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ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

Determination of the Entomopoxviruses Viral Titer by Quantitative Real Time PCR (qPCR)

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Abstract: Poxviruses are large double-stranded DNA (dsDNA) viruses that infects both human, animals and insects. Most known member of this viruses is Variola virus that caused pox disease and eradicated by vaccination. While other members still infect their hosts, it is important to detect, isolate and fight with this viruses. For this purpose, determining the titers of viruses is important. Virus titers are used for vaccine development, antiviral activity, genetic engineering, gene therapy and disease treatment studies. Despite all these uses, the methods used to determine virus titer are tedious and time-consuming. We developed a method using quantitative real-time PCR to improve the speed and efficiency of titer determination. In this method, we used a known single-copy gene from AMEV virus, cloned it into a plasmid and generated standard graphs. We measured the virus titer was unknown using the standard graph. As a result, a method was developed that can be used to determine the titer of Poxviruses in a simple, fast and effective way.

Keywords: AMEV, DNA, poxvirus, qPCR, virus titer.

Entomopoxvirüslerin Viral Titresinin Kantitatif Gerçek Zamanlı PCR (qPCR) ile Belirlenmesi

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Öz: Poksvirüsler, hem insanları hem hayvanları hem de böcekleri enfekte eden büyük çift sarmallı DNA (dsDNA) virüsleridir. Bu virüslerin en bilinen üyesi, çiçek hastalığına neden olan ve aşılama ile ortadan kaldırılan Variola virüsüdür. Diğer üyeler hala konakçılarını enfekte ederken, bu virüsleri tespit etmek, izole etmek ve onlarla savaşmak önemlidir. Bu amaçla virüslerin titrelerini belirlemek değerlidir. Virüs titreleri aşı geliştirme, antiviral aktivite, genetik mühendisliği, gen terapisi ve hastalık tedavi çalışmaları için kullanılır. Tüm bu kullanımlara rağmen, virüs titresini belirlemek için kullanılan yöntemler sıkıcı ve zaman alıcıdır. Bu çalışmada titre belirlemenin hızını ve verimliliğini artırmak için kantitatif gerçek zamanlı PCR kullanan bir yöntem geliştirilmiştir. Bu yöntemde, AMEV virüsünden bilinen tek kopyalı bir gen kullanılarak, bir plazmite klonlandı ve standart grafikler oluşturuldu. Titresi bilinmeyen virüsü standart grafik kullanarak analiz edildi. Sonuç olarak Poxvirüslerin titresi basit, hızlı ve etkili bir şekilde belirlenebileceği bir metod geliştirildi.

Anahtar kelimeler: AMEV, DNA, poksvirus, qPCR, virus titresi.

INTRODUCTION

Poxviruses are large double-stranded DNA (dsDNA) viruses that can cause infection in both human, animals and insects. It is a family of DNA viruses that replicate in the cytoplasm of both vertebrates and invertebrates (Moss, 2006). Poxviruses are divided into two subfamilies; Chordopoxvirinae, which infects vertebrates, and Entomopoxvirinae subfamily, which infects insects, have been isolated from insects belonging to the orders Lepidoptera, Orthoptera, Coleoptera, Diptera and Hymenoptera (Haller et al., 2014). Virions of entomopoxviruses are oval-shaped and 150-470 nm long and 165-300 nm wide (Sezen and Demirbağ, 2005). The Entomopoxvirus (EPV) virion consists of a central region containing dsDNA, an inner membrane covered with proteins that surround the genetic material, lateral

structures on the sides, and an outer membrane surrounding all of them.

EPVs have structural similarities with Orthopoxviruses and have been found to show gene similarities with vertebrate poxviruses (Bawden et al., 2000). *Amsacta moorei* entomopoxvirus (AMEV) is a virus that causes infection in insects belonging to the Entomopoxvirinae subfamily of the Poxviridae family (Inan et al., 2018). It is the type species of the Betaentomopoxvirus genus that infects the orders Lepidoptera (Moths) and Orthoptera (Grasshoppers). Although the natural host of AMEV is *Amsacta moorei* (Red-hairy caterpillar) it has also been isolated from agricultural pests such as *Estigmena acrea* and *Lymantria dispar* (Hall and Hink, 1990). Unlike other EPVs, AMEV can also replicate in Ld652, Sf9 and EAA-BTI cell lines (Granados and Naughton, 1975; Marlow et al., 1993; Perera et al., 2013). For this reason, studies with AMEV are important in understanding the molecular mechanisms of Entomopoxviruses.

