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Real-time PCR-based infectivity assay for the titration of turkey hemorrhagic enteritis virus, an adenovirus, in live vaccines

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A B S T R A C T

The current *in vitro* titration method for turkey hemorrhagic enteritis virus (THEV) is the end-point dilution assay (EPD) in suspension cell culture (CC). This assay is subjective and results in high variability among vaccine lots. In this study, a new *in vitro* infectivity method combining a SYBR Green I-based qPCR assay and CC was developed for titration of live hemorrhagic enteritis (HE) CC vaccines. The qPCR was used to determine the virus genome copy number (vGCN) of the internalized virus particles following inoculation of susceptible RP19 cells with 1 vaccine label dose. The measured vGCN represents the number of infectious viral particles (IVP) per 1 dose. This method was used to compare 9 vaccine lots from 3 companies in the United States. Significant lot-to-lot variations within the same company and among the various companies were found in genomic and qPCR-based infectious titer per label dose. A positive linear relationship was found between qPCR infectious titer and genomic titer. Further, considerable variations in $CCID_{50}$ titers were found among tested vaccine lots, indicating the high variability of the current titration methods. The new method provides an alternative to classical titration assays and can help reduce variation among HE vaccine products.

1. Introduction

Turkey hemorrhagic enteritis virus (THEV) is the etiologic agent of the hemorrhagic enteritis (HE) disease in turkeys. This virus, also known as turkey adenovirus 3, belongs to the family *Adenoviridae*, genus *Siadenovirus*, species Turkey adenovirus A (Davison and Harrach, 2011). Disease caused by virulent strains of THEV can be prevented by vaccination of 4½-6 weeks-of-age turkey poults with naturally occurring avirulent THEV strains (Domermuth et al., 1977; Larsen et al., 1985; Newberry et al., 1993). USDA-licensed live vaccine products propagated in cell culture (CC) are available to prevent HE. Vaccination of poults with 100 CCID per bird of CC-propagated HE vaccine is sufficient for protection against disease (Fadly and Nazerian, 1984).

Splenomegaly and a transient period of immunosuppression is caused by both virulent and avirulent (vaccine) strains of THEV in susceptible birds (Pierson and Fitzgerald, 2008; van den Hurk et al., 1994). The virus targets the spleen where it mainly replicates in B lymphocytes and induces their apoptosis. This is followed by a reduction in the numbers of IgM-bearing B cells in the spleen and peripheral blood at days 2, 3, 4, and 9 after infection, which temporarily suppresses the birds immune system and makes them vulnerable to potential secondary infections (*e.g.*, *E.*

Abbreviations: CC, cell culture; $CCID_{50}$, cell-culture infectious dose 50; CLM, complete Leibovitz's L15-McCoy's 5A media; CPE, cytopathic effect; Ct, threshold cycle; EPD, end-point dilution assay; HE, hemorrhagic enteritis disease; CC HE, cell-culture hemorrhagic enteritis vaccine; IVP, infectious viral particle(s); LOQ, limit of quantification; %CV, percentage coefficient of variation; qPCR, quantitative (real-time) polymerase chain reaction; SRLM, serum-reduced Leibovitz's L15-McCoy's 5A media; $TCID_{50}$, tissue-culture infectious dose 50; THEV, turkey hemorrhagic enteritis virus; vGCN, viral genome copy number.

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coli) that can lead to high mortality (up to 60%) (Pierson et al., 1996; Rautenschlein et al., 2000; Saunders et al., 1993; Suresh and Sharma, 1995, 1996). Poults inoculated with increasing doses of HE vaccine showed a dose-dependent increase in their relative spleen weight (Fadly and Nazerian, 1984). This indicates that the level of immunosuppression in vaccinated birds may also be dose-dependent. Therefore, for efficacious use of HE vaccines without a deleterious immune response, methods for determining an accurate titer per label dose are necessary.

Currently, two EPD assays are available for use to determine THEV infectivity titers in live vaccines. The first is an *in vivo* titration in 5–6 week old turkeys that are THEV-seronegative (Domermuth et al., 1977). This method is labor-intensive and relatively expensive to perform as a routine titration method. Birds are inoculated with serial dilutions of infectious material and assessed for splenic enlargement/mottling 3–5 days later depending on the route of inoculation. This method is used to calculate the "poult infectious dose". The second method is an *in vitro* endpoint dilution in MDTC-RP19, B-lymphoblastoid cells (RP19 cells) (Nazerian et al., 1982; Sharma, 1994). This method relies solely on visual evaluation of the cytopathic effect (CPE) observed for infected cells (*i.e.*, enlargement and ballooning of cells), the only overt manifestation of infection and viral replication. Because RP19 cells grow in suspension and determining CPE is subjective, the sensitivity and efficiency of this method is lower than those employing monolayer cultures (Nazerian and Fadly, 1982; Smither et al., 2013). Modified protocols that incorporate immunostaining to confirm the CPE scores in infected RP19 cells were developed, but they add to the cost and time necessary to complete the assay (Nazerian and Fadly, 1987; Rautenschlein and Sharma, 1999; van den Hurk, 1990). Other standard *in vitro* titration methods (*e.g.*, plaque formation and focus formation assays) cannot be applied to THEV because there are no susceptible adherent cell lines (Nazerian and Fadly, 1987). As with other traditional titration methods, these assays are laborious, require a long post-inoculation incubation period, suffer from low-throughput, poor reproducibility and sensitivity (Forsey et al., 1992; LaBarre and Lowy, 2001; Mena et al., 2003). The inherent inaccuracy associated with these traditional titration methods potentially results in poor field performance of vaccines.

