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Identification of Suitable Reference Genes for qPCR Analysis of 4T1 Mouse Mammary Tumor Cell Line

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ABSTRACT

Background: Identification of candidate reference genes for real time PCR study is a preliminary requirement to normalize experimental data and thus, deduce a reliable conclusion. Complex tissues like mouse mammary gland constitutes various cell types which makes it difficult to identify reference gene constantly expressing under different experimental conditions.

Methods: In this study we have identified suitable reference genes for 4T1 tumor cell line derived from mouse mammary tumor cells. We have studied four genes namely *Gapdh, Actb, Prdx1* and *Ctbp1* for their expression stability in CPV2.NS1 post transfected 4T1 cells by Best Keeper.

Result: By our study, three reference genes *i.e. Prdx1, Gapdh* and *Ctbp1* were found to be quite correlated with the BestKeeper index, but by considering all three criteria of selection by BestKeeper algorithm, *Prdx1* showed minimum standard deviation and coefficient of variation and was found to be ranked at first position by BestKeeper which suggests *Prdx1* to be considered as better internal control gene among all other reference genes taken in our study for qPCR based experiments in 4T1 mouse mammary tumor cell line transfected with CPV2.NS1.

Key words: Canine parvovirus, Mammary gland tumor, Non-structural gene 1, Oncolytic viral genes, Pearson correlation coefficient, Standard deviation.

INTRODUCTION

Gene expression studies in complex tissues are challenging due to the diversity of the cell types that constitute such tissues, each type with a different gene expression profile. The mouse mammary gland is one of such complex tissue that contains various cell types depicting varying gene expression under different conditions (Han *et al*., 2010). Mammary gland tumor is also an unusual physiological condition of the mammary gland in which there is an abnormal growth of cells and is quite possibly the most widely recognized oncologic disease in the world (Sleeckx *et al*., 2011).

In both humans and animals, cancer incidences are increasing day by day. After skin cancer, breast cancer is frequently reported cancer in women (Zaimy *et al*., 2017). Oncolytic viral gene therapy has recently gained significance for cancer treatment as all other traditional treatment modalities kill normal cells along with neoplastic cells. Therapeutic properties of various oncolytic genes (and their protein products) have been reported, which include VP3 (apoptin) of Chicken Infectious Anemia Virus (CIAV), E2 and E7 of Papillomaviruses; E1A 12S and 13S proteins, E3 and E4 of Adenovirus, tat of Human Immunodeficiency virus (HIV-1), Parvovirus NS1, Simian virus (SV40) large T antigen and Hemagglutinin-Neuraminidase (HN) protein of Newcastle Disease Virus (NDV). These genes, on ectopic expression, mediate targeted killing of tumor cells without causing harm to healthy cells (Gupta *et al*., 2015). Non structural gene 1 of Canine Parvovirus 2 (CPV2.NS1) is one such gene reported to have oncolytic effect and causes apoptosis through intrinsic/ mitochondrial pathway in cancer cell line and also induce anti-tumor immune response in various tumor models (Saxena *et al*., 2013; Santra *et al*., 2014; Gupta *et al*., 2016; Bhat *et al*., 2017). Quantitative real-time polymerase chain reaction (qRT-PCR) has recently emerged as a powerful tool to study gene expression in bodily tissues including the mammary glands Han *et al*. (2010).

Despite being versatile and flexible, qRT-PCR suffers some limitations in the number of genes that can be studied

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together. High throughput sequencing has however come a long way to overcome the problems by identifying gene expression changes on a large scale and by generating reproducible results. RNA sequencing is one such clear cut measure to get accurate expression data with minimum variation but it requires validation by other methods. Given its high sensitivity, qRT-PCR is most commonly used for such validation purposes but an indispensable requirement with qPCR is the selection of suitable endogenous/ reference genes to minimize variation (Sahu *et al*., 2018). Reference/Endogenous genes are those genes that are expressed uniformly in samples with different experimental conditions or treatments. Housekeeping genes are used mostly as internal control genes which normalize the variations in the qRT-PCR experiment (Tong *et al*., 2009; Köhsler *et al*., 2020). In the present study, the expression stability of four housekeeping genes namely *Prdx1, Ctbp1, Gapdh* and *Actb* was compared in the 4T1 cell line (Mice mammary tumor cell line) transfected with oncolytic Non-structural gene 1 of Canine Parvovirus 2 (CPV2.NS1).

MATERIALS AND METHODS

This study was conducted in the month of January-February, 2021 in Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly, Uttar Pradesh.

