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Research Paper

Selection of reference genes for quantitative real-time PCR analysis in chicken ovary following silver nanoparticle treatment

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ABSTRACT

Reliable results of quantitative real time PCR (qPCR) analysis require normalization of target gene expression level using reference genes (RGs). However housekeeping genes expression may vary under experimental conditions, so selection of the proper RGs is a crucial step in a qPCR analysis. Several algorithms have been developed to address this problem: geNorm, NormFinder and BestKeeper. In this study, we have used these three tools to evaluate the stability of RGs in the ovarian tissues of hens treated with silver nanoparticles. Eight genes were selected for the validation: *HPRT, HMBS, VIM, SDHA, TBP, RPL13, GAPDH* and *18S rRNA*. According to geNorm the best combination of reference genes is *SDHA* and *TPP*. NormFinder also selected *SDHA* as the most suitable gene, but in combination with *RPL13*. Analysis in BestKeeper showed that *SDHA*, *RPL13* might be the best choice in gene expression studies using the chicken ovary. In conclusion, the results obtained depend on the algorithm used and it arises from the diverse calculation strategies used in these programs. The outcome from the NormFinder is considered to be the most trustworthy and used in further qPCR analysis.

1. Introduction

Real time PCR is currently a leading method used in determination of gene expression (Bustin et al., 2005; Wong and Medrano, 2005). There are some difficulties connected with non-biological variations that need to be overcome in gene expression analysis e.g. sample preparation and storage, poor reverse transcription, mRNA quality, and cDNA concentration (Bustin, 2002; Lekanne Deprez et al., 2002; Sanders et al., 2014). Several different strategies of normalization are used to correct these sample-to-sample and run-to-run variations, but the most preferred way is normalization against reference gene/s (RG/s) (Huggett et al., 2005). Perfect RGs should be unaffected by experimental conditions and show minimal variation between tissues. It seems that the best candidates to meet these criteria are ubiquitously expressed housekeeping genes (HKGs). Over the years, the most commonly used HKGs have been β-actin (*ACTB*), glyceraldeyde-3-phosphate dehydrogenase (*GAPDH*), ribosome small subunit ribosomal RNA (*18S rRNA*), and 28S ribosomal rRNA (*28S rRNA*) (Suzuki et al., 2000). On the other hand, numerous studies argue that they are unsuitable internal controls with variable expression (Glare et al., 2002; Ohl et al., 2005; Selvey et al., 2001; Thellin et al., 1999; Toegel et al., 2007). Since it has become clear that a universal RG, one constitutively expressed regardless of conditions, does not exist, validation of RG stability in each experiment is highly required to obtain a reliable expression analysis (Kozera and Rapacz, 2013; Radonic et al., 2004).

The current approach to analysis of gene expression also demands the use of more than one RG (Bustin et al., 2009; Thellin et al., 2009) to preclude considerably large bias (Derveaux et al., 2010; Nicot et al., 2005). According to Vandesompele et al. (2002) quantification analysis using validated multiple internal controls gives the most appropriate results. The two most important algorithms for determination of the stability of RGs are geNorm and NormFinder. GeNorm creates stability ranking of tested RGs and specifies the minimum number of genes for accurate normalization of gene expression (Vandesompele et al., 2002). On the other hand, NormFinder calculates intra- and inter-group variation as well as creates a ranking of the most stably expressed genes (Andersen et al., 2004). There is another well-known statistical tool defining the variability of RGs called BestKeeper, which creates the descriptive statistics with several measures and leaves the choice of selection parameters to the experimenter (Pfaffl et al., 2004).

Literature regarding the selection of RGs in chicken tissues is scarce. Existing reports are related to infectious diseases (Kuchipudi et al., 2012; Yang et al., 2013; Yin et al., 2011; Yue et al., 2010), immunological research (Borowska et al., 2016; De Boever et al., 2008; Mitra et al., 2016) or different tissues (Bagés et al., 2015; Nascimento et al., 2015), while there are no reports associated with toxicological studies. In this type of research the selection of a proper internal control gene is extremely important due to the significant impact of

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Table 1
The summary of TaqMan Gene Expression Assays characteristic.

