Validation of suitable reference genes for quantitative expression analysis by qPCR in bovine terminal ileum and ileocecal valve

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Summary
The use of reference genes is commonly accepted as the most reliable approach to normalize qRT-PCR and to reduce possible errors in the quantification of gene expression. The aim of this study was to identify a set of reference genes suitable for gene expression analysis in the distal portion of small intestine and ileocecal valve in cattle. These sites of intestine are of interest in veterinary science as they are the main sites of inflammation caused by *Mycobacterium avium* subsp. *paratuberculosis*, agent of paratuberculosis. We employed ten PCR assays for commonly used reference genes belonging to various functional classes and then determined their expression stability. The most stable genes were *RPL13A* and *HMBS*, followed by *TFRC* and *B-ACT*. NormFinder analysis provided similar results with *B-ACT* as the best reference gene, followed by *RPL13A* and *TFRC*. This validated gene panel may be useful for studies on paratuberculosis aiming to identify genes differentially expressed by qRT-PCR.

Introduction

Quantitative PCR (qPCR) is the election technique for accurate expression profiles determination of selected gene of interest being characterized by high sensitivity, specificity and reproducibility (Bustin *et al.* 2009). Nevertheless, several variables can concur to misleading conclusions, which include biological and technical variance in the gene expression analysis: the amount and quality of the starting material, the RNA integrity, the efficiency of retrotranscription and PCR reaction, the differences in biological samples. Moreover, the presence of pseudogenes, alternative splicing, health status, storage conditions of the samples may affect the level of gene expression and the PCR efficiency (Rekawiecki *et al.* 2012). The selection of suitable reference genes is crucial to mitigate any variations arising during the experiment, because they are assumed to be characterized by constitutive and uniform expression level in all the analyzed samples, regardless of tissue differentiation, treatments or experimental design (McNeill *et al.* 2007). However, several studies indicate that the expression of reference genes varies in different tissues (Lisowski *et al.* 2008) and should be evaluated a priori to avoid biased findings (Bas *et al.* 2004). Accordingly, a proper evaluation of several reference genes should be performed to validate which and how many genes are needed before any gene expression study (Bustin *et al.* 2009, Huggett *et al.* 2005). The use of a single reference gene is strongly discourage and the use of genes traditionally considered stable has to be carefully considered. Vandesompele and colleagues (Vandesompele *et al.* 2002) demonstrated that the use of a single reference gene can imply an error of up to 20-fold in expression data. Additionally, the expression of genes assumed to be stable (i.e., *GAPDH* and *B-ACT*) can vary considerably (Bustin 2000).

The aim of this study was to identify a set of reference genes suitable for gene expression analysis in the distal portion of small intestine and ileocecal valve in cattle. These sites of intestine are of interest in veterinary science as they are the main sites of inflammation caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), the agent of paratuberculosis, a chronic and debilitating

granulomatous enteritis of ruminants, characterized by persistent diarrhea, progressive wasting, and eventual death (Coussens 2001, Dorshorst *et al.* 2006, Fanelli *et al.* 2020).

Materials and methods

Samples collection, RNA extraction and cDNA synthesis

Bovine tissue samples were collected during routine slaughtering procedures from eight Holstein Friesian cows, at least 2 years old. The distal tract of small intestine and the ileocecal valve were immediately sampled from each animal, rinsed in sterile PBS and preserved in RNAlater (Qiagen). All samples were stored at - 80 °C before RNA extraction.

Total RNA was extracted from 50 mg of bovine intestine tissue in RNAlater with the RNeasy Lipid Tissue Mini kit (Qiagen), following the manufacturer's instructions and performing the optional on-column DNase digestion with the RNase-Free DNase Set (Qiagen). Concentration and purity of the RNA were determined by spectrophotometer and fluorescent measurements, while RNA integrity was evaluated using the Bioanalyzer 2100 (Agilent Technologies). cDNA synthesis was carried out starting from 1 µg of RNA using the QuantiTect Reverse Transcription kit (Qiagen).

