Archives of Razi Institute, Vol. 62, No. 1, Winter (2007) 7-13

Plaque formation of *LaSota* pathogenic strain of Newcastle disease virus adapted in chick embryo fibroblast cells

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Received 25 Aug 2006; accepted 08 Jan 2007

ABSTRACT

In order to adapt *LaSota* strain of Newcastle disease virus (NDV) on chick embryo fibroblast (CEF) cells, 0.1 ml from *LaSota* vaccine produced in Razi vaccine and serum research institute inoculated to the CEF cells and was passaged five times in the CEF grown in Eagle's minimum essential medium (MEM). First to third passages were blind but in fourth passage cytopathic effect of virus was observed. Fifth passage viruses were propagated in SPF embryoneted eggs and then harvested allantoic fluids showed presence of NDV with the titer of 10^8 EID_{50}/ml. Plaque assays performed on this harvest using two agar overlays; one supplemented with trypsin, DEAE dextran and magnesium sulfate, the second with neutral red along with the mentioned supplements, overlaid 72 hours after first one. Eight hours later, plaques of adapted virus appeared with various sizes ranging from 1 mm to 3 mm in the diameter. Discrete plaques were observed in the 10^{-6} dilution and the calculated titer of virus was 3×10^7 pfu/ml. Agar medium overlays without above mentioned supplements obtained no plaque at any dilutions.

***Keywords*:** Newcastle disease virus, chick embryo fibroblast, plaque

INTRODUCTION

The essential prerequisite for the experimental investigation of a virus or the disease caused is the production in some laboratory host as virus cannot replicate or multiply without any live host system. Animal cell cultures have been used for cultivation of viruses since 1950 (Ahamed *et al* 2004). Different type of primary cells particularly of avian origin and certain cells lines of mammalian origin such as Median–Darby bovine kidney (MDBK) cells, LLC-MK2 (a rhesus monkey kidney cell lines) Vero cell lines and chick embryo fibroblast (CEF) being used for plaque production (Kournikakis & Jacquelin 1988, King 1993, Ahamed *et al* 2004). LLC-MK2 cells have been used by others to assay human parainfluenza viruses and have also been used in an

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NDV plaque neutralization disk test (Frank *et al* 1979, Nerome & Ishida 1982, Ishida *et al* 1985). The cell fusion / plaque type of CPE is caused by vaccinia virus and certain other poxviruses growing in MK, Vero, NCL-H2N9, and fibroblast cells. Plaques ranging from 1 mm to 6 mm in diameter, formed in 2-4 days, during which the infected cells fuse, form cytoplasmic bridging, and then disintegrate (George 1996). A technique for plaquing lentogenic strains of NDV provides a method for establishing the purity of seed stock in the production of vaccine. Titration of Newcastle disease virus (NDV) strains are more conveniently undertaken in cell culture rather than in embryonated eggs, this is relatively easy with mesogenic and velogenic strain that are cytopathic to various cell line, but is difficult with avirulant strain that are poorly cytopathic (Wanbura *et al* 2006). The ability of NDV to form plaque in cell culture is related to the virulence of the virus (Reeve *et al* 1971). The virulent strains produce greater number of syncythia than the mesogenic or avirulent strain (Ogasawaro *et al* 1992). Kournikakis and Fieldes (1988) also reported that the optimal condition for plaque assay of the avirulant *LaSota* strain of NDV was agar overlay supplementation with 2.5 µg/ml trypsin, magnasium sulfate (0.03 M) and 0.02% DEAE dextran. Umino and Khoma (1991) performed a plaque assay of an avirulant strain of NDV in primary chick kidney (PCK) cells without adding trypsin to the agar overlay medium. Harper (1988) used a combination of 5% allantoic fluid and 200 µg/ml DEAE dextran with 30 mM $MgCl_2$ as supplement to normal overlay medium and reported this to give large, clear plaques on secondary CEF monolayers infected with avirulant strain NDV which did not produce plaques without these additions. Comparative investigation between velogenic strain and 6 strains of the Newcastle disease virus belonging to various pathogenic groups in CEF cell cultures, showed that the velogenic strain were grown in this culture and produced a clear cythopathic effect without preliminary adaptation passages, however, *LaSota* and Hichner B1 did not produce cythopathic effect without adaptation (Khdzhiev 1982). Ahamed (2004) adapted NDV on African green monkey (Vero) cell line; by five times passaging of virus in this cell line and in the fifth passage obvious changes in the characteristics of cell monolayers were observed. The aim of the present study was adaptation of *LaSota* strain of Newcastle disease virus (Razi *LaSota* vaccine) to grow on CEF cells to obtain the discrete plaques for further molecular studies.