Cell culture and transfection

4T1 cells were acquired from ATCC (American Type Culture Collection; Rockville, MD) and grown in RPMI 1640 media (supplemented with 5% FBS, penicillin 100 U/ml, streptomycin 100 g/ml and L-Glutamine) (DuchefaBiocheme, Netherlands) at 37°C under 5% CO2. The CPV2.NS1 gene was available as pVIVO1.NS1 in our laboratory (Gupta *et al*., 2016). Transfection of 4T1 cells with pVIVO1.NS1 and pVIVO1 was carried out using Lipofectamine®3000 as per the manufacturer's instructions. The empty vector pVIVO1-transfected 4T1 cells were used as control samples. Cell pellet for RNA isolation and qPCR were collected from both empty vector and pVIVO1.NS1 transfected 4T1 cells at 24 hr and 48 hr post-transfection.

RNA extraction and cDNA synthesis

Total RNA was extracted from cell pellets collected in RNAlater (QIAGEN, Germany) using TRIzol reagent (Thermo Scientific, USA) as per manufacturer's guidelines. The RNA concentration and integrity was examined by Nanodrop spectrophotometer. The purity of all RNA samples was assessed by the absorbance ratios of OD260/280 and OD260/230. RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc, USA) was used for preparing cDNA from one microgram of RNA of each sample following the protocol provided in the product manual. The cDNA thus prepared were stored at -20°C till further use. The cDNA was diluted 10-fold for subsequent qRT-PCR analysis.

Primer designing and sequence of reference genes

Sequences for designing primers for all four reference genes were obtained from NCBI and primers were prepared by using software Primer Quest (https://www.idtdna.com). Primers for quality parameters were verified in Oligo Analyzer and NCBI Primer-BLAST. The sequences of all four primers are given in (Table 1).

Quantitative Real-time PCR (qRT-PCR)

Real time PCR was performed with Maxima SYBR Green qPCRMasterMix (Thermo Fisher Scientific Inc.,USA) using an Agilent AriaMx Real-Time PCR System. The thermal profile of the reaction started initially with 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15s, 56°C for 30s and 72°C for 30s. Each reaction included 5 µl of 2X SYBR Green Master mix, 0.1 µl (10 pmol) of endogenous control forward and reverse primer, 10 ng of cDNA and NFW to make up the volume to 10 µl. Finally, a melting curve was generated by increasing temperature from 95°C to 65°C for 30 sec each, followed by the final step of 95°C for 30 sec.

Gene expression stability analysis

Gene expression stability analysis of reference genes was conducted using the BestKeeper algorithm available at RefFinder (https:www.heartcure.com.au/reffinder/). Direct Cycle threshold (ct) values of samples were used as an input for the web-based tool RefFinder (Xie *et al*., 2012).

RESULTS AND DISCUSSION

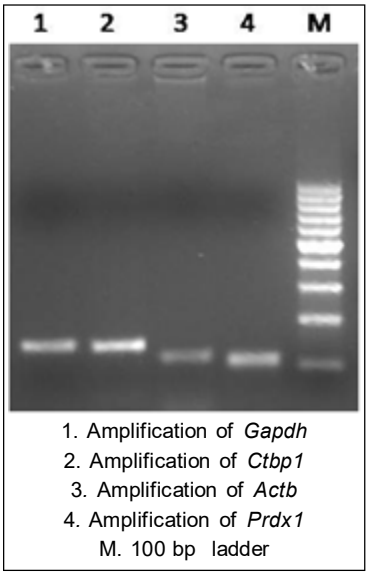
Real-time PCR is a highly sensitive method for absolute and relative quantification of gene expression. The reliability of qPCR data would further be greatly increased by including endogenous genes whose transcription level would be

Table 1: Primer Sequences of four reference gene used in validation study.

Primer	Gene symbol	Primer sequence	Size of product
Glyceraldehyde 3-phosphate dehydrogenase	*Gapdh*	F.P - GTATGACTCCACTCACG R.P -GACTCCACGACATACTC	150 bp
Peroxiredoxin 1	*Prdx1*	F.P -ACCTGGCAGTGATACCATCAAG R.P -TTCTTCTGGCTGCTCAATGC	113 bp
Beta-actin	*Actb*	F.P -GAATGGGTCAGAAGGAC R.P - CCAGATCTTCTCCATGTC	122 bp
C-terminal binding protein	*Ctbp1*	F.P -TGCCACATCCTGAACCTGTAC R.P - TAGTCCAATGATGCCCAAGGTC	147 bp