The whole genome sequencing studies of AMEV were performed in 2000 and it was shown that its genome consists of 232 kb length, encoding 279 open reading frames (ORFs) larger than 60 amino acids (Bawden et al., 2000). After that, 256 of these ORFs were subsequently shown to be active proteins by Guo and Yu (2007). Additionally, AMEV has the highest A+T content among the poxviruses with a rate of 82.2% (Bawden et al., 2000).

Success in viral infection depends on the infection capacity and viral titer in the inoculum. It is not enough to know the number of infective viruses in stock, since not all virus particles are infective due to problems and mutations that occur in the viral replication process or environmental factors such as pH, light and temperature can affect the viral infection capacity. However, in viral infection experiments, it is important to determine the titer of the infective virus particles in the stock. The most basic methods used to determine the number of infective viruses are end point dilution assay (EPDA), tissue culture infective dose 50 (TCID50) and plaque assays (Reed and Muench, 1938; Baer and Kehn-Hall, 2014; Darling et al., 1998). Using these methods, the TCID50 value is determined by EPDA, and the plaque formation unit (PFU) is determined by the plaque test. Another method that can be used to determine the number of physical virus particles is the electron microscopy technique, transmission electron microscopy (TEM) is used for this purpose. Titration methods fall into two categories: infectious or particulate (infectious plus non-infectious) titer determination (Thomas et al., 2007). In this way, the total number of viruses, not the number of infective particles, can be determined. In a study, it was observed that the number of physical virus particles was 10-100 times higher than the number of infective viruses (Bhat et al., 2022).

While counting viral titer with EPDA, the experimental success dependent to the cytopathic effects of the virus. The cytopathic effect refers to the morphological and physiological changes that occur in the cells infected by the virus and usually includes cell death, swelling or other morphological changes. But also, there are some viruses that do not show cytopathic effects in cell cultures. For example, Classical swine flu viruses (CSFV) and Rabies virus (RV) from the Rhabdoviridae family are non-cytopathic viruses (Itakura et al., 2020, Plesa et al., 2006). Also, *Murine leukemia virus* (MLV), a member of retrovirus, may not cause cytopathic effects in cell culture, but they can cause very severe and fatal infections in animals (Sliva et al., 2004). These viruses do not cause obvious morphological changes in the cells they infect, making their titers difficult to determine using traditional plaque formation methods. In this case, viruses can be identified using other diagnostic methods, such as viral particle detection or isolating and multiplying the virus.

Amsacta moorei entomopoxviruses are not producing plaques but the infection can be screened using light microscope. Virus causes cytopathic effects like viral factories in the cytoplasm and this process occurs late in the infection (approx 5 dpi). Thus, it is difficult to determine whether these effects caused by viruses or because of other stresses and needs expertise.

In the literature, it was shown that the titer of some other viruses was determined by qPCR analysis. To overcome microscope screening problems of AMEV, a qPCR based methodology was developed to quantify AMEV virus for the first time in this study.

MATERIAL AND METHOD

***Cell Line, Virus and Vector*:** *Lymantria dispar* (Ld652) insect cell line obtained from the stocks of KTU Department of Molecular Biology and Genetics, Molecular Virology Laboratory. Cells were grown in T25 flasks with medium containing 10% FBS, 45% Excell-400, 45% Grace's Insect Medium in an incubator at 28°C. (Muratoğlu et al., 2010).

Recombinant AmΔsph/gfp AMEV virus, in which the spheroidin gene was deleted and replaced with the green fluorescent protein (GFP) gene region, taken from the stocks of the Molecular Virology Laboratory of KTU Molecular Biology and Genetics Department, was used for infection experiments.

The pGAD186 vector taken from of KTU Molecular Biology and Genetics Department, Molecular Virology Laboratory stocks was used to create the standard graph in qPCR experiments.

Thawing and Passaging of Cells from Stock: Cells were frozen using freezing media (Containing 20% FBS, 10% DMSO) and stored in liquid nitrogen. Cells were removed from the liquid nitrogen tank and allowed to thaw in a water bath set at 28°C. The thawed cells were precipitated by centrifugation at 230 g for 5 minutes. Cells from the stock were dissolved in growth medium, transferred to a T25 culture dish and allowed to grow in an incubator at 28°C.

Propagation of AMEV in Cell Culture: The Ld652 cell line was used to propagate the AmΔsph/gfp AMEV virus. This recombinant virus has a green fluorescent protein (GFP) marker protein for observation of the infected cells via fluorescent microscopy. In the incubation period viral infection followed by GFP expression via Zeiss Axiovert Inverted fluorescent microscope. After that, the cell-virus suspension was centrifuged at 3,000 rpm for 10 minutes to remove the virus from cell residues. The virus suspension was stored at +4°C without exposure to light for later use.

