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Comparison between MDCK and MDCK-SIAT1 cell lines as preferred host for cell culture-based influenza vaccine production

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Abstract

Objectives To evaluate MDCK and MDCK-SIAT1 cell lines for their ability to produce the yield of influenza virus in different Multiplicities of Infection.

Results Yields obtained for influenza virus H1N1 grown in MDCK-SIAT1 cell was almost the same as MDCK; however, H3N2 virus grown in MDCK-SIAT1 had lower viral titers in comparison with MDCK cells. The optimized MOIs to infect the cells on plates and microcarrier were selected 0.01 and 0.1 for H1N1 and 0.001 and 0.01 for H3N2, respectively.

Conclusions MDCK-SIAT1 cells may be considered as an alternative mean to manufacture cell-based flu vaccine, especially for the human strains (H1N1), due to its antigenic stability and high titer of influenza virus production.

Keywords Influenza virus · MDCK-SIAT1 cells · MDCK cells · Microcarrier · Vaccine

Introduction

Increasing demands for seasonal influenza vaccine and the need for faster methods of vaccine production during flu pandemics and the threat posed by highly pathogenic avian influenza viruses, have made cell culture a suitable substrate for influenza vaccine manufacturers (Collin and De Radiguès 2009; Genzel et al. 2013; Partridge and Kieny 2013). In addition, the classical egg passages cause rapid changes in virus

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hemagglutinin (HA) amino acids, whereas cell-adapted viruses replicate with high fidelity, which are expected to have potent vaccine immunogenicity (Robertson et al. 1987; Gregersen et al. 2011).

Madin-Darby canine kidney (MDCK) and African green monkey kidney (Vero) cells are two regulatory-approved continuous cell lines being used for influenza vaccine production (Hu et al. 2011). The adherent MDCK cell line which stems from the kidney of a healthy cocker spaniel dog, was established in 1958 and it was the first cell line approved by Food and Drug Administration (FDA) in 2012 for cell culture-based influenza vaccine production (Saier and Milton 1981; Donis et al. 2014). MDCK cell culture can support replication of both human and avian influenza viruses with high efficiency because both α -2,6- and α -2,3-linked sialic acid receptors are exposed on the MDCK cells (Hatakeyama et al. 2005). However, the expression of α -2,6-linked sialic acid receptors on MDCK cells are relatively low compared with human respiratory cells. Consequently, MDCK cells are not an optimized in vitro representation model for human respiratory system. On the other hand, the HA gene is more variable in MDCK-adapted viruses (Oh et al. 2008). MDCK-SIAT1 cell has been derived from the stable transfection of MDCK cells by the cDNA of human α -2,6-sialyltransferase (SIAT1) (Matrosovich et al. 2003). As compared to original MDCK cells, MDCK-SIAT1 cells express two fold higher of 6-linked sialic acids and two fold-lower levels of 3-linked sialic acids. Over-expression of α -2,6-linked receptor can enhance the number of host cells and influenza virions interactions at the attachment step. For this reason, the higher avidity of the binding leads to fewer HA mutations which can be more reliable for several passage analysis of the human influenza virus (Lin et al. 2012).

Microcarriers are a supporting matrix for the bulk growth of adherent cells with higher yields in much lower culture volumes. The cultivation of adherent cells on microcarriers makes it relatively easy to propagate the cells in large scale and reduces culture medium and serum costs by over 50 %. In addition, the use of microcarriers in bioreactors provides better process control and a reduced risk of contamination. Cytodex 1 microcarriers are composed of cross-linked dextran matrix that is substituted with positively charged *N,N*-diethylaminoethyl (DEAE) groups to improve cell growth (Bluml 2007).

Since human strains (i.e. H1N1) recognize α -2,6-linked sialic acid receptors while avian strains (H3N2) preferentially bind to α -2,3-linked sialic acid receptors, we used both A/PR/8/34(H1N1) and Panama/2007/99(H3N2) influenza viruses as representatives to infect MDCK and MDCK-SIAT1 cells by different multiplicities of infection. The virus propagation was then determined at various time courses post-infection using hemagglutination assay and cell culture infective dose 50 (CCID₅₀) assay. In order to evaluate the scale-up merit of the cell-based virus propagation, cytodex 1 microcarrier was used to perform the microcarrier culture as well.

