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METHODOLOGY FOR QUANTIFICATION OF CIRCULATING CELL-FREE MICRORNA FROM BOVINE PLASMA FOR ANALYSIS OF MEAT QUALITY TRAITS

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SUMMARY

MicroRNAs (miRNAs) function as important genetic regulators during growth, development, and cellular processes. Circulating miRNAs, cell free or contained within exosomes, have been detected in all bodily fluids, including plasma. These excreted molecules are not well-understood and may reflect a physiological phenotype, or exert a regulatory function. To develop a means for examining the impact of circulating miRNA function on beef cattle phenotypes, we assessed parameters important for isolation of miRNAs from plasma and subsequent high-throughput expression analysis on a real-time quantitative RT-PCR microfluidics platform. . These methods will facilitate economical expression analysis of circulating microRNAs and their potential association with health status and carcass and meat quality traits in livestock.

INTRODUCTION

MicroRNAs are small (~17-27nt) RNA molecules that function as molecular rheostats to regulate gene expression as part of many physiological processes. These molecules regulate the function of entire networks of genes, increasing the complexity of genetic mechanisms (Jeffries *et al*. 2010). Several miRNAs are known to regulate skeletal muscle phenotypes, and their differential expression within muscle may reflect response to exercise or even variation in activity (Dawes *et al*. 2015, Margolis *et al*. 2016). Recent studies show that circulating miRNAs may reflect health status or response to diet (Ioannidis and Donadeu 2016; Muroya *et al.* 2016). . Thus, we expect that circulating miRNAs can be applied to beef cattle production as informative diagnostic tools for interrogating mechanisms important for muscle growth and fat deposition.

Our overall objective is to develop genomic tools that are directly applicable for food animal research. Our specific interest is to accurately measure and evaluate miRNA expression in peripheral blood and apply this tool for improvement of meat quality. During plasma isolation, lysis of erythrocytes (Kirschner *et al*. 2011, 2013), leukocytes (Al-Soud and Radstrom 2001) or activated thrombocytes (Osman and Falker 2011) can release non-target miRNAs, or iron from hemoglobin or lactoferrin into the plasma, potentially altering expression profiles or inhibiting RT-PCR. In this paper we address critical parameters for experimental handling and processing of plasma from steers on feed for isolation of intact cell-free miRNAs, free of contaminating RNA transcripts or agents inhibitory to downstream analysis. This approach is a necessary first step to enable quantitative analysis of these miRNAs, and their potential relationship to carcass traits.

MATERIALS AND METHODS

Animals, sample collection and processing. Animal handling and sample collection procedures were approved by the Texas A&M Animal Care and Use Committee (AUP #2008-234). . Blood was collected by venipuncture (Vacuette® 18 ga x 1.5 inch needles, Greiner Bio-One North America, Monroe, NC) into 10 ml BD Vacutainer® tubes containing K_2-EDTA (PN 366643, BD Diagnostics, Franklin Lakes, NJ). To assess the effect of handling, an initial pilot study was conducted. Four tubes of blood were collected from a single jugular puncture of 4 Angus steers. . All tubes were gently inverted 10 times immediately after collection. One tube from each set was shaken vigorously to mimic improper handling. Tubes were held at 4°C until processed. Blood in

"control" tubes and shaken tubes were processed within 2h of collection. The remaining 2 tubes from each steer were held and processed at 30h and 52h post-collection, respectively.

In a second study, a single tube of blood was collected from each of 88 cross-bred steers every 28d over a period of 7 months, beginning when the steers were approximately 12 months of age. Samples were kept cool and transported to the laboratory within 6 hours. Tubes of blood were centrifuged at 1300 x *g* for 10 min. Plasma was removed with care to avoid disturbing the buffy coat layer, transferred to clear, 1.5 ml tubes, and stored as 500 µl aliquots at -80°C.

Assessment and quantification of hemolysis. Hemolysis was assessed visually and quantified by spectrophotometry, according to the scale in Figure 1. This scale was adapted from a clinical laboratory protocol (http://bit.ly/2kcSm3H). Spectrophotometry was conducted as described by Kirschner *et al*. (2011, 2013), on a Bio-Rad 680 Microplate Reader (Hercules, CA).

