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Original Article

Selection of the appropriate reference genes for relative quantitative reverse transcription polymerase chain reaction (qRT-PCR) in canine pulmonary arteries

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Abstract

A normalisation of targeted genes using the appropriate reference genes is necessary for reliable quantitative reverse transcription polymerase chain reaction (qRT-PCR) experiments. This study aimed to investigate the appropriate reference genes and the numbers of the genes required for qRT-PCR in canine pulmonary arteries. Pulmonary arteries were collected from twenty-three dogs at necropsy. Candidate reference genes including ribosomal protein L32 (RPL32), ribosomal protein L13a (RPL13A), ribosomal protein S18 (RPS18), TATA box binding protein (TBP), beta-2-microglobulin (B2M), ribosomal protein S5 (RPS5), ribosomal protein S19 (RPS19) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from pulmonary arteries were examined by qRT-PCR and analysed using four major algorithms, including geNorm, NormFinder, the comparative delta-C_T method, and RefFinder. The optimal number of genes was analysed using geNorm. The ranking of the appropriate reference genes from all algorithms was evaluated by RefFinder. The appropriate reference genes for the normalisation of the targeted gene in canine pulmonary arteries are RPS19 and RPL32.

Keywords: canine, PCR, pulmonary artery, reference gene, RPL32, RPS19

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Introduction

Pulmonary hypertension (PH) is an excess of pulmonary vascular pressure; it is defined as an increase of pulmonary arterial pressure greater than 30 mmHg (MacLean and Dempsie, 2009). When PH occurs, the structures of the pulmonary arteries are remodeled. The pulmonary arterial wall thickens due to the proliferation of smooth muscle cells *(Quinlan et al.*, 2000; Delgado *et al.*, 2005). Several publications on human and animal models, such as mice and rats, have used quantitative reverse transcription polymerase chain reaction (qRT-PCR) to study the genes involve in the pathogenesis of pulmonary hypertension. When using relative qRT-PCR, the normalisation of the targeted genes' expression is necessary to eliminate variation across different samples. Reference genes that are constantly expressed in both normal and diseased conditions are used for reliable normalisation. The stability of reference genes is different in various canine tissues and organs (Brinkhof *et al.*, 2006; Peters *et al.*, 2007). Normalisation with commonly used reference genes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin, may not be suitable for all tissue types (Dheda *et al.*, 2005). Therefore, it is crucial that the appropriate reference genes for studying the tissue of interest are used (Livak and Schmittgen, 2001; Bustin *et al.*, 2009).

To the authors' knowledge, there is no report of the appropriate reference genes for canine pulmonary arteries. Therefore, this study aimed to investigate the appropriate reference genes of canine pulmonary arteries for qRT-PCR experiments.

Materials and Methods

The study protocol was approved by the Ethics Committee for Animal Experimentation, Faculty of Veterinary Science, Chulalongkorn University (number 1731039).

Sample collection: Third or fourth branched pulmonary arteries were dissected from dogs with several diseased conditions; this was done at necropsy, within 24 hours of death. Samples were immediately stored at -80˚C after collection for further RNA isolation. The pulmonary artery samples were classified into 3 groups following the major diseased condition of the lungs being examined by clinical and pathological diagnosis. The group of samples included PA tissues from dogs with normal lung tissue, dogs with pulmonary edema due to congestive heart failure (CHF) and dogs with other lung conditions without pulmonary edema.

RNA isolation and cDNA synthesis: Pulmonary artery tissues were cut using a surgical blade and sterile petri dish with dry ice under aseptic conditions. Tissues were then lysed, homogenised and extracted for total RNA using a NucleoSpin RNA kit (Macherey-Nagel, USA). After extraction, total RNA samples were treated with DNase (TURBO DNA-free Kit; Invitrogen, USA) at 37 ˚C for 30 mins to eliminate genomic DNA (gDNA) contamination. The RNA concentration was measured in DNase-treated RNA samples using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). The RNA integrity of all pulmonary artery samples was examined using 1% agarose gel electrophoresis. 28S and 18S rRNA bands were detected. The RNA integrity number (RIN) was calculated using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Treated RNA was then converted into complementary DNA (cDNA) by reverse transcription. In brief, 5 ng/µl of RNA was used to perform reverse transcription to 20 µl cDNA at 37˚C for 60 min using Omniscript RT kit (Qiagen, Germany), following the manufacturer's instructions.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR): The qRT-PCR was performed in Roter-Gene Q (Qiagen, Germany) using SYBR Green I dye (KAPA SYBR Fast qPCR Master Mix Universal, KAPA Biosystems, UK) and the specific primers. The oligonucleotide primers of eight reference genes including ribosomal protein L32 (RPL32), ribosomal protein L13a (RPL13A), ribosomal protein S18 (RPS18), TATA box binding protein (TBP), beta-2-microglobulin (B2M), ribosomal protein S5 (RPS5), ribosomal protein S19 (RPS19) and GAPDH, are shown in Table 1. The thermal cycling condition included initial denaturation at 95 ˚C for 5 mins followed by 40 cycles at 95˚C for 3 s and 60 ˚C for 25 s. The melting curve was analysed between 65 and 95˚C with the rate of 1 ˚C per second. The PCR efficiency was calculated from the standard curve of two-fold serial dilution of the standard. The acceptable PCR efficiency was 80-110% (Rocha *et al.*, 2016).

