Improved In Vitro Cultivation of Swine Influenza Virus

Margot Herman, BSc
Sigrun Haugerud, BSc
Yashpal S. Malik, PhD
Sagar M. Goyal, PhD

Department of Veterinary Population Medicine
College of Veterinary Medicine
University of Minnesota
St. Paul, MN

KEY WORDS: Swine influenza virus, cell cultures, in vitro cultivation, diethylaminoethyl (DEAE)-dextran, trypsin, centrifugation, cytopathic effects

ABSTRACT

Diagnosis of swine influenza virus (SIV) involves virus isolation in embryonated chicken eggs (ECE) and/or Madin-Darby canine kidney (MDCK) cells with trypsin added to the maintenance medium. Both trypsin and diethylaminoethyl (DEAE) dextran have been reported to enhance the attachment, entry, and replication of a number of viruses. Similarly, centrifugation of virus inoculum in shell vials has been found to be useful in growing certain viruses. In this study, we evaluated 8 different cell types for the propagation of SIV with or without the addition of trypsin or DEAE-dextran. In addition, shell vial centrifugation was evaluated to increase virus titers. The cell cultures used were MDCK, porcine turbinate (PT), swine testis (ST), St. Jude porcine lung epithelial cells (SJPL), porcine kidney (PK), feline kidney (CRFK), Vero, and horse stem cells (H-1). None of the cell cultures supported the growth of SIV with or without added trypsin, except for MDCK cells with 0.15% trypsin. The addition of DEAE-dextran did not improve the growth of SIV in any cell type. Similarly, shell vial centrifugation did not seem to be of any help. Our results indicate that the addition of trypsin is a requirement for the cultivation of SIV and that the existing system of SIV cultivation (MDCK cells with trypsin) should be continued until a better method is discovered.

INTRODUCTION

Influenza viruses are single-stranded, negative sense, segmented RNA viruses that belong to the family *Orthomyxoviridae*. Three types of influenza viruses (types A, B, and C) have been described. Influenza A is a broad host-range respiratory pathogen affecting pigs, avians, equines, and humans and is further classified into various subtypes as per antigenic and genetic differences in its surface glycoproteins. To date, 15 hemagglutinin (HA) types and 9 neuraminidase (NA) types have been described.[1]

Swine influenza virus (SIV) is a major pathogen of swine respiratory disease and is a leading cause of economic losses to pig farmers throughout the world.[2] The infection is characterized by weight loss, anorexia, rhinitis, nasal discharge, sneezing, and coughing. Currently, 3 subtypes of SIV are prevalent in the United States, namely H1N1, H3N2, and H1N2.[2,3] Early detection and subtyping of SIV are essential to managing this disease. The classical way of detecting SIV is virus isolation from suspect

pigs followed by hemagglutination-inhibition (HI) and neuraminidase (NA) assays for subtyping.

The standard method for isolation of SIV is inoculation of 10- to 11-day old embryonated chicken eggs (ECE).[4] Because of the cumbersome nature of this method, SIV isolation is now done in Madin-Darby canine kidney (MDCK) cells using trypsin as an additive.[5,6] It has been known since 1975 that the addition of trypsin to culture media stimulates the growth of influenza A viruses.[7] Recently Kessler et al.[8] reported the growth of influenza A and B viruses in the absence of trypsin in serum-free media MDCK cells. Clavijo et al.[9] recommended the use of both ECE and MDCK cells for the growth of SIV in media containing trypsin. Other studies in the past have indicated that the addition of polycations, for example, diethylaminoethyl (DEAE)-dextran, and the use of shell vial centrifugation may also increase the yield of many viruses.[10–15] We are not familiar with any study in which the growth of SIV has been evaluated in various cell lines, in the presence or absence of trypsin or DEAE-dextran, and using shell vial centrifugation. This study was therefore undertaken to fulfill this need.

MATERIALS AND METHODS

Source of Samples

A total of 5 isolates of SIV obtained from 2000 to 2002 at the Minnesota Veterinary Diagnostic Laboratory (St. Paul, MN, USA) were used in all experiments. The samples were isolated in MDCK cells, confirmed positive by reverse transcriptase-polymerase chain reaction (RT-PCR) and were stored at –70˚C until further use.

Cells and Media

The cell lines used were MDCK (National Veterinary Services Laboratories, Ames, IA, USA), St. Jude porcine lung epithelial (SJPL), porcine turbinate (PT; ATCC CRL#2528), Vero-M (ATCC CCL#81), swine testicle (ST; ATCC CRL#1746), porcine kidney (PK-15; ATCC CCL#33), Crandell-Reese feline kidney (CRFK; ATCC CCL#94), and horse stem cells (H-1). These cells were grown in Eagle's minimal essential medium containing 8% fetal bovine serum, 1% essential amino acids, 1% sodium pyruvate, 2% lactalbumin hydrolysate, 1.5% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, penicillin (100 units/mL), streptomycin (100 µg/mL), and fungizone (1 µg/mL).

