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Original article

Comparative growth kinetic study of Newcastle disease virus, infectious bursal disease virus and avian influenza virus in chicken embryo fibroblast and DF-1 cell lines

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Abstract

Viral diseases have caused devastating effect on poultry industry leading to significant losses in economy of world. In the presented study, the ability of Newcastle disease virus (NDV), infectious bursal disease virus (IBDV) and avian influenza virus (AIV) to grow in two cell lines was evaluated. Both chicken embryo fibroblast (CEF) and DF-1 cells were used and cytopathic effects (CPE) produced by these viruses were observed. The titer of virus in terms of $TCID_{50}$ was determined after 24h up to four days for each virus. The same type of CPE was observed for all viruses used in the study in both DF-1 and CEF cells. IBDV showed CPE causing rounding of cells while NDV caused formation of multicellular large nuclei, cell fusion and rounding of cells. Giant cells with inclusions and aggregation of cells with intact monolayer was observed for AIV. In growth kinetic study, higher titer of IBDV and NDV was observed in CEF cells than DF-1 cells while for AIV, DF-1 cells showed higher titer than CEF cells. These results would be useful for furthers comparative studies on growth of different cell lines of various viruses to find a suitability for vaccine production.

Key words: cytopathic effect, titer, growth kinetic, vaccine production

Introduction

Poultry industry is a substantial contributor to gross domestic product of a country. But many viral pathogens cause huge economic losses to this industry globally. Although use of vaccines seems a good effort to control these diseases but still different viruses like infectious bursal disease virus (IBDV), Newcastle disease virus (NDV), avian influenza virus (AIV) are still emerging continuously in poultry birds (Brown et al. 2018).

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Infectious bursal disease caused by IBDV is a highly contagious disease in poultry. The virus belong to family *Birnaviridae* and is double-stranded RNA virus (Méndez et al. 2017). IBDV replicates and affects the main organ of immune system called bursa of Fabricius and thus results in immunosuppression of infected birds. Surviving birds are immunocompromised and become highly susceptible to many other pathogens. IBDV is a major concern in poultry industry and is controlled by vaccination (inactive/attenuated live vaccine) (Liu et al. 2018).

Avian influenza virus (AIV) poses a great risk to poultry industry and human health. Their natural reservoirs are waterfowls which are also co-infected by other viruses (Zowalaty et al. 2011). AIV belongs to family Orthomyxoviridae and consists of RNA segmented genome, highly prone to re-assortment resulting in emergence of new strains (Zhou et al. 2000, Liu et al. 2013). The virus causes very severe infection depending upon the infecting strain leading to increased mortality (more than 80%). In recent years, many outbreaks of AIV have been occurred including Pakistan, USA, and Mexico etc. Although vaccination is being done to overcome the spread of this infection but other preventive measures like sanitation and biosecurity have also been a method of choice for eradication of AIVs (Brown et al. 2018).

Newcastle disease is a respiratory infection in wild and poultry birds. Its causative agent, NDV, is categorized in Paramyxoviridae family and is considered endemic in many parts of world. NDV is divided into three subtypes based on *in vivo* pathogenicity; lentogenic (low pathogenic), mesogenic (moderate pathogenic) and velogenic (highly pathogenic) (Alexander 2011, Ganar et al. 2014). Vaccines (both live and killed) are available and are being used to combat the infection (Mebatsion et al. 2002).

Although all of these three viruses (IBDV, AIV, NDV) grow efficiently in their specific cells but they can also be propagated in chicken embryo fibroblast cells (CEF cells) and DF-1 cells (continuous cell lines of chicken embryo fibroblasts). In our study, we used both CEF and DF-1 cells to study growth kinetic of IBDV, AIV, and NDV. This would help in understanding which cells are efficient in enhancing the yield of virus and may find use in vaccine production against these viruses.

Materials and Methods

The study was conducted at the veterinary diagnostic laboratory, University of Minnesota, USA. The materials and steps needed to be followed are described below.

