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Quantitative Real-Time PCR as an Alternative to Plaque Assay Titration for Recombinant Baculovirus Expressing Porcine Parvovirus VP2 Gene

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ABSTRACT

Baculovirus expression system having post translational modification is used for large scale production of foreign proteins. Viral titre determination is crucial for efficient protein production. Even though plaque assay and end-point dilution method are conventionally used for titre determination, a less tedious and time-saving method is required for viral titre determination. Recombinant baculovirus expressing VP2 of porcine parvovirus was transfected in SF-21 insect cells. A quantitative real-time PCR was optimized for r-baculovirus titre determination and correlated with plaque assay method for its performance. The baculovirus DNA qPCR Ct values and corresponding PFU/mL showed strong correlation having value of r =99.71 at 95% confidence interval.

HIGHLIGHTS

- qPCR is a simpler and time-saving method that can be used for baculovirus titration.
- Ct value based copy no. strongly correlates with viral titre based on plaque assay.

Keywords: Baculovirus, real-time PCR, plaque assay, insect cell, porcine parvovirus, VP2, SF-21 cell line

Baculovirus first reported as polyhedron shaped structure in mid 1800s causing polyhedrosis in silkworms. Only in 1940s with the advent of electron microscope the rod shaped virions were demonstrated. Baculovirus was first used for expression of α-interferon in insect cell larvae in 1985 (Mansouri and Berger, 2018). The prototype baculovirus *Autographa californica* multiple *nucleopolyhedrovirus* (AcMNPV) is being used as baculovirus expression vector system (BEVS) since mid-1980s (Kost and Kemp, 2016). In last 4 decades, since its advent, thousands of proteins have been expressed using baculovirus as vector. Undiluted passaging in continuous cultures could lead to mutagenesis leading to decreased concentration of polyhedravirus (Wu *et al*., 2021). In order to achieve efficient protein production appropriate multiplicity of infection has to be determined for which virus titre must be known.

Conventionally plaque assay is being done for titration of baculovirus. As this method is labour intensive, time consuming, as it requires more than 4 days for plaque formation and requires high degree of expertise for execution. End point dilution method is used for titre determination of baculovirus which require X-gal as lacZ or other reporter gene is fused to promoter of baculovirus gene (Dee and Shuler, 1997; Li *et al*., 2022). However, this method requires more than 7 days to correctly determine virus titre. Many alternative methods like a green fluorescence protein markers, using chromogen and immunostaining against expressed protein, fluidic

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system like viral counter, flow cytometry and based on viable cell size are used (Ferris *et al*., 2011; Hopkins and Esposito, 2009; Janakiraman *et al*., 2006; Kitts and Green, 1999; Mulvania *et al*., 2004). However, immune-histochemical staining would require longer incubation periods and results will vary on observers foci observing skills, fluorescence would require either engineered cell line or adding an extra tag to protein expressed and viral counter and cell size based methods would require sophisticated instruments. Hence, we need a system that can accurately, speedily and in an uncomplicated way can be applied in nearly all laboratories for totration of baculovirus. Quantitative Real-time PCR (qPCR) have been used targeting AcPMNPV genes ie-1 and gp-64 using SYBR green method and Taqman fluorescence probe method (Hitchman *et al*., 2007; Lo and Chao, 2004). In our study we report a titration method targeting the gene of recombinant viral protein being expressed in BEVS.

MATERIALS AND METHODS

Recombinant baculovirus generation

pFastBac™ vector was cloned with VP2 gene of porcine pavovirus and transformed into chemically competent DH10Bac™ cells using Bac-to-bac®, *In vitro* gen baculovirus expression system. The recombinant plasmid was isolated using PureLink® HiPure Plasmid DNA Purification Kit, Invitrogen following BAC DNA manufacturer's protocol. Insect cells SF-21 were maintained in Grace supplemented media at 27º C incubator. Cells were transfected with 1µg recombinant bacmid having porcine parvovirus VP2 containing using Cellfectin® II and Grace un-supplemented media in a 6 well cell culture plate and incubated at 27º C. Post-transfection after 5 days virus was harvested by centrifugation at 1000g for 10 minutes.

