EFFICIENT REPLICATION BUT LOW TITER GROWTH OF INFLUENZA VIRUS IN IMMORTALISED CHICK EMBRYO FIBROBLASTS CELL LINE

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ABSTRACT

Embryonated chicken eggs (ECEs) and mammalian origin Madin-Darby Canine Kidney (MDCK) cell line are culture systems of choice currently recommended for the isolation and propagation of influenza viruses. Spontaneously immortalized chick embryo cell lines (UMNSAH/DF-1) seem to be promising for the growth of influenza virus compared to ECEs because their use are less time-consuming, and they are free of virus and oncogenes compared to MDCK. The growth conditions influenza virus in UMNSAH/DF-1 cell were evaluated and optimized using Influenza A/Hong Kong/8/68 and B/Maryland/1/59 strains. Virus replication was assessed by haemadsorption, while virus yield was assayed by haemagglutination. In this study, UMNSAH/DF-1 cell was able to support the replication of both Influenza A/Hong Kong/8/68 and B/Maryland/1/59 viruses as demonstrated by positive haemadsorption reaction, but with infectious virus Haemagglutinin (HA) titre ranging from undetectable to very low suggesting that the cells are permissive to virus infection but do not release infectious virus particles. Virus replication could not be observed at first and second passage when the supernatants of infected UMNSAH/DF-1 cells were used to infect fresh confluent monolayer of UMNSAH/DF-1 cells. Lectin staining, to assess the expression of α-2, 3– and α-2, 6–linked sialic acid residues on UMNSAH/DF-1 cells, revealed that both SA receptors were expressed on uninfected UMNSAH/DF-1 cells. A sustained expression of both lectins was observed in UMNSAH/DF-1 cells after infection with influenza A/HK/8/68, but not with B/Maryland/1/59 strain. In conclusion, influenza virus replicate in UMNSAH/DF-1 cells without efficient release into the cell culture supernatant. The lectin staining used in this study was not able to completely clarify the reason for the defect in the release of the virus. This will remain unresolved until further studies are performed.

Keywords: Influenza virus - Embryo Fibroblast - Cell line.

INTRODUCTION

Influenza remains an important viral disease which requires a continuous monitoring, surveillance and molecular characterization of the circulating strains. Isolation and propagation of influenza virus remains the most important of the tools required for the surveillance and monitoring of the circulating virus strain and vaccine production. Embriyonating chicken eggs (ECEs) and cell culture are the most sensitive system currently recommended for the laboratory isolation of influenza viruses. Additionally, the yield of high titre stock of influenza virus has been traditionally achieved through propagation of the virus in ECEs making them suitable platforms for the production of vaccine(Spackman et al. 2008;Moresco et al. 2010). However, the use of ECEs is associated with a number of disadvantages and limitations; these apply not only to their use in the laboratory as a diagnostic tool, but also to their use as a platform for vaccine production. The disadvantages include the need for skilled personnel, specialized laboratory facility and equipment as well as the need for reliable fertilized eggs supply. Similarly, despite its long success in vaccine production field, ECEs have a number of problems in use. First, there is evidence that changes can occur in amino acid in the region of HA molecule following propagation of influenza virus in ECEs. Consequently, these changes lead to the generation of a strain that differs from the original circulating strain rendering the vaccine produced ineffective in controlling the epidemic. Second, the egg-based influenza vaccine process is time consuming requiring several months of advance planning in respect to eggs supply and multiple passage needed to obtain high stocks of virus. Third, egg-based influenza vaccine production represents many opportunities for biohazard exposure to the staff, who may be exposed to virus during virus harvest in allantois fluid, and to vaccine recipient, exposed to indigenous viruses, proteins contained in the eggs (Spackman et al. 2008;Moresco et al. 2010). Because of this, there has been an increasing interest in search for suitable alternatives to eggs for the propagation of influenza virus. Of these, primary cells including chicken embryo fibroblast (CEF) and chicken embyo kidney (CEK) cells were initially suggested to serve the purpose, but their further use were limited by the high cost and cumbersome process associated with their maintenance(Moresco et al. 2010). Due to their unlimited and easy supply, continuous cell lines such as MDCK cell lines have emerged as alternative to be become cell culture system of choice eliminating the use of primary cells for the propagation of influenza virus. The majority of these cells are immortalized after infection with a virus or transfection with an oncogene and may therefore suffer from chromosome alteration and be phenotypically different from the donor tissue(Moresco et al. 2010). Thus, there is an urgent need for continuous cell lines that are non-transformed and support the propagation of the influenza virus with comparable or higher sensitivity than ECEs. UMNSAH/DF-1 cell lines are one of these non-transformed cells that derived from chicken embryonic fibroblast after spontaneous immortalization (Sandig and Jordan 2015;Smith et al. 2008).

Therefore, the primary objective of this study was to evaluate and optimize growth conditions for immortalized chick embryo fibroblast (UMNSAH/DF-1) cell, a novel cell line for the propagation of both Influenza A and B viruses.

