

Cloning and identification of PK15 cells for enhanced replication of classical swine fever virus

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Abstract

Introduction: Classical swine fever virus (CSFV) causes an economically important and highly contagious disease of pigs, leading to economic losses around the world. Attenuated live vaccines with CSFV antigens have played an important role in the prevention and control of the disease. Porcine kidney 15 (PK15) cells have been widely used for the propagation of CSFV, but this cell line is not efficient or homogeneously susceptible to viral infection. **Material and Methods:** To achieve a homogeneous PK15 cell line which enabled high titre replication of CSFV, we used the limiting dilution cell cloning method. **Results:** We developed two cell clones, PK15-1A6 and PK15-3B1, which respectively have high- and low-permissive phenotypes to CSFV infection. The PK15-1A6, PK15-3B1, and PK15 parent cells showed different characteristics in cell proliferation rate, susceptibility to CSFV infection, and CSFV production. The mean virus titres per millilitre reflected by TCID₅₀ values in PK15-1A6, PK15-3B1, and PK15 parent cells were 10^{6.85}, 10^{3.63}, and 10^{4.74}, respectively. **Conclusion:** The PK15-1A6 cell clone is more permissive to CSFV infection than the PK15 parent cells. The screened high-permissive cells will be useful for CSFV propagation and vaccine development *in vitro*, and facilitate research on the pathogenicity of CSFV.

Keywords: classical swine fever virus, PK15, high-permissive cells, cell cloning, vaccine production.

Introduction

Classical swine fever (CSF), which was first recognised in 1810, is a contagious disease of pigs characterised by fever and haemorrhage with an acute or chronic process (1, 10), and it leads to economic losses around the world (6). The pathogenic agent, CSFV, is a member of the genus *Pestivirus* of the family *Flaviviridae* (8) and is hosted naturally only by domestic pigs and wild boars. The positive-sense, single-stranded RNA genome of CSFV is 12.3 kb in length, and it is composed of one long open reading frame followed by two non-translated regions, one on each side (12, 15). Field strains of this virus have been found to belong to various genotypes. Thanks to the application of live attenuated vaccines in experimental immunisations and/or accurate epidemiological surveillance (14), CSF has been controlled or completely eradicated from

domestic pigs in some countries and regions, such as Australia, New Zealand, North America, and Western Europe (5, 11). However, it still has a large effect on swine production in Asia, South America, Eastern Europe, and parts of the former Soviet Union (5). Experimental prophylactic vaccination with live attenuated vaccines (such as C-strain vaccine) and a non-vaccination eradication policy have proved to be effective ways to limit CSF epidemics (9). CSFV antigens have played an important role as vaccine components in the prevention and control of CSF (17) and it is important to produce CSFV in substantial quantities for research not only on vaccines but also on diagnosis of and treatment methods for CSFV infection. CSFV has been cultured *in vitro* in cell lines including porcine kidney cells (PK15, CPK, and SK6) and porcine, bovine, and sheep testicular cells (18). Among these lines, PK15 and ST cells are widely used for the

production of CSFV in laboratories worldwide. As Wang *et al.* described, CSFV did not induce cytopathic effects in cultured cells, and the virus titres, defined as 50% tissue culture infectious dose (TCID₅₀) per millilitre obtained from PK15 cell cultures, were usually low and in proportion to the number of cells infected by the virus (16). It is reported that there is heterogeneity in the PK15 cell population as high- and low-permissiveness to CSFV replication, and not all the cells of a PK15 population are available to be infected by the virus (4).

We aimed to achieve a homogeneous PK15 cell line of high susceptibility to CSFV infection. At the same time, we actively pursued the generation of CSFV in high titres. In this study, the growth curves and the virus titrations in the new cell lines PK15-1A6 and PK15-3B1 were measured and compared.

