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Research paper

Droplet digital PCR quantifies host inflammatory transcripts in feces reliably and reproducibly

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ABSTRACT

The gut is the most extensive, interactive, and complex interface between the human host and the environment and therefore a critical site of immunological activity. Non-invasive methods to assess the host response in this organ are currently lacking. Feces are the available analyte which have been in proximity to the gut tissue.

We applied a method of concentrating host transcripts from fecal specimens using a existing bead-based affinity separation method for nucleic acids and quantified transcripts using droplet digital PCR (ddPCR) to determine the copy numbers of a variety of key transcripts in the gut immune system. ddPCR compartmentalizes the reaction in a small aqueous droplet suspended in oil, and counts droplets as either fluorescent or non-fluorescent. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize transcript concentration.

This method was applied to 799 fecal samples from rural Malawian children, and over 20,000 transcript concentrations were quantified. Host mRNA was detected in >99% samples, a threshold for target detection was established at an average expression of 0.02 copies target/GAPDH, above which correlation coefficient between duplicate measurements is >0.95. Quantities of transcript detected using ddPCR were greater than standard qPCR. Fecal sample preservation at the time of collection did not require immediate freezing or the addition of buffers or enzymes. Measurements of transcripts encoding immunoactive proteins correlated with a measure of gut inflammation in the study children, thereby substantiating their relevance. This method allows investigators to interrogate gene expression in the gut.

1. Introduction

Optimal gut health is defined as the ability of the intestines to absorb all necessary dietary nutrients while mounting appropriate inflammatory responses to limit the dissemination of the microbes from the lumen while averting chronic local or systemic inflammation. The many microbes in the gut are thought to modulate gut health. Our awareness of the role of gut microbiota in gut health has increased exponentially in the last 5 years. The most pervasive condition associated with poor gut health worldwide is environmental enteric dysfunction (EED), which is associated with stunting [1–3]. Stunting affects 25% of the world's children, whose capacity for physical work, neurocognitive function, linear growth and immunocompetence are compromised [4]. Poor gut health has also been implicated in gastrointestinal tract cancers [5,6], autoimmune [7], mental health [8], neuro-psychological [9] and cardiovascular [10] disorders.

Unfortunately, direct assessment of gut health is invasive and expensive. The most reliable methods to assess gut health involve direct (endoscopic) visualization of the gut and biopsy, and the dual sugar absorption test [11]. Endoscopy is a resource-intensive procedure that is not well suited to mass screening, or to frequent intra-host assessment. The dual sugar absorption test, also known as the lactulose:mannitol (L:M) test, is administered by orally ingesting a solution of both sugars, and collecting all urine over a timed period of several hours. Lactulose, a disaccharide, is absorbed only through disrupted cell junctions, while mannitol, a monosaccharide, is absorbed across cell membranes and across cell junctions. Once absorbed these sugars are excreted unmetabolized in the urine. Increased L:M is indicative of disrupted architecture of the upper intestinal mucosa and poor gut health [12]. This is a theoretically sound and often used test, but does not provide information on mechanisms underlying increased permeability.

Stool is an easily acquired, but understudied, analyte that contains exfoliated enterocytes, representing gut mucosal tissue. Fecal extractions have been rarely used to analyze expression of individual host transcripts by quantitative PCR (qPCR). qPCR for host fecal

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transcripts is challenging because human mRNA is estimated to be less than 1% of total fecal RNA, which is predominantly microbial and ribosomal. mRNA in feces is also relatively degraded, and quantification can be further hampered by co-extracted inhibitors.

Here we report an improved methodology to detect fecal host mRNA using droplet digital PCR (ddPCR), as applied to stools from rural Malawian children with varying states of gut health as determined by L:M testing.

2. Materials and methods

2.1. Fecal samples

Fresh fecal samples were collected from 799 children aged 12–61 months in rural Malawi who participated in one of 3 clinical studies [13–15]. These children are from families of subsistence farmers, consume water from wells or boreholes and live in unelectrified mud huts. They are at high risk for the enteropathy associated with stunting. All of the subjects completed a carefully conducted L:M test with adequate urine collection and sugar excretion [16].

Fresh stools were collected before completion of the L:M testing using a small, clean non-absorbant, plastic diaper. The stools were immediately transferred to cryovials and flash frozen in liquid nitrogen, without buffers, enzymes or preservative solutions. Samples were transferred to a −80 °C freezer and transported to Washington University (St. Louis, MO), where they were then processed and analyzed for human fecal mRNA as outlined in Fig. 1 and detailed below.

Among the many hundreds of stool samples collected, 16 were divided into 6 aliquots and flash frozen immediately after, and 1, 2, 4, 8 and 24 h after passage. Storage prior to freezing was at ambient (Malawian) temperature, approximately 23–28 °C.

