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Short communication

Rapid quantitation of porcine epidemic diarrhea virus (PEDV) by Virus Counter

Xiangbin Zhang, Jianwei Hao, Jing Zhen, Lijuan Yin, Qianniu Li, Chunyi Xue, Yongchang Cao*

State Key Laboratory of Biocontrol, School of Life Science, Sun Yat-sen University, Guangzhou 510006, PR China

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A B S T R A C T

Virus Counter was applied for the quantitation of porcine epidemic diarrhea virus (PEDV); the working range and reproducibility of the quantification were characterized, and the performance of Virus Counter was evaluated using the plaque assay as the gold standard. In this study, the single burst curves of PEDV were first constructed with the Virus Counter or plaque assay respectively, and the results from Virus Counter and plaque assay showed similar high correlation for interval of 12–24 h ($r = 0.9581$, $p < 0.05$). Then three virus batches from independent virus propagations were harvested at interval of 16–20 h and serial-diluted; The correlation between the calculated titer and the results obtained by Virus Counter were better than those obtained by using the plaque assay. The comparison between the plaque assay data and the Virus Counter results revealed a linear relationship (slope = 1.155 ± 0.46, $R^2 = 0.9665$) with a significant Pearson correlation ($r = 0.9809$, $p < 0.0001$). The results of this study demonstrated that Virus Counter is a simple and reliable method to quantify the viral particles of PEDV.

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Porcine epidemic diarrhea (PED) is a highly contagious enteric disease that is characterized by vomiting, watery diarrhea and dehydration. A typical sign of PED occurrence in a pig farm is severe watery diarrhea that induces high death rates in piglets. The etiological agent of PED is porcine epidemic diarrhea virus (PEDV), a member of the genus Alphacoronavirus, Corinaviridae. PEDV has been affecting the world-wide swine-industry since 1978 (Pensaert and Yeo, 2006). Since late 2010, severe PEDV outbreaks have caused major economic losses in China (Chen et al., 2012; Fan et al., 2012). Recently, similar PEDV variant outbreaks in the USA and South Korea have significantly influenced the world's swine supply (Lee and Lee, 2014; Huang et al., 2013).

Methods for viral quantification can be broadly divided into traditional or more modern methods. The plaque forming unit assay and tissue culture infective dose tests represent the traditional methods and the more modern methods were represented by quantitation polymerase chain reaction and Virus Counter methods. The traditional methods are widely accepted, but they are frequently time-consuming and labor-intensive. For PEDV quantification, the traditional gold standard is the plaque assay for virus titering, which measures the concentration of infectious virus particles or plaque-forming units (pfu/mL) (Hofmann and Wyler, 1989; Mao et al., 2010). The plaque assay for PEDV is expectedly time-consuming. In addition, many factors, including cells, trypsin and slight variations influence the viral titer accuracy. To remedy the shortcomings in the traditional methods, many newer methods have been developed, such as transmission electron microscopy (TEM) (Borsheim et al., 1990), quantitative PCR (qPCR) (Kim et al., 2007) and the focus formation assay (Cruz and Shin, 2007). However, these modern methods have their own limitations that hamper their use as alternatives to the plaque assay.

Virus Counter is a newly developed flow cytometer method and can be used for the rapid quantification of total viral particles in liquid virus samples. Intact virus particles are quantified by detecting the particles containing both proteins and nucleic acids simultaneously (Stoffel and Rowlen, 2005). Therefore, Virus Counter measures the concentration of total viral particles (Borsheim et al., 1990).

PEDV quantitation was compared between the Virus Counter and plaque assay in this study. The GDS01 PEDV strain was isolated from diseased piglets (Hao et al., 2014) and was propagated according to the previously described method, with minor modifications (Hofmann and Wyler, 1988). Briefly, the growth medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with

* Corresponding author at: Rm 504#, Biobuilding, School of Life Sciences, East Campus, Sun Yat-sen University, Higher Education Mega Center, Guangzhou 510006, PR China.
E-mail address: caoych@mail.sysu.edu.cn (Y. Cao).