unwavering in the diverse experimental situations. Several approaches have been used to discover steadiness in gene expression and choose the best endogenous genes in the context of the relevant experimental conditions, but to date, there is no consensus on which technique we should rely on to study internal control gene expression stability. Among all four reference genes taken in our study, *Actb* (cytoskeletal structural protein- Beta actin) and *Gapdh* (glycolytic enzyme- Glyceraldehyde 3- phosphate dehydro genase) are traditional reference genes that are usually used for qPCR experiments whereas *Prdx*1 (peroxired oxins) and *Ctbp1* (C-terminal binding protein 1) are recently reported internal control genes for conducting qPCR based mouse mammary gland experimental studies (van de Moosdijk and van Amerongen, 2016).

For our qPCR data normalization in CPV2.NS1 transfected 4T1 mice mammary gland tumor cell line, we compared four housekeeping genes *i.e*. *Gapdh, Prdx1, Ctbp1* and *Actb* by the BestKeeper algorithm. BestKeeper calculates variation in gene expression for each reference gene based on Crossing points (CP) which is the number of cycles required to cross selected threshold fluorescence Mehta *et al*. (2010). On basis of these CP values, BestKeeper calculates standard deviation (SD) and Coefficient of variation (CV) for all housekeeping genes. By performing various pairwise correlation analyses, this expression analysis tool estimates relationships among all possible pairs of reference genes by using raw Ct values of each gene Pfaffl *et al*. (2004). Standard deviation (SD) of the endogenous genes is a key aspect to identify genes with maximum stability by BestKeeper software; the higher the coefficient of variation (CV) and standard deviation (SD) values, the more unstable the reference gene Bao *et al*. (2019). BestKeeper algorithm also assesses the correlation coefficient (r) of each reference gene under study to the BestKeeper index, as calculated from the geometric mean of the remaining reference genes. Generally, reference gene with SD values >1 are considered unstable and should be sorted out. So, overall three indicators are provided by BestKeeper to identify expression stability of a housekeeping gene which are CV, SD and correlation coefficient (r).



1. Amplification of *Gapdh*
2. Amplification of *Ctbp1*
3. Amplification of *Actb*
4. Amplification of *Prdx1*
M. 100 bp ladder

Fig 1: Amplication of four reference genes depicted in agarose gel.

Initially, four endogenous genes were amplified in the samples examined and single-band products of appropriate size were obtained by agarose gel electrophoresis (Fig 1). All genes in qRT-PCR assays produced one single peak in the melt curve analysis (Fig 2). After generating melt curve, average Ct values of triplicates of each reference gene were used for visualizing the reference gene showing stable expression and *Prdx1* Ct values were found to be varying less among both 24 and 48 hr post transfection in both control and treatment groups as compared to all other three reference gene in our study (Fig 3).

BestKeeper uses raw Ct values of all genes as input. Analysis of similar data with BestKeeper begins with the estimation of variations *i.e*., SD and CV for all the four candidate endogenous genes in the samples depicted in (Table 2 and 3). In the case of 4T1 cells with 24 hr post-transfection, not a single housekeeping genes under study showed an SD>1 indicating that all of the genes under study were appropriate enough to be considered for selecting them as endogenous genes. But in 48 hr post-

Table 2: Crossing points (CP) data of housekeeping genes by Best Keeper for 24 mhr post. transfection.

N	*Ctbp1*	*Gapdh*	*Prdx1*	*Actb*
	6	6	6	6
geo mean [CP]	21.86	17.14	21.56	16.47
AR mean [CP]	21.87	17.16	21.57	16.50
min [CP]	21.15	16.53	20.80	15.46
max [CP]	22.61	17.87	22.24	17.49
std dev [+/- CP]	0.70	0.59	0.55	0.98
CV [% CP]	3.21	3.44	2.56	5.95
min [x-fold]	-1.64	-1.53	-1.69	-2.02
max [x-fold]	1.68	1.65	1.60	2.02
std dev [+/- x-fold]	1.63	1.51	1.47	1.98