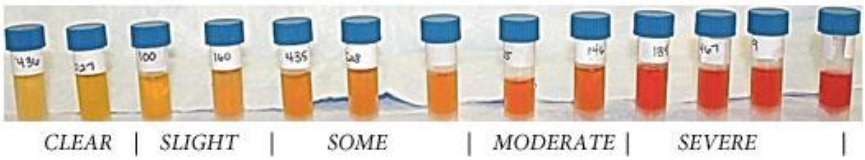

Figure 1. Representation of varying levels of hemolysis in bovine blood, ranked left to right as Clear, Slight hemolysis, Some hemolysis, Moderate hemolysis, or Severe hemolysis.

MicroRNA isolation and qRT-PCR. Procedures were conducted according to manufacturer's recommendations (Exiqon, Inc., Woburn, MA). MicroRNA was extracted from 500 µl plasma with the miRCURY™ RNA Isolation Kit-Biofluids (Exiqon). Plasma miRNA was quantified by fluorometric analysis (Qubit® microRNA Assay Kit, Qubit® 2.0 fluorometer, ThermoFisher). Extracted miRNA was reverse-transcribed (RT) into cDNA with the Universal cDNA Synthesis Kit II (Exiqon). Quantitative RT-PCR reactions (10 µl) contained 1x ExiLENT SYBR® Green master mix (Exiqon), 1x ROX (Life Technologies, Carlsbad, CA,), 1 µl primer mix and 2 µl diluted template cDNA. Primers for hsa-miR23a-3p and hsa-miR-451A (PN 204772 and 204734) were used for initial assessment of hemolysis. Amplification was carried out in the ABI 7900HT thermal cycler in 9600-emulation mode (Applied Biosystems, Inc., Foster City, CA).

Microfluidic qRT-PCR. A custom panel of 96 microRNA assays was created (Pick & Mix Panel for Fluidigm, PN 203899, Exiqon), to contain specific miRNAs expected to be present in plasma, or have relevance for muscle growth, fat deposition, and meat quality. Reverse transcription, specific target preamplification (STA) and microfluidic qPCR reactions were performed according to Exiqon's Fluidigm-BioMark recommended protocol (http://bit.ly/2ke3TUF). Plasma RNA input for RT was increased to 8 µl. Samples from severely hemolyzed plasma were excluded from further processing. As a positive control, cDNA prepared from miRNA extracted from *longissimus* muscle was included on the array, with several negative control samples. Amplification was carried out in 96.96 Dynamic Array™ Integrated Fluidic Circuits (IFC) for Gene Expression (PN BMK-M-96.96, Fluidigm, San Francisco, CA) on a Biomark HD thermal cycler. Raw amplification data were analyzed with Sequence Detection System software (SDS v.2.2.2, Applied Biosystems) or Real-Time PCR Analysis software for Biomark (Fluidigm), as appropriate.

RESULTS

Blood collection and handling procedures influence risk of hemolysis. Visual assessment of the pilot study samples confirmed that hemolysis increased if samples were handled roughly or initial processing was delayed. Shaking produced slight and moderate hemolysis and increased holding time greatly increased hemolysis compared to the control samples. In the main study, few samples

were moderately to severely hemolyzed when processed within 6 hours of collection. In cases where blood flow into the collection tube was slow, or venipuncture required more than one attempt, moderate to severe hemolysis was likely (11 of 13 records).

Effect of hemolysis on amplification of circulating microRNA. For 7 steers, plasma samples were collected over the course of the study that covered nearly the full range of hemolysis (slight to severe). Free hemoglobin was quantified by spectrophotometry in this subset of 28 samples (Figure 2A). Based on the spectrophotometric analysis, samples scored for hemolysis in the range from clear to moderate on our scale were expected to be acceptable for expression analysis. Blondal *et al*. (2013) reported that hemolysis could also be evaluated by qRT-PCR analysis of miRNAs *miR-23a* and *miR-451*. They found expression of *miR-23a* to be relatively stable in the cell-free fraction. Because *miR-451* is enriched in erythrocytes, an increase in the ratio of miR-*451* to *miR-23a* concentration would indicate hemolysis. Expression of *miR-451* and *miR-23a* in this set of samples was evaluated by quantitative RT-PCR. The difference in threshold cycle quantity (delta C_t) was calculated as described by Blondal *et al*. (2013) and expression measured by qRT-PCR was reduced in the severely hemolyzed group (Figure 2B). Based on these results severely hemolyzed samples were omitted from the microfluidic qPCR experiment.