Analysis of expression stability: The expression stability of candidate reference genes was analysed by four algorithms: geNorm, NormFinder, the comparative delta-C_T method, and RefFinder. GeNorm calculates and ranks the stability measure (M value or geNorm M), in which the lowest M value indicates the highest expression stability. GeNorm calculates the optimal numbers of the reference genes used for the normalisation of the targeted genes in qRT-PCR by pairwise variation ($V_{n/n+1}$; n=number of genes) analysis; the first V value below the cut-off value of 0.15 represents the lowest number of reference genes required for normalization (Vandesompele *et al.*, 2002). NormFinder provides mathematical models for the estimation of expression stability and to rank the candidate reference genes (Andersen *et al.*, 2004). The log-transformed C_T values of the samples are added to the models and calculated into the stability value, with the least stable value referred to as the most stable reference gene. The comparative delta-C_T method (Silver *et al.*, 2006) compares the relative expression of pairs of the candidate genes to calculate the mean and SD of the delta-C_T value between each pair of genes. The SD values of all delta- C_T value comparisons are then calculated for the mean SD values, which are used to rank the expression stability. RefFinder is an algorithm that integrates and concludes all the previously mentioned computerised programs to compare and rank the expression stability of the candidate reference genes (Xie *et al.*, 2012). This algorithm is available online at https://www.heartcure.com.au/for-researchers. In this study, the C_T values of all samples were used to

analyse the overall stability value which represented the appropriate reference genes for all diseased conditions of the pulmonary arteries. The group analysis was operated to represent the suitable reference genes for pulmonary arteries in each diseased condition as mentioned above.

Table 1 Specific primers of candidate reference genes

Gene	Accession no.	Forward primer	Reverse primer	Product size (bp)	Tm (˚C)	Reference
Ribosomal protein L32 (RPL32)	XM_022406256.1	TGGTTACAGGA GCAACAAGAAA	GCACATCAGCA GCACTTCA	100	81.54	Peters *et al.* (2007)
Ribosomal protein L13a (RPL13A)	AJ388525.1	GCCGGAAGGTT GTAGTCGT	GGAGGAAGGCC AGGTAATTC	87	79.41	Peters *et al.* (2007)
Ribosomal protein S18 (RPS18)	NM_001048082.1	TGCTCATGTGGT ATTGAGGAA	TCTTATACTGGC GTGGATTCTG	116	80.51	Peters *et al.* (2007)
TATA box binding protein (TBP)	XM_849432.5	CTATTTCTTGGT GTGCATGAGG	CCTCGGCATTC AGTCTTTTC	96	79.50	Peters *et al.* (2007)
Beta-2-microglobulin (B2M)	NM_001284479	TCCTCATCCTCC TCGCT	TTCTCTGCTGGG TGTCG	85	82.70	Brinkhof *et al.* (2006)
Ribosomal protein S5 (RPS5)	XM_533568.6	TCACTGGTGAG AACCCCCT	CCTGATTCACA CGGCGTAG	141	84.47	Brinkhof *et al.* (2006)
Ribosomal protein S19 (RPS19)	XM_005616513.3	CCTTCCTCAAA AAGTCTGGG	GTTCTCATCGTA GGGAGCAAG	95	80.69	Brinkhof *et al.* (2006)
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	AB038240.1	GCCCTCAATGA CCACTTTGT	TCCTTGGAGGC CATGTAGAC	101	80.75	Pisamai *et al.* (2016)