Material and Methods

Virus and cell culture. The CSFV strain (SD19-15, subgenotype 2.1b) used in this study was isolated previously (3). PK15 cells were purchased from the Institute of Biochemistry and Cell Biology of China (Shanghai, China) and cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) foetal bovine serum (TransGen Biotech, Beijing, China), 50 U/mL of penicillin (Gibco), and 50 µg/ml of streptomycin (Gibco). Cells were maintained at 37°C in a humidified chamber with 5% CO₂.

Immunoperoxidase monolayer assay. Cells in an exponential phase of growth were harvested and seeded for an immunoperoxidase monolayer assay (IPMA). Monolayers of PK15 cells were incubated with CSFV. The infected cells were washed with phosphate buffered solution containing 0.5% Tween-20 (PBST) and fixed with cold acetone and methanol (1:1) for 20 min at -20°C, and then allowed to air dry. After blocking with 5% non-fat milk at 37°C for 30 min, the fixed cells were incubated with specific monoclonal antibodies (kindly provided by Prof. Gaiping Zhang) for 60 min at 37°C in a humidified chamber. Cells were washed three times with PBST, and the fixed cells were incubated with HRP-conjugated goat anti-rabbit IgG (ZSGB biotechnology, Beijing, China). Finally, the cells were visualised using AEC substrate (ZSGB biotechnology) and examined under a light microscope.

Preparation of feeder cells. Peritoneal macrophages (PM) were used as feeder cells, after being obtained according to a previously published protocol (13). Briefly, Kunming mice were intraperitoneally injected with 5 mL of thioglycolate. The peritoneal exudate was collected after 72 h and diluted in DMEM containing 10% FBS. Cells at density of 10⁵ cells/100 µL were transferred into 96-well plates for 2 h. After removal of non-adherent cells, the medium was replaced, and the remaining cells were cultured for five days in the atmosphere with 5% CO₂.

Cloning of PK15 cells by limiting dilution. Well-grown PK15 cells were trypsinised and collected. Then, 100 µL of cells were transferred to 96-well plates pre-coated with PM feeder cells at a density of 5 cells/mL; seeding an average of 0.5 cells/well ensured that some wells received a single cell. Cells were incubated at 37°C for 7–14 days. When cell growth in the bottom of the wells was 50% confluent, the subclones from each plate were transferred into new 96-well plates and incubated at 37°C for several days. The cloning procedure was repeated from the beginning until a stable single cell line was established, and the PK15 clones with high susceptibility for CSFV (and hence suitability for its replication) were identified by the IPMA method described above.

CSFV titration. Monolayers of the three types of PK15 cells were incubated with a series of 1:10 diluted CSFV solutions. Forty replications of each titre were set up, as well as a negative control. The cells were cultured at 37°C for 48 h, and the titres of CSFV (TCID₅₀) replicated in each type of PK15 cells were detected by the IPMA method.

Growth characteristics of the screened PK15 cells. The screened PK15 cells were passaged to the 60th generation; each generation of the cells was collected and stored in liquid nitrogen. The cells that were passaged to the 60th generation were thawed, and the growth curves of these cells were tracked using cell counting at 4, 8, 12, 24, 36, and 48 h post seeding. Six replications at each time point were set and the parent PK15 cells were used as control cells.

Evaluation of CSFV replication ability. The screened PK15 cells were seeded into 96-well plates, and monolayers of the cells were incubated with a series of 1:10 diluted CSFV solutions. IPMA was used to detect the titres of CSFV that replicated in each type of the PK15 cells.

To accurately compare the CSFV replication abilities, a quantitative real-time PCR was performed to detect the CSFV copy numbers in the collected cultures. Briefly, the CSFV-infected cells were incubated for the indicated hours, and the cultures were harvested and stored at -80°C for further analysis. Total RNA was isolated using RNAiso Plus (catalogue number 9108, TaKaRa Bio, Kusatsu, Japan). The first-strand cDNAs were synthesised (PrimeScript RT Reagent Kit, TaKaRa Bio), and quantitative measurements were performed with Fast qPCR Mix (catalogue number RR430, TaKaRa Bio). The ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) was used to perform the quantitative PCR reaction, and the CSFV copy number was determined by referring to a standard curve. The sets of primers used to amplify a 157 bp fragment of CSFV E2 gene for quantitative PCR were designed using a previous report as a guide (2).