Cells, Media and culture conditions

CEF cells were derived from 9-day old embryonated chicken eggs and were grown using Minimum Essential Medium (MEM) (Coring®, USA) with 8% fetal bovine serum (FBS) and antibiotics (Streptopenicillin, Fungizone, Gentamicin). DF-1 cells were maintained in MEM with 10% FBS and three antibiotics (mentioned above). Both CEF and DF-1 cells were cultured at 37°C in humidified CO_2 incubator (5% CO_2). The media, MEM with 2% FBS and antibiotics, was used to maintain both CEF and DF-1 cells growth (in every media used in this study, 455 IU of Penicillin, 455 µg of Streptomycin, 1.5 µg of Fungizone and 10 µg of Gentamicin per mL of both maintenance and growth medium were used).

Viruses

IBDV (antigen variant) was locally isolated from an outbreak in birds, Minnesota. NDV (virulent LaSota strain) and AIV (H9N9) were kindly provided by Dr. Hamada Aboubakr (already isolated and confirmed in 2020 in veterinary diagnostic laboratory, Minnesota, USA).

Virus propagation and harvesting in CEF and DF-1 cells

Total five flasks (25 cm^2) with confluent monolayer of both CEF and DF-1 cells were prepared (four for infection and one as control) for propagation of each of IBDV, AIV and NDV. Flasks were infected with each above mentioned viruses (each with 4.5 $Log_{10}T$-CID_{50}/100µL dose in both CEF and DF-1 cells) and incubated in CO_2 incubator at 37°C ((Shahsavandi et al. 2013, Kang et al. 2016). The infected cell culture fluid was collected after every 24h up to four days separately for each virus. The collected fluid was centrifuged at 3000 *g* for 15 min and supernatant was saved at -80°C until further use (Mazumder et al. 2012).

Calculation of Infectious growth titer

Ten-fold serial dilution of each IBDV sample (collected at different time intervals) was performed. CEF cells (1.2×10^4 cells/well) were seeded in 96 well plate and 100 µL of each dilution was transferred to this plate. Each sample was treated in triplicate. Based on cytopathic effect, the titer of each hour sample was calculated following Karber method. The titer of AIV and NDV samples was also calculated following the above mentioned protocol (Karber 1931, Ahamed et al. 2004).

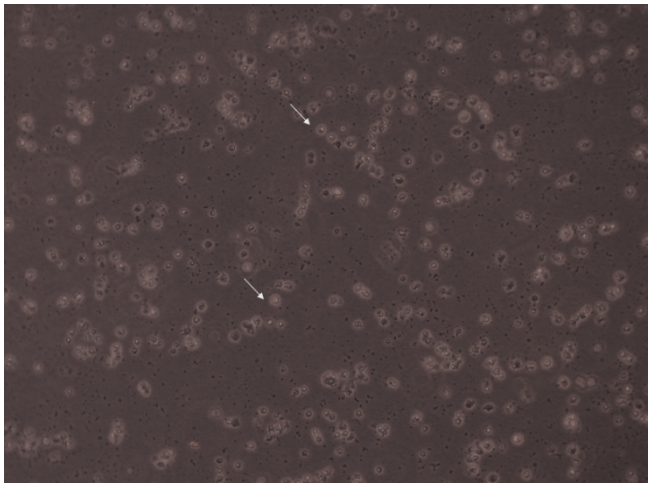

Fig. 1. Cytopathic effects of infectious bursal diseases virus (IBDV) in DF-1 cells; cells were observed as round shaped. x40

