready to use. Each plate will re- quire approximately 10 mL substrate solution. For best results, the substrate solution must be equilibrated to room temperature before use.

Preparation of 1X Stop Solution

- Dilute 2.5 mL concentrated Stop Solution in 10 mL laboratory grade (distilled or R.O.) water (1:5). Mix well. Approximately 12.5 mL Stop Solution is needed for each 96 well ELISA plate.

Elisa test procedure

Preparing the test plate

- Remove an antigen coated test plate from the protective bag and label according to the dilution plate identification.
- Add 50 µl, Dilution Buffer to all wells on the test plate.
- Add 50 µl, diluted Positive Control Serum to wells AI, A3, and HI1. Discard pipette tip.
- Using an 8 or 12 channel pipette transfer 50µl*/well* of each of the diluted serum samples and Normal Control Serum samples from the dilution plate to the corresponding wells of the coated test plate (yields a 1: 100 dilution. Discard pipette tips after each row of sample is transferred of samples to the ELISA plate should be done as quickly as possible.
- Incubate plate for 30 minutes at room temperature.

Wash procedure

- Tap out liquid from each well into an appropriate vessel containing bleach or other decontamination agent.
- Using an 8 or 12 channel pipette (or comparable automatic washing device), fill each well with approximately 300 µl, Wash Solution. Allow to soak in wells for 3 minutes; then discard contents into an appropriate waste container (waste container should contain bleach solution). Tap in- verted plate to ensure that all residual liquid is removed.
- Repeat wash procedure 2 more times.

Addition of anticonjugate IgG peroxidase conjugates, substrate and stop solution

- Using an 8 or 12 channel pipette (or transplating device) dispense 100 µl, diluted conjugate (prepared as described above) into each assay well. Discard pipette tips.
- Incubate for 30 minutes at room temperature. Wash as in steps above
- Using an 8 or 12 channel pipette (or transplating device) dispense 100 µl,

diluted Substrate Solution (prepared as described above) into each test well. Discard pipette tips.

- Incubate 15 minutes at room temperature.
- Using an 8 or 12 channel pipette (or transplating device) dispense 100 µl, diluted Stop Solution (prepared as described above) into each test well.
- Allow bubbles to dissipate before reading plate.
- Read the plate using an ELISA plate reader set at 405-410 run. Be sure to blank the reader as directed.

7. RESULT INTERPRETATION

7.1 Calculate the average Positive Control Serum absorbance (Optical Density [O.D.] using the absorbance values of wells A1, A3, and H11. Calculate the Normal Control Serum absorbance using values obtained from wells A2, H10, and H12. Record both averages.

7.2 Subtract the average normal control absorbance from the average positive absorbance. The difference is the Corrected Positive Control

7.3 Calculate a sample to positive (Sp) ratio by subtracting the average normal control absorbance from each sample absorbance. The difference is divided by the corrected positive control. Use the following equation format:

$$SP = \frac{\text{(SAMPLE ABSORBANCEHAVERAGE NORMAL CONTROL ABSORBANCE)}}{\text{CORRECTEOPOSITIVECONTROL ABSORBANCE}}$$

An NDV ELISA titer can be calculated by the following suggested equation:

LOG10 TITER = (1.464 X LOG 10 Sp) + 3.740
TITER = ANTILOG OF LOG10 TITER

Precaution/Limitation of procedure:

- High sensitivity and specificity: keeps assure of accurate test results
- Excellent reproducibility: consistent test results among batches and over time
- Long shelf life at 24 months for easy inventory management

Reporting

- A "0" NDV ELISA titer represents a chicken serum sample that contains an extremely low to insignificant NDV anti- body level compared to the NDV ELISA kit positive and nor- mal control sera.
- An NDV ELISA titer value above "0" indicates only that a chicken serum sample contains a significant and ELISA- detectable NDV antibody level compared to the NDV ELISA kit positive and normal control sera. However, these titers do not imply or ensure "protection" nor provide serologic differentiation between a vaccine response or NDV field infection.

Quality Control

- Assay Control Values: Valid NDV ELISA results are obtained when the average optical density (O.D.) value of the Normal Control Serum is less than 0.250 and

the Corrected Positive Control (CPC) value range is between 0.250 and 0.900.

- The NDV ELISA titer values obtained represent a comparison of the NDV antibody level within each field chicken serum tested and the NDV ELISA kit positive and normal control sera. Therefore, it is important to first determine that the NDV ELISA positive and normal control sera values obtained are valid as detailed above in the Quality Control section of this pamphlet before ELISA results are interpreted.

8. RISK ASSESSMENT

8.1 Handle all reagents and samples as bio-hazardous material.
8.2 Keep all reagents away from skin and eyes. If exposure should occur, immediately flush affected areas with cold water.
8.3 Wash solution, control sera, test plates, field samples and all other test kit reagents should be properly decontami-nated with bleach or other strong oxidizing agent before disposal
8.4 Take special care not to contaminate any of the test re-agents with serum or bacterial agents.
8.5 Humidity indicators are supplied with each plate. If any of the indicators exhibit a pink color, the plate may be com- promised in some way; decontaminate (i.e. wash the plate with bleach solution) and dispose of the plate.
8.6 The best results are achieved by following the protocols as they are described below, using good, safe laboratory techniques
8.7 Never pipette by mouth. Allow all reagents to come to room temperature before starting!
8.8 Store all reagents provided in the kit at 2-7°C. Reagents should not be frozen
8.9 Storage of 5X Stop Solution at refrigerated temperatures may cause the formation of a white solid. This does not affect product performance. Warm at room temperature or 37°C to dissolve before use

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TITLE: Sandwich ELISA for FMD

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1. INTRODUCTION

Foot and mouth disease (FMD) is caused by a virus of the genus Aphthovirus, family Picornaviridae. There are seven serotypes of FMD virus (FMDV), namely O, A, C, SAT 1, SAT 2, SAT 3, and Asia 1, that infect cloven- hoofed animals. Infection with any one serotype does not confer immunity against another. Of the domesticated species, cattle, pigs, sheep, goats and water buffalo are susceptible to FMD. Many species of cloven-hoofed wildlife may become infected, and the virus has occasionally been recovered from other species as well.

Infection of susceptible animals with FMDV can lead to the appearance of vesicles on the feet, in and around the oral cavity, and on the mammary glands of females. The vesicles rupture and then heal whilst coronary band lesions may give rise to growth arrest lines that grow down the side of the hoof. The age of lesions can be estimated from these changes as they provide an indicator of the time since infection

Within serotypes, many strains can be identified by biochemical and immunological tests. Virus neutralisation tests (VNTs) and ELISAs for antibodies to structural proteins are used as serotype-specific serological tests. VNTs depend on tissue cultures and are therefore more prone to variability than ELISAs; they are also slower and subject to contamination. ELISAs for detection of antibodies have the advantage of being faster, and are not dependent on cell cultures. The ELISA can be performed with inactivated antigens, thus requiring less restrictive bio-containment facilities. Sandwich Enzyme-linked immunosorbent assays (ELISA) can also be used to detect FMD viral antigens and for serotyping.

2. PRINCIPLES

Antigen capture sandwich ELISA for virus serotyping is highly sensitive method of virus detection and serotyping in clinical materials. In this test plates are coated with serotype specific rabbit polyclonal serum and viruses present in processed clinical samples are allowed to bind to capturing antibodies. Bound viruses are detected by serotype a specific tracing antibody which increases the specificity of the test. Reaction is developed by tracing antibody specific conjugated antibody and substrate solution.

3. APPLICATION

This test is employed for serotyping O, A Asia1

4. OBJECTIVE

To describe the procedures for serotyping FMD virus to O, A, Asia I

5. APPARATUS/TEST KIT/REAGENTS.

Kit Components

5.1 96-well Nunc Maxisorp ELISA plates (cat. No. 442404)
5.2 Freeze dried Anti-FMDV coating serum: Type specific anti-146S FMDV serum raised in rabbits.
5.3 Freeze dried Anti-FMDV tracing serum: type specific anti-146S FMV serum raised in guinea pig.
5.4 Anti-guinea pig conjugate: Rabbit/ goat anti-guinea pig immunoglobulin HRPO conjugate (DAKO)
5.5 FMDV inactivated antigens: sero-type specific inactivated freeze dried antigens
5.6 Chromogen: OPD (Orthophenylenediamine dihydrochloride, Sigma)
5.7 Hydrogen peroxide (30% H_2O_2 w/v)
5.8 Phosphate buffer saline vials (Sigma)
5.9 Substrate buffer capsules
5.10 Coating buffer tablets
5.11 Tween-20

Materials needed but not provided:

5.12 Precision pipettes: multi channel pipettes variable range from 50 to 200µl & Single channel pipettes variable range from 1 to 20µl, 20 to 100µl, 50 to 200µl and 200 to 1000µl along with disposable tips.
5.13 Distilled water
5.14 H2SO4
5.15 Wash bottle fitted with Immuno-washer
5.16 1 container: 1 to 2 liters
5.17 ELISA plate reader, 492 nm filter.
5.18 Photometer: Multiscan type Micro pate ELISA reader with an interference filter of 492nm and reference filter of 620nm.
5.19 Refrigerator: range of +2ºC to +6ºC
5.20 Freezer: range of -15 to -20ºC.
5.21 Incubator: warm wall incubator maintained at +35ºC to 37ºC.
5.22 pH meter: with accuracy of 0.01pH units or good quality pH strips with varying range from 2 to 10 can be used.
5.23 Glassware/ plastic ware: flasks (50-5000ml), graduated cylinders (10-2000ml), graduated pipettes (1-100ml).
5.24 Weighing balance

6. TEST PROCEDURE

6.1 **Coating of ELISA wells:** Dilute all the 4 coating serum, as suggested, with coating buffer sufficient for the number of plates to be used (2ml of working dilution per serum per plate). Dispense the diluted coating serum in 50µl volumes per well as indicated later in the plate layout. Gently tap the plates to ensure that

the liquid has covered the whole well area and incubate at 37ºC for 1 hour or 4ºC overnight. **Coating serum is diluted as per the batch wise dilution sheet.**

6.2 Wash the plate by adding washing buffer using immune-wash and discard the contents of wells by abrupt downward hand motion. Repeat the washing 3 times with 3-5 minutes of hold period between each wash. Slap the inverted microplate 3-4 times onto a dust free absorbent pad to remove all residual contents in the wells.

6.3 Dispense 50µl of test sample/ antigen per well according to plate layout. In background wells dispense 50µl of blocking buffer in place of antigen and in positive controls dispense 50µl of respective controls provided. Cover the plate with lid and incubate at 37ºC for 1 hour with intermittent gentle shaking followed by washing as described in step 2.

6.4 Dilute all the 4 tracing serum, as suggested, with blocking buffer sufficient for the number of plates required. Dispense 50µl volumes per well as indicated in plate layout.

6.5 Incubate the plates at 37ºC for 1 hour with intermittent gentle shaking followed by washing as described in step 2.

6.6 Prepare working dilution of conjugate, as suggested, in blocking buffer in sufficient volume. Dispense 50µl of diluted conjugate to all the wells of ELISA plate and incubate the plates for 1 hour followed by washing as described in step 2.

6.7 Prepare substrate solution, as described, and dispense 50µl of substrate solution to each well. Cover the plate with lid and incubate at 37ºC for 15 min in dark. Stop the color reaction by adding 50µl of stopping solution to each well.

6.8 Measure the optical density of each well at wavelength of 492nm and reference wavelength of 620nm in ELISA reader.

7. RESULT INTERPRETATION

7.1 Performance of a test can be determined from positive reaction of the known antigens employed in the test and a clear background reaction of all the serum. The interpretation of the result should be done on the basis of the corrected OD value (OD of test well OD of background well).

7.2 When a test is conducted properly, the background reaction of all the serum provided will lie between 0.02 to 0.08 OD with substrate.

7.3 If the reactivity (OD value) of the test antigen with a particular FMDV type serum is $\geq$ 0.10 (with no heterologous reactivity), then antigen in question can be identified as belonging to that type (OD limit 0-2.5).

8. WASTE DISPOSAL

Consider it as infectious agent and dispose all the materials properly after the test.

9. RISK ASSESSMENT

9.1 All the glass wares to be used should be clean and sterile.

9.2 Slight drop in pH (less than 7.2) in washing buffer can adversely affect antigen antibody reaction. Ensure correct pH every day before use.

9.3 Microbial contamination/ precipitation formation of any degree in any of the

reagents/ buffer will very much reduce the specificity of the test.

9.4 Mark the plate orientation properly with water proof marker before starting the test to avoid any confusion which may arise later.

9.5 Do not stack plates one over other in incubator.

9.6 Prepare fresh substrate solution every time.

9.7 Do not store prepared buffers more than 1 month.

9.8 Check pH of every buffer every day before starting the test.

10. TROUBLESHOOTING

NA

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TITLE: Rabies SERI ELISA

PREPARED BY: Serology section

REVISED BY: Serology section

APPROVED BY: Head, LSU

DATE: 11.06.2018

1. INTRODUCTION

Rabies is a fatal viral zoonotic disease of significant public and veterinary health threat. The causative agent of the disease, rabies virus (RABV), mainly infects warm-blooded vertebrates (both mammals and humans). The RABV infection is generally transmitted by the bite of infected animals, most commonly dogs and other wildlife carnivore species.

Rabies and rabies-related viruses are highly neurotropic members of the *Lyssavirus* genus, family *Rhabdoviridae*. Within this family of viruses are seven genotypes (gts) (1-7), namely:- classical rabies virus (gt1), Lagos bat virus (LBV, gt2), Mokola virus (MOKV, gt3), Duvenhage virus (DUVV, gt4), European bat lyssavirus type-1 and 2 (gts 5 & 6 respectively) and the Australian bat lyssavirus (ABLV, gt7) as well as unassigned viruses.

Virus factories in infected cells appear as inclusion bodies, consisting predominantly of viral capsids (Ribo-Nucleo-Protein, RNP). The histologically demonstrable rabies inclusion bodies are called “Negri bodies”. Among the laboratory tests available for routine diagnosis, the fluorescent antibody test (FAT), which detects viral antigen is the gold standard and should be used by all laboratories performing such work. A minimum of 0.5 IU/ml Rabies antibodies is required to protect against Rabies infection, according to the World Health Organisation recommendations (WHO, 1992. Expert Committee on Rabies, 8th Report. World Health Organisation, Geneva, Technical Report Series n° 824). The indirect ELISA allows a quantitative detection of Rabies antibodies in individual dog and cat serum samples.

