Short communication

Assessment of reference genes for reliable analysis of gene transcription by RT-qPCR in ovine leukocytes

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A B S T R A C T

With the availability of genetic sequencing data, quantitative reverse transcription PCR (RT-qPCR) is increasingly being used for the quantification of gene transcription across species. Too often there is little regard to the selection of reference genes and the impact that a poor choice has on data interpretation. Indeed, RT-qPCR provides a snapshot of relative gene transcription at a given time-point, and hence is highly dependent on the stability of the transcription of the reference gene(s). Using ovine efferent lymph cells and peripheral blood mono-nuclear cells (PBMCs), the two most frequently used leukocytes in immunological studies, we have compared the stability of transcription of the most commonly used ovine reference genes: *YWHAZ*, *RPL-13A*, *PGK1*, *B2M*, *GAPDH*, *HPRT*, *SDHA* and *ACTB*. Using established algorithms for reference gene normalization "geNorm" and "Norm Finder", *PGK1*, *GAPDH* and *YWHAZ* were deemed the most stably transcribed genes for efferent leukocytes and *PGK1*, *YWHAZ* and *SDHA* were optimal in PBMCs. These genes should therefore be considered for accurate and reproducible RT-qPCR data analysis of gene transcription in sheep.

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1. Introduction

The advent of next-generation sequencing has led to a vast increase in available genomic and transcriptomic data, which combined with the paucity of available reagents for analysis of protein expression in most veterinary species, has led to an increased use of quantitative reverse transcription PCR (RT-qPCR) for gene transcription profiling (Bustin et al., 2005, 2009). Despite well-documented shortcomings of this technique associated with major differences in mRNA quantity, quality and optimal assay design (Nolan et al., 2006), RT-qPCR is becoming the method of choice for RNA quantification. Measuring gene transcription levels in different tissues and cell types requires accurate normalization, most often achieved using an internal reference gene. Ideal reference genes should exhibit a relatively consistent transcription profile in different cell cycle stages and experimental conditions, as failing to do this will result in erroneous interpretation of the results (Vandesompele et al., 2002).

Large animals in general, and sheep in particular, are increasingly being used in immunological research (Davenport et al., 2014; Mahakapuge et al., 2015; Neeland et al., 2014; Scheerlinck et al., 2008). In sheep, PBMCs and lymph are the most commonly used sources of leukocytes, as they are readily and repetitively accessible and arguably represent the level of immune activity within the animal. Using pre-femoral efferent lymphatic cells and PBMCs from ten physiologically normal sheep we compared the transcriptional stability of reference genes with or without Con-A mediated T-cell activation, to determine the most appropriate reference genes in sheep leukocytes.

To identify the most consistently transcribed reference genes among the selected reference genes, we used statistical algorithms, geNorm version 3.5 (Center for Medical Genetics, Ghent University Hospital, Belgium) (Vandesompele et al., 2002) and Norm Finder (Version 20) (Andersen et al., 2004) according to developers' recommendations. GeNorm software identifies the most consistently transcribed reference genes in a set of samples tested using average pairwise variation. The outcome is expressed as an "*M*-value", which can be defined as the average pairwise variation of a particular reference gene compared to all other reference genes. The ideal reference gene for a particular tissue used in different experimental conditions should be transcribed equally in all the samples selected (i.e. minimum *M*-value). Additionally, the software also calculates pairwise variations, providing a geNorm *V*-value, which reflects levels of variation in average reference gene stability with the addition of $n+1$th reference gene (Vandesompele et al., 2002). Similarly,

the Norm Finder algorithm also identifies optimal normalization genes amongst a given set of candidates based on overall transcription variation (Andersen et al., 2004). The software provides a stability value for each gene (as a direct measure of gene transcription variation) (Andersen et al., 2004). We used eight reference genes and 10 samples for identification of the most consistently transcribed reference genes.

2. Materials and methods

2.1. Animals

Ten mature merino ewes (between 6 months and 2 years of age) were housed in the Faculty of Veterinary and Agricultural Sciences Animal Facility, The University of Melbourne. They were kept in raised pens and fed with lucerne chaff and commercial pellets while water was provided *ad libitum*. All experimental procedures were approved by the Animal Ethics Committee, The University of Melbourne.