Materials and methods

Cell line and virus strains

A/PR8/34(H1N1) and Panama/2007/99(H3N2) viruses were kindly provided, respectively, by Dr Xavier Saelens (University of Ghent, Ghent, Belgium) and Dr Anke Hueckride (University of Groningen, Groningen, Netherlands). Madin-Darby Canine Kidney (MDCK) cell line (ATCC CCL-34) and MDCK-SIAT1 cell line obtained, respectively, from National Cell Bank, Pasteur Institute of Iran and the Virology Department of University of Tehran.

Influenza virus infection of the host cells

MDCK and MDCK-SIAT1 cell lines were cultured in DMEM containing 10 % (v/v) FBS, 100 IU penicillin G/ml, and 100 μ g streptomycin sulfate/ml in 6 well microplates. The confluent cells were washed twice with phosphate buffered saline (PBS) and inoculated with 1, 0.1, 0.01, 0.001 and 0.0001 multiplicities of infection (MOIs) of Panama/2007/99(H3N2) and A/PR/8/34(H1N1). One uninfected well of each cell type in each plate was considered as control. The cell lines were maintained in 6 well microplates, incubated at 37 °C for 1 h in humidified 5 % CO₂ incubator. Following virus adsorption, the cell monolayer was washed three times with PBS, and then 3 ml fresh infection media containing 2 μ g tosyl phenylalanyl chloromethyl ketone (TPCK)-trypsin/ml (Gibco) was added to each well. Cell culture supernatants and cell lysates were harvested at 1, 2, 3, 4, 6, 8, 12, 24, 48 and 72 h post-infection and the virus titer measurements

were carried out to determine the virus yield based on the established methods (Rimmelzwaan et al. 1998).

Virus infectivity titers in MDCK and MDCK-SIAT1 cells by 50 % cell culture infective dose

To conduct the infectivity assay at different times of 1 (immediately after adsorption), 2, 3, 4, 5, 6, 7, 8, 12, 24, and 72 h post infection, the cell culture supernatants were harvested and evaluated for CCID₅₀. The 96 well microplates were seeded with MDCK and MDCK-SIAT1 cells which were grown to 60–70 % confluency overnight. All grown cells were infected with quadruplicate tenfold serial dilution of the viruses. Three days following infection, the plates were scored for cytopathic effects. CCID₅₀ values were calculated according to the Karber method (Karber 1931; Abdoli et al. 2013a, b). In addition to cytopathic effect observation, HA assay was carried out to confirm the results of CCID₅₀ as well.

Hemagglutination assay (HA)

For titering influenza viruses with hemagglutination assay, 50 µl volumes of the culture supernatants were harvested at each time point. After serial dilution with PBS, 50 µl of 0.5 % fresh chicken red blood cell suspensions were added to each well. After a gentle agitation, the plates were incubated for 30 min at room temperature (RT). The highest dilution showing complete hemagglutination pattern was taken as the end point (Kistner et al. 1998; Othman et al. 2010).

Scale-up virus propagation using Cytodex1 microcarriers

For scaling-up the cell-based influenza virus propagation, Cytodex 1 microcarriers were used. Following sterilization of Cytodex 1 microcarriers (Sigma-Aldrich) they were added at 2 g/l in siliconized spinner flasks (Cellspin Integra Biosciences). 2×10^5 cells/ml were added to each flask with fresh complete DMEM and positioned on a stirrer in 37 °C, 5 % CO₂ humidified incubator and agitated at 55 rpm for 4 h (1 min, with 20 min intervals). Finally, cells bound to the microcarriers were evaluated under microscope. Subsequently, spinner flasks were filled by complete medium up to 70 % of final volume while incubated at 37 °C in 5 % CO₂ humidified incubator.

The attached cells were inoculated with 0.1 MOI as an optimized MOI of the virus for replication on the microcarrier (Genzel et al. 2004; Abdoli et al. 2013a, 2014).

Results

Hemagglutination assay (HA)

The viral log₁₀ HA titers for both H1N1 and H3N2 from the supernatants of infected MDCK and MDCK-SIAT1 cells were calculated. For both cells and both viruses HA titers were observed as early as 12 h post-infection. As shown in Table 1, 12 h results appeared in higher MOIs; 0.1 and 1 with similar values in both cell lines. Table 2 shows 12 h results in 1 MOI in both cell lines. During 24, 48 and 72 h post-infection, all MOIs in both tables showed log₁₀ HA titers which reached the same level of HA units for each time point. At 72 h, the HA titers hit the highest point although the values were higher in H1N1 as compared to H3N2.

