Animal Science Papers and Reports vol. 38 (2020) no. 3, 225-234
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Stability of reference genes in ruminants' milk somatic cells – a review

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(Accepted March 27, 2019)

To gain insight into lactation physiology it is particularly important to know which genes control milk synthesis and its composition, as well as how these genes are regulated. Milk somatic cells are a potential source of RNA and may be used as an alternative starting material in gene expression studies instead of mammary gland biopsy or *post mortem* sample collection. The key stage in any experiment with the use of the quantitative reverse transcription polymerase chain reaction (RT-qPCR) consists in the selection of appropriate reference genes, characterised by a constant level of expression in the examined biological material in order to avoid errors and to interpret the obtained results correctly. In this review the stability of reference genes has been discussed based on various studies concerning gene expression profiling in somatic cells contained in ruminant milk. Moreover, bioinformatic tools applied to determine the stability of reference genes have been described.

KEY WORDS: internal control genes / gene expression / somatic cells / milk

To understand cellular and tissue functions at the molecular level it is essential to know which genes are activated in different cells and tissues by diverse factors. A variety of methods are applied to identify differences in gene expression, including microarray analysis and quantitative reverse transcription polymerase chain reaction (RT-qPCR). Microarray analysis allows scientists to quickly and efficiently determine expression levels of hundreds or thousands of specific genes within a cell in a single experiment. As it is quite costly the number of samples is limited. Comparing

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to microarray, RT-qPCR is less expensive [Narrandes and XU 2018] and thus is commonly used for smaller numbers of genes analysed in greater numbers of samples, while generating reliable, reproducible and biologically meaningful results. Moreover, it is used to validate results obtained from the microarray technique [Thomas *et al.* 2014]. RT-qPCR is one of the most commonly used techniques for gene expression profiling in various organs and tissues, including the mammary gland in ruminants in the course of lactation [Bionaz and Loor 2007].

However, the application of the RT-qPCR technique is burdened with certain difficulties, such as different amounts of starting material between samples, differences in RNA integrity or reverse transcriptase efficiency [Chervoneva *et al*. 2010]. In order to obtain reliable results, despite these difficulties, it is necessary to normalise the reaction involving reference genes (ICG - Internal Control Genes) [Ullmannova and Haskovec 2003]. The normalisation is based on a comparison between expression of the investigated genes of interest (GOI) and the ICG expression. This parallel analysis constitutes the point of reference indicating which expression is relatively constant under identical experimental conditions [Modesto *et al*. 2013]. Currently it is the most effective and one of the simplest methods to correct errors arising in the course of experiments concerning gene expression profiling [Kozera and Rapacz 2013]. Selection of the right genes to normalise expression in RT-qPCR is essential if the results are expected to reflect natural biological processes [Robinson *et al*. 2007].

In studies of gene expression in the mammary gland, tissue biopsy is usually used as a starting material. Unfortunately, this is an invasive and expensive procedure hindering such studies. Boutinaud *et al*. [2002], Feng *et al*. [2007] and Jacobs *et al*. [2012] have shown that gene expression in milk somatic cells (MSC) is closely correlated with gene expression in the mammary gland during lactation. It can therefore be concluded that MSC may be used as an alternative starting material to the biopsy of mammary gland tissue for gene expression profiling. So far, MSC have been used in the studies on gene expression in cattle [Bhatt *et al*. 2012, Jacobs *et al*. 2012, Varshney *et al*. 2012, Verbecke *et al*. 2015, Karthikeyan *et al*. 2016], sheep [Bonnefont *et al*. 2011], goats [Pisoni *et al*. 2010, Cremonesi *et al*. 2012, Modesto *et al*. 2013, Jarczak *et al*. 2014, Pławińska-Czarnak *et al*. 2019] and yaks [Bai *et al*. 2014].

The aim of this review is to discuss the stability of internal control gene expression, essential in gene profiling studies in ruminants' milk somatic cells using RT-qPCR.

