CULTIVATION OF INFLUENZA A VIRUS IN PRIMARY CELL CULTURE OF TURKEY EMBRYONIC FIBROBLASTS

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ABSTRACT

Cell cultures in particular the clonally selected Madin Darby Canine Kidney (MDCK) cell lines are widely used to cultivate influenza viruses because of their high susceptibility to infection and their ability to produce high number of viruses. However, these cells have been in culture for decades and are well adapted to the two-dimensional culture environment, and as a result, often differ genetically, physiologically, and phenotypically from their tissue origin. The aim of this study was to extract turkey embryonic fibroblast cells directly from tissue as a new primary cell type and then infect them with H2N3 influenza A virus to determine their susceptibility to infection. This cell type will have normal cell characters and maintain many of the important markers and functions seen *in vivo*. Results showed that the level of susceptibility to infection was comparable between turkey embryonic fibroblasts and MDCK cell line based on incubating with peroxidase labelled monoclonal IgG antibody to viral nucleoprotein. In addition, progeny virions were clearly visualized on the surface of turkey embryonic fibroblasts by using transmission electron microscope. For further confirmation, progeny virions were also detected in the infected cells following treatment with a fluorescently labelled IgG antibody specific to viral H2 protein by performing immunofluorescent technique. This study confirms that turkey embryonic fibroblast cells are susceptible to infection with influenza viruses and can be considered as a primary cell model to cultivate influenza viruses and to study their effects on cells.

1. INTRODUCTION

Influenza A viruses belong to the *"Orthomyxoviridae"* family cause dangerous outbreaks with high morbidity and mortality because they mutate more rapidly and have a wider range of hosts [1]. They were firstly isolated from swine in 1931 and later from humans in 1933 [2, 3]. Influenza A viruses infect a wide range of animals, including birds, pigs, horses, whales, seals, and humans [4, 5]. They are not identical in their morphological features. They have different shapes ranging from spherical with a size of around 100 nm to filamentous with a size often in excess 300 nm in length, and occasionally they are pleomorphic [6]. They are enveloped with surface glycoprotein spikes and a segmented RNA genome. The genome is organized into 8 segments of different lengths, which are the polymerase basic (PB1 and PB2), the polymerase acidic (PA), hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), matrix (M), and non–structural (NS) genes [7].

Influenza viruses can be isolated in embryonated chicken eggs or by using cell culture techniques, whereby a specimen is inoculated in a live culture system and the virus is then detected after a given period of incubation [8, 9]. Madin Darby Canine Kidney (MDCK) cell line is frequently used to detect viral replication by observing the cytopathic effects (CPE) on infected cells due to its high susceptibility to various influenza viruses [10, 11]. MDCK cells, like any other cell line, have been continually passaged over a long period and have acquired homogenous genotypic and phenotypic characteristics [12]. Although MDCK cells are a good source for production of large number of viruses and being considered for use in manufacture of inactivated influenza vaccines, they have limited ability to develop biologically relevant complex *in vitro* models. For decades, cell lines have played a fundamental role in scientific development, yet researchers have become increasingly careful when interpreting data generated from cell lines only. Factors such as contaminated cell lines have demanded the replace with primary cells [13, 14]. On the other hand, primary cell cultures, which are isolated directly from tissues, are closer to an in *vivo* model. Although primary cells usually have a limited lifespan, they offer a huge number of advantages compared to cell lines. Of these advantages, the use of primary cells provides more relevant results than cell lines, and pre-screened primary cells are good models to represent the signalling in vivo very closely [15].

Transmission electron microscope (TEM) has been widely used to observe and study budding of enveloped viruses including influenza virions by ultra–thin sections of cell cultures or infected tissues [16-18]. In addition, immunofluorescence technique has been employed for the detection of many viruses that cause respiratory infection including influenza A virus [19, 20].

Based on the variety of influenza A virus surface glycoproteins (antigens), the immunofluorescence assay is used for the identification and subtyping of virus strains using monoclonal antibodies against HA surface antigens [21].

The aim of this study was to extract turkey embryonic fibroblast cells from turkey embryos to grow influenza viruses in the laboratory. The aim was achieved by determination of susceptibility of this cell type to infection with influenza virus following incubation with peroxidase labelled monoclonal antibody to viral nucleoprotein (NP protein), observation of progeny virions by electron microscopy, and detection of progeny virions by immunofluorescent technique following staining with a fluorescently labelled IgG antibody specific to viral surface glycoprotein (HA protein).