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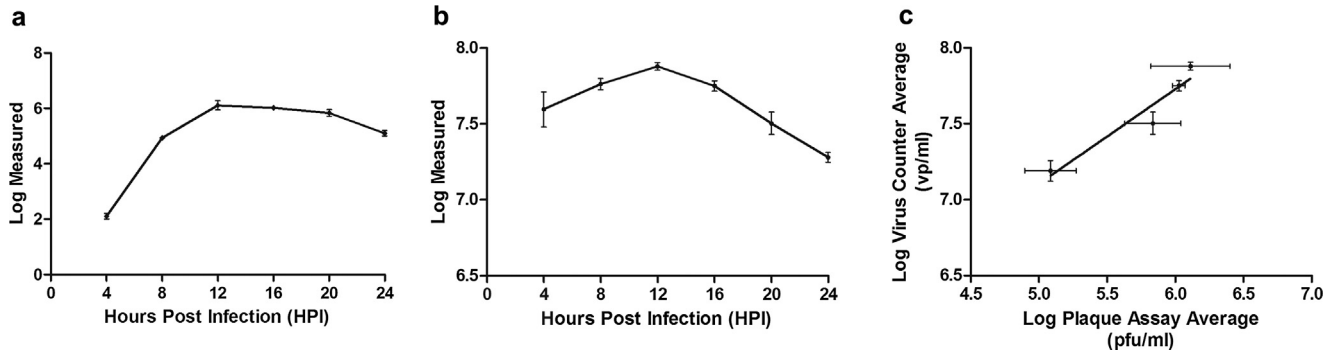

Fig. 1. Single growth curve analysis of PEDV strain GDS01 (a) by a plaque assay and (b) by Virus Counter and (c) the linear fit of the two methods using data from 12 to 24 hpi.

10% heat-inactivated fetal calf serum and antibiotics) was removed from confluent monolayer Vero cells that were washed twice with PBS and subsequently infected with a mixture of the GDS01 stock and 'infection medium' (DMEM, 0.3% tryptose phosphate broth and 10 μg ml^{-1} trypsin). After absorption for 60 min at 37 °C, the cells were washed with PBS, and then, the infection medium was added. The culture media and cells were harvested and then centrifuged for 15 min at 4500 rpm. The supernatant was stored as virus stock at −80 °C.

The single burst curve of PEDV was constructed as follows: (1) The confluent monolayer Vero cells in 6 wells plates were inoculated with GDS01 at a multiplicity of infection (MOI) of 0.05 which was tested as the optimal inoculation concentration previously; (2) Samples including cells or cell debris together with the media were collected at 4, 8, 12, 16, 20 and 24 h, respectively. The different time-point samples were collected from 6-well plates at each time point, one well sample from one plate was collected. After three cycles of freeze–thawing and centrifugation at 4000 × *g* for 30 min, the clarified supernatants of different time-point samples were stored at −80 °C until ready for use. (3) The plaque assay was performed according to a previously described method (Kim et al., 2010), and the plate were incubated for 36 h when the pfu/ml were calculated. To construct the single burst curve with the plaque assay, the 4–24 h samples were assayed at the same time to minimize the inter-experiment variations. In addition, each time-point sample was tested in triplicate to minimize the intra-experiment variations; (4) The single burst curve was constructed using Virus Counter. The samples were prepared according to Virus Counter protocol (InDevR, Boulder, CO, USA). Briefly, a diluted virus sample was stained with 160 μl of a Combo Dye solution (1:2, dye: sample ratio) (InDevR, Boulder, CO, USA) for 30 min at room temperature. The performance validation standard (PVS) and cleanliness verification fluid (CVF) were used to validate the testing process. All samples were analyzed three times over three days. The time required for analyzing one sample was about 10 min. The instrument quantification limit (IQL) for Virus Counter that defines the lowest concentration that the instrument can report with statistical confidence, normally approximately 5×10^5 vp ml^{-1}. The linear dynamic range for Virus Counter is 5×10^5 to 1×10^9 vp ml^{-1}. Both results revealed the highest titer occurred at 12 hpi (Fig. 1a and b with larger y-axis range for easier plotting). It is important to note that while Virus Counter replication analyses were performed in multiple days, the results showed high consistence. The results from the plaque assay were performed in the same day in this study. In addition, the plaque assay results for different time-point samples had 0.03007 for the average of coefficient of variation (CV) and 0.016 for the standard deviation (STDEV). In contrast, the results from Virus Counter had 0.012 for CV, and 0.008 for STDEV. In summary, in comparison with the plaque assay, Virus Counter is simpler and less time-consuming.

The single burst curve experiment was repeated three times, and all three repeats revealed the same trend, including that the highest titer occurred at 12 h. However only data from one repetition set was used for statistical analysis to further reduce the experimental influence on the data complexity.