The Internal Control Gene expression in ruminants' milk somatic cells

An optimal ICG should represent a level of expression that is not influenced by any experimental conditions. Its expression should also be constant in analysed samples and should have a similar threshold cycle to the GOI [Chervoneva *et al*. 2010]. Most often these internal controls are housekeeping genes, which participate in processes necessary for basic cell metabolism and are typically expressed at a constant and unregulated level, therefore they are considered reliable. However, numerous studies

have shown that expression of these genes varies at different experimental conditions [Kozera and Rapacz 2013]. Consequently it is crucial to identify and validate which housekeeping gene should be used for normalisation in a particular experiment. Thus, the choice of ICG must be made prior to the testing of GOI expression, while at the same time ICG must be tested in the same way with the use of the RT-qPCR technique. The genes should be selected individually for each experiment. Two mistakes can be made during the experiment design: either selection of ICG without prior validation or testing only one candidate gene. It is strongly advised to select a minimum of three genes to calculate a normalisation factor [Vandesompele *et al*. 2002]. The final number of ICG should be adapted to a particular experiment depending on its complexity.

Feng *et al*. [2007] developed an optimal method of RNA isolation from MSC to study the expression of the stearoyl-CoA desaturase (*SCD*) gene in the mammary gland in cattle. In this paper the influence of temperature and storage time of MSC on the quantity and quality of the isolated material were also investigated. RT-qPCR was applied to determine the expression level of the *SCD* gene in relation to actin beta (*ACTB*) ICG. This was confirmed by an experiment carried out by Jacobs *et al*. [2012]. The aim of the study was to evaluate the use of MSC as a source of RNA for the analysis of *SCD* gene expression in cow's mammary gland. The expression of GOI two isoforms (*SCD1* and *SCD5*) and two candidate ICG (keratin 8 - *KRT8* and *ACTB*) was measured in MSC and in tissue collected by biopsy. In both cases *KRT8* was the most stable ICG.

Some studies have been carried out on gene expression in MSC in subsequent lactation stages. Varshney *et al*. [2012] evaluated the stability of nine ICGs in MSC in zebu (*Bos indicus*). Samples were collected from Sahiwal cows (N=18) in three lactation phases: 1st (25±5 days; N=6), 2nd (160±15 days; N=6) and 3rd (275±25 days; N=6). Among the candidate ICGs, the following combination proved to be most stable: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), protein phosphatase 1 regulatory inhibitor subunit 11 (*PPP1R11*), *ACTB*, ubiquitin C (*UBC*). In turn, Bai *et al*. [2014] carried out a similar experiment in MSC in yaks (*Bos grunniens*). Ten ICGs were evaluated during lactation. Milk samples (N=6) were collected at 3±1, 28±4, 80±7, 150±7 and 180±7 days after calving. The most stable was the combination of the ribosomal protein S9 (*RPS9*), *PPP1R11*, ubiquitously expressed prefoldin like chaperone (*UXT*) and the mitochondrial ribosomal protein L39 (*MRPL39*) genes. Jarczak *et al*. [2014] analysed optimal ICG in MSC and in whole blood of healthy (N=13) and CAEV infected (N=13) goats. Samples were collected at 7, 30, 100 and 240 days after kidding. In goat's MSC the most stable ICG were peptidylprolyl isomerase A (*PPIA*) and ribosomal protein lateral stalk subunit P0 (*RPLP0*). In addition, Pławińska-Czarnak *et al*. [2019] demonstrated that *RPLP0* was the most stable for milk fat globules, while it was 18S ribosomal RNA (*RN18S1*) for MSC in samples collected 70 days after parturition from two goat breeds: Polish White Improved and Polish Fawn Improved.

In order to understand immune response to pathogens, research has been conducted based on experimental infection with bacteria causing *mastitis* in ruminants.