Statistical analysis. Data shown as the mean ± SD were analysed using one-way ANOVA followed by LSD multiple comparison by SPSS 20.0 software (IBM SPSS, Armonk, NY, USA). A P value less than 0.05 was considered statistically significant.

Results

Cloned PK15 cell line highly permissive to CSFV infection. In order to achieve a substantially homogeneous high-permissive PK15 cell line, the limiting dilution method was used first to obtain single cells, which were drawn from the heterogeneous parent PK15 cells that contained cell populations with different susceptibilities to CSFV infection. Then, each cell clone derived from a single cell was infected with CSFV and was screened to test its susceptibility to CSFV infection by the number of IPMA-positively stained cells thereafter. Two clones of PK15 cells (PK15-1A6 and PK15-3B1) were screened, of which PK15-1A6 showed more positive cells and higher susceptibility to CSFV infection, while PK15-3B1 showed fewer positive cells than the parent PK-15 (Fig.1). Our results indicated that the PK15-1A6 cell clone was the most easily infected by CSFV.

Characteristics of the screened PK15 cells. The screened PK15-1A6 cells were cultured and detected to ascertain the stability of their high permissiveness to CSFV infection. The cell line still presented good cellular morphology and a steady high permissiveness to CSFV infection after 60 passages. To further characterise the growth of PK15-1A6, PK15-3B1, and the parent cells, their growth curves were depicted. As shown in Fig. 2, the high-permissive PK15-1A6 showed more potent proliferation ability as determined by the

number of cells when compared with the parent PK15 cells or the PK15-3B1 clone. The PK15-3B1 clone showed the slowest growth rate (Fig. 2). At 48 h after seeding, the number of cells of clone PK15-1A6 exceeded those of the PK15 parent and PK15-3B1 cells by approximately 50% and 100%, respectively.

CSFV multiplication in the screened cells. We used the IPMA method to evaluate the CSFV multiplication in the infected PK15-1A6 cells, PK15-3B1 cells, and parent cells. CSFV was serially passaged in the parent cell line and two derivative clones. The virus in the supernatant and cells was collected and stored at -80°C until the susceptibility was determined. A mean virus titre of $10^{6.85}$ TCID₅₀/mL was detected in PK15-1A6 cells, which was significantly higher than the titre of $10^{4.74}$ TCID₅₀/mL produced from the parent PK15 cells ($P < 0.01$) and the $10^{3.63}$ TCID₅₀/mL generated from PK15-3B1 cells (Fig. 3). Our results indicated that the PK15-1A6 cell line could prove itself more effective for CSFV multiplication in high titres.

Replication of CSFV in the screened cells. To compare the influence of cell susceptibility on the replication of CSFV in PK15 cells with high or low susceptibility, cells infected with CSFV were fixed and tested using the IPMA method at different time points. IPMA positive cells were identified as early as 12 h post infection (hpi) in the PK15-1A6 clone and the parent PK15 cells, while no positive cells were found in the PK15-3B1 clone until 18 hpi (Fig. 4).