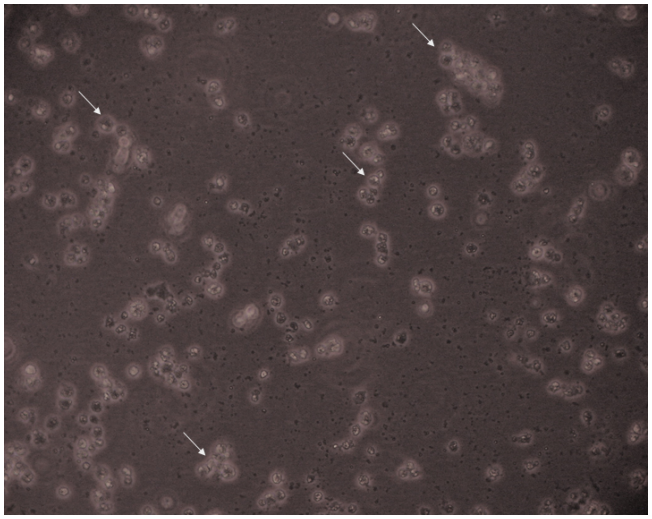

Fig. 2. Cytopathic effect shown by Newcastle disease virus (NDV) in chicken embryo fibroblast (CEF) cells; the virus showed the same type of CPE in both DF-1 and CEF cells in the form of aggregation and rounding of cells. Also the formation of multicellular large nuclei was observed. x40

Statistical analysis

Each of the above experiment was done in triplicate and the data was represented as mean value obtained in triplicate assay for each time intervals. The data was analyzed using one-way analysis of variance (ANOVA).

Results

Primary chicken embryo fibroblast cells and the secondary cell lines called duck fibroblast were used in the present study and their morphology was compared. It was observed that DF-1 cells grow more slowly than CEF cells but the main difference observed in their morphology was that CEF cells are long spindle/fiber shaped than DF-1 cells. All the three viruses (IBDV, NDV and AIV) were propagated in both types of cell lines and CPE of each virus was recorded. Regarding the CPE of the viruses used in this study, IBDV showed similar type of CPE in both CEF and DF-1 cells causing rounding of cells (Fig. 1). NDV showed CPE in form of aggregated cells with round shape and formation of multicellular large nuclei were found among both DF-1 and CEF cells (Fig. 2). In case of AIV, aggregation and giant cells with inclusions, keeping the monolayer somewhat intact, was observed (Fig. 3).

All the three viruses, IBDV, NDVB, AIV, were propagated in both types of cell lines and titer of each virus was determined after every 24h up to 96h. A higher titer of AIV was observed in DF-1 cells as compared to CEF cells at various hours of post infection (PI) (Fig. 4). In case of NDV, it showed higher titer in CEF cells than DF-1 cells. After 72h PI, the virus titer was found to be maximum (6.89 $Log_{10}TCID_{50}$ / 100 µL) (Fig. 5). In growth kinetic study of IBDV, we found that CEF cell had more potential to give high growth titer of IBDV as compared to DF-1 cells (Fig. 6).

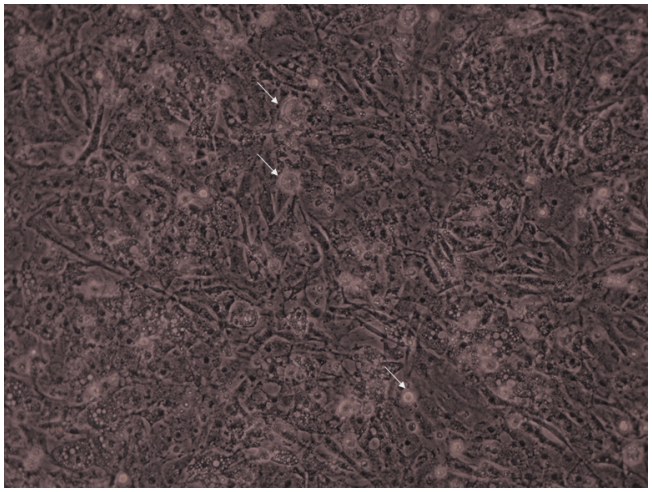

Fig. 3. Observed cytopathic effects of avian influenza virus (AIV) in chicken embryo fibroblast cells; the clear giant cells with inclusions were observed although the monolayer was intact. Some aggregated cells were also observed. x40

