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Reference genes for livestock gene expression profiling – Literature review

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SUMMARY

Quantitative real-time polymerase chain reaction (qPCR) is an essential tool for understanding animal cell's response to developmental progression or to different experimental conditions at gene expression level. However the reliability of this method heavily lies on proper normalization (measuring a target and a reference gene's expression from the same sample to correct for technical related variations).

Our literature review aimed to summarize the articles addressing the most important livestock species in regards of reference gene stability used as normalizers for quantitative real-time polymerase chain reaction experiments. Stably expressing reference genes were categorized into 14 distinct groups according to gene function. The number of reference genes tested and the publication numbers according to years and the ranking algorithms were also noted.

Counting showed that genes encoding ribosomal protein components are ranked as most stable in majority of cases and therefore should be taking into account for qPCR stable normalizer gene finding experiments.

Keywords: *gene expression, normalization, quantitative real-time polymerase chain reaction*

ÖSSZEFOGLALÁS

A kvantitatív valós-idejű polimeráz láncreakció (qPCR) elengedhetetlen eszköze a génexpressziós kísérleteknek, ezekkel közelebb kerülhetünk a gének, az állat fejlődése, vagy bizonyos kísérleti körülményekre adott válaszának megértésében. A módszer megbízhatósága nagyban függ azonban a megfelelő normalizációtól (a célgénünk és egy ún. referencia gén expressziójának mérése egyazon mintából, a technikai variációk minimalizálása érdekében).

Az irodalmi összefoglalónk célja volt összegezni azokat a publikációkat, melyek a legfontosabb haszonállatokkal végzett kísérletekben stabil referencia gén keresésre irányultak. A stabilan kifejeződő géneket 14 eltérő kategóriába soroltuk azok funkciója alapján. A referencia gének stabilitásával foglalkozó cikkek számát az publikálás éveinek függvényében, a rangsoroló algoritmusokat, valamint a tesztelt referencia gének számát az egyes publikációkban szintén megvizsgáltuk.

Eredményeink alapján a fehérjeszintézisben szerepet játszó riboszóma alegységeket kódoló gének nagyobb arányban rangsorolódnak stabilnak a többi kategóriával szemben ezért ajánlott lehet minden esetben stabilitás vizsgálatuk a qPCR előkísérletekben.

Kulcsszavak: *gén expresszió, normalizáció, kvantitatív valós-idejű polimeráz láncreakció*

INTRODUCTION

Quantitative real-time polymerase chain reaction (qPCR) is an essential tool for understanding animal cell's response to developmental progression or to different experimental conditions at gene expression level. Relevant gene expressional changes are often small and can be hindered by technically related variations. These variations arise from unequal starting sample amounts, dissimilar RNA isolation efficiencies, inaccurate RNA concentration measurement before reverse transcription, etc. Therefore results obtained by this method heavily relies on proper normalization. The most common technique correcting for technically related variations is the use of reference genes (Pfaffl 2001).

Reference genes (or formally called housekeeping genes) are genes which encodes gene products essential for every cells basic cellular functions like metabolism (carbohydrate, lipid and so on), transcription, translation, protein folding, signal transduction etc. This assumes that, these genes shows stable and constant level unregulated by experimental effects to maintain these important functions. The popular equation for gene expression ratio determination which takes into account the normalisation with reference genes is published by Pfaffl (2001). Applicability of them can only be useful if their expression is not affected by the experimental condition itself. Therefore in the last decade several algorithms were developed to test reference gene stability and an emerging number of articles published which test them under different experimental conditions.