Viral Titration by plaque assay

Plaque assay was done as per by King and Possee in 1992 (Dunigan, 1993). SF-21 cells in log phase were sub-cultured using 0.8×10^6 cells in 2 mL Grace supplemented media with 10 % fetal bovine serum. After cells were attached in 2-3 hours, media was removed infected with serially 10 fold diluted from 10^{-1} to 10^{-8} in media along with cell control containing media. After 1 hour incubation, the inoculum was removed and overlaid with 2 mL agarose overlay containing 0.8 % low gelling agarose insect cell culture (Sigma-Aldrich) in media was. After 15-20 minutes 800 µL of Grace supplemented media was placed over it to prevent drying of agarose. The plaques were counted after 84 h. post-infection using neutral red staining.

The viral titre was calculated using the formula—

Plaque forming unit (PFU)/mL= Average no. of plaques × dilution factor/ volume of virus dilution

Recombinant Baculovirus DNA extraction

In BSL-2 cabinet 200 µL of viral supernatant was collected 72 h post-infection for DNA isolation. Tenfold dilution of viral supernatant was made in 1X PBS and extraction was done using QIAamp® DNA Mini Kit following manufacturer's protocol. Briefly 15 µL of proteinase K and lysis buffer was added and mixed properly and incubated at 56ºC for 1.5 hr. Followed by adding of 200 µL 100 % ethanol, brief vortexing and spin. The sample was then placed in QIAamp® min column and centrifuged at 8000 g for 1 minute. Columns were then washed with wash solution A and B. Residual wash solution was removed by centrifuging at 12, 000 g for 2 minutes. The DNA was eluted using 25 µL elution buffer.

Primer designing

Primer targeting 152 bp sequence (Table 1) of recombinant VP2 porcine parvovirus using Primer3plus® software and analysed using OligoAnalyzer™ Tool, IDT technologies available online. The primers were synthesized commercially by Eurofins® genomics.

Table 1: Details of primers designed in the study

Sl. No.	Primer name	Sequence	Size	Amplicon size
1	PPV-515 F	GCTTAATGGTCGCACTAGAC	20	152 bp
2	PPV-666R	TGTTGGTGGATTTAGGTTTC	20	

Quantitative PCR conditions

Real-time reaction mixture was made using 2x Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix, Agilent Technologies. Final reaction volume of 10 µL contained 2.5mM $MgCl_2$, 0.4 µL primer from 10 µM stock concentration, dNTPs and double stranded DNA binding SYBR Green dye for detection. For standard curve generation for quantification purpose, plasmid DNA of known concentration containing VP2 target 152 bp sequence was serially 10 fold diluted.

The incubation was carried-out 95 ºC for 3 minutes followed by 40 cycles each of denaturation at 95 ºC for 5 seconds, amplification at 49.5 ºC for 15 seconds, Melt curve at 65 ºC-95 ºC with 0.5 ºC resolution and 5 second soak time. Results were visualized using Agilent AriaMx™ software.

Data Analysis

qPCR standard curve generation, graph plotting and data analysis was done using Graph Pad Prism software. Regression analysis was done to check efficiency of qPCR and to extrapolate titre based on Ct value. Correlation analysis was done based on Ct value and plaque assay.

RESULTS AND DISCUSSION

In the present study, real-time PCR based rapid quantification of recombinant baculovirus expressing VP2 gene of PPV was optimized and its suitability as an alternative to plague assay for titration/quantification of recombinant baculovirus was investigated. The traditional virus titration method like plaque assay alone and with the combination of specific antibody in the immunostaining, immunofluorescence has been successfully applied for titration of wild and recombinant baculovirus expressing foreign protein (Basak *et al*., 2021; Knudson, 1979; Qingsheng and Yuanyuan, 2022). However, plaque assay is very cumbersome, time consuming (> 4 days), and requires high degree of expertise for execution. In contrast, real-time PCR based quantification of baculovirus expressing target gene is convenient, quick and fairly reproducible methods as compared to plaque assay. Real-time PCR based quantification of bluetongue virus, african swine fever virus, canine distemper virus, orf and other pox viruses in the cell culture system has already been reported and results were highly correlated with the infectivity assays based titration methods such as plaque assay or end point dilution methods like $TCID_{50}$, EID_{50}, $FFID_{50}$ (Brown *et al*., 2020; Khanal *et al*., 2022; Steinrigl *et al*., 2010; Zhao *et al*., 2013). However, research works related the utilization of qPCR for quantification of recombinant baculovirus is very limited (Hitchman *et al*., 2007; Lo and Chao, 2004; Mahammad *et al*., 2013). The present study was conducted to explore the possibility of qPCR based alternative assays for quantification of recombinant baculovirus expressing VP2 protein of porcine parvovirus.