Firstly, Ld652 cells were counted using a hemocytometer. The counted cells were diluted to $1x10^6$ cells and seeded to a T-25 culture dish. Cells were expected to be 90%-100% confluent. Then the medium covering the entire surface was removed, 1 ml of infection mixture including virus was added onto the cells. The infection continued for 2 hours by shaking the flask at 10-minute intervals. At the end of the incubation period, the infection mixture was removed, and the cells were washed with PBS to remove unattached viruses, then 5 ml of fresh medium was added to the cells. Cells were incubated in an incubator at 28 °C for 5 days.

In the incubation period viral infection followed by green fluorescent protein expression via Zeiss Axiovert Inverted fluorescent microscope. After signs of infection were observed in most of the cells, T25 flask placed in the refrigerator at -80 ºC. The cells were kept at -80 ºC overnight and then kept at room temperature to thaw quickly. The thawed cells were subjected to three freeze-thaw cycles of one hour each. After that, the cell-virus suspension was centrifuged at 3,000 rpm for 10 minutes to remove the virus from cell residues. The virus suspension was stored at +4°C for later use.

Determining the Concentration of the Replicated Virus: The concentration of the replicated virus was determined by the endpoint dilution method (EPDA) (Reed and Muench, 1938).

In the experiment, 10 microcentrifuge tubes were numbered 1 to 10 and 90 µl of the growth medium was added into the tubes. 10 µl sample from the virus stock was placed in the first tube and diluted 1/10 by serial dilution until the ninth tube. The 10th tube was used as a control and no virus was added to this tube. Ld652 cells were suspended to contain $2x10^6$ cells/ml. 90 µl of the cell suspension was added into each tube. Then tubes were mixed thoroughly by pipetting and ensured homogeneity. In each row of a Terasaki plate (60 wells), 10µl of the same concentration of cell virus suspension was added from A to F. The Terasaki plate (3 replicates) was placed in a bag and incubated in an incubator at 28°C. Incubation was continued for 5 days until signs of infection appeared.

Preparation of Competent Cells and Propagation of the pGAD-186 Vector by Transformation: The pGAD-186 vector was used to create the qPCR standard graph. This vector has 8327 bp length and contains viral replication A28-like protein of AMEV genome (amv186) that is essential for viral growth and has only one copy in each virus genome. DH5α competent cells prepared for propagation of pGAD-186 vector. Heat shock transformation method was used for transformation.

To prepare competent cells, DH5α overnight culture was performed. The next day, measurements were taken on a spectrophotometer and the bacterial density was diluted to 0.1 OD. The dilutions were incubated in incubator with shaker until the OD value reached between 0.45-0.55. When the desired value was reached, the culture was centrifuged at 4500 rpm at +4°C for 5 minutes. The supernatant was removed, and the pellet was dissolved with 10 ml of cold $CaCl_2$ (100mM) and kept at ice for 30 minutes and the suspension was centrifuged at 4500 rpm at +4°C for 5 minutes. The supernatant was removed, and the pellet was dissolved with 2 ml of cold $CaCl_2$. Competent cells are kept at +4°C for 2 hours.

In heat shock transformation, pGAD186 plasmid DNA was added to the previously prepared competent cells. The tubes were kept on ice for 30 minutes. Heat shock was applied at 42°C for 2 min. It was kept on ice for 1 minute. A tube containing 1ml LB Broth was prepared for each sample. Samples were transferred to tubes containing LB Broth and gently pipetted. Cells were incubated at 37°C for 1-1.5 hours. At the end of the incubation, the cells were precipitated by centrifugation at 6000xg for 3 minutes. The supernatant was removed, leaving approximately 50 µl, and the pellet was dissolved in the remaining liquid. Bacteria were planted on LB agar solid medium containing ampicillin. Petri dishes were incubated in an incubator at 37°C for 16 hours.

Plasmid Isolation: After transformation, bacterial colonies growing on medium containing ampicillin were selected and used for plasmid isolation. Plasmid was isolated using the protocol of E.Z.N.A. Endo-free plasmid DNA mini kit 1 (D6948-02), plasmid DNA isolation kit from Omega Bio-tek.

pGAD186 plasmid validated stocks were incubated for 12-16 hours. After incubation, the culture was precipitated by centrifugation at 10,000× g for 1

minute at room temperature. The supernatant was removed and 250 µl Solution 1 RNase A was added to the pellet. The pellet was mixed thoroughly by pipetting. The suspension was transferred to a clean centrifuge tube and 250µl Solution 2 was added. The tube was inverted several times to obtain clear lysate. 2-3 minutes of incubation may be required. 350µl of Solution 3 was added and inverted several times until a white precipitate formed. It was centrifuged at 13,000x g for 10 min. A white pellet was formed. The DNA mini column was placed in a 2ml collection tube. After centrifugation, the cleared supernatant was carefully transferred to the column. It was centrifuged for 1 minute at maximum speed. The supernatant was discarded, and the collection tube was reused. 500µl HBC buffer was added and centrifuged for 1 minute at maximum speed. The supernatant was discarded, and the collection tube was reused. 700µl DNA wash buffer was added and centrifuged for 1 minute at maximum speed. The supernatant was discarded and centrifuged for 2 minutes at maximum speed to dry the column. The column was transferred to a 1.5 ml eppendorf and 30-100µl Elution buffer was added directly to the column membrane. Mixture incubated for 1 minute at room temperature and then centrifuged for 1 minute at maximum speed.