2. PRINCIPLES

The reaction is composed of three steps:

Each serum sample is placed in a well sensitised with inactivated Rabies viral antigens. Antibodies present in the sample bind to the viral antigens coated at the bottom of the well.

After a wash step, Protein A/peroxidase conjugate is added. It fixes to the immunoglobulins (antibodies) previously captured, forming a complex: (Rabies Ag) - (Ab anti-Rabies) - (Protein A/peroxidase)**.**

Excess conjugate is eliminated by a wash step. The enzyme linked to the complex is revealed by addition of a substrate which is transformed into a coloured product. After stopping the reaction, the optical densities are measured. The presence or absence of antibodies is determined by using threshold values obtained from the positive control.

3. APPLICATION

The SERI ELISA Synbiotics is used for the detection of Anti-Rabies antibodies in Dog and Cat or Cat individual serum by indirect immunoenzymatic technique.

4. OBJECTIVE

To describe the procedure for the detection of Anti-Rabies antibodies in Dog and Cat or Cat individual serum by indirect immunoenzymatic technique.

5. APPARATUS/TEST KIT

5.1 Microplate containing six 16-well strips sensitized with Rabies antigens
5.2 Conjugate : Protein A/peroxidase (**CJ**) (10X concentrated)
5.3 Buffered peroxidase substrate (**PS**)
5.4 Negative control (**N**) (10X concentrated)
5.5 Positive control (**P**) (10X concentrated)
5.6 Sample diluent (**SD**)
5.7 Wash solution (**W**) (10X concentrated)
5.8 Conjugate diluent (**CD**)
5.9 WHO reference serum
5.10 Distilled or demineralized water
5.11 Adjustable or set pipettes to measure and deliver between 0 to 1000 µl. Measurement deviations must be 10% for volumes10 µl and 5% for all other volumes.
5.12 Graduated cylinders (100 ml and 1000 ml).
5.13 Manual, automatic or semi-automatic washing device for micro-titration plates.
5.14 Microplate reader, fitted with filters for bichromatic reading at 450 and 630 nm. It is also possible to use a monochromatic reader fitted with a 450 nm filter.
5.15 Incubator at +37 ± 3℃.

6. PROCEDURE

6.1 Carefully set up the distribution and identification of controls and samples
6.2 Prepare the sera to be tested. Dilutions are performed in the kit sample diluent (SD). Dilutions should be performed as follows: the samples are first pre-diluted at 1:10 in a blank microplate (10 µl of sample in 90 µl of SD)
6.3 For serum titration, a set of seven dilutions of the WHO standard serum should be performed either in tubes or in microplates with an initial dilution of 1:10 and 1:100 then 1:25, 1:60, 1:80, 1:170, 1:400 and 1:800.
6.4 The preparation of he WHO standard serum range of dilutions must be carried out as follows: 1:100 (not deposit in the test plate): 10 µl WHO 1:10 + 90 µl SD.

WHO dilution	Preparation
1:10	25 µl of WHO + 225 µl of sample
1:25	40 µl of 1:10 dil + 60 µl of SD
1:60	25 µl of 1:10 dil + 125 µl of SD
1:80	20 µl of 1:10 dil + 140 µl of SD

1:170	10 µl of 1:10 dil + 160 µl of SD
1:400	25 µl of 1:100 dil + 75 µl of SD
1:800	20 µl of 1:100 dil + 140 µl of SD

Control distribution

- Controls are not ready-to-use and should be diluted at 1:10.
- Dispense 90 µl of sample diluents, and add 10 µl of the kit negative control into wells A1 and A2, and 10 µl of the positive control to wells B1 and B2.

Distribution of samples and WHO serum dilutions

- Dispense 90 µl of sample diluent, add 10 µl of either 1:10 sample pre-dilution or each WHO serum dilution from 1:10 to 1:800 into the test wells and mix gently
- Strips should always be placed on the frame so that both washer and reader can be used.
- Cover the wells with adhesive film, cut to the necessary length by the number of strips used.
- Mix the plate manually by gently shaking or by using a plate agitator.
- Incubation of the plate**:** 1 hour ± 5 min. at +37 ± 3℃.

	1	2	3	4
A	N 1:10	N 1:10	WHO 1:8000	WHO 1:8000
B	P 1:10	P 1:10	S1 1:100	S1 1:100
C	WHO	WHO 1:100	S2 1:100	S2 1:100
D	WHO	WHO 1:250	S3 1:100	S3 1:100
E	WHO	WHO 1:600	S4 1:100	S4 1:100
F	WHO	WHO 1:800	S5 1:100	S5 1:100
G	WHO	WHO 1:1700	S6 1:100	S6 1:100
H	WHO	WHO 1:4000	S7 1:100	S7 1:100

Antibody quantification (final dilution)
Over OD values may be observed for the 1:100 WHO dilutions. In this case, use the following WHO dilutions to perform the regression curve.

Washing

- Wash buffer: dilute the concentrated washing solution (W) 1:10 in distilled or demineralized water.
- Carefully remove the adhesive film and wash 4 times.

Addition of conjugate

- Dilute the concentrate (CJ) 1:10 in the conjugate diluent (CD). 2 ml are needed for one strip, meaning 200 µl of CJ in 1.8 ml of CD.
- Add 100 µl of diluted conjugate to all the wells and cover with a new piece of adhesive film.
- Incubation of conjugate: 1 hour ± 5 min. at +37 ± 3℃.
- Carefully remove the adhesive film and wash 4 times.

Addition of the substrate

- Add 100 µl of buffered peroxidase substrate (PS) per well. Do not cover

with adhesive film at this stage. Mix by gentle shaking the plate manually or use a plate agitator to ensure correct homogenisation.

- Incubate for 30 ± 5 min. at laboratory temperature (+23 ± 5°C), shielded from light.

Addition of the Stop Solution

- Add 50 µl of stop solution (S) per well.
- Mix by gentle shaking the plate manually or by using a plate agitator. Make sure that no bubbles occur in the wells.

Measure of the optical density

- Measure the optical density (OD) bichromatically at 450 and 630 nm or monochromatically at 450 nm (in the yellow band).
- Reading bichromatically is strongly recommended. Should a monochromatic reader be used, ensure the cleanliness of the bottom of the wells prior to reading.

7. RESULT INTERPRETATION AND REPORTING

Titer calculation using the regression curve

7.1 (We recommend the use of an Excel spreadsheet. Synbiotics will provide you with a ready-to-use file upon request)

7.2 Calculate the average OD value for each sample tested and each WHO serum dilution.

7.3 Calculate the Neperian logarithm (ln) value for each average OD and the ln value of the Rabies Ab concentration for each WHO dilution (from 6.7 to 0.0223 IU/ml, without taking into account the 1:100 testing dilution factors).

7.4 Plot the ln (OD) (Y-axis) as a function of the ln (Rabies Ab concentration) (X-axis) in order to draw the reference curve for the *WHO* standard serum.

7.5 Using all individual results obtained for the *WHO* standard serum dilutions, perform a linear regression curve between ln Rabies Ab concentrations (expressed in EU/ml) (equivalent units per ml) and ln (OD), to establish the corresponding mathematics model: ln [Rabies Ab concentration (EU/ml)]= a + b * ln OD

7.6 For each tested sample, calculate the average OD value and then the Rabies antibody concentration of the sample expressed as «equivalent units per ml» (EU/ml), from the established model: Sample Rabies Ab concentration (EU/ml) = $e^{(a + b^* \ln OD)}$

Rabies antibodies quantities are reported as protected or non-protected.

EXAMPLE

* Positive control: well B1 = 0.610; well B2 = 0.690 Average OD P = 0.650

* Negative control: well A1 = 0.190; well A2 = 0.210 Average OD N = 0.200

* Sample 1: OD 1 = 1.790, OD 2 = 1.750 Average OD = 1.770

* Sample 2: OD 1 = 0.350 OD 2 = 0.390 Average OD = 0.370

* Test validation: OD P = 0.650 > 0.300 Average OD N = 0.200 < 0.50 x 0.650 = 0.325, therefore valid test.

Samples (final dilution)	Ab	OD 1	OD 2	averag	ln	ln
WHO 1:100	6.7	over	over	over	1.9021	-
WHO 1:250	2.233	1.280	1.237	1.259	0.8033	0.2299
WHO 1:600	0.67	0.809	0.751	0.780	-0.4005	-0.2485
WHO 1:800	0.447	0.600	0.620	0.610	-0.8052	-0.4943
WHO 1:1700	0.2233	0.406	0.425	0.416	-1.4992	-0.8783
WHO 1:4000	0.067	0.214	0.217	0.216	-2.7031	-1.5348
WHO 1:8000	0.0223	0.148	0.154	0.151	-3.8032	-1.8905
Sample 1	unknow	1.790	1.750	1.770	unknow	0,5710
Sample 2	unknow	0.350	0.390	0.370	unknow	-0,9943

Mathematics Model:

ln [Rabies Ab] = 0.255 + 2.063 * ln OD

* Test validation: correlation coefficient r = 0.996 > 0.95, therefore valid test

* Rabies Ab concentration in Sample 1:

$e^{(0.255 + 2.063 * \ln OD)} = e^{(0.255 + 2.063 * 0.5710)} = 4.19$ EU /ml protected

* Ab Concentration in sample 2:

$e^{(0.255 + 2.063 * \ln OD)} = e^{(0.255 + 2.063 * -0.9943)} = 0.17$ EU /ml not protected (FAVN confirmation).

8. WASTE DISPOSAL

Since the kit and the samples are highly infectious materials, dispose off properly following a SOP.

9. RISK ASSESSMENT

9.1 Place all reagents at laboratory temperature for at least 1 hour prior to use.
9.2 Handle all reagents and samples as biohazardous material.
9.3 Keep all reagents away from skin and eyes. If exposure should occur, immediately flush affected areas with cold water.
9.4 Never pipette by mouth.
9.5 Avoid inter sample contamination during sample collection, storage or transport. Use separate disposable pipette tips for each sample.
9.6 Avoid contamination of the substrate solution with metallic ions, oxidizing agents or detergents. Make sure that all containers are clean. Do not use the same container or the same pipette tip for the conjugate and the substrate.

9.7 It is recommended to dispose reagents and contaminated material according to the applicable regulations. The safety data sheets for the product are available upon request.

10. TROUBLESHOOTING

10.1 The results of each test run (or for each plate) are valid
10.2 When the optical density (OD) obtained with the positive control is 0.300, and
10.3 When the optical density (OD) obtained with the negative control is 0.50 x OD P
10.4 When the correlation coefficient between the Neperian logarithm (ln) ODs and ln Rabies Ab concentrations for the *WHO* standard serum is 0.95.

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TITLE: Rose Bengal Test (RBT) for *Brucella*

PREPARED BY: Serology section

REVISED BY: Serology section

APPROVED BY: Head, LSU

DATE: 11.06.2018

1. INTRODUCTION

Brucella is a small Gram-negative bacterium (0.4-0.8 µm in diameter and 0.4-3.0 µm in length) which is non-flagellated, and non-spore forming. Four species are pathogenic to human: *Brucella abortus*, *Brucella melitensis*, *Brucella suis* and *Brucella canis*. All four species are exciters of Brucellosis, a disease characterized by undulating fever. Depending on exciter the disease is also called Morbus Bang (*B. abortus*) or Malta fever (*B. melitensis*).The pathogens are transmitted from animals, which are mainly affected. The infection is caused by contact with ill animals or their excrements as well as by non-pasteurized milk and milk products like fresh cheese from sheep or goat. Main entrances are skin wounds, conjunctives and digestive tract. The intact pathogens are transported by granulocytes into local lymph nodes, from where they spread haematogenous. All kind of organs can be infected.

2. PRINCIPLES

The Rose Bengal test is a simple agglutination test in which a standardized antigen with added rose bengal dye is mixed with serum on a standardized, clear glass or plastic substrate. After standardised mixing for a standard time at a standard temperature, the degree of agglutination is estimated over a light box in comparison with positive controls.
The Rose Bengal test is relatively simple to perform and interpret and can be done by laboratories with basic resources.

3. APPLICATION

The Rose Bengal test is a spot agglutination test used to screen herds for *Brucella abortus*. For the diagnosis of individual cattle the test is oversensitive. This test is also used to test for *B. suis*.

4. OBJECTIVE

To describe the procedures for screening the herd against Brucellosis.

5. APPARATUS/TEST KIT/REAGENTS

5.1 *B.abortus* Rose Bengal antigen.
5.2 *B.abortus* positive reference control serum
5.3 *B.abortus* negative reference control serum
5.4 Agglutination plates, flat, Perspex
5.5 Pipettors and tips
5.6 Spatula for mixing serum and antigen

5.7 Timer

6. TEST PROCEDURE

6.1 Bring antigen and test sera to room temperature ($20^0C \pm 5^0C$).
6.2 Pipette 25-30 µl of test serum, and positive and negative reference control serum onto a labelled agglutination plate.
6.3 Add 25-30 µl of antigen to each well. One plate at a time is set up to minimise delay between the addition of antigen to the first and last serum.
6.4 Mix antigen and sera with a mixer / spatula, wiping the implement between each mix.
6.5 Place the agglutination plate on a tray rocker (approximately 30 oscillations / minute) and mix for 4 min.
6.6 Read the results using a light box.
6.7 Read results of reference sera first, then the remaining wells.

7. RESULT INTERPRETATION AND REPORTING

A scoring system is used to allow correlation with the CFT results. The following allows distinction of degrees of reaction:

Negative	No agglutination, no "ringing", a uniform pink color.
1+	Barely perceptible agglutination and/or some "ringing". Also any doubtful reaction.
2+	Fine agglutination, definite ringing, and some clearing.
3+	Coarse clumping, definite clearing.

Results are recorded as Negative or Positive in standard laboratory report format.

8. WASTE DISPOSAL

The samples and the kits items contain infectious materials hence, should be disposed properly.

9. RISK ASSESSMENT

All Positive and Suspect samples are retested to confirm results using *B. abortus* ELISA test. It can infect the handlers if adequate precautions are not taken.