Gene symbol	Assay ID	Context sequence	Amplicon size (bp)
SDHA	Gg03330765_m1	GCAGAAGACAATGCAAAGCCATGCT	103
RPL13	Gg03348054_m1	TTATGCCGATCAGGAACGTTTTCAA	66
TBP	Gg03366488_m1	CAGGAGCAAAAAGCGAGGAACAGTC	82
HMBS	Gg03317022_m1	GCTTTGAGATTGTTGCCATGTCTAC	87
VIM	Gg03360311_m1	GGAAACTAGAGATGGACAGGTTATT	85
GAPDH	Gg03346982_m1	TCGTCAAGCTTGTTTCCTGGTATGA	107
HPRT1	Gg03338899_m1	GATATCGGCCAGACTTTGTTGGATT	89
CYP19A1	Gg03346001_m1	ATTGAAACTGTTATGGGTGACAGAG	76
18S rRNA	Eukaryotic 18S rRNA Endogenous Control		187

SDHA – Succinate dehydrogenase complex flavoprotein subunit A, *RPL13* – 60S ribosomal protein *L13*, *TBP* – TATA-binding protein, *HMBS* – Hydroxymethylbilane synthase, *VIM* –vimentin, *GAPDH* – Glyceraldehyde 3-phosphate dehydrogenase, *HPRT1*– Hypoxanthine-guanine phosphoribosyltransferase, *CYP19A1* – Cytochrome P450, family 19, subfamily a, polypeptide 1, *18S rRNA* – 18S ribosomal RNA.

experimental factors (Arukwe, 2006). The increasing application of silver nanoparticles (AgNPs) in the contemporary world creates the necessity to verify their safety for humans and the environment. AgNPs are now considered as new additives in poultry nutrition and used as disinfectants and odorant limiting agents (Farzinpour and Karashi, 2013; Pineda et al., 2012). In females of domestic birds the number of eggs produced, reproductive rates and breeding results depend on the number of oocytes produced in the ovary, and any anomalies in this area are highly undesirable by breeders. Therefore, the aim of this study was to select the best RGs using normalization of gene expression in the ovaries of hens treated with AgNPs. Eight candidate RGs were analyzed: hypoxanthine-guanine phosphoribosyltransferase (*HPRT),* hydroxymethylbilane synthase *(HMBS),* vimentin *(VIM),* succinate dehydrogenase complex flavoprotein subunit A *(SDHA),* TATA-binding protein *(TBP),* 60S ribosomal protein L13 *(RPL13), GAPDH,* and *18S rRNA*. Their stability was assessed using geNorm, NormFinder and BestKeeper software tools.

2. Materials and methods

All procedures were performed with the permission of the Local Animal Ethics Committee in Krakow (no. 9/2015). Hy-Line Brown chickens at the age of 25 weeks were kept in individual cages with free access to water and feed, on a 14L:10D lighting schedule. Chickens received *per os* 1 ml/kg b.w. of colloidal AgNPs in two sizes: 13 nm and 50 nm and in concentrations of 10 ppm or 100 ppm, respectively. The control group was treated with the reference solution in which AgNPs were suspended. After 14-day administration of AgNPs, the chickens were decapitated and prehierachical and hierarchical ovarian follicles were obtained. Granulosa and theca layers were separated from hierarchical ovarian follicles and stored separately. Tissues were snap-frozen in liquid nitrogen immediately after isolation (c.a ~2–3 min) and stored at −80 °C up to 3 months until RNA extraction from all tissues. All collected samples of ovarian tissues were analyzed together to select the best reference gene for the whole ovary; i.e. from each treatment group (13 nm, 50 nm, control group) we randomly chose 4 samples from prehierachical follicles, 5 granulosa layers from F2 follicles and 5 theca layers from F2 follicles (i.e. 14 biological replicates in each treatment group). It means, that 42 (i.e. 14 replicates × 3 groups) biological repetitions were used in the whole reference gene selection analysis.

