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

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Short Report

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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 14dps 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.

Introduction

Messenger RNA levels may constitute good indicator of physiological and/or immune status of cells, addressing the host responses under different environmental conditions, 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, 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 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), RNeasy Protect Animal Blood Tubes (Qiagen, Venlo, Netherlands), Tempus Blood RNA tubes (Applied Biosystems, Foster City, CA) and RNeasy Lysis Reagent (ThermoFisher, Waltham, MA). Although, all these reagents, except for RNeasy Lysis reagent, require proper commercial RNA extraction kits, and the costs related to these reagents may be very expensive, especially for experiments including a large sampling size.

A second important drawback associated to most of these blood collection devices is due to the high amounts of globin mRNA, 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 specific cell subsets since all blood cells are lysed when added to the stabilizing reagent.

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, RNeasy LS Reagent, RNeasy Lysis 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 RNeasy Lysis 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 32h, but when whole blood samples were stored at low temperature (4°C), the RNA integrity remained stable at duration of 40 hours [12]. 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 hours [12, 13] or at low temperature [13].

Most of studies targeting 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 hours, 1 day, 2 days, 3 days, 7 days and 14 days. The RNA extractions were performed using the protocol described by Jiang et al. [14], with some modifications. Each tube containing 5 mL of whole blood was centrifuged at 2,000 x g at 4 °C 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 2,000 x 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 2,000 x 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 sec and incubated at RT for 5 min. The sample was centrifuged for 15 min at 12,000 x 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 x g at 4 °C. The pellet was washed with 1 mL 75% ethanol and centrifuged at 7,500 x 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/analyser>) and BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE=Search&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-gamma 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 [15], NormFinder [16] and BestKeeper [17], 2004). In addition, a final classification of most stable genes was provided by using RankAggregated tool which applied a Monte Carlo cross-entropy algorithm as previously described [18].

RT-qPCR assay

The qPCR reactions were carried out on the Rotor gene Q equipment (Qiagen, Hilden, Germany) for a final volume of 10 μ L that contained: 5 μ L of the QuantiNova SYBR[®] Green RT-PCR Kit (Qiagen) master mix, 0.3 μ L (10 mM) of each primer, 2.4 μ L of ultra-pure water (Sigma-Aldrich) and 2 μ L of cDNA. One cycle of enzymatic activation of 95 °C was programmed for 2 minutes followed by 35 cycles of 95 °C for 10 seconds (denaturation) and 60 °C for 30 seconds (annealing/extension). After amplification, a melting temperature analysis was generated by raising the incubation temperature from 60 °C to 95 °C in 0.5 °C increments with a hold step of 5 sec 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 using "PROC GLM" procedure and the means were compared by Tukey test ($p < 0.05$), using the SAS (SAS Institute, Cary, NC).

Regarding the RT-qPCR results, the analysis was performed using a mixed model described by Steibel et al. [19], 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 (2h, 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. (2009) 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 ± 135.7 ng/ μ L, 1.88 ± 0.04 and 8.9 ± 0.7 , respectively. The mean results per storage interval are presented in Table 1, while individual values are presented in Supplementary information 1. Regarding RNA concentrations and RIN means, there were no significant differences between 2 hps to 3 dps intervals, while for $A_{260/280}$ mean ratios, unaltered values were observed between 2 hps and 2 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 and CD4 genes, and at 14 dps for B2M, GAPDH, YWHAZ and CD4 genes (Figure 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 was observed for both CD4 and IFN- γ at 7 dps and 14 dps compared to 2 hps group (Figure 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 [14] 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, 20-22].

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 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.

Animal welfare statement

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).

Declarations

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Competing Interest/Conflicts of interest

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

Availability of data and material

All experimental data may be available if requested.

Authors' contributions

R.G. and C.H.O.: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing; H.N.O, L.M.K., A.E.V.F. and M.C.S.O.:

Conceptualization, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. B.T.A.: Methodology. All authors read and approved the final manuscript.

Consent to participate

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

Consent for publication

The authors declare that they consent publication of this manuscript.

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Tables

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: CCCAGATCATGTTTCGAGACCTT R: GAAGGGCGTACCCCTCATAGAT	NM_173979.3	449-604	156	3/4	90.8	This study
B2M	F: CAGACCTGTCTTTTCAGCAAGGA R: GCTTACAGGTCTCGATCCCACT	NM_173893.3	249-396	148	3/4	95.0	[23]
GAPDH	F: CTGGAGAAACCTGCCAAGTATG R: CTTGACAAAGTGGTCGTTGAGG	NM_001034034.2	793-975	183	9/10	96.5	This study
PPIA	F: ATTTATGTGCCAGGTTGGTGAC R: AGATGCCAGGACCTGTATGCTT	NM_178320.2	188-303	116	4/5	97.9	[23]
YWHAZ	F: GAAAGGGATTGTGGACCAG R: GGCTTCATCAAATGCTGTCT	NM_174814	467-650	183	4/5	97.5	This study
CD4	F: GGTGATGAAAGTGACTAAGTCCCC R: CTCCGGCTGTGTACCAACTTC	NM_001103225.1	939-1080	142	6/7	95.8	[23]
IFN- γ	F: CAGAGCCAAATTGTCTCCTTC R: ATCCACCGGAATTTGAATCAG	NM_174086	298-465	167	3/4	96.9	This study

Table 2. Means ($\pm$ SD) of RNA integrity number (RIN), RNA concentration (ng/ μ L) and RNA purity level estimated by 260/280 nm ratio.

Interval	RIN	RNA concentration (ng/ μ L)	260/280 nm ratio
2hps	9.3 $\pm$ 0.16 ab	404.00 $\pm$ 108.52 ab	1.92 $\pm$ 0.02 ab
1dps	9.5 $\pm$ 0.21 a	463.87 $\pm$ 120.26 a	1.94 $\pm$ 0.02 a
2dps	9.5 $\pm$ 0.14 ab	478.07 $\pm$ 130.28 a	1.89 $\pm$ 0.05 abc
3dps	8.8 $\pm$ 0.66 ab	469.60 $\pm$ 147.00 a	1.85 $\pm$ 0.06 bc
7dps	8.5 $\pm$ 0.95 bc	240.53 $\pm$ 63.57 bc	1.84 $\pm$ 0.06 bc
14dps	7.6 $\pm$ 0.63 c	157.00 $\pm$ 72.3 3c	1.83 $\pm$ 0.05 c

Means followed by different letters in the same column differ significantly ($p \leq 0.05$)

Figures

Figure 1

Raw Cq means (upper graph) and gene expression mean values (bottom graph) for all evaluated genes (B2M, PPIA, GAPDH, ACTB, YWHAZ, IFN-γ and CD4) obtained at different storage intervals of whole bovine blood samples at common fridge. Asterisks indicate significant differences compared to the samples processed immediately after blood collection (2hps). Different letters in the same group of bars (per gene) differ significantly ($p \leq 0.05$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [RawCqvalues.xlsx](#)
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- [Supplementaryinformation2.docx](#)
- [Supplementaryinformation3.docx](#)