Short communication

A GeNorm algorithm-based selection of reference genes for quantitative real-time PCR in skin biopsies of healthy dogs and dogs with atopic dermatitis

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ABSTRACT

Quantitative real time PCR (Q-PCR) is the method of choice to study mRNA expression levels. Since Q-PCR is very sensitive, normalization of the data with stably expressed reference genes if of utmost importance. The stability of reference genes depends on the tissue and the species of interest. Therefore, evaluation of the stability of reference genes must be performed for each new tissue and species under study. The stability of B2M, GAPDH, HPRT, SRPR, hnRNPH, GUSB, RPL8, RPS5, and RPS19 was analyzed with the GeNorm software in snap frozen canine skin biopsies. Healthy dogs ($n = 7$) and dogs with confirmed atopic dermatitis ($n = 28$) were included. Lesional and non-lesional skin was analyzed. The study indicated that the most appropriate reference genes in canine skin are the ribosomal gene products RPL8, RPS5 and RPS19 besides GUSB and HPRT. As little as three reference genes will reveal highly reliable Q-PCR calculations.

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1. Introduction

Molecular genetic tools are more and more advanced and their applications in the veterinary field are rapidly increasing. This has, amongst others, been greatly improved by the in-depth sequencing of the complete dog genome (Parker et al., 2004; Lindblad-Toh et al., 2005) and a single-nucleotide polymorphism (SNP) data base, which contains 2.5 million SNPs (Sargan et al., 2007). Even commercial, dog-specific micro-arrays are available to perform functional genomic studies in order to dissect affected signalling pathways in diseases or to predict the clinical outcomes of a therapy. This kind of high-throughput gene expression profiling requires the use of high quality mRNA and high quality internal controls. Quantitative real time PCR (Q-PCR) is the method to verify independently the differential expressions as measured with micro-array studies. Moreover, Q-PCR has been used in most of the mRNA expression studies done in veterinary research the last 5–10 years. Since Q-PCR is an extremely delicate and sensitive technique, numerous variables (e.g. RNA integrity, enzymatic efficiency) need to be controlled in such a gene expression analysis. Evaluation of internal controls that take into account all the variability's, has been limited to internal organs mainly (Brinkhof et al., 2006; Etschmann et al., 2006; Peters et al., 2007). However, no validation of reference genes in canine skin is available. Therefore, we evaluated nine well-known reference genes in healthy skin and non-lesional and lesional skin of dogs suffering from atopic dermatitis (AD). The stability of B2M,

GAPDH, HPRT, SRPR, hnRNPH, GUSB, RPL8, RPS5, and RPS19 was analyzed with the GeNorm software as done previously for companion animal reference genes (Brinkhof et al., 2006; Etschmann et al., 2006; Peters et al., 2007). This selection was based on reference genes already described and evaluated in companion animals such as dogs and cats (Brinkhof et al., 2006; Penning et al., 2007), horse skin (Bogaert et al., 2006), dolphin skin (Spinsanti et al., 2006) and human skin samples (de Kok et al., 2005). This low number of papers on this specific subject further shows that the evaluation of reference genes for skin specimen is at its infancy.

2. Materials and methods

2.1. Dogs

All privately owned atopic dogs ($n = 28$) presented to the Department of Clinical Sciences of Companion Animals, Utrecht University, fulfilled the diagnostic criteria for atopic dermatitis (Willemse, 1986; Prélaud et al., 1998). This group included 19 Labradors retrievers and one of each of the following breeds: Flat-coated Retriever, Gordon setter, Boxer, Vizsla, French bulldog, Jack Russell Terrier, Podenco Canario, Dachshund and German Shepherd. Female and male dogs were equally represented. Five healthy male Beagle dogs and two healthy female mongrel dogs were included as control animals. Punch biopsies (6 mm) were obtained under general anesthesia (medetomidine: 20 µg/kg body weight and propofol: 1–2 mg/kg body weight). Healthy control and non-lesional skin specimens were all taken from the lateral thorax, whereas the lesional biopsies were obtained from affected predilection sites. After collection, the skin biopsies were immediately snap-frozen in liquid nitrogen and stored at −70 °C until used for RNA isolation. All samples were obtained after written consent of the dog owner. The procedures were approved by the Utrecht University Animal Experiments Committee as required under Dutch legislation.