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METHOD

Viruses and cells

The Virus strains used in this study were made of influenza A/Hong Kong/8/68 ((H3N2), (ATCC number VR-544) and Influenza B/Maryland/1/59 (ATCC number VR-296), purchased from LGC Standards; Middlesex, United Kingdom (UK). Initially, viruses were inoculated in Specific Pathogen Free (SPF) Embryonated hens' eggs in order to produce working stocks of the virus.

Immortalised chick embryo fibroblast UMNSAH/DF-1 (ATCC® CRL-12203™) cells required for cultivation experiments and viral propagation were purchased from American Type Culture Collection; Manassas, VA, USA. UMNSAH/DF-1 is a cell line obtained after spontaneous immortalization of primary chicken embryonic fibroblasts (CEF) cells from 10 day old East Lansing Line (ELL)-0 eggs as described earlier (Sandig and Jordan 2015).

Passage of stock viruses in Eggs

SPF eggs (from Rhode Island Red chickens) were purchased from Henry Stewart & Co limited, UK. Immediately on delivery to the laboratory, eggs were incubated at 37°C in a humidified atmosphere; eggs were turned and humidity level was checked daily. Prior to inoculation on day seven of incubation, using a candle, eggs were examined for embryo development and viability or presence of cracks. Virus stocks were thawed at room temperature and serially diluted ten-fold with sterile phosphate buffered saline (PBS) supplemented with 5% (v/v) antibiotic (containing 10,000 units of penicillin and 10mg streptomycin, Sigma Aldrich; Ayrshire, UK), and 0.1ml of each dilution were inoculated, in triplicate, via allantoic sac route into 7 to 9 day-old eggs using a 25 gauge 16 mm (5/8 inch) needle. Three ECEs were also inoculated with 0.1ml PBS containing antibiotic to serve as negative control. After 24 and 48 hours of incubation at 37°C in a humidified atmosphere, eggs were removed from the incubator and chilled at 4°C for 2 hours in order to kill the embryo and minimize bleeding. Using a Pasteur pipette and tongue depressor, the allantoic fluid was then aseptically harvested, pooled per dilution and centrifuged at 5000 x g for 10 min to pellet debris including some red blood cells. Haemagglutination assay (see section 2.5.2) was performed on the clarified supernatants to confirm the presence of virus and to determine the virus titer. Allantoic fluids positive for haemagglutination were aliquotted and frozen to -20 °C freezer until use as stock virus for the infection of the cell.

Cell culture and viral infection

Following the supplier's standard protocol (ATCC), immortalised chick embryo fibroblast (UMNSAH/DF-1) cells were sub-cultured from 75 cm2 cell culture flasks; cells were cultured in growth medium: Dulbecco's modified Eagle's medium (DMEM) (Sigma Aldrich; Ayrshire, UK) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin and nonessential amino acids. Cells were allowed to grow at 37°C in a 5% CO2 incubator for 24 hours until they become 90% confluent, after which they were detached with PBS containing trypsin at 0.025% (w/v) and resuspended in DMEM growth medium. The cell suspension was counted using a vital stain, trypan blue (T 8154, Lot RNBC 0 242, Sigma Aldrich; Ayrshire, UK) at a 2/3 dilution, in an Improved Neubauer haemacytometer. An appropriate volume of fresh DMEM growth medium was added to achieve a concentration of 2.5 x 105 cells/ml for seeding into 24-well plates. The 24 well plates, each containing 1 ml of cell suspension, were then incubated for 24 hours at 37ºC in a humidified atmosphere of 5% CO2. After 24 hours, growth medium was removed and confluent monolayer cells inoculated with 200µl of ten-fold serial dilutions of virus in 2% FCS supplemented DMEM maintenance medium, then incubated for 1 hour at 37ºC in a humidified atmosphere of 5% CO2 to facilitate virus adsorption to the cells. For each virus dilution, one well was inoculated with 2% FCS supplemented DMEM maintenance medium as negative control. After 1 hour, the inoculum was discarded, replaced with 1 ml of fresh 2% FCS supplemented maintenance medium and incubated at 37ºC in a humidified atmosphere of 5% CO2. Virus growth in the DF-1 cell culture was demonstrated by performing haemadsoprtion (see section 2.5.1) at 24 hour, 48 hour, and 72 hour post-infection as described below.

The effect of serum on the virus propagation was evaluated using serum-free DMEM media as virus growth medium. For this, infection of the confluent UMNSAH/DF-1 cell was carried out as described above, but with the exception that a serum free DMEM medium was used as maintenance and virus growth medium. The levels of viral replication and extracellular virus yield were assessed by performing haemadsoprtion (HAd) and haemagglutination (HA) assays at various times during incubation.