Reference genes, primer design and qPCR

Ten genes usually used as references in qPCR experiments were selected as candidate normalizers (Table I). They were tested on a serial 10-fold-dilutions of pooled cDNA to determine primer efficiency, slope value and correlation coefficient. cDNAs of the eight samples were pooled and 10-fold diluted until 1:100,000 then a five-point calibration curve was constructed for each gene. The qPCR reactions were performed in 25 µl-total volume containing: 1X iTaq Universal SYBR Green Supermix (Biorad), 11 µl of Nuclease free water, 200 nM of each primer and 1 µl of cDNA. The reactions were set up in triplicates and loaded on a Mx3005P qPCR System (Agilent Technologies) with the following thermal cycle: 95 °C for 5 min; 44 cycles of 95 °C for 30 sec, 56 °C for 45 sec and 72 °C for 30 sec; a melting curve analysis of 95 °C for 1 min and 56 °C for 30 sec. Amplification directly from genomic DNA and no-template control were included to recognize /

Table I. *Details of candidate reference genes assays.*

Gene symbol	Gene name	Function	Accession No.	Reference	Primer Sequence	Amplicon size
B-ACT	Beta-actine	Cytoskeletal structural protein	AY141970.1	Liu *et al.* 2016	F:GCACAATGAAGATCAAGATCATC R:CTAACAGTCCGCCTAGAAGCA	173
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Glycolytic enzyme, Oxidoreductase in glycolysis and gluconeogenesis	U85042.1	Reist *et al.* 2003	F: GTCTTCACTACCATGGAGAAGG R: TCATGGATGACCTTGGCCAG	197
HMBS	Hydroxymethyl-bilane synthase	Heme biosynthesis	NM_001046207.1	Lecchia *et al.* 2012	F: GAGAGGAATGAAGTGGACCTAG R: GCATCATAGGGGCTCTCCC	110
PGK1	Phosphoglycerate kinase 1	Glycolytic enzyme, polymerase α cofactor protein	NM_001034299.1	Modesto *et al.* 2013	F: GGAAGGGAAGGGAAAAGATGC R: TCCCCTAGCTTGGAAAGTGA	92
PPIA	Peptidylprolyl isomarase A (cyclophilin A)	Accelerate the folding of proteins	NM_178320.2	De Maria *et al.* 2010	F: GCCCCAACACAAATGGTTCC R: CCCTCTTTCACCTTGCCAAAG	95
RPL13A	Ribosomial protein L13A	Member of ribosome proteins	NM_001076998.2	Modesto *et al.* 2013	F: CCCTGGAGGAGAAGAGAAAGG R: AATTTTCTTCTCGATGTTCTTTTCG	104
RPS9	Ribosomial protein S9	Member of ribosome proteins	DT860044.1	Janovick-Guretzky *et al.* 2007	F: CCTCGACCAAGAGCTGAAG R: CCTCCAGACCTCACGTTTGTTC	62
SF3A1	Splicing factor 3 subunit 1	Structural component of the splicing system	NM_001081510.1	Lecchia *et al.* 2012	F: CCTTACCATGCCTACTACCGG R: CACTTGGGCTTGAACCTTCTG	144
TFRC	Transferrin receptor	Transferrin receptor	NM_001206577.1	Modesto *et al.* 2013	F: TGGAAAAATCAGTTTTGCTGAA R: GTCCAAAAACTGGAAGATTTGC	124
YWHAZ	Tyrosine 3-monooxygenase	Signal transduction by binding to phosphorylated serine residues on a variety of signaling molecules	NM_174814.2	Modesto *et al.* 2013	F: CTGAACTCCCCTGAGAAAGC R: CTGCTTCAGCTTCGTCTCCT	165

to exclude genomic DNA contamination. Stability of the ten candidate reference genes was evaluated by algorithms geNorm and NormFinder available in the GenEx software (bioMCC). GeNorm sequentially eliminates the gene that shows the highest variation relative to all other genes, generating an M-value that should be lower than 1.5 (Vandesompele *et al.* 2002). Specifically, the lower is the M-value, the more stable is the gene. NormFinder compares the individual genes to a global average expression of all the genes in all samples, estimating a standard deviation (SD) for each reference gene. It also calculates the accumulated SD by using multiple reference genes: in this case the random variation among their expression is partially cancelled by SD reduction. Plotting the SD from different number of reference genes according to their stability, allows to identify a minimum in the accumulated SD, indicating the number of reference genes giving the lowest SD.