Additives

Lyophilized trypsin (LS003740; Worthington Biochemical Co, Lakewood, NJ, USA) was diluted in Hanks balanced salt solution to prepare a 1% stock solution and stored at –20˚C. A 1% stock solution of DEAE-dextran was prepared (Sigma-Aldrich, St. Louis, MO, USA) in distilled water followed by autoclave sterilization and storing at 4˚C until use.

Effect of Additives on Cell Viability

To determine the effect of trypsin and DEAE-dextran on cell viability, all cell lines were cultured in 24-well plates followed by the addition of maintenance media containing 7 concentrations of trypsin (0%, 0.05%, 0.025%, 0.1%, 0.15%, 0.2%, and 0.25%) or 4 concentrations of DEAE-dextran (0%, 0.05%, 0.1%, and 0.2%) in duplicate wells. The cells were incubated at 37˚C and observed for signs of toxicity, such as floating dead cells or peeling of monolayers up to 3 days. The concentrations at which the cells showed normal morphology were used to determine the effect of additives on growth of SIV.

Effect of Additives on Growth of SIV

All 5 isolates of SIV were inoculated in duplicate in monolayers of cells prepared in 24-well plates with 1 well of each pair serving as a negative control (containing phosphate buffered saline instead of the virus). Maintenance medium containing optimal concentration of trypsin or DEAE-dextran was added to 1 of the 2 virus-inoculated wells. The second well served as trypsin or

DEAE-dextran control. The cells were incubated at 37°C and observed daily for cytopathic effects (CPE). Two blind passages were given. Six days after the second passage, cells were harvested and frozen and thawed for HA titration. The experiments were repeated 3 times and mean HA titers of the harvested virus were determined.

Effect of Shell Vial Centrifugation on Virus Growth

To determine the effect of shell vial centrifugation on SIV growth, the method of Tahir and Goyal[16] was followed. Briefly, cells (1 × 10^5/mL) were grown in a shell vial. The cells were incubated at 37°C until 80% confluency was achieved. The shell vials containing each of the cells were inoculated with 0.2 mL of 1 of the 5 SIV isolates. The vials were centrifuged at 700 x g for 60 min at 30°C. The monolayers were washed twice with HBSS followed by the addition of 0.5 mL maintenance medium to each vial. Mock infected vials were included as negative controls. Inoculated vials were incubated at 37°C in a 5% CO_2 atmosphere and examined daily for virus-specific CPE.

Hemagglutination Test

The HA test was performed as per the method of Hierholzer et al.[17]

RESULTS

Effect of Additives

The cell lines exhibited variable tolerance to trypsin and DEAE-dextran. The MDCK and H-1 cells tolerated up to 0.2% trypsin, while Vero, PK-15, and SJPL cells withstood 0.05% and CRFK and ST cells tolerated only 0.025% trypsin. The PT cells were highly susceptible to trypsin and did not survive even for a short time at 0.025% concentration. The concentrations that did not produce deleterious effects on cells were used to assess the effect of trypsin on virus growth. Trypsin and DEAE-dextran did not show any effect on SIV growth in any of the cell cultures, except the MDCK cells. The latter cells showed CPE within 3 to 6 days postinfection in the presence of 0.15% trypsin. All samples were blind passaged twice but none showed CPE. In MDCK cells the HA results demonstrated that SIV was present only in MDCK. In addition, HA titers in the presence of trypsin reached a maximum of 1: 2048 (average titer, 1: 870) while without trypsin the titers were 1: 4 to 1: 8 (average titer, 1:2.6).

DEAE-dextran at a concentration of 0.1% and above created a false-positive reading, as all cell lines inoculated with virus isolates showed high HA titers (1:512 to 1:2048) at 0.1% and 0.2% concentrations. However, 0.05% or 0% concentration of DEAE-dextran showed no positive titer. The false-positive behavior of DEAE-dextran at concentrations at 0.1% and above was further confirmed by observing the nonspecific agglutination of red blood cells (RBCs) even in the absence of any virus isolate. All negative controls (cells without virus) showed agglutination of RBCs nonspecifically.

The SJPL cells were found to be extremely slow growing compared with other cell lines. PT was the least hardy of the cell types, showing great difficulty in surviving when exposed to minimal amounts of trypsin (0.025%). SJPL, Vero, PK-15, and CRFK were healthy and did not demonstrate any difficulty in surviving at 0.025% to 0.05% trypsin. MDCK and H-1 were the hardiest cell lines and tolerated high levels of trypsin up to 0.2%.