3. RNA isolation, cDNA synthesis and Q-PCR

Total RNA was isolated using a combination of the TRIzol reagent (Invitrogen, Breda, The Netherlands) and the RNeasy Mini Kit (Qiagen, Leusden, The Netherlands) according to the manufacturer's instructions. In short, the skin tissue was disrupted and homogenized in TRIzol reagent using a Biopulverizer (Biospec #59013, Biospec, Inc., Bartlesville, OK) and Ultra-turrax (T8, IKA® Labortechnik GmBH, Staufen, Germany). The TRIzol manufacturer's instructions were followed until the water-phase was obtained after the chloroform step. Subsequently, the procedure continued with RNeasy columns for clean-up of the RNA including the optional on-column DNase digestion (Qiagen Rnase-free DNase kit). RNA was dissolved in 30 µl of RNase free water and was quantified spectrophotometrically using Nanodrop ND-1000 (Isogen Life Sciences, IJsselstein, The Netherlands). cDNA synthesis and Q-PCR conditions were as described previously (Brinkhof et al., 2006). Information about the primers used is depicted in Table 1. To reduce chances to amplify traces of genomic DNA, the primers were positioned in different exons. Calculations to estimate the expression stability and the pair wise variation were performed with the freely available GeNorm program (http://medgen.ugent.be/~jvdsomp/genorm; Ohl et al., 2005).

4. Results and discussion

Samples were screened for contamination with genomic DNA by Q-PCR of non-reverse-transcribed RNA templates. No-template controls were included to test for other contaminations. All controls were negative.

GeNorm-based evaluation of canine reference genes revealed a stable expression of ribosomal gene products (RPS5, RPS19 and RPL8) and HPRT and GUSB as most stably expressed non-ribosomal gene products. B2M and GAPDH turned out to be rather unstably expressed reference genes (Fig. 1). Consequently, conclusions in scientific papers based on only one reference gene, especially if it concerns GAPDH, must be read with caution. In contrast, a combination of one or two ribosomal with one or two non-ribosomal gene products will result in highly accurate normalizations. Moreover, determination of the lowest number of reference genes needed for reliable data indicated that little improvement will be obtained with more than four independent reference genes (Fig. 2). Furthermore comparing dolphin, horse and human studies

Table 1
Details of primers and reaction conditions used.

	Accession number	Forward primer 5′ → 3′	Reverse primer 5′ → 3′	Product length (bp)	Ta (C°)
β-Glucuronidase (BGLR)(GUSB)	NM_001003191	AGACGCTTCCAA/GTACCCC	AGGTGTGGTGTAGAGGAGCAC	103	62.0
Ribosomal protein S5 (RPS5)	XM_533568	TCACTGGTGAG/AACCCCCT	CCTGATTCACACGGCGTAG	141	62.5
Ribosomal protein S19 (RPS19)	XM_533657	CCTTCCTCAAAAA/GTCTGGG	GTTCTCATCGTAGGGAGCAAG	95	61.0
Hypoxanthine phosphoribosyltransferase (HPRT)	AY283372	AG/CTTGCTGGTGAAAAGGAC	TTATAGTCAAGGGCATATCC	114	56.0
Heterogeneous nuclear ribonucleoprotein H (hnRNPH)	XM_538576	CTCACTATGATCCACCACG	TAGCCTCCATAAC/CTCCAC	151	61.2
Ribosomal protein L8 (RPL8)	XM_532360	CCATGAAT/CCTGTGGAGC	GTAGAGGGTTTGCCGATG	64	55.0
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM_001003142	TGTCCCCACCCCCAATGTATC	CTCCGATGCCTGCTTCACTACCTT	100	58.0
b-2-Microglobulin (B2M)	XM_535458	TCCTCATCCTCCTCGCT	TTCTCTGCTGGGTGTCG	85	61.2
SRPR	X03184	GCTTCAGGATCTGGACTGC	GTTCCCTTGGTAGCACTGG	81	61.2