On both figures (Fig. 1a and b), the PEDV single burst curve could be divided into two sections. The first section, including the time points before 12 hpi, usually represents the virus eclipse period and the exponential phase. The second section represents 12–24 hpi, during which the viruses are released from the cells and more completed viruses are produced. Generally, viruses are harvested during the second section for research or industry productions. In this study, the linear fit of the Virus Counter results in comparison to the plaque assay data for 12–24 hpi is shown in Fig. 1c. Pearson correlation analyses were performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA) and revealed that the Virus Counter results correlated with the plaque assay titers ($r = 0.9581$, $p < 0.05$). The relative error in the Virus Counter results ranged from 0.04 to 0.13 log vp mL^{-1} units, while the relative error in the plaque assay results ranged from 0.04 to 0.29.

To evaluate the reproducibility of the above results, three batches of PEDV were harvested from independent virus cultures during the interval of 16–20 h when 80–90% of cells revealed cytopathic changes. They were designated as batches 'a' 'b' and 'c' and the titers from plaque assays for these batches were as follows: 1, 2×10^5 (pfu ml^{-1}) for 'a'; 2, 7×10^5 (pfu ml^{-1}) for 'b'; 3, 4×10^5 (pfu ml^{-1}) for 'c'. The serial-dilution of the PEDV stocks is listed in Table 1. The intended titer values listed for each sample are based on the titer and dilution factor. All of the analysis results shown in Table 1 are represented by the mean of the replicates ($N = 3$) as analyzed by the plaque assay and the Virus Counter; the listed error represents the standard errors.

The linear fit of each batch's results, relative to the intended titer was calculated, respectively. Pearson correlation analysis of the results showed that both Virus Counter ($r = 0.9923$–0.9987) and the plaque assay ($r = 0.9650$–0.9928) correlated well with the intended titer values. The linear regression fit of the Virus Counter results to the intended titer was conducted for batches and the results indicated that the R^2 values for Virus Counter (0.9795–0.9956) were higher than that of plaque assay results (0.8982–0.9233). The average and STDEV values for inter CV of the three batches were 0.008 ± 0.003 for 'a', 0.01 ± 0.006 for 'b' and 0.006 ± 0.002 for 'c', respectively.

Further, to minimize the discrepancy between the three batches, the direct correlation between the Virus Counter and plaque assay results was evaluated by combining the plaque assay titer values from the three diluted sample sets. The results shown in Fig. 2 indicate that the linear regression analysis of the log of the average Virus Counter data (vp ml^{-1}) versus the log of plaque assay

Table 1
Samples used in this study and obtained results.

Sample ID[a]	Intended titer[b] (pfu/ml)	Virus Counter (vp/ml)	Tested titer (pfu/ml)
1a	1×10^5	$(3.8 \pm 0.7) \times 10^7$	$(1.2 \pm 0.7) \times 10^5$
2a	5×10^4	$(1.9 \pm 0.3) \times 10^7$	$(3.3 \pm 2.3) \times 10^4$
3a	1×10^4	$(1.8 \pm 0.2) \times 10^6$	$(8.7 \pm 5.0) \times 10^3$
4a	5×10^3	$(6.6 \pm 0.3) \times 10^5$	$(2.7 \pm 1.2) \times 10^3$
5a	1×10^3	<IQL[c]	$(8.0 \pm 3.5) \times 10^2$
6a	5×10^2	<IQL[c]	$(3.3 \pm 1.2) \times 10^2$
1b	6×10^5	$(2.7 \pm 1.0) \times 10^8$	$(6.0 \pm 2.0) \times 10^5$
2b	3×10^5	$(1.9 \pm 0.3) \times 10^8$	$(3.3 \pm 1.2) \times 10^5$
3b	6×10^4	$(1.4 \pm 0.1) \times 10^7$	$(4.0 \pm 2.0) \times 10^4$
4b	3×10^4	$(5.4 \pm 0.4) \times 10^6$	$(8.7 \pm 5.0) \times 10^3$
5b	6×10^3	$(9.5 \pm 1.5) \times 10^5$	$(6.7 \pm 2.3) \times 10^3$
6b	3×10^3	$(6.7 \pm 0.9) \times 10^5$	$(3.3 \pm 1.2) \times 10^3$
7b	6×10^2	<IQL[c]	$(4.0 \pm 3.5) \times 10^2$
1c	4×10^5	$(2.1 \pm 0.2) \times 10^8$	$(4.0 \pm 1.7) \times 10^5$
2c	2×10^5	$(1.0 \pm 0.8) \times 10^8$	$(2.6 \pm 1.5) \times 10^5$
3c	4×10^4	$(1.2 \pm 0.2) \times 10^7$	$(4.6 \pm 1.5) \times 10^4$
4c	2×10^4	$(3.8 \pm 0.3) \times 10^6$	$(1.3 \pm 0.6) \times 10^4$
5c	4×10^3	$(6.4 \pm 0.4) \times 10^5$	$(4.3 \pm 2.5) \times 10^3$
6c	2×10^3	<IQL[c]	$(1.2 \pm 0.7) \times 10^3$
PVS (+) control	PVS passed[e]	–[d]	–[d]
SDB (−) control	SDB passed[e]	–[d]	–[d]

