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Development of gp64 Gene based Real-time Quantitative PCR Assay for Rapid and Accurate Determination of Baculovirus Titer

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ABSTRACT

The present study was conducted to develop a simple and rapid quantitative real-time PCR (qPCR) assay for determination of baculovirus titer. Recombinant baculovirus expressing CE1E2 structural proteins of classical swine fever virus (CSFV) along with green fluorescent protein (GFP) reporter developed in our lab was used for the study. Sf21 cells were infected with tenfold dilution (10^{-1} to 10^{-10}) of baculovirus stock and GFP fluorescence was visualized. The titer (5.75×10^7 $TCID_{50}$/mL) calculated by Reed and Muench method was taken as a standard stock to develop qPCR. DNA was isolated from baculovirus stock and checked for amplification of 175 bp baculovirus gp64 by standard PCR. Different dilutions of isolated DNA (10^{-1} to 10^{-7}) from P3 baculovirus stock were used as template and gp64 primers were used to determine the titer by qPCR. A linear relationship was obtained from 10^0 to 10^6 $TCID_{50}$ per 100 µL (Y = -3.34 X + 40.19, r^2 = 0.97). Using this equation, titer of unknown recombinant baculovirus stock was calculated to be $5.7 \times 1\ 0^7$ per mL. Intra and inter assay coefficient of variations for qPCR results were less than 5%. The titration of baculovirus by this qPCR assay can be completed within 2-3 hrs compared to 10-12 days in the end point dilution method. To conclude, SYBR Green based qPCR titer estimation is a reliable, rapid and accurate assay for the titration of baculovirus and comparable to traditional end point dilution method.

HIGHLIGHTS

- This study describes SYBR Green based qPCR assay for baculovirus titer determination using gp64 gene with recombinant baculovirus expressing CE1E2 structural proteins of CSFV as a template.
- This assay applicable to any baculovirus titre determination is rapid, reliable and comparable to traditional end point dilution method.

Keywords: CSFV, Baculovirus titer, end point dilution, SYBR Green, qPCR

The baculovirus vector expression system (BEVS) has revolutionized the field of recombinant protein production over decades, enabling efficient and high-yield expression of recombinant proteins, particularly in insect cells (Hong *et al.,* 2022). One of the unique advantages of BEVS is its ability to perform post-translational modifications especially N- and O- linked glycosylation which is similar to higher eukaryotic cells (Tsai *et al.,* 2020). With its scalability and versatility BEVS has become a widely used tool for biomanufacturing and research purposes.

Maximizing the expression levels of recombinant proteins in BEVS demands careful optimization of multiple parameters. One crucial factor that greatly impacts protein expression is multiplicity of infection (MOI), which signifies the optimal ratio of viruses to host cells required for efficient infection (Qi *et al.,* 2015). However, it is crucial to determine the ideal MOI level for maximum protein expression, as using excessively high MOI can trigger rapid cell death and a consequent reduction in

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protein production. Therefore, precise optimization of MOI is indispensable for unlocking the full potential of BEVS in achieving high-level recombinant protein expression. Furthermore, continuous usage of high MOI results in generating defective interfering particles (Gomez *et al.,* 2014). On the other hand, the cells infected with MOI lower than desired level may result in reduced protein yield. When insect cell expression system is used for production of recombinant proteins, baculovirus titer determination is crucial to optimize the MOI. Accuracy of titration method is a major concern because in case of overestimated titer, lower MOI ensues that causes non-synchronous infection and lesser protein expression. On the contrary, underestimated titer results in severe metabolic burden, lesser protein yield because of high MOI infection of cells (Roldao *et al.,* 2008).

When recombinant baculovirus applications continue to diversify, it is becoming increasingly important to develop precise and rapid method for baculovirus titer determination. The most frequently used conventional methods for the titration of baculovirus stocks are end-point dilution (Lynn, 1992 and Cresta *et al.,* 2021) and plaque assay (Hink and Vail, 1973; Smither *et al.,* 2013; Mendoza *et al.,* 2020). In both methods, it is important to seed and infect the cells with different dilution of viral particles. These two methods are based on formation of occlusion bodies' detection and it takes at least 7-8 days for titration. Furthermore, conventional approaches are regarded as laborious, time-consuming and error prone; that involve expertise in cell culture for handling of viruses.

Later, reporter genes such as ß-galactosidase (Yahata *et al.,* 2000) and green fluorescent protein (GFP) genes were used for detecting viral infection (Cha *et al.,* 1997 and Hopkins and Esposito, 2009). While these reporters enhanced detection efficiency, they did not dramatically reduce the time and effort required to obtain the titer. Over the years, several methods were developed to decrease the time and effort needed for titer determination such as flow cytometry assay (Shen *et al.,* 2002; Lothert *et al.,* 2022), magnetic cell sorting software (MACS) (Philipps *et al.,* 2004) and microfluidic bioanalyzer (Malde and Hunt, 2004). Although these developments allowed an attempt, the problems faced in performing large quantities of baculoviral titrations have still not diminished.