Primer sequences, product sizes and optimal primer melting temperatures of canine reference genes used in this study.
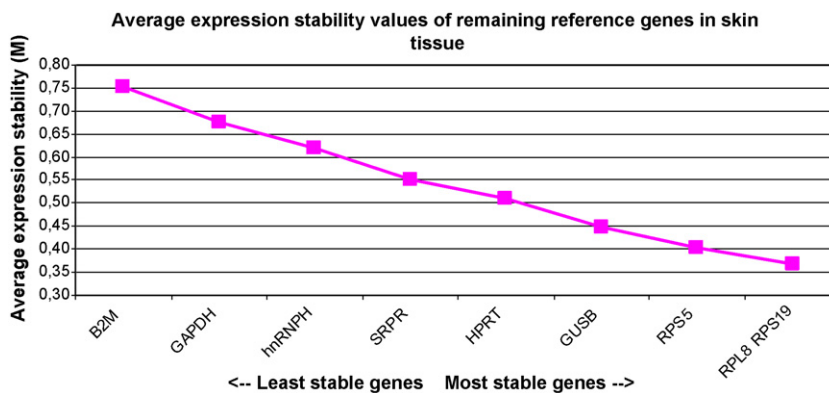
Fig. 1. Average expression stability values of remaining reference genes. The geNorm program (http://medgen.ugent.be/~jvdesomp/genorm) calculates the gene expression stability (M) of one gene based on the average pair wise variation between all studied reference genes. The highest M values characterize genes with the least stable expression, indicative for a less optimal reference gene. Step-by-step elimination of the least stable gene generates a ranking of reference genes according to their M values and finally results in the identification of the two most stable genes (Ohl et al., 2005).
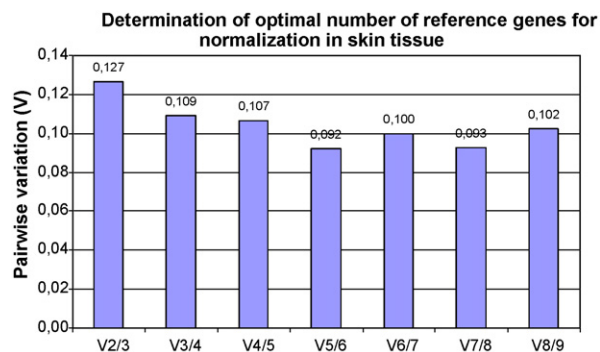

Fig. 2. Determination of the optimal number of reference genes for normalization. The geNorm program (http://medgen.ugent.be/~jvdesomp/genorm) calculates the normalization factor assessing the optimal number of reference genes for generating the M factor by calculating the pair wise variation V. The pair wise variation between these genes defines the variable V (Ohl et al., 2005). The lower the variable V is, the less variation. $V3/4$ indicates the variation in normalization factor with 3 vs. 4 reference genes.

on skin reference genes showed conflicting data about the stability of B2M (good in horses, poor in dolphins) and HPRT (poor in dolphins and horses, the best in people). This clearly consolidates our opinion that the use of one single reference gene without prior evaluation of its stability for the tissue/species of interest, can result in misinterpretation of the data.

This manuscript is the first, to our knowledge, that describes the evaluation of a large number of well-known canine reference genes in skin tissues. The most appropriate reference genes in canine skin are ribosomal gene products and GUSB and HPRT. As little as three reference genes will reveal highly reliable Q-PCR calculations. In this respect canine skin tissue is comparable to other canine tissues studied in reference gene evaluations.

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