2. MATERIALS AND METHODS

2.1 VIRUSES

A stock of low pathogenic avian influenza H2N3 viruses (A/mallard duck/England/7277/06) was used in this study. This virus strain has a spherical morphology determined largely by specific amino acids of the M protein and by observation under electron microscope [22]. Viruses were propagated in the allantoic cavity of Dekalb white hen's eggs provided by Henry Stewart & Co. Ltd, UK.

2.2 CELL CULTURES

Turkey embryonic fibroblast cells (primary cell cultures) were extracted from 10.5-day-old turkey embryos provided by Henry Stewart & Co. Ltd, UK. The eggshells were opened and the embryos were pulled out with a sterile curved forceps and tweezers. The embryos were placed in a Petri dish and rinsed with phosphate buffer saline (PBS). The limbs, heads, and internal organs were removed, and the bodies were moved to new Petri dishes containing PBS. The embryos were digested in 0.25% trypsin in dissociation medium composed of: F12 Hams + 1% penstrep + 1.5% fungizone, and incubated at 37°C for about 1 hour. Large undigested pieces were removed using a cell strainer. The remaining tissue suspensions were centrifuged at 400 x g for 5 minutes. Cells were then seeded into cell culture flasks and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum, and supplemented with 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin).

Madin Darby Canine Kidney (MDCK) cells, a genetically modified cell line and high susceptible to infection with various influenza strains, were used to grow the viruses as a control.

2.3 SUSCEPTIBILITY OF TURKEY FIBROBLAST CELLS TO INFECTION WITH THE VIRUS

Monolayers of MDCK and turkey embryonic fibroblast cells were grown in 96-well plates for 12 hours at 37°C. Cells were infected with H2N3 virus subtype in triplicate at multiplicity of infection of 1.0 in serum free infection medium supplemented with 100 U/ml penicillin and 100 µg/mL streptomycin, 2% Ultroser G, and 500 ng/ml TPCK trypsin, and incubated for 2 hours at 37°C. Following 2 hours of incubation, the cells were carefully washed three times with PBS, to remove residual virus inoculum, followed by addition of fresh medium. Cells were incubated for a further 4 hours at 37°C and then fixed with 1:1 acetone:methanol. The infected cells were detected by determining of viral nucleoprotein expression using a primary mouse monoclonal antibody followed by visualization with Envision+ HRP. Cells expressing viral nucleoprotein (positive cells) were visualized by using inverted microscope.

2.4 ELECTRON MICROSCOPY (EM)

Turkey embryonic fibroblast cells were grown on Thermanox plastic coverslips in 24-well plates for 12 hours at 37°C in DMEM medium supplemented with 10% faecal calf serum and 1% antibiotics. They were infected with H2N3 virus strain (which has a spherical morphology) in infection media at multiplicity of infection of 1.0 for 2 hours at 37°C. Cells were then washed three times with PBS and fresh medium was added, and then further incubated for 6 hours at 37°C. Cells were then fixed with electron microscopy fixative buffers (3% glutaraldehyde in 0.1 M sodium cacodylate buffer). They were then rinsed twice (3 minutes each) in 0.1 M cacodylate buffer and then were placed in 1% osmium tetroxide in the same buffer for 1hour, then rinsed in distilled water for 5 minutes. They were then dehydrated in graded ethanol series, culminating in two changes in propylene oxide. The samples were then infiltrated, polymerized with resin and sectioned. They were stained with ethanolic urenyle acetate and then examined using a Tecnai bio twin digital transmission electron microscope run at 100Kv.

2.5 IMMUNOFLUORESCENCE

Turkey embryonic fibroblast cells were grown on glass coverslips (19 mm diameter) in 12-well plates for 12 hours at 37°C. The cells were then infected with H2N3 virus strain in infection medium at multiplicity of infection of 1.0 for 2 hours at 37°C. Cells were then washed three times with PBS and fresh medium was added, and further incubated for 6 hours at 37°C. Cells were washed with PBS, then fixed with 4% paraformaldehyde for 5 minutes at room temperature, and then rinsed with PBS. They were blocked with 1% bovine serum albumin for

1 hour at room temperature and incubated with polyclonal antibody specific to the H2 antigen (chicken H2N3 antiserum) for 1 hour at room temperature. After 3 times washing (5 minutes each) in PBS, cells were incubated in the dark for 1 hour at room temperature with a secondary antibody (goat anti-chicken IgG antibody) labelled with green fluorescent Alexa Fluor® 488. Cells were then washed 3 times, allowed to air dry, and mounted with Prolong Gold Anti-Fade Reagent with 4 ,6-diamidino-2-phenylindole (DAPI). Cells were then viewed using a Leica DM 5000B epifluorescence imaging system.