10. TROUBLESHOOTING

NA

11. REFERENCES

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Veterinary Laboratory User's Guide. Edited by R A McKenzie, ARI.

TITLE: SAT for Salmonella

PREPARED BY: Serology section

REVISED BY: Serology section

APPROVED BY: Head, LSU

DATE: 11.06.2018

1. INTRODUCTION
Infections with *Salmonella pullorum* can result in acute systemic disease and a high incidence of mortality in young poultry. *Salmonella enteritidis* is the cause of food-borne infections via faecal contamination or more directly from eggs. *Salmonella pullorum gallinarum* causes severe infectious diseases in young poultry. This bacterium is spread from generation to generation via the eggs.

2. PRINCIPLES
The Standard Tube Agglutination Test (STAT) is a simple, tube agglutination test in which a standardized antigen solution is incubated with test serum. Specific *Salmonella* antibody if present will agglutinate this antigen causing precipitation and clearing compared with a standard tube.
The results of the agglutination tests are expressed in international units (IU), which are defined by reference to OIE Standard.

3. APPLICATION
The test is used for screening the poultry flock against salmonella. Agglutination reveals the presence of circulating antibodies.

4. OBJECTIVE
To describe the procedure for conducting Standard Tube Agglutination Test (TSAT).

5. APPARATUS/TEST KIT
5.1 Plastic tubes (5 ml volume).
5.2 Pipettors and tips
5.3 1 ml and 10 ml Pipettes
5.4 37^0C Incubator

6. REAGENTS, SOLUTION AND BUFFER
6.1 *Salmonella sp.* Antigen
6.2 0.5 % (w/w) Phenol Saline (See Media Manual)
6.3 *Salmonella sp.* Positive control serum
6.4 *Salmonella sp.* Negative control serum

7. PROCEDURE

7.1 Prepare a General Serology Work Sheet for the test serum samples (plus positive and negative reference control serum).

7.2 Each test serum and the negative reference control serum is tested at serum dilutions of 1/10, 1/20 and 1/40 (See Fig 1). These dilutions are the final serum dilution after addition of antigen. The positive reference control serum is tested at 1/10, 1/20, 1/40, 1/80, 1/160 and 1/320.

7.3 Prepare three (3) tubes for each test serum and the negative reference control serum and six (6) tubes for the positive reference control serum.

7.4 Add 0.8 ml Phenol Saline to Tube 1 and 0.5 ml Phenol Saline to all other tubes for each sample.

7.5 Add 0.2 ml of test serum and reference control serum to the respective tubes (Tube 1) and mix thoroughly

7.6 Transfer 0.5 ml of diluted serum from Tube 1 to Tube 2 and mix thoroughly.

7.7 Transfer 0.5 ml of diluted serum from Tube 2 to Tube 3, mix and discard 0.5 ml. For the positive reference controls continue on to Tube 6 and then discard 0.5 ml.

7.8 Add 0.5 ml of antigen to all tubes and mix thoroughly.

7.9 NB. The antigen is used at ten times the dilution as used in the CFT and is diluted with phenol saline (i.e if the antigen is used at 1:100 in the CFT it is used at 1:10 in the SAT).

7.10 Incubate the tubes at 37^0C for 20 ± 1 hour

8. RESULT INTERPRETATION

8.1 Read results of the reference sera first. If the positive and negative reference results are satisfactory proceed to next step. If reference results are unsatisfactory, tests are repeated. Responses to further test failures are determined by detailed examination of the various control elements in the procedure.

8.2 The tubes are examined without being shaken against a black background. By comparison with the positive control, read and record the degree of clearing in the test sera tubes.

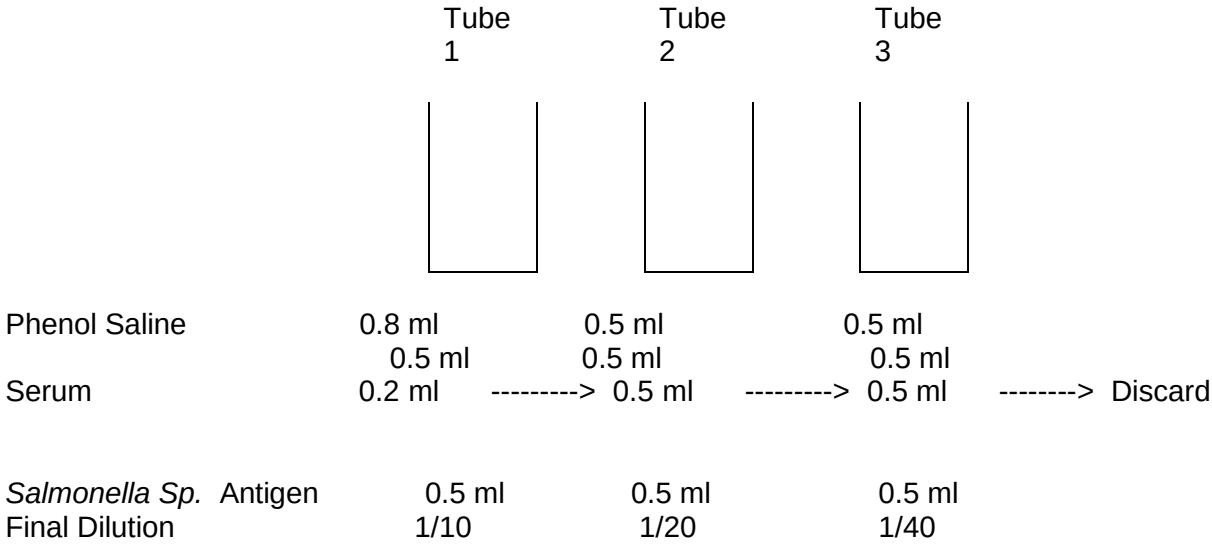
8.3 Read tubes as follows:

Result	Agglutination	% Clearing
Negative	No agglutination	no clearing
1+	Some agglutination	25% clearing
2+	Marked agglutination	50% clearing
3+	Nearly complete agglutination	75% clearing
4+	Complete agglutination	100% clearing

8.4 Convert the agglutination reading to International Units [IU] (Appendix B).

8.5 Retest all positive test samples using a six (6) tube dilution series (1/10 – 1/320).

Figure 1.



9. WASTE DISPOSAL

Consider the material as infectious waste and dispose properly as per the SOP.

10. RISK ASSESSMENT

The antigen may be infectious hence, proper disinfection should be carried out after the tests and the materials should be handled with precaution.

11. TROUBLESHOOTING

NA

12. REFERENCES

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13. Appendix: Conversion of SAT Titres to International Units (IU)/ml.

Final dilution of serum	End point reading	IU/ml
1/10	1+	17
	2+	20
	3+	23
	4+	27
1/20	1+	34
	2+	40
	3+	47
	4+	53
1/40	1+	67
	2+	80
	3+	93
	4+	106
1/80	1+	134
	2+	160
	3+	186
	4+	212
1/160	1+	268
	2+	320
	3+	372
	4+	424
1/320	1+	536
	2+	640
	3+	744
	4+	848
1/640	1+	1072
	2+	1280
	3+	1488
	4+	1696

TITLE: SAT for Mycoplasma

PREPARED BY: Serology section

REVISED BY: Serology section

APPROVED BY: Head, LSU

DATE: 11.06.2018

1. INTRODUCTION

Mycoplasma infections in chickens and turkeys are characterized by respiratory rales, coughing, and nasal discharge and clinical signs are usually slow to develop in chickens. Air sacculitis can be a significant cause of condemnations at slaughter. *Mycoplasma* infections in chickens and turkeys may result in acute to chronic infectious synovitis and chickens may also exhibit subclinical upper respiratory tract infections and air sacculitis. Turkey infections are characterized by lameness, swollen joints, and reduced weight gain.

2. PRINCIPLES

The SAT is a simple, tube agglutination test in which a standardised antigen solution is incubated with test serum. Specific *Mycoplasma* antibody if present will agglutinate this antigen causing precipitation and clearing compared with a standard tube.
The results of the agglutination tests are expressed in international units (IU), which are defined by reference to OIE Standard.

3. APPLICATION

It is used to screen poultry against Mycoplasma infection.

4. OBJECTIVE

To describe the procedure for carrying out simple tube agglutination test for *Mcoplasma.*

5. APPARATUS/TEST KIT

5.1 Plastic tubes (5 ml volume).
5.2 Pipettors and tips
5.3 1 ml and 10 ml Pipettes
5.4 37^0C Incubator

6. REAGENTS, SOLUTION AND BUFFER

6.1 *Mycoplasma sp.* antigen
6.2 0.5 % (w/w) Phenol Saline (See Media Manual)
6.3 *Mycoplasma sp.* positive control serum
6.4 *Mycoplasma sp.* negative control serum

7. PROCEDURE

7.1 Prepare a General Serology Work Sheet for the test serum samples (plus positive and negative reference control serum).

7.2 Each test serum and the negative reference control serum is tested at serum dilutions of 1/10, 1/20 and 1/40 (See Fig 1). These dilutions are the final serum dilution after addition of antigen. The positive reference control serum is tested at 1/10, 1/20, 1/40, 1/80, 1/160 and 1/320.

7.3 Prepare three (3) tubes for each test serum and the negative reference control serum and six (6) tubes for the positive reference control serum.

7.4 Add 0.8 ml Phenol Saline to Tube 1 and 0.5 ml Phenol Saline to all other tubes for each sample.

7.5 Add 0.2 ml of test serum and reference control serum to the respective tubes (Tube 1) and mix thoroughly

7.6 Transfer 0.5 ml of diluted serum from Tube 1 to Tube 2 and mix thoroughly.

7.7 Transfer 0.5 ml of diluted serum from Tube 2 to Tube 3, mix and discard 0.5 ml. For the positive reference controls continue on to Tube 6 and then discard 0.5 ml.

7.8 Add 0.5 ml of antigen to all tubes and mix thoroughly.

7.9 NB. The antigen is used at ten times the dilution as used in the CFT and is diluted with phenol saline (ie if the antigen is used at 1:100 in the CFT it is used at 1:10 in the SAT).

7.10 Incubate the tubes at 37^0C for 20 ± 1 hour

8. Result Interpretation

8.1 Read results of the reference sera first. If the positive and negative reference results are satisfactory proceed to next step. If reference results are unsatisfactory, tests are repeated. Responses to further test failures are determined by detailed examination of the various control elements in the procedure.

8.2 The tubes are examined without being shaken against a black background. By comparison with the positive control, read and record the degree of clearing in the test sera tubes.

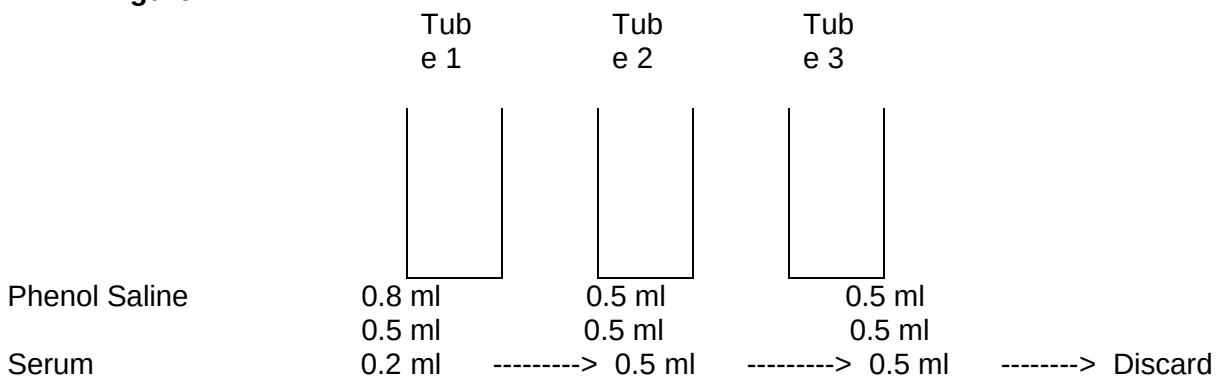
8.3 Read tubes as follows:

Result	Agglutination	% Clearing
Negative	No agglutination	no clearing
1+	Some agglutination	25% clearing
2+	Marked agglutination	50% clearing
3+	Nearly complete agglutination	75% clearing
4+	Complete agglutination	100% clearing

8.4 Convert the agglutination reading to International Units [IU] (Appendix B).

8.5 Retest all positive test samples using a six (6) tube dilution series (1/10 – 1/320).

Figure 1.



Calculation and Expression of Results

- Interpretation of results with Bovine serum:

Negative	<50 IU/ml
Suspect	>50 and <100 IU/ml
Positive	>100 IU/ml

- Results are entered as Negative, Suspect or Positive with the International Units [IU] attached.
- Data recording and document tracking is described in the Specimen Receipt/Processing document.
- Quality assurance procedures and repeat testing policies are outlined in the Quality Assurance of Test Methods document.

9. WASTE DISPOSAL

Since the kit contains antigen, it should be properly disinfected after the tests and dispose properly.

10. RISK ASSESSMENT

NA

11. TROUBLESHOOTING

NA

12. REFERENCES

Alton, G.G., Jones, L.M., Angus, R.D. and Verger, J.M. *Techniques in the Brucellosis Laboratory.* Institut National de la Recherche Agronomique; Parris. 1988.

Corner, L.A. Bovine Brucellosis: Serology. *In* "Standing Committee on Agriculture and Resource Management, *Australian Standard Diagnostic Techniques for Animal Diseases*", Eds Corner, L.A. and Bagust, T.J., 1993.

Veterinary Laboratory User's Guide. Edited by R A McKenzie, ARI.