Effect of Shell Vial Centrifugation

All cells showed signs of poor health within 3 days of incubation after shell vial centrifugation. Of all the cell types, CRFK cells appeared to survive the best. All mock-infected control cells and virus-infected cells were negative for HA, except for MDCK and ST cells, which showed extremely low (1:2 to 1:4) HA titers.

DISCUSSION

The growth of SIV in vitro to high titers is necessary so that vaccines against SIV can be produced economically. In addition, iso-

lation of influenza viruses from clinical samples is important in surveillance and pathogenesis studies. Due to the cumbersome nature of ECE cultivation and continued use of only 1 cell line (MDCK) for in vitro growth of SIV, we considered it necessary to evaluate other cell culture systems for the growth of SIV.

Some of the cell lines (MRC-5, Vero, ST, SJPL, PK, and BHK 21) used in this study have been reported to grow influenza A and B viruses with variable success.[7–9,14,18–22] MDCK with trypsin is considered to be better than amniotic cultivation of influenza viruses.[7,9,14] In a recent study Clavijo et al.[9] found ECE to be better than MDCK and recommended that both ECE and MDCK containing trypsin to be used for SIV isolation. Vero cells were reported to be the least sensitive for influenza A and B viruses by Demidova et al.[19] and Orstavik[20] but Govorkova et al.[21] found them to be the most suitable hosts for these viruses. Orstavik[20] demonstrated that addition of trypsin to medium was essential for obtaining high virus titers, but Kessler et al.[8] obtained high titers of influenza viruses even in the absence of trypsin. A new cell line from porcine lung epithelium (St. Jude porcine lung epithelial cell line; SJPL) has been used to grow influenza A and B viruses and was found to be better than MDCK.[22] In the present study, no cell lines other than MDCK were successful in growing SIV either in the presence or absence of trypsin.

DEAE-dextran and shell vial centrifugation have previously been shown to increase the yield of many viruses. It is postulated that polycations such as DEAE-dextran increase the nonspecific attachment of virus to cells.[10,12–15,23] The addition of DEAE-dextran increased retrovirus growth by 3-fold,[13] while a 17.5- to 37.5-fold increase was seen in herpes virus growth.[15] In the present study, higher concentrations of DEAE-dextran caused nonspecific agglutination of erythrocytes in the HA test even in the absence of virus. In our study, concentrations above 0.05% showed false-positive readings, whereas wells that were inoculated and maintained at lower concentrations of DEAE-dextran gave no positive HA readings, indicating the toxic nature of higher DEAE concentrations. Similar results have been reported by Kaplan et al.[11] in which concentration of 50 μg/mL was found to be toxic to BHK 21 cells. Our results are not in agreement with any of the previous studies on herpes virus, retrovirus, encephalitis viruses, enteroviruses and arboviruses.[10,12–15,24] One of the reasons for the failure of DEAE-dextran to support the growth of SIV could be the highly unstable nature of the influenza virus and requirement of a specific cell surface receptor for attachment. Polycations are reported to enhance the nonspecific binding of the virus to cell surface molecules.[25] Therefore, we conclude that DEAE-dextran might be useful for other viruses but not for the growth of SIV.

Shell vial centrifugation was also ineffective because most of the cells were not able to tolerate the centrifugation process. Minimal positive results obtained with shell vials do not support their use as a functional SIV testing method. The results of this preliminary study demonstrate that the currently available MDCK cells with 0.15% trypsin is a good host system for SIV. Studies with a large number of SIV of different subtypes are needed to draw final conclusions, and a search should continue for an optimum cultivation system for SIV.

REFERENCES

1. Scholtissek C, Hinshaw VS, Olsen CW. Influenza in pigs and their role as the intermediate host. In: Nicholson KG, Webster RG, Hay AJ, eds. *Textbook of Influenza*. Oxford, England: Blackwell Science, Ltd., UK;1998:137–145.
2. Swenson SL, Vincent LL, Lute BM, et al. A comparison of diagnostic assays for the detection of type A swine influenza virus from nasal swabs and lungs. *J Vet Diagn Invest*. 2001;13: 36–42.
3. Choi YK, Goyal SM, Joo HS. Prevalence of swine influenza virus subtypes on swine farms in the United States. *Arch Virol*. 2002;147:1209–1220.
4. Bachmann PA. Swine influenza. In: Pensaert MB, ed. *Virus Infections of Pigs*. London: Elsevier Science;1989:193–207.