Results

Animals: Twenty-three dogs were enrolled in the study, with an age range of 1-16 years old and a body weight range of 2.2-17.2 kg. Twelve dogs were females (five intact [22%] and seven sterile females [30%]). Eleven dogs were males (eight intact [35%] and three castrated [13%] males). There were 9 Poodles (39.13%), 3 Shih Tzus (13.04%), 2 mixed breeds (8.70%), 1 Cocker Spaniel (4.35%), 1 Siberian Husky (4.35%), 1 Yorkshire Terrier (4.35%), 1 Chihuahua (4.35%), 1 Pomeranian (4.35%), 1 Miniature Pinscher (4.35%), 1 Schnauzer (4.35%), 1 Dalmatian (4.35%) and 1 Golden Retriever (4.35%). Classification of the samples following the diseased condition of the lung following clinical and pathological diagnosis revealed that 4 dogs had normal lung tissue, 10 dogs had pulmonary edema due to CHF and 9 dogs had other lung conditions without pulmonary edema including 3 dogs with lung metastasis due to gastric signet ring cell carcinoma (1), squamous cell carcinoma (1), hemangiocarcinoma (1) and 6 dogs had pneumonia. Information on the dogs included in the study is presented in table 2.

Table 2 Information on dogs included in the study.

	Normal lung	Pulmonary edema due to congestive heart failure	Other lung conditions without pulmonary edema
N (%)	4 (17.39)	10 (43.48)	9 (39.13)
Age (years)	8 (7.25-14.00)	13.50 (10.00-16.00)	11.00 (8.50-13.50)
Gender (female/male)	1/3	6/4	5/4
Weight (kg)	4.35 (2.60-24.55)	4.50 (3.33-7.83)	5.90 (3.85-8.03)

Data was expressed as median and interquartile range.

qRT-PCR validation: All reactions were run in triplicate. The melting temperatures (Tm) of all genes are shown in Table 1. The reaction efficiency from two-fold serial dilution ranged between 90 and 109% The correlation coefficients (R^2) were between 0.985 and 0.990.

Reference gene analysis: The expression stability value and ranking of all genes calculated by all algorithms is described in Table 3. GeNorm analysis of the expression stability of the candidate reference genes described that RPS19 and RPS18 were the most stable genes using the combination of Ct values of all diseased condition. GeNorm evaluation of all three groups suggested that RPS19 was the most stable reference gene while other suitable genes were RPS5 for dogs with normal lung tissue and other lung conditions without pulmonary edema, and RPS18 for dogs with pulmonary edema due to CHF. According to a pairwise variation calculation of geNorm, all $V_{n/n+1}$ values were lower than the threshold of 0.15. Therefore, $V_{2/3}$ determined that at least two suitable reference genes were required for the accurate normalisation of the targeted gene expression for canine pulmonary arteries in the relative qRT-PCR experiment. (Fig 1).

NormFinder showed that RPS19 and RPL32 were the most stable reference genes for PA tissues of all dogs, dogs with normal lung tissue and dogs with other lung conditions without pulmonary edema. In the pulmonary edema due to CHF group, RPL13A and RPS18 were the most suitable reference genes, whereas RPS19 and RPL32 were in the third and fourth ranks, respectively.

The comparative delta-Ct method suggested that RPS19 was the appropriate reference gene of pulmonary artery tissues analysed by both overall data and data separately into groups. RPS18 was another

proper gene for pulmonary artery tissue of all sample analysis and samples of normal lung tissue group, on the other hand, RPL13A was the most suitable gene for pulmonary artery tissue of dogs with pulmonary edema due to CHF and other lung diseased condition without pulmonary edema.

RefFinder illustrated that RPS19 was the proper reference gene for both overall and all group analysis. Another suitable gene of all sample analysis and pulmonary edema due to CHF group was RPS18, whereas the second suitable reference genes for normal lung tissue and other lung condition groups were RPS5 and RPL32, respectively.

Table 3 Ranking and expression stability value of the candidate reference genes calculated by four algorithms