Time point measurement of virus infectivity titers in cells by 50 % cell culture infective dose

Tables 3 and 4 summarize the influenza viruses' infectivity titers from both MDCK and MDCK-SIAT1 cells. The results of virus infectivity assay at 1 and 0.1 MOIs for H1N1 showed the presence of infective virus at supernatant of cell culture of both cell lines immediately (t1) post-infection. However, these traces of eluted viruses could be found, as false positive by CCID₅₀. At lower MOIs, for cell inoculation with lower titers of the virus, no eluted virus could be observed in this time point. Virus progeny production was detected at 8, 12, 24 and 48 h post-infection for H1N1 in both cell lines and 12, 24, 48 and 72 h post-infection for H3N2 in both cell lines.

Virus propagation and cell attachment on Cytodex-1 microcarrier beads

The cellular yield for both cells on microcarrier cultures were 2×10^6 cells/ml after 4–5 days of growth with 2 g/l of the solid microcarriers. At 72 h post-infection, the H1N1 yielded 10⁸ CCID₅₀/ml and 40960 HA unit/ml on MDCK-SIAT1 and 10⁸ CCID₅₀/ml and 81920 HA unit/ml on MDCK cells. The yield

Table 1 Kinetic of log₁₀ HA titer/50 µl of MDCK and MDCK-SIAT1 cell supernatant for H1N1

Time of post-infection (h)	Cell line used for virus production									
	MDCK					MDCK-SIAT1				
	MOI (H1N1)					MOI (H1N1)				
	0.0001	0.001	0.01	0.1	1	0.0001	0.001	0.01	0.1	1
4	–	–	–	–	–	–	–	–	–	–
6	–	–	–	–	–	–	–	–	–	–
8	–	–	–	–	–	–	–	–	–	–
12	–	–	–	2.107	2.709	–	–	–	2.107	2.709
24	1.204	2.107	2.408	2.709	3.010	1.806	2.709	2.709	2.709	3.010
48	2.709	2.709	2.709	2.709	2.709	3.010	2.709	2.709	2.709	2.709
72	3.010	3.010	3.010	3.311	3.311	3.311	3.311	2.709	3.010	3.010

MOI Multiplicity of infection

Table 2 Kinetic of log₁₀ HA titer/50 µl of MDCK and MDCK-SIAT1 cell supernatant for H3N2

Time of post-infection (h)	Cell line used for virus production									
	MDCK					MDCK-SIAT1				
	MOI (H3N2)					MOI (H3N2)				
	0.0001	0.001	0.01	0.1	1	0.0001	0.001	0.01	0.1	1
4	–	–	–	–	–	–	–	–	–	–
6	–	–	–	–	–	–	–	–	–	–
8	–	–	–	–	–	–	–	–	–	–
12	–	–	–	–	1.505	–	–	–	–	1.806
24	–	0.903	2.107	1.505	2.107	–	–	1.505	2.107	1.505
48	0.903	2.107	2.408	1.505	2.107	0.903	1.806	1.806	2.107	1.806
72	2.107	2.408	2.709	1.505	2.709	1.505	2.408	2.107	2.107	1.806

MOI Multiplicity of infection

of H3N2 virus following 72 h post-infection was 10^{5.5} CCID₅₀/ml and 10240 HA unit/ml on MDCK-SIAT1 cell and 10^{6.5} CCID₅₀/ml and 20480 HA unit/ml on MDCK cell.

Discussion

Human influenza virus has a high morbidity and mortality worldwide. An effective way to prevent influenza pandemics is vaccination. Considering world population of more than 6.5 billion, pre-pandemic vaccines should be available for the same

number of people. (Osterhaus 2007). The selection of preferred hosts for vaccine production is one of the basic steps to improve the viral yield. Viral yield in cell culture-based production is crucially important, due to its higher virus yield and faster production procedure compared to egg-based system. During influenza pandemics, this translates into more vaccine being available in a limited period of time, hence protecting more people. The viruses produced by cell culture-based system are more similar to the primary human isolates compared to the egg-adapted ones. This is because the cultivation of influenza viruses in eggs leads to the selection of antigenic variants (Schild

