

ISSN 1682-8356
ansinet.org/ijps



INTERNATIONAL JOURNAL OF
POULTRY SCIENCE

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Assessment of Pathogenic Potential of Avian Influenza Viruses by MDCK Cell Culture

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Abstract: The influenza viruses are sub-classified in to two pathotypes of Highly Pathogenic Avian Influenza (HPAI) and Low Pathogenic Avian Influenza (LPAI) viruses on the basis of the pathogenicity of AIV in domestic poultry. Samples of H5N1 (7966/06 and 7972/06) and the H9N1 (5844/05) viruses were also grown on MDCK cell. All these produced cytopathic effect within 72 h. Normally, the nonpathogenic AIV does not produce CPE in MDCK cells. However, CPE can be produced if trypsin is incorporated while, culturing the viruses. In the present study, without the addition of trypsin the viruses produced CPE. On FAT both cytoplasmic and nuclear florescence was observed. It is also known that the viruses which produce CPE in absence of trypsin are pathogenic. It proved beyond that the H5N1 and H9N1 viruses isolated in the present study were pathogenic based on cell culture study.

Key words: Avian influenza H5N1, MDCK, CPE, India

INTRODUCTION

Avian influenza viruses replicate in a limited number of cell cultures. Chicken Embryo Fibroblasts (CEF) are the most commonly used primary cell cultures, where, as the frequently used continuous cell line is the Madin-Darby Canine Kidney (MDCK) followed by Primary Rhesus Monkey (PMK) cultures. The ability of influenza viruses to produce plaques in CEF and MDCK cells in the absence of trypsin correlates with pathogenicity because it indicates that the HA of that strain is readily cleaved by cell proteases, where as Low Pathogenic viruses cannot form plaques in the absence of trypsin because the HA remains uncleaved. The addition of trypsin to the cells will accomplish the cleavage and allow plaquing (Bosch *et al.*, 1981). Virus culture using Primary Rhesus Monkey (PMK) or Madin-Darby Canine Kidney (MDCK) cells is the currently accepted 'Gold-standard' for the laboratory diagnosis of influenza virus (Gavin and Thomson, 2003). After culture isolation, most influenza isolates are definitively identified using immuno assays or immunofluorescence test (Murphy and Webster, 1996). However, traditional virus isolation and identification takes time and virus culture results are generally available in four to five days. Immunofluorescence Assays (IFA) generally has very high sensitivity, but is laborious and require expertise in fluorescence microscopy. Immunofluorescence test for the detection of viral antigen in tissue impression smear was evaluated by Selleck *et al.* (2002) and gave good result in brain and pancreatic impression smear for rapid diagnosis test for HPAI virus infection of poultry. In

the present study the pathogenic potential of two H5N1 and one H9N1 was calculated by MDCK cell culture with FAT.

MATERIALS AND METHODS

The confluent MDCK monolayer in 25 mL flask was washed with 2 mL of GMEM medium and 1 mL of trypsin (0.5 µg/mL) was added to the cells. The flask was incubated in 37°C for 3-5 min. and observed continually under microscope for rounding of cells. When, the rounding of cells was nearly 90%, trypsin was discarded and the cells were resuspended in GMEM medium with 10% FBS and 1X antibiotics keeping the split ratio as 1:4.

The MDCK cells were subcultured in 48 well cell culture plate by adding cells of around 15,000 in each well of the plate, cells counted by using haemocytometer, as WBC counting. Plate was kept in 5% CO₂ incubator overnight and then observed under microscope to confirm that there was 60-80% monolayer should form. Serial dilution of all the virus isolates (7966/06, 7972/06 and 5844/05) was made in 1X PBS viz. 1:10 1:100, 1:1000, 1:10000, 1:100000, 1:1000000, 1:10000000 and 1:100000000. Monolayer was washed twice with the GMEM medium, 150 µL of the diluted virus was dispensed on to the wells B 1-8, C 1-8 and D 1-8. The cells were incubated for 1 h in a 5% CO₂ incubator at 37°C for the cells to adsorb the virus. After 1 h the cells were washed thrice with GMEM medium and 1 mL of GMEM with 10% FBS (complete media) was added to all the wells of B, C and D row. About 1 mL of GMEM with

10% FBS (complete media) was added to the wells A 1-8 (Cell control). The plate was incubated at 37°C for 3 days in 5% CO₂ incubator and observed daily twice for the development of cytopathic effect.

The medium was removed and the monolayer was washed once with GMEM and added 1 mL of 0.5% crystal violet solution (with 10% formalin) into all the wells; kept 30 min for proper staining. After 30 min decant the stain from plate and washed with 1 mL of sterile distill water. Discard the water completely from plate and kept the plate inverted for drying for 20 min. then observe plate directly under inverted microscope.

Infected MDCK cells with infectious bacterial free virus isolates (7966/06, 7972/06 and 5844/05) along with cell control in 48 wells plate, observed upto 72 h for CPE. After 72 h decant the media, wash the plate once with 200 µL of sterile 1X PBS, discard PBS and wash with 200 µL of 80% acetone in water. Then, 400 µL of 80% acetone was added to all the wells and incubated for 30 min at room temperature, discard the acetone and kept the plate 1 h for drying in slant position. Then, 150 µL of reference serum (1: 200 diluted) was added to all the wells, incubated for 40 min at 37°C and wash the plate twice with 1X PBS. About 150 µL of Antichicken FITC conjugate (1:100 dilutions in PBS) was added and incubated the plate at 37°C for 30 min, then wash the plate twice with 1X PBS and observe the plate directly under florescent microscope.

