ORBIVIRUS ISOLATION IN EMBRYONATED CHICKEN EGG		
Date: 22/06/2021	Document code: GL-LCV-13	*Rev. 02*

1. SCOPE

To describe the isolation of Orbivirus (African horse sickness virus, Bluetongue virus and Epizootic haemorrhagic disease virus) in embryonated chicken eggs from clinical samples of animals.

This procedure is applicable to samples obtained accordingly to the PROCEDURE 1 (SAMPLE PREPARATION) from:

-Whole blood collected on anticoagulant (EDTA)

-Tissue samples, specially spleen, lung and lymph nodes

2. MATERIALS AND EQUIPMENT

Material and reagents

Plasticware (sterile): 2 ml and 15 ml conical tubes

Sterile disposable plastic syringes (0,5 – 1ml)

Syringe needles: approximately 29G

Manual drill or awl

Scalpel blade

Spatula or cotton swab (to distribute paraffin)

Racks to hold eggs and tubes

Sterile Petri dish

Container biosanitary waste

Container needle waste

Alcohol 70%

Paraffin (to close hole in egg)

Phosphate buffer saline (PBS1X)

Virucidal disinfectant (Virocid 1:50)

Sterile mineral oil

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Positive control: stock Virus, previously growth and full titrated

Embryonated chicken eggs (9-11 days old)

Equipment

Laminar Flow Cabin Type II

Eggs incubator 33 +/- 2ºC Wet: 40 – 60%

Egg candling lamp

Refrigerator (2 - 8º C)

Heater (to maintain paraffin liquid)

3. PROCEDURE

Orbivirus replicates in embryo´s tissues and therefore the intravenous (IV) route is 100 - 1000 times more sensitive than yolk sac route (Goldsmit & Barzilai, 1968).

Preparation of the assay (day/s before)

- Calculate the number of 9 -11 days old embryonated eggs needed to test the samples and organize the distribution of samples. Consider that to analyse each sample in at least three eggs is highly recommended.
- Prepare the samples to test according to PROCEDURE 1 (SAMPLE PREPARATION). Store in refrigeration until inoculation.

Preparation of reagents

- Paraffin: heat until become liquid and maintain

Performance of the assay

- Several 9 -11-day-old ECEs are examined for viability with a strong light source in a dark room. Only viable eggs are selected.

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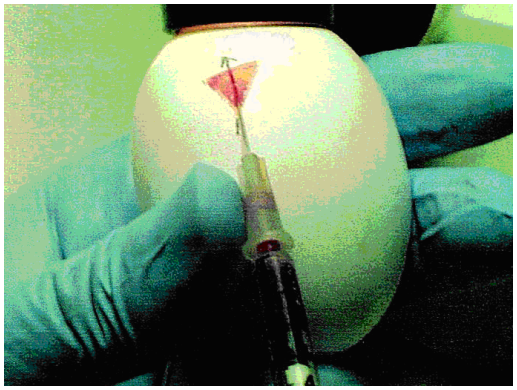
- Mark with a pencil indicating an appropriate (a straight section with no branches) blood vessel to be used for inoculation by drawing a triangle on the shell. The direction of blood flow should also be indicated on the shell so that the inoculum will flow with the blood.
- Moreover mark on the shell the identification of the sample to inoculate, to ensure traceability.
- Using the manual drill, the triangle drawn on the shell is cut by drilling along the pencilled lines just deep enough to go through the shell, but not through the underlying shell membrane.
- The drilled out triangle can be removed by using a sterile scalpel blade. The shell membrane must remain intact.
- Apply sterile mineral oil on the membrane to clear it, making a 'window' into the interior of the egg.
- Inject 0,1 ml of the inoculum using a 1-ml plastic syringe with a 29G needle. For proper IV inoculation of the embryo, the egg is illuminated from above in a dark room employing the egg candling lamp. PBS1X is used as mock inoculum.
- The injection is made by inserting the needle at the right direction (towards embryo) at an acute angle while the egg is held in the other hand beneath the light source. The needle is removed slowly and no bleeding should be present. If bleeding occurs, the egg is discarded and the inoculation is repeated on another egg.
- Positive control must be prepared and handled always after samples and mock inoculum in each phase. Prepare positive control using PBS1X by adding the appropriate volume of stock virus to achieve i.e. 100 $TCID_{50}$.