In previous study, conducted by Hitchman *et al*. (2009), P1 passaged virus has been titrated by qPCR. In the present study, P3 recombinant baculovirus was used for quantification to rule out any residual transfected DNA in the early passaged virus (P1and P3) and also for determination of multiplicity of infection (MOI) of P4 virus in High-five insect cell lines. Insect cell line particularly, SF-9 and SF-21 cells have been recommended for transfection and production of recombinant baculovirus for subsequent infection to cell lines (High-five /Tn5) recommended for protein production (Unger and Peleg, 2012). Several reports have been indicated that level of protein expression can be increased by appropriately determining the multiplicity of infection (MOI) for High-five /Tn5 insect cell line (Wilde *et al*., 2014). Therefore, titration of P3 passage virus was conducted in the present study and same has been used for calculation of MOI of P4 virus in high Five cells. Plaque visualization was done by using neutral red dye as it stains live cells and plaques can be visualized during formation. The time of plaque visualization was fixed after visualization from 48-96 h and at 84 h having distinct plaques. A titre of 1.6×10^8 titre was obtained after 84 h.

For real-time PCR, PPV-VP2 protein gene primers were designed and product size (152 bp) and specificity was checked with gel electrophoresis (Fig. 1). Viral DNA was isolated from serial 10 fold dilution made from virus titrated with plaque assay After 40 cycles of qPCR run distinct Ct values were observed across different viral titres (Table 2). Melt curve analysis was done and it showed all specific products in uniform peak at 83.5 ºC melting temperature with negative template control showing primer dimer peak occasionally which was absent in positive templates (Fig. 2). This indicated qPCR protocol was accurate. Using standard plasmid, standard curve plot was made for Ct

value against DNA copies (10 log scale). To meet the basic requirements of standard curve at least 5 dilutions were used in the study. The curve showed a linearity across different DNA copies with r^2 value of 99.35, p value <0.0002, with equation y = -3.027X + 37.7 (Fig. 3). Further this equation was used to find the DNA copy no. of *Ct* value of different 10 fold viral dilution ranging from 10^7 PFU/mL to 10^2 PFU/mL.

Table 2: Threshold cycle (Ct) value of quantitative real-time PCR with log10 DNA copies calculated with standard curve and log10 PFU/mL of corresponding viral titre dilution used for quantitative real-time PCR

Sl. No.	Ct value	log10 DNA copies	log10 PFU/mL
1	15.31	7.39676247	7
2	17.77	6.58407664	6
3	20.33	5.73835481	5
4	23.54	4.67789891	4
5	25.79	3.9345887	3
6	27.57	3.34654774	2

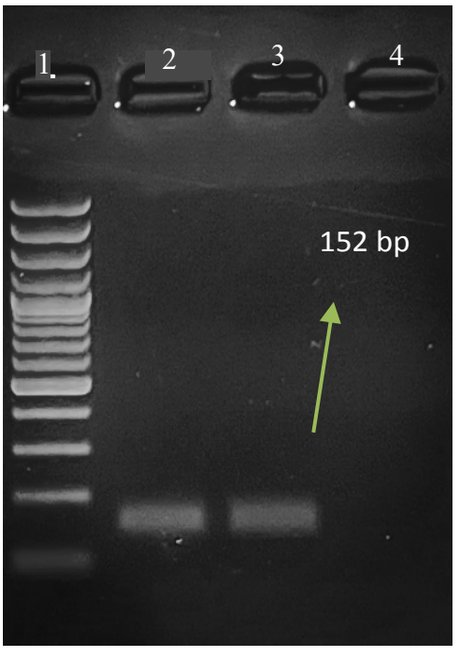

Fig. 1: PCR amplification using primers 515F and 666R. **(1)** DNA ladder (Puregene 100 bp-3 kbp). **(2)** Standard plasmid **(3)** r-baculovirus DNA **(4)** Non-template control

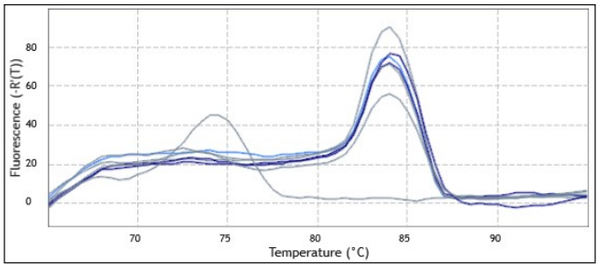

Fig. 2: Confirmation of specificity of qPCR products by melt-curve analysis. All positive products formed peak at 83.5 ºC and negative template control showed primer dimer peak at different temperature