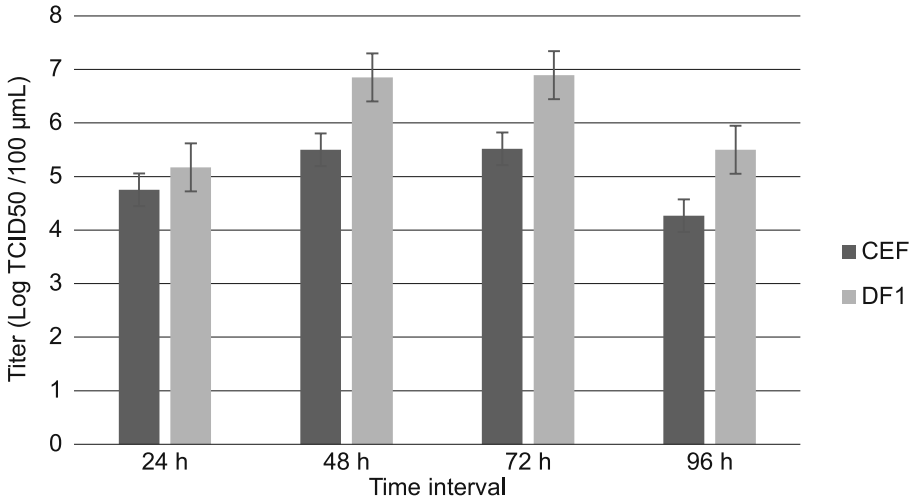

Fig. 4. Graph showing growth kinetic values of AIV in both DF-1 and CEF cells; a high titer was observed in DF-1 cells in comparison to CEF cells.

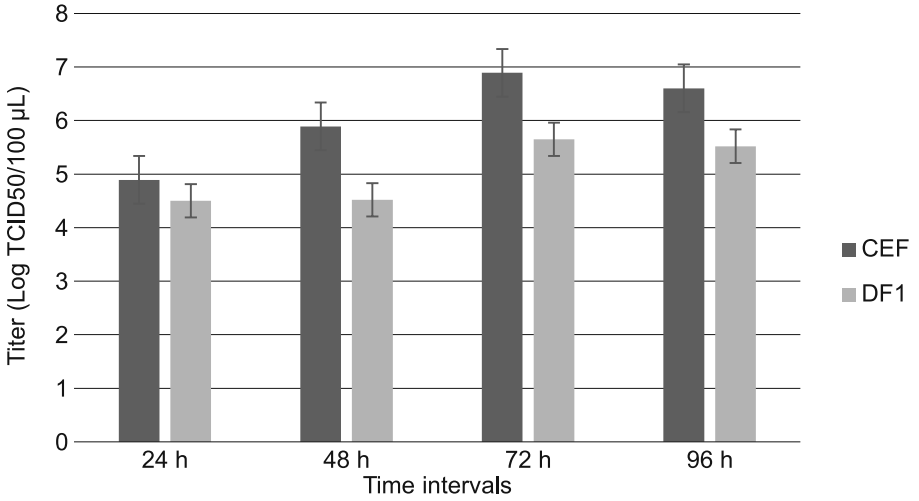

Fig. 5. Comparative growth titer of NDV in both DF-1 and CEF cells; CEF cells exhibited more potential for growth of NDV than DF-1 cells.