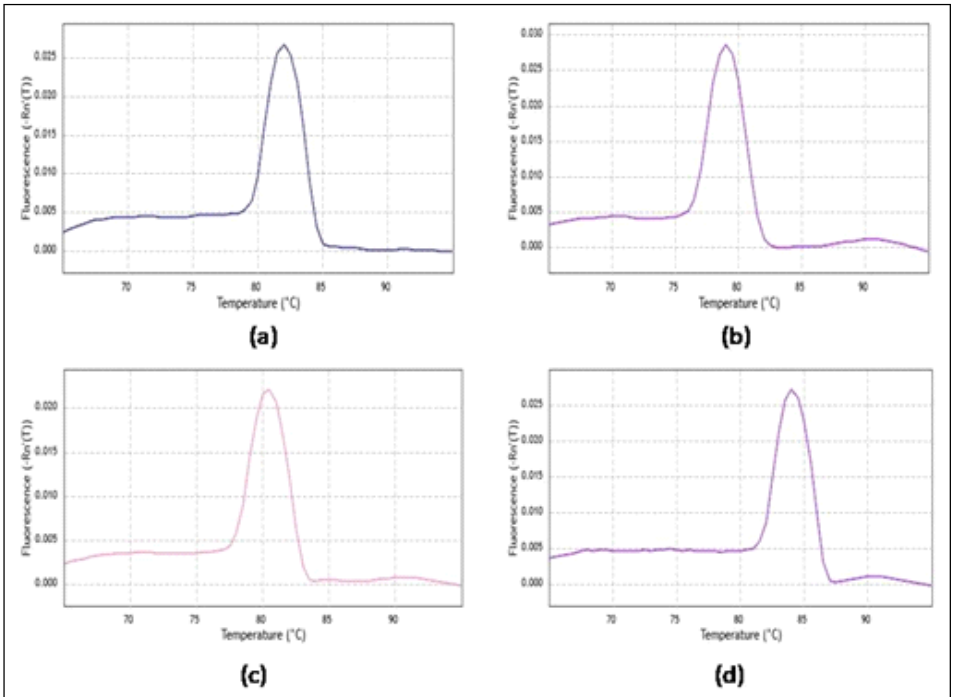
Table 3: Crossing points (CP) data of housekeeping Genes by Best Keeper for 48 hr post transfection.

N	*Ctbp1*	*Gapdh*	*Prdx1*	*Actb*
	6	6	6	6
geo mean [CP]	21.00	17.87	21.71	16.85
AR mean [CP]	21.02	17.88	21.71	16.89
min [CP]	19.89	17.30	21.31	15.51
max [CP]	22.14	18.42	22.14	18.22
std dev [+/- CP]	0.98	0.38	0.30	1.26
CV [% CP]	4.65	2.13	1.37	7.47
min [x-fold]	-2.16	-1.49	-1.32	-2.52
max [x-fold]	2.20	1.46	1.35	2.59
std dev [+/- x-fold]	1.97	1.30	1.23	2.40

transfection, *Actb* had SD more than 1 which eliminates this reference gene from our consideration.

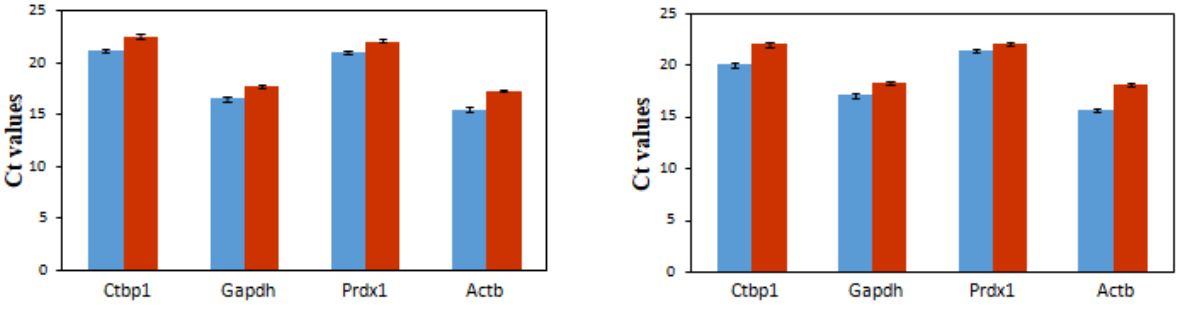
Further data processing involved consideration of CV and Pearson correlation coefficient (r) of all candidate reference genes. In 4T1 cells with 24 hr post-transfection, the Pearson correlation coefficient for all reference genes was quite high which shows a strong resemblance among inter-genes and with BestKeeper index (Table 4). But by taking CV as criteria for selection of endogenous gene, two genes *i.e.*, *Ctbp1* and *Actb* with the highest variation can be excluded. Of the remaining two genes *i.e.*, *Gapdh* and *Prdx1*, minimum variation was shown by *Prdx1* which suggest the significance of this gene for taking as an endogenous control for qPCR experiments.