Therefore, in this study we report a simple and incredibly quick method for baculovirus titer determination with quantitative real-time PCR (qPCR). The qPCR has been shown to evaluate the quantity of DNA with great precision and sensitivity (Gregorova *et al.,* 2022). It has been used earlier to determine the titer of herpes simplex virus, adenovirus, and adeno-associated virus (Virok *et al.,* 2017; Gallaher and Berk, 2013; Dobnik *et al.,* 2019).

In our previous study (Bisht *et al.,* 2018), a recombinant baculovirus encoding FLAG-tagged protein and GFP reporter as bicistronic messenger (CSFV CE1E2-IRES-GFP) was developed and detection of GFP reporter expression using fluorescent microscope was shown to be useful in end point titer determination. In the present study, qPCR was developed using gp64 gene primers for titer determination of this recombinant baculovirus.

MATERIALS AND METHODS

Cell culture and production of recombinant baculovirus stock

Spodoptera frugiperda 21 (*Sf*21) insect cells (Invitrogen) were revived and maintained in a 25cm^2 flask under non-CO_2, non-humidified conditions at 27°C using SFM II medium fortified with Penicillin, Streptomycin, and Amphotericin.

Recombinant bacmid constructed by ligating Classical Swine Fever Virus (CSFV) CE1E2 gene fragment along with IRES (internal ribosomal entry site) and GFP (green fluorescent protein) into pFastBac 1 plasmid in our previous study (Bisht *et al.,* 2018) was successfully transformed and cloned into *E. coli* DH10Bac cells. Recombinant bacmid DNA was extracted by using alkaline-lysis method, eluted in 20 µL of elution buffer and stored at -4°C for transfection procedure.

Recombinant baculovirus stocks were prepared using Bac-to-Bac® Baculovirus Expression System on the basis of site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) propagated in *E. coli* as described in manufacturers protocols. Briefly, 8×10^5 cells/well was seeded in 35mm 6 well plates (TPP tissue culture plate). Transfection was done by infecting cells with mixture of cellfectin II and recombinant bacmid DNA. Five days post-transfection, recombinant baculovirus (P0)

containing cell culture medium was collected, centrifuged at 5000 rpm for 5 min and supernatant was stored as P0 stock at 4°C. 40µL of P0 stock was used to infect 2×10^6 cells/well to prepare P1 stock. After 5th day of post infection, the culture medium containing recombinant baculovirus (P1) was clarified and 20µL was used to infect 2×10^6 cells/well to prepare baculovirus stock P2. On the 5th day of infection, recombinant baculovirus (P2) containing cell culture medium was clarified and 0.1 mL was used to inoculate Erlenmeyer shake flask containing 50 mL of Sf21 cells (2×10^6 cells/mL) and incubated at 27°C, 100 rpm. On 4th day of infection, recombinant baculovirus stocks (P3) were harvested, clarified by centrifugation at low speed and titrated by GFP reporter based baculovirus titration method described in our earlier study (Bisht *et al.,* 2018) as well as by quantitative real time PCR method developed in present study as described below:

Titration of recombinant baculovirus

End point method using GFP reporter

P3 baculovirus stock was diluted from 10^{-1} to 10^{-10} with SFM II media to infect Sf21 cells. 70,000 cells/well were seeded into 96 well plate and infected with 100 µL of each dilutions of virus in quadruplets. On 7th day post-infection, the plate was examined under fluorescence microscope for detection of Green fluorescence indicating the presence of baculovirus. The titer of baculovirus stock was calculated by Reed and Muench method as reported earlier (Bisht *et al.,* 2018).

Viral DNA extraction

200 µL of P3 baculovirus was taken from the stock to be titrated and used for viral DNA extraction using QiAamp DNA mini kit as per manufacturer's protocol. Briefly, 20 µL of protease was added to 200 µL of baculovirus sample. After proper mixing, virus was lysed by addition of 200 µL of lysis buffer and then incubated at 56°C for 10 min. 200µL of ethanol (96-100%) was added to the sample, then transferred to the spin column and centrifuged at 8000 rpm for 1 min followed by two washings of the column with 500 µL of washing buffer 1 and 2, centrifuged at 8000 rpm for 1 min, 14000 rpm for 3 min, respectively. Finally, to remove the residual buffer, spin column was centrifuged at 14000 rpm for 1 min and DNA was extracted by addition of 200 µL elution buffer and centrifugation at 8000 rpm for 1 min.

Titration of baculovirus by standard PCR

PCR was performed with gp64 primers to visualize amplification of gp64 gene (175 bp) using DNA isolated from different dilutions (10^{-1} to 10^{-8}) of baculovirus stock P3. PCR reaction mixture (25 µL) contained 10µL of isolated DNA (template), 0.5 µL (50pmoles/µL) each of gp64 forward (5'-CACCACACGTGCAACAAATC-3) and reverse (5'- GAATCATACTCACGCCGTCT-3') primer, 2.5 µL of 10X buffer, 0.75 µL of 50 mM $MgCl_2$, 0.5µL of dNTPs, 0.2µL of platinum Taq polymerase (5 U/µL), 10.5 µL of nuclease free water employing the cycle conditions of 94°C for 2 min followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min and final extension at 72°C for 5 min. 10 µL of amplified PCR products were loaded in 1% agarose gel along with 100 bp DNA ladder.

