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Evaluation and validation of reference gene stability during Marek's disease virus (MDV) infection

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A B S T R A C T

Quantitative RT-PCR (qRT-PCR) is widely used in the study of relative gene expression in general, and has been used in the field of Marek's disease (MD) research to measure transcriptional responses to infection and/or vaccination. Studies in the past have either employed cellular β-actin (BACT) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal reference genes, although the stability of their expression in the context of Marek's disease virus (MDV) infection has never been investigated. In the present study, we compared the stability of five reference genes (BACT, 28S RNA, 18S RNA, GAPDH, Peptidyl-prolyl-isomerase B [PPIB], a.k.a. cyclophilin B) as standard internal controls in chicken embryo fibroblast (CEFs) cultures infected with either MD vaccine or oncogenic MDV1 viruses. We further extend these analyses to reference gene stability in spleen lymphomas induced by infection of commercial broiler chickens with a very virulent plus MDV1 (vv+ TK-2a virus). Two excel based algorithms, (Bestkeeper and Normfinder) were employed to compare reference gene stability. Bestkeeper and Normfinder analysis of reference gene stability in virus- and mock-infected cells, showed that 28S RNA and PPIB displayed higher stability in CEF infections with either oncogenic or vaccine viruses. In addition, both Bestkeeper and Normfinder determined 28S RNA and PPIB to be the most stably-expressed reference genes *in vivo* in vv+ TK-2a-induced spleen lymphomas. Furthermore, Bestkeeper and Normfinder analyses both determined BACT to be the least stable reference gene during MDV infection of CEF with oncogenic viruses, vaccine viruses, as well as in vv+ TK-2a-induced spleen lymphomas.

1. Introduction

Quantitative reverse-transcription primed PCR (qRT-PCR) is widely used for the quantification of cellular mRNA expression, as well as viral RNA replication (Mackay et al., 2002). qRT-PCR offers several advantages over other methodologies for the accurate quantification of mRNA levels in terms of sensitivity, dynamic range, and capacity for multiplexing (Huggett et al., 2005). On the other hand, the sensitivity and robustness of qRT-PCR can be affected by RNA quality and quantity, the presence of inhibitors that co-purify with RNA, the efficiency of the reverse transcription reaction, and the accuracy of pipetting (Bustin and Nolan, 2004). In addition, poor assay design, experimental conditions, and inappropriate normalization strategies compromise the integrity of the resulting data (Bustin, 2010). Various methods of normalization have been proposed, such as normalization to sample size (cell number), total RNA, and the use of standard internal reference (housekeeping) gene expression (Huggett et al., 2005; Talaat et al., 2002).

Normalization to cell number is not possible if the experimental sample is a whole tissue. Normalization to total RNA can be affected by variation in extraction efficiencies, with the final yields being quite low in either quantity or quality. In addition, normalization to total RNA does not take into account the variation in the efficiency of reverse transcription or PCR amplification (Stahlberg et al., 2004). For qPCR, normalization to total DNA poses an additional drawback, due to the presence of multiple haplotypes or genome integrations in tumor cells (Huggett et al., 2005; Kaufer and Flamand, 2014; Talaat et al., 2002) and the presence of multiple copies of particular loci in replicating bacteria in comparison to non-replicating bacteria (Rocha, 2004). Normalization to internal control reference genes has been the most popular and reliable method of normalization, due to the fact that it considers and precludes the error due to initial RNA/cDNA loading, as well as the variation in the efficiency of

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reverse transcription reaction (Huggett et al., 2005). Normalization to the geometric mean of multiple reference genes has also been suggested as an accurate way of normalization, although it is not always possible to use multiple reference genes due to limitations in sample availability, increased expense, and the possible variability in one of the selected reference genes. This method, however, is considered to be the most rigorous method of normalization.

The concept of using reference genes for normalization is based on the assumption that the expression of these genes remains constant under any experimental condition, although this may not be the case in reality. Hence, fluctuations in the expression of reference genes selected as internal controls produce erroneous biological results, leading to the misinterpretation of data (Thellin et al., 1999). Based on the published literature, expression of various reference genes varies among different tissues, under different experimental conditions, at various developmental stages, or during various physiological or disease states (Dheda et al., 2004). Therefore, identification and validation of ideal reference genes with constant or stable expression levels under the given experimental condition is important to produce biologically-relevant data (Schmittgen and Zakrajsek, 2000).

qRT-PCR has been widely used to define cellular responses to Marek's disease virus (MDV) infection or vaccination, and also to elucidate mechanisms of MDV pathogenesis and tumor development (Abdul-Careem et al., 2008a,b,c, 2007, 2009; Garcia-Camacho et al., 2003; Kaiser et al., 2003; Lian et al., 2012; Morgan et al., 2001). Additionally, qRT-PCR has also been used for the relative or absolute quantification of viral genome loads or viremia levels, in an attempt to correlate the amount of lytic or latent virus to various factors associated with the outcome of disease, such as host genotype, vaccination status, cellular or viral gene expression, and viral shedding via feather follicular epithelium (Abdul-Careem et al., 2006; Baigent et al., 2005; Gimeno et al., 2011; Islam et al., 2006). Chicken embryo fibroblast (CEF) cultures or fibroblast-derived cell lines (DF1, OU-2, SOgE) have been used for the propagation of MDVs, and for the study of MDV-encoded genes (Hunt et al., 2001; Levy et al., 2005; Parcells et al., 1994; Schumacher et al., 2002).

