National One Health Mission (NOHM)

National BSL-3 Laboratory Network

Standard Operating Procedures (SOPs) for Investigation of Environmental Samples in BSL-3 Facility

INDEX

Purpose: This SOP describes the procedure for extraction of RNA using Trizol reagent and the QIAmp viral RNA extraction kit**.**

Scope : This SOP is applicable for extraction of RNA from precipitates of environmental water samples.

Requirements:

1. Reagents

a) Trizol reagent
b) Chloroform
c) QIAmp Viral RNA kit
d) Molecular biology grade 70% ethanol

2. Equipment

a) Bench Top refrigerated centrifuge
b) Vortex mixer
c) Micropipettes

3. Consumables

a) Tissue Paper
b) Spirit cotton
c) Blotting paper
d) Sterile 1.7 ml. micro centrifuge tubes
e) Discarding beaker
f) Tube racks
g) Sterile filter tips

Procedure:

1. Add 750 µl Trizol Reagent in 1.5 ml micro centrifuge tube (Use freshly prepared aliquot every time).
2. Take 250 µl each of pre, supernatant and precipitate samples in separate sterile 1.5 ml tubes, containing the Trizol Reagent.
3. Mix by pulse vortexing for 15 seconds and incubate at room temperature for 10 minutes.

4. Briefly centrifuge to remove drops from inside of the lid.
5. Add 200 µl Chloroform in each tube.
6. Vortex the sample and put it back on ice for five mins. Repeat the step three times.
7. Spin the sample at 10956 x g for 15 minutes at 4°C.
8. Take fresh sterile1.5 ml micro centrifuge tubes and label them.
9. Add supernatant to the labeled collection tubes.
10. Add same amount of 70% ethanol to supernatant. Mix by pulse vortexing for 15 seconds & briefly centrifuge to remove drops from inside of the lid.
11. Take columns provided with RNA Extraction Kit and label them.
12. Transfer the above sample to the columns. Spin the columns at 3578 x g for 1 minute at room temperature. Repeat same with remaining sample.
13. Transfer the column to new collection tube provided with the kit.
14. Add 500µl of buffer AW1 to the column and centrifuge at 3578 x g for 1 minute at room temperature. Discard filtrate.
15. Add 500 µl of buffer AW2 to the column and centrifuge at 10956 x g for 3 minutes at room temperature.
16. Discard filtrate and centrifuge column at 10956 x g for 1 minute (Dry spin).
17. Discard filtrate with collection tube and place column in labeled 1.5 ml micro centrifuge tube.
18. Elute the RNA in 50 µl Elution Buffer in 1.5 ml micro centrifuge tube.
19. Store at +4 °C for short period and at -80 °C for a longer period.

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Purpose: Precipitation of Avian Influenza viruses using the novel method using potassium aluminium sulphate

Scope: This is applicable for testing of environmental water samples.

Requirements:

1. **Reagents**

a) Potassium aluminium sulphate (ER, Qualigens)
b) Milk powder (Nestle)
c) Sodium Hydroxide (ER, Qualigens)

2. **Equipment**

a) Sterile Separating funnel and stand for separating funnel
b) Weighing balance
c) Bench centrifuge
d) Vortex mixer
e) Micropipettes

3. **Consumables**

a) Sterile centrifuge tubes, graduated conical 50 ml
b) Sterile 1.7 ml micro centrifuge tubes
c) Sterile 10 ml serological pipettes

Procedure:

Preparation of virus precipitation kit

Reagent A: Weigh 0.2 gm of potassium aluminium sulphate and dissolve in 10 ml of distilled water to attain a final concentration of 0.02%.

Reagent B: Weigh 0.1 gm of milk powder and dissolve in 10 ml of distilled water to attain a final concentration of 0.01%.

Reagent C: 1N Sodium Hydroxide (NaOH) is used to adjust the pH of water to 6.5-7.0.

Precipitation of avian influenza viruses

1. If tap water is used for standardization purpose, keep the water overnight at ambient temperature in an open container to remove residual chlorine, if any.
2. For surface water samples, there is no need of step no. 1.
3. Take 1 L of the water sample in a separating funnel.
4. Measure the pH of the water. Adjust pH in the range of 6.5 to 7.0 with Reagent C, if necessary.
5. After a thorough mixing of water by rigorous stirring, collect 1 mL of the water suspension (pre-sample) in a sterile micro centrifuge tube.
6. Add 10 ml each of Reagent A and Reagent B to the water and mix thoroughly.
7. After addition of these reagents, measure the pH of the water suspension and re-adjust if necessary to the range 6.5 to 7.0 with Reagent C.
8. Keep the water overnight at ambient temperature (20-28 ^{o}C) for precipitation.
9. Collect supernatant (1 ml) in a sterile micro centrifuge tube for testing, to ensure settling of the virus in the precipitate.
10. Collect the precipitate in a sterile 50 ml conical centrifuge tube and centrifuge at 894 x g for 2 min at 4 °C to obtain a pellet.
11. Discard the supernatant and resuspend the precipitate in 5 ml sterile distilled water (pH 6.5) and homogenize the precipitate using a vortex mixer.
12. Dilute the resuspended precipitate 10-fold in sterile distilled water and use 250 µL of the diluted precipitate samples for extraction of the viral RNA. Refer AI/SOP/T09 for RNA extraction.
13. Decontaminate the separating funnel and other material by autoclaving.

