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Validation of putative reference genes for gene expression studies in heat stressed and α-MSH treated melanocyte cells of *Bos indicus* using real-time quantitative PCR

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ABSTRACT

Normalization of cellular mRNA data using internal reference gene (IRG) is an essential step in expression analysis studies. MIQE guidelines ensure that the choice and appropriateness of IRG should be validated for particular tissues or cell types and specific experimental designs. The objective of the present study was to assess 15 IRGs from different functional classes that could serve as best IRGs for *Bos indicus* (Tharparkar cattle) melanocyte cells under heat stress and hormonal treatment. We implemented the use of geNorm, NormFinder and BestKeeper algorithm to measure the stability of the gene transcript. A total of 15 IRGs (ACTB, BZM, EEF1, GAPDH, GTP, HMBS, HPRT, RPL22, RPL4, RPS15, RPS18, RPS23, RPS9, UBC and UXT) from different functional classes were evaluated. Pair wise comparisons using geNorm revealed that HPRT and RPS23 were the most stable combination of IRGs with M-value of 0.29 followed by UXT (0.30) and RPL4 (0.31). The NormFinder analysis also identified the same set of stably expressed genes (UXT, RPL4, RPS23 and HPRT); however, the rank order was little different. The UXT gene showed lowest crossing point SD and CV values of 0.30 and 1.17, respectively indicating its maximum expression stability through BestKeeper analysis. The present study indicated that, ACTB and HMB were not reliable IRGs for melanocytes cells on account of their lower expression stability. Current study further revealed that UXT, HPRT and RPS23 are the best IRGs for normalization of qPCR data in *Bos indicus* melanocyte cells under heat stress and hormonal treatment.

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1. Introduction

Thermal stress is a major concern for all livestock keepers in most of the tropical countries. The animals exposed to thermal stress try to acclimatize to adverse conditions with the help of phenotypic responses to environmental changes [1]. High ambient temperatures, both directly and indirectly affect production and health of farm animals resulting in significant loss to animal production systems [2,3]. The skin being largest organ of body has the function to protect the internal body structure from a hostile external environment of varying pollution, temperature, humidity and radiations. Melanocytes are the cells that produce melanin, a pigment in the skin, eyes, and hair. This skin pigment is the most important photo-protective factor, since melanin, besides functioning as a broadband UV absorbent, has antioxidant and radical

List of abbreviations: α-MSH, Alpha melanocyte stimulating hormone; IRGs, Internal reference genes; ACTB, b-actin; B2M, Beta 2-microglobulin; EEF1, Eukaryotic translation elongation factor 1 alpha 1; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GTP, GTP binding protein; HMBS, Hydroxymethylbilane synthase; HPRT1, Hypoxanthine phosphoribosyltransferase; RPL22, Ribosomal protein L22; RPL4, Ribosomal protein L4; RPS15, Ribosomal protein S15; RPS18, Ribosomal protein S18; RPS23, Ribosomal protein S23; RPS9, Ribosomal protein S9; UBC, Ubiquitin C; UXT, Ubiquitously expressed transcript.

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scavenging properties [4]. Quantitative real time polymerase chain reaction (qPCR) is an effective tool to measure gene expression which can also be used in characterizing the transcriptional response of melanocyte cells. Reference genes, frequently termed as "housekeeping genes," are used to normalize the expression results for differences in cDNA quantity between different specimens enabling comparisons between genes of interest across treatments [5]. In order to act as a reference, a housekeeping gene's expression should remain unchanged regardless of the treatment [6]. Previously, it was assumed that issues related to experimental variation and normalization could be resolved by using total amount of cells/tissue RNA and use of 18S or 28S ribosomal RNA as an internal control [7]. However, these approaches were reported to have serious limitations [8]. Use of internal reference genes (IRGs) or housekeeping genes with constant expression levels between samples in response to experimental treatment or physiological state are now being considered as effective strategies for normalization of qPCR data to account for the experimental variations [8–11]. To date, not even a single gene or set of genes have been termed as universal IRGs that can be used for all types of experimental conditions and protocols. Few genes that were stably expressed under particular experimental conditions have also been observed to vary and regulated otherwise. Therefore, each new experimental condition and species under study requires proper evaluation of IRGs prior to qPCR normalization in order to avoid variations and errors [12–16]. The information on cattle melanocytes is meagre and to our knowledge, no reports are available on identification of suitable IRGs in melanocyte cell line of cattle. Understanding that the choice of suitable IRGs is crucial for accurate expression profiling of target genes, the present study was aimed to identify the most appropriate reference genes for analysis of transcriptional response of cattle melanocyte cells under heat stress and with α-MSH treatment.

2. Material and methods

2.1. Ethical permission

The work under study was approved by the Institutional Animal Ethics Committee (IAEC) constituted as per the article number 13 of the CPCSEA–rules, laid down by Government of India norms regarding the ethical treatment of animals.