RESULTS AND DISCUSSION

One H9N1 (accession no.5844/05) and two H5N1 (accession no. 7966/06 and 7972/06) viruses were passaged in the MDCK cell lines maintained in laboratory. The Cytopathic effect including rounding and shrinkage of cells, plaque formation and finally detachment of cells from the surface of culture plate were noticed in MDCK cells (Fig. 2 and 3). All the three isolates showed CPE after 24 h of inoculation, but the extent of CPE is varied between the isolates. The CPE shown by H9N1 virus is different (duration and type of CPE) as compared with the CPE of H5N1 influenza viruses (Table 1). Both the H5N1 virus isolates 7966/06 and 7972/06 gave the titer of 10⁶ and 10⁷, respectively. The H9N1 isolate gave the titer of 10⁴ after 72 h. No CPE was observed in the cell controls up to 72 h.

Fluorescence was observed in all three isolates infected in MDCK cell culture plate. The both cytoplasmic and nuclear fluorescence were observed up to the dilution of 10⁶, 10⁷ and 10⁴ from isolates 7966/06, 7972/06 and 5844/05, respectively. There was no fluorescence in the control wells.

The ability of influenza viruses to produce cytopathic effects in tissue culture cells such as Madin Darby Canine Kidney (MDCK) cells in the absence of trypsin correlates with pathogenicity because it indicates that the HA of that strain is readily cleaved by cell protease.



Fig. 1: Uninfected MDCK cell line (40X)

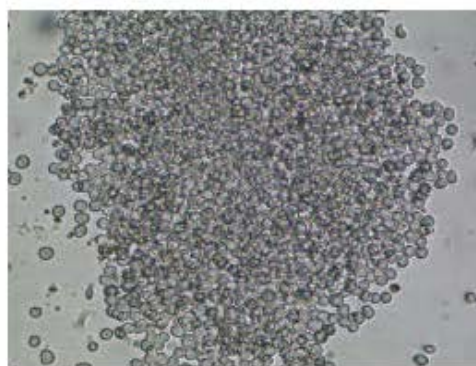


Fig. 2: MDCK cells infected with AIV (H5N1) showing rounding, degeneration and detachment of cells (40X)

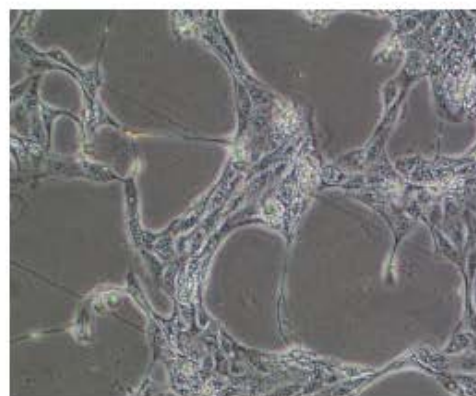


Fig. 3: MDCK cells infected with AIV (H5N1) showing large plaques (40X)

In contrast, low pathogenic viruses cannot form plaques in the absence of trypsin because the HA remains uncleaved. The addition of trypsin to the cells will accomplish the cleavage and allow plaquing (Bosch *et al.*, 1981). The two H5N1 isolates (accession no. 7966/06 and 7972/06) and one H9N1 (5844/05) were

Table 1: Titration of virus isolates 7966/06, 7972/06 and 5844/05 in MDCK cells

Days	Dilutions	10-1	10-2	10-3	10-4	10-5	10-6	10-7	10-8
One	Control	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	7966/06	+++	+++	++	Nil	Nil	Nil	Nil	Nil
	7972/06	+++	+++	+++	+	Nil	Nil	Nil	Nil
	5844/05	++	++	Nil	Nil	Nil	Nil	Nil	Nil
Two	Control	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	7966/06	+++	+++	+++	+++	++	+	Nil	Nil
	7972/06	++	+++	+++	+++	+++	+++	+	Nil
	5844/05	+++	+++	+++	+++	++	Nil	Nil	Nil
Three	Control	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	7966/06	+	++	++	+++	+++	++	Nil	Nil
	7972/06	+	++	++	+++	+++	+++	+++	+
	5844/05	+++	+++	+++	+++	++	+	Nil	Nil

Nil- No CPE , + - Mild CPE, ++ - Moderate CPE, +++ - More CPE

passed in MDCK cell line. The cytopathic effects were noticed in MDCK cell lines on the first day itself and increased cytopathic effects were noticed upto 3rd day when cultured in the absence of trypsin. Thus, the viruses used in the present study could be, as per the OIE (2001) guideline, highly pathogenic based on their cytopathic effect in cell culture. The ability of the virus to produce CPE in cell culture is considered important to classify the virus as pathogenic or non-pathogenic. In view of the above criteria Banks *et al.* (1999) classified the H5N1 isolate from a turkey as non-pathogenic although the virus had multiple basic amino acids at the cleavage site of HA gene. On fluorescence antibody staining of MDCK cell culture plate infected with 3 virus isolates (7966/06 7972/06 and 5844/05) produced both cytoplasmic and nuclear fluorescence. This indicates the virus multiplies in the cell cultures. We have known that the viruses which produce CPE in absence of trypsin are pathogenic. It proved beyond that the H5N1 and H9N1 viruses isolated in the present study were pathogenic based on cell culture study.

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