Isolated plasmid DNAs were visualized on a 1% agarose gel and stored at -20°C.

DNA Isolation and Visualization of DNA with Agarose Gel Electrophoresis: Viral DNA isolation was performed from the viral fluid, which was multiplied in cell culture and separated from the contaminant by centrifugation. Viral DNA was extracted using the kit (MasterPure™ DNA Purification Kit, MCD 85201). The protocol of the kit was modified and followed for isolation.

1 µl Proteinase K and 150 µl 2x T and C Lysis solution were mixed and 150 µl Virus fluid was added. Incubated in a 65°C heater block for 15 minutes by vortexing every 5 minutes. The sample was then cooled to 37 °C. After the samples were cooled to 37 °C, 1 µl of 5 µg/µl RNase A was added and incubated at 37 °C for 30 minutes. After incubation, the samples were kept in ice for 3-5 minutes. 175 µl of MPC Protein precipitation solution was added and vortexed for 10 seconds. It was then centrifuged at 4°C 10,000 × g for 10 min to precipitate impurity. The supernatant was transferred to a new tube and 500 µl isopropanol was added and mixed by inverting 30-40 times. It was then centrifuged at 4°C, 10,000 × g for 10 minutes. Isopropanol was removed by pipetting without disturbing the pellet. The pellet was washed twice with 70% ethanol. Alcohol was removed and the pellet was dissolved in 35 µl TE buffer. The concentration of isolated DNAs was measured in the nanodrop (Thermo). The isolated DNAs were visualized on a 1% agarose gel at 95V. Imaging of DNAs run on agarose gel were visualized under UV light (BioRad ChemiDoc MP Gel Imaging System).

Determining Standard Graphs by Real Time PCR Using pGAD186 Plasmid: In our study, we aimed to determine the copy number of viral DNA using the qPCR technique. For this purpose, qPCR standard graphs were created using a plasmid (pGAD186) containing a viral gene *amv186* that has only one copy in the viral genome. This vector has 8327 bp length. The vector was isolated after cloning in *E.coli DH5α* cells. The copy number of the isolated plasmid was determined using the following formula and 10-fold serial dilutions of this plasmid was prepared (Palacios et al., 2017).

$$\text{DNA (copy number)} = \frac{6.02 \times 10^{23} \left(\frac{\text{copies}}{\text{mol}}\right) \times \text{concentrationg (g/µ)}}{\text{Number of Basepairs (Vector + Insert)} \times 660 \text{ (g/mol)}}$$

To determine copy number of the amv186 gene the primers AMV186-qFW (5' TTAACTGTTTTTGATCCCAACG 3'), AMV186-qRV (5' AAAAATCCAAAAGTTGAAACTGC 3') were used. The reaction mixture prepared with adding 0.4 µl forward and 0.4 µl reverse primers, 1 µl Template DNA, 10 µl qPCR mixture and 8.2 µl ddH2O.The quantitative PCR conditions were followed as: 95 °C 3', then 39 cycles of 95 °C 10", 60 °C 30" while fluorescent measured in each cycle. To create melting curve: the temperature of the tube increased from 65°C to 95°C in increments of 0.5°C.

Validation of the Stock Viral Titer Using qPCR: Using the standard graph, the amount of viral samples of unknown quantity was quantitatively determined. DNA isolated from the viral fluid was amplified by qPCR. The amount of viral DNA was determined using the standard graph. Thus, the number of virus particles in the isolated volume was determined. To determine the number of viruses per ml, the total volume isolated was calculated by multiplying the total volume isolated by the dilution factor. In our study, we isolated DNA from 150µl of viral fluid. The number of viral particles in 150µl was determined using the standard graph. To compare with the result obtained in EPDA, the number of virus particles per ml was calculated by multiplying by the dilution factor. The results obtained were compared with each other.

RESULTS

Propagation of AMEV in Cell Culture: Our fluorescent microscopy studies showed that AmΔsph/gfp AMEV was propagated in Ld652 cells (Figure 1). Under a fluorescent microscope, GFP protein was visualized 2 days after infection. The produced virus was obtained by freeze-thaw technique and centrifugation.

