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pH but Not Hypoxia Affects Neonatal Gene Expression: Relevance for Housekeeping Gene Selection

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Abstract

Objective—To identify a candidate neonatal housekeeping gene and to determine the effects of pH and PaO₂ on the stability of newborn gene expression in physiologically hypoxic and acidotic newborn blood.

Methods—Quantitative (RT)-PCR amplification was performed for 4 commonly used housekeeping genes (*GAPDH*, *β-actin*, *cyclophilin*, *28S rRNA*) on extracted RNA. Blood gas analyses determined pH and PaO₂ levels.

Results and Conclusions—*β-Actin* was least variable and *GAPDH* was most variable housekeeping gene studied. pH negatively correlated with gene expression levels. PaO₂ levels did not significantly affect gene expression. These results inform selection of housekeeping genes for neonatal mRNA research.

Keywords

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR); housekeeping gene; newborn gene expression

INTRODUCTION

Identifying constitutively-expressed housekeeping genes is essential for proper normalization of mRNA expression, particularly when using semi-quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) amplification, which relies on comparative gene expression for quantification. Although qRT-PCR is arguably the most sensitive technique available for mRNA quantification, multiple variables including sample quality, amplification efficiency, operator variability and subjectivity in data analysis can limit its accuracy and therefore its biological applicability (1). Selection of the ideal housekeeping gene(s) depends on many factors, including cell or tissue type, pathological conditions, and whether the experimental conditions simulate normal physiology.

In contrast to other human tissue, newborn blood is physiologically hypoxic and relatively acidotic. These physiologic conditions introduce new variables to consider when identifying

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a proper housekeeping gene. Furthermore, a stable, constitutively-expressed housekeeping gene for newborn blood studies has yet to be identified. Here, we examined the stability of four commonly used housekeeping genes, glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*), *cyclophilin A*, β -*actin* and *28S rRNA*, in neonatal whole blood samples with varying degrees of hypoxia and acidosis. Additional analysis included determination of the effects of physiologic hypoxia and acidosis on neonatal gene expression.

MATERIALS AND METHODS

The Tufts-New England Medical Center Institutional Review Board approved this study. After obtaining informed parental consent, umbilical whole blood samples (7.5 cc) were obtained in PaxGene™ (PreAnalytiX, Hombrechtikon, Switzerland) tubes immediately following cesarean delivery at term (n=15). Samples were stored at room temperature for >2 hours but < 30 hours prior to RNA extraction according to the PaxGene™ blood RNA kit (PreAnalytiX) manufacturer's protocol. During extraction, on column DNase digestion (RNase-Free DNase Set, Qiagen, Hilden, Germany) was performed to eliminate DNA contamination. Quality of extracted RNA was assessed by the presence of peaks at 18S and 28S with the Agilent™ Bioanalyzer 2100 (Foster City, CA, USA) prior to qRT-PCR.

One-step qRT-PCR was performed on extracted total RNA for four commonly used housekeeping genes: *GAPDH*, β -*actin*, *cyclophilin A*, and *28S rRNA*. All samples were run on the Perkin-Elmer Applied Biosystems 7900 Sequence Detector with the TaqMan® One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems, CA) as previously described (2). PCR plates were run for two housekeeping genes in batches of five samples. All samples were run in triplicate, with negative controls run on each plate. Amplification primer and probe sequences were chosen either if they had been previously reported in the literature (*GAPDH*) (3) or with *Primer Express*® software v1.0 (*cyclophilin A*, *28S rRNA*, β -*actin*) (Table 1).

Calibration curves for all sequences were prepared with serial dilutions from commercially available total RNA (Applied Biosystems, CA). Curves were optimized based upon the following criteria: a slope of -3.3 (± 0.4); y-intercept of 40 cycles (± 5 cycles); curve correlation coefficient of ≥ 0.98 . All unknown values for each sample fell within the upper and lower limits of detection on each curve. Thresholds were manually adjusted for each curve by the same operator to minimize variation.

The thermal cycle profile for all transcripts was as follows: the reaction was initiated at 48°C for 30 minutes for the uracil N-glycosylase to act, followed by reverse transcription at 60°C for 30 minutes. Following a 5 minute denaturing cycle at 95°C, 40 cycles of PCR were performed with 20 seconds of denaturing at 94°C, then 1 minute at 60°C for annealing and extension.

An additional 0.5cc of whole blood was collected at time of delivery from the same umbilical vessel punctured to obtain blood for RNA analysis. Blood was drawn into a heparinized syringe and placed immediately on ice to minimize the known consumption of oxygen by leukocytes. A blood gas analysis was performed on the Bayer™ Rapidpoint 405 Blood Gas Analyzer (Bayer Diagnostics Corporation, Medfield, MA, USA) within 15 minutes of acquisition. PaO₂ and pH were considered in the statistical analysis.

Statistical analysis was performed using SAS software (version 9.1.3). Parameters to estimate batch to batch variation were computed using the *proc mixed* procedure using a random effect model. Coefficients of variation were calculated to determine the least variable gene. Spearman correlation analysis was carried out to estimate the relations of gene expression with pH and PaO₂ levels while adjusting for sample, batch, and gene.

