National Institute of Hygiene and Epidemiology

No1- Yersin Street - Hanoi-Vietnam

Report for evaluation the inactivation performance of Daikin's streamer technology to avian influenza A/H5N1.

(# 1 Evaluation to clade 1-HN30408)

Performance period: March,10th to Arp, 16th 2009

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1. Objective

The goal of study is to provide information of effective available of Daikin's streamer technology to against highly pathogenic avian influenza A/H5N1 viruses (HPAI/H5N1)

2. Materials and Method

2.1.Materials

- Chamber (be provided by Daikin).
- Petri dish (50mm diameter).
- Timer.
- Plastic consumables: pipette 5ml, 10 ml,
- Media: DMEM, BSA fraction V, Penicilline-Streptomycine, Hepes solution…
- Chemical: Acetone (Merck), Sulfuric acid (H2SO4), PBS pH7.4
- Stock virus

2.2.Methods:

2.2.1 Virus titration

- **Stock Virus :**
  - Virus strains clade 1- HN 30408
- **Virus Titration**
  1. Thaw an ampoule of virus. Micro neutralization test uses only a virus that has been freeze-thawed once.
  2. Dilute virus 1/100 in *virus diluents* (100 µl virus + 9.9 ml virus *diluents*).
  3. Add 100 µl of *virus diluents* (with or without TPCK-trypsin, 2 µg/ml*) to all wells, except column 1, of a 96-well tissue culture plate. (Perform titration of

virus in quadruplicate cultures).

4. Add 146 µl virus of 1/100 working virus (2) to column 1. Perform 1/2 $\log_{10}$ dilutions of virus
5. Transfer 46 µl serially from column 1→2→3→…11. Dilutions will be 10^{-2},$10^{-2.5}$,10^{-3}…10^{-7}. Column 12 is cell control. Incubate virus at 37°C in 5% CO_2 for 1 hr.
6. Results :

   - Virus strains clade 1- HN 30408 : 10.000 $TCID_{50}$/ ml

2.2.2. Experimental performance

a. Setting up streamer system.

- Put 5 ml of virus solution with concentration from 10.000 $TCID_{50}$/ ml into Petri dishes.
- Remove the ceiling board from the chamber
- Make sure the streamer be turn off
- Remove the ceiling board of streamer
- Set up 4 Petri dishes of virus solution into the chamber.
- Return the ceiling board of streamer
- Cover the chamber by ceiling board of the chamber.
- Turn on the streamer.

Take out Petri dish at 1 hour different of incubation (1, 2, 3, 4 hours).

b. Evaluation the efficient of streamer system by checking titer of viruses

- Collect viruses from Petri dishes following different times.
- Dilute virus 1/100 in *virus diluents* (100 µl virus + 9.9 ml *virus diluents*).

- Add 100 µl of *virus diluents* (with or without TPCK-trypsin, 2 µlg/ml*) to all wells, except column 1, of a 96-well tissue culture plate. (Perform titration of virus in quadruplicate cultures).
- Add 146 µl virus of 1/100 working stock to column 1. Perform 1/2 $\log_{10}$ dilutions of virus
- Transfer 46 µl serially from column 1→2→3→...11. Dilutions will be 10^{-2},$10^{-2.5}$,10^{-3}...10^{-7}. Column 12 is cell control. Incubate virus at 37°C in 5% CO_2 for 1 hr.

Preparation of MDCK Cells

- Check MDCK cell monolayer (should be 70-95% confluent). **Do not allow to overgrow**. Typically, a confluent 162 cm^2 flask should yield enough cells to seed up to 7-10 microtiter plates (~2X10^7 cells/flask). Split confluent monolayer 1:10 two days before use for optimum yield and growth. (*CELLS MUST BE IN LOG PHASE GROWTH FOR MAXIMUM VIRUS SENSITIVITY*).
- Gently rinse monolayer with 5 ml *trypsin-EDTA* and remove.
- Add 4-5 ml *trypsin-EDTA* to cover the cell monolayer.
- Lie flask flat and incubate at 37°C in 5% CO_2 until monolayer detaches
- Add 5-10 ml of *MDCK medium* to each flask, remove cells and transfer to centrifuge tube.
- Wash cells 1-2X with PBS (5 min at 300 x g).
- Resuspend cells in *virus diluents* and count cells with a hemacytometer
- Adjust cell number to 1.5x10^5 cells/ml with *virus diluent*.

- Add 100 µl cells to each well of microtiter plate.
- Incubate cells overnight at 37°C, 5% CO_2 (18-22 hrs).

Assay and Determination of 50% Tissue Culture Infectious Doses ($TCID_{50}$)

- Remove medium from plate.
- Wash each well with 200 µl PBS.
- Remove PBS (Do not allow wells to dry out) and add 100 µl / well of *cold fixative.*
- Cover with lid and incubate at room temperature for 10 min.
- Remove *fixative* and let plate air-dry.
- Perform ELISA .
- Calculate the mean absorbance (OD) of the cell controls.
- Any test well with an OD > 2 times OD of cell control wells (CC) is scored positive for virus growth.
- Once all test wells have been scored positive (+) or negative (-) for virus growth, the titer of the virus suspension can be calculated by the method of Reed and Muench. This will determine $TCID_{50}$ per 100 µl volume.
- Dilute the virus suspension so that 50 µl contains 100 $TCID_{50}$. (Initial virus titration will determine if addition of TPCK-trypsin to virus diluents is optimal for virus infection of MDCK cells).

c. ***Evaluation the efficient of streamer system by checking appearance of viruses***

- **Preparation of cell culture flats**
  - Check the MDCK cells with microscope at 40X magnification.
  - Decant growth medium into a beaker and wash two times with 5ml PBS (-) and a time with (D-MEM) containing 2 µg/ml of TPCKtrypsin.
- **Inoculation of cell culture flats**

  - Inoculate 250 µl of each virus collected from different time of experiments into a MDCK flat.
  - Allow inoculate to adsorb for 60 minutes at 37^0C.
  - Add 5ml of complete media (D-MEM) containing 2 µg/ml of TPCK-trypsin with bovine serum albumin fraction V (BSA).
  - Observe daily for cytopathogenic effect (CPE) among 7 days
  - If CPE does not appearance, the test will be repeated 2 more time.

