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Monitoring of a virulent turkey hemorrhagic enteritis virus on MDTCRP-19 cells by real time PCR

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A B S T R A C T

Turkey hemorrhagic enteritis virus (THEV) is one of Siadenoviruses associated with splenomegaly, bloody diarrhea and death in turkey poults. MDTCRP-19 cells are appropriate host for THEV propagation. The high yield of THEV nucleic acid was intended in molecular identification and genetic engineering, so RT-PCR hexon gene based was used to monitor THEV-A nucleic acid on MDTCRP-19 cells at two different multiplicity of infection (MOI) 10& 20 infectious virus particle (IVP) for 1-144 h post inoculation (P.I) that aid to detect the suitable time to harvest high yield of viral nucleic acid either from cell associated harvest or cell free harvest. The highest yield of viral nucleic acid was $3.4x10^8$ viral genome copy number (VGCN)/ µL as cell associated harvest at 120 h P.I. with 20 MOI whereas; the maximum viral nucleic acid yield was $8.9x10^7$ VGCN / µL as cell associated harvest at 144 hr P.I. with 10 MOI. We concluded that the maximum yield of hexon gene for molecular biology studies was obtained at 120hr P.I. from cell associated harvest with 20 MOI.

Key words: *Turkey hemorrhagic enteritis virus , real time PCR , MDTCRP-19 cells*

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1. INTRODUCTION

Turkey adenovirus 3(TAdV-3) is also known as Turkey hemorrhagic enteritis virus (THEv).THEV is non-enveloped icosahedra capsid that enclosed non segemented DNA with diameter 70-90 nm (Tolin & Domermuth, 1975, van den Hurk, 1992, Beach et al., 2009). It is one of family Adenoviridae viruses. THEV belongs to the genus Siadenovirus (Adams et al., 2014). From previous studies MDTC-RPl9 cells were the susceptible host for propagation and isolation of virulent and avirulent THE viruses (Nazerian and Fadly, 1982).

In last few decades, sequential events occurred in understanding the HE pathobiology and virus genetics. The THEV diagnosis ways have been changed from traditional methods to the molecular, with higher sensitivity and specificity. (Dhama et al., 2017). The availability of viral nucleic acid might help in several molecular studies sequencing, restriction enzyme mapping, infectious clone preparation etc. Real time quantitve PCR(qPCR) allows sensitive , specific and reproducible quantification of viral nucleic acids as, qPCR is able to measure the generated product during each cycle of PCR process which directly proportional to the amount of template prior to the start of the PCR process(Arya et al ,2005). As adenovirus (AdV) hexon constitutes the major virus capsid protein. The epitopes located on the hexon protein are targets of neutralizing antibodies in vivo, serve in the recognition by cytotoxic T cells, and provide the basis for the classification of adenoviruses as hexon protein contain surface specific protein encoded by hypervariable regions of hexon gene so, hexon gene might be important for classification and recognition of individual serotype (Ebner et al., 2005). So qPCR assay was used to detect high yield of hexon gene of THEV-A on MDTCRP-19cells that might help in molecular studies on Turkey hemorrhagic enteritis virus.

2. MATERIALS AND METHODS

2.1. *Cell culture and virus:*

MDTCRP-19 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA).These cells are Marek's disease virus-induced B-lymphoblastoid cell line of turkey origin (Nazerian et al., 1982). They were grown in suspension cultures at 41°C, 5% CO2 in 1:1 McCoy's 5A / Lebovitz L-15 medium supplemented with 10% Fetal Bovine Serum (FBS), 20% chicken serum (ChS), 5% tryptose phosphate broth (TPB), and 1% penicillin (pen)/ streptomycin (strep) solution. They were used for induction of THEV-A infection at 10 and 20 MOI. The inoculated MDCTRP-19 cells were maintained in 1:1 McCoy's 5A / Lebovitz L-15 medium supplemented with 2.5% FBS, 20% ChS, 1.2% TPB, and 1% pen/strep solution (SRLM).

Avirulent turkey hemorrhagic enteritis virus (THEV-A) was obtained from Dr. Peirson at center for one health research Virginia-Maryland College of Veterinary Medicine, Virginia Tech. THEV-A was obtained as lyophilized; cell cultured-generated vaccine of *(Avian Adenovirus Type 2, Pheasant strain)* which is commercially available by ADENOMUNE® II Ceva company.