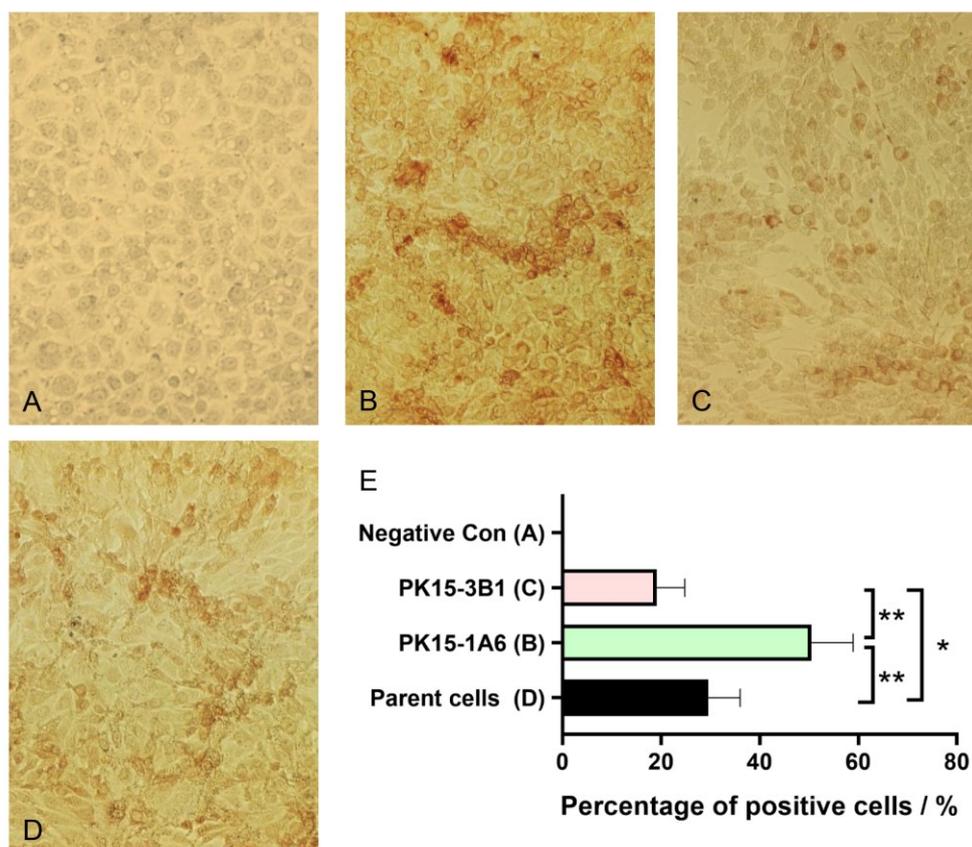


Fig. 1. The percentages of CSFV-infected cells in the parent cell line and cloned PK15 cells. A – mock-infected PK15 cells (negative control); B – high-permissive (clone 1A6); C – low-permissive (clone 3B1); D – infected parent PK15 cells; E – percentages of positively stained cells. The cells were infected with CSFV and stained with CSFV-specific monoclonal antibodies (400 \times). Five visual fields were randomly selected to be photographed in each group. The percentages of positively stained cells in the photos were counted and shown as mean \pm SD. * $P < 0.05$, ** $P < 0.01$

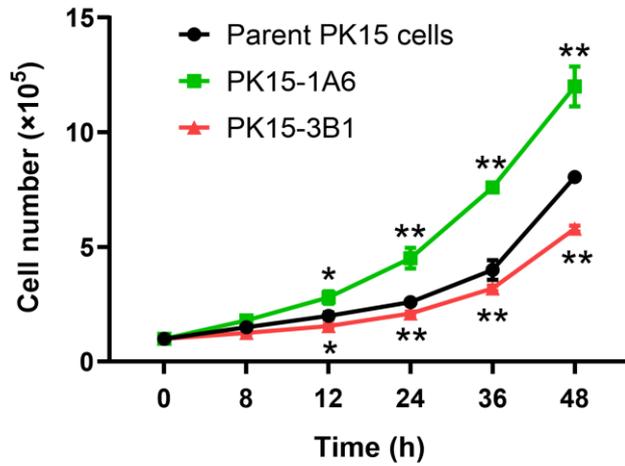


Fig. 2. Growth curves of parent PK15, clone PK15-1A6, and PK15-3B1 cells. The cells were trypsinised and counted at 8, 12, 24, 36, and 48 h post seeding. The data represent the mean ± SD of three independent experiments performed. The differences between the number of parent cells and that of PK15-1A6 cells are shown above the squares. The differences between the number of parent cells and that of PK15-3B1 cells are shown below the triangles. * P < 0.05, ** P < 0.01