Titration of baculovirus by Quantitative real time PCR assay

Real time PCR was performed in different dilutions of isolated DNA (10^{-1} to 10^{-7}) from P3 baculovirus stock and no template control in quadruplicates. Each reaction mixture contained 12.5 µL of Agilent brilliant III ultra-fast SYBR green QPCR master mix, 100 nM of each forward and reverse gp64 primer, 10 µL of isolated DNA as template, 0.3 µL of 1:500 diluted reference dye in a final volume of 25 µL reaction. The intensity of fluorescence of the reporter label was normalized to the Rhodamine derivative ROX, supplied as a passive reference label in the buffer solution. DNA amplification was carried out with the cycle conditions of 95°C for 3 min followed by 40 cycles of 95°C for 5 sec, 62°C for 20 sec and one cycle of dissociation as 95°C for 1 min, 62°C for 30 sec, 95°C for 30 sec in a Agilent Stratagene MX3005P system.

The SYBR Green qPCR standard curve was generated by plotting known viral titer (10^6 to 10^0 per 100 µL) against real time PCR threshold cycle number (CT). The results were employed for calculation of SYBR Green qPCR efficiency and quantification of titer in the unknown baculoviral stocks.

RESULTS

Baculovirus titer determination using End point dilution assay with GFP reporter

Here, a ten-fold dilution (10^{-1} to 10^{-10}) of baculovirus P3 stock was used to infect the *Sf* 21 cells. When cells were examined under inverted fluorescence microscope for visualizing GFP on the 7th day of post infection, control (uninfected) cells didn't express detectable GFP, while in infected cells, in all the four wells revealed GFP expression from 10^{-1} to 10^{-5} dilution, three out of four wells in 10^{-6} dilution, two out of four well in 10^{-7} dilution were expressing GFP whereas no expression of GFP was observed from 10^{-8} to 10^{-10} dilution. Based on the GFP expression, end point dilution method titer was calculated by Reed and Muench formula and it was found to be 5.75 × 10^7 $TCID_{50}$/mL (Table 1).

Table 1: Calculation of baculovirus titer by End point dilution method using Reed and Muench formula

Dilution	No. of Positive wells	No. of negative wells	Cumulative positive wells	Cumulative Negative wells	Ratio (Positive/ total)	Ratio %
10^{-1}	4	0	25	0	25/25	100
10^{-2}	4	0	21	0	21/21	100
10^{-3}	4	0	17	0	17/17	100
10^{-4}	4	0	13	0	13/13	100
10^{-5}	4	0	9	0	9/9	100
10^{-6}	3	1	5	1	5/6	83.3
10^{-7}	2	2	2	3	2/5	40
10^{-8}	0	4	0	7	0/7	0
10^{-9}	0	4	0	11	0/11	0
10^{-10}	0	4	0	15	0/15	0

Proportionate distance = (83.3-50)/ (83.3-40) = 33.3/43.3 = 0.76

Log 10 $TCID_{50}$/100 ul = Log dilution + (PD × Log dilution factor)

Log 10 $TCID_{50}$/100 ul = 6 + (0.76 × Log 10) = 6 + 0.76 = 6.76

Log 10 $TCID_{50}$/ml = 7.76

Titer = Antilog 7.76 = 5.75 × 10^7 $TCID_{50}$/ml

Titer of P3 stock was determined using End point dilution method by infecting Sf21 cells with tenfold dilutions (10^{-1} to 10^{-10}) of P3 in 96 well plate. 7th day of post infection presence of GFP was examined under fluorescence microscope. All four wells were positive for GFP from 10^{-1} to 10^{-5} dilution, three out of four wells in 10^{-6} dilution, two out of four wells in 10^{-7} dilution was expressing GFP, no GFP was observed from 10^{-8} to 10^{-10} dilution. Based on the expression of GFP, titer was calculated using Reed and Muench as 5.75 × 107 TCID 50/mL.

Standard PCR

In standard PCR, DNA isolated from different dilutions (10^{-1} to 10^{-8}) of the baculovirus P3 stock were used for amplification using gp64 gene specific primers at optimum thermal conditions. Amplification of 175 bp (gp64) PCR product was observed in 10^{-1} to 10^{-6} dilution, no amplification was observed in 10^{-7} and 10^{-8} dilution (Fig 1), indicated that this PCR could detect as low as 10 $TCID_{50}$/mL.