13. Appendix: Conversion of SAT Titres to International Units (IU) /ml.

Final dilution of serum	End point reading	IU/ml
1/10	1+	17
	2+	20
	3+	23
	4+	27
1/20	1+	34
	2+	40
	3+	47
	4+	53
1/40	1+	67
	2+	80
	3+	93
	4+	106
1/80	1+	134
	2+	160
	3+	186
	4+	212
1/160	1+	268
	2+	320
	3+	372
	4+	424
1/320	1+	536
	2+	640
	3+	744
	4+	848
1/640	1+	1072
	2+	1280
	3+	1488
	4+	1696

TITLE: ELISA for Infectious Bursal Disease

PREPARED BY: Serology Section

REVISED BY: Serology Section

APPROVED BY: Head, LSU

DATE: 11.06.2018

1. INTRODUCTION

Infectious bursal disease, **IBD** (also known as Gumboro disease, infectious bursitis and infectious avian nephrosis) is a highly contagious disease of young chicken caused by infectious bursal disease virus (IBDV), characterized by immunosuppression and mortality generally at 3 to 6 weeks of age. It is economically important to the poultry industry worldwide due to increased susceptibility to other diseases and negative interference with effective vaccination. In recent years, very virulent strains of IBDV (vvIBDV), causing severe mortality in chicken. Infection is via the oro-fecal route, with affected bird excreting high levels of the virus for approximately 2 weeks after infection.

2. TEST PRINCIPLES

This assay is designed to measure the relative level of antibody to IBD in chicken serum. Viral antigen is coated on 96-well plates. Upon incubation of the test sample in the coated well, antibody specific to IBD forms a complex with the coated viral antigens. After washing away unbound material from the wells, a conjugate is added which binds to any attached chicken antibody in the wells. Unbound conjugate is washed away and enzyme substrate is added. Subsequent color development is directly related to the amount of antibody to IBD present in the test sample.

3. APPLICATION

The Infectious Bursal Disease virus antibody test kit idexx is used for detection of antibody against IBD virus in chicken serum.

4. OBJECTIVE

To outline the procedure for conduction the immunoassay for detection of antibody against IBDV in chicken serum.

5. APPARATUS/TEST KIT/REAGENTS

5.1 IBD Antigen Coated Plates
5.2 Positive Control
5.3 Negative Control
5.4 Conjugate
5.5 Sample Diluent
5.6 TMB Substrate
5.7 Stop Solution

6. PROCEDURE

All the reagents must be allowed to come to 18-26^{o}C before use. Mix reagents by gently inverting or swirling.

6.1 Obtain coated plate and record the sample position.
6.2 Dispense 10 µL of the un diluted Positive control into duplicate wells
6.3 Dispense 10 µL of the un diluted Negative control into duplicate wells
6.4 Dispense 10 µL of samples in to appropriate wells. Samples may be tested in duplicate but a single well is acceptable. None of the samples are diluted for testing.
6.5 Cover the plate and incubate for 30 minutes (± 2minutes) at 18 - 26^{o}C.
6.6 Wash each well with 350 µL of distilled water 3-5 times. Aspirate completely.
6.7 Dispense 100 µL conjugate in to each well.
6.8 Incubate for 30 minutes at at 18 - 26^{o}C.
6.9 Repeat step 6
6.10 Dispense 100 µL of TMB substrate into each well.
6.11 Incubate at 18 - 26^{o}C for 15 minutes ((± 2minutes).
6.12 Dispense 100 µL of stop solution into each well.
6.13 Read the results at a wavelength of 650 nm.

7. RESULT INTERPRETATION and REPORTING

$$\text{SP ratio} = \frac{\text{Sample mean - NC}}{\text{PC-NC}}$$

Negative - SP ratio ≤ 0.20

Positive - SP ration ≥ 0.20

8. WASTE DISPOSAL

Consider it as infectious agent and dispose properly.

9. RISK ASSESSMENT

NA

10. TROUBLESHOOTING

NA

11. REFERENCES

Infectious Bursal Disease Virus Ab test kit int 06-02470-08 manual

TITLE: ELISA for Infectious Bovine Rhinotrachitis

PREPARED BY: Serology Section

REVISED BY: Serology Section

APPROVED BY: Head, LSU

DATE: 11.06.2018

1. INTRODUCTION
Infectious Bovine Rhinotracheitis is a contagious disease cause by type 1 Heroes virus (BHv-It is most often characterized by respiratory syndrome associating cough, runny nose, and high body temperature and brochopulmonary complications. More rarely it can also present ocular, nervous or genital forms (Bovine Pustular Vulvo-vaginitis). It can also result in abortion and neonatal mortality.

2. TEST PRINCIPLES
Microplates are coated with an ultra-purified BHV-1 lysate. Samples to be tested are diluted and incubated in the wells. Upon incubation of the test sample in the coated wells, BHV-1 specific Antibodies from immunocomplexes with BHV-1 Antigen.

3. APPLICATION
The Infectious Bovine Rhinotracheitis Antibody test kit (idexx Trachitest serum screening) is used to detect the antibody to bovine hepes virus 1 (BHV-1), the virus of the Infectious Bovine Rhinotracheitis, in individual serum or plasma samples and pools up to 10 serum samples from bovines.

4. OBJECTIVE
To describe the procedure for carrying out the enzyme immunoassay for detection of antibody against BHV-1.

5. APPARATUS/TEST KIT/REAGENTS

5.1	BHV-1 Antigen coated plates	10
5.2	Positive control	2ml
5.3	Negative control	2ml
5.4	(Anti-Ruminant IgG-HRPO) conjugate	110 ml
5.5	Sample diluent	100ml
5.6	TMB substrate N 12	100ml
5.7	Stop solution N3	100ml
5.8	Wash concentrate (10X)	480ml

Materials Required but Not provided

5.9 Pipettes (10-1000µl)

5.10 Disposable pipette tip
5.11 500m; graduated cylinder for wash solution
5.12 96-well plate reader equipped with 450nm filter
5.13 Distilled or de-ionized water
5.14 Device for delivery and aspiration of wash solution
5.15 A trap for retaining aspirate and disinfectant
5.16 Humid chamber/incubator capable of maintaining a temperature + 370C (+30C)
5.17 Plate sealer lids
5.18 Vortex mixer

6. PROCEDURE

All the reagents must be allowed to come to 18-26^{o}C before use. Mix reagents by gently inverting or swirling.

6.1 Dispense 90µL of samples diluents into each well of the microtiter plate.

6.2 Add 10 µL of the undiluted samples and control into the appropriate wells of the micro titter plate. (Final dilution = 1:10).

6.3 Mix the contents within each well by gently shaking the microtiter plate briefly.

6.4 **Short Incubation**- Cover the plate with lid and incubate for 60 minutes (± 5 mins) at 37^{0}C in a humid chamber.

6.5 **Overnight Incubation**- Cover the plate with lid and incubate for 4 – 18 hours at 2 - 8^{0}C in a humid chamber.
6.6 Wash each well with 300 µL of wash solution 3 times. Avoid the plate dying between plate wash. Tap each plate onto absorbent material after the final wash to remove any residual wash fluid.
6.7 Dispense 100 µL conjugate in to each well.
6.8 Incubate for 60 minutes at 37^{o}C in a humid chamber using plate covers.
6.9 Repeat step 6
6.10 Dispense 100 µL of TMB substrate N.12 into each well.
6.11 Incubate at 18-26^{o}C for 15 minutes.
6.12 Dispense 100 µL of stop solution N.3 into each well.
6.13 Read the results at a wavelength of 450 nm.

7. RESULT INTERPRETATION and REPORTING

7.1 The OD of the Positive control should not exceed 2.00.
7.2 The OD of the Negative control should not exceed 0.500.
7.3 The difference between Positive OD and Negative OD must be ≥ 0.300.

8. WASTE DISPOSAL

The samples and the reagents after use should be treated as infectious agent and dispose it properly.

9. RISK ASSESSMENT

NA

10. TROUBLESHOOTING

NA

11. REFERENCES

IBR-06-40659-01 manual

TITLE: ELISA test for Equine Infectious Anaemia (EIA)

PREPARED BY: Serology Section

REVISED BY: Serology Section

APPROVED BY: Head, LSU

DATE: 11.06.2018

1. INTRODUCTION
Equine infectious anaemia (EIA) virus causes a persistent infection in horses, resulting in periodic episodes of fever, anaemia, thrombocytopenia, leukopenia and weight loss. The virus may be transferred in utero or horizontally by biting flies, contaminated needles or mother's milk. Once a horse is infected with EIA, it will test positive for antibody to the virus in serological tests and remain infected for life.
The IDEXX cELISA EIA equine infectious anemia competitive ELISA test is a rapid convenient and specific test for the detection of EIA antibody in horse serum.

2. TEST PRINCIPLES
The IDEXX cELISA kit contains microtiter pre-coated with monoclonal antibody specific for p26, the major group specific antigen of equine infectious anemia virus.

3. APPLICATION
The kit is used for EIA antibody in equine serum.

4. OBJECTIVE
To describe the ELISA procedure for EIA.

5. APPARATUS/TEST KIT/REAGENTS
- 5.1 Anti-EIA Antibody Coated Plate
- 5.2 Positive control
- 5.3 Negative control
- 5.4 EIAV antigen Conjugate
- 5.5 TBM substrate
- 5.6 Stop Solution

6. PROCEDURE
All the reagents must be allowed to come to 18-26oC before use. Mix reagents by gently inverting or swirling.

- 6.1 Dispense 100µL of each serum samples into appropriate wells.
- 6.2 Dispense 100µL of Negative Control and 100µL of Positive into their respective wells.
- 6.3 Add 50 µL of EIAV antigen Conjugate into all wells.
- 6.4 Mix the contents by gently shaking the microtiter plate briefly.

6.5 Incubate uncovered for 30 minutes (± 2 mins) at 370C.
6.6 Wash each well with plate washer. Tap each plate onto absorbent material after the final wash to remove any residual wash fluid.
6.7 Dispense 100 µL of TMB substrate into each well. Mix thoroughly 10 times.
6.8 Incubate at 18-26oC for 15 minutes. (± 1 mins)
6.9 Dispense 100 µL of stop solution into each well.
6.10 Read the results at a wavelength of 650 nm.

7. RESULT INTERPRETATION

- Positive control must be ≥ 0.150 OD
- Positive control must be ≤ 70% of Negative control OD.

8. WASTE DISPOSAL

Dispose properly as per the SOP/guidelines

9. RISK ASSESSMENT

NA

10. TROUBLESHOOTING

NA

11. REFERENCES

TITLE: Antibody ELISA for Classical Swine Fever Virus

PREPARED BY: Serology section

REVISED BY: Serology section

APPROVED BY: head, LSU

DATE: 11.06.2018

1. INTRODUCTION
Classical Swine Fever Virus (CSFV), Bovine viral Diarrhoea Virus (BVDV), and Border Disease Virus (BDV) are the 3 members of the genus Pestivirus within the family Flaviviridae. CSF causes serious losses in the pig industry since it is highly contagious and pathogenic, and can cause widespread deaths.

2. TEST PRINCIPLES
IDEXX CSFV Ab is IDEXX's immunoassay for the detection of CSFV specific antibodies in swine serum or plasma. The assay is a blocking ELISA which utilizes Microplates coated with CSF antigen.

3. APPLICATION
To test for antibody against Classical Swine Fever Virus (CSFV) in pig serum.

4. OBJECTIVE
To describe the procedure for conducting antibody ELISA for CSFV.

5. APPARATUS/TEST KIT/REAGENTS
- 5.1 CSFV Antigen coated plate
- 5.2 Positive control
- 5.3 Negative control
- 5.4 Conjugate
- 5.5 Sample diluents
- 5.6 TMB Substrate N.12
- 5.7 Stop Solution N.3
- 5.8 Wash Concentration (10X)

6. PROCEDURE

All the reagents must be allowed to come to 18-26oC before use. Mix reagents by gently inverting or swirling.

6.1 Dispense 50 µL of the sample diluents to all well to be tested and to the control wells.

6.2 Dispense 50 µL of Negative Control and 50µL of Positive into duplicate wells

6.3 Dispense 50 µL of the serum or plasma to all the remaining wells.

6.4 Mix the content gently by taping the plate

6.5 Incubate 2 hours (± 5 mins) or overnight (12-18 hours) at 18-26^{0}C with either option. The plates should be tightly sealed or incubated in a humid chamber using plate covers to avoid any evaporation.

6.6 Wash each well with 300 µL of wash solution 3 times. Avoid the plate dying between plate wash. Tap each plate onto absorbent material after the final wash to remove any residual wash fluid.

6.7 Add 100 µL of Conjugate into all wells.

6.8 Mix the contents by gently shaking the microtiter plate briefly. The plates should be tightly sealed or incubated in a humid chamber using plate covers to avoid any evaporation

6.9 Incubate for 30 minutes (± 2 mins) at 18-26^{0}C.

6.10 Repeat step 6

6.11 Dispense 100 µL of TMB substrateN.12 into each well.

6.12 Incubate 10 minutes. (± 1 mins) at 18-26^{o}C away from direct sunlight.

6.13 Dispense 100 µL of stop solution into each well.

6.14 Read the results at a wavelength of 450 nm.

7. RESULT INTERPRETATION and REPORTING

- ✓ Negative OD- >0.500
- ✓ Positive OD- < 50

$$\text{Blocking \%} = \frac{\text{Sample mean - NC}}{\text{PC-NC}}$$

- ✓ **Negative Blocking % =≤ 30**
- ✓ **Suspect Blocking % = < 40**
- ✓ **Positive Blocking % = ≥ 40**

8. WASTE DISPOSAL

9. RISK ASSESSMENT

NA

10. TROUBLESHOOTING

NA

11. REFERENCES

IDEXX CSFV Ab 06-43230-09

TITLE: Classical Swine Fever Virus Antigen test

PREPARED BY: Serology section

REVISED BY: Serology section

APPROVED BY: head, LSU

DATE: 11.06.2018

1. INTRODUCTION
Classical swine Fever Virus (CSFV), Bovine viral Diarrhoea Virus (BVDV), and Border Disease Virus (BDV) are the 3 members of the genus Pestivirus within the family Flaviviridae. CSF causes serious losses in the pig industry since it is highly contagious and pathogenic, and can cause widespread deaths.

2. TEST PRINCIPLES
A microtitration format has been configured by immobilizing specific monoclonal antibodies on the plates. CSFV AG of the sample is captured on the plates.

3. APPLICATION
IDEXX CSFV AG serum Plus is IDEXX's enzyme immunoassay for the detection of CSFV antigens in swine serum, plasma and tissue (preferably tonsil, spleen, mesenteric lymph node or kidneys) samples

4. OBJECTIVE
To outline the procedure of IDEXX CSFV AG serum Plus is IDEXX's enzyme immunoassay for the detection of CSFV antigens in swine serum, plasma and tissue (preferably tonsil, spleen, mesenteric lymph node or kidneys) samples.

