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VISUAL EXPERIMENT

In vitro antiviral activity of *Lactobacillus plantarum* using SPF embryonated eggs and hemagglutination assay

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ABSTRACT

Serum pathogen-free (SPF) embryonated egg for culturing virus has three main steps: a) candling of egg, b) inoculation of egg and c) harvesting of inoculated egg. Furthermore, hemagglutination assay consists of only one step. The method is easy to perform. However, this experiment must be performed in a class 2 biosafety cabinet.

INTRODUCTION

More than 200,000 hospitalizations and around 36,000 deaths are associated with influenza infection every year in the United States. As a consequence of consecutive mutations, new influenza viral strains emerge that further complicate the process of drug and vaccine development. The unavailability of cost-effective and efficient methods for the propagation of virus limits the research and hence, further impedes the process of discovering a potent cure. Propagation of influenza virus using serum pathogen-free (SPF) chicken eggs as the source is being recognized as one of the promising, frequently – used, cost effective and efficient method. Therefore, high yield titer of viral stocks obtained from SPF chicken eggs, has resulted in its extensive use in research laborites especially in drug and vaccine production. However, the ability to reproduce in eggs differ with strains of influenza virus. Avian influenza viruses replicated in embryonated chicken eggs. During the incubation phase, the virus duplicates in the cells and make up the chorioallantoic membrane (CAM) — also called the chorialantois. The new viral particles are then produced and released into the allantoic fluid. In this paper we demonstrate how influenza virus is cultured in SPF embryonated chicken eggs and also substantiate the activity of anti-viral drugs by hemagglutination test.

MATERIALS AND EQUIPMENT

Disposables

Glue or varnish

Needle (size: 22 gauge, 11/2 inch)

SPF embryonated chicken egg [purchased from (강남농장) Kang Nam Nong Jang, Nam Won City, Korea]

Sterile plastic pipettes (10 mL)

Sterilized tubes (15 mL) and rack

Syringe (1 mL)

Tips for micropipettors

Virus [Influenza virus H1N1 (A/ Korea/01/2009); (105.5 EID50/100 µL)].

General equipment and glassware

Automatic pipettor for serological pipettes

Egg candler

Egg incubator

Egg puncher

Humidifier to maintain 80% humidity environment

Measuring cylinder (100 mL; sterile one)

Multichannel pipettor (12 channel and 20-200 µL)

Single channel pipettor (2-200 µL and 100-1000 µL)

Sterilized forceps

Major equipment

Desktop centrifuge

Freeze (-70°C for storage facility)

pH meter

Refrigerator

Solution

Alcohol (70%)

Ethanol:	30 mL
Distilled water:	70 mL

Chicken red blood cells (0.5% v/v)

Red blood cells:	0.5 mL
Phosphate buffer saline:	99.5 mL

Phosphate buffer saline, pH 7.2, (1x):

NaCl	8 g
KCl	0.2 g
Na2HPO	4 1.15 g
KH2PO	4 0.2 g

Make up to 1 liter with distilled water and adjust pH to 7.2

V-form shape 96 well microplate

VIDEO CLIPS

In vitro antiviral activity test using SPF embryonated eggs: 7.5 min

Hemagglutination test: 7.3 min

METHOD 1: PREPARATION OF VIRUS INOCULUM

Candling of SPF egg

1. Examine the SPF embryonated chicken egg with the help of an egg candler for any cracks or porous shell.

2. Discard eggs having cracks or any damage.
3. Label each egg with a specific identification number.
4. Incubate the eggs for 11 days in an egg incubator pre-adjusted at 35°C.

Inoculation of egg

5. Examine the pre-incubated egg again with egg candler before virus inoculation.
6. Discard infertile or damaged egg.
7. Place the egg with blunt end up into egg tray. Wipe the egg with 70% ethanol.
8. Punch a small hole in the shell over the air sac without damaging the egg using an egg puncher.
9. Locate the embryo and inoculate 100 µL of virus by inserting the needle into the hole to amniotic cavity.
10. Inoculate in two more eggs in same fashion using same syringe and needle. For statistical analyze and better results inoculate three eggs per specimen.
11. Discard disposable into a proper safety container.
12. Seal the egg hole with glue or varnish and incubate at 35 to 37°C for 5 days.

Harvesting of inoculated egg

13. Before harvesting egg is chilled at 4°C for 4 hours or overnight.
14. Label sterilized plastic tube one per egg and arrange tubes in a rack.
15. Clean off the top of each egg with 70% ethanol.
16. Break the egg shell over the air sac carefully with sterile forceps. Push aside the allantoic membrane with the forceps.
17. Aspirate the allantoic fluid with the help of 1 mL or 10 mL pipette and place in a pre-labeled plastic tube (one per egg). Combine the amniotic fluid from the three eggs into one tube.
18. To remove any blood or cells, centrifuge harvested fluids at 3,000 rpm for 5 min.

[The above protocol was followed from WHO manual on Animal Influenza Diagnosis and Surveillance with some slight modification]

Check the activity of bacterial strains or drugs

The mixture of bacterial cell-free supernatant or drug and IFV H1N1 ($10^{5.5}$ EID_{50}/0.1 mL) are injected into allantoic cavity of 11 days old SPF embryonated eggs and inoculated for 5 days at 35°C on 80% humidity. Follow by step 16 as mentioned above.

METHOD 2: HEMAGGLUTINATION TEST

1. Mix chicken RBS with 1x PBS (0.5% v/v).
2. Add 100 µL of samples (A, B, C, D), in first row of wells marked as A, B, C, D, respectively.
3. From 2nd to last row of microplate add 50 µL PBS, and perform 1:2 dilution.
4. Add 50 µL of diluted chicken RBS in each well.
5. Incubate at room temperature without disturbing the plate for 30 min.
6. Observe wells for positive and negative agglutination.

[For hemagglutionation assay 2-fold dilution of treated samples are made in 50 µL with phosphate buffered saline in V-form shape 96 well microplate. Furthermore, serially diluted samples are reacted with

equal volume of 0.5% chicken red blood cell at room temperature to allow hemagglutination reaction (Seo et al., 2012, Rather et al., 2015)].

DISCUSSION

Influenza contributes by a large extent to global burden of disease, therefore, rigorous work continues into understanding the pathogenesis of the disease. In order to accelerate research in this field several methods have been developed for the propagation of influenza virus. Here, we describe a technique to produce influenza virus in chicken eggs. The advantage of this method is that it is highly reproducible and results in large quantities of high titer influenza viral stocks, which is often necessary for *in vitro* studies. The method is also useful to screen the antiviral activities of different bacterial strains or drugs.

REFERENCES

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Seo BJ, Rather IA, Kumar VJR, Choi UH, Moon MR, Lim JH, Park YH. Evaluation of *Leuconostoc mesenteroides* YML003 as a probiotic against low-pathogenic avian influenza (H9N2) virus in chickens. J Appl Microbiol. 2012; 113: 163-71.

PRECAUTION

Take proper safety measures before handling Influenza viruses.

This experiment must be performed in a class 2 or higher Bio safety cabinet.

Only store specimens at -70^{o}C. Influenza viruses are unstable at -20^{o}C.

Avian influenza grows well at 35^{o}C or 37^{o}C.

Avoid any possible contamination.