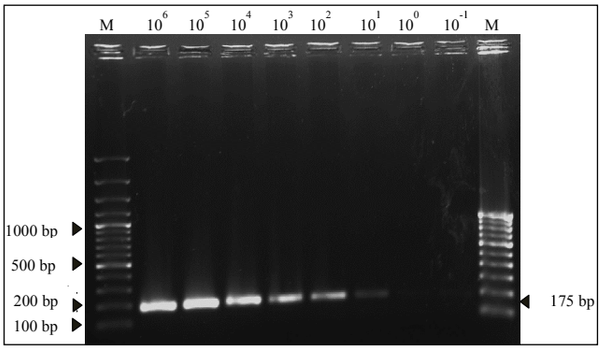

Fig. 1: Determination of Baculovirus titer using PCR

DNA isolated from different 10 fold dilutions (10^6/ml to 10^{-1} $TCID_{50}$/ml) of baculovirus P3 stock was used as template in PCR to detect gp64 gene encoded by recombinant baculovirus. Amplification of 175bp PCR product was observed in 10^{-1} to 10^{-6} dilutions, indicated that PCR could detect as low as 10 $TCID_{50}$/mL.

SYBR Green based Quantitative real time PCR (qPCR)

Ten-fold diluted isolated DNA from baculovirus P3 stock with gp64 primer and SYBR Green dye were used to generate the standard curve. As shown in Fig. 2a, this assay can detect upto 10 viruses per reaction which indicates its sensitivity. The standard curve (Fig. 2b) generated by

using mean threshold cycle number (Ct) on Y axis against different dilutions of known titer baculovirus stock on X axis demonstrated linear relationship (Y = 3.34 Log X + 40.19. $r^2 = 0.97$). Using this equation, titer of an unknown baculovirus stock was calculated to be 5.7×10^7 $TCID_{50}$/mL.

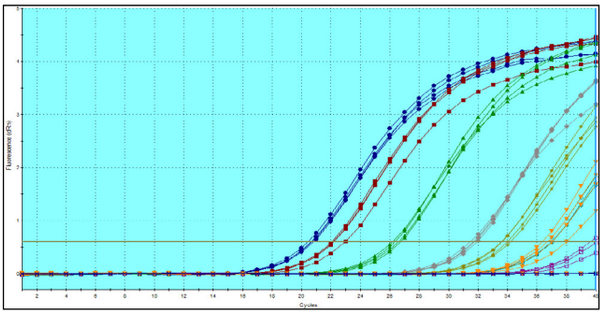

Fig 2a: Sensitivity of the SYBR Green I based real-time PCR assay. Amplification plot by using cycle number on X axis versus fluorescence on Y axis of known viral titer (10^6 to 10^0 per 100µL) plotted with four replicates

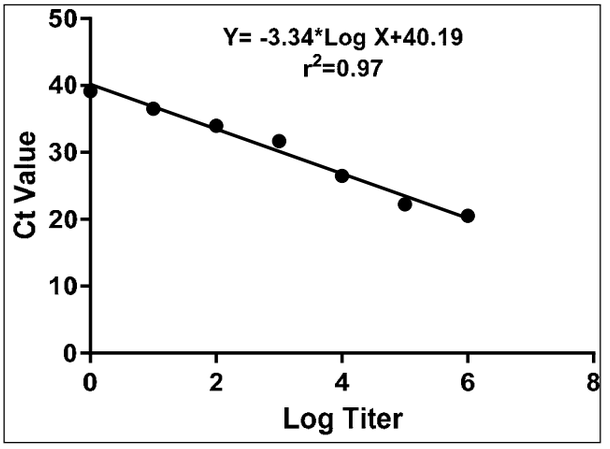

Fig 2b: Standard curve plot for calculation of SYBR Green qPCR efficiency and quantification. The SYBR Green qPCR standard curve was constructed by plotting known viral titer (10^6 to 10^0 per 100 µL)against real time PCR threshold cycle number (CT).

The assay was repeated three times using quadruplicates for evaluating assay reproducibility. Intra-assay reproducibility was assessed by using each dilution at four replicates and for inter-assay, same assay was repeated in three independent runs. For each dilution mean, standard deviation (SD) and coefficient of variation (CV) were calculated. Intra-assay CV values ranged between 0.37 to 1.84% (Table 2, Fig. 3) for and inter-assay CV ranged between 0.33 to 2.52% (Table 3, Fig. 4).

Table 2: Intra assay variability of real-time PCR

Replicates	10^6	10^5	10^4	10^3	10^2	10^1
1	20.68	22.95	26.2	31.5	33.59	37
2	20.73	22.05	26.36	31.92	33.98	36.73
3	20.82	22.22	26.61	31.7	33.63	36.99
4	20.46	22.15	26.74	31.75	34.21	37.02
Ct mean ± SD	20.67 ± 0.15	22.34 ± 0.41	26.48 ± 0.24	31.72 ± 0.17	33.85 ± 0.29	36.93 ± 0.14
CV (%)	0.74	1.84	0.92	0.55	0.87	0.37

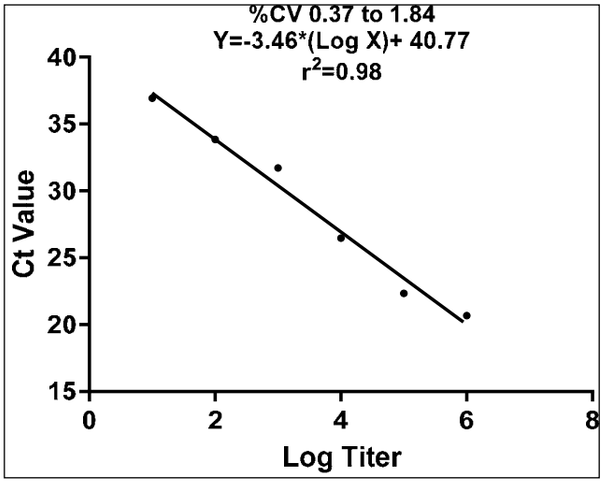

Fig. 3: Intra assay Repeatability of Ct values obtained from SYBR Green based qPCR

