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Research Article

THE EFFECT OF TEMPERATURE OSCILLATION ON INSECT CELL GROWTH AND BACULOVIRUS REPLICATION FOR PRODUCTION OF RECOMBINANT S1 DOMAIN OF THE PORCINE EPIDEMIC DIARRHEA VIRUS SPIKE PROTEIN

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ABSTRACT
Porcine epidemic diarrhea (PED) is an enteric disease in swine caused by an alpha coronavirus. It affects swine of all ages causing acute diarrhea and can lead to severe dehydration and death in suckling piglets. Temperature oscillation can enhance cell viability of sf9 insect cells and baculovirus production of occlusion bodies (OB) and extracellular virus (ECV) compared with constant temperature in stationary culture and suspension culture. The optimal oscillation range was 24 to 28°C. At this temperature oscillation, the viability of uninfected and infected sf9 cells can be maintained much longer than at 28°C. Although the rate of virus infection was a little low at 24 to 28°C, the final cell infectivity was similar to that at a constant temperature of 28°C. The production of OB was increased from 13.4 to 17.4/cell in stationary culture and from 13.9/cell to 18.1/cell in suspension culture. The titer of ECV was increased from 87 to 114 PFU/cell in stationary culture and from 79 to 114 PFU/cell in suspension culture.

Keywords: *Spodoptera frugiperda Cells, Baculovirus, Temperature, PEDV*

INTRODUCTION
Porcine epidemic diarrhoea (PED) is a highly contagious infectious disease caused by a coronavirus, porcine epidemic diarrhoea virus (PEDV). It causes acute and watery diarrhoea in pigs of all ages although the most severe signs are reported in piglets less than two weeks old, in which diarrhoea leads to severe dehydration and is associated with mortalities which can reach up to 100 % in affected litters (Pensaert & De Bouck, 1978). The PEDV genome is composed of the 50 untranslated region (UTR), at least seven open reading frames (ORF1a, ORF1b, and ORF2 through 6), and the 30 UTR (Kocherhans *et al.,* 2001). The two large ORFs 1a and 1b make up the 50 two-thirds of the genome and encode the non-structural replicase genes. The remaining ORFs in the 30 terminal region code for four major structural proteins: the 150-220 kDa glycosylated spike (S) protein, the 20–30-kDa membrane (M) protein, the 7-kDa envelope (E) protein, and the 58-kDa nucleocapsid (N) protein (Duarte *et al.,* 1994; Lee & Lee, 2014).
The S protein of PEDV is a type I membrane glycoprotein composed of 1,383 to 1,386 amino acids (Bosch *et al.,* 2003), depending on the strain. It contains a putative signal peptide (aa 1–24), a large extracellular region, a single transmembrane domain (aa 1,334–1,356), and a short cytoplasmic tail. Although PEDV has an uncleaved S protein because it lacks a furin cleavage site, the S protein can be divided into S1 (aa 1-735) and S2 (736 - the last aa) domains based on homology with S proteins of other coronaviruses (Duarte & Laude, 1994; Jackwood *et al.,* 2001; Lee *et al.,* 2010a; Sturman & Holmes, 1984). Like other coronavirus S proteins, the PEDV S protein is known to play a pivotal role, interacting with the cellular receptor to mediate viral entry and inducing neutralizing antibodies in the natural host (Bosch *et al.,* 2003; Chang *et al.,* 2002). More precisely, previous studies have shown that the S1domain includes the main neutralizing epitopes and the receptor-binding region (Lee *et al.,* 2011; Sun *et al.,*

2007). Furthermore, along with the full-length S gene, the S1 portion is known to be a suitable region for determining genetic relatedness among the different PEDV isolates and for developing differential diagnostic assays (Chen *et al.,* 2014; Lee *et al.,* 2010b). Considering these molecular and biological features of the S1 domain, it would be an appropriate target for developing effective vaccines against PEDV.