3. RESULTS

3.1 MDCK AND TURKEY EMBRYONIC FIBROBLAST CELLS SUSCEPTIBILITY TO H2N3

The susceptibility of MDCK cells and turkey embryonic fibroblast cells to infection was assessed fallowing infecting them with H2N3 virus subtype at multiplicity of infection of 1.0. Similar level of infection was obtained following infection of the two types of cells with the virus. Uninfected controls did not show any indication of infection (Figure 1).

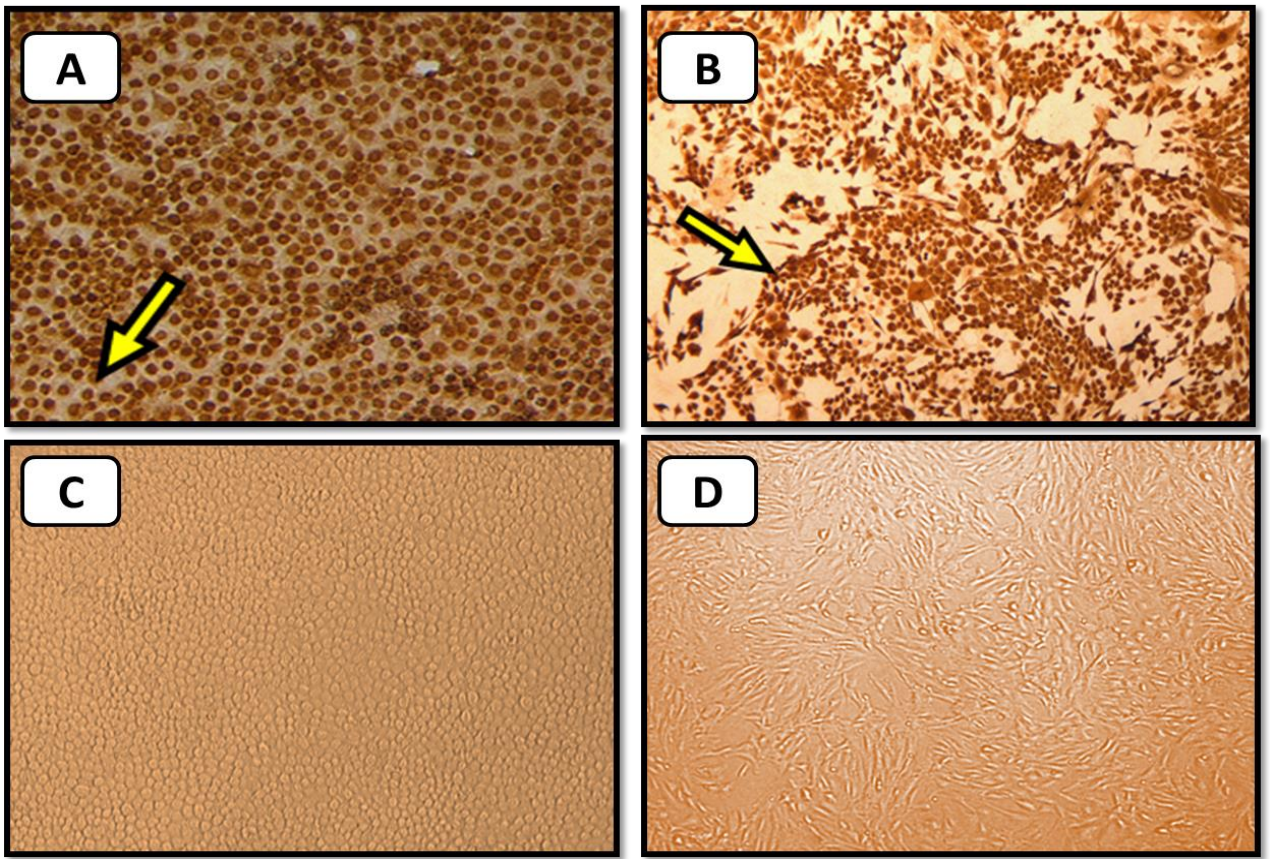

Figure 1: Susceptibility of Madin Darby Canine Kidney (MDCK) cells and turkey embryonic fibroblast cells to H2N3 influenza.