Ranking	1	2	3	4	5	6	7	8
All diseased conditions (N=23)								
geNorm	RPS19	RPS18	RPS5	RPL32	RPL13A	B2M	GAPDH	TBP
	0.249	0.267	0.274	0.325	0.350	0.502	0.598	0.722
NormFinder	RPS19	RPL32	RPS18	RPL13A	GAPDH	RPS5	B2M	TBP
	0.003	0.003	0.004	0.005	0.007	0.008	0.010	0.010
Comparative ΔCt	RPS19	RPS18	RPL13A	RPL32	GAPDH	RPS5	B2M	TBP
	0.432	0.455	0.517	0.538	0.631	0.636	0.650	1.102
RefFinder	RPS19	RPS18	RPL32	RPS5	RPL13A	GAPDH	B2M	TBP
	1.316	2.115	2.449	3.310	4.472	6.481	6.701	7.737
Normal lung tissue group (N=4)								
geNorm	RPS5	RPS19	RPS18	B2M	RPL32	RPL13A	TBP	GAPDH
	0.188	0.203	0.24	0.323	0.397	0.489	0.565	0.64
NormFinder	RPS19	RPL32	RPS18	RPS5	RPL13A	B2M	GAPDH	TBP
	0.003	0.006	0.006	0.008	0.009	0.010	0.011	0.015
Comparative ΔCt	RPS19	RPS18	B2M	RPL32	RPS5	GAPDH	RPL13A	TBP
	0.388	0.438	0.452	0.475	0.503	0.516	0.649	0.818
RefFinder	RPS19	RPS5	RPL32	RPS18	B2M	RPL13A	TBP	GAPDH
	1.316	1.682	3.344	3.464	4.427	6.236	6.435	7.445
Pulmonary edema due to congestive heart failure group (N=10)								
geNorm	RPS19	RPS18	RPL13A	RPL32	RPS5	GAPDH	B2M	TBP
	0.215	0.217	0.228	0.283	0.317	0.455	0.571	0.749
NormFinder	RPL13A	RPS18	RPS19	RPL32	GAPDH	RPS5	B2M	TBP
	0.001	0.001	0.001	0.002	0.008	0.009	0.014	0.015
Comparative ΔCt	RPL13A	RPS19	RPS18	RPL32	GAPDH	B2M	RPS5	TBP
	0.389	0.439	0.439	0.554	0.628	0.754	0.789	1.221
RefFinder	RPS18	RPS19	RPL32	RPL13A	RPS5	GAPDH	B2M	TBP
	1.495	2.000	2.828	3.000	3.976	6.000	7.238	7.737
Other lung conditions without pulmonary edema group (N=9)								
geNorm	RPS5	RPS19	RPS18	RPL32	RPL13A	B2M	TBP	GAPDH
	0.230	0.237	0.243	0.291	0.307	0.415	0.531	0.602
NormFinder	RPL32	RPS19	RPS18	RPL13A	B2M	RPS5	GAPDH	TBP
	0.003	0.004	0.005	0.006	0.007	0.008	0.010	0.011
Comparative ΔCt	RPL13A	RPS19	RPS18	B2M	RPL32	RPS5	GAPDH	TBP
	0.391	0.391	0.412	0.478	0.490	0.529	0.646	0.800
RefFinder	RPL32	RPS19	RPS5	RPS18	RPL13A	GAPDH	TBP	B2M
	1.682	2.115	2.943	4.120	4.213	4.757	6.086	6.447

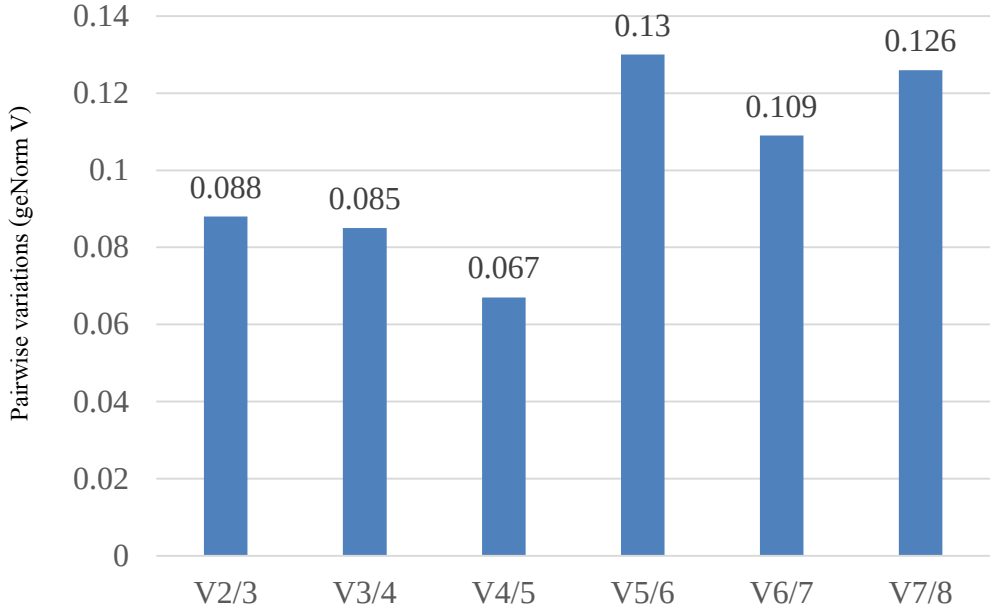

Figure 1 The optimal number of reference genes of the canine pulmonary artery required for normalisation analysed by pairwise variation (geNorm V). The cut-off value is 0.15.

