Original Article

Optimization of Microcarrier-based MDCK-SIAT1 Culture System for Influenza Virus Propagation

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Abstract:

Introduction: The preparation of seasonal influenza virus vaccines and especially its large-scale production requirement after the emergence or reemergence of a pandemic will need an alternative host cell system due to current suboptimal methods and the insufficiency of embryonated chicken eggs needed for producing them. In response to the vital and increasing demand for alternative means for influenza vaccine production, a cell line culture on microcarriers could be a potential alternative to the egg-based production. **Methods:** Influenza A/PR/8/1934 H1N1 was purified and quantified by plaque assay. The purified virus with 0.01 multiplicity of infection (MOI) was inoculated on Madin-Darby canine kidney-Siat1 (MDCK-SIAT1) cell line. Cytodex-1 microcarrier beads (2 g/l and 2.0×10^5cells/ml) were used in a spinner flask to culture MDCK-SIAT1cells .The culture medium was harvested and clarified and the virus yield was quantified by 50% cell culture infective dose ($CCID_{50}$) and hemagglutination assays. Next, the virus was concentrated and purified by ultra-filtration and ultra-centrifugation, respectively. **Results:** MDCK-SIAT1cells attached to the microcarriers and the cell numbers were increased efficiently. The cellular yield from the microcarrier culture was 2×10^6 cells/ml after 4-5 days. The yield of the virus titer measured by $CCID_{50}$ and hemagglutination assays after the clarification was 108 $CCID_{50}$/ml and 40960 HA unit/ml, respectively. **Conclusion:** MDCK-SIAT1cells may be considered as a new substrate for the production of influenza vaccines. Using Cytodex-1 microcarrier beads can be an appropriate strategy to improve the viral yield and to lower the cost of influenza vaccine production. *Vac Res, 2014, 1 (1): 35-38*

Keywords: Influenza virus, Cytodex 1, Vaccine, MDCK-SIAT1 cells.

INTRODUCTION

Influenza has had significant historical impact and continues to pose a serious world-wide threat to public health [1]. The production of influenza virus vaccines is dependent on the availability of embryonated eggs for the virus propagation. This is an extremely cumbersome process which conveys many major drawbacks in respect to the selection of the virus variants and the presence of adventitious viruses. In the 1980s, it was known that the growth of influenza viruses in chicken eggs result in selection of receptor-binding variants with mutation at the antibody binding site of the *hemagglutinin* gene and in many cases it can lead to changed viral antigenic properties [2- 4]. In addition, eggs have to be available in large quantities, particularly in pandemic situations. Any disruption in the egg supply can lead to delays in vaccine production. On the other hand, some influenza strains grow more slowly or less robustly than others and some viral vaccine strains, given the origin of some influenza viruses in birds, can be toxic to the eggs. In such situations, the egg-based influenza vaccine production approaches would be impractical [5- 6].

These limitations put emphasis on the necessity for a cell line-based production system that could replace eggs in the production of influenza virus vaccines. In this respect, cell culture-based production systems such as Madin-Darby canine kidney-Siat1 (MDCK-SIAT1) cells, are extremely attractive for a number of reasons. First, in MDCK-SIAT1 cells, the selection of receptor-binding variants does not take place. Second, they

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create an easier supply of the substrate which in case of a pandemic is of particular importance. Third, the viruses produced represent more closely the wild-type virus in comparison with the egg-adapted viruses and as a consequence, vaccines derived from the mammalian cell-grown viruses induce a higher cross-reactive protective immune response. Fourth, the risks of contamination by adventitious viruses are significantly reduced, making the use of thiomersal (a mercury-containing compound used to maintain a sterile vaccine production line), dispensable. Fifth, the allergic reactions induced by egg proteins are absent [6- 9]. MDCK-SIAT1 cell line has been derived by the stable transfection of MDCK cells with the cDNA of human α-2, 6-sialtransferase (SIAT1) by Matrosovich and colleague [10].