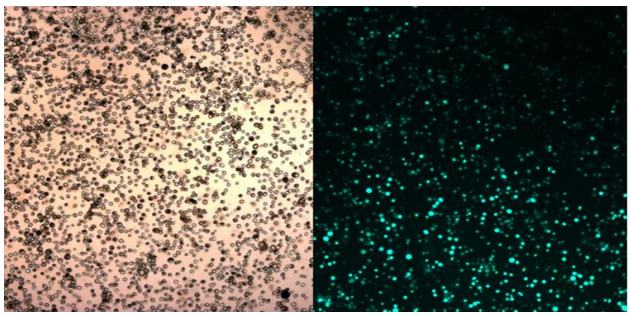

Figure 1. Fluorescence microscope image (Zeiss Axiovert) of the gfp protein produced by AmΔsph/gfp AMEV day 5 post infection (dpi). A: Image of Ld652 cells under halogen light, 50x. B: Image of gfp protein produced by recombinant virus under a fluorescent microscope, 50x.

Determination the Concentration of Virus: The concentrations of viruses were determined by the EPDA method. A + (positive) or – (negative) value was given for each well according to the infection status seen from the fluorescence microscope. Virus concentration (titer) was calculated by the formula in Reed and Muench, (1938) and values were obtained.

Titer (pfu/ml)=10(a+x) x 100/ml

n=highest dilution at which the infection value is 50% or greater than 50%;

a=log n; b=% of n dilution,

c = % value below n dilution.

x = (b-50%)/(b-c).

According to the calculations, the titer of the produced AmΔsph/gfp AMEV virus suspension was calculated as $6*10^6$ pfu /ml.

Plasmid Isolation: The accuracy of the isolated plasmid DNA was screened in agarose gel electrophoresis (Figure 2).

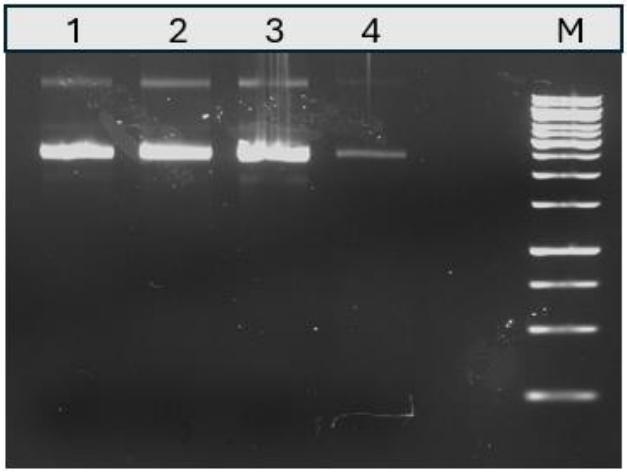

Figure 2. Agarose gel electrophoresis image of the isolated pGAD186 plasmid DNA. M: Marker, 1,2,3: İsolated pGAD186 plasmid DNA, 4: Stock pGAD186 plasmid DNA.

DNA Isolation and Visualization of DNA with Agarose Gel Electrophoresis: DNA of the AMEV virus were isolated using a kit (MasterPure™ DNA Purification Kit). Isolated DNAs were used as template in a PCR reaction and the amplicon were run on a gel containing 1% agarose at 95 volts and screened (BioRad ChemiDoc MP Gel Imaging System) (Figure 3).

Real-Time PCR Concentration Graph: To create a standard graph from plasmid DNA, firstly the copy number of the plasmid DNA were determined using the formula. Our calculations showed that the copy number of the 99.2 ng/µl pGAD186 plasmid stock was found to be 10,835,742,268. Based on this 10-fold serial dilutions using stock plasmid DNA were made and plasmid DNA copy numbers of each standard dilution were calculated (Table 1).

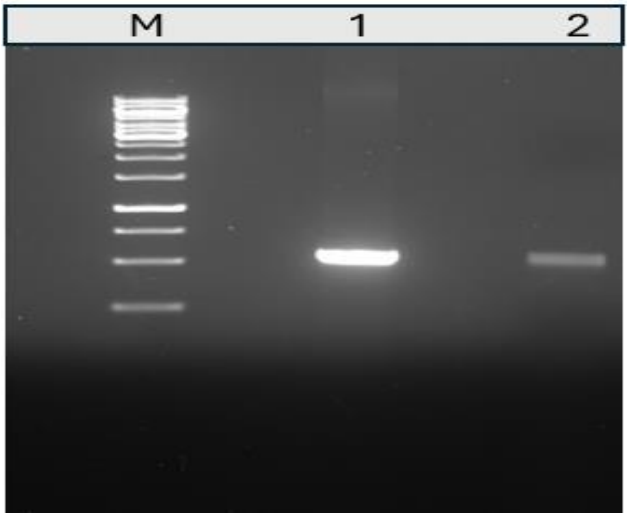

Figure 3. Agarose gel electrophoresis image of isolated viral DNA. M: Marker,1: Stock AMEV DNA, 2: İsolated Viral DNA

Table 1. Copy number calculations of each dilution made using pGAD186 Vector.