RESULTS AND DISCUSSION

Mean arterial oxygen tension (PaO_2) was 22.4 mmHg (range: 10.6–34.1). Mean pH was 7.29 (range: 7.11–7.37). β -actin showed the least variability in expression, while *GAPDH* was the most variable as measured by log-transformed quantitative values (Figure 1A). *Cyclophilin A* amplified earlier than all other housekeeping genes as measured by threshold cycle (C_T), and therefore had the highest concentration in newborn whole blood. *28S rRNA* amplified the latest and had the lowest concentration (Figure 1B). pH negatively correlated with gene expression levels ($r^2 = -0.33$; $p = 0.01$) (Figure 2). Although PaO_2 levels did not significantly affect gene expression levels ($r^2 = 0.03$; $p = 0.79$) (Figure 3), PaO_2 was positively correlated with pH ($r^2 = 0.59$; $p < 0.001$).

The use of qRT-PCR is an essential component for transcriptomic studies, either as a direct measurement of a specific gene's expression or as a confirmatory method following microarray analyses. However, several authors have recently highlighted the limitations of qRT-PCR, suggesting the use of multiple housekeeping genes for normalization (4), or the utilization of a computational model for accurate qRT-PCR expression profiling (5). Physiologic conditions are another important factor to consider when identifying appropriate housekeeping genes for the normalization of mRNA expression. The purpose of our study was to identify a stable housekeeping gene for newborn blood given its inherent physiological variability.

In this study, β -actin demonstrated the least variability and *GAPDH* had the most variability across multiple neonatal whole blood samples with varying degrees of hypoxia and acidosis. The primer and probe sequences for *GAPDH* used in this study are identical to sequences cited in over 1000 recent research articles (www.google scholar.com). Despite the prevalence of *GAPDH* in the literature, our study suggests that for newborn blood obtained following delivery, *GAPDH* is not the housekeeping gene of choice. Similar studies have shown that *GAPDH* shows varying expression in both peripheral blood and hypoxic tissue (6,7). β -actin is a more preferable choice for the normalization of mRNA in newborn whole blood, as it appears more stable in the unique physiological environment of neonatal blood.

Of the genes studied, *cyclophilin A* had the highest concentration and *28S rRNA* had the lowest concentration in neonatal whole blood. This was an interesting finding for two reasons. First, *cyclophilin A* gene expression has been shown to vary significantly in different gestational tissues and is expressed in low levels in the placenta (8). Our findings suggest that in newborn blood, *cyclophilin A* levels are high, and support the hypothesis that its expression in the fetus is tissue dependent. Second, given that ribosomal RNA comprises the majority of total RNA, one might expect that *28S rRNA* would have the highest measurable expression levels. Since we did not find this, it is possible that the choice of amplicon sequence contributed to the low concentration levels observed.

Interestingly, physiologic hypoxia does not appear to alter gene expression, unlike a previous study in which simulated hypoxia had a direct effect on the expression of similar housekeeping genes (6). Zhong and colleagues showed that while levels of *28S rRNA* were constant under simulated hypoxic conditions, *GAPDH*, β -actin, and *cyclophilin A* varied widely with hypoxia. In the present study, we had clear documentation of hypoxia obtained from the same samples used for gene expression. We theorize that the lack of variation of gene expression seen in our study was due to the fact that hypoxia in neonatal blood is normal and is not part of a pathological or simulated process.

Conversely, pH had a significant effect on gene expression. This may be a result of the more pathologic transient acidosis that occurs at the time of delivery. *In utero*, fetal blood is not acidotic (mean umbilical artery pH: 7.35; mean umbilical vein pH: 7.38) (9). However, a

transient acidosis occurs during delivery (mean umbilical pH at term: 7.28–7.29) (10) that is likely due to the impaired blood flow to the fetal-placental unit at time of delivery. We speculate that this transient acidosis at time of newborn delivery is a more pathological process compared to the chronic physiologic hypoxia of the newborn, and as such has a direct effect on gene expression.

In summary, neonatal blood is a unique human specimen that is physiologically hypoxic and acidotic, and deserves special consideration when used in transcriptomic studies. Our study has identified one constitutively expressed gene, *β-actin*, that remains stable under varying physiological conditions, and could be used preferentially as a housekeeping gene in studies that use neonatal blood samples acquired at delivery. The use of *GAPDH* as a normalization gene should be avoided in newborn whole blood. While physiologic hypoxia does not affect gene expression levels, the more pathologic transient acidosis that occurs at delivery, significantly impacts housekeeping gene expression in newborn blood. It is critical to consider the effect of pH on neonatal gene expression in future transcriptomic studies.