Table 3: Intra assay variability of real-time PCR

Replicates	10^6	10^5	10^4	10^3	10^2	10^1
1	20.67	22.34	26.48	31.72	33.85	36.93
2	20.18	21.33	25.7	31.52	32.6	36.5
3	20.42	22.23	26.43	31.67	33.96	36.38
Ct mean ± SD	20.42 ± 0.25	21.97 ± 0.55	26.2 ± 0.44	31.63 ± 0.1	33.47 ± 0.76	36.6 ± 0.29
CV (%)	1.19	2.52	1.67	0.33	2.26	0.79

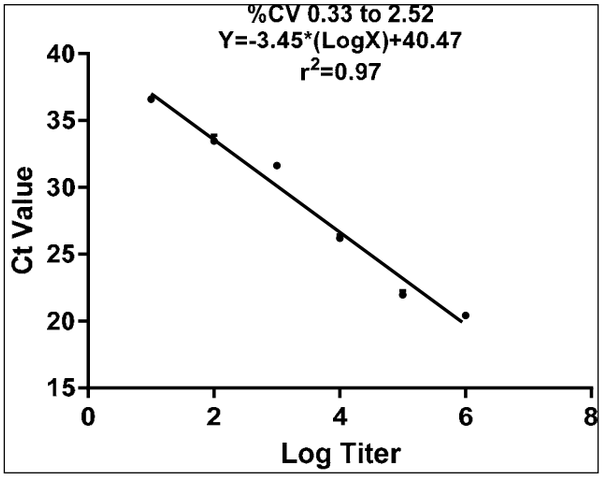

Fig. 4: Inter assay Repeatability of Ct values obtained from SYBR Green based qPCR

DISCUSSION

Classical Swine Fever (CSF) stands out as a highly significant viral disease that has re-emerged within the swine population and is currently of utmost concern. CSF is deemed a notifiable disease by the World Organisation for Animal Health (OIE) due to its significant impact on both the economy and sanitary conditions (OIE, 2020). The members of the *Suidae* family are the exclusive natural hosts for CSF virus (CSFV) and the disease affects both domestic and wild pigs (Everett *et al.,* 2011; Postel *et al.,* 2018).

CSFV is a single-stranded, enveloped, positive sense RNA virus with a diameter of 40- 60 nm. It belongs to *Pestivirus* genus and *Flaviviridae* family (Simmonds *et al.,* 2017). It has single open reading frame flanked by non-translated region (Lefkowitz *et al.,* 2018). It possesses four structural proteins includes Core (C) protein, three envelope glycoproteins such as E1, E2, E^{rns} and 8 non-structural proteins which includes Npro, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Lowings *et al.,* 1996; Chander *et al.,* 2014). Among these E2, E^{rns}, NS3 are capable of inducing detectable antibodies upon infection and E2, E^{rns} producing neutralizing antibodies (Zhang *et al.,* 2006). The bacmid containing CSFV C, E1, E2 glycoprotein gene fragments along with IRES and GFP reporter gene was used in this study.

BEVS is popularly used for high level recombinant protein expression and the protein is similar to the higher eukaryotic cells (Tsai *et al.,* 2020). The robustness and efficiency of the production workflow play a pivotal role in determining the success of the BEVS. To achieve this, precise control over the critical process parameters is imperative. Among these parameters, MOI stands out as the most crucial and influential factor. However MOI is important for optimizing the protein expression and determination of baculovirus titer is necessary to optimize MOI. End point dilution method (Lynn, 1992; Bisht *et al.,* 2018 and Cresta *et al.,* 2021) and plaque assay (Hink and Vail, 1973; Smither *et al.,* 2013; Mendoza *et al.,* 2020) are conventional methods to determine the baculovirus titer based on detection of occlusion body formation in infected cells. In endpoint dilution, the infection with baculovirus particles prompts the generation of early viral proteins, ultimately leading to the activation of the integrated polyhedron promoter and subsequent expression of GFP. The release of viral particles and the infection of adjacent cells results in easily detectable small foci of green cell using a fluorescent microscope (Lei *et al.,* 2021). Although the two conventional methods for determining titer are still commonly used, they have several drawbacks. These include being labor-intensive, time-consuming, error-prone and requiring expertise in cell culture and virus handling. Additionally, when it comes to titrating large numbers of baculovirus stocks, these methods are entirely ineffective.

