How long does the mRNA remains stable in untreated whole bovine blood?

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Abstract

Background High quality and quantity of messenger RNA (mRNA) are required for accuracy of gene expression studies and other RNA-based downstream applications. Since RNA is considered a labile macromolecular prone to degradation, which may result in falsely altered gene expression patterns, several commercial stabilizing reagents have been developed aiming to keep RNA stable for long period. However, for studies involving large number of experimental samples, the high costs related to these specific reagents may constitute a barrier.

Methods and results In this context the present study was designed aiming to evaluate the stability of mRNA in whole bovine blood collected in EDTA tubes during storage at common fridge (4 °C). Whole blood samples were collected from six Holstein calves and submitted to RNA extraction in each different interval: immediately after blood sampling (< 2 h), at 1-day post-sampling (dps), 2 dps, 3 dps, 7 dps and 14 dps intervals. RNA integrity and purity were evaluated, and RT-qPCR assays were run using seven different genes (B2M, ACTB, PPIA, GAPDH, YWHAZ, CD4 and IFN-γ) aiming to evaluate the presence of altered gene transcription during storage. All extracted RNA samples presented high purity, while optimal integrity and unaltered gene expression were observed in whole experimental group up to 3 days of storage.

Conclusion Bovine blood RNA remained stable in K3EDTA tubes for 3 days stored at common fridge and can be successfully and accurately used for gene expression studies.

Keywords RNA integrity · Gene expression · Cattle · Stability · Storage · Fridge

Introduction

Messenger RNA levels may constitute a good indicator of physiological and/or immune status of cells, addressing the host responses under different environmental conditions and targeting the better understanding of mechanisms of host–pathogen interactions.

Several studies targeting the quantification of gene expression profiles have been performed to better understand human and veterinary diseases [1–8]. In this context, blood specimens are the most common applied matrix since peripheral blood may be considered less invasive and valuable source of RNA in vertebrates [9]. However, the RNA blood is highly vulnerable to degradation, which may consequently influence on the results of RNA studies to varying level [9].

The appropriated storage of samples, before the RNA extraction procedure, is of utmost importance to keep the RNA quality. Therefore, different methods have been applied to avoid RNA degradation. Snap frozen using liquid nitrogen (−180 °C) is one of the most common ways, though cryopreservation is not always possible or practical. Stabilizing reagents are also commonly applied, such as PAXgene blood RNA tubes (PreAnalytix, Qiagen/BD, Hombrechtikon, Switzerland), RNAprotect Animal Blood Tubes (Qiagen, Venlo, Netherlands), Tempus Blood RNA tubes (Applied Biosystems, Foster City, CA) and RNAlater stabilization reagent (Thermofisher, Waltham, MA). Although, all these reagents, except for RNAlater reagent, require proper commercial RNA...
with RNAlater or Paxgene Blood RNA tubes [9].

A second important drawback associated to most of these blood collection devices is due to the high amounts of globin mRNA in human blood, released from reticulocytes during total cell lysis immediately after sample collection, which may lead to decreased RT-qPCR detection sensitivity of rare mRNA transcripts and/or increases of signal variation on microarrays [10]. Additional globin depletion steps were performed to circumvent these problems, but this strategy was pointed out as inefficient, besides the requirement of related new costs [11]. Finally, most of these reagents are also not applicable for studies targeting the specific cell subsets since all blood cells are lysed when added to the stabilizing reagent. On the other hand, very low levels of globin mRNA transcripts were found in total RNA isolated from equine and bovine peripheral blood, therefore, the need for globin depletion in these species may be not required [12].

Different pre-treatment methods for RNA extraction were compared for human blood specimens, from whole blood or buffy coat isolation, followed or not by RNA stabilization with Trizol Reagent, TRizol LS Reagent, RNAlater or Paxgene Blood RNA tubes, wherein the method using a buffy coat pre-separation added directly to Trizol Reagent yielded the highest RNA integrity and quality for RNA extraction, emphasizing here the superior quality compared to pre-treatments with RNAlater or Paxgene Blood RNA tubes [9].