Results

The concentration of the RNA extracted from the eight samples by fluorimeter analysis ranged from 550 ng/µl to 1,000 ng/µl. RNA purity was assessed as A260/280 ratio (range: 1.5-2.0) and A260/230 ratio (range: 1.0-1.7). RNA integrity was checked by determining RIN values, which were between 6.6 and 7.9.

qPCR experiments showed that all the reference geneassays were expressed in the eight samples and provided a single sharp peak in the melting curve profile corresponding to an amplicon of expected size on agarose gel. Only the melting curve for *SF3A1* gene assay showed a secondary peak for the last three dilution points, due to primer dimer formation.

Correlation coefficients and efficiencies for all the standard curves were higher than 0.97 and above 90%, respectively. Cq values for the reference genes varied from 18 to 33.

Data analysis with geNorm ranked the reference genes according to their M-values in decreasing order (Figure 1, Table II). All the genes reached M-values less than 1.5 except for *YWHAZ* and *GAPDH*. Most of the genes showed stable expression with M-values less than 1. For adequate data analysis, reference genes with M-values less than 1 should be use for a comparison of minor differences in gene expression (Hellemans *et al.* 2007). The most stable genes were *RPL13A* and *HMBS*, followed by *TFRC* and *B-ACT*, while the less stable were *GAPDH* and *YWHAZ*. NormFinder analysis provided similar results with *B-ACT* as the best reference gene, followed by *RPL13A* and *TFRC*; *YWHAZ* and *GAPDH* showed the highest SD values (Figure 2). NormFinder showed that the lowest number of reference genes for the best evaluation of gene expression is three: in fact, the accumulated SD from *B-ACT*, *RPL13A* and *TFRC* was 0.4446 and its increment with the addition of the fourth gene *PGK1* was negligible (Figure 3).

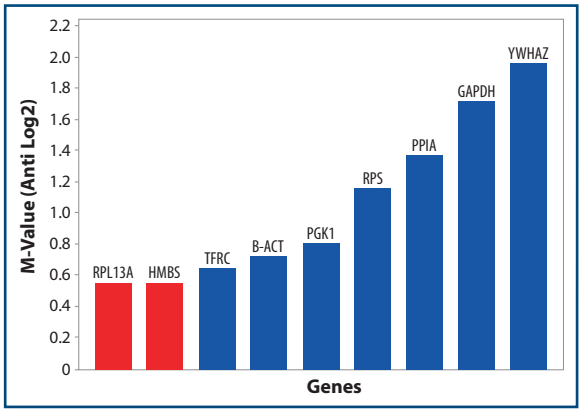

Figure 1. *Ranking of candidate reference genes according to their expression stability by geNorm after stepwise exclusion of the worst scoring genes.*

Discussion

RNA yields and quality were adequate for qPCR and similar to results obtained by other studies on bovine intestinal RNA (Weber *et al.* 2016, Lecchi *et al.* 2012). A good quality RNA is fundamental for gene expression analysis to avoid wrong conclusion, but it could be a problem for matrices such as intestine. It is very important the preservation of the tissue immediately after the sampling: for this reason, samples are often immediately frozen in liquid nitrogen (Hempel *et al.* 2016, De Luca *et al.* 2014, Weber *et al.* 2016). The samples for this study could not be snap-frozen, but RNA was preserved by immediately adding RNAlater solution that avoids RNA degradation with acceptable quality and yield for qPCR, as it was done also by Lecchi and colleagues (Lecchi *et al.* 2016).