Later on, various reporter genes such as β-galactosidase (Yahata *et al.,* 2000) or green fluorescent protein (GFP) genes (Cha *et al.,* 1997 and Hopkins and Esposito, 2009) were utilized to simplify the identification of viral infections. While these reporter genes improved detection sensitivity, they did not significantly reduce the time and effort required to obtain a titer. In an effort to optimize the efficiency of titer determination, several methods were developed to substantially decrease the time required for the process like immunological assay (Paul and Gisele, 1999) and antibody based assay (Konow *et al.,* 2002). These methods can determine the titer of baculovirus stock within 48 hours and 10 hours respectively. However, cell seeding, virus dilution, and infection remain time-consuming and error-prone prerequisites for a later 10 or 48 hours titer assessment in all of these methods.

Despite the advancements made, the titration of baculovirus remains a significant hindrance in the protein production process, particularly when dealing with a large number of recombinant viruses in a high throughput format.

Hence, SYBR Green I based qPCR, an alternative approach that gives accelerated and less complicated way to accurate recombinant baculovirus titer determination was further examined in this study. In comparison to other baculovirus titration methods, the SYBR Green I qPCR assay developed in this study provides several advantages. These include quicker results, decreased labor requirements and the ability to handle large volumes of samples effectively. A conventional PCR assay based on amplification of gp64 gene was developed previously for baculovirus titer determination in our lab (Bisht *et al.,* 2018). However, conventional PCR methods have limitations in terms of quantification and may occasionally generate non-specific products of similar sizes. Unlike conventional PCR, a qPCR-based assay eliminates the requirement for gel electrophoresis of the reaction product. The qPCR enables the real-time monitoring of reaction results, allowing for prompt and unambiguous interpretation. Conventional PCR is generally effective in detecting specific targets, but it can produce false negatives when the viral content is low. The qPCR offers enhanced sensitivity and accuracy, making it particularly advantageous when dealing with lower viral content.

In our study, Gp64 gene, the envelope glycoprotein of Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV) was selected as amplification target because gp64 gene is specific to baculovirus AcMNPV and it is essential for receptor binding and membrane fusion during virus entry (Blissard and Wenz, 1992).

The qPCR technology has established itself as a reliable and effective method for rapidly determining the titers of various viruses, including adenoviruses, adeno-associated viruses, herpes simplex virus and retroviruses (Virok *et al.,* 2017; Gallaher and Berk, 2013; Dobnik *et al.,* 2019). The TaqMan probe technology was adopted by all of these above mentioned viral assays. The utilization of fluorescently labeled probes like TaqMan probes proves to be costly and introduces additional complexity to both the design and parameters of the amplification reactions (Wang *et al.,* 2017).

SYBR Green I based qPCR simplifies the process by detecting a fluorescent signal using the chimeric fluorescent dye, SYBR Green I, thereby removing the requirement for a specific probe. The sensitivity of the SYBR Green I assay is highly comparable to the published sensitivity of 50 copies for the TaqMan probe based qPCR assay (Gallina *et al.,* 2006). The SYBR Green I method provides information about the PCR reaction's amplification through the generation of a melting curve. This melting curve and Tm value analysis provide whether the resulting product of the reaction corresponds to the intended target. The SYBR Green I method overcomes problems associated with probe contamination, including issues such as false positive results caused by substandard quality or weak fluorescence signals as well as false negative results resulting from a probe-template mismatch, leading to a lack of fluorescent signal or low detection rate (Wang *et al.,* 2017).

In the present study, a quick and precise SYBR Green I based qPCR method was established for classical swine fever virus (CSFV) C, E1, E2 glycoprotein gene containing recombinant baculovirus titration. The qPCR titer analysis depends on the intensity of fluorescence signal obtained from accumulated product in each amplification cycle. Viral titers are estimated by comparing the signal intensity obtained for known viral concentration standards with the signal intensity of the viral stock sample (Brandolini *et al.,* 2021). A significant correlation was noticed between titer determinations using conventional end-point dilution and Q-PCR techniques. Our findings demonstrate that qPCR can significantly decrease the time required for titer determination, reducing it from 10-12 days using end-point dilution to just 2-3 hours. Moreover, seeding of cells on plates or 10-fold dilution of the stocks of viruses and infection is not necessary. These advantages promote the use of qPCR over other techniques for routine determination of baculovirus titre, despite its higher cost.

To determine baculovirus titer determination by using qPCR standard curve and formula are needed. This standard curve was repeatedly established without having large variations in our laboratory (Fig 2b). The standard curve demonstrates a remarkable amplification efficiency of 98% which lies well within the acceptable range of efficiency. (Ruiz-Villalba *et al.,* 2021). As per Table 2 and 3 for intra assay and inter assay, CV values found to be less than 5% suggesting reliability of this assay (Islam *et al.,* 2004; Araujo *et al.,* 2020).

In conclusion, this study reports simple and precise SYBR Green based qPCR assay for baculovirus titer determination and the titer is comparable to conventional end point dilution method. Because of its rapid, reliability and accuracy, we can employ it for titration when large numbers of samples are involved. Moreover, only 2-3 hrs are required to obtain the titer which is very less as compared to 10-12 days of conventional endpoint dilution method.

