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Technical Note

Validation of reference genes in the feline endometrium

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ABSTRACT

The aim of the study was to find the most stable reference genes from: *ACTB*, *GAPDH*, *RPL30*, *CYC*, *RPL17*, *RPS7* and *YWHAZ* in the feline endometrium. Three free software packages, geNorm, NormFinder and BestKeeper were used. In geNorm analysis, the most stable gene was *RPS7* (at a primer concentration 1000 nM) or *YWHAZ* (500 and 250 nM). According to NormFinder and BestKeeper, *ACTB* (at all examined primer concentrations) followed by *RPS7* and *CYC* were the most stable genes. Based on geNorm results at least two genes from among *RPS7*, *RPL30*, *ACTB* or *YWHAZ* should be chosen for Real Time-PCR result normalization.

1. Introduction

The domestic cat is a valuable model for studying molecular mechanisms that link hormonal contraception in carnivorous animals to the development of endometritis–pyometra complex. At present, studies are often based on molecular techniques including gene expression methods e.g., real-time PCR (RT-PCR). The sensitivity of the method depends on applying a suitable reference gene. The expression of a reference gene should be constant in all cells, in different developmental stages and under all experimental conditions [1]. However, many genes being used as reference genes do not meet these requirements. In view of the limited information on the selection of reference genes in felines, the aim of the present study was to find the most stable reference gene from among *β-actin* (*ACTB*), *glyceraldehydes 3-phosphate dehydrogenase* (*GAPDH*), *60S ribosomal protein L30* (*RPL30*), *cyclophilin* (*CYC*), *60S ribosomal protein L17* (*RPL17*), *40S ribosomal protein S7* (*RPS7*) and *tyrosine 3-monooxygenase* (*YWHAZ*) in the endometrium of: (1) luteal phase queens, (2) hormonally treated queens, and (3) queens with symptoms of pyometra. To establish stability expression of the genes, three free software packages, geNorm, NormFinder and BestKeeper were used in the study.

2. Materials and methods

All procedures were approved by the Local Animal Care and Use Committee in Olsztyn, Poland (No. 60/2010/DTN). A total

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Table 1 – Characteristic of primers used for real-time PCR.

Gene	Gene name	Function	Accession number	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Ref.
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Glycolytic enzyme	NM 001009307	AGTATGATTCCACCCACGGCA	GATCTCGCTCCTGGAAGATGGT	[14]
RPL17	60S ribosomal protein L17	Ribosomal protein	AY738264	CTCTGGTCATTGAGCACATCC	TCAATGTGGCAGGGAGAGC	[14]
RPL30	60S ribosomal protein L30	Ribosomal protein	AY700577	CCTCGGCAGATAAATTGGACTGTC	TGATGGCCCTCTGGAATTTGAC	[14]
RPS7	40S ribosomal protein S7	Ribosomal protein	AY800278	GTCCCAGAAGCCGCACTTTGAC	CTCTTGCCCACAATCTCGCTCG	[14]
YWHAZ	Tyrosine 3-monooxygenase/ tryptophan 5-monooxy-genase activation protein	Binds to phosphatydylserine containing proteins	EF458621	GAAGAGTCCTACAAAGACAGCACGC	AATTTTCCCCTCCTTCTCCTGC	[14]
CYC	Cyclophilin	Isomerase involved in protein folding	AY029366	CCTTCTGTAGCTCGGGTCAG	CTTGGAGGGGAGGTAAGGAG	[2]
ACTB	β-Actin	Cytoskeletal protein	AB051104	ATCAAGGAGAAGCTGTGCTACGT	CGTTGCCGATGGTGATCA	[3]

All primers were purchased from Genomed (Warszawa, Poland).

of 11 domestic shorthair female cats (6–36 months old) were enrolled in the study. The cumulative information provided by inspection of the ovaries at ovariohysterectomy (OHE) [2] performed in the local veterinary clinics, blood progesterone concentration and information from the owner, were used to stage the estrous cycle of each animal. No pharmacological treatment was performed to provoke ovulation in the animals. Cats were assigned to: (1) luteal phase (LP) group ($n = 3$); (2) hormonally treated with octane medroxyprogesterone (OMP, Depo-Promone, Pfizer Animal Health, Louvain-la-Neuve, France) group ($n = 5$), in which animals had been treated with OMP (5 mg/animal/week) for one month to prevent an estrus behavior; and (3) pyometric (PYO) group ($n = 3$).

The uteri were washed immediately after surgery with saline, placed into fresh sterile saline (4 °C) and transported to the laboratory within 1 h. Uterine horns were cut longitudinally, pieces of endometrium (weighted 20–30 mg) were prepared, washed in fresh saline, kept overnight (4 °C) with RNAlater (Ambion Biotechnologie GmbH, Wiesbaden, Germany), and stored at −80 °C. Total RNA was isolated using TRIZOL-Reagent (Sigma–Aldrich, St. Louis, MO, USA). The RNA content was measured with a Nano-Drop™ 1000 Spectrophotometer (Thermo, Wilmington, DE, USA). The RNA quality was confirmed by electrophoresis on 1.5% agarose gel. Prior to reverse transcription, genomic DNA contamination was removed by treatment with DNase (Sigma–Aldrich). Reverse transcription was performed using the ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA) [3]. In brief, each RT reaction mixture (volume 20.5 μL) contained: 1 μg of RNA in 12 μL of reaction mix, 4 μL of reaction buffer, 2.5 μL of $MgCl_2$ (final concentration 3 mM), 1 μL of dNTP Mix (0.5 mM final concentration of each dNTP) and 1 μL of ImProm-II™ Reverse Transcriptase.

Real-time PCR was carried out in an automated fluorometer Viia 7 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using FAST-SYBR Green Master Mix (Applied Biosystems). RT-PCR was performed in 96-well plates. The sequences of specific primers and the GenBank accession numbers are depicted in Table 1. Each reaction mixture (20 μL) contained 4 μL of cDNA (39 ng); 1000 nM, 500 nM or 250 nM forward (2 μL) and reverse (2 μL) primers; 10 μL FAST-SYBR Green Master Mix; and 2 μL nuclease free water. Amplification was carried out as follows: denaturation for 20 s at 95 °C, followed by 40 cycles at 95 °C for 1 s and 60 °C for 20 s. After RT-PCR, melting curves were acquired in temperature from 60 °C to 95 °C to ensure that a single product was amplified in the reaction.

To determine the most stable reference gene, three free software packages that use different algorithms were used: geNorm [4], NormFinder [5] and BestKeeper [6]. GeNorm software package determines stability of gene expression by calculating the stability value (M). The low M value (<1.5) determines the most stable gene [4]. In addition, geNorm software determines the optimal number of reference genes needed for gene expression normalization. A pairwise variation (V) was calculated for two sequential normalization factors NF_n and NF_{n+1} (n = number of reference genes). When variation is lower than 0.15, using an additional reference gene is not required. NormFinder software package calculates gene expression stability based on the intra- and the inter-group variation. Genes characterized by the lowest gene expression stability value (ρ) are most stable [5]. BestKeeper software package estimates relations of possible reference gene pairs by performing numerous pairwise correlation analyses. Reference gene is assessed based on the average threshold cycle value (C_t) obtained from RT-PCR analysis, standard deviation (SD), coefficient of variance (CV) and coefficient of correlation (r). A good stability gene is characterized by low (<1) standard deviation value [6].

Differences in mRNA levels among the examined groups (LP, OMP, PYO) were analyzed by the Kruskal–Wallis test followed by the Newman–Keuls multiple comparison test using the statistical software program GraphPad6 (GraphPad