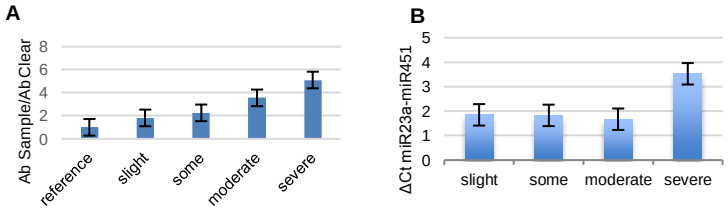

Figure 2. (A) Free hemoglobin in plasma, measured by spectral absorbance at 414 nm and expressed as a ratio of absorbance compared to a clear reference sample. (B) ΔC_t (miR-23a-3p – miR-451a). Severely hemolyzed samples exhibited a 1.8-fold increase in contaminating miRNA abundance compared to those scored as slight ($p<0.05$).

Microfluidic qPCR. Amplification was visually assessed on heat maps for each IFC, and UniSP2 and UniSp6 spike in controls were verified. Seventy-six miRNA assays amplified in at least 1 sample type; 59 amplified cDNA from both plasma and skeletal muscle, 7 amplified only plasma, and 10 amplified only muscle. Poor signals or amplification failure was observed for 17 miRNA assays. MicroRNAs are not abundant in plasma and recovery can be somewhat variable (Brunet-Vega *et al.*, 2015). Modification of the protocol to increase the input volume resulted in amplification signal sufficient for quantification on the Fluidigm IFC platform. While expression was expected to vary across the 240 experimental samples extracted from plasma samples, amplification signal was detected for all 68 assays. Expression failed across all assays for only 2 samples. In 17% of the samples, amplification signal was weak and expression was detected for less than a third of the assays. However, hemolysis status (clear to moderate) was not correlated with general amplification robustness.

DISCUSSION

Proper handling reduces likelihood of plasma hemolysis. Clinical recommendations for human blood collection suggest use of a 21 ga needle, filling the tube to proper capacity, gentle inversion, maintaining ambient temperature between collection and processing, and timely processing (within 2-4 hours) to separate the plasma from the cells (Tuck *et al*. 2009). We found that use of 18 ga needles for cattle, with gentle handling of blood tubes and storage to protect from high heat was

sufficient for suitable plasma collection for microRNA isolation. Storage on ice was avoided to prevent activation of platelets, release of miRNA, and contamination of the cell-free fraction (Osman and Falker 2011). Our results indicate prompt processing may be the most important factor in handling, and that plasma should be processed within 6 hours of collection. .

Severe hemolysis may impact data negatively. Several studies have reported methods of quantifying hemolysis and the effect of hemolysis on downstream analysis (Blondal *et al.* 2013; Kirschner *et al.* 2011, 2013). We found that severe hemolysis did alter RT-PCR amplification. By restricting sample quality to hemolysis scored no greater than "moderate," we found that expression measurement via the Fluidigm platform was not inhibited by low level hemolysis.

Significance. This is, to our knowledge, the first description of methodology for use of the Fludigm microfluidics platform expression analysis of circulating microRNAs in biofluids from cattle. Adaptation of a protocol for analysis of microRNA from plasma (Exiqon) resulted in sensitivity sufficient for this platform. This method provides an economical tool to enable PCR-based high-throughput expression analysis of microRNAs. These results are a first step toward systematic evaluation of circulating microRNAs that may play important regulatory roles on growth and development. While not a discovery technique such as RNA-sequencing, this type of approach provides a relatively simple and more economical method for analysis of low-abundance targets over time. We anticipate that investigation of expression patterns of circulating miRNA in relation to carcass and meat quality traits may result in key insights for regulation of muscle growth and fat deposition. Importantly, we expect this approach will provide a new tool for improving meat quality and other desirable phenotypic traits.

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