Total RNA was isolated using silica membrane technology with commercial kit Extractme Total RNA according to the manufacturer's instructions (DNA-Gdańsk, Poland). RNA quality and quantity were measured using NanoDrop Lite (Thermo Scientific, Madison, USA) and checked on 1% agarose gel electrophoresis. The A260/A280 ratios were quantified to assess the quality of the extracted RNA, for all analyzed samples were between 1.96 and 2.

Two μg of total RNA was reverse transcribed using a RevertAid RT Transcription Kit (Thermo Scientific) following manufacturer's recommendations. First strand cDNA was synthesized using random primers for 5 min at 25 °C followed by 60 min at 42 °C; reaction was terminated by heating at 70 °C for 5 min.

Quantitative real time PCR (qPCR) was performed on 96-well plates in a StepOne Plus thermocycler (Applied Biosystems, Foster City, CA, USA). Each reaction was performed in duplicate in a total volume of 10 μl. One ul of 10-fold diluted cDNA was added to 9 μl of reaction mix containing 5 μl TaqMan Gene Expression Master Mix (Applied Biosystems), 3.5 μl nuclease free water, and 0.5 μl TaqMan Gene Expression Assay (Applied Biosystems). Thermal cycling conditions were as follows: 2 min at 50 °C, 10 min at 95 °C and 40 cycles of denaturation for 15 s at 95 °C, and 1 min of annealing at 60 °C. Standard curves from 10-fold serial dilutions of pooled cDNA were generated to determine amplification efficiency for each tested gene. Amplification efficiencies were as follows: *HPRT* – 97%, *HMBS* – 96%, *VIM* – 99%, *18S rRNA* – 95%, *GAPDH* – 100%, *SDHA* – 95%, *RPL13*–102%, *TBP* – 99%. No template controls with nuclease free water were prepared in each plate in order to check reagent contamination. TaqMan Gene Expression Assays containing primers and probe were chosen from predesigned assays offered by Applied Biosystems (Table 1). Designed probes spanned exon–exon junctions in order to avoid amplification of genomic DNA. Moreover, we used minus-reverse transcriptase controls to assesses the amount of DNA contamination for each gene. 2% agarose gel electrophoresis was prepared in order to verify the amplicons size of each tested gene and it revealed that all gene expression assays amplified a single PCR product with the expected size (Fig. 1).

Statistical analysis was carried out using three available software programs: geNorm, NormFinder and BestKeeper. GeNorm calculates the gene expression stability value (M) for the reference gene as the average pairwise variation V for that gene with all other tested reference genes. It creates a ranking of stable RGs by stepwise elimination of genes with the highest M value, determining the two best RGs. GeNorm also indicates the minimum number of genes that should be used for normalization (Vandesompele et al., 2002). This algorithm is sensitive to co-regulation so selected genes must stem from different biological pathways (Andersen et al., 2004). The input data for geNorm were the quantities obtained through transformation of Ct values according to the delta Ct method ($Q = E^{(minCt - sampleCt)}$).

NormFinder is a model based tool, which calculates the M of RGs taking into account intra and inter-group variations. It also suggests the two most stable genes and provides their combined stability value. This model is considered less susceptible to co-regulation (Andersen et al., 2004). Analysis in NormFinder was made on Ct values converted to linear scale according to formula $E^{-\Delta Ct}$ and then log-transformed by the program itself.

BestKeeper creates descriptive statistics with multiple factors, i.e. the geometric mean (GM), arithmetic mean (AM), minimal (Min) and maximal (Max) values, standard deviation (SD), and coefficient of