Sample Name	Concentration (Plasmid DNA)	Number of Copies /µl
Std-1	99.2ng/µl	10,835,742,268
Std-2	9.92ng/µl	1,083,574,226.8
Std-3	0.992 ng/µl	108,357,422.68
Std-4	99.2pg/µl	10,835,742.268
Std-5	9.92 pg/µl	1,083,574.2268
Std-6	0.992 pg/µl	108,357.42268
Std-7	99.2fg/µl	10,835.742268
Std-8	9.92 fg/µl	1,083.5742268
Std-9	0.992 fg/µl	108.35742268

Validation of the Stock Viral Titer Using qPCR: Based on the standard graph (Figure 4), the amount of unknown virus was calculated.

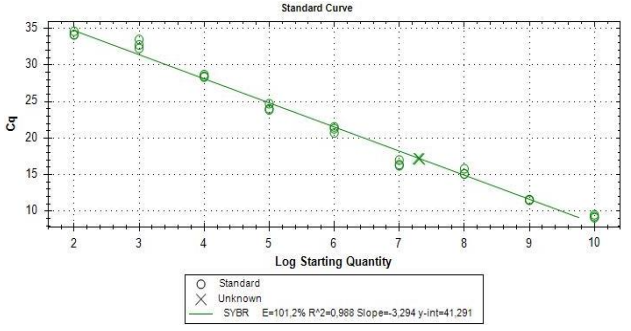

Figure 4. Standard curve graph of the pGAD186 vector generated using qPCR.

The amount obtained from the Cq values was determined by converting the isolation volume to ml and multiplying by the dilution factor. Isolation was made from 150 µl of viral fluid. The result was multiplied by 6.67 to complete to ml volume. The number of virus particles in 1 ml was calculated as **$1.3*10^8$** based on the data obtained from the qPCR standard graph and the described calculations. qPCR-based titer determination and EPDA testing were compared using different virus stocks and similar results were obtained. Thus, the sustainability of the technique was established.

DISCUSSION

Poxviruses are double-stranded DNA (dsDNA) viruses. These DNA viruses have a wide host range depending on the organism they infect. Variola virus, which causes smallpox in humans, is one of these viruses and has had a fatal effect on humans for many years. Poxviruses that cause infection in insects are called Entomopoxviruses (EPV). To be able to track replication in cell culture, recombinant AMEV (AmΔsph/gfp) is used in which the *spheroidin* (*sph*) gene, which has no effect on virus replication in cell culture, is deleted and replaced with a green fluorescent protein gene (GFP) were used in the experiments. (Palmer et al., 1995) Entomopoxviruses has potential to be used as an agent in the biological control of insect pests that cause significant losses in agricultural areas and forests (Sezen and Demirbağ, 2005). Also, EPVs have the potential to be used as gene expression and gene transfer vectors. Due to these features, intensive studies are being carried out on Amsacta moorei entomopoxvirus (AMEV), which is an important type of EPVs. The concentration of the replicated virus was determined by the end-point dilution method (EPDA) used in the study. Although this method takes a long time, it is a reliable method and is still used in the literature. (Muratoğlu et al., 2010; Reed and Muench, 1938).

There are routinely used methods to determine virus concentration. These can be listed as plaque assay, TCID50, EPDA, hemagglutination assay, flow cytometry and electron microscopy (Baer and Kehn-Hall, 2014; Darling et al., 1998). For many years, the plaque assay and the endpoint dilution method (EPDA) have been the most accurate and reliable methods used to determine virus concentration. However, the results obtained from these methods vary in different laboratories. The reliability of these methods decreases due to differences in chemicals and protocols used. At the same time, the time and workload required for the application of these methods, which are based on determining the amount of virus in cell culture, is quite high.

Many DNA and RNA viruses can be quantified rapidly and accurately by using Real Time quantitative PCR. Lo and Chao (2004) determined the titer of baculovirus by RT-PCR method. Thomas et al. (2007) determined adenovirus titer by RT-PCR method. Fajardo et al. (2017) quantified 5 different viruses in grapevines from viral RNAs. Keyaerts et al. (2006) and Jiang et al. (2004) determined the amount of SARS-Coronavirus virus in infected organisms using an RT-PCR-based method. The titer of SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), which caused a worldwide pandemic in 2019, was determined by a one-step RT-PCR study by Jiang et al (2021).