Regarding the stabilization of cellular RNA in blood stored at room temperature, some studies have demonstrated RNA degradation in whole blood samples longer than 32 h, but when whole blood samples were stored at low temperature (4 °C), the RNA integrity remained stable at duration of 40 h [13]. Since transcription and translation can continue after collection of samples, the final RNA composition may present differences compared to RNA content at the moment of collection [8]. In this context, altered transcription levels were also observed in whole blood samples stored at room temperature after 24 h [13, 14] or at low temperature [14].

Most of studies targeting the RNA stabilization in whole blood samples were performed in human specimens, and to our knowledge there are no similar investigations for bovine whole blood. Thus, the aim of this study was to evaluate the storage time that RNA remains stable in bovine whole blood, regarding purity, integrity and unaltered gene transcription profiles at common fridge (4 °C).

### Material and methods

#### Sample collection and storage intervals in common fridge

Thirty-six blood samples (six samples per animal) were collected from the jugular vein from six Holstein calves, using a vacuum system (Vacutainer®, Becton Dickinson) containing EDTA anticoagulant. After collection, the blood samples were stored in a refrigerator at 4 °C until the moment of analysis. This study was approved by the Ethics Committee on Animal Experimentation of the Instituto de Zootecnia (Protocol Nr. 298–2020).

#### Extraction of total RNA from whole blood

The thirty-six blood samples were divided into six groups and were submitted to RNA extraction at the following intervals post-sampling (ps): 2 h, 1 day, 2 days, 3 days, 7 days and 14 days (during whole experimental period, all the samples were free from hemolysis). The RNA extractions were performed using the protocol described by Jiang et al. [15], with some modifications. Each tube containing 5 mL of whole blood was centrifuged at 2000×g for 10 min. The layer of plasma was discarded, and 0.5 mL of buffy coat layer was aspirated, transferred into a new 2 mL microtube and 1.5 mL of RBC lysis buffer was added. The tube was vigorously shaken and incubated at room temperature (RT) for 15 min prior to centrifugation at 2000×g for 10 min (4 °C). Supernatant was discarded, and 1.0 mL RBC lysis buffer was added to the cell pellet, followed by incubation at RT for 10 min. After centrifugation at 2000×g for 10 min, at 4 °C, supernatant was discarded, WBC pellet lysed and homogenized with 1.0 mL Qiazol® Reagent (Qiagen, Hilden, Germany). The lysate was transferred into a 1.5 mL microtube and incubated at RT for 5 min. Then 0.2 mL of 1-Bromo-3-chloropropane was added to the lysate, followed by vigorously shaken for 15 s and incubated at RT for 5 min. The sample was centrifuged at 15 min at 12,000×g at 4 °C. The upper layer was transferred to 1.5 mL fresh tube, mixed with 0.5 mL 100% isopropanol alcohol, and incubated at RT for 10 min. The mixture was centrifuged for 10 min at 12,000×g at 4 °C. The supernatant was removed. The pellet was washed with 1 mL 75% ethanol and centrifuged at 7500×g for 5 min. at 4 °C. The pellet was air dried and dissolved in 50 μL TE-buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, pH 8.0). The recovered RNA was stored at −80 °C.

The concentration and purity of the RNA samples were determined using a BioDrop spectrophotometer (BioDrop uLITE, Integrated Scientific Solutions Inc., Walnut Creek,
CA, USA). RNA integrity was verified with Agilent RNA 6000 Nano Kit (Agilent Technologies) in an Agilent 2100 Bioanalyzer instrument (Agilent Technologies) for determination of RIN (RNA Integrity Number) and by RNA analysis in 1% gel electrophoresis.