2.2. Isolation and culture of melanocyte cells

Skin biopsy specimens were aseptically collected from the ear pinna of healthy three *Bos indicus* heifers (Tharparkar cattle) in the sterile DPBS and transported to the laboratory for further processing. The tissues were washed thoroughly with DPBS and transferred into a cell culture dish containing 2 ml culture medium (melanocyte basal medium) supplemented with Bovine Pituitary Extract (0.004 ml/ml), Basic Fibroblast Growth Factor (recombinant human) (1 ng/ml), Insulin (recombinant human) (5 μg/ml), Hydrocortisone (0.5 μg/ml), Phorbol Myristate Acetate (10 ng/ml), Penicillin (100 U/ml) and Streptomycin (100 μg/ml). The tissue was minced into small pieces (about 1 mm in size) using a sterile surgical blade and washed five times in the culture medium followed by seeding into 60 mm tissue culture dishes. The small amount of medium sticking to the tissues was sufficient to nourish them till their attachment. The dishes were incubated for 4–8 h in a 5% CO_2 incubator and observed at different intervals so as not to allow them to dry out completely. Upon attachment of tissues, sufficient culture medium was added to the dishes and incubated in a 5% CO_2 incubator at 37 °C. Tissue explants were regularly observed for proliferation of melanocyte and were removed aseptically when sufficient number of cells had proliferated and formed a monolayer on the cell culture dishes. After reaching 70–80% confluence, the melanocyte cells were sub-cultured by partial trypsinization. The cells were subjected to 25–30 continuous passages for establishment of melanocyte cell line. The melanocyte cells were routinely evaluated for sterility by growing them in antibiotic free media. For heat and α-MSH treatment, melanocytes were divided into the following four groups each in triplicate: Group 1 (control): was kept at 37 °C, Group 2: was kept at 37 °C with 50 ng/ml α-MSH, Group 3: was kept at 42 °C and Group 4: was kept at 42 °C with 50 ng/ml α-MSH for 3 h.

2.3. Characterization of melanocytes

2.3.1. Morphological examination

Purity of cells at different time intervals after initiation of culture was performed by microscopic observation of cell morphology. Cells were classified morphologically as melanocytes based on a characteristic dendritic morphology with multiple long processes. For the visualization of tyrosinase activity, a specific histochemical marker of melanocytes, was used which further confirmed the purity and phenotypic characteristics of melanocytes [17].

2.4. Total RNA extraction and cDNA synthesis

Total RNA was isolated from all the 4 groups of melanocytes (triplicate of each group) at 3 h of culture using RNeasy Mini Kit (Qiagen India Pvt. Ltd.) according to the manufacturer's protocol. RNA integrity was assessed in 1.5% agarose gel electrophoresis by observing rRNA bands corresponding to 28S and 18S. Possible genomic DNA contamination in RNA preparation was removed by using the RNase-Free DNase treatment (Qiagen India Pvt. Ltd.). Purity of the RNA was checked in a Bio Spec Nandrop (Shimadzu, Japan) with the ratio between 2 and 2.1 at OD 260 nm–280 nm. For each group including all the replicates, about 200 ng of RNA was used for cDNA synthesis using Revert Aid First strand cDNA synthesis Kit (Fermentas, USA) by reverse transcription (RT) PCR according to the manufacturer's protocol. The RT reaction was carried out at 65 °C for 5 min, 42 °C for 60 min, 70 °C for 5 min in a thermal cycler (Bio-Rad, USA).

2.5. Primer designing and validation

The primers were selected either from literature or designed using Primer express 3.0 software (Applied Biosystems, Carlsbad, CA, USA) with minimum amplicon size ranging between 50 and 119 bp. Prior to qPCR, primers specificity was further confirmed in a 20-μL PCR reaction using the same protocol described for qPCR except for the final dissociation protocol. 5 μL of the PCR product was run in 2.4% agarose gel. Primer details for all genes have been given in Table 1.

2.6. Quantitative PCR (qPCR) assay

The quantitaive PCR reaction was carried out on cDNA in duplicate (for each group with $n = 6$) in a Real-Time PCR system (Applied Biosystem 7500, USA). Reactions were carried out in a 96-well plate. Each reaction was performed in a final volume of 15 μL containing 4 μL diluted cDNA, 0.5 μL of 200 nM of each specific primer, 7.5 μL of 2X SYBR Green Master mix (Thermo Scientific, USA) and 2.5 μL DNase/RNase-free water. For each gene, samples were run in duplicate along with six-point relative standard curve and the non-template control (NTC). The first segment of the amplification cycle comprised of a denaturation programme of 5 min at 95 °C, followed by the second segment that comprised of

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