Bonnefont *et al*. [2011] carried out transcriptional profiling using a specific ovine microarray in sheep of two lines: susceptible and resistant to experimental provocation by *Staphylococcus epidermidis* and *Staphylococcus aureus*. Data obtained in microarray analysis were validated by RT-qPCR with the use of ICG ribosomal protein L9 (*RPL9*), hypoxanthine phosphoribosyltransferase (*HPRT*), succinate dehydrogenase complex flavoprotein subunit (*SDHA*) and *GAPDH*. Cremonesi *et al*. [2012] tested an early immune response to controlled *S. aureus* infection with the use of MSC and whole blood from goats as the RNA source. Microarray profiling was verified by RT-qPCR. Ribosomal protein L13a (*RPL13A*) turned out to be the most stable out of the five ICGs. Likewise, Modesto *et al*. [2013] determined the optimal combination of ICG in MSC of healthy and *S. aureus* infected goats. The authors tested ten commonly used ICG and two GOI. Stability unaffected by changes in conditions of induced infection was shown for a set of the following genes: glucose-6-phosphate dehydrogenase (*G6PD*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*) and *ACTB*. In addition, Pisoni *et al*. [2010] carried out gene expression profiling in MSC from the mammary gland infected with *S. aureus* in goats using specific bovine microarrays. The reference point for GOI in RT-qPCR was *GAPDH*. Similarly, Tao and Mallard [2007] conducted a study, in which *S. aureus* infection had been induced and followed by a microarray analysis of the immune-endocrine pathway in cows with mastitis. The MSC and blood mononuclear cells were used as the source of RNA. The *GAPDH* was used as ICG in RT-qPCR. Verbecke *et al*. [2015] also analysed the influence of experimental intra-mammary provocation in Bovinae. The authors investigated relative expression of the chemokine (C-X-C motif) receptor 1 (*CXCR1*) gene and candidate ICG in heifers with c.980GG genotypes after calving and the influence of this polymorphism (c.980A>G, GenBank: NM_001105038.1) on *CXCR1* expression in MSC. Previous studies showed that this polymorphism is related to immunity against udder inflammation [Leyva-Baca *et al*. 2008]. Ten potential ICGs were assessed in the analysis, the most stable being *UBC*, ribosomal protein S15a (*RPS15A*) and *ACTB*. The *CXCR1* gene and the influence of its polymorphism on immune response to pathogen attack was also studied by Beecher *et al*. [2012]. The aim of their work was to characterise inborn immune response to experimental infection with *Streptococcus dysgalactiae ssp*. *dysgalactiae* in the bovine. The expression of genes involved in the immune response was examined by RT-qPCR and verified with ubiquitin-conjugating enzyme E2 D2 (*UBE2D2*) as ICG. Lee *et al*. [2006] determined the expression profile of selected inflammatory cytokines in MSC from cows experimentally infected with *S. aureus* and *Escherichia coli*, whereas in a study by Alluwaimi and Cullor [2002] it was in MSC in the middle and final lactation phases from experimentally infected cows. Expression of the tested cytokines was normalised based on ICG expression, *ACTB* and *GAPDH*, respectively. Likewise, Bhatt *et al*. [2012] analysed expression of inflammatory cytokines in response to udder inflammation in healthy and diseased Kankrej and Gir cows and in *Bos taurus* x *Bos indicus* hybrids. The relationship between MSC count, total bacteria load and expression level of individual cytokines

Table 1. Candidate internal control genes used in RT-qPCR analysis in ruminants' milk somatic cells

Species	Candidate internal control genes[1]	Reference
Cattle	***GAPDH***, ***PPP1R11***, ***ACTB***, *B2M*, *RPS15A*, *UXT*, *MTG1*, *RN18S1*, ***UBC***	Varshney *et al*. 2012
	ACTB, *B2M, H2A, HPRT1, PPP1R11,* ***RPS15A***, *SDHA, TBP,* ***UBC***, *YWHAZ*	Verbecke *et al*. 2015
	KRT8, *ACTB*	Jacobs *et al*. 2012
	GAPDH, *ACTB*	Bhatt *et al*. 2012
	GAPDH, ***UBQ***	Baumert *et al*. 2009
	GAPDH	Tao and Mallard 2007, Fonseca *et al*. 2009, Alluwaimi and Cullor 2002
	UBB	Karthikeyan *et al*. 2016
	UBC	Pfaffl *et al*. 2003
	ACTB	Lee *et al*. 2006, Feng *et al*. 2007
	UBE2D2	Beecher *et al*. 2012
Goats	***ACTB***, *GAPDH,* ***G6PD***, *PGK1, RN18S1, RPL13A, SDHA,* ***YWHAZ***, *TUBB, TFRC*	Modesto *et al.* 2013
	ACTB, GAPDH, HMBS, ***RPL13A***, *YWHAZ*	Cremonesi *et al*. 2012
	ACTB, GAPDH, ***PPIA***, *RN18S1, UBQLN1,* ***RPLP0***	Jarczak *et al*. 2014
	RPLP0, ***RN18S1***	Pławińska-Czarnak *et al*. 2019
	GAPDH	Pisoni *et al*. 2010
Sheep	***RPL9***, ***HPRT***, ***SDHA***, ***GAPDH***	Bonnefont *et al*. 2011[2]
Yaks	*ACTB, B2M, GAPDH, GTP,* ***MRPL39***, ***PPP1R11***, ***RPS9***, *RPS15,* ***UXT***, *RN18S1*	Bai *et al*. 2014