2.2. THEV-A infection on MDTCRP-19 cells

A virulent THEV was inoculated in 5×10^5 cell suspension/ mL of MDTCRP-19 cell line with 10, 20 infectious virus particle (IVP) as multiplicity of infection (M.O.I.) .The inoculated cell suspension was distributed in 24 multi well tissue culture plate, where each well contain 1mL of inoculated cell suspension then the plate was incubated at 41°C and 5% CO2. The inoculated MDTCRP-19 cell line were harvested at different time intervals began with 1hr and ended at144 h P.I. The cell culture media were harvested to represent cell free virus and cell pellets as cell associated virus. The cell medium was separated by centrifuging the cell suspension at 3000 RPM for 5 min and frozen at - 20°C, while the cell pellet was washed three times with Serum reduced Leibovitz McCoy's (SRLM) media to remove unbound virus particles, Cell pellets were suspended in 1mL of medium and frozen at -20°C.

2.3. *Preparation of cell free and cell associated harvest for titration as infectious genome copy number:*

According to Mahsoub et al., (2017) MDTCRP-19 cells were suspended as (5×10^5cell/mL) in 1.5 ml micro centrifuge tubes. Cells in triplicate were then individually inoculated with 100 μL dose of each cell free and cell associated harvest at different time

interval. The inoculated cells were then transferred into the wells of a round-bottom 96-well culture plate and incubated at 41°C for 1 h (time sufficient for virus entry). Post-incubation cells were transferred from the wells of culture plate to 1.5 mL micro centrifuge tubes and pelleted by centrifugation at 3000 rpm for 7 min at 4°C.Cells were then washed three times with 200 μL aliquots of SRLM media to remove unbound virus particles and free-floating viral DNA. Cell pellets were then resuspended in 200 ul of PBS and stored at −20°C until DNA extraction. The total DNA was extracted from infected MDTCRP-19 cells using QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. The DNA was eluted in 50 μL of DNase/RNase-free distilled water. The qPCR assay was performed on extracted DNA using 1 μL per reaction.

2.4. Preparation of qPCR standards:

The recombinant PCR2.1 TOPO plasmid that contain hexon gene of THEV was obtained from Dr. Mahsoub (Mahsoub et al., 2017). .The recombinant plasmid was used in qPCR standards Preparation. Depending on Staroscik, 2004 plasmid copy number was calculated using this formula: pCR2.1-hex copies/ μL = (plasmid concentration [ng/ μL] × 6.022 × 1023)/ (nucleotide length × 1 × 109× 650). Different dilution from 10 till 10^6 copy number/ul was done from the original stock and small aliquots were made and stored at−80°C for future use in creation of standard curves.

2.5 Primer design and Oligonucleotide synthesis for qPCR:

The oligonucleotide primers for qPCR were designed based on the published sequence of hexon gene of the Virginia a virulent strain of THEV (GenBank accession number AY849321). Forward and reverse primers were able to generate 115-bp amplicon for the qPCR assay. Their sequences and positions with in the THEV genome are shown in Table (1). They were designed using Primer3 software and manufactured by Sigma-Aldrich.

2.6. SYBR green based Real-time PCR assay (qPCR):

The qPCR reaction was prepared in final volume 25ul reaction; each reaction contained 0.5 μL of each of forward and reverse primers at 10 μM, 10.5 μL DNase/RNase-free distilled water, 1 μL of DNA template and12.5 μL of 2 x SYBR Green I master mix (SensiMix TMSYBR®& Fluorescein Kit; Bioline, Taunton, MA) in Eppendorf TM real-time PCR Tube Strips (white color, 8 tubes/ strip) with Master clear TM Cap Strips (Fisher Scientific). The DNA amplification and data analysis were carried out using I Cycler® thermal cycler instrument and its software (Bio-Rad, Hercules, CA).Using these cycling conditions of 95°C for 10 min for enzyme activation, and 40 cycles of 95°C as denaturation temperature for 15 sec, 56°C for 15 sec as annealing temperature and 72°C for 15 sec as extension temperature. The melting curve analysis was applied under these conditions 1 cycle of denaturation at 95°C for 1 min, 1 cycle of annealing at 55°C for 1 min, and 80 cycles of temperature increment at a rate of 0.5°C/10 sec starting at 55°C to verify the specificity of the amplified products (Mahsoub et al., 2017).in addition to; Non-template control (NTC; water only) reaction was also included as a negative control to detect reagent contamination and primer-dimer formation.