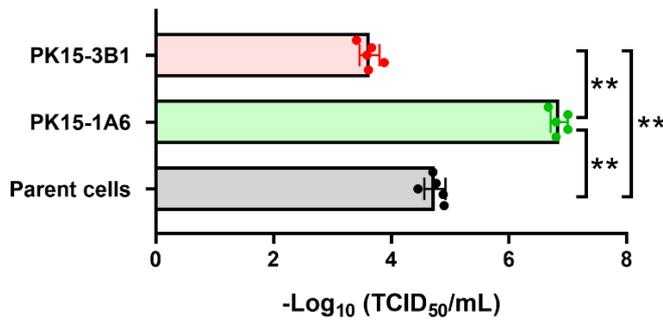


Fig. 3. Mean CSFV titres produced by the parent PK15, PK15-1A6, and PK15-3B1 cells. The replication abilities of CSFV in each type of PK15 cells (TCID₅₀) were detected by the IPMA method. ** P < 0.01

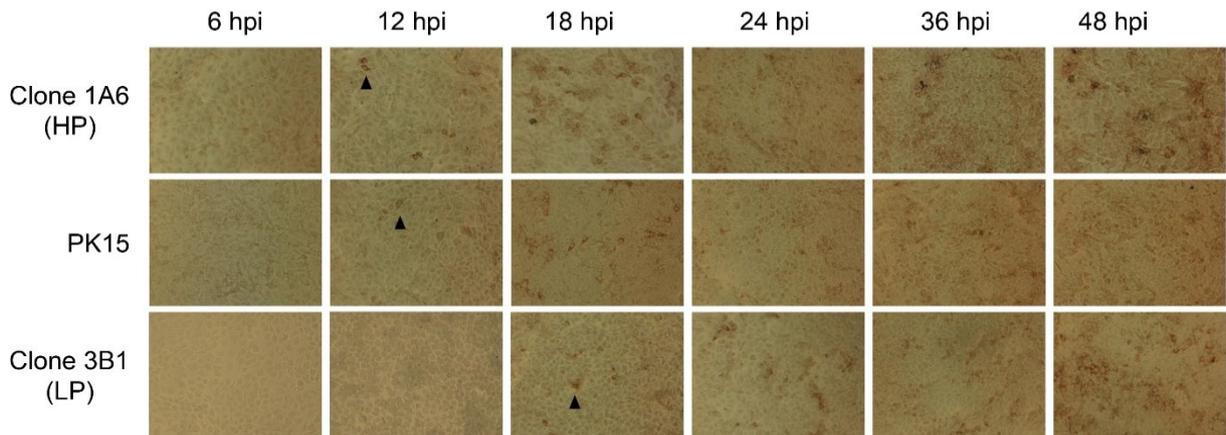


Fig. 4. IPMA results demonstrating the replication abilities of CSFV in high- and low-permissive PK15 cells at different time points. Different cell lines were fixed at 6, 12, 18, 24, 36, and 48 h post CSFV infection and stained with CSFV-specific antibodies followed by HRP-conjugated secondary antibodies (400×)

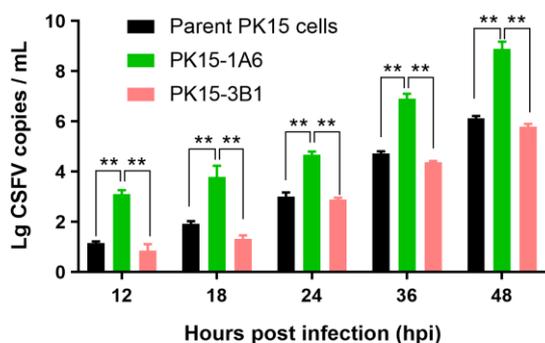


Fig. 5. The synthesis of CSFV genome in parent PK15 cells and PK15-1A6 (HP) and PK15-3B1 (LP) cell lines. The genomic RNA extractions from infected cells were quantitated by RT-PCR (amplification of partial E2 gene). The copy numbers of CSFV were determined by referring to a standard curve. ** $P < 0.01$