Beside human and laboratory animals publications related to farm animals started to address reference gene stability in the last decade as well. Chapman and Waldenström (2015) analysed the literature to see which are the most commonly used reference genes in vertebrate gene expression studies and found that *ACTB* (used in 38% of studies) encoding beta-actin a cytoskeletal structure protein and *GAPDH* (37%) glyceraldehyde 3-phosphate dehydrogenase takes part in glycolysis with *RN18S* (12%) encoding 18S ribosomal RNA and their results showed that only 15 percent of total publications tested a panel of potential reference genes for stability before used as normalizer. However the aforementioned genes are the most commonly used ones, this does not mean that these are always stable under all conditions. Bionaz and Loor (2007) found that *ACTB* and *GAPDH* are the less stably reference genes when investigated in cattle mammary gland. While Bougarn et al. (2011) found that *ACTB* is the

most stable expressing reference gene in cattle mammary epithelial cells unstimulated and stimulated with mastitis pathogens. Similarly *RN18S* ranked better as other candidates in chicken lung cells infected with influenza virus (Kuchipudi et al. 2012), but ranked poor in similar tissue when infected with avian infectious bronchitis virus (Fan et al. 2012). These examples shows the importance of stability testing. Testing algorithms freely available by Andersen et al. (2004), Chervoneva et al. (2010), Pfaffl et al. (2004), Silver et al. (2006) and Vandesompele et al. (2002).

Our literature review aimed to summarize the articles addressing the most important livestock species in regards of reference gene stability used as normalizers for quantitative real-time polymerase chain reaction experiments. Stably expressing reference genes were categorized into distinct groups and investigated if one of the category is ranked better over others across all the various experimental situations.

MATERIALS AND METHODS

A literature review was performed using Web of Science v5.23.2 bibliographic database (available at https://webofknowledge.com/) with keywords for TOPIC "cattle or chick* or goat or horse or pig or rabbit or sheep AND reference gene" and obtained a total of 118 publications. Experimental condition(s), the investigated tissue type(s) the most stable and the least stable two reference genes were collected and shown in *Table 1* as a representative table including chicken related experiments and a summary in *Table 2* for the rest of the aforementioned species. The type and the number of applied methods were also noted and counted. When the authors did not calculate the final ranks for each gene, we did it with geometric averaging of the different available rankings and finally rank them as the geometric mean with smallest value being most stable (as recommended by Chen et al. 2011). Gene products were classified using the European Bioinformatics Institute protein database (can be found at http://www.ebi.ac.uk/). One-way analysis of variance (ANOVA) and Tukey post hoc test were applied to compare the means of reference genes tested in each year using GraphPad Prism 6 (La Jolla, California, USA). Classification was the following: cat I.: cytoskeletal components, cat II.: signal transduction, cat III.: transcription, cat IV.: protein degradation, cat V.: mitochondrial electron transport chain, cat VI.: ribosomal protein component, cat VII.: ribosomal RNA, cat VIII.: protein folding, cat IX.: carbohydrate metabolism, cat X.: nucleotide metabolism, cat XI.: protein transport, cat XII.: chromosome organization, cat XIII.: heme biosynthesis.

Table 1.

Example table representing publications addressing Gallus gallus related experiments

Samples	Experimental conditions	Genes with the most stable expression	Genes with the least stable expression	Genes used	Algorithms used	Reference
Four tissues	Cross-tissue examination	*RPL32* (VI)* *B2M* (X) *SDHA* (V)	*TBP* (III) *YWHAZ* (II)	5	BestKeeper, Comparative ΔCq, geNorm, NormFinder	Bagés et al. (2015)
Lymphoid organs	Cross-tissue examination	*TBP* (III) *GAPDH* (IX) *RN28S* (VII)	*B2M* (X) *GUSB* (o) *TUBAT* (I)	7	BestKeeper, geNorm, NormFinder	Borowska et al. (2016)
Blood	Lipopolysaccharide administration	*UB* (IV) *G6PDH* (IX)	*ACTB* (I) *HPRT* (XI) *GAPDH* (IX)	5	BestKeeper, geNorm	De Boeve et al. (2008)
Nine different tissue	Control and infected with avian infectious bronchitis virus	*UB* (IV) *GAPDH* (IX)	*ACTB* (I) *G6PDH* (IX) *RN18S* (VII)	5	geNorm	Fan et al. (2012)
Lung-derived cells	Influenza virus infection	*RN18S* (VII) *GAPDH* (IX)	*ACTB* (I)	3	BestKeeper, NormFinder	Kuchipudi et al. (2012)
Pectoralis major	Four levels of lysine supplementation	*HMBS* (XIV) *ACTA1* (I) *HPRT* (XI)	*UBC* (IV) *B2M* (X) *TFRC* (II)	13	BestKeeper, Comparative ΔCq, geNorm Excel, geNorm SAS, NormFinder	Nascimento et al. (2015)
Embryo fibroblasts	Avian leucosis virus infection	*RPL30* (VI) *SDHA* (V) *HPRT* (XI)	*B2M* (X) *ACTB* (I *TUBB* (I)	11	geNorm	Yang et al. (2013)
Embryo fibroblasts	H5N1 infected and control	*YWHAZ* (II) *RPL4* (VI) *ACTB* (I)	*ALB* (I) *RPL30* (VI) *TUBB* (I)	11	geNorm	Yue et al. (2010)