3. RESULTS

3.1. *Analytical sensitivity of qPCR standards:*
qPCR standards analytical sensitivity was as shown in fig (1)

3.2. *The yield of THEV-A hexon gene monitoring on MDTCRP-19 cells:*
The viral nucleic acid yield of cell-associated and cell free harvests at input of 20 IVP per cell is shown in Fig (2). Approximately $4x10^4$ to $2x10^5$VGCN/ μL were found in both the fluid-phase and cell-associated harvests at 1 hr PI. Through the following hours, the viral nucleic acid yield gradually decreased.

The viral nucleic acid yield was rapidly increased in cell-associated harvest between 18- 24hr P.I. and continued to raise almost exponentially until 120 h P.I. with maximum yield $3.4x10^8$VGCN / µL .whereas ; the cell associated viral nucleic acid yield at 144 h P.I. was $2.3x10^8$ VGCN / µL. On the other side the viral nucleic acid yield of cell free virus harvest was exponentially increased at 18-24 h P.I. with growing until 72 h P.I. with maximum viral nucleic acid yield $3x10^6$ VGCN / µL at 144 h P.I.

The results of inoculation of MDTCRP-19 cells with 10 MOI are shown in Fig. 3. Both cell associated and cell free viral nucleic acid yield was gradually decreased until 18 h P.I. followed by exponential increment with maximum at144hr P.I. $8.9x10^7$ VGCN/ µL in cell associated harvest. In the other aspect; the cell free yield around 18-24 h P.I. gradually increased, with the plateau occurring by approximately72h P.I. This peak was tailed with decline in viral nucleic acid yield at 96h P.I. until reaching to the maximum at 144h P.I. 5.5x 10^6 VGCN/ µL.

Table (1): Nucleotide sequences of the primers used in the standard and real-time PCR assays.

Real time PCR primers	Sequence 5` to 3 `	Position	TM	Product size (bp)
THEVqPCRhexF	GGCATGGGCAACTATCCTAA	14726	63.6 ˚ C	
THEVqPCRhexR	GAACACTGCCAAAACCCATC	14840	64.3˚C	115

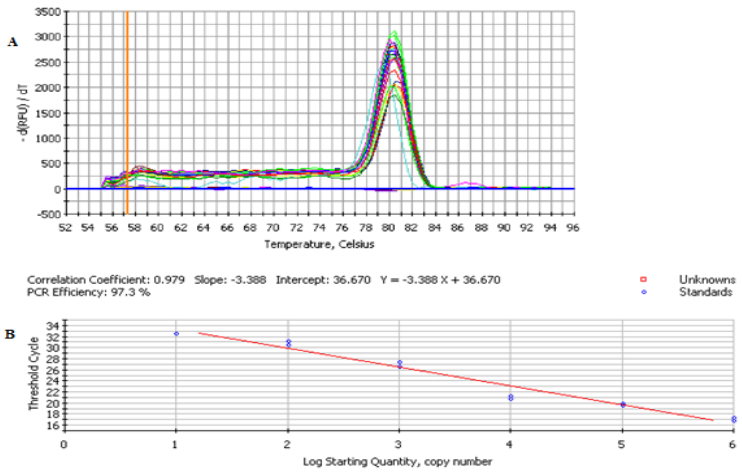

Figure (1): Determination of the analytical sensitivity of TAd-3 virus real time PCR based on 10 fold dilution series of recombinant plasmid. A) Melting curve B) standard curve Spreadsheet Data for SYBR-490 for different dilution of standard samples in duplicate.

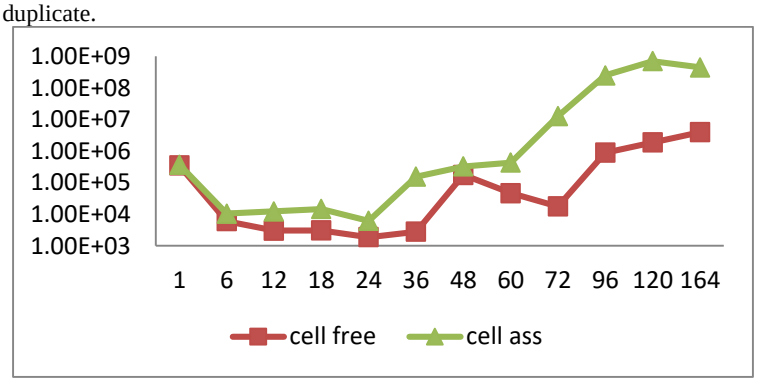

Figure (2): THEV-A based hexon nucleic acid yield monitoring at different time interval with 20 MOI.