We also detected the CSFV titres in the infected cells by quantitative real-time PCR and compared the replication curves. The results showed that significantly higher CSFV copy numbers were detected in the PK15-1A6 clone than those in the parent PK15 cells or the PK15-3B1 clone at all time points ($P < 0.01$) (Fig. 5). At 48 hpi, the CSFV copy number generated in PK15-1A6 cells had increased to $10^{8.89}$ copies/mL, which was much higher than those in the parent PK15 ($10^{6.13}$ copies/mL) and PK15-3B1 cells ($10^{5.79}$ copies/mL).

Discussion

CSFV infection causes a highly contagious disease and the swine industry suffers severe economic losses on its account (6). Systematic prophylactic vaccination with live attenuated vaccines (such as C-strain vaccine) and non-vaccination eradication policies have proved to be effective ways to control CSF epidemics, vaccination demonstrating itself to be the most effective way to prevent and control CSF (9). PK15 cells have been widely used for the propagation of CSFV. However, it was reported that this cell line is not efficient or homogeneous when it is to be used for viral infection (4). To achieve a homogeneous PK15 cell line which could generate CSFV in high titres and facilitate research on vaccines, diagnostics, and therapies for CSF, a stable high-permissive cell line for CSFV infection is needed. After an extensive literature review, we found that no study had been conducted clarifying the efficiency and heterogeneity of a PK15 cell population when infected by CSFV.

Usually, the PK15 porcine kidney cell line is selected to cultivate CSFV *in vitro* in laboratories. The viral titre produced by replication and propagation of CSFV in *in vitro*-cultured cell lines is usually low and does not cause cytopathic effects (16). Our data in this study indicated that the parent PK15 cell line contains cell populations with different susceptibilities to CSFV infection. After limiting dilution cell cloning, we screened two cell clones from PK15 parent cells, namely, the high-permissive PK15-1A6 and low-

permissive PK15-3B1. PK15 cells are established cell lines, besides being susceptible to CSFV, are known to also be to other swine-origin viruses. It is reported that in a PK15 cell culture infected with porcine circovirus 2 (PCV2), about 20% of PK15 cells comprised the group susceptible to the virus (19). It is proven that the lower virus titres which are generated from cells infected with CSFV are related to the heterogeneity of the cell line. There are many reports about the proliferation characteristics of CSFV in different cells, but there had been no cell line that could significantly enhance the propagation of CSFV *in vitro* before this research. In this study, we developed a homogeneous PK15 cell line (PK15-1A6) which showed high susceptibility to CSFV infection and could generate CSFV in high titres, and limiting dilution cell cloning was the approach which gave success. According to previous reports, this technique has been employed to generate high- and low-permissive cells to porcine reproductive and respiratory syndrome virus from the MA-104 cell line (7) and to PCV2 from the PK15 cell line (19). Our results showed that the high-permissive PK15-1A6 showed higher proliferation ability as determined by the cell number when compared with the parent PK15 cells or the PK15-3B1 clone. Further studies need to be conducted to identify whether the rate of virus replication is correlated with the growth rate of the cell line.

Through the whole study, we screened monoclonal PK15 clones that had different proneness to CSFV infection and further confirmed the heterogeneity of the parent PK15 cell line. The PK15-1A6, PK15-3B1, and PK15 parent cells are different in cell proliferation rate, susceptibility to CSFV infection, and virus production. The study found that the PK15-1A6 cell clone is more susceptible to CSFV infection than the parent PK15 cells. Therefore, the PK15-1A6 cell clone will be more advantageous for CSFV replication *in vitro*, production of vaccines, and diagnostic and research applications. Further studies will be conducted to learn the mechanism of the differences in susceptibilities to CSFV infection among PK15-1A6, PK15-3B1, and PK15 parent cells.

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