**DNase digestion and cDNA synthesis**

RNA samples were treated with RQ1 RNase-Free DNase (Promega, Wisconsin, US, cat. # M6101), following the manufacturer’s recommendations. The cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription kit with RNase inhibitor (ref. No. 4374967, Applied Biosystems, CA, USA), following the manufacturer’s information, using OligodT primers (IDT, Coralville, IA, USA). In addition to the RNase treatments, the absence of genomic DNA amplification in the RNA samples was confirmed by qPCR assays containing treated RNA instead cDNA.

**Primer design and analysis of the genes**

The primers for all evaluated genes were designed using the PrimerQuest software (http://www.idtdna.com/PrimerQuest/Home/Index). The specificities and qualities of the primers were tested using an online tool NetPrimer (http://www.premierbiosoft.com/netprimer/), OligoAnalyzer IDT (https://www.idtdna.com/calc/analyzer) and BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGETYPE=BlastSearch&LINKLOC=blasthome) and Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Details of specific primer sets are shown in Table 1.

The expression profiles of seven genes were evaluated: beta-actin (ACTB) which encodes one of the two non-muscle cytoskeletal actins, beta-2-microglobulin (B2M) which encodes a component of MHC I molecule, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) which encodes an enzyme of glycolysis pathway, peptidylprolyl isomerase A (PPIA) encodes a protein responsible by catalyzes of the cis–trans isomerization of proline imidic peptide bonds involved in many biological processes (intracellular signaling, transcription, inflammation and apoptosis), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) which encodes a central hub protein for many signal transduction pathways, cluster of differentiation 4 (CD4) which encodes a molecule expressed in the surface of T cells, macrophages, monocytes, dendritic cells and neutrophils and interferon-gama cytokine (IFN-γ) which encodes a soluble cytokine member of type II class of interferons. The algorithms used to identify the most stable genes were geNorm [16], NormFinder [17] and BestKeeper [18]. In addition, a final classification of most stable genes was provided by using RankAggregated tool which applied a Monte Carlo cross-entropy algorithm as previous described [19].

**RT-qPCR assay**

The qPCR reactions were carried out on the Rotor gene Q equipment (Qiagen, Hilden, Germany) for a final volume of 10 μL that contained: 5 μL of the QuantiNova SYBR® Green RT-PCR Kit (Qiagen) master mix, 0.3 μL (10 μM) of each primer, 2.4 μL of ultra-pure water (Sigma-Aldrich) and 2 μL of cDNA (approximately 50 ng). One cycle of enzymatic activation of 95° C was programmed for 2 min followed by 35 cycles of 95° C for 10 s (denaturation) and 60° C for 30 s (annealing/extension). After amplification, a melting temperature analysis was generated by raising the incubation temperature from 60 to 95 °C in 0.5 °C increments.

Table 1 Sequences of oligonucleotides used in the RT-qPCR assays, location, amplicon size, exon boundary, efficiency, and reference