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REFERENCES

1. Bustin SA, Nolan T. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. *J Biomol Tech* 2004;15:155–166. [PubMed: 15331581]
2. Livak KJ. Allelic discrimination using fluorogenic probes and the 5' nuclease assay. *Genet Anal* 1999;14:143–149. [PubMed: 10084106]
3. Ng EKO, Tsui NBY, Lam NYL, Chiu RWK, Yu SCH, Wong SC, Lo ES, Rainer TH, et al. Presence of filterable and nonfilterable mRNA in the plasma of cancer patients and healthy individuals. *Clin Chem* 2002;48:1212–1217. [PubMed: 12142376]
4. Tricarico C, Pinzani P, Bianchi S, Pagleirani M, Distatne V, Pazzagli M, Bustin SA, Orlando C. Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies. *Anal Biochem* 2002;309:293–300. [PubMed: 12413463]
5. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;7:0034.1–0034.11.
6. Zhong H, Simons JW. Direct comparison of GAPDH, *β-actin*, cyclophilin, and 28S rRNA as internal standards for quantifying RNA levels under hypoxia. *Biochem Biophys Res Commun* 1999;259:523–526. [PubMed: 10364451]
7. Pachot A, Blond JL, Mouglin B, Miossec P. Peptidylpropyl isomerase B (PIPB): a suitable reference gene for mRNA quantification in peripheral whole blood. *J Biotechnol* 2004;114:121–124. [PubMed: 15464605]
8. Sehringer B, Zahradnik HP, Deppert WR, Simon M, Noethling C, Schaefer WR. Evaluation of different strategies for real-time RT-PCR expression analysis of corticotrophin-releasing hormone and related proteins in human gestational tissues. *Anal Biochem* 2005;383:768–775.
9. Cunningham, FG.; Gant, NF.; Leveno, KJ.; Gilstrap, LC., III; Hauth, JC.; Wenstrom, KD., editors. *Williams Obstetrics*. New York: McGraw-Hill Co, Inc.; 2001.
10. Yeomans ER, Hauth JC, Gilstrap LC 3rd, Strickland DM. Umbilical cord pH, PCO₂, and bicarbonate following uncomplicated term vaginal deliveries. *Am J Obstet Gynecol* 1985;151:798–800. [PubMed: 3919587]

A

B

Figure 1.**Figure 1A.** Log-transformed quantitative values in pg/5 μ L (Log QT) for each gene.**Figure 1B.** Threshold cycle (C_T) reflects the cycle number at which the fluorescence generated within a reaction crosses the threshold. It is inversely correlated to the logarithm of the initial copy number. The C_T value assigned to a particular well reflects the point during the reaction at which a sufficient number of amplicons have accumulated. In both figures, the boxes mark the interval between the 25th and 75th percentiles. The lines inside the boxes denote medians. The whiskers denote the interval between the 10th and 90th percentiles. The circles represent outliers.

Figure 2. Correlation between gene expression level (logarithm scale) and pH with fitted linear regression line for each gene: A. *GAPDH*; B. *β -actin*; C. *Cyclophilin A*; D. *28S rRNA*.

Figure 3.
Correlation between gene expression level (logarithm scale) and PaO₂ with fitted linear regression line for each gene: A. *GAPDH*; B. *β-actin*; C. *Cyclophilin A*; D. *28 rRNA*.

Table 1

Genes and Sequences Used for RT-PCR

Gene	Accession Number	Amplicon Sequences	Melting temperature (T _m) (°C)	G-C content (%)
<i>β-actin</i>	NM_001457	Sense: 5'-GGAAGGTCCGTGCGAGG-3'	Sense: 59	Sense: 71
		Antisense: 5'-GAGACGGAGCAGGTCCCA-3'	Antisense: 58	Antisense: 67
		Probe: 5'-(6-FAM)-CAAAATCGAGTGCTCCGACAATGGTG-(TAMRA)-3'	Probe: 68	Probe: 50
<i>Cyclophilin A</i>	NM_021130	Sense: 5'-GCCATGGAGCGCTTTGG-3'	Sense: 60	Sense: 65
		Antisense: 5'-TCCACAGTCAGCAATGGTGATC-3'	Antisense: 60	Antisense: 50
		Probe 5'-(6-FAM)-TCCAGGAATGGCAAGACCAGCAAGA-(TAMRA)-3'	Probe: 69	Probe: 52
<i>GAPDH</i>	NM_002046	Sense: 5'-GAAGGTGAAGGTCGGAGTC-3'	Sense: 53	Sense: 58
		Antisense: 5'-GAAGATGGTGATGGGATTTC-3'	Antisense: 53	Antisense: 45
		Probe: 5'-(6-FAM)-CAAGCTTCCCGTTCTCAGCC-(TAMRA)-3'	Probe: 61	Probe: 60
<i>28S rRNA</i>	NM_032014	Sense: 5'-ACACGCGCCGCACTACA-3'	Sense: 60	Sense: 65
		Antisense: 5'-TCCAGGTTACCTGTGTGCAGC-3'	Antisense: 60	Antisense: 57
		Probe: 5'-(6-FAM)-CGCCACCGTAAAGGCTGGCT-(TAMRA)-3'	Probe: 68	Probe: 67