Insect cell culture is an emerging technology for the production of biologicals, including recombinant proteins and biological insecticides. The optimization of insect cell culture systems for the production of insect viruses, primarily those from the Baculoviridae family, has been the focus of many studies (Goodman & Mcintosh, 1994). One of the key points is how to enhance the insect cell growth and baculovirus production through the improvement of culture conditions. Temperature is an important factor affecting insect cell growth and baculovirus replication (Johnson *et al.,* 1982). The temperature range of 25 to 30°C is favorable to insect cell growth, and the optimal range is 27 to 28°C (Agathos *et al.,* 1990). However, the number of occlusion bodies (OB) produced per cell increased at 25 and 32°C compared with 28°C. This shows that the optimal temperature for cell growth is not necessarily the same as that for the production of virus. Most present studies of insect cell culture use a constant temperature in the culture process. This work reports a novel operation in the culture process, temperature oscillation, and studies its effect on insect cell growth and baculovirus replication.

MATERIALS AND METHODS

Cell Line and Virus Stock

The sf9 insect cell line (*Spodoptera frugiperda*) was purchased from Invitrogen Company (USA). Cells were grown in TC-100 medium (Gibco) supplemented with NaHCO3 (0.5 g/liter) 10% (vol/vol). The recombinant baculovirus expressing the full length PEDV-S1 gene of high titer virus (10^8 pfu/mL as determined by plaque assay), was obtained and sustained by Vaccine and Adjuvant lab, Graduate Institute of Animal vaccine Technology, National Pingtung University of Science and Technology, Pingtung, Taiwan.

Culture Conditions and Assay

For stationary cultures, 25-cm2 tissue culture flasks (Nunclon) with 5 ml of medium were used and the seeding density was $2.8x10^5$ cells/ml. For suspension cultures, 150-ml spinner flasks, each with a 50-ml working volume, were used and the seeding density was $3.0x10^5$ cells/ml. The agitation rate was set at 80 rpm. Cell density was determined with a hemocytometer, and viability was assessed by Trypan Blue (0.2%) exclusion. Cells were inoculated with baculovirus at a multiplicity of infection of 0.5 on the 3rd day of culture. Virus was harvested 4 days post infection. Total virus production, including the titer of extracellular virus (ECV) and the number of OB, was determined by end point assay (9) and counting with a hemocyometer, respectively. The percentage of infected cells was obtained by microscopic counting of cells with polyhedral inclusion bodies. The counts of OB and cells were replicated five times in each experiment.

Temperature Oscillation

Cells were grown at 28°C for 2 days and then transferred to different temperature oscillation conditions. The upper temperature limit was kept at 28°C, and the lower limit was 20, 22, 24, or 26°C. Based on preparatory experiments, the switching interval of oscillation was set at 12 h. The culture at a constant temperature of 28°C was used as a control for all comparisons made in this study since 28°C was optimal for baculovirus production among four constant temperatures: 22, 24, 26, and 28°C (data not shown).

RESULTS AND DISCUSSION

Effect of Temperature Oscillation on Uninfected Insect Cell Growth

Figure 1 shows the growth of uninfected insect cells in stationary cultures with different temperature oscillations. The 28°C culture, the culture oscillating between 26 and 28°C, and the culture oscillating between 24 and 28°C had approximately the same maximal viable cell densities ($18.4x10^5$, $18.8x10^5$, and $18.9x10^5$ cells/ml, respectively). The maximal cell density was reached at the 4th day in the 28°C culture

but was not reached until the 5th day in the culture oscillating between 26 and 28°C and the 6th day in the culture oscillating between 24 and 28°C. The cell growth phase in the culture oscillating between 24 and 28°C was about 2 days longer than that in the culture at a constant 28°C. The maximal cell densities of cultures oscillating between 22 and 28°C and those oscillating between 20 and 28°C were not as high as that of the 28°C culture ($16.4x10^5$ and $14.3x10^5$ cells/ml versus $18.4x10^5$ cells/ml), whereas the viable cell density did not reduce at the 7th day in these two cultures. The effect of temperature oscillation on insect cell growth in suspension cultures is shown in Figure 2. The culture with oscillation between 24 and 28°C gave the highest viable cell density ($25.4x10^5$ cells/ml) among all the cultures, and its cell growth phase was 2 days longer than that at 28°C. These results indicated that temperature oscillation can prolong the cell growth phase. Oscillation between 24 and 28°C was optimal for promoting high cell viability without decreasing the maximal cell density.