Note: *roman numbers in parentheses represents a category number described in materials and methods, o means other

Table 2.

Summary table representing genes ranked as the most commonly stable or most commonly unstable in the further examined species

Species	Gene with the most stable expression	Genes with the least stable expression	References
cattle *(Bos taurus)*	*RPS9* (VI)*,(*n*=4) *YWHAZ* (II), (*n*=4)	*ACTB* (I), (*n*=6)	Anstaett et al. (2010), Baddela et al. (2014), Bionaz and Loor (2007), Bonnet et al. (2013), Bougarn et al. (2011), Brym et al. (2013), De Ketelaere et al. (2006), Emam et al. (2015), Fredericksen et al. (2015), Goossens et al. (2005), Hosseini et al. (2009), Janovick-Guretzky et al. (2007), Kadegowda et al. (2009), Khan et al. (2014), Lecchi et al. (2012), Lisowsk et al. (2008), Liu et al. (2015), Luchsinger et al. (2012), Macabelli et al. (2014), Mihi et al. (2011), Ontsouka et al. (2004), Ostrowska et al. (2014), Pérez et al. (2008), Rekawiecki et al. (2012), Rekawiecki et al. (2013), Robinson, et al. (2007), Ross et al. (2010), Saremi et al. (2012), Schoen et al. (2014), Spalenza et al. (2011), Verbeke et al. (2015), Walker et al. (2009), Zhao et al. (2016)
goat (*Capra hircus*)	*YWHAZ* (II), (*n*=3)	*RN18S* (VII), (*n*=3)	Bai et al. (2014), Bonnet et al. (2013), Finot et al. (2011), Frota et al. (2009), Jarczak et al. (2014), Manjunath et al. (2015), Modesto et al. (2013), Najafpanah et al. (2013), Zhang et al. (2013), Zhu et al. (2015)
horse (*Equus caballus*)	*UBB* (IV), (*n*=3)	*ACTB* (I), (*n*=3) *GAPDH* (IX), (*n*=3)	Ahn et al. (2010), Beekman et al. (2011), Bogaert et al. (2006), Bruynsteen et al. (2013), Cappelli et al. (2008), Cieslak et al. (2015), Hjertner et al. (2013), Kayis et al. (2011), Klein et al. (2011), Looijen et al. (2016), Paris et al. (2011), Smits et al. (2009), Zhang et al. (2009)
pig (*Sus scrofa*)	*RPL4* (VI), *(n=4)*	*ACTB* (I), *(n=5)*	Chooi et al. (2013), Cinar et al. (2012), Erkens et al. (2006), Facci et al. (2011), Feng et al. (2010), Gu et al. (2011), Huang et al. (2016), Kuijk et al. (2007), Lee et al. (2015), Li et al. (2011), Li et al. (2016), Manjarin et al. (2011), Martínez-Giner et al. (2013), Martino et al. (2011), McBryan et al. (2010), McCulloch et al. (2012), Monaco et al. (2010), Muráni et al. (2007), Nesvadbová et al. (2011), Nygard et al. (2007), Park et al. (2015), Pierzchała et al. (2011), Piórkowska et al. (2011), Ropka-Molik et al. (2012), Skovgaard et al. (2007), Svobodová et al. (2008), Tramontana et al. (2008), Uddin et al. (2011), Wang et al. (2014), Wang et al. (2015), Xiang-Hong et al. (2011), Zhang et al. (2012)
rabbit (*Oryctolagus cuniculus*)	*YWHAZ* (II), *(n=3)*	*B2M* (X), *(n=2)*	Llobat et al. (2011), Ma et al. (2015), Mamo et al. (2008), Nachar et al. (2014), Peng et al. (2012)
sheep *(Ovis aries)*	*GAPDH* (IX), (*n*=3)	*ACTB* (I), (*n*=5) *YWHAZ* (II), (*n*=5)	Budhia et al. (2006), Garcia-Crespo et al. (2005), Jiang et al. (2015), Lyahyai et al. (2009), Mahakapuge et al. (2016), O'Connor et al. (2013), Passmore et al. (2009), Paten et al. (2014), Peletto et al. (2011), Pereira-Fantini et al. (2016), Puech et al. (2015), Serrano et al. (2011), Vorachek et al. (2013), Xu et al. (2015), Zang et al. (2011), Zaros et al. (2010)