[1]Bolded abbreviations refer to stable expression of reference genes in milk somatic cells. When more than one ICG were bolded in a row a combination of those genes was used in the analysis.
[2]Seven candidate internal control genes were tested; rejected ICGs were not mentioned.
Gene symbol and gene name (biological function): *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase (carbohydrate metabolism); *PPP1R11* = protein phosphatase 1 regulatory inhibitor subunit 11 (protein phosphatase inhibitor); *ACTB* = actin beta (cytoskeletal structural protein); B2M = beta-2-microglobulin (immune system); RPS15A = ribosomal protein S15a (structural constituent of ribosome); *UXT* = ubiquitously expressed prefoldin like chaperone (gene transcription regulation); *MTG1* = mitochondrial ribosome associated GTPase 1 (regulation of the mitochondrial ribosome); *RN18S1* = 18s ribosomal RNA (cytosolic small ribosomal subunit, translation); *UBC* = ubiquitin C (protein degradation); *H2A* = histone 2 alpha (DNA modification); *HPRT1* = hypoxanthine phosphoribosyltransferase (purine metabolism); *SDHA* = succinate dehydrogenase complex flavoprotein subunit A (Electron transporter in the tricarboxylic acid cycle and respiratory chain); *TBP* = TATA-box binding protein (transcription factor); *YWHAZ* = tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (signal transduction); *KRT8* = keratin 8 (cellular structural integrity); *UBQ* = ubiquitin (polyubiquitin precursor); *UBB* = ubiquitin B (protein binding); *UBE2D2* = ubiquitin-conjugating enzyme E2 D2 (degradation of short-lived and abnormal proteins); *G6PD* = glucose-6-phosphate dehydrogenase (cytosolic enzyme producing NADPH); *PGK1* = phosphoglycerate kinase 1 (glycolytic enzyme, polymerase α cofactor protein); *RPL13A* = ribosomal protein L13a (inflammation processes); *TUBB* = tubulin beta class I (major constituent of microtubules); *TFRC* = transferrin receptor (erythropoiesis and neurologic development); *HMBS* = hydroxymethylbilane synthase (hydroxymethylbilane synthase activity); PPIA = cyclophilin A (acceleration of protein folding); UBQLN1 = ubiquilin 1 (protein degradation mechanisms); RPLP0 = ribosomal protein lateral stalk subunit P0 (ribosomal protein); *RPL9* = ribosomal protein L9 (ribosomal protein); *GTP* = GTPase-like protein (cytoskeletal reorganization, cell polarity, cell cycle progression, gene expression); MRPL39 = mitochondrial ribosomal protein L39 (mitochondrion-specific ribosomal protein); *RPS9* = ribosomal protein S9 (ribosomal protein).

was also investigated. Out of the candidate ICGs the *GAPDH* was selected, while the second potential ICG (*ACTB*) showed an unstable pattern of expression. Baumert *et al*. [2009] evaluated the immunological competence of MSC in cows' stimulated by a lipopolysaccharide derived from *E. coli* under ex-vivo conditions. The vitality and diversity of MSC were determined along with the level of mRNA in the case of several immunomodulating factors. Their expression was normalised in relation to two ICGs: *GAPDH* and ubiquitin (*UBQ*).

Similar studies on the immune response were conducted without experimental induction of the pathogens. Fonseca *et al*. [2009] assessed the relative expression of factors related to the mechanisms of immune response to mastitis in healthy and with clinical mastitis cows of the Holstein and Gir breeds. To analyse this relative expression of immune response genes based on RNA extracted from MSC the authors decided to select *GAPDH* as the ICG of interest. Karthikeyan *et al*. [2016] analysed the pattern of gene expression of innate immune response in hybrid cows (*Bos taurus* x *Bos indicus*) with a sub-clinical form of mastitis in mid-lactation. The authors studied the expression of five GOI in MSC in relation to one ICG, the ubiquitin B (*UBB*) gene. Pfaffl *et al*. [2003] examined the synthesis of selected immunological factors in MSC, blood cells and milk gland tissues of cows with different levels of somatic cell count (SCC). The gene expression was normalised against *UBC* as ICG.

The most frequently used ICGs in gene expression studies in MSC in ruminants have been *GAPDH*, *ACTB*, *UBC* and the protein phosphatase 1 regulatory inhibitor subunit 11 (*PPP1R11*). The candidate ICG used in the analysis of gene expression in ruminants' MSC are presented in Table 1.