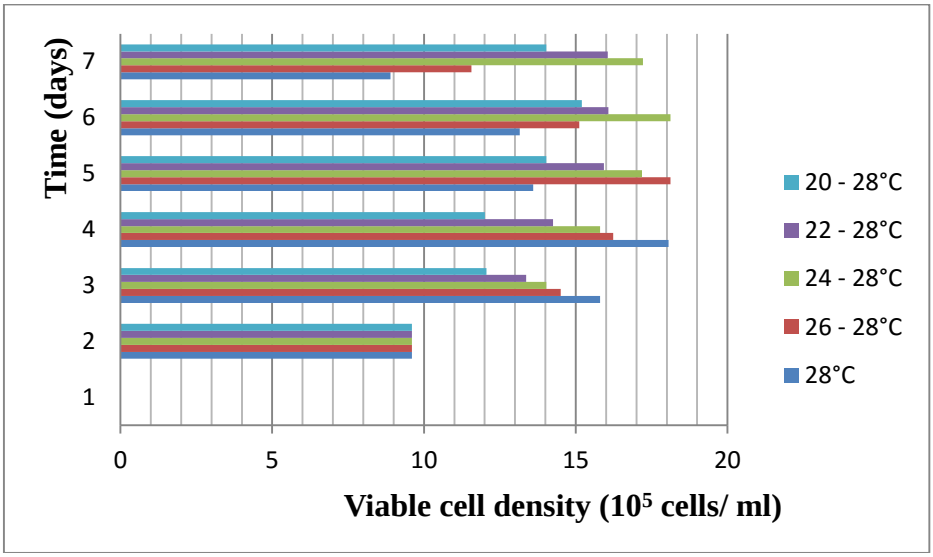

Figure 1: Growth of Uninfected Insect Cells at Four Temperature Oscillations and Bone Constant Temperature in Stationary Culture (Five Replicates were Done)

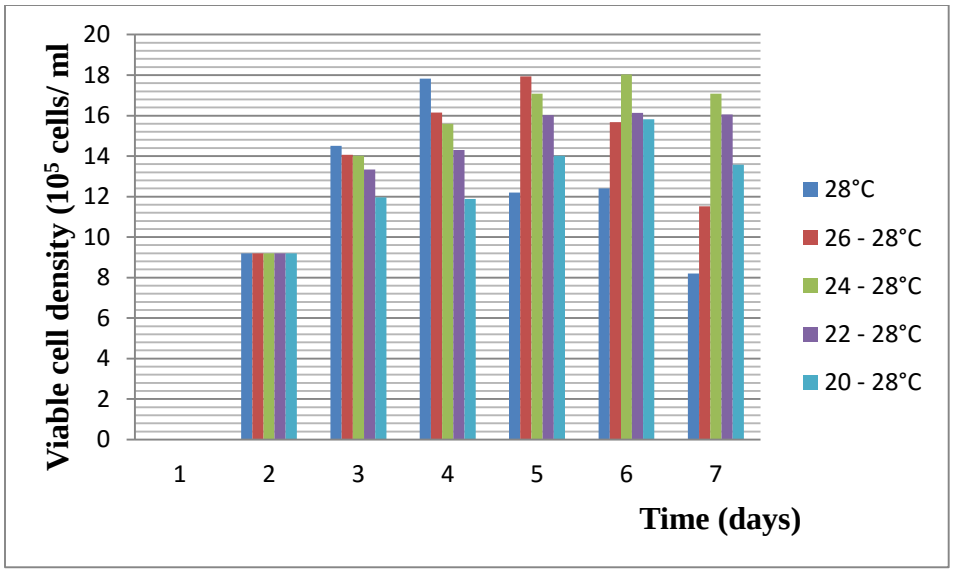

Figure 2: Growth of Uninfected Insect Cells at Four Temperature Oscillations and One Constant Temperature in Suspension Culture (Five Replicates were Done)

Effect of Temperature Oscillation on Infected Insect Cell Growth

The growth of infected insect cells in stationary cultures at different temperature oscillations is shown in Figure 3.