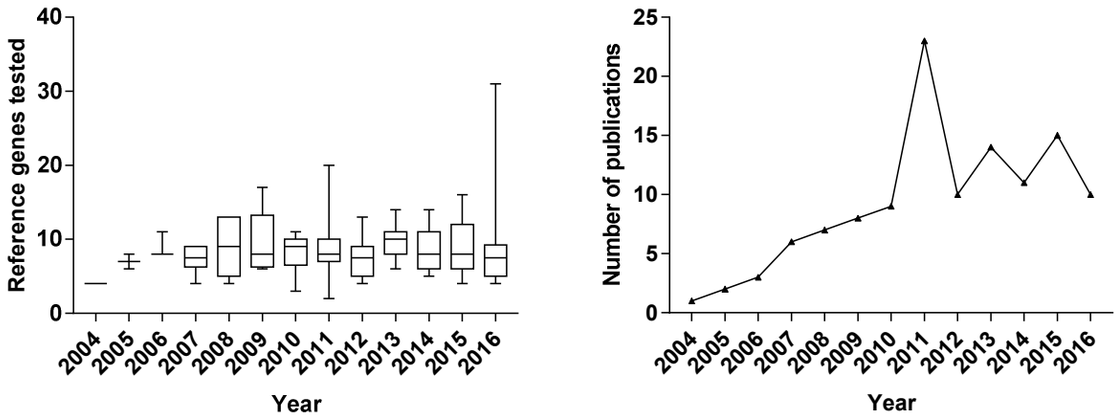
Note: *roman numbers in parentheses represents a category number described in materials and methods, o means other, n summarize the events when the given gene ranked as stable or unstable

RESULTS AND DISCUSSION

We processed 118 publications overall including the following species: cattle (33), chicken (8), goat (10), horse (13), pig (32), rabbit (5) and sheep (16). No differences were found in the number of reference genes tested depending on publication date, but an increasing number of publications started to address reference gene stability after 2008 in livestock species *(Figure 1)*, which can be a result of increased awareness for proper normalization due to a publication contains important guidelines which was published at 2009 (Bustin et al. 2009).

When we counted how many methods were used we found that most often a single method was applied in 44.44% of the cases, two in 25.64%, three 20.51%, four 6.84%, and 2.56% used five algorithm to test reference gene stability. In majority of cases the method called geNorm (Vandesompele et al. 2002) were used in 47.08% of the cases followed by NormFinder (Andersen et al. 2004) 28.75% BestKeeper (Pfaffl et al. 2004) 16.25% the comparative ΔCq method (Silver et al. 2006) 4.58%, others like descriptive statistic and principal component analysis in 3.33%. of papers The aforementioned most common algorithms use either descriptive statistics or multivariate analysis of variance.