Haemadsorption

Haemadsorption was performed on the supernatant of the infected cells at 24 hour, 48 hour, and 72 h following incubation using 0.5% chicken red blood cells. Adult chicken red blood cells (RBCs) were purchased from TCS Biosciences Ltd; Buckingham, UK. Prior to the experiment, chicken RBCs were washed three times in PBS suspension by centrifugation. After the last wash to achieve a clean supernatant, the pellet was resuspended in PBS at a final concentration of 0.5% (v/v) and kept at 4°C. The maintenance medium was harvested from the inoculated well, and then 500 µl of cold 0.5% adult chicken blood cells was added and incubated for 10 minutes at 4°C. Following incubation, the RBC suspension was removed and the infected cells were gently washed with chilled PBS to remove unbound red blood cells. Finally, the infected cells were microscopically observed for the adhesion of blood cells suggesting a positive haemadsorption and evaluated for positivity grading scale as per the following grades: no expression (−), extremely weak expression (+/−), weak expression (+), moderate expression (++), slightly strong expression (+++), strongest expression (++++). Haemadsorption images (Fig. 3) were captured using a Nikon COOLPIX 995 digital camera. The harvested supernatants from the well showing haemadsorption were collected for serial passaging in a fresh confluent monolayer of UMNSAH/DF-1 cells while viral titer was measured on supernatants of the infected cells by HA assay as described below.

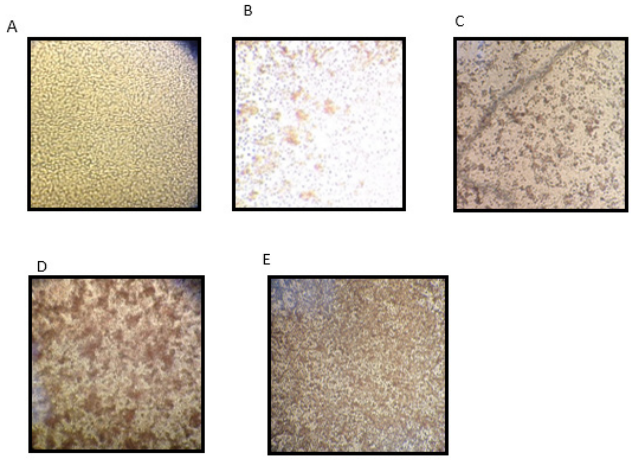

Figure 3 Combination of light field inverted microscopy images of haemadsorption reaction of influenza virus strains in UMNSAH/DF-1 cells as per the grading score. A: Negative (-) haemadsorption reaction in UMNSAH/DF-1 cells, B: Weak (+) haemadsorption reaction in UMNSAH/DF-1 cells, C: Moderate (++) haemadsorption reaction in UMNSAH/DF-1 cells, D: slightly strong (+++) haemadsorption reaction in UMNSAH/DF-1 cells and E: strong (++++) haemadsorption reaction in UMNSAH/DF-1 cells.

Haemagglutination assay

To determine the virus titer, supernatants of infected cell showing haemadsorption were assayed for haemgglutination according to a method previously described (Killian 2008). Briefly, 25 µL of PBS was dispensed in each well in use of 96-well V-bottomed microtitre plates. Subsequently, the first well of each row of column was filled with 25µl of the supernatant of infected cells from which a two-fold serial dilution was carried out through each line for the entire plate. After serial dilution, using a multichannel pipette, each well received additional 25µL of PBS followed by the addition of 50µL of 0.5% chicken RBCs. For each plate, two wells filled with 50µL of PBS and 50µL of 0.5% chicken RBCs served as negative controls. The plate was then gently tapped on its side for mixing and allowed to stand for about one hour at room temperature. The plate was inclined at 45 degrees and read visually for the occurrence of the haemagglutination which consisted of the appearance of a diffuse pattern of settling on the well bottom. Haemagglutination titre was obtained by considering the highest dilution that resulted in the complete agglutination of RBCs.

Lectin staining

Lectin staining was performed to assess the expression of α-2,3– and α-2,6–linked sialic acid (SA) residues on uninfected confluent monolayers UMNSAH/DF-1 cells and 24, 48 hours following infection with both influenza A and B virus. Two biotinylated labeled lectins were selected for use in this evaluation: Maackia amurensis agglutinins (MAA II) which is specific for α-2,3- gal-linked sialic acid and Sambucus nigra agglutinin (SNA) which is specific for α-2,6-gal-linked sialic acid. Both lectins were purchased from Vector Laboratories Inc; Burlingame, CA, USA

UMNSAH/DF-1 cell culture and infection on spot slide

Prior to cell culture on spot slide, 3-well 12 mm spot slides (lot: 473589; BIOTANG Inc., Lexington, MA, USA) were sterilized by immersion in 100% industrial methanol solution (IMS) for 15 minutes followed by 5 minutes exposure to UV light radiation. Then, 150µl of cell suspension at a concentration of 2.5 x 105 cells/ml was seeded in each 12 mm spot and placed in a humid chamber at 37ºC in 5% CO2 for 24 hours. After 24 hours post incubation, cell suspension was removed from each spot, with one slide immediately fixed overnight in acetone, while the remaining slides were used for viral culture. For infecting the cells, 150 µl of 10-1 ten-fold virus dilution was spotted into Confluent monolayer cells on slide and incubated in moist chamber for 1 hour at 37ºC in 5% CO2 for the virus to absorb. On each slide, one spot was inoculated with 150 µl DMEM maintenance medium to serve as negative control. DMEM maintenance media was then spotted on the slide and allowed to incubate in the same condition. Slides were removed from the incubator at 24 hours and 48 hours post infection and fixed overnight in dry acetone prior lectin staining as described below.