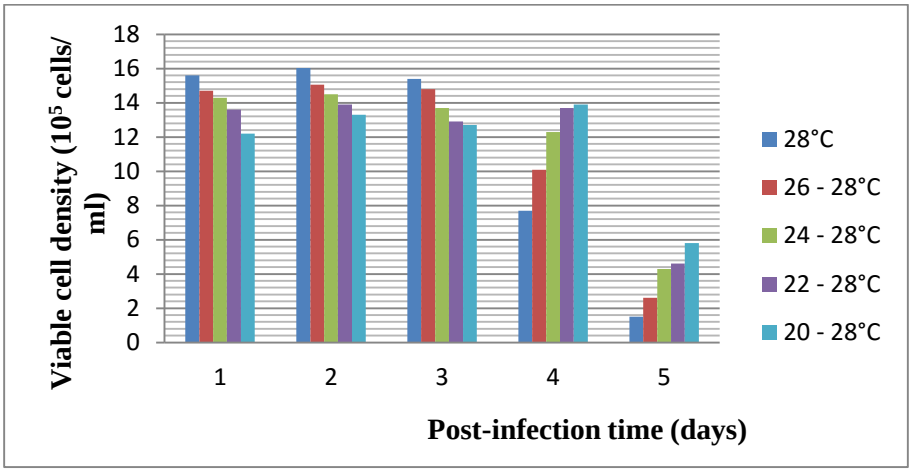

Figure 3: Growth of Infected Insect Cells at Four Temperature Oscillations and One Constant Temperature in Stationary Culture (Five Replicates were Done)

The viability of infected cells declined quickly at 28°C and slightly more slowly with oscillation between 26 and 28°C at 1 day post infection, whereas the viability of other cultures did not change much in the first 3 days post infection. The culture with oscillation between 24 and 28°C kept the highest level of cell viability at 3 days post infection. This suggested that cultures with temperature oscillation can maintain high levels of cell viability, even post infection. Results with suspension cultures were similar to those with stationary cultures (Figure 4). Temperature oscillation was beneficial for maintaining infected cell viability. When the lower limit of temperature oscillation was below 24°C, the viable cell density could be maintained for 3 days.

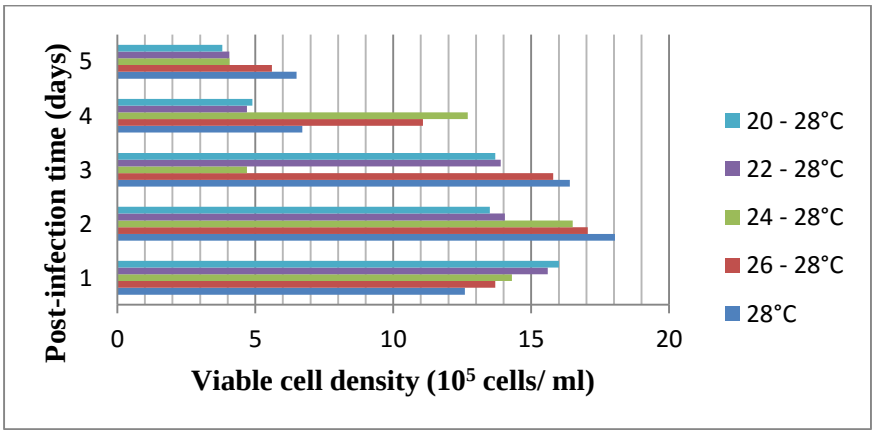

Figure 4: Growth of Infected Insect Cells at Four Temperature Oscillations and One Constant Temperature in Suspension Culture (Five Replicates were Done)