The cell lines used in the production of vaccines are either suspension cells or anchorage-dependent cells. Microcarrier systems are suitable for anchorage-dependent cells which require attachment and spreading of the cells [11]. The need to establish a better method of culturing anchorage-dependent cells in large scale prompted the development of the microcarriers. The first microcarrier culture was demonstrated by Van Wezel in 1967 with diethyl-aminoethyl (DEAE) Sephadex A50 particles, originally designed as column-packing beads for ion exchange chromatography [12]. The first industrial-scale product (*i.e*. inactivated polio vaccine) produced in a microcarrier culture was subsequently introduced by Van Wezel in 1972 [13].

In microcarrier cell culture technology, anchorage-dependent animal cells are grown on small spherical surfaces (100 to 300 µm diameter) and at density of 1.02-1.05 g/cm3 that are maintained in stirred suspension cultures. Owing to their extremely high surface area to volume ratio, the microcarriers are an attractive alternative to conventional monolayer cell culture techniques, such as roller-bottle, stirred-tanks, perfusion or air-lift methods [14-15].

Cytodex-1 is a general purpose microcarrier, formed by substituting a cross-linked dextran matrix with positively charged DEAE groups, distributed throughout the matrix. It is particularly suitable for most established cell lines and for production of viruses or cell products from cultures of primary cells and normal diploid cell strains [14]. It is interesting to note that for many cell types, the initial attachment rate to charged microcarriers, such as Cytodex-1 is faster than to those coated with collagen which has an excellent surface for the cell adhesion [16].

In this study, influenza A/Puerto Rico/8/34 (H1N1) virus was propagated on MDCK-SIAT1 cell line and was expanded on the Cytodex-1 microcarrier. The efficiency of virus growth was evaluated by $CCID_{50}$ and hemagglutination assays. The supernatants were concentrated and purified by ultrafiltration and ultracentrifugation procedures, respectively.

MATERIALS AND METHODS

Cell line and virus strains. MDCK-SIAT1 cell line was kindly provided by Dr. Talat Mokhtari-Azad (Department of Virology, School of Public Health, Tehran University of Medical Sciences). Influenza virus A/Puerto Rico/8/34 (H1N1) was kindly provided by Dr. Xavier Saelens (University of Ghent, Ghent, Belgium).

Preparing the microcarriers. Cytodex-1 microcarrier beads (Sigma-Aldrich, Sweden) were washed with sterile PBS (pH 7.2) by allowing them to settle to the bottom of the bottle and aspirating the supernatant just above the carriers, followed by replacing the buffer with fresh PBS. The microcarrier beads were then disinfected by autoclaving (120°C, 15 min) and washing was repeated with complete medium. After resuspending the micro-carrier beads in complete medium (Dulbecco's modified Eagle's medium; DMEM, Gibco) containing antibiotics (100 IU /ml penicillin and 100 µg /ml streptomycin), they were allowed to equilibrate to the temperature and pH in a humidified incubator (37°C, 5% CO2) [17].

Seeding the spinner flask. The prepared microcarrier beads were dispensed (2 g/l) to a siliconized spinner flask (Cell Spin, Integra Biosciences, Germany). MDCK-SIAT1 cells (2×105/ml) were added to the flask then complete medium was added to approximately half of the final volume. The flask was placed on a stir plate in an incubator (37°C, 5% CO2), agitated with a 1 min on and 20 min off cycle for 4 h at 55 rpm. After 4 h, a half-ml sample was removed and viewed under a microscope to confirm the cells attachment. Subsequently, complete medium was added up to 70% of the full volume of the flask. Finally, the flask was placed on the stir plate in the incubator as above at 50 rpm as shown in Fig. 1[18- 20].