Lectin staining

Lectin staining was carried out using a previously described method (Jones et al. 1992) with some modifications. In brief, on the day of lectin staining, slides were immersed in 99% IMS for 5 minutes, after which endogenous peroxidase activity was blocked by treating the slides with methanol containing 30% hydrogen peroxidase (H2O2) and 1M HCl at room temperature for 30 minutes. After three washes of 2 minutes in 0.5M tris-buffered saline (TBS), the slides were incubated for 30 minutes at room temperature with biotinylated MAA II (10 µg/mL) and SNA (10 µg/mL) (Vector Laboratories Inc; Burlingame, CA, USA) in TBS supplemented with 1mM CaCl, followed by three washes of 5 minutes in TBS containing CaCl. One slide was incubated with TBS instead of lectin as negative control to exclude non-specific lectin staining. Avidin-peroxidase (A3151-1MG, Sigma Aldrich; Ayrshire, UK) at 5µg/mL concentration diluted in 0.125M TBS was added to slides and allowed to incubate at room temperature for 1 hour. After three washes of 5 minutes in TBS, slides were incubated with NovaRed substrate solution (Vector Laboratories Inc; Burlingame, CA, USA) for 3 minutes and counterstained by 0.25% methyl green for 1 minute. Slides were allowed to completely dry before being mounted using DePex and for viewing under light microscopy of brown staining indicating cells expression of receptors. Sides were carefully read and receptors distribution within the cells was evaluated on the basis of the percentage of positive expression in cells within in a microscopy field. Lectin staining images were captured using a Nikon COOLPIX 995 digital camera.

RESULTS

Determination Virus stock and optimum working virus dilution

To generate working stocks of the virus used in this thesis, influenza A/Hong Kong/8/68 and B/Maryland/1/59 viruses of haemagglutinin titre (HA): 128 units/0.025mls was selected from previously stored virus and inoculated in ECEs and virus yield assessed by HA assay in allantoic fluid. After the first inoculation attempt, out of six ECEs replicate inoculated with 10-4, 10-5 and 10-6 dilution for both viruses, only allantoic fluid harvested from ECEs inoculated with influenza A/ Hong Kong/8/68 at dilution 10-4 could result in virus yield

as measured by HA assay (data not shown). To assess whether the poor virus yield in ECEs was due to low virus level, HA assay was performed on original virus stocks, while loss in infectivity of virus stock was assessed for their ability to grow in the UMNSAH/DF-1 cell culture, as demonstrated by haemadsoprtion at different time points during incubation. Data from the latter experiment were also used to determine optimal virus inoculum, as well as the optimal period between cell infection and maximum period of culture supernatant collection to be considered during the subsequent evaluation of UMNSAH/DF-1 cells for influenza virus propagation.

It was demonstrated that the HA titre from the current HA assay remained unchanged with virus titre similar (HA: 128 units/0.025mls) to the previous HA titre. This finding was consistent for both influenza A/HK/8/68 and Influenza B/Maryland/1/59 virus stocks. Similarly, both viruses were able to produce clear haemasdoprtion when inoculated in UMNSAH/DF-1 cell indicating a conserved infectivity (data not shown). These findings implied that the poor growth in eggs may not be related to the level of virus nor infectivity in the original virus stocks. Thus, a second eggs inoculation attempt with minor modification was carried out, using low virus dilution (10-2 to 10-4), 7-days old ECEs and harvesting the allantoic fluid 48 hour post inoculation. In this second inoculation attempt, the use of low inoculation dilution resulted in the harvest of allantoic fluids with HA titres ranging from 4 units/0.025mls to 128 units/0.025mls (data not shown).

To optimize the working virus dilution and incubation time, ten-fold dilutions ranging from 10-1 to 10-10 from the same original influenza A/HK/8/68 and Influenza B/Maryland/1/59 virus stock were inoculated in 24 well plates containing confluent monolayer UMNSAH/DF-1 cells; haemadsorption and HA assay of supernatant was assessed at 24, 48, 72, 96, and 120 hour for 5 consecutive days post-virus infection. This experiment revealed that the virus dilution 10-1 was the most efficient infectious dose that displayed a strong haemadsorption 24 hours post-infection with both influenza A/HK/8/68 and B/Maryland/1/59 viruses. After 72 hours post virus infection, cells degeneration occurred without efficient further virus growth as demonstrated by the absence of haemadsorption (data not shown). Based on these findings, virus dilution of 10-1 revealed to be the appropriate infectious dose for the evaluation while 72 hours represented the optimal time period for incubation post-virus infection.