Table II. *Gene expression stability measures determined by geNorm for each candidate reference gene.*

Gene name	M-value
YWHAZ	1.959
GAPDH	1.712
PPIA (cyclophilin A)	1.364
RPS9	1.153
PGK1	0.799
B-ACT	0.714
TFRC	0.643
HMBS	0.552
RPL13A	0.552

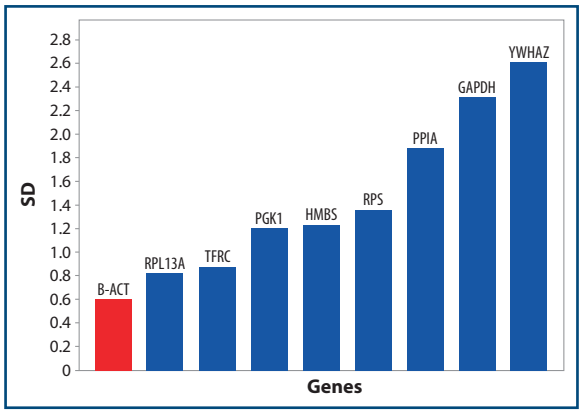

Figure 2. *Ranking of candidate reference genes according to their expression stability by NormFinder.*

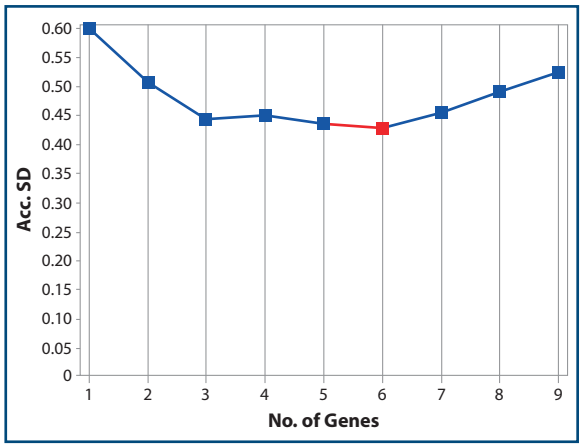

Figure 3. *NormFinder: accumulated standard deviation (SD) for the determination of the optimal number of reference genes.*

For the set up of optimal qPCR conditions, the ten reference genes were tested on pooled cDNA samples and genomic DNA. The selected primer pairs spanned two exons, in order to generate specific melt-curve profile for cDNA and genomic DNA amplifications and allow the identification of possible DNA contamination in the samples. The presence of a single peak on the melt-curve profile and of a single band of the expected size on agarose gel electrophoresis confirmed the gene specific amplifications (Peletto *et al.* 2011).

The identification of a list of appropriate reference genes is basilar for a proper determination of gene expression, to reduce the variability introduced during the different steps of the experiment (Sahu *et al.* 2018). The ideal reference geneshould show a stable expression in the tissue under different experimental conditions, so it is necessary to identify and validate reference genes for each type of sample and for each condition (Bustin *et al.* 2009). Genes such as *GAPDH*, *B-ACT* and *18S rRNA* have been used as reference genes in a great number of studies in the last decades without validation, but their expression can vary considerably, influencing the validity of the results (Thelling *et al.* 1999, Vandesompele *et al.* 2002). NormFinder identified *B-ACT*, *RPL13A* and *TFRC* as the best reference genes for qPCR analyses in the terminal tract and ileocecal valve of the bovine small intestine, while for geNorm *RPL13A* and *HMBS*, followed by *TFRC* and *B-ACT*, were the most stable (Figures 2 and 3). Both the software gave similar results, with small differences in the ranking of the reference genes, due to the different algorithms used to calculate variability. Therefore, the comparison of reference gene rankings from different software/algorithms is advisable for robust results (Modesto *et al.* 2012).