Inoculation. Image from CLAVIJO A et al 2000

- After inoculating each egg, the 'window' is closed with liquid paraffin employing a spatula or a cotton swab.

<u>Eggs incubation and visualization</u>

- The eggs are incubated for 7 days at 33°C.

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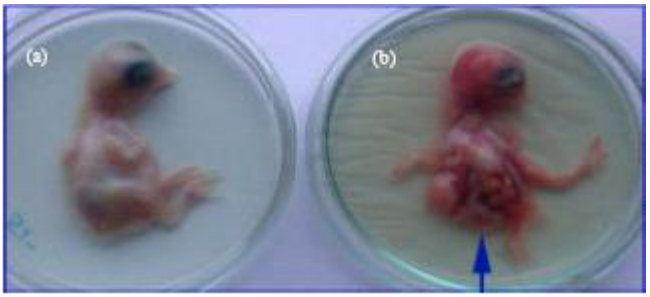
- Examine the eggs daily, by candling lamp and record any death. Eggs dying within 24 h post-inoculation are discarded (death is probably due to bacterial contamination or trauma from inoculation).
- Store the dead eggs at refrigeration, until they are processed. If the embryo, after 7 days of incubation, has not died, it will be kept refrigerated for a period of between 4 and 24 hours, to promote the death of the embryo.

Processing of dead embryonated eggs

- The eggs are disinfected with 70% alcohol and the shell is removed through the air chamber using tweezers and forceps, taking care not to damage the membrane.
- Using a decontaminated forceps, the entire embryo is removed to a sterile Petri dish. Carry out this process with care not to break the yolk sac, as it can affect cell culture, if subsequent inoculation in cell culture is carried out. Egg remains are disposed of in the waste container.
- Using sterile scissors, cut the head and sternum and take the brain, liver, spleen, heart and lungs to continue processing. If the size and state of the embryo does not allow it, then remove beak and legs as possible and process the whole embryo.
- Continue the processing of organs/embryo such as it is described in the PROCEDURE 1 (SAMPLE PREPARATION).



Not affected (a) and haemorrhagic (b) embryo chicken.
SEKAR. P., et al. May 2008

- Analyze the homogenated tissue in a confirmatory test (routinely by serogroup specific rRT-PCR)

4. ANALYSIS AND INTERPRETATION OF RESULTS

Ideally:

-Eggs inoculated with the mock inoculum (negative control) should remain viable after 7 days of incubation, resulting negatives in the confirmation technique.

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-The positive control eggs die within 3 to 6 days, being in a haemolytic state, and the presence of Orbivirus in embryos is positive in the confirmation technique.

NOTE: it is recommended to inoculate several eggs per each sample to increase the chance of isolate the virus, not as a quality control.

However:

-Death or survival of the embryo does not always guarantee failure or success in virus isolation, therefore, it is not an adequate test acceptance criterion.

-On the other hand, the technical difficulty of IV inoculation and the fact that it is an "in vivo" system does not guarantee a positive result of the positive control in 100% of inoculated eggs.

For these reasons, test is accepted if the negative control eggs are negatives and a percentage of positive control eggs are positives in the confirmatory technique.

Results:

Confirmation of presence of viable virus in tissues of the embryo is always mandatory. A serogroup specific quantitative rRT-PCR is used for this purpose:

- Virus isolation is considered as **POSITIVE** when rRT-PCR is positive and the Ct value is significantly lower than Ct value of sample inoculated. In case of any doubt, it is recommended to carry out a passage in cell culture.
- Virus isolation is considered as **NEGATIVE** when rRT-PCR is negative.

Virus neutralization test is a classic method of confirmation and typing, which requires virus producing CPE in mammal cells and much more time than rRT-PCR. This method is used in EURL as an alternative method (not routinely).

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5. REFERENCES

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Chapter African horse sickness (Infection with African horse sickness virus)

Chapter: Bluetongue (Infection with Bluetongue virus)

Chapter: Epizootic haemorrhagic disease (Infection with Epizootic haemorrhagic disease virus)

SEKAR. P., et al. Optimization and characterization of bluetongue virus in embryonated chicken egg. Advanced Biotech. (May 2008)

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