Since current methods are not available to accurately titrate THEV, there is a need for a quantitative virus titration method. In recent years a new trend has emerged for quantification of infectious virus particles by infectivity methods that integrate qPCR technology, both SYBR Green and TaqMan, with cell culture systems. The use of such methods throughout the multiple phases of vaccine development and assessment has been recently reviewed (Wolf et al., 2007), highlighting their importance relative to current vaccine production technologies. qPCR-based potency estimation and infectivity titration have been successfully employed for a variety of human and animal DNA and RNA viruses including orf virus (genus: *Parapoxvirus*, family: *Poxviridae*), adeno-associated viruses (*Dependoparvovirus*, *Parvoviridae*), camelpox and buffalopox viruses (*Orthopoxvirus*, *Poxviridae*), rotaviruses (*Rotavirus*, *Reoviridae*), measles virus (*Morbillivirus*, *Paramyxoviridae*), mumps virus (*Rubulavirus*, *Paramyxoviridae*), rubella virus (*Rubivirus*, *Togaviridae*), and adenoviruses (*Mastadenovirus*, *Adenoviridae*) (Ammour et al., 2013; Bora et al., 2012; Mayginnes et al., 2006; Prabhu et al., 2012; Ranheim et al., 2006; Rohr et al., 2005; Wang et al., 2005). The aim of the present study was to establish a rapid and quantitative method for the infectivity titration of THEV in live vaccines using a combination of RP19 cell culture and qPCR assay.

2. Materials and methods

2.1. Cells and growth conditions

MDTC-RP19 (RP19), a B-lymphoblastoid cell line established from a Marek's disease virus-induced tumor in turkeys (Nazerian et al., 1982), was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and used in the infection experiments. The cells were grown as suspension cultures in 1:1 complete Leibovitz's L15-McCoy's 5A (CLM) media, supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 20% chicken serum (CS; Sigma-Aldrich), 5% tryptose phosphate broth (TPB; Millipore, Solon, OH), and penicillin [100 I.U./ml] and streptomycin [100 μg/ml] (ATCC), at 41 °C in a humidified atmosphere with 5% CO_2. When infected with THEV, the cells were maintained in 1:1 serum-reduced Leibovitz's L15-McCoy's 5A (SRLM) media, supplemented with 2.5% FBS, 5% CS, 1.2% TPB, and penicillin [100 I.U./ml] and streptomycin [100 μg/ml].

2.2. Primer design and synthesis

Oligonucleotide primers were designed based on the published sequence of the hexon (*hex*) gene of the Virginia avirulent strain of THEV (GenBank accession number, AY849321). Primers were designed using Primer3 software (v. 0.4.0) (Koressaar and Remm, 2007; Untergasser et al., 2012), to generate a 445-bp amplicon for the conventional PCR and a 115-bp amplicon for the qPCR assays described below. Primer sequences and positions within the THEV genome are shown in Table 1. The primers were synthesized by Sigma-Aldrich (St. Louis, MO). The preliminary specificity of the qPCR primer set was assessed by using the basic local alignment search tool (Altschul et al., 1990) and then through melting curve analysis. The qPCR primers THEVqPCRhexF (G + C content 50%; 3′ complementarity = 1; 20 bp) and THEVqPCRhexR (G + C content 50%; 3′ complementarity = 0; 20 bp) were specific to THEV *hex* after running pairwise alignment against the available GenBank nucleotide database using BLASTn. The BLASTn report showed a calculated E-value of 4e-04; 100% identity; and 100% query coverage for both primers with the THEV *hex* accessions available on GenBank nucleotide database. No significant sequence similarity was found with the turkey genome, or other viral or bacterial nucleic

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

Name	Sequence (5′–3′)	Position[a]	Tm (°C)	Amplicon size (bp)
Conventional PCR primers				
THEVhexfragF (forward)	GCGGATTTGATGAGTAGGAATC	14594	59.9	445
THEVhexfragR (reverse)	TATAACGCCCGCCAATATGT	15038	60.2	
Real-time PCR primers				
THEVqPCRhexF (forward)	GGCATGGGCAACTATCCTAA	14726	59.9	115
THEVqPCRhexR (reverse)	GAACACTGCCAAAACCCATC	14840	60.4	

Tm, melting temperature. bp, base pair.

[a] Oligonucleotide primers were designed based on the published sequence of the hexon gene of the Virginia avirulent strain of THEV (GenBank accession number AY849321). Positions of primers are relative to the published genome sequence. Primers were designed using the online software Primer3 (http://frodo.wi.mit.edu/primer3/) and were manufactured by Sigma-Aldrich.