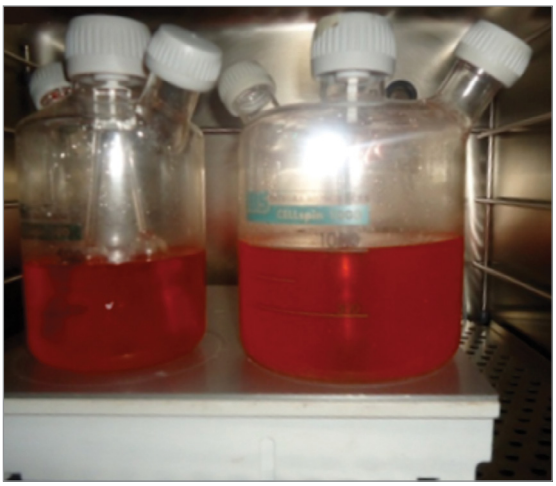

Fig. 1. MDCK-SIAT1 cell growth on Cytodex-1 microcarriers in spinner-flask cultivation

Feeding the microcarrier culture and harvesting the supernatants. The culture was monitored by counting the cells, determining the cell's viability, measuring its glucose and lactate concentrations and visually inspecting its pH indicator. For feeding the culture, the spinner flasks were transferred from the incubator to a laminar flow hood and after allowing the microcarrier beads to settle-down, half the supernatant volume was carefully aspirated and replaced with fresh complete medium and the flasks were incubated as above.

Virus inoculation. Following quantification of the virus by plaque-forming unit assay, approximately 80% of the cell culture growth medium was replaced with complete DMEM medium without serum, with 2 µg/ml L-1-tosylamido-2-phenyle-

thyl chloromethyl ketone (TPCK)-treated trypsin (Sigma). The MDCK-SIAT1 cells were infected with PR8 virus at a MOI of 0.01. Finally, the virus stocks were collected from the microcarrier culture supernatants at 96 h post-infection [21].

Clarification and hemagglutination (HA)-assay. Cell derbies and Cytodex-1beads were completely removed by 3 rounds of centrifugation (5000 rpm, 10 min). The clarified supernatants, considered as the source of the influenza virus, were diluted in two-fold serial dilutions with PBS and 50 µ1 of each dilution were added to 50 µl of a 0.5% suspension of chicken red blood cells in a U-shaped microtitre plate. After gentle agitation, the plates were left undisturbed for 30 min at room temperature and then read. The amount of HAU corresponded to the reciprocal value of the highest virus dilution that showed full hemagglutination [22].

Determination of infectious viral particles. After harvesting the supernatants, the infectious viral particles were determined in a 96 well plates by measuring 50% cell culture infective dose ($CCID_{50}$/ml). Initial cell density of about 1.0×10^5 cell/cm2 was inoculated with 10 fold serial dilution (100 µl of each dilution) of the virus samples in quadruplicates. The logarithmic virus titer, log10 ($CCID_{50}$/ml), for individual virus samples was done 48-72 h post-infection by the Spearman-Karber formula [23].

Concentration and purification. Tangential Flow Filtration (TFF) ultra-filtration set (Millipore, USA) was used for the concentration of the clarified supernatants using a 300- kDa molecular weight cut-off membrane filter. The purification was carried out by stepwise sucrose equilibrium density gradient ultracentrifugation (10 - 60% ; w/v) for (100'000 x g, 4°C, 1.5 h). The final purified virus particles were harvested from the boundary of the two sucrose layers and dialyzed against HBS at 4°C overnight using a Slide-A-Lyzer dialysis cassette with 10-kDa protein MW cut-off to remove the residual trace of sucrose [24].

RESULTS

As shown on Fig. 2, MDCK-SIAT1 cells attached and grew well on microcarrier beads.