2.3. Data analysis

- TCID $_{50}$ results of invidual Petri dish to be collected and analysis due to evaluate the efficient of the streamer during 4 hours of incubation.

- CPE observation of invidual Petri dish to be collected and repeat 2 more time in case CPE negative due to make sure the virus be inactivated after treatment by streamer.

Due to the unknown pathogenic potential of avian/human viruses, all experiments involving live virus will be carried in Biosafety level 3 laboratories at High-tech center of National Institute of Hygiene and Epidemiology (HTC-NIHE).

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3. Results

3.1. Evaluation effective of DAIKIN's streamer technology by $TCID_{50}$ testing.

3.1.2. Avian influenza A/H5N1 clade 1.

Time of incubation	HN 30408 (clade 1)		Reducing (%)
	$TCID_{50}$	The number of object	
0 hour	$10^{-2.91}$	813	0
1 hours	$10^{-1,35}$	24	97
2 hours	$10^{-0,35}$	2	99,8
3 hours	Neg	0	100
4 hours	Neg	0	100

3.1. Evaluation effective of DAIKIN's streamer technology by cytopathogenic effect (CPE) on MDCK cells.

- **HN 30408 (April 8, 2009)**

Virus Incubation	24 hrs		48 hrs		72 hrs		96hrs		day 4		day 5		day 6		day 7		CC	
	CPE	%	CPE	%	CPE	%	CPE	%	CPE	%	CPE	%	CPE	%	CPE	%	CPE	%
0 hour	-		-		-		+/-	10	+/-	10	+	30	+	50	+	60	-	0
1 hour	-		-		-		-		-		-		+	20	+	30	-	0
2 hours	-		-		-		-		-		-		-		+/-	10	-	0
3 hours	-		-		-		-		-		-		-		-		-	0
4 hours	-		-		-		-		-		-		-		-		-	0

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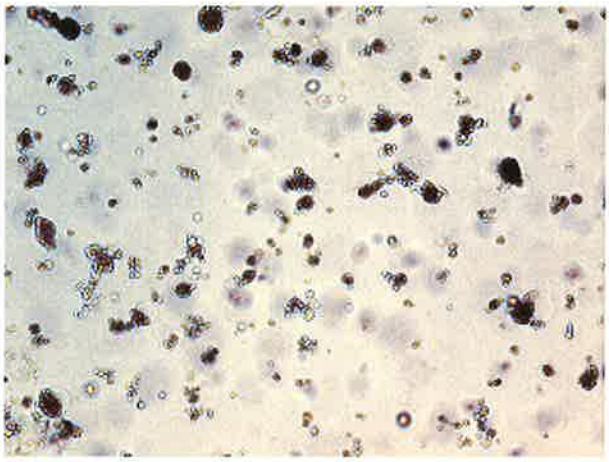
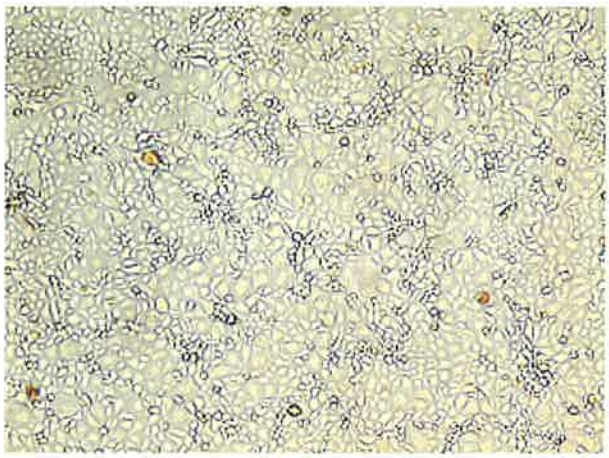
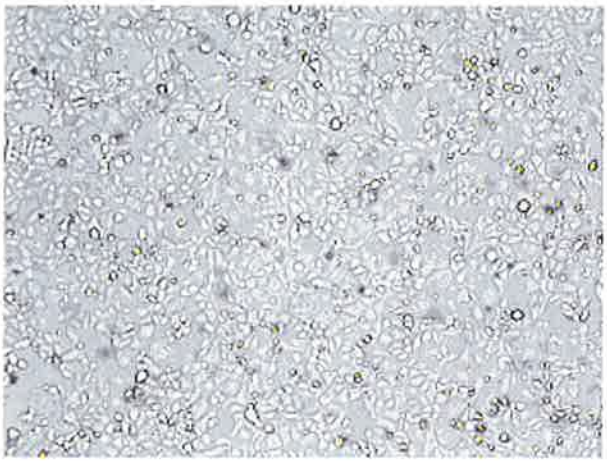
7rd day: 30408-0 hour



7rd day: 30408-3 hour



7rd day: 30408 Cell control



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4. Conclusion

- DAIKIN's streamer technology has completely destroyed (100%) avian influenza viruses A/H5N1 clade 1 after 3 hours of incubation.