[a] The letter within the sample ID indicates the dilution used. 'a', 'b' and 'c' indicate different virus batches.
[b] Intended titer values were determined from dilution of PEDV stock that had been previously titered by the plaque assay.
[c] Indicated values that were below the Virus Counter's limitation.
[d] Indicates a normalized count or zero count for that sample.
[e] The performance validation standard (PVS) and sample dilution buffer (SDB) were used to validate the testing process. The Virus Counter could be ready to use only when the two fluid pasted on the machine.

average results (pfu ml^{-1}) yields a reasonable fit, with aslope of 1.167 ± 0.03, and the R^2 value was indicated in Fig. 2. In addition, the Pearson correlation test also revealed a statistically significant correlation ($r = 0.9809$, $p < 0.0001$).

Accurate and time-saving virus quantitation methods are important for virus research. Viral concentrations include two aspects: infectious particles and total particles. The ratio of infectious particles to the total number of particles is a key parameter in virus research and is a variable number that is influenced by many elements, such as cells, reagents, virus growth period, and so on. Traditional virus titer methods, such as the plaque forming assay, mainly focus on infectious particle calculations, whereas other methods discuss the total concentration. In addition, infectious titer methods are also influenced by such factors, and the results always reveal inconsistencies. Therefore, new methods are needed to better address the above problems.

In this study, the Virus Counter method was proven to be a simple and faster method for analyzing infectious titers at the 12–24 h interval. It should be noticed that the two methods revealed the largest discrepancy at the 4 h time point. It is clear that the 4–8 hpi period was the exponential phase of the virus life cycle and thus may induce higher variability between the infectious particle amount and total viral particles. To test this, the N protein amounts at two time points (4 hpi and 8 hpi) were simply compared with semi-quantitative SDS-PAGE using the diluted BSA as the standard sample. The N protein amounts of the two time points revealed that the trend was more like that of the Virus Counter than that of the plaque assay (data not shown); thus, it is possible that the discrepancy might be induced by the exponential phase of the virus. However, the result needed further clarification because the SDS-PAGE based method was only semi-quantitative. To address the limitation of lacking total particle determination experimental methods, such as TEM, it is difficult to say that the result indeed calculated the total viral particles accurately.

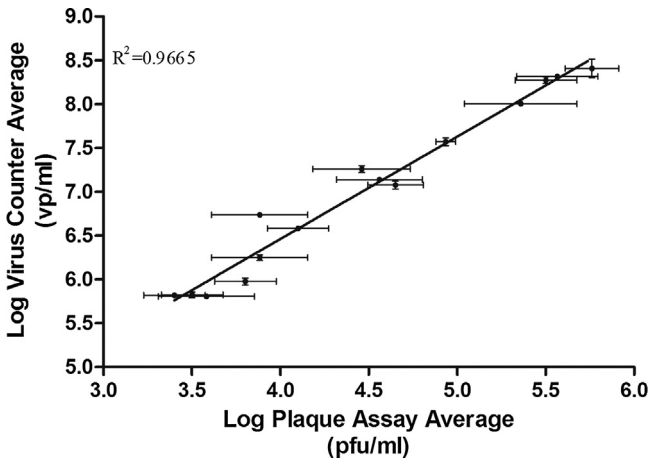

Fig. 2. Correlation between the virus counter and plaque assay methods for titration of a 10-fold serial dilution of PEDV.