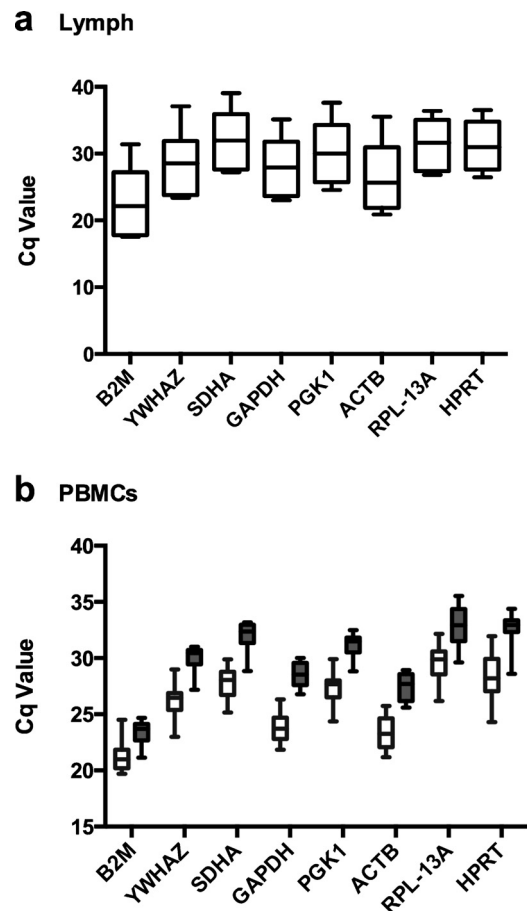### 2.2. Efferent lymphatic cannulation and lymph collection

Surgery was carried out under general gas anesthesia (Isoflurane, Henry Schein Company, Melville, USA). Contralateral efferent pre-femoral lymphatic ducts were cannulated as described elsewhere (Windon et al., 2000). Lymph samples from 10 sheep were collected seven days after the surgery. Lymph was centrifuged at 400g for 5 min after which the cellular fraction was immersed in 1 mL of RNAlater® (Ambion, USA) and kept at 4 °C for 24 h; cells were then transferred to −20 °C for long-term storage.

2.3. Collection of PBMCs from sheep

Thirty mL of blood was collected from the jugular vein of 10 clinically normal sheep into heparinized (100 μL of 5000 IU/mL) tubes. PBMCs were isolated as previously described (Scheerlinck et al., 2006). PBMCs were re-suspended in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FCS (Invitrogen, Carlsbad, CA), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine (Invitrogen, Carlsbad, CA) and 50 μM 2-β-Mercaptoethanol (Sigma-Aldrich, Sydney, Australia). In a volume of 0.2 mL, 2×10^6 cells were cultured for 72 h in the presence or absence of 5 μg/mL Concanavalin A (Con A; Pharmacia, Uppsala, Sweden). After 72 h the cells were harvested, pelleted and re-suspended in 1 mL of RNAlater® and stored for 24 h at 4 °C, after which they were transferred to −20 °C until required.

Fig. 1. C_q values of each gene in efferent lymphatic cellular fraction (a) and in PBMCs (b). Clear box and whisker plots in graph b show C_q values of PBMCs after 72 h Con A stimulation and solid box and whisker plots show C_q values of PBMCs without T cell stimulant.

2.4. RNA extraction and cDNA synthesis

Total RNA was isolated using RNeasy Mini kit (Qiagen). RNA quantification was performed using a NanoDropND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). RNA quality was assessed on a 1% non-denaturing agarose gel in Trisborate-EDTA buffer. Complementary DNA (cDNA) was synthesized from 100 ng of total RNA using QuantiTect® reverse transcription

Table 1
Reference genes and primer sequences used for qRT-PCR.

Gene symbol	Gene name	GenBank accession number	Forward (F) and reverse (R) primer sequence (5′–3′)	Amplicon size (bp)	Amplification efficiency (E)
ACTB	Beta-actin	NM_001009784.1	F CAAAGACCTCTACGCCAACAC R GACTCGTCGTACTCCTGCTTG	225	1.98
B2M	Beta 2 microglobulin	NM_001009284.2	F TTCTGTCCCACGCTGAGTTC R GGTGCTGCTTAGAGGTCTGC	118	2.05
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_001190390.1	F TCCGGGAAGCTGTGGCGTGA R GGGATGACCTTGCCCACGGC	92	2.09
HPRT	Hypoxanthine-guanine phosphoribosyltransferase 1	XM_004022693.1	F TCCAGTTTCGCTAATGACACA R AGGACCCCTCGAAGTGTTG	138	2.10
PGK1	Phosphoglycerate kinase 1	NM_001142516.1	F TCTGCCAAAGAAGGCTGGAG R AAAGGCCATTCCACCACCAA	187	2.09
RPL-13A	60S ribosomal protein L13a	XM_004015371.1	F TGTCGTAAGTGTGCTGTGGA R CACAACCTTCCGACCCAGAA	147	1.97
SDHA	Succinate dehydrogenase complex subunit A, flavoprotein	XM_004017097.1	F CCGAAGCAGGTTTCAACACG R TCACGGTGTCGTAGAAGTGC	141	2.02
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein,zeta polypeptide	NM_001267887.1	F GACTGGGTCTGGCCCTTAAC R TCCGATGTCCACAATGTCAAGT	193	2.07