Cell infection is detected by immunostaining for viral nucleoprotein antigen to influenza A virus. (A) MDCK and (B) turkey embryo fibroblast cells show comparable susceptibility to infection with the virus (6 hours post infection at multiplicity of infection of 1.0). (C) MDCK and (D) turkey fibroblasts uninfected controls did not show staining with antibody.

3.2 EM IMAGING OF INFECTED TURKEY EMBRYONIC FIBROBLAST CELLS WITH H2N3

Progeny virions were clearly observed on the surface (the budding site) of turkey embryonic fibroblast cells. The majority of viruses budding from cells were spherical in shape (like the parent viruses) and about 100 nm in diameter (Figure 2).

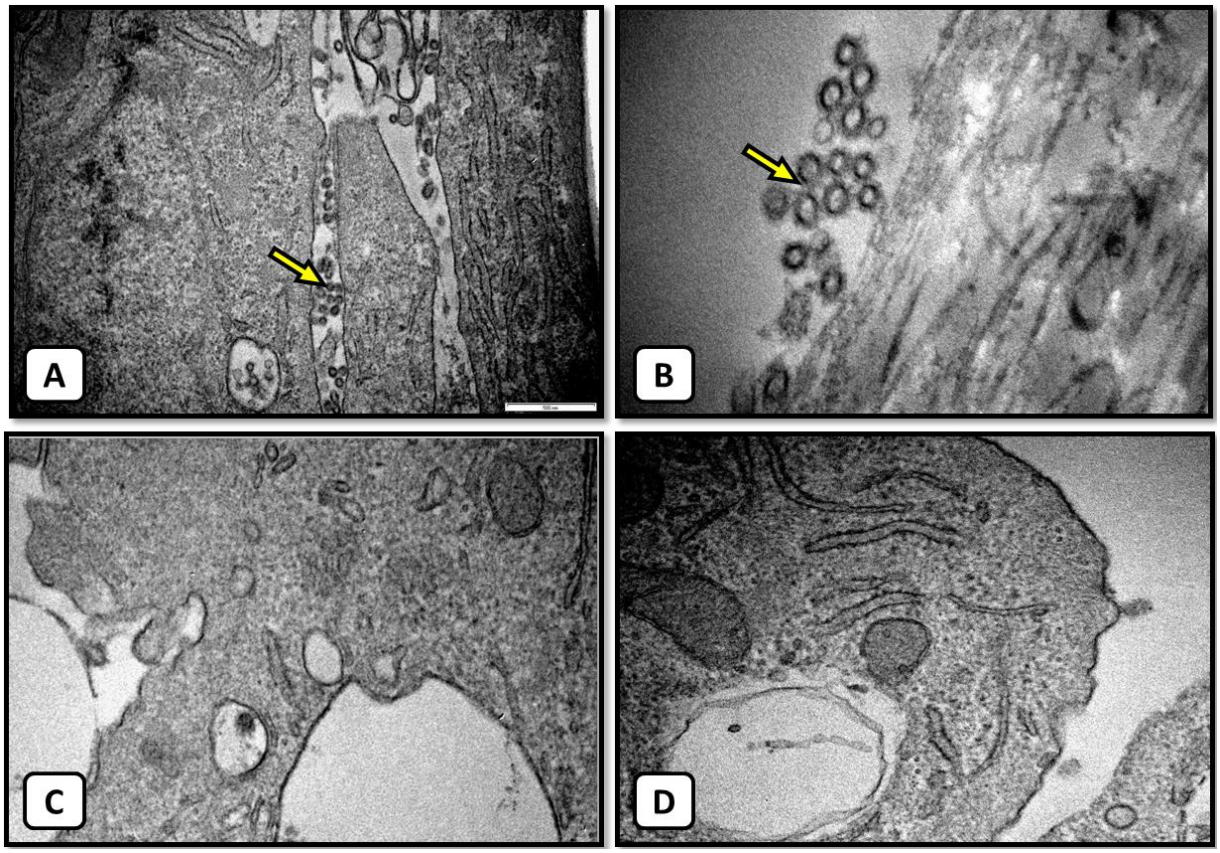

Figure 2: Budding of Influenza A virions from infected turkey fibroblasts.