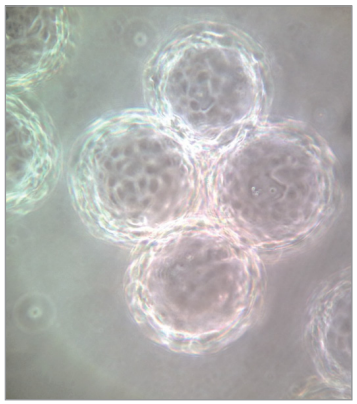

Fig. 2. Cell Attachment of MDCK-SIAT1 cell on Cytodex-1microcarrier beads inside a spinner flask after 80 h, viewed under microscope at 20X magnification

The cellular yield from the microcarrier cultures containing 2 g/l of solid carriers was 2×10^6 cells/ml after 4-5 days of growth. The yield of the virus titer for cells grown on Cytodex-1 yielded 10^8 $CCID_{50}$/ml and hemagglutination assay indicated 40960 HA unit/ml. The virus infectious particles were obtained after 70-h incubation post inoculation. Different Cytodex-1 concentrations from 1 to 3 g/l and initial cell seeds from 0.5×10^5 to 3.0×10^5 cells/ml were tested in a 2-l spinner flask, in order to achieve reliably high final cell densities higher than 2×10^6 cells/ml. However, the maximum cell numbers and the growth behavior of MDCK-SIAT1cells showed that a starting condition with 2 g/l Cytodex-1 and 2.0×10^5 cells/ml seeding cell density were the optimal conditions to obtain the maximal yield. The Influenza virus buoyant density in sucrose was 1.19-1.21 g/ml; therefore, we used 10% and 60% step gradient sucrose for purification of the virus.

DISCUSSION

Influenza virus is one of major health concerns that has had substantial impacts on the human society as well as the poultry industry. Vaccination remains as one of the most effective strategies to mitigate this viral disease. The results of several studies have shown that the passage of the virus in eggs can modify the composition of the antigenic sites of hemagglutinin, making it different from the wild-type virus [2- 4]. The immune responses to such modified hemagglutinin sites would not match exactly to the structure of the circulating virus, leading to inefficacy of the egg-based vaccines [25]. Owing to the ever more obvious limitations of embryonated eggs for manufacturing influenza vaccines, the search for a stable pipeline and a reliably scalable cell culture system for manufacturing influenza vaccines have drawn substantial attention, particularly in the pandemic situations. MDCK, PER.C6, and Vero cells are the only recommended cell lines for manufacturing influenza vaccines by the World Health Organization [6]. In this study, MDCK-SIAT1 cells were selected to be used because this cell line was shown to yield high titers of influenza virus without changing antigenic sites of the hemagglutinin protein [9, 26].

The microcarrier culture is a popular option for cell expansion because of the ease of scaling-up and its readily extendable capacity (up to 10'000 liters). The micro-carriers provide a high surface area to volume ratio for the growth of the anchorage-dependent cells in unit batch cultures. At least, 18 different types of microarriers of various formulations and coating are now commercially available. The reason for selecting Cytodex-1 microcarrier beads in this study was a previous report by Julia A. Tree and colleagues (2001) which indicated their suitability for production of influenza virus from MDCK cells that resulted in high yields up to $0.8\text{-}1.3\times10^9$ PFU/ml and 5.0×10^4 HAU/ml [27]. Our results were quantitatively comparable in terms of cell density to those obtained by Montagon *et al*. [28] who reported a cell density level of 1.6×10^6 MDCK cells/ml on 1 g/l of Cytodex-1. Moreover, our obtained virus titre was similar to Kessler et al. [29] who have demonstrated greater influenza virus particles, yielded from MDCK cells when grown on Cytodex-1 (8.5 Log $CCID_{50}$) in trypsin-free conditions compared to porous microcarriers (7–7.5 log $CCID_{50}$). In a similar approach, Genzel *et al*. [21] have also shown a titer of 2.4 log HA/100µl of Equine influenza strain A/Equi 2 (H3N8) from MDCK cells grown on Cytodex-1 microcarriers.

Interestingly, Mendonca *et al*. [30] and Frazzati-Gallina *et a*l. [31] have shown that Cytodex-1 microcarriers could be used

to collect several supernatant harvests from Vero cells grown under continuous or batch culture conditions when infected with rabies virus. They have also reported that medium renewal of Vero cell culture at a high flow rate resulted in the highest cell density (2.5×10^6 cells/ml) on Cytodex-1. In our follow-up study, we intend to use this method to collect several supernatant harvests from MDCK-SIAT1 cells grown on Cytodex-1 carriers, in order to increase the virus yield from several shedding of the influenza infectious particles.