End of the document

Isolation of avian influenza viruses in embryonated chicken eggs

Standard Operating Procedure (SOP)

(Avian Influenza)

Objective: Isolation of Avian Influenza viruses from fecal dropping, cloacal swabs, organs of migratory birds, fowls etc. by egg inoculation.

Requirements:

10-12 days old fertile chicken eggs (Specific Pathogen Free)
Sample processed in VTM

Equipment:

1. -80 °C freezer
2. Egg incubator
3. 2-8 °C Refrigerator
4. Egg candler etc.
5. Egg driller
6. Sterile scissors, forceps
7. Micropipettes and tips

Consumables:

1. 1 and 2 ml disposable syringes
2. Cryobox, 2ml storage vials
3. 50 ml sterile tubes
4. Ice

Principle:

In an embryonated chicken egg, immediately under the shell is the shell membrane, a tough fibrous material which lines entire shell but is readily separable from it, at the blunt end of the egg, the shell membrane forms the air sac. The chorioallontoic membrane is highly vascular and serves as the respiratory organ of the embryo lies directly under the shell membrane throughout the entire egg. The chorioallontoic membrane is separated from the amnion by the allantoic cavity which contains about 5-10 ml of allantoic fluid. The amniotic membrane forms a sac which encloses the embryo, contains approx. 1 ml of the amniotic fluid. Attached to the embryo is the yolk sac containing nutrients for the developing embryo.

Egg inoculation is done usually by four different routes viz.
1) Amniotic route 2) Allantoic route,
3) yolk sac route and 4) Chorioallantoic membrane route.

The allantoic route is preferred for inoculation of avian-origin samples. This route is employed for primary isolation of Influenza A and B viruses. Virus introduced in the allantoic cavity multiplies in the endodermal cells of the chorioallontoic membrane with subsequent release into the allantoic fluid so that both the membrane and the fluid can be used as a source of virus.

Precautions:

1. Strictly follow guidelines for working in the BSL3 laboratory
2. Check eggs by candling before proceeding with inoculation.

Procedure
Before entering the BSL3 laboratory:

1. Candle and select 10-12 days old active and well vascularized chicken embryos.
2. Make a pencil mark on an area over the air sac near the membrane boundary opposite the embryo where there are few or no major blood vessels.
3. Label the Eggs with E number.
4. Carry all consumables required inside the BSL3 laboratory.

Egg Inoculation:

1. Candle and mark the 9 to 11-day-old embryonated chicken eggs. Check for viability. Identify the position of the embryo.

2. Switch on biological safety cabinet (BSC). Let the flow run for 10 min. before starting the work.

3. Keep all the required material inside the BSC in place.

4. Arrange eggs and corresponding specimen vials to be inoculated in corresponding order. Spirit swab all the eggs. Make a small hole, using sterile needle or push-pin, in the air sac region at the opposite side of the embryo.

5. Mock inoculate control egg with transport medium.

6. Wipe the mouth of the sample vial with spirit. Take out 0.2ml of sample from specimen vial in a sterile 1 ml insulin syringe fitted with needle (26-gauge, 1 inch) and deposit the inoculum gently into the respective eggs, withdraw the needle and seal the holes completely with the help of fevicol (glue).

7. Wipe the gloves with spirit before inoculating each sample. If wetting of the gloves with specimen is suspected, discard and wear fresh gloves and proceed further.

8. Incubate these eggs at 37 °C and observe daily for dead eggs. Separate dead eggs and keep at 4 °C after incubation period of 72 hrs transfer all the eggs to 4 °C even if they are alive.

9. Fumigate the BSC after each use.

Harvesting of eggs

1. Chill the egg overnight at 4 °C or for 2 hours at -20 °C to kill the embryo and to induce contraction of the blood vessels to prevent bleeding.

2. Place the chilled egg in egg rack with blunt end up and wipe the area with spirit swab and break shell over the air sac with sterile scissors and forceps.

3. Harvest the allantoic fluid with a sterile pipette or syringe into a 50 ml tube.

4. Aliquot the harvested fluid in 0.5 ml aliquots in 2.0 ml cryo vials.

Quality Control

SPF eggs obtained from commercial sources are passed through quality control checks

Documentation

1. E-Card for Egg Inoculation

E.No. **Age – 10 days old** **Date** **By**

Specimen no. **Material**

Passage

DIL **in** **Dosage** **ML** **Route-allantoic**

							HA T I T E R			
	0	1	2	3	4		Horse RBC S	Fowl RBC S	H.A.No.	Date
0						1				
1						2				
2						3				
3						4				
4						5				
5						6				
6										

Keep at + 4°C

Isolation of avian influenza viruses in the Madin Darby Canine Kidney (MDCK) cell line

Standard Operating Procedure (SOP)

(Avian Influenza)

OBJECTIVE: Isolation of Avian Influenza viruses from fecal dropping, or other environmental samples. by cell Culture.