5. APPARATUS/TEST KIT/REAGENTS
5.1 Anti-E^{rns}mAB coated plate
5.2 Positive control
5.3 Negative control
5.4 Conjugate
5.5 Tissue Soaking Buffer Concentrate (2X)
5.6 TMB Substrate N.12
5.7 Stop Solution N.3
5.8 Wash Concentration (10X)

6. PROCEDURE

Sample preparation

Use preferably fresh tissue but, if necessary, tissue can be stored frozen. Process one or two tissue from each animal submitted, preferably tonsil, spleen, mesenteric lymph node or kidneys.

a) With scissors, cut tissue into small pieces (250mg0
b) Place the tissue into appropriate centrifuge or an Eppendorf tube ad add 1mL Tissue soaking Buffer. Vortex at 18-26^{0}C for 1-2 hours.
c) Centrifuge at 1500xg for 10 minutes and use 50 µL of the clean supernatant for the testing as described in the test procedure.

Please use the overnight incubation protocol for tissue samples

All the reagents must be allowed to come to 18-26^{o}C before use. Mix reagents by gently inverting or swirling.

6.1 Dispense 50 µL of detection solution in to all wells to be tested and to the control wells.

6.2 Dispense 50 µL of Negative Control and 50µL of Positive into duplicate wells

6.3 Dispense 50 µL of the samples to all the remaining wells.

6.4 Mix the content gently by taping the plate

6.5 Incubate Sample

A. **Serum and plasma**: Incubate for 2 hours (± 5 mins) at 37^o C. The plates should be tightly sealed or incubated in a humid chamber using plate covers to avoid any evaporation.
B. **Tissue samples:** Incubate overnight (12-18 hours) at 2-8oC. The plates should be tightly sealed or incubated in a humid chamber using plate covers to avoid any evaporation.

6.6 Wash each well with 300 µL of wash solution 5 times. Avoid the plate dying between plate wash. Tap each plate onto absorbent material after the final wash to remove any residual wash fluid.

6.7 Add 100 µL of Conjugate into all wells.

6.8 Incubate for 10 minutes (± 3 mins) at 18-260C away from sun light.

6.9 Repeat step 6

6.10 Dispense 100 µL of TMB substrateN.12 into each well.

6.11 Incubate 10 minutes. (± 1 mins) at 18-26oC away from direct sunlight.

6.12 Dispense 100 µL of stop solution into each well.
6.13 Blank the spectrophotometer on air
6.14 Read the results at a wavelength of 650 nm.

7. RESULT INTERPRETATION and REPORTING

For the assay to be valid, the difference (P-N) between the positive and negative control mean must be greater than or equal to 0.150 OD.

- Negative mean= ≤ 0.25

  - ✓ **Sample with S-N values equal or less than 0.300 are classified as Negative.**
  - ✓ **Sample with S-N values greater than 0.300 are classified as Positive**

8. WASTE DISPOSAL

Consider the samples and reagents as infectious material and dispose after proper disinfection.

9. RISK ASSESSMENT

NA

10. TROUBLESHOOTING

NA

11. REFERENCES

IDEXX CSFV AG serum Plus , CSFV-06-43230-09 manual

REVISED BY:

APPROVED BY:

DATE:

12. INTRODUCTION

.

13. TEST PRINCIPLES

14. APPLICATION

15. OBJECTIVE

16. APPARATUS/TEST KIT/REAGENTS

17. PROCEDURE

18. RESULT INTERPRETATION and REPORTING

19. WASTE DISPOSAL

20. RISK ASSESSMENT

21. TROUBLESHOOTING

22. REFERENCES

TITLE: Haemagglutination test for Newcastle Disease

PREPARED BY: Serology section

REVISED BY: Serology section

APPROVED BY: Head, LSU

DATE: 11.06.2018

1. INTRODUCTION

Newcastle disease is an infection of domestic poultry and many other bird species with virulent Newcastle disease virus. NDV synonymous with avian paramyxovirus serotype 1 (PMV-1), is an RNA virus and the most important of the 9 known PMV serotypes as a pathogen for poultry. NDV can be isolated from oropharyngeal or cloacal swabs or tissues from infected birds by inoculation of the allantoic cavity of 9- to 11-day-old embryonated chicken eggs.

Infection is confirmed by recovery of a hemagglutinating virus that is inhibited with NDV antiserum or by detection of NDV RNA by reverse transcriptase PCR. A rise in NDV antibody titer by hemagglutination-inhibition or ELISA of paired serum samples indicates NDV infection.

2. PRINCIPLES

Haemagglutination (HA) is the agglutination of red blood cells (RBC), which can be caused by some viruses. This method is done to find presence or absence of haemagglutinin. The property of some viruses to agglutinate the red blood cells of poultry and certain other animals is used in a range of systems to detect viruses and to titrate antibodies. Haemagglutinating properties of viruses are most often used in serology to detect and titrate antibodies by using the haemagglutination inhibition test (HI). However before the HI test can be carried out, the haemagglutinating strength of the virus must be determined. Doubling dilutions of antigen are mixed with washed RBC and allowed time to agglutinate; the dilution at which agglutination stops is considered 1 haemagglutinating unit (1 HAU). By counting back two doubling dilutions the dilution is reached which contains 4 HAU and this is the strength of antigen usually used in the HI test. Imagine that neat virus solution has 100 agglutinating units, then a 1:2 dilution will have 50, and a 1:4 will have 25 etc. A point will be reached when there is less than 1 unit in the diluted virus and at this point the RBC will no longer agglutinate. In other words it takes atleast one unit to agglutinate RBC. For e.g. if a 1:64 dilution was the last dilution to have atleast one unit then 1:32 must be 2 unit and 1:16 must be 4 HA units. Therefore 1:16 is the dilution of antigen (virus), which must be used in subsequent HI test.

Haemagglutination inhibition (HI) is the inhibition of HA, for example by using hyperimmune serum specific to a haemagglutination virus. In this test, wells contain equal volumes and strengths of antigen, an equal volume of 1% RBC and the same volume of doubling dilutions of test serum (antibody). Haemagglutination properties of the viruses are blocked by antibody binding to the virus. Blocking takes place up to the dilution of serum (antibody)

where there is no longer sufficient antibody to combine with, and eliminate the virus. At this dilution virus will be free to agglutinate RBCs.

3. APPLICATION

The test is used to assess the efficacy of Newcastle disease vaccine in laboratory and field trials and also to assess the level of Newcastle disease virus antibodies in the field.

4. OBJECTIVE

To outline the method of the HA and HI.

5. APPARATUS/TEST KIT

HA

5.1 Antigen
5.2 V bottom microtitre plate and lid
5.3 Micropipette and tips
5.4 1% RBC
5.5 Discard tray Microtitre plate recording sheet
5.6 Phosphate Buffer solution
5.7 Glucose 2.05 g
5.8 NaCl 0.42 g
5.9 Trisodium citrate 0.80 g
5.10 Distilled water 100 ml

HI

5.11 Thawed serum samples in racks
5.12 V bottom microtitre plates
5.13 Lids for microtitre plates
5.14 1% washed red blood cells
5.15 V-bottom reagent trough
5.16 25 µL single and multichannel pipettes and tips
5.17 Microtitre plate recording sheet
5.18 Newcastle disease virus antigen diluted to contain 4 HA units
5.19 Standard positive and negative serum

6. PROCEDURE

6.1 Dispense 25 µL PBS into each well of a plastic V-bottomed microtitre plate.
6.2 Add 25 µL antigen to first well and mix well
6.3 Make two-fold serial dilutions along the row until the last well. Discard the 25 µL this well.
6.4 Each well now contains a dilution of 1 in 21 22 23… and so on
6.5 Add 25 µL of 1% red blood cells to each well
6.6 Mix by tapping the plate gently and allow to stand at room temperature for 45 minutes.
6.7 Record on microtitre recording sheets the position of samples in corresponding wells of the microtitre plates.
6.8 Read settling patterns

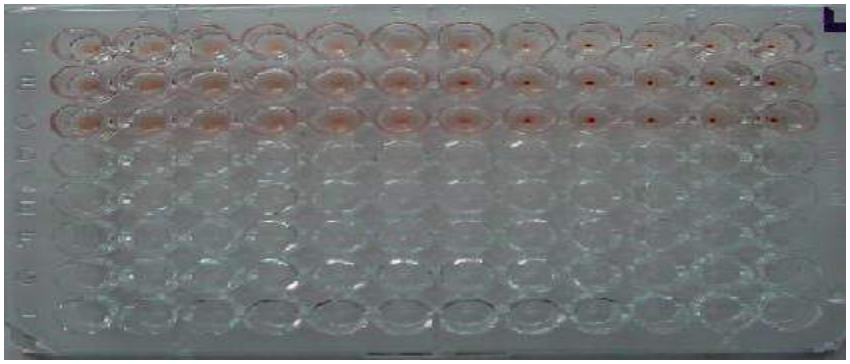
6.9 HA is determined by tilting the plate and observing the presence or absence of tear shaped streaming of the RBC.
6.10 Calculate the number of plates required to titrate and test antigen and test all the serum samples including the positive and negative serum. Check the plates are clean and number each plate with a marker pen.
6.11 Fill each well of each plastic V-bottomed microtitre plate with 25 µL of PBS.
6.12 Dispense the serum samples. Shake before dispensing. Place 25 µL of a sample into the first well and the last (control) well of each row of a microtitre plate.
6.13 Use a multichannel pipette to make two-fold serial dilutions along the row until the second last well from the end. Discard the 25 µL taken from this well. Do not the dilute the last well, which is the serum control.
6.14 Add 25 µL of the 4 HA dilution of antigen to each well excluding the control wells in the last column.
6.15 Mix gently and allow standing for 30 minutes at room temperature.
6.16 Add 25 µL of 1% chicken red blood cells (RBC) to each well including the control wells in the last column.
6.17 Gently tap the sides of the microtitre plates to mix the reagents. Stack the plates and cover with a lid and let the microtitre plates stand at room temperature for 45 minutes.
6.18 Read the settling patterns for each serum sample. Read the control serum well first then read the patterns in the other wells.
6.19 Record the patterns observed in each well on a microtitre plate-recording sheet.
6.20 Determine the end point. This is the point where there is complete haemagglutination inhibition. The agglutination is assessed more exactly by tilting the plates. Only those wells in which the RBC 'stream' at the same rate as the control wells (containing 25 µL RBC and 0.05 ml PBS only) should be considered as showing inhibition.
6.21 Record the antibody level foe each sample. This is expressed as a log base 2. For convenience, the titre is often recorded as just the log index. For example a titre of 26 would be recorded as 6.

7. RESULT INTERPRETATION

7.1 The setting patterns of single and agglutinated red blood cells are different. Single cells roll down the sides of the V-bottom well and settle as a sharp button. Agglutinated cells do not roll down the sides of the well to form a button. Instead they settle as a diffuse film.
7.2 Identify the end point. This will be last well to show complete haemagglutination of the red blood cells. In the haemagglutination titration the last well that shows complete agglutination is the well that contains one HA unit. One HA unit is the minimum amount of virus in the assay that will cause complete agglutination of the red blood cells.
7.3 *Interpretation*
- The HI titre is the highest dilution of serum causing complete inhibition of 4HAU of antigen.

- In the wells where antibodies are present there will be haemagglutination inhibition. The red blood cells will settle at a bottom.
- In the wells where antibodies are absent, the red blood cells will agglutinate.
- The end point is not always easy to determine. Look at the size of the button as an indication of the degree of haemagglutination inhibition. Use the control well as a point of comparison. Be consistent in deciding which well shows complete haemagglutination inhibition. The validity of results should be assessed against a negative control serum, should not give a titre >22, and a positive control serum for which the titre should be within one dilution of the known titre.
- Negative HA result (-) = a sharp button
- Positive HA result (+) = a diffuse film

Figure: Photograph of a plate showing HA test (note the diffuse film till well no 6)



8. WASTE DISPOSAL

Consider as infectious material and dispose off properly.

9. RISK ASSESSMENT

Newcastle disease viruses, whether virulent field viruses or live vaccine, can produce a transitory conjunctivitis in humans, but the condition has been limited primarily to laboratory workers and vaccination teams exposed to large quantities of virus.

10. TROUBLESHOOTING

10.1 Antibodies not detectable for several days of infection
10.2 Limited value because of routine use of vaccine

11. REFERENCES

Alexander DJ (1997). Newcastle disease and other avian Paramyxoviridae infections. In: Calnek BW, Barnes HJ, Beard CW, McDougald LR, Saif YM (ed), Disease of poultry, 10th edn. Iowa State University Press, Ames Iowa, pp 541–570.

Anon (1971). Methods for examining poultry biologics and for identifying and quantifying avian pathogens. Newcastle disease, p. 66. National Academy of Science, Washington, D. C.

TITLE: AGID for New Castle Disease (NCD)

PREPARED BY: Serology section

REVISED BY: Serology section

APPROVED BY: Head, LSU

DATE: 11.06.2018

1. INTRODUCTION

Newcastle disease is an infection of domestic poultry and many other bird species with virulent Newcastle disease virus. NDV synonymous with avian paramyxovirus serotype 1 (PMV-1), is an RNA virus and the most important of the 9 known PMV serotypes as a pathogen for poultry. NDV can be isolated from oropharyngeal or cloacal swabs or tissues from infected birds by inoculation of the allantoic cavity of 9- to 11-day-old embryonated chicken eggs.

Infection is confirmed by recovery of a hemagglutinating virus that is inhibited with NDV antiserum or by detection of NDV RNA by reverse transcriptase PCR. A rise in NDV antibody titre by hemagglutination-inhibition or ELISA of paired serum samples indicates NDV infection.

2. PRINCIPLES

Agarose plates are poured with 18mL of agarose. Wells of 6.5 mm diameter with a 3mm distance between wells are cut in agar plates in patterns of 7 wells consisting of a circular centre-well and six surrounding circular wells forming the points and centre of a regular hexagon. Six patterns may be cut around the periphery of the agar plate using a template for spacing. Antigen is placed in the centre well. Sera to be tested and reference antiserum are placed in the 6 surrounding wells. Antigen and serum antibody diffuse toward each other through the agar. Where specific antibody and antigen meet at approximately equal concentration, a visible precipitin line forms.