| Gene   | Primers (5’-3’) | Accession number | Location (nt) | Amplicon size (bp) | Exon boundary | Efficiency | Reference     |
|--------|----------------|------------------|---------------|--------------------|---------------|------------|---------------|
| ACTB   | F: CCCAGATCATGTTGACAGACCT | NM_173979.3 | 449–604       | 156                | 3/4           | 90.8       | This study    |
|        | R: CAGAGGGCTACCTCATAG     |               |               |                    |               |            |               |
| B2M    | F: CAGACCTGTTCTTACGAAAG   | NM_173893.3 | 249–396       | 148                | 3/4           | 95.0       | [24]         |
|        | R: GCTCAGGCTCCGTACCTCAT   |               |               |                    |               |            |               |
| GAPDH  | F: CTGGGAGAACCTGCGAAGATG  | NM_001034034.2|793–975      | 183                | 9/10          | 96.5       | This study    |
|        | R: CTTGACAAACCTGCTTGAGG   |               |               |                    |               |            |               |
| PPIA   | F: ATTTATGTCGAGGAGTGGAC   | NM_178320.2 | 188–303       | 116                | 4/5           | 97.9       | [24]         |
|        | R: AGATGCGAGCCTGATGTGTT   |               |               |                    |               |            |               |
| YWHAZ  | F: GAAGGGGTTGTTGACCACAG   | NM_174814 | 467–650       | 183                | 4/5           | 97.5       | This study    |
|        | R: GCTTCTACAAAGGGTCTCT    |               |               |                    |               |            |               |
| CD4    | F: GGTGATGAAAGTGACTAAGAGC  | NM_001103225.1| 939–1080     | 142                | 6/7           | 95.8       | [24]         |
|        | R: CTCGCGCTGTGTCACAACCTTC |               |               |                    |               |            |               |
| IFN-γ  | F: CAGAGCCAAAATGGCTCTCTTC | NM_174086 | 298–465       | 167                | 3/4           | 96.9       | This study    |
|        | R: ATGCACCAGAATTGGAATCAG   |               |               |                    |               |            |               |
with a hold step of 5 s at each increment. All reactions were run in duplicate.

**Statistical analysis**

The results of RNA concentrations, 260/280 nm ratios and RNA integrity number (RIN) were analyzed regarding the normality of data distribution. Then, the data were analyzed by mixed models using the PROC MIXED procedure using the SAS (SAS Institute, Cary, NC). The mixed model included repeated measures from the same animal, and the effect of the interval was used as fixed effect. The structure of the (co)variance matrix used in this model was the heterogeneous compound symmetry (CSH).

Regarding the RT-qPCR results, the analysis was performed using a mixed model described by Steibel et al. [20], with some modifications, to jointly analyze the expression of target and reference genes: $Y_{gikr} = T_{gi} + D_{ik} + e_{gikr}$.

Where $Y_{gikr}$ is the Cq obtained of the $g$th gene (target genes: B2M, ACTB, PPIA, GAPDH, YWHAZ and IFN-γ, or the geometric mean of the Cq of the three reference genes used: B2M, GAPDH and PPIA) from the $r$th well (referring to the technical replicate), in a sample obtained from animal $k$ of treatment $i$ (2 h, 1dps, 2dps, 3dps, 7dps and 14dps). $T_{gi}$ is the effect of treatment $i$ in the expression of gene $g$, $D_{ik}$ is a random sample-specific effect (common to both genes), and $e_{gikr}$ is a residual term. The sample-specific effect, $D_{ik}$, captures differences among samples that are common to both genes, particularly those that affect total mRNA concentration, such as differential extraction or amplification efficiencies among samples. The model implemented in this study was adjusted using the “Mixed” procedure of the SAS (SAS Institute, Cary, NC). The comparisons between treatments were made through the assembly of contrasts according to Steibel et al. [20] that allowed the hypotheses to be tested at values equivalent to fold change. Comparisons were performed between 2 hps and other intervals, aiming to evaluate if storage time led to altered gene expression.

**Results**

**RNA quality and integrity during blood storage at common fridge**

The total RNA concentration, $A_{260/280}$ ratio averages and RIN were 368.8 ± 43.2 ng/μL, 1.88 ± 0.01 and 8.9 ± 0.18, respectively. The mean results per storage interval are presented in Table 2, while individual values are presented in Supplementary information 1. All the samples presented optimal 260/280 nm ratios (> 1.8), while optimal RIN values were observed up to 3 dps (Table 2). The RNA integrity was also observed by the gel electrophoresis, presenting clearly visible 18S and 28S ribosomal RNA bands with no smeared bands (Supplementary information 2).

**Gene regulation**

Raw Cq values from all evaluated genes remained unaltered during whole blood storage up to 3 dps, while significant differences were observed at 7 dps for GAPDH gene, and at 14 dps for B2M, GAPDH, PPIA, YWHAZ, ACTB and CD4 genes (Fig. 1).