Effect of Temperature Oscillation on Cell Viability and Virus Infectivity

To investigate the effect of temperature oscillation on virus infection, cell viability and virus infectivity were examined in the 28°C culture, the culture with oscillation between 24 and 28°C, and the culture with oscillation between 20 and 28°C. Figures 5 show the cell viabilities and virus infectivities in stationary cultures and suspension cultures, respectively. There was much similarity between the stationary culture

and the suspension culture, except the infection rate in the suspension culture was a little higher than that in the stationary culture. This can be explained by the fact that the movement of released virus particles from infected to noninfected cells in suspension culture is easier than that in stationary cultures (Kioukia *et al.*, 1995). When the rate of virus infection was high, the cell viability dropped rapidly at 28°C. Compared with the culture at 28°C, the culture with oscillation between 24 and 28°C had higher cell viability and a slightly lower rate of virus infection. In the culture with oscillation between 20 and 28°C, virus infectivity remained below 80% and cell viability was nearly 40% at the 4th day post infection. More time was probably needed to attain a higher infectivity in this oscillation culture.

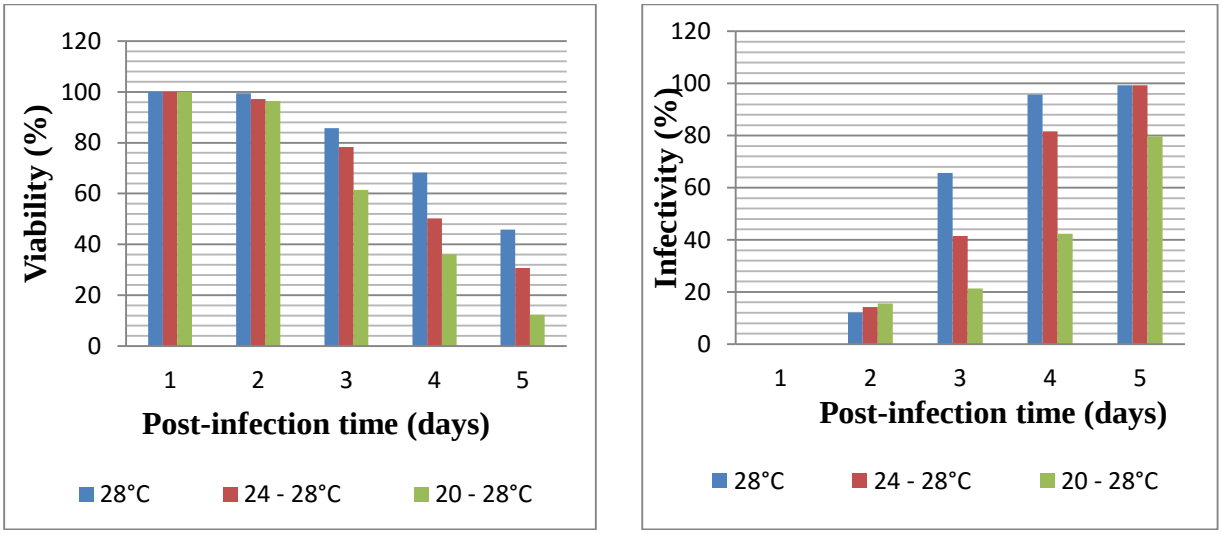

Figure 5: Cell Viability and Virus Infectivity at Two Temperature Oscillations and One Constant Temperature in Stationary Culture

Effect of Temperature Oscillation on Virus Production

Table 1 indicates the virus productions in both stationary and suspension cultures under different temperature conditions. Among all experiment conditions, the oscillation between 24 and 28°C was optimal for virus production in terms of the titer of ECV and the number of OB. The virus production in the culture with oscillation between 24 and 28°C was significantly higher than that of the 28°C culture by the t test for independent samples (Table 1).