3. APPLICATION

The Agar Gel Immunodiffusion test (AGID) is a simple and reliable technique for the diagnosis of New Castle Disease (NCD).

4. OBJECTIVE

To outline the method of AGID for NCD.

5. APPARATUS/TEST KIT

5.1 Agar plates for NCD AGID test (Labelled NCD -See Media Manual).
5.2 NCD Reference Sera (standardised).
5.3 NCD Positive Control Sera. The NCD Reference Sera is used as the Positive Control Serum if a separate Positive Control is not provided.
5.4 NCD Negative Control Serum (standardised). Kit Negative antisera is used if provided. If not Foetal Bovine Serum known to be negative to NCD is suitable.

5.5 NCD Antigen (standardised).
5.6 Well cutter
5.7 Template – locates 6 x 7 well patterns on the agarose plate.
5.8 Vacuum pump.
5.9 50 µL pipettor.
5.10 Humidified ambient temperature (15-25°C) incubating chamber.
5.11 Fibre optic light source.

6. PROCEDURE

6.1 Samples are placed on the plate according to the plate layout described in 6.11 below. Details of each sample or control are recorded against the appropriate Specimen Number on the work sheet to indicate the position of the sample on each plate. Samples 1-18 are placed on Plate 1, samples 19-36 on Plate 2 etc. Reagent sera wells are not included in the numbering system as recorded on the work sheet.
6.2 Remove the required number of NCD plates from sealed plastic bags stored at 4°C.
6.3 Use the template, if necessary, to locate a maximum of 6 x 7 well patterns around the periphery of the NCD plate. The area in the centre of the plate should not be used.
6.4 With the location marker on the well cutter directed to the outer edge of the agarose plate, firmly push the well cutter through the agarose.
6.5 Repeat this procedure for all 6 positions on the plate.
6.6 Suck the agarose plugs from all wells on the plate using a vacuum pump with a pipette tip attached to the end of a hose. Be careful to avoid lifting the agarose from the plastic plate as this may result in leakage of reagents under the agarose.
6.7 Number the centre of the base of each plate sequentially. Label Plate 1 on its lid with the date and the type of AGID (i.e. NCD). Mark the location of one well with an easily recognisable spot on the rim of the plastic plate adjacent to it. This will be the first sample position for the plate.
6.8 Samples are placed sequentially from the marked well in a clockwise pattern using every second well only. The outermost well is the first sample well for each pattern (Well 1 in 6.11 below).
6.9 Wells are loaded with 50µL of reagents or sample according to the following:
   i. Central Well: NCD Antigen
   ii. Wells 1, 3 and 5: Test Samples or Controls
   iii. Wells 2, 4 and 6: Reference Serum
6.10 Three test sera are tested per 7-well pattern. Thus, with a maximum of 6 patterns per plate, a maximum of 18 sera may be tested per plate.
6.11 Plates are placed in foam boxes with moist paper towel in its base to maintain a humid atmosphere and are incubated at ambient temperature (15-25°C) for 24-72 hours.
6.12 AGID tests performed on different days are placed in different boxes to ensure plates do not get mixed up and that samples can be traced back to the appropriate work sheet.
6.13 The NCD AGID test is read after 24-72 hours incubation. If distinct lines of identity can be seen at the time of viewing the result can be reported.
6.14 Plates are read using a fibre optic light source in a darkened room against a dark background. The light is adjusted to an angle of approximately 45° to the bottom of the

plate. Precipitin lines should be clearly visible where specific antibody and antigen meet at approximately equal concentration.

7. RESULT INTERPRETATION

For antibody detection, antibody precipitation turns the reference line inwards towards the antigen well.

The strength of the test reaction is determined by comparison with the reference line.

Interpretation

Negative: The control lines continue into the test sample well without bending or with a slight bend away from the antigen well and toward the positive control serum.

- **Weak positive (1+):** The control line bends slightly towards the antigen and may or may not form a complete line between the antigen and the test serum. This reaction occurs very close to the test well. These reactions require careful observation.
- **Positive (2+-3+):** The control line joins with and forms a continuous line with the line formed between the test serum and antigen.
- **Very strong positive (4+):** The control lines will turn toward the antigen well before they reach the well containing the test serum and continue on as a broad or hazy between test serum and antigen. This line is situated very near the antigen well especially if the plate is observed at 24 hours. This reaction should not be confused with those obtained by use of inactive or weak control agents which produce short, faint, control lines extending equal distances on each side of the antigen, but not continuing into the test wells. A more distinct line will usually form if the very strong positive samples are diluted and retested.

8. WASTE DISPOSAL

Consider as infectious material and dispose off properly.

9. RISK ASSESSMENT

Newcastle disease viruses, whether virulent field viruses or live vaccine, can produce a transitory conjunctivitis in humans, but the condition has been limited primarily to laboratory workers and vaccination teams exposed to large quantities of virus.

10. TROUBLESHOOTING

Non-specific lines: These lines are observed between the antigen and test serum well. However, the reference positive control lines will pass through the non-specific line and continue on into the test serum well of negative sera. The non-specific line does not form a continuous line with the reference positive control lines. The reference positive control lines will form more acute angles with a non-specific line than with an NCD-specific line of identity. The non-specific lines are formed by sample-antibody reactions with antigens other than with NCD p26. A sample serum may produce a specific NCD line as well as a non-specific line. Care must be taken to be certain a specific reaction is not obscured by a non-NCD line. Retesting such samples and observing the reactions at frequent intervals may facilitate making determinations if the samples are positive or negative.

Haze Around Well: Occasionally a haze, due to lipids or other material in the serum, will form around the test serum well that may obscure the reference positive control lines near the sample well. If the test is read at 24 and 48 hours, sometimes the results can be determined before the haze obscures the reaction.

- Semi quantitative
- Moderate sensitivity
- Subjective interpretation
- Requires 24 hours
- Further testing of positives
- Antibodies not detectable for several days

11. REFERENCES

Veterinary Laboratory User's Guide. Edited by R A McKenzie, ARI.

TITLE: ELISA for detection of antibody to Mycoplasma mycoides (CBPP)

PREPARED BY: Serology section

REVISED BY: Serology section

APPROVED BY: Head, LSU

DATE: 11.06.2018

1. INTRODUCTION

Mycoplasma mycoides subsp. mycoides (Mmm) is the causative agent of contagious bovine pleuropneumonia (CBPP), an insidious, infectious and highly contagious disease of cattle and water buffaloes.

Serological techniques for the detection of antibodies to Mmm have been examined using enzyme linked immunosorbent assay. It was applied for the detection of antibodies to Mmm in sera of cattle at least 19 months after recovery from an infection and 23 months from the period of vaccination.

2. PRINCIPLES

The principle of the test is:

2.1 The microplates wells are coated with an MmmSC lysate.

2.2 Serum samples to be tested are diluted and mixed with the specific monoclonal antibody (Mab 117/5) in a dilution plate or “pre-plate”. This mixture is then transferred into the MmmSCcoated microplate. Any specific antibodies present in the test sera will bind to the MmmSC antigen, competing with the Mab for thespecific epitope.

2.3 After washing, an anti-mouse IgG serum conjugated to horseradish peroxidase (HRP), which will bind to any Mab fixed to the wells, is added. If specific MmmSC antibodies are present in the bovine sera, they will displace the Mab and the conjugate will not be able to bind.

2.4 Following another series of washes, the HRP substrate (TMB) is added, forming a blue compound that will turn to yellow when the reaction is stopped. The intensity of the color is an inverse measure of the proportion of MmmSC antibodies present in the test sera.

The cut-off point is calculated using the results obtained from a monoclonal control (Cm, 0% inhibition) and a conjugate control (Cc, 100% inhibition). Positive and negative control sera are delivered within the kit. They must be included in each microplate in order to validate the results.

3. APPLICATION

The kit is used to detect antibody against *Mycoplasma mycoides* in serum of cattle.

4. OBJECTIVE

To describe the procedure for serological detection of *Mycoplasma mycoides sub species mycoides in cattle.*

5. APPARATUS/TEST KIT/REAGENTS

5.1	96-well coated microplate	
5.2	Wash concentrate (20x)	2 x 100ml bottle
5.3	Dilution buffer 24	3 x 120ml bottle
5.4	Strong positive control (CP ++)	1 x 0.5ml vial
5.5	Weak positive (CP +)	1 x 0.5ml vial
5.6	Negative control (CN)	1 x 0.5ml vial
5.7	Monoclonal anti-MmmSC antibody (Mab 117/5) freeze dried	1 x 1 ml
5.8	Anti-mouse IgG HRP-conjugated	1 x 1.2ml
5.9	TMB substrate solution 3	1 x 120ml bottle
5.10	Stop solution (0.5M H_2SO_4)	1 x 120ml bottle

Materials required but not provided:

5.11 Refrigerator +5°C (+3°C) and freezer -16C
5.12 Incubator at +37C (+3C)
5.13 Plate agitator
5.14 Microplate reader
5.15 Microplate washing system that distributes 300µl per well (optional)
5.16 Vortex or similar (optional)
5.17 Precision micropipettes and multichannel micropipettes (the precision required must be lower than or equivalent to 10% for volumes lower or equal to 10µl and to 5% for all the other volumes indicated)
5.18 Disposable micropipette tips
5.19 Reagent reservoirs for multichannel pipettes
5.20 96-well microplates for dilutions
5.21 Microplate covers (lid, aluminium foil or adhesive)
5.22 Distilled water. The water used for the reconstitution of controls and of wash solution can be produced by a conventional distillation system or any other high-performance water purification system (reverse osmosis, resin or activated charcoal purification...)
5.23 Disinfectant for safe disposal of test sera (when required)

6. TEST PROCEDURE

6.1 DISTRIBUTION OF TEST AND CONTROL SERA Prior to transferring the sera into the coated microplate, they are diluted and mixed with the monoclonal anti-MmmSC antibody (Mab 117/5) in a 96-well microplate ,called the “pre-plate”, which should be made out of normal plastic without any absorption activity.

a) Distribution of controls and test sera

These dilutions must be carried out just before the test: - Distribute 100µl "Dilution buffer 24" in all the wells of the pre-plate. - Distribute110µl "Dilution buffer 24" in wells A1 and A2 (Conjugate control, Cc) - Distribute 11µl of the three control samples: CP++ in B1, B2, C1, C2 and CP+ in D1, D2, E1, E2 and CN in H1, H2. - Distribute11µl of each of the test sera in the remaining wells (A3 to H12, as required). b)

b) Reconstitution and distribution of the monoclonal antibody (Mab 117/5)
Reconstitute the Mab 117/5 with 1 ml of distilled water. Dilute the necessary quantity of the Mab to 1/120 in "Dilution buffer 24" (i. e.: 100µl Mab in 11.9 ml "Dilution buffer 24" for one plate). - Distribute110µl diluted Mab 117/5 in all the wells except for A1 and A2. Note: If the whole amount is not immediately used, the monoclonal antibody must be stored in aliquots at a temperature ≥16 ^{0}C (before dilution in “Dilution buffer 24”).

c) Incubation of the serum/Mab mixture
- Mix by pipetting and transfer 100µl of the serum/Mab mixture from the pre-plate into the coated plate by using a multi-channel pipette (see fig. 1). - Cover the plate with a lid, aluminium foil or adhesive and incubate for 1 hour (+/- 5 minutes) at 37°C (±3°C) under gentle agitation.

6.2 WASHING
a) Dilute a bottle of "Wash concentrate (20x)" in 1900 ml of distilled water. This solution is hereafter called the "wash solution". The dilution can be carried out before the disappearance of the crystals which may appear at +5C (± 3C) as long as the whole 100 ml bottle is used. However, if the whole amount is not to be used immediately, prepare only 400ml of wash solution per plate tested (i. e.: dilute 20 ml “Wash concentrate (20x)” in 380 ml water).
b) Empty the contents of the plate by inversion or by another manual or automatic method.
c) Fill all the wells of the plate with wash solution; then empty them again.
d) Repeat step c) (a total of 2 washes).
Note: When performing the washes manually, empty the plates completely by tapping them upside-down on an absorbent towel.

6.3 DISTRIBUTION OF THE CONJUGATE
a) Dilute the conjugate to 1/100 in "Dilution buffer 24" (i.e.: 120 l conjugate in 11.88 ml “Dilution buffer 24” for one plate).
b) Distribute100µl of diluted conjugate in each well.
c) Cover the plate with a lid, aluminium foil or adhesive and leave to incubate for 30 minutes (± 3 minutes) at 37C (±3C) under gentle agitation.

6.4 WASHING
a) Empty the content of the plate by returning or by another manual or automatic method.
b) Fill all the wells of the plate with the wash solution; then empty them again.
c) Repeat step b) twice (a total of 3 washes). Note: The care brought to the last washing is essential for a good implementation of the test. If the washing is manual, the plate can be inverted and tapped on an absorbent support, in order to completely empty the wells after the last washing.

6.5 REVELATION
a) Distribute 100µl of ready to use "TMB substrate 3" per well.

b) Cover the plate and incubate for 30 minutes (±10 minutes) at +37C (±3C) under gentle agitation.
c) Add 100µl of "Stop solution" per well.
d) Gently shake the plate until the colored solution is homogenized. Wipe carefully the bottom of the plate.
Notes:
1. A 30–minute incubation with the substrate provides the O.D. values given in the paragraph "INTERPRETATION" when implemented in our laboratories. However, the rate of color development can be slightly modified by different factors (quality of the washes and of the water used, pipetting precision, temperature of the reaction...). Depending on working conditions, assay development may result in OD values higher or lower than those expected. Therefore, the user may stop the reaction after 30 minutes 10 minutes.
2. Reading may be performed up to 1 hour after the reaction has been stopped as long as the plates are kept in the dark.