Cells were infected with avian H2N3 at multiplicity of infection of 1.0 for 8 hours. Electron micrographs show the presence of spherical virions (indicated by arrows) budding from the surface of infected turkey fibroblasts (A and B). Uninfected controls (C and D) showed no virions.

3.3 VIRUS DETECTION BY FLUORESCENT MICROSCOPY

Virions budding from the surface of turkey embryonic fibroblast cells were detected by immunofluorescence microscopy following the infection of cells with the virus. Photographs were taken in two steps, detection of viral HA, and then were merged with DAPI to stain the nucleus. Viruses released were clearly observed on the surface of the infected cells while the uninfected cells showed no evidenced of virus budding (Figure 3).

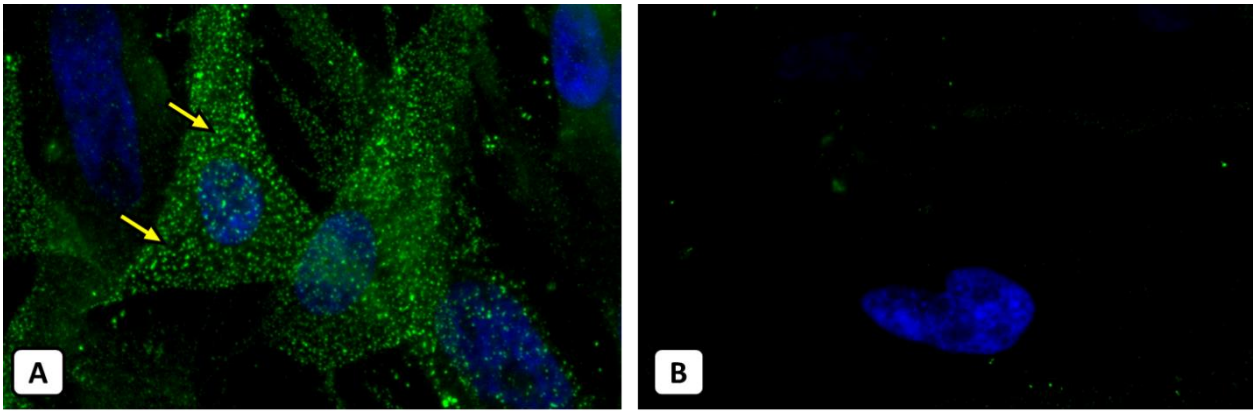

Figure 3: Observation of H2N3 in turkey embryonic fibroblast cells by immunofluorescent microscopy. Cells were infected with virus and then incubated with fluorescently labelled IgG antibody to stain surface HA protein (green). Figure A shows spherical virions on the surface of the cells (indicated by arrows). Uninfected controls (Figure B) showed no virions. Cell nuclei (blue) were clearly observed in infected and uninfected cells.

4. DISCUSSION

In this study, primary turkey embryonic fibroblast cells were extracted from turkey embryos and then infected with influenza A virus, and the results were assessed by performing immunocytochemistry, electron microscopy, and immunofluorescence techniques. Results revealed that turkey embryonic fibroblast cells have high susceptibility to infection with the virus similar with the most common cell line used to isolate influenza viruses. In addition, progeny virions were clearly visualised on the surface of the infected primary cells using electron and immunofluorescence microscopes.

A previous study showed that chicken and duck embryonic fibroblast cells are also susceptible to infection with influenza viruses. Chicken embryo fibroblasts tend to produce virions similar to their parents in their molecular and morphological features. In contrast, viruses produced from duck embryo fibroblasts are not consistent in morphological features with the parent viruses [22]. In the current study, the viruses produced from turkey fibroblasts were spherical in shape, which were similar to the known spherical form of H2N3 strain used to infect the cells. The type of cell culture that produces viruses morphologically consistent with virus inoculum

can be considered as a good cell model to cultivate viruses. Such a cell type may support virus replication more efficiently than other cell types.