Currently, PEDV quantification methodologies mainly include plaque assay and $TCID_{50}$. Infectious titer assays are time consuming and labor intensive; the plaque assay for PEDV can take up to 4 days. Other methods, such as quantitative RT-PCR (qPCR), significantly shorten the analysis time; however, there are still shortcomings for PEDV quantitation. qPCR requires primers and probes specific to each virus of interest; thus, each virus requires a set of specially designed primers. Even for the same type of virus, the primer or probes need to be adjusted for use. It should be noticed that the primers and probes used to detect PEDV previously were not perfect matches with new PEDV genogroups (Kim et al., 2007). The specific primers and a TaqMan probe were designed to construct the PEDV single burst curve and further quantify samples. Although the largest copy amount was identified at 12 h, the Pearson values between qPCR and the plaque assay or Virus Counter were 0.7996 and 0.9173, respectively, at the 12–24 h interval. In addition, the incompatible results from different batches hampered further comparisons; the qPCR method seems to be too sensitive because slight time changes could cause great deviation.

These experiments showed that the Virus Counter method is a simple and reliable alternative method to analyze the amount of PEDV particles. For determining the infectious titer, the correlation range was identified. For determining total particles, other virus quantification evidence revealed a good relationship between the

Virus Counter and total particles and this experiment also showed the same trend. In conclusion, the merits of the Virus Counter allow for an extensive application range in virus particle quantification. Due to lack of specificity, Virus Counter methods cannot be used in swine diagnostics at this point. Once highly specific monoclonal antibodies have been identified and labeled, the dual-signal detection system could be used to achieve this goal. The simple operation of the Virus Counter will make it easily adaptable for use in laboratories or for industry production.

Conflict of interest

There are no conflicts of interest for any of the authors.

References

Borsheim, K.Y., Bratbak, G., Heldal, M., 1990. Enumeration and biomass estimation of planktonic bacteria and viruses by transmission electron microscopy. Appl. Environ. Microbiol. 56, 352–356.

Chen, F., Pan, Y., Zhang, X., et al., 2012. Complete genome sequence of a variant porcine epidemic diarrhea virus strain isolated in China. J. Virol. 86, 12448.

Cruz, D.J., Shin, H.J., 2007. Application of a focus formation assay for detection and titration of porcine epidemic diarrhea virus. J. Virol. Methods 145, 56–61.

Fan, H., Zhang, J., Ye, Y., et al., 2012. Complete genome sequence of a novel porcine epidemic diarrhea virus in south China. J. Virol. 86, 10248–10249.

Hao, J.W., Xue, C.Y., He, L.L., et al., 2014. Bioinformatics insight into the spike glycoprotein gene of field porcine epidemic diarrhea strains during 2011–2013 in Guangdong, China. Virus Genes, http://dx.doi.org/10.1007/s11262-014-1055-y

Hofmann, M., Wyler, R., 1988. Propagation of the virus of porcine epidemic diarrhea in cell culture. J. Clin. Microbiol. 26, 2235–2239.

Hofmann, M., Wyler, R., 1989. Quantitation, biological and physicochemical properties of cell-adapted porcine epidemic diarrhea coronavirus (PEDV). Vet. Microbiol. 20, 131–142.

Huang, Y.W., Dickerman, W., Pineyro, A.P., et al., 2013. Origin, evolution, and genotyping of emergent porcine epidemic diarrhea virus strains in the United States. mBio, http://dx.doi.org/10.1128/mBio.00737-13

Kim, H.Y., Eo, E.Y., Park, H., et al., 2010. Medicinal herbal extracts of Sophorae radix, Acanthopanaicis cortex, Sanguisorbae radix and Torilis fructus inhibit coronavirus replication in vitro. Antivir. Ther. 15, 697–709.

Kim, S.H., Kim, I.J., Pyo, H.M., et al., 2007. Multiplex real-time RT-PCR for the simultaneous detection and quantification of transmissible gastroenteritis virus and porcine epidemic diarrhea virus. J. Virol. Methods 146, 172–177.

Lee, S., Lee, C., 2014. Outbreak-related porcine epidemic diarrhea virus strains similar to US strains, South Korea, 2013. Emerg. Infect. Dis. 20, 1223–1226.

Mao, Y.Y., Zhang, G.H., Ge, J.W., et al., 2010. Isolation and characteristics of virus culture of porcine epidemic diarrhea virus LJB/03. Bing Du Xue Bao 26, 483–489.

Pensaert, M.B., Yeo, S.G., 2006. Porcine epidemic diarrhea. In: Straw, B.E., Zimmerman, J.J., D'Allaire, S., Taylor, D.J. (Eds.), Swine Disease. Blackwell Publishing, Ames, pp. 367–372.

Stoffel, C., Rowlen, K.L., 2005. Design and characterization of a compact dual channel virus counter. Cytometry A 65, 140–147.