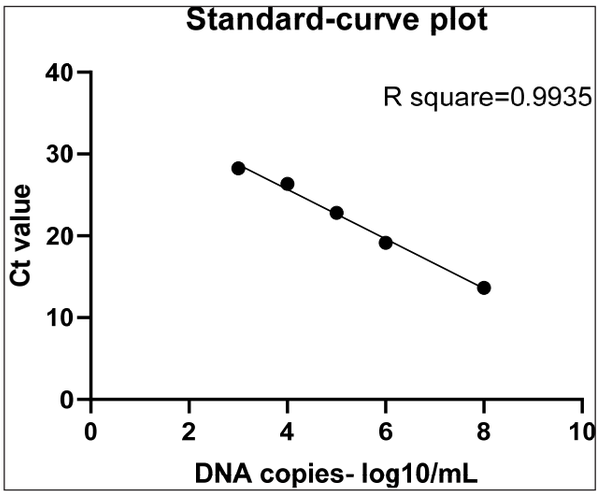

Fig. 3: Standard curve generated using plasmid containing VP2 sequence. 10 fold serial dilutions of plasmid with known copy no plotted in 10 log scale in x-axis and corresponding threshold value/Ct value plotted in y-axis

Data from 3 repeated sets of qPCR were used for log10 DNA copies calculation. The log10 DNA copies ranged from 7.186 to 3.357 respectively. Correlation analysis was done using Ct equivalent of different viral titre with DNA (10 log scale) copies (Fig. 4). The Pearson correlation coefficient, r was 99.71 at 95 % confidence interval. This indicates a good correlation between conventional plaque assay method and qPCR. A linear regression, r^2 of 0.9942, p value <0.0001 was obtained. The test was found significant at alpha level 0.05.

Our study results imply that quantitative real-time PCR could replace the 5 day long conventional procedure

for baculovirus titration. In conventional technique cell seeding has to be done and cells should be able to form confluent monolayer failing which plaques cannot be visualized. Moreover, virus dilutions have to be made from stock virus which could lead to human error. qPCR involves column based DNA extraction and use of qPCR master mix and rest all protocol will be done by machine which will increase the precision . The standard curve generated using Ct value based log DNA copies and viral titre in PFU/mL can also be used to test r-baculovirus in 4 ºC and -80 ºC. This method can be used in different baculovirus systems. This method also reduces the cost of baculovirus titration assay which involves expensive insect cell culture media, use of sophisticated agarose for insect cell culture. Being based on mechanical analysis and having wide range of quantification (10^7 to 10^2), this method could hasten the precise virus titre estimation for getting optimum protein production using BEVS.

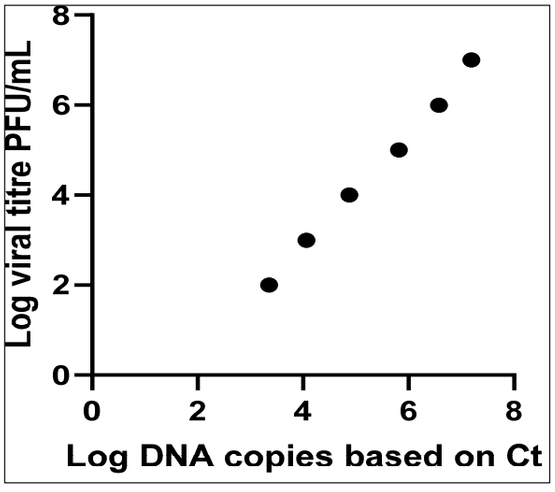

Fig. 4: Correlation between plaque assay based viral titre and log DNA copies based on Ct value. Ct value for each dilution of titre was determined by qPCR based on which log DNA copies were calculated by standard curve

CONCLUSION

qPCR assay was developed for rapid r-baculovirus determination. The assay could aid in determination of viral tire in simple way using baculovirus DNA isolation kit and qPCR protocol which takes less than 3h for getting results. The correlation analysis also implies qPCR method can satisfactorily replace plaque assay for r-baculovirus titre determination.

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