MATERIALS AND METHODS

Virus. A commercial vaccine (*LaSota* strain) batch No 13 was obtained from poultry vaccine production department, Razi vaccine and serum research institute, Tehran, Iran and kept at 4 °C till used. A vial of lyophilized vaccine (4000 dose/vial) was reconstituted with 4 ml sterile phosphate buffered saline (PBS), and stored at -70 °C in 0.2 ml aliquots.

Reagents. 0.2% bovine serum albumin (Sigma) was added to PBS, prepared in triple glass distilled water and sterilized using 0.22 µm, filter.

Culture media. The minimum essential medium (MEM) Eagles (Sigma) supplemented with 5% (*v/v*) fetal calf serum (Sigma) inactivated at 56 °C for 30 minutes, 100 U/ml penicillin G (sigma), 2 µg/ml gentamycin sulfate (Gibco), 0.25 mg/ml glutamine and 0.2% sodium bicarbonate was used as growth medium for CEF cell culture (Kournikakis and Jacquelin Filds 1988).

Agar overlay medium. Consisted of Eagle's MEM, 0.7% Bacto-Agar (Difco), 2.5 µg/ml trypsin (Gibco), 0.2% sodium bicarbonate (Sigma), 0.02% DEAE dextran, 0.03 M magnesium sulfate (Sigma), 0.25 mg/ml glutamine, 100 U/ml penicillin G (sigma) and 2 mg/ml gentamycine sulfate (Gibco). This overlay medium was used as the first and second overlay. The second one also contained

neutral red at a concentration of 1:10000 (Kournikakis & Filds 1988). Other media used for agar overlay consisted of Eagle's minimum essential medium (MEM), supplemented with 5% (*v/v*) inactivated fetal calf serum (Sigma), 100U/ml penicillin G (sigma) 2 µg/ml gentamycine sulfate (Gibco), 0.25 mg/ml glutamine, 0.2% sodium bicarbonate and 0.7% Bacto-Agar (Difco), without trypsin and DEAE dextran.

Chick embryo fibroblast culture. Ten days old embryonated eggs (SPF eggs–Lohmans Co) were used to prepare CEF monolayer. Briefly the eggs were candled, air sac was marked and embryos were aseptically removed from the eggs, washed in cooled PBS and head, legs, wings and viscera were cut and discarded. The rest of the body portion was further washed, cut into small pieces and trypsinised with 10 ml of 0.25% trypsin solution in PBS for 30 minutes. Trypsin was inactivated by adding 0.5 ml fetal calf serum and the cell suspension was filtered through two layers sterile cheese cloth, washed three times using cold incomplete Medium and centrifuged at 1200 rpm for 15 minutes. The cell sediment was mixed with growth complete medium and adjusted on 5×10^5 cells/ml and 4 ml of such cell suspension was dispensed in plate having 4 cm diameter (Nunc Co) and incubated at 37 °C in CO_2 incubator with 5% CO_2. The confluent CEF monolayers were obtained after 24 hours (Harper *et al* 1989, Swayne *et al* 1998, Kournikakis & Jacquelin 1988, Ahamed *et al* 2004).

Virus titration of *LaSota* vaccine. Virus stock propagated 48 hours in allantoic cavity of 10-days old chicken eggs at 37 °C. Fifty percent egg infection dose (EID_{50}) titers were determined by serial dilution of viruses in SPF eggs (Lohman), and endpoints were calculated by Reed and Munch formula (Reed & Munch 1938).