The use of a single ICG to normalise expression is currently not considered to be sufficient [Dundas and Ling 2012]. From three to five genes are typically used [Thellin *et al*. 2009, Derveaux *et al*. 2010]. Due to experimental variability, the optimal number and choice of ICG must be confirmed experimentally for specific tissues or cell types under specific experimental conditions [Dundas and Ling 2012]. Since there are no universal ICG, it is very important to carefully select a small set of ICGs with optimal properties. The authors pointed out that in many experiments on gene expression in MSC only one ICG was used, which seems inadequate according to Vandesompele *et al*. [2002], who showed that in expression studies using a single reference gene errors can increase 20-fold, thus negatively affecting mRNA expression and altering the final results.

Bioinformatic tools used to determine Internal Control Gene stability

Currently there are a number of bioinformatic tools applied to analyse data facilitating determination of the most appropriate ICG for specific experimental conditions. These programmes are based on various algorithms used to analyse variability in tested gene expression [Hendriks-Balk *et al*. 2007, Robinson *et al.* 2007, Dundas and Ling 2012]. The most frequently used bioinformatic tools include geNorm [Vandesompele *et al*. 2002], BestKeeper [Pfaffl *et al*. 2004] and NormFinder

[Andersen *et al*. 2004].

The geNorm is a popular programme used for the determination of the most stable ICG from the set of candidate genes under consideration. It was designed as a VBA (Visual Basic Application) for Microsoft Excel. The algorithm determines the expression stability of unverified ICGs on the basis of a gene-stability measure called the M value assigned to each gene. The ICGs under consideration are compared in pairs. Their stability is determined based on the geometric mean of expression for each combination. This allows to organise the tested genes depending on their stability of expression from the most stable (the lowest values of M) to the least stable (the highest M values). With geNorm it is also possible to determine the optimum number of ICGs required for standardisation under specific experimental conditions [Vandesompele *et al*. 2002].

The BestKeeper is a software used to evaluate the stability of expression of both potential ICG and GOI. It is used for a simultaneous analysis of ten ICG and the genes under consideration. This algorithm performs the analysis in several steps based on CT (or CP) values for each gene expression in the RT-qPCR reaction. On its basis the geometric mean, the arithmetic mean, the minimum and maximum values, the standard deviation and the coefficient of variation are calculated for each gene. These parameters are used respectively by the algorithm at each selection stage. The first step is to assess variability of expression for a particular candidate gene. The genes characterised by a very low stability are excluded from further calculations. Then a correlation analysis of pairs is carried out for each pair out of the ten ICG. This is the source for the so-called the BestKeeper Index, an indicator calculated based on the geometric mean of the CT value for the ICG. The next step involves a comparison by pair correlation analysis again, this time comparing the BestKeeper Index values with all the ICGs. The described method is useful when comparing genes with both similar and very different levels of expression [Pfaffl *et al*. 2004].

The NormFinder has also been written as a Visual Basic Application (VBA) for Microsoft Excel. The characteristic feature of the algorithm is the ability to analyse any number of samples organized in any number of groups. This enables an estimation of the general variability for each candidate gene expression, as well as inter-group variability that describes gene expression stability between the groups. The software is based on a linear mathematical model which, on the basis of individual gene behaviour and its influence on other genes determines its stability within the group. As a precondition it is recommended to use a minimum of eight samples per group and at least three candidate genes with similar expression levels. It is also highly recommended to select genes belonging to different functional classes in order to reduce the risk of mutual regulation. The strategy presented above may be applied to assess suitability of any ICG for any type of experimental project [Andersen *et al*. 2004].

So far no ideal tool has been developed that would help to select the most optimal ICG for specific experimental conditions. In order to obtain the best results it is recommended to use at least three ICGs [Bustin *et al*. 2009, Derveaux *et al*. 2010],

three different validation programmes [Kozera and Rapacz 2013] and analyse at least three samples. For each sample three replicates should be made [Kozera and Rapacz 2013]. This is the so-called Best 3 rule.

Conclusions

To select optimal ICGs it is important to remember that the set of suggested reliable ICGs is specific to each individual experiment and may be used if the same experiment is being performed. If something changes in the experimental model, the proposed genes should be tested by means of statistical algorithms to assess their reliability under new experimental conditions. Otherwise, minor changes in expression may be impossible to detect and the final results of the analysis cannot be regarded as meaningful.

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