Figure 1: **Mean number of reference genes tested in livestock animal related publications (left) and number of publications regarding the topic in the last 13 year (right)**



Note: boxes represent the interquartile interval (25–75%) with median value; whiskers represent minimum and maximum values. ANOVA multiple comparisons test showed no significant differences at α=5%, P resulted as >0.9598 after each comparison. *due to lack of adequate number of cases only one value is showed for 2004.

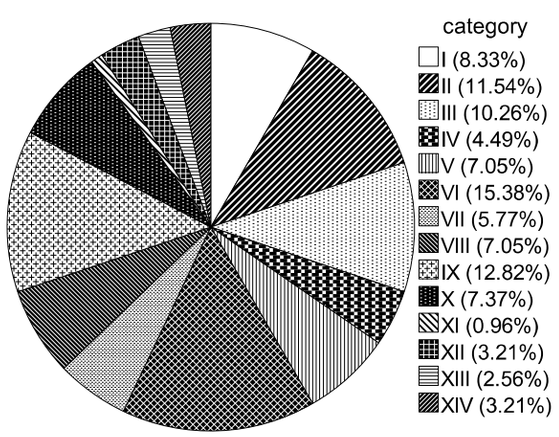
Currently there is no scientifically consensus which method provides the best results, but geometric averaging of the rankings derived from each method, followed by re-ranking can increase robustness (Kozera and Rapacz 2013).

The most commonly used method amongst all for reference gene stability testing is geNorm. This method first calculate the pairwise variation for each reference gene with all other reference genes is calculated as the standard deviation of the logarithmic transformed expression ratios. This followed by the calculation of a reference gene stability value (M value) which is the average pairwise variation of a single reference gene. The lower of this value, the more stably expressed is a reference gene. All of the popular methods can easily be conducted on Microsoft Excel. Popularity of geNorm may be due to this was the first reference gene selector algorithm published as early as 2002.

Results showed that publications addressing reference gene stability in livestock appeared as early as 2004 two year after the release of geNorm (Vandesompele et al. 2002) and continued to grow in number in the next years. Classifying the reference genes into distinct classes resulted that ribosomal protein encoding genes *(Figure 2)* ranked better over other categories. Inversely counting was also done with the least stable ones which resulted as genes encoding glycolytic enzymes counted 52 times (17.82%) as most unstable expressed genes.

Thorrez et al. (2008) meta-analysed some publicly available microarray datasets derived from 22 different tissues. Ribosomal protein encoding genes are most stably expressed amongst of all the investigated genes but found that they exhibit important tissue dependent variation in mRNA expression and therefore they cannot be considered as universal reference genes. Similarly according to our review we found that reference genes encoding ribosomal proteins ranked most frequently as stable, otherwise counting was also done with the least stable ones and resulted as ribosomal encoding genes are also the fourth most unstable gene category in some instances (35 case, 12.03%), this observation also confirms that genes in this category are cannot be considered as universal reference genes.

Figure 2: **Proportion of different functional categories as stable reference genes**



Note: category 6 (ribosomal proteins encoding genes) with 15.38% represents the highest percentage over other categories. Total gene count was n=313. Classification was the following: cat I.: cytoskeletal components, cat II.: signal transduction, cat III.: transcription, cat IV.: protein degradation, cat V.: mitochondrial electron transport chain, cat VI.: ribosomal protein component, cat VII.: ribosomal RNA, cat VIII.: protein folding, cat IX.: carbohydrate metabolism, cat X.: nucleotide metabolism, cat XI.: protein transport, cat XII.: chromosome organization, cat XIII.: heme biosynthesis, XIV.: other.

CONCLUSION

Literature review of publications investigating reference gene stability in livestock species revealed the gene types which are most often ranked as stable in qPCR experiments. The highest percentage represents genes that encode ribosomal proteins. We recommend

that genes encoding ribosomal components should be included for pilot experiments aiming to identify stable reference genes under the particular experimental conditions. This should be done prior in order to reliable quantify the target gene's expression.

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