Discussion

Relative qRT-PCR is one of the most acceptable techniques for the investigation of targeted gene expression in many studies. Accurate normalisation with the expression of reference genes that are suitable for each interested tissue is necessary for the relative quantitation of RNA data (Bustin *et al.*, 2009).

Several researchers have used GAPDH as the standard reference gene for the normalisation of qRT-PCR results. However, this study presented that the stability values of GAPDH of canine pulmonary arteries were mostly in the bottom three ranking. In accordance with previous studies which have suggested that GAPDH is not a suitable reference gene for several tissues, such as the ovine pulmonary artery and lungs (Passmore *et al.*, 2009), pulmonary artery of chicken embryos (Lin and Redies, 2012) and canine lung, liver, kidney, heart, spleen, lymph node, muscle and prostate gland (Brinkhof *et al.*, 2006; Peters *et al.*, 2007).

Differences between species, tissues and animal health status may result in differences in the appropriate reference genes; the appropriate reference gene of the same tissue type may be dissimilar in different species. A previous study (Passmore *et al.*, 2009) revealed that the most stable reference genes for the ovine pulmonary artery are beta-actin (*ACTB*) and peptidylprolyl isomerase A (*PPIA*). However, the present study demonstrated that RPS19 and RPS18 are the most appropriate reference genes for the canine pulmonary artery analysed from the C_T data of all samples. Based on these findings, similar sets of the appropriate reference genes cannot be used for cross-species investigations. Similarly, the appropriate reference genes vary between tissue types. A previous study reported that the most stable genes for canine heart tissues are RPL13A, RPL32 and TBP. RPS18 and RPL32 are the most stable genes for canine lung tissues (Peters *et al.*, 2007). Previous studies suggested reference genes for each tissue type in dogs (Brinkhof *et al.*, 2006; Peters *et al.*, 2007), showing that the suitable reference genes of each tissue type are different. It is best to use reference genes that are appropriate for each tissue type to provide a more accurate PCR result. Additionally, the health status of subjects also affects the rank of appropriate reference genes (van Rijn *et al.*, 2014). In this study, RPS19 was mostly presented in the top two rankings of all samples and all groups calculated by all four algorithms, while the second stable reference genes varied following the diseased conditions between RPS18, RPS5 and RPL32. RPS18 was predominately expressed in PA tissues of overall analysis and evaluation of pulmonary edema due to CHF group, whereas RPS5 was mainly expressed as the top two stable gene in the PA of dogs with normal lung tissue, and RPL32 was mainly expressed in PA of dogs with other diseases without pulmonary edema. The present study recruited dogs with several diseased conditions. Candidate genes with the highest expression stability in several diseased conditions are the most appropriate genes to be used as reference genes for normalization which are RPS19 and RPS18. However, both RPS19 and RPS18 encode the protein components of 40S subunit of ribosomes, therefore, changes in this ribosomal subunit may affect the expression stability of these genes and cause defective normalisation (Peters *et al.*, 2007). To prevent unreliable normalisation, Peters *et al.* (2007) suggested that the gene in next stability ranking which is not located in the similar subunit or organelle can be chosen. Consequently, RPL32 can be used for normalisation of the qRT-PCR experiment of canine PA together with RPS19.

A major limitation of this study was the use of tissue samples from necropsies. Post-mortem change may affect RNA quality. To minimise the RNA degradation, we tried to collect tissue samples immediately after the dogs had died. Another limitation of the study was the wide age range and the

varying size of the breeds of dogs included in the study that might affect the gene expression.

This study investigated the appropriate reference genes of the canine pulmonary artery for normalisation of the targeted genes in qRT-PCR experiments; it suggested that the two suitable reference genes for the canine pulmonary artery, required for normalization, are RPS19 and RPL32, which should be used in combination for samples with several diseased conditions. Differences in expression stability ranking through analyses via different algorithms resulted from different approaches to the data; consequently, a combination of several methods should be used.

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