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Research paper

A method for isolating and analyzing human mRNA from newborn stool

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ABSTRACT

Efforts to characterize the human transcriptome have largely been limited to blood, urine, and tissue analyses (i.e., normally sterile materials). We report here an extraction protocol using commercially available reagents to obtain high-yield, reverse-transcribable RNA from human stool. Quantitative reverse transcriptase polymerase chain reactions demonstrated minimal intra-specimen but considerable intra-subject variability over time of transcripts for interleukin-6 (IL-6), IL-8, epidermal growth factor (EGF), calprotectin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This technique now expands opportunities to use the human fecal transcriptome to characterize gastrointestinal pathophysiology.

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1. Introduction

The human transcriptome, defined as the totality of messenger RNA transcripts made by a set of cells or tissues, holds considerable promise for use in studies of human physiology in health and disease. Studies of the human transcriptome have largely been confined to blood cells (Mohr and Liew, 2007), urine (Roos et al., 2008), and soft tissue (Farber and Lusis, 2008). Efforts to characterize transcripts in heterogeneous substances such as stool have been limited to attempts to detect specific transcripts relating to gastrointestinal malignancy in adults (Ahmed et al., 2007; Ahmed et al., 2004), and bacterial virulence genes in human pathogens shed by patients and bovine hosts (Rashid et al., 2006; Sjoling et al., 2006). Attempts to study mRNA in other gastrointestinal substances, such as saliva, have also been variably successful (Kumar et al., 2006; Setzer et al., 2008).

Published techniques to isolate RNA from human stool have used methods and reagents that are not commercially available (Rashid et al., 2006; Alexander and Raicht, 1998), or that do not consistently remove polymerase chain reaction (PCR) inhibitors (Ahmed et al., 2004). PCR inhibition, presents a particular challenge when attempting to analyze nucleic acid in stool or environmental samples. The problem appears to be that reagents used in downstream reactions, such as polymerases in PCR, are inhibited by substances carried over from the stool (Abu Al-Soud and Radstrom, 1998). Kang et al. (2009) recently compared several techniques to isolate RNA from murine stool, and concluded that a combination of bead-beating and silica column extraction is the best method to examine pooled samples from multiple time points.

Here we describe our efforts to purify mRNA from human stool that can then be used for reverse transcription and quantification. We extend the work of Kang et al. (2009) by reporting the degrees of intra-specimen, and intra-host variability of mRNA, presence and viability of selected host transcripts, and the utility of the silica-based method to remove potential inhibitors of downstream PCR.

2. Methods and materials

2.1. Subjects and specimen handling

Stool was obtained weekly from three infants born after 27, 27, and 29 weeks estimated gestations. All stools were collected with a sterile, disposable wood spatulas (Cardinal

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Health, Dublin, OH, USA) from diapers after the stools were freshly passed, and then placed into a 125 mL sterile specimen container (Cardinal Health, Dublin, OH, USA) and stored at 4 °C until transported to the laboratory within 4 h of collection. Immediately after receipt in the laboratory, non-homogenized specimens were divided into three ~100 mg aliquots, which were then immediately flash frozen in separate 1.2 mL Nalgene screw top vials (Thermo Fisher Scientific, Inc., Hampton, NH, USA) by immersion in an ethanol–dry ice bath, which were then stored at −80 °C until further analysis. RNA was extracted between one and 4 weeks after the samples were frozen. Three samples from three different time points for each of the three patients were chosen to assess intra-specimen, and intra-individual variability in mRNA content over time. For positive controls, we used either total RNA from adult human leukocytes (gift of David Hunstad, M.D.) extracted from whole human blood using the Qiagen Mini Kit, according to the manufacturer's instructions, or total RNA isolated from *Escherichia coli* 055: H7, strain TB182A (Bokete et al., 1993) stock cultures using a Qiagen RNeasy spin column according to the manufacturer's instructions. All human subjects research was approved by The Human Research Protection Office of Washington University School of Medicine.