Adaptation of *LaSota* strain of NDV. To adapt the virus, 100 µl of virus with the titer of 10^8 EID_{50}/ml was added to the center of a confluent CEF monolyer in a plate. PBS was added to the another confluent CEF monolyer as negative control. The plate was set on a level surface for 1 hr in an atmosphere of 5% CO_2 and 80% relative humidity. After 1 h, 4 ml MEM growth medium supplemented with 5% (*v/v*) fetal calf serum (Sigma), 100 U/ml pencillin G (sigma), 2 µg/ml gentamycine sulfate (Gibco), 0.25 mg/ml glutamine, 0.2% sodium bicarbonate and 2.5 µg/ml trypsin was added to the confluent monolayer cells and kept in CO_2 incubator at 37 °C with 5% CO_2 for 3 days. After 3 days 0.5 ml of 0.25% of trypsin solution in PBS was added to CEF cells monolayer and incubated at 37 °C in CO_2 incubator. CEF cells were detached after 30 minutes and centrifuged at 600 ×g for 15 minutes. The supernatant centrifuged again at 50000 ×g for 45 minutes, and then 100 µl PBS was added to the virus pellet and inoculated to CEF cells using same media and techniques describe above. Subsequent passages were done as well. Infected monolayers were examined twice a day under an inverted microscope for any change as compared to control uninfected cells (Beard *et al* 1970, Nagai 1973, Nagai *et al* 1976, kournikakis & Filds 1988, Madhan *et al* 2005). 0.2 ml of the adapted virus from fifth passage was inoculated to 10 days old SPF embryonated chicken eggs for propagation and after 48 hrs, the allantoic fluid was harvested and (EID_{50}) titers were determined by serial titration of viruses in SPF eggs (Lohman). The endpoint was calculated by the methods of Reed and Munch (1938).

Rapid HA test. 25 µl of treated amniotic fluids were mixed with 25 µl of 5% suspension of chicken red blood cells, with a plastic stick on the center of a clean glass slide, while 25 µl of 5% suspension of chicken red blood cells were mixed with 25 µl PBS on the left side of the same slide as negative control and 25 µl of 5% suspension of chicken red blood cells were mixed with 25 µl NDV solution at right side as positive control and rotated for one minute. Clear and consistent HA was considered as the positive reaction.

Plaque assay. The plaque assay was performed for the *LaSota* vaccine strain of NDV as described before (Barhonna & Hanson 1967, Nagai *et al* 1973, Nagai *et al* 1976, Kournikakis & Fields 1988). Briefly, confluent monolayers of CEF cells grown in plates were washed twice with 3.0 ml of PBS/BSA. 0.2 ml of each dilution (10^{-4} through 10^{-10}) (10^{8} EID_{50}) of adapted virus was inoculated to the CEF monolayers in quadruplicate. 0.2 ml PBS inoculated to one well was used as negative control. The inoculated plates were kept in CO_2 incubator under 5% CO_2 and 80% humidity at 37 °C for 1 hour. Agar overlay medium with supplements was then added to the cells infected with 10^{-4}-10^{-10} dilutions of adapted viruses in duplicate and agar overlay medium (42 °C) without trypsin, DEAE dextran and magnesium sulfate was added to other two plates inoculated with above dilution of viruses (10^{-4}-10^{-10}). Plates were gently agitated to ensure distribution of the overlay medium and the overlay medium were allowed to solidify by placing the plates on a level surface at room temperature for 15 minutes. All of the plates incubated for 72 hours at 37 °C in CO_2 incubator with 5% CO_2 and 80% humidity. Seventy two hrs after infection a second 2 ml agar overlay medium containing 1:10000 neutral red (Difco) was added to each plate. 80 h after addition of the first layer of agar overlay the plaques with different shapes and size were visible. Selected discrete plaques were harvested with the insulin syringe, suspended in 0.2 ml of sterile PBS, inoculated into embryonated SPF egg and incubated for 48 hours. These propagated viruses were harvested, HA test was carried out and HA positive allantoic fluids was collected and stored at -70 °C for molecular investigations (Ahamed *et al* 2004, kournikakis & Jacqueline 1988).

RESULTS

To get confluent monolayer chick embryo fibroblast 5×10^{5} cells/ml were found to be satisfactory and gave confluent monolayer after 24 h incubation at 37 °C under 5% CO_2.