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REFERENCES

Araujo, R.V., Suntheimer, F.F., Gold, A.S., Berlin, L.R. and Colpitts, T.M. 2020. One-step RT-qPCR assay for ZIKV RNA detection in Aedes aegypti samples: a protocol to study infection and gene expression during ZIKV infection. *Parasitol. Vectors.,* **13**: 128.

Bisht, D., Bhattacharya, S., Rajan, L.S., Rana, M., Bachan, R., Rama, T. and Gupta, P.K. 2018. A Rapid Method for Titration of Recombinant Baculovirus Stock Based on Bicistronic Expression of Green Fluorescent Protein (GFP) Reporter. *Int. J. Curr. Microbiol. Appl. Sci.*, **7**(12): 1141-1149.

Blissard, G.W. and Wenz, J.R. 1992. Baculovirus gp64 envelope glycoprotein is sufficient to mediate pH-dependent membrane fusion. *J. Virol.*, **66** (11): 6829-6835.

Brandolini, M., Taddei, F., Marino, M.M., Grumiro, L., Scalcione, A., Turba, M.E., Gentilini, F., Fantini, M., Zannoli, S., Dirani, G. and Sambri, V. 2021. Correlating qRT-PCR, dPCR and Viral Titration for the Identification and Quantification of SARS-CoV-2: A New Approach for Infection Management. *Viruses*, **13**: 1022.

Cha, H.J., Gotoh, T. and Bentley, W.E. 1997. Simplification of titer determination for recombinant baculovirus by green fluorescent protein marker. *Biotechniques*, **23**(5): 782-786.

Chander, V., Nandi, S., Ravishankar, C., Upmanyu, V. and Verma, R. 2014. Classical swine fever in pigs: Recent developments and future perspectives. *Anim. Health Res. Rev., **15*:** 87–101.

Cresta, D., Warren, D.C., Quirouette, C., Smith, A.P., Lane, L.C., Smith, A.M. and Beauchemin, C.A.A. 2021. Time to revisit the endpoint dilution assay and to replace the TCID50 as a measure of a virus sample's infection concentration. *PLoS Comput. Biol*., **17**: E1009480.

Dobnik, D., Kogovšek, P., Jakomin, T., Košir, N., Tušek Žnidaric, M., ˇLeskovec, M., Kaminsky, S.M., Mostrom, J., Lee, H. and Ravnikar, M. 2019. Accurate quantification and characterization of adeno-associated viral vectors. *Front. Microbiol*., **10**: 1570.

Everett, H., Crooke, H., Gurrala, R., Dwarka, R., Kim, J., Botha, B., Lubisi, A., Pardini, A., Gers, S., Vosloo, W. and Drew, T. 2011. Experimental infection of common warthogs (*Phacochoerus africanus*) and Bushpigs (*Potamochoerus larvatus*) with Classical Swine Fever Virus. I: Susceptibility and Transmission. Transbound. *Emerg. Dis.,* **58**: 128–134.

Gallaher, S.D. and Berk, A.J. 2013. A rapid Q-PCR titration protocol for adenovirus and helper dependent adenovirus vectors that produces biologically relevant results. *J. Virol. Methods*, **192**(0): 28–38.

Gallina, L., Dal, P.F., Mc Innes, C.J., CardetiG, G.A., Battilani, M., Ciulli, S. and Scagliarini, A. 2006. A real time PCR assay for the detection and quantification of orf virus. *J. Virol. Methods,* **134**:140–145.

Gomez, A.C., Sanchez-Miron, F.A., Garcıa-Camacho., Molina-Grima, E. and Chisti, Y. 2014. Protein production using the baculovirus-insect cell expression system, *Biotechnol. Prog.,* **30** (1).

Gregorova, P., Heinonen, M.K. and Sarin, L.P. 2022. An improved RT-qPCR method for direct quantification of enveloped RNA viruses. *MethodsX*, **9**: 101737.

Hink, W.F. and Vail, P.V. 1973. A plaque assay for titration of alfalfa looper nuclear polyhedrosis virus in a cabbage looper (TN-368) cell line. *J. Invertebr. Pathol.*, **22**(2): 168-174.

Hong, M., Li, T., Xue, W., Zhang, S., Cui, L., Wang, H., Zhang, Y., Zhou, L., Gu, Y., Xia, N. and Li, S. 2022, Genetic engineering of baculovirus-insect cell system to improve protein production. *Front. Bioeng. Biotechnol*., **10**: 994743.

Hopkins, R.F. and Esposito, D. 2009. A rapid method for titrating baculovirus stocks using the Sf-9 Easy Titer cell line. *Biotechniques***, 47**: 785–787.

Islam, A., Harrison, B., Cheetham, B.F., Mahony, T.J., Young, P. L. and Walkden-Brown, S.W. 2004. Differential amplification and quantitation of Marek's disease viruses using real-time polymerase chain reaction. *J. Virol. Methods*, **119** (2): 103-113.

Konow, M.S., Dojima, T., Toriyama, M. and Enoch, Y.P. 2002. Development of an antibody-based assay for determination of baculovirus titer in 10 h. *Biotechnol. Prog.*, **18**: 647- 651.