Given the highly cell-associated nature of MDV, infection with MDV is associated with global expression changes in both mRNA and ribosomal RNA levels (Morgan et al., 2001). Target gene normalization based on varying housekeeping gene can therefore produce erroneous results. Hence, it is important to validate the stability of a housekeeping gene, to ensure that the selected reference gene is unaffected by MDV infection both in cell culture and *in vivo*.

In the current study, we have evaluated the relative stability of five commonly-used reference genes: chicken β-actin (BACT), 28S RNA, 18S RNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and peptidyl-prolyl-isomerase B (PPIB) as standard internal controls by assessing consistency of their expression in MDV-infected CEF cultures and in MDV-induced splenic lymphomas.

2. Materials and methods

2.1. Cells and viruses

For the propagation of MDV oncogenic and vaccine viruses, secondary chicken embryo fibroblasts (CEF) were prepared from 10-day-old specific pathogen free (SPF) embryos (Sunrise Farms, Inc. Catskill, NY). Secondary CEF were propagated in M199 medium supplemented with 3% filtered calf serum, L-glutamine, 1X PSN (Penicillin/Streptomycin/Neomycin) and 1X fungizone (Life Technologies, Carlsbad, CA) and maintained at 37 °C in 5% CO_2. CEF cultures were infected with 5000 PFU of each virus, in triplicate, and harvested upon the appearance of plaques at five days post-infection. The MDV1 strains used were: CU2 (a mildly-virulent MDV, obtained originally from Dr. K.A. Schat, Cornell University), RB-1B (a vvMDV, originally obtained from Dr. K.A. Schat), rMd5 (originally obtained from Dr. Sanjay Reddy, Texas A & M University), and T KING (TK-2a, a vv + MDV, originally obtained from Dr. John K. Rosenberger, University of Delaware) (Tavlarides-Hontz et al., 2009). Vaccine viruses used were commercially-produced HVT, SB-1, and CVI-988, all provided by Merial, Inc., Gainesville, GA.

2.2. In vivo *lymphoma isolation*

Solid lymphoma masses were isolated from unvaccinated commercial broiler chickens (Hubbard × Cobb) infected via contact with the vv + MDV (TK-2a strain)-inoculated shedder chickens during a vaccine efficacy study. The bird experiment described here followed a natural MDV infection model known as "shedder model" that has already been described elsewhere (Tavlarides-Hontz et al., 2009). Briefly, one-day-old commercial broiler chickens, grouped as "shedders" were infected intra-abdominally with 200 PFU of TK-2a-infected CEF, and were placed on wood shaving-based litter in a multi-room chicken house equipped with independent heating, ventilation, feeders, and waterers. Two weeks post-placement of shedders, one-day-old unvaccinated and vaccinated contact chickens were placed in contact with infected shedders. Frank lymphomas were obtained from spleens (n = 4) during necropsy of the contact-exposed chickens at the end of the seventh week post-placement, and were excised from the surrounding non-lymphomatous spleen tissue. In addition, as an infected positive control, phenotypically non-lymphomatous adjacent tissue sections (n = 4) were also isolated from the lymphoma-containing spleens.

To serve as negative controls, healthy normal spleens (n = 3) were isolated from unvaccinated and unchallenged chickens, housed separately. Spleen samples were collected into RNA later (Ambion Inc., Austin, TX) and stored at −80 °C for further RNA purification. The animal experiment from which these samples were obtained was conducted in accordance to Ag Animal Care and Use Committee (AACUC) guidelines of University of Delaware registered as Vaccine Trial protocol: (22) 04-15-10a.

2.3. qRT-PCR analysis

Total RNA was isolated from mock- or MDV-infected cells and spleen tissues using Qiagen RNA/DNA/Protein Kit according to the manufacturer's instructions (Qiagen, USA). Total RNA quality (260/280 ratio) and quantity (at 260 nm absorbance) were measured using an Agilent Nanodrop spectrophotometer. RNA samples with OD 260/280 and OD 260/230 > 2 only were selected for further experimentation. For each sample, 1 μg of total RNA was reverse transcribed with random hexamers using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), as recommended by the manufacturer's protocol (Step 1: 25 °C for 10 min; Step 2: 37 °C for 120 min; Step 3: 85 °C for 5 min; Step 4: 4 °C storage).

The final cDNAs were diluted 10 fold with nuclease-free water and 1 μl of the final diluted cDNA was used in a 20 μl reaction composed of 10 μl iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA), 8.2 μl of nuclease free water, and 250 nM of each forward and reverse primer. Quantitative Real Time PCR (qRT-PCR) was performed using SYBR green chemistry, as recommended by the manufacturer (Bio-rad Laboratories) on the MyiQ2 Two Color Real Time PCR Detection System (Bio-rad Laboratories, Hercules, CA). Following amplification and collection of raw fluorescence data, melt curve analysis was performed to exclude the possibility of