Virus replication and infection titres

Viral replication of influenza A/HK/8/68 and B/Maryland/1/59 in confluent monolayer UMNSAH/DF-1 cells was assessed by haemadsorption while the virus yield in supernatant of infected DF-1 cell was assayed by haemagglutination. Based on the results obtained from the initial optimization experiment (see section 3.1), cell infection was carried out using serial ten-fold dilutions ranging from 10-1 to 10-6 of eggs-grown influenza A/HK/8/68 (HA titre: 16 units/0.025mls) and Influenza B/Maryland/1/59(HA titre: 16 units/0.025mls and 128 units/0.025mls) and efficient replication monitored at 24 hour, 48 hour, and 72 hour post-infection. The result of virus replication and virus yield of the influenza viruses in UMNSAH/DF-1 cell evaluated are presented in the table 1. As demonstrated by haemadsorption, there was a clear evidence of virus replication up to an infectious dose of 10-3 for the influenza B/Maryland/1/59 of eggs-HA titre of 128 units/0.025mls, while influenza virus A/HK/8/68 of HA titre of 16 units/0.025mls, displayed sign of virus replication at an infectious dose of only 10-1. By contrast, the influenza B/Maryland/1/59 128 of HA titre of 16 units/0.025mls showed negative haemadsorption reactions for all infectious doses and throughout the 3 days incubation. As shown in Figure 3 (see section2.5.1), both influenza viruses replicated to the high extent at dilution 10-1, as evidenced by a diffuse haemadsorption reaction. At subsequent dilutions (10-2 and 10-3) of the eggs-grown influenza B/Maryland/1/59 128 of higher HA titres, however, haemadsorption reaction was rather weak and did not increase throughout the 3 days post-virus infection.

In contrast, Virus replication could not be observed at first and second passage using the supernatants of infected UMNSAH/DF-1 monolayers cells to infect fresh confluent monolayer of UMNSAH/DF-1 cells. No haemadsorption reaction was observed throughout the 3 days incubation during the two passages for both influenza viruses.

With respect to HA titre as a measure of virus yield, all infected UMNSAH/DF-1 cells showed negative HA titre with exception of the supernatants of UMNSAH/DF-1 cells infected with the dilution 10-1 of the influenza B/Maryland/1/59(HA: 128 units/0.025mls), which was able to show a virus yield of 2 units/0.025mls. This finding of negative HA titre remained constant at first and second passage and despite centrifugation of UMNSAH/DF-1 cells infected supernatant prior to HA assay. Taken together, these observations imply absence of release of the completed virion, and thus a negative HA titre in the supernatant.

Table 1 Virus replication and infectious titre in UMNSAH/DF-1 cells infected with influenza A/HK/8/68 and B/Maryland/1/59 viruses

Hours post infection	Infectious dose	A/Hong Kong/8/68		B/Maryland/1/59		B/Maryland/1/59	
		HAd[a] reaction	HAU/0.0025 ml	HAd reaction	HAU[b]/0.0025 ml	HAd reaction	HAU/0.0025 ml
24	10^{-1}	++++[c]	0	++++	0	++++	2
	10^{-2}	-	0	-	0	++[d]	0
	10^{-3}	-	0	-	0	+[e]	0
	10^{-4}	-	0	-	0	-	0
	10^{-5}	-	0	-	0	-	0
	10^{-6}	-	0	-	0	-	0
48	10^{-1}	++++	0	++++	0	++++	2
	10^{-2}	-	0	-	0	++	0
	10^{-3}	-	0	-	0	+	0
	10^{-4}	-	0	-	0	-	0
	10^{-5}	-	0	-	0	-	0
	10^{-6}	-	0	-	0	-	0
72	10^{-1}	++++	0	++++	0	++++	2
	10^{-2}	-	0	-	0	++	0
	10^{-3}	-	0	-	0	+	0
	10^{-4}	-	0	-	0	-	0
	10^{-5}	-	0	-	0	-	0
	10^{-6}	-	0	-	0	-	0

[a]HAd: haemadsorption reaction; [b]HAU: Haemagglutinating Units; [c] ++++: strong reaction; [d] ++: Moderate reaction; [e] +: weak reaction and [f] -: No reaction

Finally, investigations were also performed to evaluate the effect of serum on the influenza virus propagation in UMNSAH/DF-1 cells. When serum-free DMEM media was used as virus growth medium, no difference in virus replication and virus yield was observed (data not shown).

Determination of α-2, 3– and α-2,6–linked sialic acid receptors

In attempt to further elucidate the reason of low level of virus released in the supernatant following replication in UMNSAH/DF-1 cells and the possibility of lack or inhibition of viral neuraminidase activity the distribution of receptors on uninfected to infected UMNSAH/DF-1 cells was analysed. Lectin staining was carried out to assess the expression of α-2, 3– and α-2, 6–linked sialic acid residues on uninfected DF-1 cells and 24, 48 hours following infection with both influenza viruses influenza A/HK/8/68 and B/Maryland/1/59, using Maackia amurensis agglutinins (MAA II), which is specific for α-2, 3- gal-linked sialic acid and Sambucus nigra agglutinin (SNA), which is specific for α-2,6-gal-linked sialic acid. As shown in figures 4 and 5, it was found that both receptors were expressed on uninfected UMNSAH/DF-1 cells. Interestingly, there was a marked increase in the expression of both receptors demonstrated by a marked lectin staining at 24 and 48 hours post infection with influenza A/Hong Kong/8/68 (Fig. 4 and 5) As for the cell infected with influenza B/Maryland/1/59, however, no clear difference in lectin staining could be detected between the uninfected and infected UMNSAH/DF-1 cells 24 and 48 hours following infection (Fig. 2 and 5), which correlates with the negative haemadsorption reaction findings during virus propagation.