In conclusion, the aim of this work was the development and optimization of influenza virus propagation in Cytodex-1 microcarrier beads using MDCK-SIAT1 culture for the first time. Cytodex-1 and MDCK-SIAT1 cell line provided an efficient and robust condition for large-scale production of viruses that may replace embryonated eggs and other cell lines for manufacturing of influenza virus vaccines.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

REFERENCES

1. Rappuoli R, Del Giudice G. Influenza vaccines for the future. 2nd ed. Springer Science & Business Media, 2010.
2. Gregersen JP, Schmitt HJ, Trusheim H, Bröker M. Safety of MDCK cell culture-based influenza vaccines. Future Microbiol, 2011; 6 (2): 143-52.
3. Oxford JS, Corcoran T, Knott R, Bates J, Bartolomei O, Major D, Newman RW, Yates P, Robertson J, Webster RG, Schild GC. Serological studies with influenza A (H1N1) viruses cultivated in eggs or in a canine kidney cell line (MDCK). Bull World Health Organ, 1987; 65 (2): 181-7.
4. Robertson JS, Bootman JS, Newman R, Oxford JS, Daniels RS, Webster RG, Schild GC. Structural changes in the haemagglutinin which accompany egg adaptation of an influenza A (H1N1) virus. Virology, 1987; 160 (1): 31-7.
5. Hagenaars N, Mastrobattista E, Glansbeek H, Heldens J, van den Bosch H, Schijns V, Betbeder D, Vromans H, Jiskoot W. Head-to-head comparison of four nonadjuvanted inactivated cell culture-derived influenza vaccines: effect of composition, spatial organization and immunization route on the immunogenicity in a murine challenge model. Vaccine, 2008; 26 (51): 6555-63.
6. Lee I, Kim JI, Park MS. Cell Culture-based Influenza Vaccines as Alternatives to Egg-based Vaccines. J Bacteriol Virol, 2013; 43 (1): 9-17.
7. Genzel Y, Reichl U. Continuous cell lines as a production system for influenza vaccines. Expert rev vaccines, 2009; 8 (12): 1681-92.
8. Pau MG, Ophorst C, Koldijk MH, Schouten G, Mehtali M, Uytdehaag F. The human cell line PER. C6 provides a new manufacturing system for the production of influenza vaccines. Vaccine, 2001; 19 (17-19): 2716-21.
9. Oh DY, Barr IG, Mosse JA, Laurie KL. MDCK-SIAT1 cells show improved isolation rates for recent human influenza viruses compared to conventional MDCK cells. J Clin Microbiol, 2008; 46 (7): 2189-94.
10. Matrosovich M, Matrosovich T, Carr J, Roberts NA, Klenk HD. Overexpression of the α-2, 6-sialyltransferase in MDCK cells increases influenza virus sensitivity to neuraminidase inhibitors. J Virol, 2003; 77 (15): 8418-25.
11. Reuveny S. Research and development of animal cell microcarrier cultures. PhD. thesis, The Hebrew University Jerusalem, 1983.
12. van Wezel AL. Growth of cell-strains and primary cells on micro-carriers in homogeneous culture. Nature, 1967; 216 (5110): 64-5.
13. van Wezel AL. New trends in the preparation of cell substrates for the production of virus vaccines. Prog Immunobiol Stand, 1970; 5: 187-92.
14. Healthcare GE, Biosciences A. Microcarrier cell culture: Principles and methods. ed, GE Healthcare/Amersham Biosciences: 2005.
15. Reuveny SHAUL. Microcarrier culture systems. Large-Scale Mammalian Cell Culture Technology, 1990; 271-341.