Lefkowitz, E.J., Dempsey, D.M., Hendrickson, R.C., Orton, R.J., Siddell, S.G. and Smith, D.B. 2018. Virus taxonomy:

the database of the International Committee on Taxonomy of Viruses (ICTV). *Nucleic Acids Res*., **46**: D708–D717.

Lei, C., Yang, J., Hu, J. and Sun, X. 2021. On the Calculation of TCID50 for Quantitation of Virus Infectivity. *Virol. Sin.*, **36**: 141–144.

Lothert, K., Eilts, F. and Wolff, M.W. 2022. Quantification methods for viruses and virus-like particles applied in biopharmaceutical production processes. *Expert Rev. Vaccines.,* **21**: 1029–1044.

Lowings, P., Ibata, G., Needham, J. and Paton, D. 1996. Classical swine fever virus diversity and evolution. *J. Gen. Virol.*, **77**(6)**:** 1311-1321.

Lynn, D.E. 1992. Improved efficiency in determining the titer of the *Autographa californica* baculovirus non-occluded virus. *Biotechniques*, **13**: 282–285.

Malde, V. and Hunt, I. 2004. Calculation of baculovirus titer using a microfluidic-based bioanalyzer. *Biotechniques*, **36**(6): 942-946.

Mendoza, E.J., Manguiat, K., Wood, H. and Drebot, M. 2020. Two Detailed Plaque Assay Protocols for the Quantification of Infectious SARS-CoV-2. *Curr. Protoc. Microbiol.*, **57**.

OIE. 2020. World Organization for Animal Health 'OIE-Listed Diseases, Infections and Infestations in Force in 2020.

Paul, A.K. and Gisele, G. 1999. An immunological assay for determination of baculovirus titer in 48 h. *Anal. Biochem.,* **268**, 173-178.

Philipps, B., Forstner, M. and Mayr, L. M. 2004. Baculovirus expression system for magnetic sorting of infected cells and enhanced titer determination. *Biotechniques*, **36**(1): 80-83.

Postel, A., Austermann-Busch, S., Petrov, A., Moennig, V. and Becher, P. 2018. Epidemiology, diagnosis and control of classical swine fever: recent developments and future challenges. *Transbound. Emerg. Dis.,* **65**: 248-261.

Qi, J., Liu, T., Pan, J., Miao, P. and Zhang, C. 2015. Rapid baculovirus titration assay based on viable cell side scatter (SSC). *Analytica Chimica Acta*, **879**: 58-62.

Roldão, A., Carrondo, M.J.T., Alves, P.M. and Oliveira, R. 2008. Stochastic simulation of protein expression in the baculovirus/ insect cells system. *Comp. Chem. Eng*., **32**: 68–77.

Ruiz-Villalba, A., Ruijter, J.M. and van den Hoff, M.J.B. 2021. Use and Misuse of Cq in qPCR Data Analysis and Reporting. *Life*, **11**: 496.

Shen, C.F., Meghrous, J. and Kamen, A. 2002. Quantitation of baculovirus particles by flow cytometry. *J. Virol. Methods*, **105**(2): 321-330.

Simmonds, P., Becher, P., Bukh, J., Gould, E.A., Meyers, G., Monath, T., Muerhoff, S., Pletnev, A., Rico-Hesse, R., Smith, D.B. and Stapleton, J.T. 2017. ICTV virus taxonomy profile: Flaviviridae. *J. Gen. Virol.,* **98**: 2-3.

Smither, S.J., Lear-Rooney, C., Biggins, J., Pettitt, J., Lever, M.S. and Olinger, G.G. Jr. 2013. Comparison of the plaque assay and 50% tissue culture infectious dose assay as methods for measuring filovirus infectivity. *J. Virol, Methods*, **193**: 565–571.

Tsai, C.H., Wei, S.C., Lo, H.R. and Chao, Y.C. 2020. Baculovirus as versatile vectors for protein display and biotechnological applications. *Curr. Issues Mol. Biol.*, **34**: 231–256.

Virok, D.P., Eszik, I.O., Mosolyg , O.T., Onder, K.E., Val, E., Buri, A. and Katalin. 2017. A direct quantitative PCR-based measurement of herpes simplex virus susceptibility to antiviral drugs and neutralizing antibodies. *J. Virol. Methods*, http://dx.doi.org/10.1016/j.jviromet.2017.01.007

Wang, Y., Yang, K., Bai, C., Yin, D., Li, D., Qi, K., Wang, K. and Li, Y. 2017. Development of a SYBR Green I real-time PCR for the detection of the orf virus. *AMB Expr*, **7**: 21.

Yahata, T., Andriole, S., Isselbacher, K.J. and Shioda, T. 2000. Estimation of baculovirus titer by β-galactosidase activity assay of virus preparations. *Biotechniques*, **29** (2): 214-215.

Zhang, F.Q., Yu, M., Weiland, E., Morrissy, C., Zhang, N., Westbury, H. and Wang, L.F. 2006. Characterization of epitopes for neutralizing monoclonal antibodies to classical swine fever virus E2 and Erns using phage-displayed random peptide library. *Archiv. Virol.*, **151**(1): 37-54.