Table 1: Baculovirus Production under Different Temperature Conditions in Stationary and Suspension Cultures

Culture	Temperature (°C)	No. of OB Produced		ECV Titer	
		10^7/ ml	Cell^{-1}	10^7 PFU/ ml	PFU/ cell
Stationary	28	2.12 ± 0.062	13.16 ± 0.17	13.35 ± 0.23	85.00 ± 1.63
	26-28	2.17 ± 0.037	14.60 ± 0.22	14.57 ± 0.17	97.67 ± 1.25
	24-28	2.42 ± 0.076	17.37 ± 0.12	16.03 ± 0.17	112 ± 1.63
	22-28	1.70 ± 0.016	12.57 ± 0.48	11.87 ± 0.17	91.40 ± 0.54
	20-28	1.31 ± 0.026	10.93 ± 0.25	9.37 ± 0.40	78.93 ± 0.90
Suspension	28	2.62 ± 0.056	13.53 ± 0.74	14.70 ± 0.86	78.00 ± 2.16
	26-28	2.63 ± 0.021	14.90 ± 0.24	16.30 ± 0.65	89.20 ± 0.62
	24-28	2.87 ± 0.050	18.00 ± 0.10	18.08 ± 0.13	113.67 ± 1.25
	22-28	1.65 ± 0.051	13.23 ± 0.25	11.75 ± 0.23	94.21 ± 0.60
	20-28	1.36 ± 0.054	11.93 ± 0.48	9.33 ± 0.45	80.20 ± 2.43

Values are mean productions of OB (based on five replicates) and of ECV (based on three replicates) 6 standard deviations. For values followed by the same letter, the means are not significantly different (P <0.05), as determined by the t test for independent samples.

The titer of ECV was increased 18.4% on a volumetric basis and 31.0% on a cellular basis in the stationary culture. These increases were 23.0 and 44.3%, respectively, in the suspension culture. The number of OB was increased 16.7% on a volumetric basis and 30.0% on a cellular basis in the stationary culture, and these increases were 10.7 and 30.2%, respectively, in the suspension culture. The culture with oscillation between 26 and 28°C also produced more ECV and OB on a cellular basis than the culture at 28°C did. Compared with the 28°C culture, the virus production of the culture with oscillation between 22 and 28°C was no less on a cellular basis and less on a volumetric basis. The culture with oscillation between 20 and 28°C culture produced less virus than the 28°C culture at the 4th day post infection. Since the virus infectivity was below 80% at that time, the virus production in the former culture might be increased if its time were prolonged.

Our results show that temperature oscillation can prolong the cell growth phase of uninfected and infected sf9 cells in stationary and suspension cultures. The optimal oscillation for promoting a long cell growth phase without decreasing the maximal cell density was between 24 and 28°C. At temperatures below 22°C, cells grew too slowly (Reuveny *et al.*, 1993) and did not reach a cell density as high as that reached in the 28°C culture.

An additional reason for the long phase of cell viability post infection was the low infection rate due to the low temperature (Reuveny *et al.*, 1993). As quick infection at high temperatures leads to quick cell lysis, implementing a suitable temperature oscillation was able to increase baculovirus production. There are two possible explanations for this: either infected insect cells survive longer at temperatures oscillating from low to high so that they can produce more virus or the slow infection that results from temperature oscillation might leave some viable cells to divide, causing secondary infection (Licari & Bailey, 1991, 1992) and thus, increasing virus production.

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REFERENCES

Agathos S, Jeong YH & Venkat K (1990). Growth Kinetics of Free and Immobilized Insect Cell Cultures. *Annals of the New York Academy of Sciences* **589**(1) 372-398.

Bosch BJ, van der Zee R, de Haan CAM & Rottier PJM (2003). The coronavirus spike protein is a class I virus fusion protein: Structural and functional characterization of the fusion core complex. *Journal of Virology* **77**(16) 8801-8811.

Chang SH, Bae JL, Kang TJ, Kim J, Chung GH, Lim CW and Jang YS (2002). Identification of the epitope region capable of inducing neutralizing antibodies against the porcine epidemic diarrhea virus. *Molecules and Cells* **14**(2) 295-299.

Chen Y, Shi Y, Deng H, Gu T, Xu J, Ou J and Wang C (2014). Characterization of the porcine epidemic diarrhea virus codon usage bias. *Infection, Genetics and Evolution* **28** 95-100.