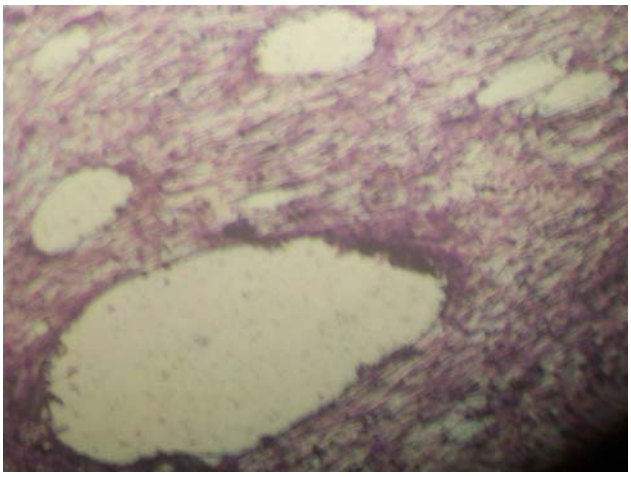

Figure 1 discrete plaques with various size and shape were found in chick embryo fibroblast infected with adapted *LaSota* strain of NDV dilution 10^{-6}.

CEF cell culture was maintained in MEM supplemented with 5% FCS for 7 days. The calculated titer of Razi *LaSota* vaccine strain virus and adapted virus by the methods of Reed and Munch were $10^{9.2}$ EID_{50}/ml and 8 log 10 EID 50/ml respectively. In this study 5 serial passages of the *LaSota* strain of NDV confirmed its successful adaptation, however; in the first to third passages, virus did not produce any clear change in CEF cells and viruses were just started to be adapted & infect the CEF cells, nevertheless, complete cythopatic effect (CPE) of *LaSota* strain of NDV on CEF were obvious in fourth and fifth passages. In fourth passage, cells gradually started to change in the shape and nucleus and a few CEF cells aggregated in several places on the monolayer. Six discrete plaques (Figure 1) obtained in 10^{-6} dilution of adapted viruses (3×10^{7} PFU/ml) with 1-3 mm diameter at 80 h post inoculation and in the 10^{-7}-10^{-10} dilution did not obtain any plaque(Table 1). Dilution of 10^{-5} produced 35 plaques (1.75×10^{7} PFU/ml) but not discrete plaque, at 10^{-4} dilution plaques were too much that were not countable (Figure 2).

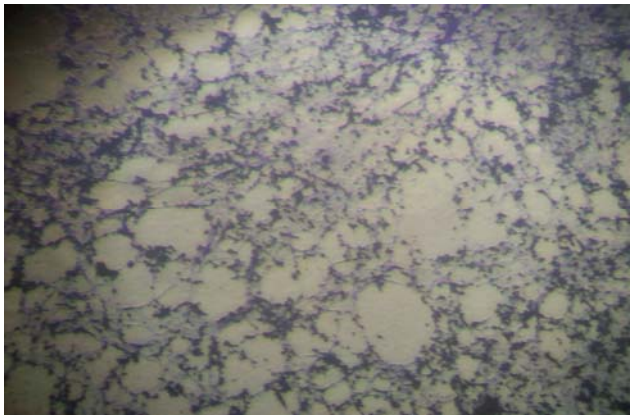

Figure2. Uncountable plaques in Chick embryo fibroblast infected with adapted *LaSota* strain NDV in 10^{-4} dilution.

Table 1 Plaque counts of (Razi) *LaSota* vaccine strain of NDV.

Dilution	Cell	overlay	Plaques (No.)	Titer (PFU/ml)
10^{-5}	CEF	Agar+DEAE+MgSO4	35	1.7×10^{7}
10^{-6}	CEF	Agar+DEAE+MgSO4	6	3×10^{7}
10^{-7}	CEF	Agar+DEAE+MgSO4	N*	-
10^{-8}	CEF	Agar+DEAE+MgSO4	N*	-

*No plaques were obtained.