In the case of 4T1 cells with 48 hr post-transfection, the reference gene having SD>1 *i.e.* Actb was previously eliminated. Further data analysis with *Gapdh*, *Ctbp1* and *Prdx1* was done for their expression stability. Pearson correlation coefficient was high for all these remaining three genes but deviation from the mean (CV) was highest for *Ctbp1* which warrants the exclusion of this gene (Table 5). Between *Gapdh* and *Prdx1*, the Correlation coefficient washigh for both, but *Prdx1* due to minimum variation and $p<0.001$ can be considered as a better option among both endogenous genes for qPCR based studies in 4T1 mouse mammary tumor cell line. The ranking of four candidate reference genes on the basis of variation from the most stable to the least stable for both 24 and 48 hr is depicted in Fig 4.



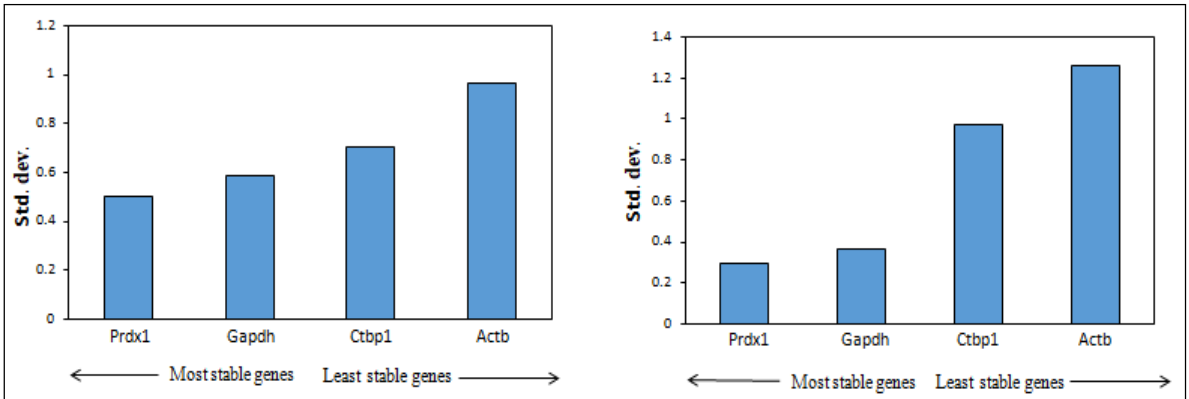
(a) Melt Curve of *Gapdh;* (b) Melt Curve of *Prdx1*; (c) Melt Curve of *Actb*; (d) Melt Curve of *Ctbp1*.

Fig 2: Single peak observed by each reference gene in Melting Curve Analysis.



(a) 24 hour post transfection; (b) 48 hour post transfection.

Fig 3: Visualization of reference gene with stable expression in both control (Blue colour) and treatment (Red colour) groups at both 24 and 48 hr post transfection by qPCR.



(a) 24 hour post transfection; (b) 48 hour post transfection.

Fig 4: Ranking of most stable to least stable reference genes by BestKeeper.

Table 4: Best Keeper correlation analysis (24 hr post transfection).

BestKeeper vs.	*Ctbp1*	*Gapdh*	*Prdx1*	*Actb*
Coeff. of corr. [r]	0.997	0.994	0.978	1.000
p-value	0.001	0.001	0.001	0.001

Table 5: Best Keeper correlation analysis (48hr post transfection).

BestKeeper vs.	*Ctbp1*	*Gapdh*	*Prdx1*	*Actb*
Coeff. of corr. [r]	0.999	0.965	0.974	0.998
p-value	0.001	0.002	0.001	0.001

So, in conclusion, three reference genes *i.e. Prdx1, Gapdh* and *Ctbp1* were found to be quite correlated with the BestKeeper index, but by considering all three criteria of selection by BestKeeper algorithm, *Prdx1* showed minimum variation and was found to be ranked at first position by BestKeeper in both time intervals which suggests *Prdx1* to be considered as better internal control gene in 4T1 mouse mammary tumor cell line transfected with CPV2.NS1 for qRT-PCR experiments.

Author contributions

RA and AKT conceived the idea for the article and prepared the first draft of the manuscript. RA and WAM searched the relevant literature. AT and SS helped in drafting the manuscript. BS and SM contributed by critically revising its contents. All the authors have read and approved the final version of the manuscript and agree to be accountable for its contents.

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Conflict of interest

The authors declare no conflict of interest.

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