2.2. Isolation of fecal RNA

Fecal nucleic acid extractions were prepared using NucliSENS® easyMAG® system (bioMerieux, Durham, NC) and a modified version the protocol of Agapova et al. [17]. Approximately 200–300 mg of frozen stool and 8–10 disruption beads zirconium/silica 23 mm (Research Products International Corp.) was homogenized in 1 ml of easyMAG® lysis buffer using MP FastPrep-24 tissue homogenizer (MP Biomedicals) (60 s, 6.5 m/s,) two times, and then incubated (room temperature, 15 min). Debris was pelleted by centrifugation (10 min, 14,800*g*), and the clarified supernatant was loaded into wells of the easyMAG® cartridges, avoiding visual particulates. EasyMAG® lysis buffer was added to fill the remaining volume to 2 ml, 50 µl NucliSENS® easyMAG® magnetic silica were added. Samples were then extracted following the manufacturer's instructions for Protocol A using the offboard lysis option.

2.3. Droplet digital PCR detection of transcripts

Quantitative polymerase chain reaction assays were performed using duplexed FAM and VIC TaqMan assays in a droplet digital PCR system (QX100; Bio-Rad Laboratories, Inc, Hercules, CA) [18]. Duplicate reactions of 20 µl were prepared using 7.76 µl total nucleic acids, 10 µl ddPCR Supermix for Probes (BioRad), 0.08 µl SuperScript III Reverse Transcriptase (200 U/µl, Invitrogen Corporation, Carlsbad, CA), 0.16 µl RNase OUT (40 U/µl, Invitrogen), and 1 µl of each 20 × TaqMan Gene Expression Assay (Applied Biosystems, Carlsbad, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) assays were performed for each sample on each plate for normalization of all other targets. PCR reactions were dispersed into droplets using the QX100 droplet generator per the manufacturer's instruction (BioRad Laboratories) and transferred to a 96-well PCR plate. End point PCR was performed in a C1000 Touch thermal cycler (BioRad) with the following conditions:

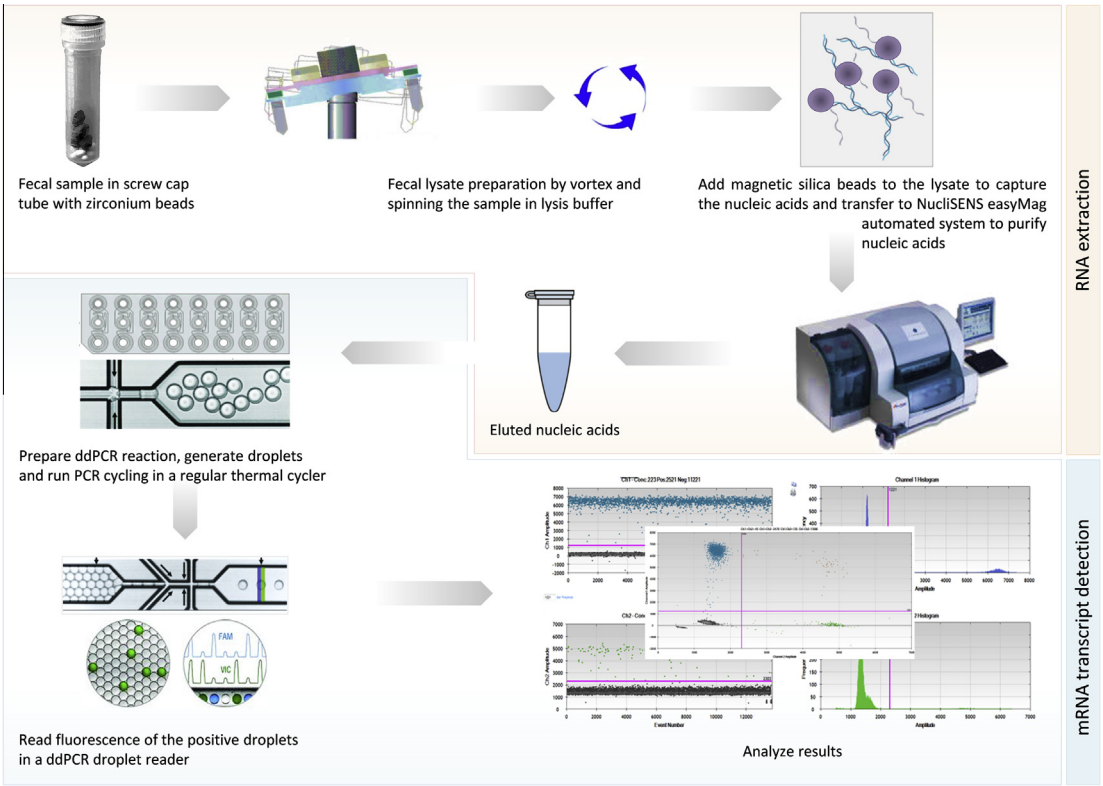

Fig. 1. Methodology used to isolate and detect fecal host transcripts.