Table 3 CCID₅₀/50 µl in MDCK and MDCK-SIAT1 cell supernatant for H1N1

Time of post-infection (h)	Cell line used for virus production									
	MDCK					MDCK-SIAT1				
	MOI (H1N1)					MOI (H1N1)				
	0.0001	0.001	0.01	0.1	1	0.0001	0.001	0.01	0.1	1
1	0	0	0	10 ^{1.5}	10 ²	0	0	0	0	0
2	0	0	0	10 ^{1.5}	10 ²	0	0	0	10 ^{1.5}	10 ^{1.5}
3	0	0	0	10 ^{1.5}	10 ^{2.5}	0	0	10 ^{1.5}	10 ^{1.5}	10 ³
4	0	0	0	0	10 ³	0	0	0	0	10 ^{1.5}
5	0	0	0	10 ^{3.75}	10 ^{1.5}	0	0	0	0	10 ^{2.5}
6	0	0	0	10 ^{1.5}	10 ^{3.5}	0	0	0	0	10 ^{1.5}
7	0	0	0	10 ^{2.75}	10 ⁵	0	0	0	10 ^{2.25}	10 ^{2.5}
8	0	0	10 ^{1.5}	10 ^{4.5}	10 ⁶	0	10 ^{1.5}	10 ^{2.5}	10 ^{3.5}	10 ^{3.5}
12	0	10 ^{2.5}	10 ^{4.5}	10 ^{5.5}	10 ⁷	0	10 ^{1.5}	10 ⁴	10 ^{4.5}	10 ^{4.5}
24	10 ^{5.5}	10 ^{6.5}	10 ^{6.5}	10 ^{7.5}	10 ^{7.5}	10 ^{4.5}	10 ^{6.5}	10 ^{7.5}	10 ^{7.5}	10 ^{5.5}
48	10 ⁸	10 ⁸	10 ⁸	10 ⁸	10 ^{7.5}	10 ^{6.5}	10 ⁷	10 ⁷	10 ^{5.5}	10 ⁵

MOI Multiplicity of infection

Table 4 TCID₅₀/50 µl of MDCK and MDCK-SIAT1 cell supernatant for H3N2

Time of post-infection (h)	Cell line used for virus production									
	MDCK					MDCK-SIAT1				
	MOI (H3N2)					MOI (H3N2)				
	0.0001	0.001	0.01	0.1	1	0.0001	0.001	0.01	0.1	1
1	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	10 ¹	0	0	0	0	0
12	0	0	0	10 ²	10 ²	0	0	0	0	0
24	10 ^{4.5}	10 ^{5.5}	10 ³	10 ^{4.5}	10 ⁴	10 ^{2.5}	10 ⁴	10 ²	0	0
48	10 ^{3.5}	10 ⁵	10 ⁵	10 ³	10 ³	10 ^{3.5}	10 ^{4.5}	10 ^{3.5}	10 ⁵	10 ⁵
72	10 ^{3.5}	10 ⁵	10 ⁴	10 ³	10 ³	10 ^{3.5}	10 ⁵	10 ^{3.5}	10 ⁴	10 ⁴

MOI Multiplicity of infection

et al. 1983; Oxford et al. 1991) and for some strains, it would be even impossible to cultivate them in eggs. A recent example is influenza H3N2 A/fujian/411/02 virus which was unable to replicate in eggs and it was

difficult to produce a match vaccine to control its infection (Del Giudice et al. 2006; Widjaja et al. 2006). A cell culture-based vaccine production platform provides significant advantages in all of these