REQUIREMENTS:

MDCK cell line (less than passage level 25)

EQUIPMENT REQUIREMENTS:

1. Inverted Microscope
2. CO_2 Incubator
3. Laminar flow
4. 37 °C water bath
5. -80 °C deep freezer
6. Refrigerator

CONSUMABLES REQUIREMENTS:

1. T25 flasks
2. Pipettes
3. MEM
4. TPCK (0.5 mg/ml)
5. Nystatin (1.25 X 10^4 units/ml)
6. 100 ml sterile bottle
7. N-95 Mask
8. Gloves
9. Spirit Swab
10. Discard jars

VIRUS GROWTH MEDIUM: Add 0.2 ml. TPCK trypsin, (final conc. 2 µg/ml.) and Nystatin (final conc. 50 units/ml) to 50 ml. MEM in sterile 100ml bottle.

PROCEDURE:

Isolation of Avian Influenza virus from field specimens is carried out in two parts. First part can be carried out in clean cell culture laboratory in BSL 2 cabinet. The second part of infecting the bottles is carried out in BSL 3 laboratory.

PART I:

1. 48-72 hrs. T 25 flask of MDCK cell line is examined under the microscope for uniform monolayer formation and make sure that the cells are healthy, confluent and free from microbial contamination.

2. Remove MEM from fridge and TPCK trypsin vials and Nystatin vials from -20 °C freezer.

3. Switch on BSC UV for 15 min. before starting the work. After 15 min. switch off laminar UV and switch on laminar flow, light and exhaust. Let the flow run for 10 min. before starting the work.

4. Remove aliquoted specimens from -80^{0}C freezer, thaw quickly at room temperature and place on wet ice.

5. Wipe media bottles and vials with spirit swab.

6. Discard growth medium from T25 flask with monolayer MDCK culture. Wash bottle with 2ml growth medium twice.

PART II:
PRELIMINARY PREPARATION BEFORE ENTERING THE BSL3 LABORATORY:

a. Arrange the samples to be inoculated in order on a rack with ice.
b. Label the culture flasks with specimen no.
c. Carry all required things such as micro tip box, ice and virus growth medium, inside the BSL3 laboratory.

Procedure:
1. Switch on the BSC. Let the flow run for 10 min. before starting the work. Swab the work area with spirit.

2. Keep all the required material inside the BSC in place.

3. Arrange the flasks and corresponding specimen vials to be inoculated in corresponding order.

4. Mock inoculate control bottle with transport medium.

5. Wipe the mouth of the sample vial with spirit. Take out 0.5ml of sample from specimen vial and add gently into the flask. Make sure lid is replaced tightly. Spread the sample

evenly on the cell sheet by tilting the flask.

6. Before inoculating the next specimen, wipe gloves with spirit and proceed. If wetting of the gloves with specimen is suspected, discard and wear fresh gloves and proceed further.

7. Allow the inoculum to adsorb for 1 hr. at 37^{o}C with intermittent shaking.

8. After 1 hr. add 4ml of virus growth medium to each bottle. Incubate at 37^{0}C temperature and 5% Co2.

9. Fumigate the BSC after each use.

Analysis and Interpretation

Observe the flask daily for following:

a) **TOXICITY:** Cell cultures show rapid degeneration within 1-2 days of inoculation. Subject such sample to HA test. If HA activity is observed, subject to 2nd passage.

b) **CONTAMINATION:** Cell culture medium shows high turbidity or fungal culture. Discard such culture and Millipore filter second aliquot of sample before inoculation.

c) **CYTOPATHIC EFFECT:** Cells become progressively granular, swollen and round. Amount of floating cells increases significantly. The CPE is graded from 1+ to 4+ i.e. from 25% to 100%. Keep such samples at 4^{o}C till the H.A. is performed.

d) **NO EFFECT:** Cells remain healthy. Harvest supernatant and perform H.A. Pass the supernatant from the entire flask to fresh T25 flask for second passage on 7th day.

Documentation:

K-Card for Tissue Culture Inoculation

K FLU No.----------Date----------By----------

Specimen No.----------Material----------

Vol.----------ml. Dil.---------- Bottle/T25FL/S.V.-------Cell Culture----------

Medium-MEM(E)+Nyst+ 2 ug C.T./ml+ ina. FCS----------Passage---------

	Number of days																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
No of bottles	1																		
	2																		
	3																		
	4																		
	5																		

HA TITER				
	Horse RBCs	Fowl RBCs	H.A. No.	Date
1				
2				
3				
4				
5				

Quality Control

1. Over a number of passages, MDCK cells might lose their susceptibility to respiratory viruses. For this reason, the laboratory should keep a stock of cells at a low passage level frozen in liquid nitrogen with 7.5% Dimethyl Sulfoxide (DMSO) and 15% fetal bovine serum.

2. After 15-20 passages of MDCK cells, a new vial of cells must be thawed out, if sensitivity of the isolation system is to be maintained.

3. At intervals, the sensitivity of the MDCK cell line must be assessed using positive control virus of known titre.

4. Cell lines should be free of mycoplasma contamination.