2.2. RNA extraction

A 100 mg portion of frozen stool was placed into a 1.5 mL Eppendorf tube to which 600 µL of room temperature phosphate buffered saline (pH 7.4) had been added. This tube was then vortexed until the contents were well suspended. 100 µL of this suspension was aspirated using a 1 mL blue pipette tip, and transferred to a fresh 1.5 mL Eppendorf tube. One mL of RNA Bee (Tel-Test, Inc. Friendswood, TX) was added to this suspension. Immediately thereafter, we added 200 mg of acid washed glass beads and performed "bead-beating" with a Fast Prep FP120 (Thermo Savant) for 45 s at a speed setting of 6.5 in three different pulses, with two-minute intervals between pulsations. We then added 200 µL of chloroform to this suspension, and mixed the samples by inverting them several times before placing them on ice for 15 min. The samples were then centrifuged (13,000 g, 15 min). The clear, colorless upper (aqueous) phase was easily differentiated from the blue-green lower (organic) phase above a pellet of glass beads and stool debris. The aqueous phase (usually 500–700 µL) was aspirated and placed into a fresh 1.5 mL Eppendorf tube; the organic phase and pellets were discarded.

Ethanol (1:1 volume, 70%) was added to each sample. The resultant mixture was applied to an RNeasy spin column (Qiagen), which was then centrifuged, and washed twice with RPE buffer, per the manufacturer's instructions. Bound RNA was eluted with 50 µL of RNase-free water. DNase digestion was not performed. Our early work demonstrated no difference in RNA yield or effect on RT-PCR after DNase digestion, so we eliminated this step from our current protocol. When multiple samples were purified simultaneously, a QiaVac 24 Plus (Qiagen) was used to wash the corresponding silica membranes in parallel.

The time to simultaneously purify aqueous RNA from 5 to 20 samples from frozen samples was approximately 2 h.

To assess effects of temperature and time from production to freezing on RNA yield, three aliquots of one stool sample were exposed to different pre-extraction intervals (0, 1, 2, 4, 12, and 24 h) at 20 °C or 4 °C, prior to freezing. RNA was isolated and analyzed as with the other samples.

2.3. Assessments of RNA yield and quality

Specimens were first analyzed on a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). RNA concentration was expressed as a function of the mass of the stool from which it was purified. Subsequently, a portion of each purified sample was diluted to ~5 ng/µL, and 1 µL of this diluted RNA solution was placed into an Agilent 5200 Bioanalyzer (Agilent Technologies), and quantified in duplicate. The electropherogram was examined and the RNA integrity number (RIN) was computed for each sample using the supplied software (Schroeder et al., 2006).

2.4. Quantitative reverse transcription PCR

We used quantitative reverse transcriptase PCR (qRT-PCR) to measure the ratios of various host mRNAs. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), interleukin-6 (IL-6), IL-8, S100A8 (a calprotectin subunit), and epidermal growth factor (EGF) were used in separate reactions, and in triplicate, for all samples. All primers were obtained from Applied Biosystems, Inc. (product numbers: Hs99999905_m1 [GAPDH], Hs99999032_m1 [IL-6], Hs00171403_m1 [IL-8], Hs00374264_g1 [S100A8], Hs01099990_m1 [EGF], respectively). These particular mRNAs were chosen for study because we postulated that these transcripts might be present in the stools of normal or developing (GAPDH, EGF) or inflamed (IL-6, IL-8, S100A8) intestines. Each primer was designed to straddle splice sites to avoid amplifying residual DNA. Copy number standards were prepared according to the manufacturer's instructions. Ten µL reactions were prepared using 3 µL of RNA (concentrations adjusted to ~200 ng RNA per reaction), 5 µL TaqMan 2X Environmental Master Mix, 0.5 µL 20X primer mix, 0.1 µL SuperScript III Reverse Transcriptase, 0.2 µL RNase inhibitor, and 1.2 µL RNase-free H_2O (all reagents from Applied Biosystems, Inc.). Amplifications were performed for 40 cycles in a 7500 Fast Real Time PCR System (Applied Biosystems, Inc.). Copy numbers for each transcript in each sample were calculated using 7500 Fast Real Time PCR System Sequence Detection Software v. 1.3.1 (Applied Biosystems, Inc.). Positive controls consisted of purified human RNA from blood and negative controls consisted of RNase-free H_2O. Positive controls amplified effectively with each primer, and negative controls did not amplify above threshold values for any primer pair. To assure PCR products of correct size were obtained, and to reiterate that RNA was indeed amplified rather than residual genomic DNA, a single aliquot from each sample was run on a 2% agarose gel. Each amplicon's size was estimated by an appropriately-sized marker lane run in parallel. All PCR products matched the expected amplification size in the documentation for each primer used.

We normalized the IL-6, IL-8, S100A8, and EGF transcripts to a constitutively expressed transcript, GAPDH. Transcript to GAPDH ratios were compared within the same sample, within the same patient, and among different patients to establish

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