In our study, we used the quantitative RT-PCR technique to determine the amount of AMEV, which is in the same family as Variola virus, which causes fatal infections in humans. We chose a single-copy gene of the virus genome for the generation of standard graphs. A plasmid vector containing this gene was created and used to create a standard graph. A standard formula was used to determine the plasmid DNA copy number. According to the result obtained from this formula, 10-fold dilutions were made starting from the stock. Cq values of these dilutions were determined by real-time PCR. Based on these values, a standard plasmid DNA copy number graph was created. With this standard graph, the number of virus particles in the stock was quantitatively determined as $1.3*10^8$ per mililiter. According to the EPDA, the titer of the virus suspension was calculated as $6*10^6$ pfu /ml.

CONCLUSION

In this work, infection of AmΔsph/gfp AMEV was observed in cell culture and visualized under a fluorescent microscope. In order to increase the efficiency of virus propagation, the cells that did not lyse were exploded by the freeze-thaw method and the virus particles inside were allowed to pass into the liquid. The concentration of AmΔsph/gfp AMEV propagated by the EPDA method was determined.

To generate a standard graph, the pGAD186 plasmid was used to improve the accuracy of the standard curve. qPCR reactions were performed using the BioRad CFX Connect Real-Time PCR Detection System. Standard graphs were created from plasmid DNA and the number of physical virus particles in the unknown amount of virus fluid was calculated using this standard graph. The number of infective virus particles determined by the EPDA method, and the number of physical virus particles determined by the qPCR method were compared. In conclusion, this study has shown that the amount of AMEV virus can be successfully determined using qPCR. With this methodology, the viral titer of all entomopoxviruses,

which capable of infecting cell culture can be determined with using a set of primers belonging to any viral gene

REFERENCES

Baer, A. & Kehn-Hall, K. (2014). Viral concentration determination through plaque assays: Using traditional and novel overlay systems. *Journal of Visualized Experiments*, ***93***, e52065, 1-10. DOI: 10.3791/52065

Bawden, A.L., Glassberg, K.J., Diggans, J., Shaw, R., Farmerie, W. & Moyer, R.W. (2000). Complete genomic sequence of the Amsacta moorei entomopoxvirus: Analysis and comparison with other poxviruses. *Virology*, ***274***, 120-139. DOI: 10.1006/viro.2000.0449

Bhat, T., Cao, A. & Yin, J. (2022). Virus-like particles: measures and biological functions. *Viruses*, ***14***, 383. DOI: 10.3390/v14020383

Darling, A. J., Boose, J. A. & Spaltro, J. (1998). Virus assay methods: accuracy and validation. Biologicals : *journal of the International Association of Biological Standardization*, ***26***(2), 105-110. DOI: 10.1006/biol.1998.0134

Granados, R.R. & Naughton, M. (1975). Development of Amsacta moorei entomopoxvirus in ovarian and hemocyte cultures from estigmene acrea larvae. *Intervirology* ***5***, 62-68. DOI: 10.1159/000149881

Guo, F.B. & Yu, X.J. (2007). Re-prediction of protein-coding genes in the genome of Amsacta moorei entomopoxvirus. *J Virol Methods*, ***146***, 389-392. DOI: 10.1016/j.jviromet.2007.07.010

Hall, R. L. & Hink, W. F. (1990). Physical mapping and field inversion gel electrophoresis of Amsacta moorei entomopoxvirus DNA. *Archives of virology*, ***110***(1-2), 77-90. DOI: 10.1007/BF01310704

Haller, S.L., Peng, C., McFadden, G. & Rothenburg, S. (2014). Poxviruses and the evolution of host range and virulence. *Infection, Genetics and Evolution*, ***21***, 15-40. DOI: 10.1016/j.meegid.2013.10.014

Inan, C., Muratoglu, H., Arif, B.M. & Demirbag, Z. (2018). Amsacta moorei entomopoxvirus encodes a functional heparin-binding glycosyltransferase (AMV248). *Virus Genes* ***54***, 438-445. DOI: 10.1007/s11262-018-1561-4

Itakura, Y., Matsuno, K., Ito, A., Gerber, M., Liniger, M., Fujimoto, Y., Tamura, T., Kameyama, K. ichiro, Okamatsu, M., Ruggli, N., Kida, H. & Sakoda, Y. (2020). A cloned classical swine fever virus derived from the vaccine strain GPE− causes cytopathic effect in CPK-NS cells via type-I interferon-dependent necroptosis. *Virus Res.*, ***276***. DOI: 10.1016/j.virusres.2019.197809