Duarte M & Laude H (1994). Sequence of the spike protein of the porcine epidemic diarrhoea virus. *Journal of General Virology* **75**(Pt 5) 1195-1200.

Duarte M, Tobler K, Bridgen A, Rasschaert D, Ackermann M & Laude H (1994). Sequence analysis of the porcine epidemic diarrhea virus genome between the nucleocapsid and spike protein genes reveals a polymorphic ORF. *Virology* **198**(2) 466-476.

Goodman CL & Mcintosh AH (1994). *Production of Baculoviruses for Insect Control Using Cell Culture*, (CRC Press, Boca Raton, FL).

Jackwood MW, Hilt DA, Callison SA, Lee CW, Plaza H & Wade E (2001). Spike glycoprotein cleavage recognition site analysis of infectious bronchitis virus. *Avian Diseases* **45**(2) 366-372.

Johnson D, Boucias D, Barfield C & Allen G (1982). A temperature-dependent developmental model for a nucleopolyhedrosis virus of the velvetbean caterpillar, Anticarsia gemmatalis (Lepidoptera: Noctuidae). *Journal of Invertebrate Pathology* **40**(2) 292-298.

Kioukia N, Al-Rubeai M, Zhang Z, Emery A, Nienow A & Thomas C (1995). A study of uninfected and baculovirus-infected Spodoptera frugiperda cells in T-and spinner flasks. *Biotechnology Letters* **17**(1) 7-12.

Kocherhans R, Bridgen A, Ackermann M & Tobler K (2001). Completion of the porcine epidemic diarrhoea coronavirus (PEDV) genome sequence. *Virus Genes* **23**(2) 137-144.

Lee D-K, Cha S-Y & Lee C (2011). The N-terminal region of the porcine epidemic diarrhea virus spike protein is important for the receptor binding. *Korean Journal of Microbiology and Biotechnology* **39**(1) 40-50.

Lee DK, Park CK, Kim SH & Lee C (2010a). Heterogeneity in spike protein genes of porcine epidemic diarrhea viruses isolated in Korea. *Virus Research* **149**(2) 175-182.

Lee DK, Park CK, Kim SH & Lee C (2010b). Heterogeneity in spike protein genes of porcine epidemic diarrhea viruses isolated in Korea. *Virus Research* **149**(2) 175-182.

Lee S & Lee C (2014). Outbreak-related porcine epidemic diarrhea virus strains similar to US strains, South Korea, 2013. *Emerging Infectious Diseases* **20**(7) 1223-1226.

Licari P & Bailey J (1991). Factors influencing recombinant protein yields in an insect cell–bacuiovirus expression system: Multiplicity of infection and intracellular protein degradation. *Biotechnology and Bioengineering* **37**(3) 238-246.

Licari P & Bailey J (1992). Modeling the population dynamics of baculovirus-infected insect cells: Optimizing infection strategies for enhanced recombinant protein yields. *Biotechnology and Bioengineering* **39**(4) 432-441.

Pensaert M & De Bouck P (1978). A new coronavirus-like particle associated with diarrhea in swine. *Archives of Virology* **58**(3) 243-247.

Reuveny S, Kim Y, Kemp C & Shiloach J (1993). Effect of temperature and oxygen on cell growth and recombinant protein production in insect cell cultures. *Applied Microbiology and Biotechnology* **38**(5) 619-623.

Sturman LS & Holmes KV (1984). Proteolytic cleavage of peplomeric glycoprotein E2 of MHV yields two 90K subunits and activates cell fusion. In: *Molecular Biology and Pathogenesis of Coronaviruses,* 25-35 (Plenum Press, Springer, New York, USA).

Sun DB, Feng L, Shi HY, Chen JF, Liu SW, Chen HY & Wang YF (2007). Spike protein region (aa 636789) of porcine epidemic diarrhea virus is essential for induction of neutralizing antibodies. *Archives of Virology* **51**(3) 149-156.