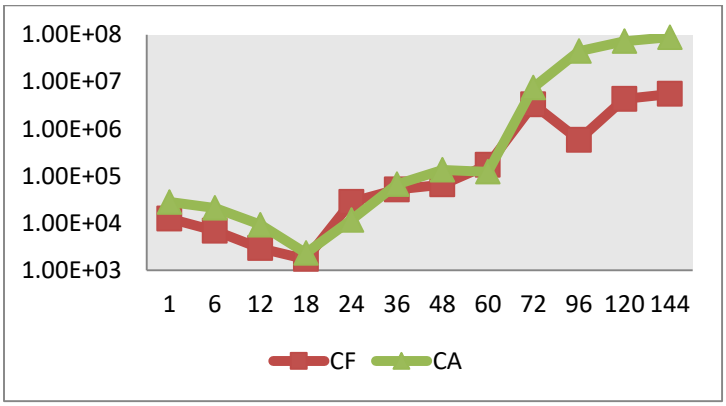

Figure (3): THEV-A based hexon nucleic acid yield monitoring at different time interval with 10 MOI.

4. DISCUSION

Hemorrhagic enteritis virus (HEV), an adenovirus associated with acute hemorrhagic gastro-intestinal disease of 6–11-week old turkeys causing depression, intestinal damage and hemorrhages followed by death of infected birds. THEV has immunosuppressive nature, opening the gate to secondary bacterial infections in affected flocks (Pierson & Fitzgerald, 2013). THEV is fastidious to grow in cell culture system. There are a lot of difficulties were encountered the in vitro replication of THEV because of narrow range of susceptible cells. The MDTC-RPl9 cells were the susceptible host for propagation and isolation of virulent and avirulent HE viruses (Nazerian and Fadly, 1982).

qPCR is a routinely method to detect and quantify many different viruses as hepatitis B virus (Qian et al ,2005) and ORF V(Gallina et al , 2006). There are many advantages of this approach are rapidity as the entire process requires only approximately 3 h , simplicity, the use of common reagents available in most molecular biology laboratories (Espy et al., 2000; Liu et al.,2013; Mohamed et al., 2013; Niesters, 2001). This study provides information on viral nucleic acid replication pattern in cultures of MDRP-19 cells. As, quantitative PCR (qPCR) is used to detect, characterize and quantify nucleic acids for numerous applications. The qPCR was used to observe THEV-A nucleic acid on MDCTRP-19 at different interval of inoculation. Newly developed quantitive PCR method were used for assessing the THEV-A nucleic acid in relation to tissue culture infectivity assay as infectious viral genome copy number (IVGCN). Comparing with other biological titration assay the qPCR is rapid and reproducible as the viral nucleic acid can be detected after 1h P.I., and this is correlated with biological activity as the only biologically active virion is counted as part of their titer determination (Gallaher and Breck, 2013, Mahsoub et al., 2017).

To our knowledge ours is the only THEV-A nucleic acid monitoring on MDTC-RP-19 cells. It was demonstrated that THEV-A replicating successfully in MDCTRP-19 cells .the maximum THEV-A nucleic acid yield was achieved by 120 h P. I. with 20 MOI and 144 h P. I. with 10 MOI. The THEV-A infection curves were exhibited that the yield cell associated harvest nucleic acid was rather higher than the cell free one. As TAV-3 replicate successfully in MDTCRP-19 cells and forming CPE in MDTCRP-19 cells (Nazerian and Fadly, 1982) The result of these study were contributed to that was reported by Van den Hurk, 1990, where the virus particle were observed in the nuclei of infected cells at 18-24hr after infection of the turkey blood leukocyte cells using electron microscopy and mostly of produced avirulent THEV were cell associated, as THEV-A replicate in the nucleus.

As SYBR Green I real-time PCR depend on fluorescent signal detection. The need of a specific probe eliminated in SYBR Green I based assay. It does not require the design of a separate probe, which can be complex and expensive comparing with TaqMan probe method. Moreover, the SYBR Green I method provides information regarding the amplification of the PCR reaction in the form of the melting curve. Using the melting curve and Tm value, could assess whether the product of the reaction is the intended target. Lastly, the SYBR Green I method also eliminates problems related to probe contamination, in which substandard quality or weak fluorescence signals cause false positive results, or mismatch of the probe with the template, where the lack of a fluorescent signal or low detection rate leads to false negative results (Gallina et al., 2006, Wang et al.,2017). In conclusion, our results demonstrate qPCR based on SYBR green allows sensitive and accurate assessment of THEV-A copy number at THEV-A DNA level as the high yield of THE-V nucleic acid based on hexon gene is 120h P.I. with 20MOI and 144h P.I. with 10 MOI.

6. REFERANCES

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