The plaque size and shape were found to be varied under agar over layer with additives. Cell cultures that inoculated with adapted virus and agar overlaied without DEAE-dextran and magnesium sulfate and trypsin did not show any discrete plaques at any dilution. Plaques were referred to a necrotic patch in the cellular monolayers, visible cells stained red, while the necrotic areas caused by viral infection did not stain. A minimum of 4 hrs was necessary for the color contrast to be appeared in CEF monolayers. Six various plaques were collected and propagated for further molecular investigations.

DISCUSSION

Although different type of cells are used to cultivate and propagate NDV, but CEF cells is the most popular throughout the world to grow NDV (Ahamed *et al* 2004). Khadzhiev (1982) reported some of the velogenic and mesogenic strains produced clear cytophatic effect on the CEF cells, but without adaptaion passages *LaSota* strain did not produce plaques on CEF cells. Ahamed (2004) after 5 serial passages of wild NDV on vero cell line obtained cytopathic effect and finally plaque.

Mesogenic and velogenic strain of NDV produce cythopathic effect to various cell lines, but it is difficult with avirulant isolates that are poorly cythopathic (Wambura 2006). Virulant NDV can be different by its ability to replicate in most avian and mammalian cell types without the addition of trypsin. Although all NDV isolates can replicate in chicken embryo kidney cells, but lentogenic strains require trypsin for replication in avian fibroblast or mammalian cell types (Seal *et al* 1995). Lentogenic viruses can replicate only in areas with trypsin-like enzymes such as the respiratory and intestinal tracts, whereas virulent viruses can replicate in a range of tissues and organs resulting in systemic infection (Ogasawaro *et al* 1992). Lentogenic strains such as the *Hichner B1* and *LaSota* strains are widely used as the live vaccines against Newcastle disease (Peeters *et al* 1999). During replication NDV particles are produced with a precursor glycoprotein, F0 which has to be cleaved to F1 and F2 for the virus particles to be infectious (Rott & Klenk 1988, Berinstein *et al* 2001). This post translation cleavage is mediated by host cell proteases. Trypsin is capable of cleaving F0 for all NDV strains and in vitro treatment of noninfectious virus will induce infectivity (Nagai 1976, Olav *et al* 2003). Kournikakis and Fildes (1988) reported trypsin was required for plaque formation by avirulant NDV, and plaque size varied directly with trypsin concentration and some degradation of the cell monolayer was seen at 5 µg/ml trypsin concentration and they used 2.5 µg/ml as the optimal trypsin concentration for that assay. In the present study 2.5 µg/ml trypsin, 0.03 M magnesium sulfate and 0.02%

DEAE dextran used in plates infected with adapted viruses, obtained discrete plaques different in shape and size. The presence of DEAE-dextran in the overlay medium provided a significant improvement in the virus titer (Kounikakais & Filds 1988). Some inhibitory such as sulfate polysaccharides is present in agar, which combine with virus particle to form non infectious complex. DEAE-dextran acts by combining with the sulfate polysaccharides to neutralize their inhibitory effect. Barahona and Hanson (1968) showed that lentogenic strain of NDV are cytopathic but fail to produce plaques in CEF cells within 96 hours in absence of magnesium and dimethlaminoethyl (DEAE). They also found that velogenic strain of NDV when grown on CEF monolayer usually exhibit, clear plaques (2 to 4 mm). Scholer and Hanson (1968) showed that NDV forms plaques with several sizes ranging from 0.5 to 4.0 mm in diameter. Kounikakais and Filds (1988) cultured avirulent strain of NDV in LLC-MK2 cells, plaques were visible after 2 days and maximum virus titer was reached in 3 days. Lentogenic strains such as the Hichner B1 and *LaSota* strains are widely used as the live vaccines against Newcastle disease so plaque provide from lentogenic strains is important. (Peeters *et al* 1999*)* Advantage of the plaque assay is the possibility to detect mixed virus population in an isolate.

This technique for plaque formation by lentogenic *LaSota* strain of Newcastle disease virus provides a method for establishing the purity of seed stock used in the production of vaccine. The present assay is useful tool for demonstration of discrete plaques using adapted NDV virus within 3 days on CEF monolayer covered with modified overlay media containing magnesium ions, DEAE dextran and ionoagar to a certain purity.

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