Marlow, S.A., Billam, L.J., Palmer, C.P. & King, L.A. (1993). Replication and morphogenesis of Amsacta moorei entomopoxvirus in cultured cells of Estigmene acrea (salt marsh caterpillar). *The Journal of general virology*, ***74***(Pt 7), 1457-1461. DOI: 10.1099/0022-1317-74-7-1457

Moss B. (2006). Poxvirus entry and membrane fusion. *Virology*, ***344***(1), 48-54. DOI: 10.1016/j.virol.2005.09.037

Muratoğlu, H., Nalçacıoğlu, R. & Demirbağ, Z. (2010). Transcriptional and structural analyses of Amsacta moorei entomopoxvirus protein kinase gene (AMV197, pk). *Ann. Microbiol.*, ***60***, 523-530. DOI: 10.1007/s13213-010-0082-8

Palacios, J. del C.A., Barreto, C.A.B., Lara, J.S.M. & Navas, Á.M.L. (2017). Standardization of DNA Residual Quantification Method of Vero Cell Rabies Vaccine for Human Use. *Open Med. Chem. J.*, ***11***, 66-80. DOI: 10.2174/1874104501711010066

Palmer, C.P., Miller, D.P., Marlow, S.A., Wilson, L.E., Lawrie, A.M. & King, L.A. (1995). Genetic modification of an entomopoxvirus: deletion of the spheroidin gene does not affect virus replication in vitro. *The Journal of general virology*, ***76***(Pt 1), 15-23. DOI: 10.1099/0022-1317-76-1-15

Perera, S., Krell, P., Demirbag, Z., Nalçacioğlu, R. & Arif, B. (2013). Induction of apoptosis by the Amsacta moorei entomopoxvirus. *Journal of General Virology* ***94***, 1876-1887. DOI: 10.1099/vir.0.051888-0

Plesa, G., McKenna, P.M., Schnell, M.J. & Eisenlohr, L.C. (2006). Immunogenicity of cytopathic and noncytopathic viral vectors. *Journal of virology*, ***80***(13), 6259-6266. DOI: 10.1128/JVI.00084-06

Reed, L.J. & Muench, H. (1938). A Simple Method of Estimating Fifty Percent Endpoints. *American Journal of Hygiene*, ***27***, 493-497.

Sezen, K. & Demirbağ, Z. (2005). Entomopoksvirüsler ve Biyolojik Kontrol. *Turkish Journal of Parasitology*, ***29***(4), 280-286.

Sliva, K., Erlwein, O., Bittner, A. & Schnierle, B.S. (2004). Murine leukemia virus (MLV) replication monitored with fluorescent proteins. *Virol. J.*, ***1***. DOI: 10.1186/1743-422X-1-14

Fajardo, T. V. M., Vanni, M. F. & Nickel, O. (2017). Absolute quantification of viruses by TaqMan real-time RT-PCR in grapevines. *Ciência Rural*, ***47***(6), 1–5. https://doi.org/10.1590/0103-8478cr20161063

Jiang, S. S., Chen, T. C., Yang, J. Y., Hsiung, C. A., Su, I. J., Liu, Y. L., Chen, P. C. & Juang, J. L. (2004). Sensitive and Quantitative Detection of Severe Acute Respiratory Syndrome Coronavirus Infection by Real-Time Nested Polymerase Chain Reaction. *Clinical Infectious Diseases*, ***38***(2), 293–296. https://doi.org/10.1086/380841

Jiang, Y., Zhang, S., Qin, H., Meng, S., Deng, X., Lin, H., Xin, X., Liang, Y., Chen, B., Cui, Y., Su, Y. H., Liang, P., Zhou, G. Z. & Hu, H. (2021). Establishment of a quantitative RT-PCR detection of SARS-CoV-2 virus. *European Journal of Medical Research*, ***26***(1), 1-7. DOI: 10.1186/s40001-021-00608-5

Keyaerts, E., Vijgen, L., Maes, P., Duson, G., Neyts, J. & Van Ranst, M. (2006). Viral load quantitation of SARS-coronavirus RNA using a one-step real-time RT-PCR. *International Journal of Infectious Diseases*, ***10***(1), 32-37. DOI: 10.1016/j.ijid.2005.02.003

Lo, H. R. & Chao, Y. C. (2004). Rapid titer determination of baculovirus by quantitative real-time polymerase chain reaction. *Biotechnology progress*, ***20***(1), 354-360. DOI: 10.1021/bp034132i

Thomas, M.A., Lichtenstein, D.L., Krajcsi, P. & Wold, W.S. (2007). A real-time PCR method to rapidly titer adenovirus stocks. *Methods in molecular medicine*, ***130***, 185-192. DOI: 10.1385/1-59745-166-5:185