5. Erickson GA, Krauss S, Landgraf JG, Swenson SL, Greer-Graham SC. New approaches to swine influenza virus isolation. *Proceedings of the 42nd Annual Meeting of the American Association of Veterinary Laboratory Diagnosticians.* San Diego, CA;1999:45.
6. Reina J, Fernandez-Baca V, Blanco I, Munar M. Comparison of Madin-Darby canine kidney cells (MDCK) with a green monkey continuous cell line (Vero) and human lung embryonated cells (MRC-5) in the isolation of influenza A virus from nasopharyngeal aspirates by shell vial culture. *J Clin Microbiol.* 1997;35:1900–1901.
7. Tobita K, Sugiura A, Enomote C, Furuyama M. Plaque assay and primary isolation of influenza A viruses in a established line of canine kidney cells (MDCK) in the presence of trypsin. *Med Microbiol Immunol.* 1975;30:9–14.
8. Kessler N, Thomas-Roche G, Gerentes L, Aymard M. Suitability of MDCK cells grown in a serum free medium for influenza virus production. *Dev Biol Stand.* 1999;98:13–21.
9. Clavijo A, Tresnan DB, Jolie R, Zhou EM. Comparison of embryonated chicken eggs with MDCK cell culture for the isolation of swine influenza virus. *Can J Vet Res.* 2002;66: 117–121.
10. Pagano JS, Vaher A. Enhancement of infectivity of poliovirus RNA with diethylaminoethyl-dextran. *Arch Ges Virusforsch*. 1965;17:456–464.
11. Kaplan MM, Wiktor TJ, Maes RF, Campbell JB, Koprowski H. Effect of polyions on the infectivity of rabies virus in tissue culture: construction of a single-cycle growth curve. *J Virol.* 1967;1:145–151.
12. Pattyn SR, De Vleesschauwer L. Plaque production by group A Arboviruses. II. Enhancing effect of DEAE-dextran on plaque numbers in chick embryo cells. *Acta Virol.* 1969;13:26–33.
13. Lee SG, Kim S, Robbins PD, Kim BG. Optimization of environmental factors for the production and handling of recombinant retrovirus. *Appl Microbiol Biotechnol.* 1996;45:477–483.
14. Merten OW, Manugueraa JC, Hannoun C, van der Werf S. Production of influenza virus in serum free mammalian cell cultures. *Dev Biol Stand*. 1999;98:23–37.
15. Scott GM, Ratnamohan VM, Rawlinson WD. Improving permissive infection of human cytomegalovirus in cell cultures. *Arch Virol*. 2000;145:2431–2438.
16. Tahir RA, Goyal SM. Rapid detection of pseudorabies virus by the shell vial technique. *J Vet Diagn Invest.* 1995;7:173–176.
17. Hierholzer JC, Suggs MT, Hall EC. Standardized viral hemagglutination and hemagglutination-inhibition tests. II. Description and statistical evaluation. *Appl Microbiol*. 1969;18:824-33.
18. Potgieter LN, Stair EL, Morton RJ, Whitenack DL. Isolation of wine influenza virus in Oklahoma. *J Am Vet Med Assoc*. 1977;171:758-760.
19. Demidova SA, Gadashevich VN, Kirillova FM, EL-Shinnavi IM, Rovnova ZI. Isolation and study of influenza A viruses in different cell cultures. *Vopr Virusol.* 1979;4:346–352.
20. Orstavik I. Susceptibility of continuous lines of monkey kidney cells to influenza and Parainfluenza viruses in the presence of trypsin. *Acta Pathol Microbiol Scan.* 1981;89:197–183.
21. Govorkova EA, Kaverin NV, Gubareva LV, Meignier B, Webster RG. Replication of influenza A viruses in a green monkey kidney continuous cell line (Vero). *J Infect Dis.* 1995;172:250–253.
22. Seo SH, Goloubeva O, Webby R, Webster RG. Characterization of porcine lung epithelial cell line suitable for influenza virus studies. *J Virol.* 2001;75:9517–9525.
23. Hudson JB, Misra V, Mosmann TR. Cytomegalovirus infectivity: analysis of the phenomenon of centrifugal enhancement of infectivity. *Virol.* 1976;72:235–243.
24. Sato K, Inaba Y, Miura Y, Tokuhisa S, Matumoto M. Inducement of cytopathic changes and plaque formation by porcine haemagglutinating encephalomyelitis virus. *Vet Microbiol.* 1983;8:521–530.
25. Bailey CA, Douglas KM, John L. Effects of DEAE-dextran on infection and hemolysis by VSV: evidence that nonspecific electrostatic interactions mediate effective binding of VSV to cells. *Virology*. 1984;133:111–118.