7. RESULT INTERPRETATION

The Optical density (OD) to be read in an ELISA reader at 450 nm and the cut off points to be calculated to validate the results. All sera with percentage Inhibition (PI) > 50% should be considered as positive. Sera with PI between 40-50% to be considered doubtful and those sera with PI less than 40% should be considered as negative (cELISA-Version P05410/02 from CIRAD / Institut POURQUIER).

$$\frac{\text{OD Sample} - \text{OD negative}}{\text{OD Positive serum} - \text{OD negative serum}} \times 100 = \text{OD\%}$$

$$\frac{\text{OD Cm} - \text{OD Test}}{\text{OD Cm} - \text{OD Cc}} \times 100 = \text{PI (Percentage inhibition)}$$

VALIDATION CRITERIA

- The reaction is considered valid when the following criteria are obtained:
- The OD of **Cm** must be between 0.5 and 2.0 (preferably near 1.0)
- The OD of **Cc** must be below 0.3
- The PI of CN must be equal to or lower than 35%
- The PI of CP+ must be between 50 and 80%
- The PI of CP++ must be between 60 and 90%

8. INTERPRETATION

8.1 Sera with a percentage of inhibition equal to or lower than **40%** are considered negative.
8.2 Sera with a percentage of inhibition between **40 and 50%** are considered doubtful.
8.3 Sera with a percentage of inhibition equal to or greater than **50%** are considered positive.

9. WASTE DISPOSAL

Consider as infectious and toxic materials hence, dispose the samples and kits properly after the test.

10. RISK ASSESSMENT

10.1 Never pipette by mouth.
10.2 Stop dilution contains 0.5M H_2SO_4 that causes serious burns.
10.3 Avoid contact of the substrate (TMB) with skin mucous membrane and eyes.
10.4 Control sera contains sodium azaide and may be toxic if ingested.

11. TROUBLESHOOTING

NA

12. REFERENCES

Antibodies to Mmm CBPP serum is based on competitive ELISA - Version P05410/02 from CIRAD / Institut POURQUIER.

TITLE: ELISA for detection of Bovine Viral Diarrhoea Virus (BVDV) Antigen

PREPARED BY: Serology section

REVISED BY: Serology section

APPROVED BY: Head, LSU

DATE: 11.06.2018

1. INTRODUCTION
Bovine Viral diarrhoea virus (BVDV) are one of the member virus of Pestivirus with in the family Flaviviridae. BVDV is one of the most important pathogenic virus in cattle causing considerable losses in dairy and beef industries worldwide. The typical symptom of BVDV infections are diarrhoea, fever followed by reduction in milk production.

2. PRINCIPLES
IDEXX BVDV Ag/Serum Plus is an enzyme immunoassay designed to detect BVDV antigens in serum bovine serum, plasma, whole blood and ear notch tissue samples. A microtitration format has been configured by immobilising specific monoclonal antibodies for BVDV (Ems) on the plates. BVDV Ag of the sample is captured on the plates. After incubation of the test sample in the well, captured BVDV Ag is detected by specific antibodies and a horseradish-peroxidase conjugate. Next unbound conjugate is washed away and a substrate/chromogen solution is added. In the presence of enzyme, substrate id converted into a product which reacts with chromogen to generate blue colour. Upon addition of the stop solution, a yellow colour is generated. The absorbance at a single wavelength of 450nm or a dual wavelength of 450nm and 650nm is measured using spectrophotometer. The corrected OD value of the sample is calculated by using the absorbance obtained with the test sample and corrected for the absorbance of the negative control.

3. APPLICATION
IDEXX BVDV Ag/Serum Plus is IDEXX's enzyme immunoassay for the detection of BVDV antigens in bovine serum, plasma, whole blood and ear notch tissue samples.

4. OBJECTIVE
To describe the procedure for IDEXX BVDV Ag/Serum Plus is IDEXX's enzyme immunoassay for the detection of BVDV antigens in bovine serum, plasma, whole blood and ear notch tissue samples.

5. APPARATUS/TEST KIT/REAGENTS

1	Anti-E^{ms}mAb Coated Plates	2	5	30
2	Positive Control	1.6 mL	2 mL	6.5 mL
3	Negative Control	1.6 mL	2 mL	6.5 mL

4	Conjugate	25 mL	60 mL	350 mL
5	Ear Notch Tissue Soaking Buffer	80 mL	2×80 mL	2× 480 mL
6	TMB Substrate N. 12	20 mL	60 mL	400 mL
7	Stop Solution N. 3	20 mL	60 mL	400 mL
8	Wash Concentrate (10 ×)	125 mL	480 mL	3 × 480 mL
9	Detection Solution	15 mL	30 mL	180 mL

Materials required but not provided:

1. Centrifuge (capacity 2000xg)
2. Precision micropipettes and multichannel micropipettes (the precision required must be lower than or equivalent to 5% for all the other volumes indicated)
3. Disposable micropipette tips
4. Microplate shaker
5. Distilled water or deionized water
6. Microplate washer
7. Microplate covers (lid, aluminium foil or adhesive)
8. 96-well microplates reader equipped with a single wavelength filter of 450nm or dual wavelength of 450nm and 650nm.
9. Humid chamber/incubator capable of maintaining temperature of about $\pm 37^0$C ($\pm 3^0$C)
10. Tubes for soaking ear notch tissue samples.

6. TEST PROCEDURE

Preparation of Reagents

Wash Solution

The Wash Concentrate (10×) should be brought to 18-26^{0}C and mixed to ensure dissolution of any precipitated salts. The wash solution (10×) must be diluted 1 to 10 with distilled/deionized water before use (e.g., 30 mL of wash Concentrate (10 ×) plus 270 mL of water per plate to be assayed). When prepared under sterile conditions, the Wash Solution can be stored for one week at 2-8^{0}C.

Preparation of Samples

Fresh or frozen serum, plasma, whole blood or ear notch tissue samples can be tested.

Ear Notch Tissue Samples

. Use ear notch tissue plugs (samples) of 2-3 mm diameter in size (e.g., sampled by applying ear tags with attached ear notch tissue sampling device).
. If applicable, ear notch samples can be remain in the sampling device for incubation.
. **Note:** Fresh, humid, desiccated or frozen ear notch tissue samples can be tested.
. Add 150-250ul of IDEXX Ear Notch Soaking Buffer to the ear notch tissue. Make sure the tissue sample is completely covered with the solution (gently tap or mix).
. Seal the tube and allow the samples to soak in soaking buffer between 12 and 24 hours at 18-26^{0}C or over weekend up to 72 hours at 18-26^{0}C (or at 2-8^{0}C) in a humid chamber.
. Aspirate 50ul of the soaking buffer foe testing.

Note: Remaining soaking buffer can be separated from the ear notch tissue sample and stored frozen (-20^{0}C) for later testing or for retesting.

Test Procedure

All reagents must be allowed to come to 18-26^{0}C before use.

Reagents should be mixed by gently swirling or vortexing. Use a separate pipette tip for each sample.

1. Obtain coated plates and record the sample position on a worksheet.
2. Add 50uL of the Detection antibodies to each well. A multichannel pipette (8-12 Channel) can be used for this step.
3. Add 50 µL Negative Control into the appropriate wells.
4. Add 50 µL Positive Control into the appropriate wells.
5. Add 50 µL samples into the remaining wells. Use a separate pipette tip for each sample.
6. Mix the content of the microwells by gently tapping the plate or use a shaker for microtiter plates.
7. Incubate for 2 hours (± 5 mins.) at 37^{0}C (± 3^{0}C) or overnight (12-18 hours) at 2-80C (in a refrigerator). With either option, the plates should be tightly sealed or incubate in a humid chamber using plate covers to avoid any evaporation.
8. Aspirate the liquid contents of all the wells into an appropriate waste reservoir.
9. Wash each well with approximately 300 µL of Wash Solution five times. Aspirate the liquid contents of all the wells after each wash. Following the final aspiration, firmly tap residual wash fluid from each plate onto absorbent material. Avoid plate drying between washes and prior to the addition of the next reagent.

   Important: Control carefully that no traces of blood are left on the walls or edges of the wells**.** Additional 2-3 washes can be necessary to remove the blood before proceeding to the next step.
10. Dispense 100 µL of Conjugate into each well.
11. Incubate for 30 minutes (±2 min.) at 18-26^{0}C.
12. Repeat steps 8 and 9.
13. Dispense 100 µL of TMB Substrate N. 12 solution into each well.
14. Incubate for 10 minutes (±1 min.) at 18-26^{0}C in darkness. Begin timing after the first well is filled.
15. Dispense 100 µL of stop solution N. 3 into each to stop the reaction. Add the stop solution in the same order as the substrate solution was added in step 13.
16. Blank the spectrophotometer on air.
17. Measure and record the absorbance of the samples and controls at 450 nm or using a dual wavelength of 450 nm and 650 nm.
18. Calculate the results.

For the assay to be valid, the difference (P-N) between the Positive Control mean (PCX) and the Negative Control mean (NCX) must be greater then or equal to 0.150 optical density (OD). In addition, the Negative Control mean (NCX) must be less then or equal to 0.250 OD.

For invalid assays, techniques may be suspect and the assay should be repeated following a through review of the package insert. The presence or absence of BVDV antigen in the sample is determined by the corrected OD value (S-N) for each sample.

See the Calculations section for examples.

Note: IDEXX has instrument and software systems available that calculate means and S-N and provide data summaries.

Calculation

Calculation of Negative Control mean (NCX)

NCX= NC1 A450 + NC A450/2

Example:

0.056+0.060/2=0.058

Calculation of Positive Control mean (PCX)

PCX= PC1 A450 + PC2 A450/2

Example:

1.100 + 1.090/2 =1.095

Calculation of test samples

S-N= Sample A_{450}- NCX

Example sample A_{450}=1.558

S-N1.558-0.058= 1.500

9 RESULT INTERPRETATION

Serum, Plasma and Whole Blood Samples

Samples with S-N values equal or less than 0.300 are classified as **negative** for BVDV Ag.

Samples with S-N values greater than 0.300 are classified as **positive** for BVDV Ag. **Positive** results from this assay are valid for calves for any age. Circulating high titers of maternal BVDV antibodies might interfere with the detection of BVDV antigen in serum, plasma and whole blood. Detection of BVDV antigen in serum, plasma and whale blood samples can be less sensitive after antibody intake via colostrum. “False-negative” results can occur after colostrum intake (“diagnostic gap”). In order to exclude influence of colostrum antibodies, it is recommended to test calves before colostrum intake. Negative results of calves after colostrum intake should be confirmed by re-testing at age of more than 30 days. Refer to regulation in your country if different from this description.

Ear Notch Tissue Samples

Samples with S-N values equal or less than 0.200 are classified as **negative** for BVDV Ag.

Samples with S-N values greater than 0.200 but equal or less than 0.300 are considered **suspect** and should be retested.

Samples with S-N values greater than 0.300 are classified as **positive** for BVDV Ag. **Suspect** samples should be retested by using another 50 µL of the same soaking buffer. If there is less than 50 µL remaining, retesting can be done by soaking a second time the ear notch tissue sample with 250 µL of new soaking buffer between 12 and 24 hours at 18-26^{0}C in a humid chamber. If the sample tests are suspect again, a blood sample

should be taken and tested by IDEXX BVDV Ag/Serum ELISA Plus, virus isolation or PCR for BVDV. If there is any doubt about the status of a valuable, positive live animal, retest another sample collected 7-14 days after the initial sample was collected to confirm persistent infection. We recommend that positive results obtained with whole blood should be confirmed using serum or plasma from the same animal. When testing ear notch samples, there is no diagnostic gap.

10 WASTE DISPOSAL

All wastes should be properly decontaminated prior to disposal. Dispose as per the SOP.

11 RISK ASSESSMENT

- Control, TMB substrates and wash concentrate solutions can cause eye irritation
- Stop solution can cause severe skin burns and eye damage

12 TROUBLESHOOTING

NA

13 REFERENCES

IDEXX BVDV Ag/Serum Plus manual version 06-43860-08

TITLE: ELISA test for *Leptospira interrogans serovar hardjo*

PREPARED BY: Serology section

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APPROVED BY: Head, LSU

DATE: 11.06.2018

1. INTRODUCTION
Leptospirosis is a contagious disease of animals and human caused by infection with the spirochete *Leptospira*. Diagnosis of Leptospira through isolation of bacteria is cumbersome and time consuming henace, the detection of antibody is the method of choice.
The ELISA kit (Cypress) is used for detection of antibodies in serum and milk against *Leptospira interrogans serovar hardjo (L. Hardjo).*

2. PRINCIPLES
A microtitre plate is coated with inactivated antigen. Serum/milk are samples are dispensed in coated wells of the microtitre plate. Antibodies directed against L. hardjo that are present in the test samples will bind to the antigen during incubation. The bound antibodies are detected using an anti-bovine monoclonal antibody conjugated to the enzyme horseradish-peroxidase. Subsequently, the bound conjugate is visualized by incubation with the chromogen (TMB) substrate. After the chromogen (TMB) substrate incubation addition of the stop solution will terminate the colour. The OD can be measured in an ELISA reader. Test results are expressed in percentage positivity (PP).

3. APPLICATION
The ELISA kit (Cypress) is used for detection of antibodies in serum and milk against *Leptospira interrogans serovar hardjo (L. Hardjo).*

4. OBJECTIVE
To describe the procedure for detection of antibodies in serum and milk against *Leptospira interrogans serovar hardjo (L. Hardjo).*

5. APPARATUS/TEST KIT/REAGENTS
Materials provided in the kit

5.1 Five coated test plates
5.2 Conjugate, 30x concentrated
5.3 Lyophilized reference serum 1 (=positive serum)
5.4 Lyophilized reference serum 2 (=negative serum)
5.5 Lyophilized reference serum 3 (=weak positive serum)
5.6 Lyophilized horse serum
5.7 Dilution buffer, 5x concentrated
5.8 Chromogen (TMB) substrate solution (ready to use)

5.9 Demineralized water
5.10 Washing solution, 200x concentrated
5.11 Stop solution

Additional materials required

5.12 Dummy plates to make pre-dilutions of milk and serum samples. U-bottom shaped plates are recommended, however, also other non-binding plates or tubes can be used
5.13 General laboratory equipment
5.14 Plate reader 450nm filter
5.15 Plate washer (optional)
5.16 Plate sealer

6. TEST PROCEDURE

1. Solutions to be made in advance
   a. Dilution buffer working solution
      - The concentration dilution buffer must be diluted 5 times (1 part concentrated dilution buffer + 4 parts demineralised water); e.g. for 1 plate prepare 55 mL (add 11 mL concentrated dilution buffer to 44 mL demineralised water). The total volume of dilution buffer that can be prepared is 300 mL. The dilution buffer working solution is stable for 4 hours at room temperature (20-25^{0}C).

   b. Horse serum
      - Reconstitute horse serum 100 times with 3, 5 demineralised water (supplied in the kit). The reconstitute horse serum can be stored at -20^{0}c until expiry date.

   c. ELISA buffer
      - Dilute reconstituted horse serum 100 times in dilution buffer working solution; e.g. for 1 plate prepare 40 ml (add 400µl horse serum to 39.6 ml dilution buffer working solution). The ELISA buffer can be stored up to 4 hours at room temperature (20-25^{0}C).

   d. Conjugate dilution
      - Dilute the concentrated conjugate solution 30 times in dilution buffer working solution; e.g. for 1 plate prepare 12 ml (add 400µl horse serum to 11.6 ml dilution buffer working solution).
      - NOTE: The conjugate dilution is not stable, prepare just before use.

   e. Reference sera
      - Reconstitute each reference serum with 0.5 ml demineralised water (supplied in the kit).
      - Reconstituted reference sera are preferably aliquot and can be stored at -20^{0}C until expiry date. Mix sera gently after thawing and do not refreeze.

   f. Reconstitution of lyophilised reagents should be performed as follows:
      1. Equilibrate the vials at room temperature
      2. With the vials in an upright position, tap the vial gently against the worktop to ensure that the content is on the bottom of the vial.