16. Peshwa MV, Wong K, Hu WS. Microcarrier culture. Encyclopedia of Bioprocess Technology, 1999.
17. Bleckwenn NA, Shiloach J. Large-Scale Cell Culture. Current Protocols in Immunology, 2004; 59: A. 1U. 1-A. 1U. 44.
18. Abdoli A, Soleimanjahi H, Kheiri MT, Jamali A, Sohani H, Abdoli M, Rahmatollahi HR. Reconstruction of H3N2 influenza virus based virosome in vitro. Iran J Microbiol, 2013; 5 (2): 166-71.
19. Liu CC, Guo MS, Lin FH, Hsiao KN, Chang KH, Chou AH, Wang YC, Chen YC, Yang CS, Chong PC. Purification and characterization of enterovirus 71 viral particles produced from vero cells grown in a serum-free microcarrier bioreactor system. PLoS One, 2011; 6 (5): e20005.
20. Kistner O, Barrett PN, Mundt W, Reiter M, Schober-Bendixen S, Dorner F. Development of a mammalian cell (Vero) derived candidate influenza virus vaccine. Vaccine, 1998; 16 (9): 960-8.
21. Genzel Y, Behrendt I, König S, Sann H, Reichl U. Metabolism of MDCK cells during cell growth and influenza virus production in large-scale microcarrier culture. Vaccine, 2004; 22 (17-18): 2202-8.
22. Abdoli A, Soleimanjahi H, Tavassoti Kheiri M, Jamali A, Jamaati A. Determining Influenza Virus Shedding at Different Time Points in Madin-Darby Canine Kidney Cell Line. Cell J, 2013; 15 (2): 130-5.
23. Szretter KJ, Balish AL, Katz JM. Influenza: propagation, quantification, and storage. Curr Protoc Microbiol, 2006; 15G.1.1-15G. 1.22.
24. Noori M, Ghorbani Sh, Jamali A, Shenagari M, Hashemi H, Saghiri R, Kazemi N, Tavassoti-Kheiri M. Construction of Influenza A/H1N1 Virosomal Nanobioparticles. Iran J Virol, 2012; 5 (2): 13-18
25. Wood JM, Oxford JS, Dunleavy U, Newman RW, Major D, Robertson JS. Influenza A (H1N1) vaccine efficacy in animal models is influenced by two amino acid substitutions in the hemagglutinin molecule. Virology, 1989; 171 (1): 214-21.
26. Li D, Saito R, Suzuki Y, Sato I, Zaraket H, Dapat C, Caperig-Dapat IM, Suzuki H. In vivo and in vitro alterations in influenza A/H3N2 virus M2 and hemagglutinin genes: effect of passage in MDCK-SIAT1 cells and conventional MDCK cells. J Clin Microbiol, 2009; 47 (2): 466-8.
27. Tree JA, Richardson C, Fooks AR, Clegg JC, Looby D. Comparison of large-scale mammalian cell culture systems with egg culture for the production of influenza virus A vaccine strains. Vaccine, 2001; 19 (25): 3444-50.
28. Montagnon B, Vincent-Falquet JC, Fanget B. Thousand litre scale microcarrier culture of Vero cells for killed polio virus vaccine. Promising results. Dev Biol Stand, 1983; 55: 37-42.
29. Kessler N, Thomas-Roche G, Gérentes L, Aymard M. Suitability of MDCK cells grown in a serum-free medium for influenza virus production. Dev Biol Stand, 1999; 98: 13-21.
30. Mendonca R, Ioshimoto LM, Mendonça RM, De-Franco M, Valentini EJ, Beçak W, Raw I, Pereira CA. Preparation of human rabies vaccine in VERO cell culture using a microcarrier system. Braz J Med Biol Res, 1993; 26 (12): 1305-17.
31. Frazzati-Gallina NM, Paoli RL, Mourão-Fuches RM, Jorge SA, Pereira CA. Higher production of rabies virus in serum-free medium cell cultures on microcarriers. J Biotechnol, 2001; 92 (1): 67-72.