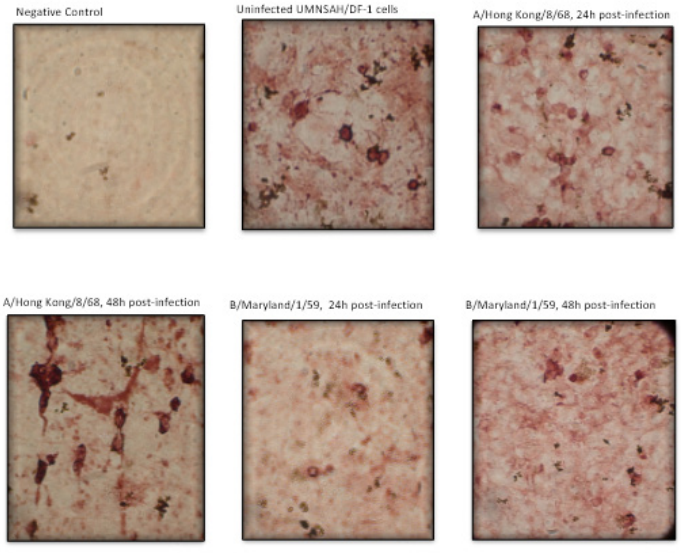

Figure 4 Comparison of lectin staining of uninfected and infected UMNSAH/DF-1 cells with MAA Lectin (specific for α-2,3-linked sialic acid (SA) receptors). The expression of presence α-2,3-linked SA receptors is indicated by a brown color. A slight increase in the expersion of MAA lectin in UMNSAH/DF-1 cells infected with influenza A/HK/8/68 at 24 and 48 hours post-infection. No difference seen after infection with influenza B/Maryland/1/59.

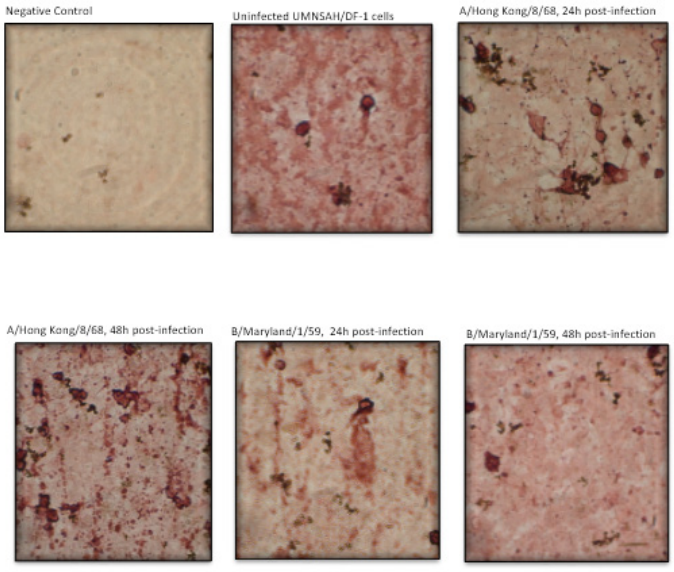

Figure 5 Comparison of lectin staining of uninfected and infected UMNSAH/DF-1 cells with SNA lectin (specific for α-2,6-linked sialic acid (SA) receptors). The expression of presence α-2,6-linked SA receptors is indicated by a brown color. Similar findings of increase in the expersion of MAA lectin was observed in UMNSAH/DF-1 cells infected with influenza A/HK/8/68 at 24 and 48 hours post-infection while no difference appeared after infection with influenza B/Maryland/1/59.

DISCUSSION

For several years, isolation and propagation of influenza virus has been successfully achieved using ECEs, primary cells such as CEF and CEK cells, and recently, continuous cell lines such as MDCK; both as a vaccine production platform and in some cases as diagnostic tools in laboratory(Spackman et al. 2008;Moresco et al. 2010). UMNSAH/DF-1 cell lines are non-transformed cells but with the characteristic of continuous cell line that derived from chicken embryonic fibroblast after spontaneous immortalization. UMNSAH/DF-1 cell lines have been shown to exhibit variable sensitivities and rate of successes with respect to the propagation of both influenza A and B viruses (Sandig and Jordan 2015;Smith et al. 2008). The present study was designed to evaluate and optimize growth conditions for UMNSAH/DF-1 cells, a novel non-transformed cell lines derived from chicken embryonic fibroblast, for the propagation of both Influenza A and B viruses.

Prior to the UMNSAH/DF-1 cell evaluation, both influenza A/HK/8/68 and B/Maryland/1/59 viruses were initially passaged in ECEs to generate the required virus stocks for the experiments. Although this was not part of the study's primary objective, the use of ECEs for passage of both influenza viruses was revealed to yield titres ranging from low to undetectable by HA assay resulting in a high number of ECEs discarded in our study. This is not a surprising finding as in several previous studies; the generation of high virus stock in eggs has been achieved after multiple passages in more than 2 eggs(Tree et al. 2001;Woolcock et al. 2001).