The B2M, PPIA and GAPDH genes were the most stable, then they were used for normalization of Cq results, while YWHAZ and ACTB were considered the least stable genes (Supplementary information 3). There was no altered expression for all evaluated genes between samples stored from 2 hps to 3 dps, while altered gene expression were observed for both CD4 and IFN-γ at 7 dps and 14 dps compared to 2 hps group (Fig. 1).

**Discussion**

The accurate transcriptional quantification and high reproducibility of RT-qPCR assays are crucial for the understanding of biological regulation when gene expression studies are performed. In this context, proper standardization of sample handling and posterior procedures are also required for those experiments. Therefore, the present study aimed to evaluate the storage time that RNA remains stable in bovine whole blood samples without stabilizers addition, regarding purity, integrity, and unaltered gene transcription profiles at common fridge (4 °C).

The extraction method was able to provide RNA at high yields and integrity rates from whole blood samples stored up to 3 days at common fridge, while the RNA purity was reduced longer than 3 dps, although during whole experimental period the estimated purity was kept at optimal ratios (> 1.8). Three methods of RNA extraction from blood.
samples were previously compared [15] and it was verified that the method, also applied in the present study, produced the highest yield, whereas lower yields were obtained by the other two methods. One advantage of specific processing of WBC, performed in our study, is associated to decreased globin interference effects on PCR sensitivity. In this context, several studies have demonstrated that the abundance of globin genes in whole blood may mask the underlying biological differences between whole blood samples [10, 11, 21–23].

The RNA integrity was fully maintained up to 3 dps in whole blood stored at common fridge, while at 7 dps, one (1/6) sample presented RIN value (6.8) below recommended (> 7.5), and at 14 dps, three (3/6) samples presented RIN values below recommended (6.6, 7.4 and 7.4). However, even for these samples presenting low RIN values, the values were close to the minimum recommended.

Altered gene expression were also observed for genes which encode both a surface T cell molecule (CD4) and a soluble cytokine member (IFN-γ) from 7 dps of storage of whole blood samples, while all the genes routinely used as reference genes here evaluated (B2M, GAPDH, PPIA, YWHAZ and ACTB), which are constitutively regulated, remained unaltered during whole experimental period. Besides, the raw Cq values from all evaluated gene were also unaltered up to 3 dps, highlighting the absence or prominent RNA degradation of transcriptional changes during this interval. Therefore, we concluded that whole bovine blood samples can be stored up to 3 dps for studies targeting gene expression profiles. However, further investigations are necessary.
highly recommended in other cattle breeds, bovine ages and for other target genes, aiming to reproduce our experimental findings.

There are scarce studies available focused on assessing the RNA integrity and gene expression during storage of whole blood samples. To our knowledge this was the first report to evaluate the storage time that RNA kept stable in untreated whole bovine blood at common fridge, without addition of any RNA stabilizing reagents, and may constitute a valuable information for studies targeting bovine mRNA in blood samples, especially when restricted funding source is available. Moreover, further studies, which may include microarray or RNAseq analysis may better addresses the transcriptional changes which may occur during untreated whole blood storage.

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Author contributions  RG and CHO: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing—original draft, Writing—review & editing. HNO, LMK, AEVF and MCSO: Conceptualization, Supervision, Validation, Visualization, Writing—original draft, Writing—review & editing. BTA: Methodology. All authors read and approved the final manuscript.

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Data availability  All experimental data may be available if requested.

Declarations

Conflict of interest  The authors declare that there is no competing interest or conflicts of interest.

Informed consent  The authors declare that they consent to participate of this manuscript.

Consent for publication  The authors declare that they consent publication of this manuscript.

Research involving human and animal rights  All the animals handling in accordance with the ethical principles and guidelines adopted by the Brazilian College of Experimentation. All procedures were approved by the Ethical Committee on Animal Experimentation of the Instituto de Zootecnia (Protocol Nr. 298–2020).

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