The results of this study partially confirmed the conclusions by Lecchi and colleagues (Lecchi *et al.* 2012), regarding the evaluation of suitable reference genes in a collection of 33 different bovine tissues comprising small intestine, cecum and colon. They analyzed the expression of seven genes, and *SF3A1*, *HMBS*, *B-ACT* were the three most stable. This study differed from the previous because it focused on a specific portion of the small intestine, near the ileocecal valve, and on the ileocecal valve itself. Our results confirmed *B-ACT* and *HMBS* as optimal reference genes; *SF3A1* was also included in our gene panel, but it was discarded for the presence of an unspecific peak in the melt-curve profile of the higher dilution points, probably due to primer dimer formation. This result is apparently in contrast with the results by Lecchi and colleagues (Lecchi *et al.* 2016), but in that study the standard curve for the qPCR analysis was performed with a 4-fold serial dilution, while, in our study, 10-fold serial dilutions were used. This technical difference in the experiment setup results in Cqs > 32 at the last dilution points, and the *SF3A1* expression is probably low in the studied intestinal sites, thus resulting in unspecific amplification products at the highest dilutions. So *SF3A1* is not helpful as reference gene for qPCR analysis of genes with a low level of expression in bovine terminal ileum tract and ileocecal valve.

Our analysis demonstrated that *YWHAZ* and *GAPDH* are not suitable for optimal normalization, but they were instead acceptable for Lecchi and colleagues (Lecchi *et al.* 2012). It is important to take into consideration that these conclusions derived by the analysis of the stability of reference genes on 33 different bovine tissues together and are not focused on a particular one, as in this work. Considering the gastrointestinal tract, *GAPDH* resulted stable in abomasums, duodenum, jejunum and cecum of lactating and not lactating cows (Connor *et al.* 2010): it could be considered a good reference gene for gene expression analysis in intestinal tissues, but this conclusion is not true for

the ileocecal tract. So, these evidences highlighted the importance of stability analysis of the reference genes, in order to take in account all the possible biological variation related to the expression level of genes for normalization.

In this study, the analysis on the selected samples identified other two stable genes, *TFRC* and *RPL13A*. These genes are not frequently used as reference genes for gene expression analysis in bovine samples and there is few information in literature. Both of them were chosen for stability analysis in bovine neutrophils: *RPL13A* showed a stable expression in these cells (Crookenden *et al.* 2017), while *TFRC* was discarded from subsequent analyses because of a high standard deviation (Vorachek *et al.* 2013). In a recent gene expression study aiming to identify putative biomarkers in MAP-infected cattle, *TFRC* resulted up-regulated in bovine whole-blood, suggesting caution in the use of this gene as reference gene. Also, the use of *RPL13A* had to be carefully considered, because rRNA gene expression may not be a good estimation of the total mRNA (Vandesompele *et al.* 2002), so the exclusive use of rRNA genes as reference genes should be avoided. Analyses of expression stability on somatic cells from goat milk (Modesto *et al.* 2013) and ovine whole-blood (Peletto *et al.* 2011) defined *TFRC* and *RPL13A* as the least reliable controls. In contrast, the high stability of *TFRC* and *RPL13A* was demonstrated in spleen, liver, cecum and cecal tonsil of turkeys, independently from the infection by *Histomonas meleagridis* (Mitra *et al.* 2016).

As reported by several studies, the expression stability of genes to be used as reference genes needs to be carefully validated to ensure reliable data, and the use of software such as geNorm and NormFinder could help in the selection of suitable genes for each specific sample type/experimental condition. An universal reference gene did not exist and changing sampling site or experimental condition could lead to different conclusions: actually, not all the reference genes previously identified in the bovine small intestine were confirmed as stable in the sampling sites of our study. On the basis of our results, *B-ACT*, *RPL13A*, *TFRC* and *HMBS* are stable reference genes for normalization of gene expression data in the terminal tract of the bovine small intestine and ileocecal valve. This validated gene panel may be useful for studies on paratuberculosis aiming to identify differentially expressed genes by quantitative PCR.

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