The improved virus yield observed during the second inoculation attempt using high infectious doses inoculum (10-2 to 10-4) and a 7-days old ECEs and virus harvested at 48 hour post-inoculation suggested that the right infectious dose, coupled with the right age of the embryo at the time of inoculation, and the optimal virus harvesting time may represent important

factors in the efficient propagation in ECEs system(Szretter et al. 2006;Zarkov 2006).

The virus yield may also be affected by different uncontrollable factors that included the strain or subtypes of influenza virus which define host specificity, virus-embryo interaction and difference in embryos or batches of ECE used(Zarkov 2006;Parvin et al. 2015). Incubation conditions such as temperature and humidity in the incubator represent additional important factors that may influence virus yield from ECEs(Lang et al. 2011;Khalili et al. 2013). On the other hand, skills are required during the whole process, but especially during candling of eggs, to evaluate embryo viability before inoculation. Finally, the presence of specific receptors on the lining tissues of the inoculated route of the embryo has been reported to impact the efficient viral growth in ECEs(Feldmann et al. 2000;Ito et al. 1997). This may partly explain the unsatisfactory virus yield, with particularly B/Maryland/1/59 strain, a human virus known to preferentially bind to 2, 6-linked sialic acids absent within the allantois lining. For this virus, the inoculation of the amniotic cavity would have been chosen as there is a degree of 2, 6-linked sialic acids expression within the amniotic lining required for the initial virus binding(Rogers and Paulson 1983;Ito et al. 1997;Connor et al. 1994).

Overall, it should be noted that despite their wide recognition as efficient system for the influenza virus propagation, ECEs may not always be consistent with expected high virus yield, thus caution should always be taken considering the above mention interfering factors.

After optimization of the infectious dose and the incubation time, evaluation planned in the current study was undertaken and viral replication of influenza A/HK/8/68 and B/Maryland/1/59 in UMNSAH/DF-1 cells was assessed by haemadsorption while the virus yield in in supernatant of infected UMNSAH/DF-1 cell was assayed by haemagglutination. In this study, it was found out that both influenza viruses were able to produce a positive haemasdsorption reaction in UMNSAH/DF-1 cells indicating efficient virus replication. This study demonstrates that UMNSAH/DF-1 cells are capable of supporting the propagation of avian and human influenza. Of particular note, the replication of the two strains of influenza virus in UMNSAH/DF-1 cells was achieved without the strains being previously adapted to the cell line.

However, undetectable to low virus yield could be obtained by HA assay from supernatant of wells showing viral replication. Additionally, attempts to passage both influenza viruses in UMNSAH/DF-1 cells were performed without success. These results are in agreement with those obtained in a recent study, which also showed that UMNSAH/DF-1 cells did not support the growth of influenza viruses(Lombardo et al. 2012). Similar observations were also found in a previous project (Kuugbee, 2012) comparing UMNSAH/DF-1 cells to ECEs and other mammalian cells, but with the only difference that, in that study, it was not possible to demonstrate haemadsorption reactions in all the infected UMNSAH/DF-1 cells (unpublished data). The findings of negative HA titre in the present study and other studies mentioned above are surprising since a high virus growth comparable to egg propagation would be expected from a cell line as a result of their similar characteristics and advantages to primary chicken embryonic fibroblasts, their tissue of origin.

Although, our results of negative HA titres match those observed in the above studies, they do not support the previous research where similar cell lines were revealed to be efficient in supporting the propagation of influenza viruses (Lee et al. 2008;Smith et al. 2008;Coussens et al. 2011). In these studies, DF-1, a cell line similar to UMNSAH/DF-1 cell, was found to be comparable to ECEs and other cell lines such as MDCK for the cultivation of different strains of influenza virus and the yield of high virus titre.

Consistent with earlier reports(Szretter et al. 2006), it is interesting to note that the infection of UMNSAH/DF-1 cells with high infectious dose of influenza B/Maryland/1/59 with a high virus has resulted in a very low HA titre in this study, suggesting that the virus replication may depend on initial infection dose, and thus partly explaining heterogeneous results of various studies. The possible interference of high molecular weight anti-protease contained in the serum on the virus replication, through competition with cell protease, has also been suggested by some investigators to be the cause of differences in results observed in previous studies(Travis and Salvesen 1983;Zhirnov et al. 2002). In order to demonstrate this assumption in our study, investigations were performed to evaluate the effect of serum on the influenza virus propagation in UMNSAH/DF-1 cells. However, no difference in virus replication and virus yield could be observed using serum-free DMEM as maintenance medium.

To further explain the variable results obtained in studies evaluating cell substrates for virus propagation, it is important to understand the behaviour of the different influenza virus subtypes during replication and the permissiveness characteristics of the cell line in use.