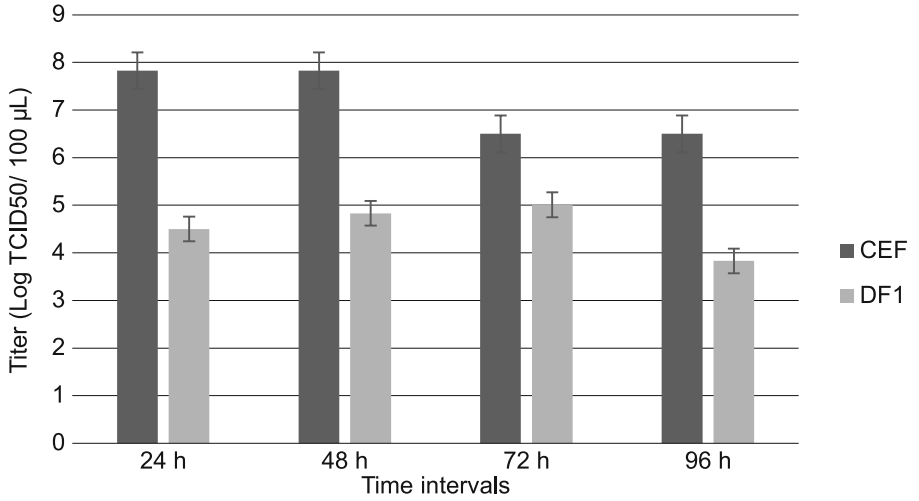

Fig. 6. Growth kinetic values of IBDV; CEF cells showed more suitability for higher growth titer for IBDV than DF-1 cells.

Discussion

Both DF-1 and CEF cells have ability to grow rapidly but DF-1 cells grow more slowly than CEF cells as the cell density of CEF cells increases more rapidly than DF-1 cells after each 24 h. The main difference observed in their morphology was that CEF cells were more longer spindle shaped than DF-1 cells. Regarding the CPE of the viruses, IBDV showed similar type of CPE in both CEF and DF-1 cells causing rounding of cells. These results were found to be similar with the findings of previous study (Rekha et al. 2014). Aggregated cells with round shape and formation of multicellular large nuclei were observed as CPE of NDV in both DF-1 and CEF cells. A previous study also supported these results (Kamal et al. 2015). In case of AIV, aggregation and giant cells with inclusions, keeping the monolayer somewhat intact, was observed. Similar CPE was also reported by other authors in MDCK and other cells (Hamilton et al. 2011).

Different cell lines are used to propagate AIV, IBDV and NDV. As previously described, AIV grow efficiently in chicken embryonated eggs but it seemed to be laborious and consuming. However, growth kinetic of AIV with high titer in DF-1, CEF, MDCK and tracheal organ cultured cells have also been reported (Moresco et al. 2010). In our study, AIV shows more potential to grow in DF-1 cells, compared to CEF cells. However, after 72h of PI, titers of 6.89 $Log_{10}TCID_{50}$/100 µL and 5.5 $Log_{10}TCID_{50}$/100 µL were observed in DF-1 and CEF cells, respectively (increase in 1 Log $TCID_{50}$), indicating that AIV grow more rapidly with high value of titer in DF-1 cells than CEF cells. Lee et al. (2008) described DF-1 cells as an important biological system other than chicken eggs for propagation of AIV. They evaluated QT-6, and DF-1 cells instead of MDCK and primary CEF cells. It was concluded that DF-1 may be considered another avian cell lines suitable for propagation of AIV with high titer.

NDV has potential to grow in chicken embryonated eggs and different avian cell lines. In our study, NDV cultured in CEF cells showed higher titer than DF-1 cells. Maximum titer of the virus (6.89 $Log_{10}TCID_{50}$ / /100 µL) was observed after 72h PI. No comparative growth kinetic study on NDV using DF-1 and CEF cells has been done before. Tan et al. (2016) used DF-1 cells for study of NDV. Growth kinetic study of NDV was also performed using chicken broilers by other authors (Ara et al. 2009). They also used CEF cells for *in vitro* growth of NDV but no results were reported about comparative growth kinetic study.

A comparative study of IBDV was performed by other authors (Rekha et al. 2014) in which DF-1 and CEF cells were used to determine suitable cells giving higher titer of virus. They reported DF-1 cells as suitable avian cells to grow IBDV in high titer. These results were contrary to our findings. In our results, CEF cells show more potential to give high growth titer of IBDV as compared to DF-1 cells. Our study may be helpful for further investigations to compare various cell lines suitable for virus replication.

Conclusions

In view of the above mentioned results, the viruses were grown in these cell lines successfully, and CEF cells are suitable to obtain NDV and IBDV in high titer and may be considered for vaccine production. But in case of AIV, DF-1 are better than CEF to obtain high growth kinetic. The authors recommend further verification of the use of these cell lines with different strains

of these viruses (virulent/attenuated, pathogenic/apathogenic) prior to their application.

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