It is well known that influenza viruses have high affinity to replicate in epithelial cell layers *in vivo* in particular the upper and lower respiratory tract of mammals [23, 24] and the respiratory and intestinal epithelium of birds [25, 26]. In addition, viruses have been successfully cultivated *in vitro* in epithelial cells extracted from respiratory organs and intestine [27, 28]. Moreover, the epithelial Madin Darby Canine Kidney (MDCK) cells have been the best cell type to propagate influenza viruses [29]. In this study, although the cells used was a non-epithelial cell type, the viruses were cultivated successfully with a high susceptibility to infection almost similar with MDCK cells. This supports the use of turkey embryonic fibroblasts to cultivate influenza viruses and to study virus-cell interaction *in vitro*.

Influenza A viruses have been infecting poultry worldwide and may cause severe disease with high economical losses [30]. In addition, the viruses were successfully isolated from infected turkeys in embryonated chicken eggs, Vero and MDCK cell lines [31]. Although many researchers have chosen to work with cells lines as they are generally highly proliferative and easier to culture and transfect, most cell lines have been in culture for decades, they often differ genetically and phenotypically from their tissue origin, and show altered morphology [32]. In contrast to cell lines, primary cells, which are isolated directly from tissues, have a finite lifespan and limited expansion capacity, have normal cell morphology, and maintain many of the important markers and functions seen *in vivo* [15]. Therefore, the use of directly isolated primary cells such as turkey embryonic fibroblasts will have a role for studying the effect of viruses on cells because of their similarity to tissue characteristics *in vivo.* Therefore, it is highly recommended to use primary cells to study host cell activity and changes such as cell viability, innate immune response, and cell apoptosis following infection with the virus.

In summary, based on the findings gained from this study, it appears plausible that primary cell culture of turkey embryonic fibroblast cells is a good cell model for cultivation of influenza A viruses *in vitro*. Further studies are required to understand the impact of influenza viruses on turkey cells in more detail, which may lead to potential new findings.

استزراع فيروس الانفلونزا نوع أ في المزارع الخلوية الاولية للخلايا الجنينية الليفية للديك الرومي

الخلاصة

تستخدم المزارع الخلوية وبصورة خاصة الخلايا المكلوّنة نوع MDCK والمستخلصة من كلية الفصيلة الكلبية على نطاق واسع لتمنية وعزل فيروسات الانفلونزا ودراسة خصائصها بسبب قابليتها العالية لتقبل الاصابة وانتاجها اعدادا كبيرة من الفيروسات. مع ذلك، هذه الخلايا كانت وما زالت تزرع في المختبر لعقود وتكيفت بشكل كبير مع البيئات المختلفة، ونتيجة لذلك، فهي غالبا ما تختلف وراثيا وفسلجيا ومظهريا عن الأنسجة الاصلية المستخلصة منها. الهدف من هذه الدراسة هو عزل نوع جديد من الخلايا الاولية وهي الخلايا الليفية الجنينية للديك الرومي مباشرة من الأنسجة ومن ثم إصابتها بفيروس الإنفلونزا نوع H2N3 لمعرفة مدى تقبل هذه الخلايا للاصابة وانتاج فيروسات جديدة. هذا النوع من الخلايا ستكون له نفس مواصفات الخلية الطبيعية والعديد من العلامات الهامة والوظائف الموجودة في الجسم الحي. اظهرت النتائج أن هذا النوع من الخلايا له القابلية للاصابة بالفيروس كما في خلايا نوع MDCK اعتمادا على التصبيغ بواسطة الاجسام المضادة نوع IgG والمتخصصة للارتباط بالبروتين النووي للفيروس. بالإضافة إلى ذلك، تم مشاهدة الفيروسات المتحررة على سطح الخلايا الجنينية الليفية للديك الرومي بوضوح باستخدام المجهر الإلكتروني. لمزيد من التأكيد، تم الكشف عن الفيروسات المتحررة أيضا من الخلايا المصابة بعد معاملتها مع اجسام مضادة نوع IgG معلّمة بمادة الفلورسين والتي تمتلك خاصية الارتباط مع البروتين الفيروسي نوع H2 باستخدام تقنية التألق المناعي. تؤكد هذه الدراسة على أن الخلايا الجنينية الليفية للديك الرومي لها القابلية للإصابة بفيروسات الأنفلونزا ويمكن اعتبارها كنموذج لتنمية هذه الفيروسات ودراسة آثارها على الخلايا.

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