The first step in the influenza virus life cycle involved the entry of the virus into the cell which is enabled by the binding of the HA to the sialic acid (SA) on the surface of the host cell. Influenza virus HA molecules differ in their specificity to bind to two SA molecules, with avian influenza viruses binding to SA α2-3 linkages, and human influenza viruses recognizing preferentially the SA with α2-6 linkages(Connor et al. 1994;Matrosovich et al. 1997;Samji 2009). This receptor affinity represents an important determinant the efficiency of influenza virus attachment and to initiate viral replication cycle. Consequently, the distribution of receptors on the cell surface is among numerous factors that may have a large impact on viral attachment(Suzuki et al. 2000). In addition to demonstrating the absorption of RBC in UMNSAH/DF-1 cells infected with the two viruses, it was also demonstrated (figures 2 and 3) that both receptors are expressed on uninfected UMNSAH/DF-1 cells, indicating that UMNSAH/DF-1 cells may potentially support the growth of both influenza viruses. This finding seems to be consistent with other research which found widespread distribution of both receptor types in chickens from which UMNSAH/DF-1 cells are derived (Costa et al. 2012;Yu et al. 2011).

The cleavage of the SA and release of the infectious progeny viruses from the cell surface represent another crucial step in the replication of influenza virus following virus assembly and budding. This last stage of the viral replication, enabled by the enzymatic activity of the NA, is of paramount importance in ensuring acceptable virus yields in cell substrates (Rossman and Lamb 2011;Nayak et al. 2009;Samji 2009). In the present study, in view of the striking finding of contrast in the UMNSAH/DF-1 cells infected with influenza viruses between the positive haemadsorption reaction and negative HA titre, it could conceivably be hypothesised that there is possible inhibition of viral neuraminidase activity. The investigation of the expression of SA receptors, as visualised by lectin staining using MAA and SNA, on uninfected UMNSAH/DF-1 cells compared with the expression on infected UMNSAH/DF-1 cells revealed a sustained expression of both lectins in infected UMNSAH/DF-1 cells observed with influenza A/HK/8/68. These data support, at least in part, the hypothesis that the defect in the maturation and release of the influenza A/HK/8/68 could be attributed to the inhibition of viral neuraminidase activity in UMNSAH/DF-1 cells. This is particularly interesting since earlier studies, using ex vivo explants of human and various animal tissues such chicken, duck, and pigs, have showed a deceased or no expression of SA receptor in infected areas, reflecting their successful cleavage by neuraminidase activity during infection (Trebbien et al. 2011;Yao et al. 2008;Costa et al. 2012).

However, the findings of the lectin staining in the present study are not sufficient to explain the reasons as to why viral particles were not realised after successful cell infection. There are several possible explanations for the findings of the present study for which the lectin staining by its self would not be able to provide. Thus, a note of caution is due in assessing the distribution of influenza receptors by lectin staining since the observed increased in receptor expression could be attributed to variable sensitivity and specificity of lectins, and hence detecting unspecific cell signals to infection. In support of this, variable sensitivity and specificity have observed in a previous study using lectins from different manufacturers(Nicholls et al. 2007). Furthermore, it is well known that a high expression of SA receptors in a cell and/or a high affinity of viral HA to SA receptors can interfere with the activity of viral neuraminidase(Benton et al. 2015). In this situation, an enhanced activity of neuraminidase would be necessary to cleave the receptor and allow virus release. It is also established that attachment of influenza virus might occur via wide spectrum of glycan receptors other than SA residues, whose identification is needed to understand the mechanism during viral attachment and release events(Chen et al. 2011). Finally, in addition to the receptors affinity, other cellular and viral factors underlying host-virus interaction need to be explored to fully understand events during viral replication. This will allow us to further explain reasons as to why replication of influenza in UMNSAH/DF-1 cell lines could not result in high virus yield comparable ECEs, despite it being species of origin of the cell lines and possessing relatively similar host-related factors.

Taken together, our observations on the distribution of SA receptors based on lectin staining, while preliminary, raise intriguing hypothesis questions regarding the nature and extent of viral neuraminidase activity, which will remain unconfirmed until further experiments using more robust methods are undertaken.

In conclusion, the evaluation and optimization of UMNSAH/DF-1 cell revealed the cell line to display irregular behaviour with regards to the propagation of influenza virus. UMNSAH/DF-1 cell was demonstrated to support the replication of both Influenza A/HK/8/68 and B/Maryland/1/59 viruses with infectious virus titre ranging from undetectable to very low. It can therefore be suggested that efficient virus attachment and replication occur in UMNSAH/DF-1 cell, but a defect in the maturation and release of virus is likely impaired by a lack of neuraminidase activity. This hypothesis is supported, in part, by the finding of our lectin staining showing the persistence of receptors on infected cells. However, further studies, using neuraminidase activity assay(Sandbulte et al. 2009) are needed to confirm the absence of neuraminidase activity which contributes to the low levels of infectious virus in cultured UMNSAH/DF-1 cells. It would be also interesting to assess the expression of SA receptors in a more specific ways using soluble haemagglutinin and after pre-treatment of UMNSAH/DF-1 cell with neuraminidase(Nicholls et al. 2008).

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