3. Open the vial
4. Add the specific amount of demineralised water (see label on vial).
5. Allow the lyohilised material to dissolve.
6. Replace the stopper on the vial and gently agitate so that any remaining dry material will be dissolved.
7. Allow the lyophilised material to stand at least 15 minutes at room temperature. Occasionally gently invert vial.

g. Washing solution

- Dilute the concentrated washing solution 200 times in demineralised water; e.g. for 1 plate prepare 400ml diluted washing solution (add 2ml concentrated washing solution fluid to 388ml demineralised water). The total volume of diluted washing solution that can be prepared is 12 litres. The diluted washing solution is stable for 1 week when stored at room temperature (20-25^{0}C).

Test procedure

6.1 **Pre-treatment of test plates (for milk samples only)**

a. Dispense 100 µl ELISA buffer to all wells of the test plates(s).
b. Seal or cover the test plates and incubate for 1 hours (± 2 minutes) at 37^{0}C.
c. Discard the ELISA buffer and wash the test plate(s) 6X with washing solution.

6.2 Pre-diluted(1:20 of reference sera

a. Dispense 190µl ELISA buffer to the wells of column 1 of the dummy plate.
b. Dispense 10 µl of the reference 1 to the wells C1 and D1 of the dummy plate.
c. Dispense 10 µl of the reference 2 to the wells E1 and F1 of the dummy plate.
d. Dispense 10 µl of the reference 3 to the wells G1 and H1 of the dummy plate.

6.3 Pre-dilution of test sera

a. Dispense 190 µl ELISA buffer to the wells of columns 2 to 12 of the dummy plate.
b. Dispense 10 µl of test serum to the wells of columns 2 to 12 of the dummy plate according to your test schedule.
c. Shake the dummy plate.

6.4 Dispensing of the reference samples to the test plate

a. Dispense 100 µl buffer to wells A1 and B1 (blanks) of the test plate(s).
b. Dispense 90 µl ELISA buffer to the remaining wells of column 1 (C1 to H1) of the test plates.
c. Transfer 10 µl of 1:20 pre-diluted reference 1 from the dummy plate to wells C1 and D1 of the test plates (final dilution 1:200)
d. Transfer 10 µl of 1:20 pre-diluted reference 2 from the dummy plate to wells E1 and F1 of the test plates (final dilution 1:200)
e. Transfer 10 µl 1:20 pre-diluted reference 3 from the dummy plate to wells G1 and H1 of the test plates (final dilution 1:200).

6.5 Dispensing of test samples

a. Only for serum samples

Dispense 90 µl ELISA buffer to the remaining empty wells of the test plate.
Transfer 10 µl of the pre-diluted sera from the dummy plate to the appropriate wells of the test plate (final dilution 1:200) according to your test schedule (e.g. sample 1 in A2, S2 in B2, etc).
Serum samples can be titrated by making two-fold dilutions in dilution buffer.
Cover, then shake the test plates gently and incubate for 1 hour (± 5 minutes) at 37^{0}C.
Continue from 6.

b. Only for individual Milk samples

Dispense 75 µl ELISA buffer to the remaining empty wells of the test plate.
Dispense 25 µl milk of a test plate (to achieve a final dilution 1:4) according to your test schedule. Take defatted milk from underneath the creamy layer.
Cover, then shake the test plate gently and incubate for 1 hour (±5 minutes) at 37 ^{0}C.
Continue from 6.

c. Only for Bulk milk samples

Dispense 100 µl of (undiluted) milk samples to the remaining empty wells of the plate according to your test schedule. . Take defatted milk from underneath the creamy layer.
Cover, then shake the test plate gently and incubate for 1 hour (±5 minutes) at 37 ^{0}C.
Continue from 6.

6.6 Incubation with conjugate

Wash the plate 6 times with diluted washing solution. Dispense 100 Cover, then shake the test plate gently and incubate for 1 hour (±5 minutes) at 37 ^{0}C.
Dispense 100 µl of diluted conjugate to all wells.
Cover the test plate and incubate for 1 hour (±5 minutes) at 37 ^{0}C.

6.7 Incubation with chromogen (TMB substrate solution)

Wash the test plate 6 times with diluted wash buffer solution
Dispense 100 µl of the chromogen solution to all wells.
Incubate the plates for 15 minutes at room temperature (20-25 ^{0}C.
Add 100 µl stop solution
Agitate the plate to mix the content of the well of the test plate prior to measuring.

6.8 Reading the test and calculating the result

Measure the optical density (OD) of the wells at 450 nm within 15 minutes of stopping the colour development.
Calculate the mean OD_{450} value of the blank (well A1 and B1)
Calculate the corrected OD_{450} value of all samples by subtracting the mean OD_{450} of the blanks.

Calculate the percentage positivity (PP) of the reference samples 2, 3 and the test samples according to the formula given below.
The corrected OD_{450} values of all samples are expressed as percentage positivity (PP) r relative to the corrected mean OD_{450} value of reference 1 in wells C1 and D1.

$$\textbf{Percentage positivity (PP)} = \frac{\textbf{Corrected } OD_{450} \textbf{ test sample}}{\textbf{Corrected } OD_{450} \textbf{ reference serum 1}} \textbf{ X 100 \%}$$

Calculate the PP of the test samples

Validation Criteria

The mean of the blanks must be < 0.150.
The corrected OD_{450} of reference 1 must be ≥ 1.000.
The mean percentage positivity (PP) of reference 2 must be < 20%.

The mean percentage positivity (PP) of reference 3 must be between 20% and 60 %.
The above-mentioned criteria have to be met in order to validate the results of test samples.

7. RESULT INTERPRETATION

- Interpretation of the percentage positivity of the test samples

Serum samples
PP < 20 %: Test sample is Negative for L. Hardjo specific antibodies.
PP 20 % - 45 %: Inconclusive (antibodies may be present)
PP > 45 %: Test sample is positive to L. Hardjo specific antibodies

Milk (individual and bulk) samples
PP < 40 %: Test sample is Negative for L. Hardjo specific antibodies.
PP 40 % - 60 %: Inconclusive (antibodies may be present)
PP > 60 %: Test sample is positive to L. Hardjo specific antibodies

Bulk milk samples with a doubtful test result must be retested.
When an inconclusive result is confirmed it may be followed by collection blood samples of the infected herd.

8. WASTE DISPOSAL

Treat it as the infectious material and dispose accordingly.

9. RISK ASSESSMENT

9.1 Handle all reagents and samples as bio-hazardous material.
9.2 Keep all reagents away from skin and eyes. If exposure should occur, immediately flush affected areas with cold water.

9.3 Wash solution, control sera, test plates, field samples and all other test kit reagents should be properly decontami-nated with bleach or other strong oxidizing agent before disposal
9.4 Take special care not to contaminate any of the test re-agents with serum or bacterial agents.
9.5 The best results are achieved by following the protocols as they are described below, using good, safe laboratory techniques.
9.6 Never pipette by mouth. Allow all reagents to come to room temperature before starting.
9.7 Control, TMB substrates and wash concentrate solutions can cause eye irritation
9.8 Stop solution can cause severe skin burns and eye damage

10. TROUBLESHOOTING

Humidity indicators are supplied with each plate. If any of the indicators exhibit a pink color, the plate may be com-promised in some way; decontaminate (i.e. wash the plate with bleach solution) and dispose of the plate.

11. REFERENCES

Leptospira interrogans serovar Harjo, Insert Ref. VB066 2009

TITLE: Antibody ELISA for Classical Swine Fever Virus

PREPARED BY: Serology section

REVISED BY: Serology section

APPROVED BY: head, LSU

DATE: 11.06.2018

1. INTRODUCTION

This diagnostic kit is designed to detect antibodies directed against the nucleoprotein of the Peste des Petits Ruminants (PPR) virus. It can be used with sheep and goat serum or plasma.
The test uses technology developed by a FAO reference laboratory (CIRAD-EMVT, Montpellier,France)

2. TEST PRINCIPLES

The wells are coated with purified recombinant PPR nucleoprotein (NP). The samples to be tested and the controls are added to the microwells. Anti-NP antibodies, if present, form an antibody-antigen complex which masks the NP epitopes. An anti-NP-peroxidase (HRP) conjugate is added to the microwells. It fixes to the remaining free NP epitopes, forming an antigen-conjugate -HRP complex. After washing in order to eliminate the excess conjugate, the substrate solution (TMB) is added. The resulting coloration depends on the quantity of specific antibodies present in the sample to be tested.
In the absence of antibodies, a blue solution appears which becomes yellow after addition of the stop solution. In the presence of antibodies, no coloration appears.
The microplate is read at 450nm.

3. APPLICATION

To test for antibody against Peste des Petits Ruminants (PPR) virus in sheep/goat serum.

4. OBJECTIVE

To describe the procedure for conducting antibody ELISA for PPR.

5. APPARATUS/TEST KIT/REAGENTS

5.1 PPRV Antigen coated plate
5.2 Positive control
5.3 Negative control
5.4 Conjugate 10X
5.5 Dilution Buffer
5.6 Substrate solution
5.7 Stop Solution
5.8 Wash Solution

6. PROCEDURE

Allow all the reagents to come to room temperature (210C ± 50C) before use. Homogenize all reagents by inversion or Vortex.

6.1 Dispense 25 µl of Dilution Buffer 13 to each well.

6.2 Dispense 25µl of the Positive Control to wells A1 and B1.

6.3 Dispense 25µl of the Negative Control to wells C 1 and D1

6.4 Dispense 25µl of each sample to be tested to the remaining wells.

6.5 Incubate 45 min ± 4 min at 37^0C (± 30C)

6.6 Wash each well 3 times with approximately 300µl of the Wash Solution. Avoid drying of the wells between washings

6.7 Prepare the Conjugate 1X by diluting the Conjugate 10X to 1/10 in Dilution Buffer 4.

6.8 Add 100µl of the Conjugate 1X to each well. Incubate 30 min at ± 3 min at 21^0C(± 50C).

6.9 Wash each well 3 times with approximately 300µl of the Wash Solution. Avoid drying of the wells between washings.

6.10 Add 100µl of the Substrate solution to each well. Incubate 15 min at ± 2 min at 210C(± 50C) in the dark

6.11 Add 100µl of the Stop solution to each well in order to stop the reaction.

6.12 Read and record the OD at 450nm.

7. RESULT INTERPRETATION and REPORTING

The test is validated if

- ✓ The mean value of the negative control ODnc is greater than 0.7

  OD nc > 0.700

- ✓ The mean value of the positive control is less than 30% of the OD nc

  OD pc/OD nc < 0.3

Interpretation

For each sample, calculate the competition percentage (S/N %)

$$S/N\ \% = \frac{OD\ sample}{OD\ nc} X\ 100$$

Samples Presenting a S/N %

- ✓ Less than or equal to 50% are considered Positive
- ✓ Greater than 50% and less than or equal to 60% are considered Doubtful
- ✓ Greater than 60% are considered Negative

Result	**Status**
S/N % ≤ 50 %	Positive
50% < S/N %≤ 60%	Doubtful
S/N % > 60%	Negative

8. WASTE DISPOSAL

9. RISK ASSESSMENT

NA

10. TROUBLESHOOTING

NA

11. REFERENCES

Development of competitive ELISA for detection antibodies to the Peste des petits ruminants virus uing a recombinant nucleoprotein. Libeau G, Prehaud C, Lancelot R, Colas F, Guerre L, Bishop DH, Diallo A, Res Vet Sci. 1995 Jan; 58(1): 50-5.

Test categorization (serology/ virology/ Molecular)

Sl. No.	Procedure / SOP	DVL	SVL/ TVH	RLDC/ NVH	NCAH
1	Rapid antigen detection tests - Rapid Ag detection for Influenza Type A - Rapid Ag detection for H5 - Rapid Ag detection for rabies - Rapid Ag detection for IBD - Rapid Ag detection for NCD - Rapid Ag detection for PPR - Rapid Ag detection for CD - Rapid Ag detection for CPV - Rapid Canine *Leptospira* antibody test Rapid antibody detection tests - Rapid Ab detection for FMD - Rapid Ab detection for EI - Rapid Ab detection for Brucella	X	X	X	X
2	Fluorescent Antibody Technique-FAT for rabies		X	X	X
	Immunoperoxidase Test: Rabies immunoperoxidase antigen detection(RIAD)			X	X
3	ELISA –NSP FMD, IBR, BVD, JD, ALC, CSF Ab, PRRS, rabies, NCD, IBD, CSF Ag, Mycoplasma (CBPP) Typing ELISA (Sandwich) for FMD – Type O, A and Asia1, LPB FMD, Equine Infectious anemia			X	X
5	PCR Conventional PCR: - Brucella, HS, FMD rtPCR: - AI Type A,H5N1, NCD, H7, N9, N8, Rabies			X	X
6	Agglutination tests - HA/HI for NCD		X	X	X
	- AGID	X	X	X	X
	- SAT/RBT etc. for *Salmonella*, *Brucella*, mycoplasma, etc.				X