areas, thus becomes an attractive alternative to the conventional egg-based production system. The preferred hosts for vaccine manufacturing are generally MDCK, Vero and MDCK-SIAT1 cells as they are highly permissive to influenza virus multiplication which can produce flu virions at levels comparable to or even higher than eggs. The growth of influenza virus on a cell line depends on the presence of suitable surface receptors. MDCK-SIAT1 cells over-expressing α -2,6-Sialyltransferase improve the primary isolation and growth of influenza A viruses (Govorkova et al. 1995). Hatakeyama et al. (2005) have demonstrated that human influenza viruses show improved growth in clinical samples and generated much clearer plaques in cells exposing high amount of SA α 2,6 Gal compared to MDCK cells. These results may stem from low level expression of SA α 2,6 Gal on parental MDCK cell surface compared to those on human airway cells. The fact that some influenza viruses grew more than 100-fold in over-expressing β -galactoside α 2,6-sialyltransferase I (ST6Gal I) cells in clinical specimens compared to the parental MDCK cells, suggests that the viruses replicated in MDCK cells may be mutated due to growth in a suboptimal receptor environment. Durocher and Butler (2009) demonstrated increased level of influenza A virus propagation from over-expression of α -2,6-Sialyltransferase in PER.C6 cells compared to unmodified cell line. This supports the utilization of cell lines over-expressing α -2,6-sialic acid for the isolation and recovery of human influenza viruses (Durocher and Butler 2009). Matrosovich and colleagues have indicated that a typical non-egg adapted human H1N1 influenza virus can bind with more avidity to transfected MDCK cells compared to the parental MDCK cells. They also reported that the expression of six linked sialic acid receptors makes the cell surface receptors more accessible for the virus attachment. SIAT1 catalyses the formation of 6-sialyl (*N*-acetyl-lactosamine) [Neu5Ac (α 2,6) Gal (β 1,4) GlcNAc], the high-affinity receptor which is determinant of human influenza A and B viruses attachment. They illustrated that clinical isolates of H1N1 and H3N2 influenza A viruses and type B viruses were more responsive to the NA inhibitor osteltamivir carboxylate in SIAT1-transfected cells than MDCK cells. Their data have supported the hypothesis that the low antiviral response of clinical isolates to NA inhibitor in MDCK cells is due to low level expression of 6-linked

receptor. Furthermore, Vero-SIAT1 cells produce higher titers of influenza A and B viruses than the unmodified parental Vero cells. Stable transfection with SIAT1 genes also increases the concentration of NeuAc α 2,6 Gal receptors on the surface of Vero cells approx. 7-fold (Li et al. 2010). According to Oh et al. (2008), the viruses isolated from MDCK-SIAT1 cells had fewer amino acid variations in the HA-1 domain of the HA gene following several passages as compared to MDCK cells.

In this study, we attempted to compare parental MDCK and SIAT1 expressing MDCK cells in virus yield production and to examine their capacity to propagate influenza A (H1N1 and H3N2) viruses. The reason why we used PR8 as H1N1 candidate is that, to generate influenza virus vaccine seeds, two RNA segments representing HA and NA antigens are selected from the recommended strains, while the remaining six RNA segments originate from A/Puerto Rico/8/34 (PR8; H1N1) using reassortment or reverse genetics approaches. PR8 backbone has enhanced the yield of H1N1, H3N2, H5N1 and H7N9 propagation in African green monkey kidney and Madin–Darby canine kidney cells. Therefore, PR8 vaccine backbone exhibits an advance for development of seasonal and pandemic influenza vaccine (WHO 2005; Abt et al. 2011; Ping et al. 2015).

The optimized MOIs to infect the cells on plates and microcarrier were obtained 0.01 and 0.1 for H1N1 and 0.001 and 0.01 for H3N2, respectively. The obtained results confirmed our expectations by showing that MDCK-SIAT1 cells could support the same level of H1N1 influenza virus growth but produced 1 log fewer H3N2 virus titer compared to MDCK cells. The results of the previous study mentioned H1N1 (human strains) viruses propagated on MDCK-SIAT1 cells had fewer HA mutations because of enhanced α -2,6-linked receptor levels which consequently supports high avidity of interactions between ligand of human influenza viruses and the cell receptors (Lin et al. 2012). Therefore, our data indicate that MDCK-SIAT1 cells are more applicable than parental MDCK cells for propagation of human influenza viruses. We highlight that for H1N1 propagation, in MDCK cells higher MOIs but in MDCK-SIAT1 cells lower MOIs showed comparable results which is more worthwhile to use MDCK-SIAT1. But for H3N2 propagation, no significant difference was detectable between MDCK and MDCK-SIAT1. Overall, the MDCK-SIAT1 cell

line has the capacity to be introduced as a preferred candidate and promising host for cell-based human influenza vaccine manufacturing. This system will open a broad perspective which might be generalized to trigger and facilitate future studies of the virus-host interactions and viral pathogenesis for other cell lines as well. It is given that, for efficient multiplication and high yield of influenza virus, both internal proteins (polymerases PB1, PB2 and PA) and surface proteins (HA and NA) of virus are necessary. Therefore, it would be useful to test the replication of different PR8 reassortants in MDCK and MDCK-SIAT1 cells in the future study, which may represent different patterns of replication with two different cell lines.

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