Impact of Intestinal Parasites on Microbiota and Metagenomic Influences on Gene Encoding Cobalamin Pathway

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Abstract

Background: Approximately 30% of children worldwide are infected with gastrointestinal parasites. Depending on the species, parasites can disrupt intestinal bacterial flora affecting nutritional status. We implemented a multi-parallel quantitative real-time PCR and whole-genome sequencing analysis for bacterial microbiota and *Ascaris lumbricoides*, *Ancylostoma duodenale*, *Necator americanus*, *Strongyloides stercoralis*, *Trichuris trichiura*, *Cryptosporidium* species, *Entamoeba histolytica*, and *Giardia lamblia*. Stool samples were collected from 37 asymptomatic children from rural Argentina. We focused this study on *Giardia* screening. Separate analyses were done for uninfected, *Giardia* only, *Giardia*/helminth coinfections, and helminth only groups.

Results: For *Giardia* only infected children, DNA sequencing data showed a decrease in microbiota biodiversity compared to those uninfected that correlated with increasing *Giardia* burden. Clustering was statistically significant using Shannon alpha diversity. A non-significant increase in diversity was observed for helminth only infections with a compensatory decrease in *Giardia*/helminth coinfections. In *Giardia* only infections, microbiome taxonomy shifted from *Firmicutes* towards increasing proportions of *Prevotella*, with the degree of shift related to the intensity of infection compared to uninfected. An abundance of *Prevotella* bacteria was decreased in the helminths only group but increased for *Giardia*/helminth coinfections. Metagenomic analysis of the bacterial microbiota showed that the proportion of non-vitamin B12 producing bacteria (*Prevotella*) was higher in the *Giardia* group. Total cobalamin synthesis was decreased in the *Giardia*-infected only group compared to the control and helminth-infected group.

Conclusion: Our data provide evidence for an effect of parasitic infections allowing permissive growth of anaerobic bacteria such as *Prevotella*, suggesting an altered capacity of vitamin B12 biosynthesis and potential impact on growth and development in children.

Background

Gastrointestinal (GI) parasites are estimated to infect more than two billion people throughout the world[1]. Both soil-transmitted helminths (STH) (*Ascaris*, hookworm, *Strongyloides*, *Trichuris*) and protozoa (*Giardia*, *Cryptosporidium*, *Entamoeba histolytica*) are prevalent in resource-limited areas [2, 3]. Symptoms include chronic diarrhea, severe anemia, and can lead to intestinal obstruction. Economically disadvantaged children have recurrent infections leading to growth and cognitive delays due to malnutrition[4]. These children have more difficulties in school and, subsequently, in the job market[4]. The cycle continues when they remain in poverty and have their children[4]. The link between intestinal helminths and malnutrition leading to growth stunting and anemia has been found by others[5–12], and a new Global Burden of Disease Study points to evidence that hookworm is a leading cause of anemia in resource-poor settings[13]. Valuable information from the Global Enteric Multicenter Study (GEMS) also reveals an unexpected global health impact caused by protozoa [14, 15]. There are few studies attributing giardiasis to growth delays and no published studies showing the impact on the human intestinal

microbiome using multi-parallel real-time quantitative (qPCR) detect the presence of *Giardia* and quantitating the burden of infection[16]. To date, most studies examining intestinal parasitism have not been able to study intestinal worms and protozoa simultaneously and to successfully dissect the relative contribution of each of the significant intestinal helminth or protozoan pathogens to specific diseases. The current state of diagnosing GI parasites uses the subjective method of microscopy. Depending on the parasite, single stool microscopy exam sensitivity ranges from 50–80%[17]. As a result, large numbers of infected children are not being diagnosed and treated.

Gastrointestinal parasites cause intestinal inflammation, malabsorption, and microbiome changes. With advances in Next Generation DNA sequencing, we are now able to determine a broader range of intestinal microbiota. The microbiome is associated with digestion, nutrition, and health, but alterations in biodiversity can increase disease states and induce intestinal inflammation[18]. There is a lack of literature studying the relationship between GI parasites impact on intestinal microbiome[19]. These few studies have discordant results of the impact parasites have on microbiota biodiversity[20, 21].

There is increasing evidence that specific GI parasites (*Ascaris*) may increase the biodiversity of intestinal microbiota[21]. Since the burden of parasitic infection is directly correlated to morbidity and disease, the load may also impact the intestinal microbiota[21]. Our qPCR quantitates the burden of helminths and protozoa, determining the correlation of burden to changes in intestinal microbiota biodiversity.

Alterations in intestinal microbiota alter bacterial metabolisms, such as vitamin B12, reducing the capacity for human use. Unique to only bacteria is the synthesis of vitamin B12 (cobalamin)[22–24]. Vitamin B12 is a crucial coenzyme for humans cannot produce it, and it must be derived exogenously[25].

Results

Parasite DNA intensity

Table 1. Metadata of research subjects (Geometric mean, minimum, and maximum) Parasite DNA intensity associated with the intestinal parasite detection by stool qPCR as described by Rubén et al.

Groups (number of subjects)	Age Range	Male	Female	<i>Giardia</i> DNA (fg/μl)	<i>Ascaris</i> DNA (fg/μl)	<i>Ancylostoma</i> DNA (fg/μl)	<i>Necator</i> DNA (fg/μl)	<i>Strongyloides</i> DNA (fg/μl)
Control (5)	4.5 (3 to 6)	3	2	0	0	0	0	0
<i>Giardia</i> (13)	5.6 (4 to 7)	6	7	1.12 (0.012 to 20657)	0	0	0	0
<i>Giardia</i> Helminths (7)	6.8 (4 to 8)	4	3	0	1.062	164.8 (12.95 to 556.4)	0.249 (0.03 to 12.47)	39.59 (9.97 to 157.1)
Helminths (12)	5.1 (3 to 7)	6	6	0	6.82 (4.133 to 9.67)	10627.8 (2320 to 59963)	2.839 (0.119 to 63.8)	0.01357

Diversity of intestinal microbiota

Giardia >1 fg/μl group had a significant loss of biodiversity compared to the non-infected children using Shannon alpha diversity (*Giardia*-infected > 1 fg/μl 2.346; non-infected group 3.253, $p = 0.0317$). *Giardia* < 1 fg/μl (3.253), *Giardia*/helminth (3.118), helminths (3.407), with differences between helminth-infected and *Giardia*/helminth groups ($p = 0.0018$). *Giardia* >1 fg/μl had more diversity than the helminth only groups. ($p = 0.0003$). *Giardia* >1 fg/μl showed an increase in diversity to all other groups ($p = 0.0017$) (Figure 1A).

Measuring bacterial diversity in relation to increasing *Giardia* DNA intensity of burden (fg/μl) showed an inverse correlation of bacterial diversity to *Giardia* DNA (fg/μl) (Spearman $r = -0.5491$, $p = 0.0244$) (Figure 1B).

Bacterial overgrowth

In *Giardia* infections, microbiome analysis data shows a decrease in biodiversity in the infected parasite group compared to those non-infected, a bias toward increased *Prevotella*, with the degree of change related to the intensity of infection (Figure 4). *Giardia*-infected children had significantly higher proportions of the genus *Prevotella* bacteria directly correlating to above 1 fg/μl *Giardia* DNA versus No Parasite group ($p = 0.037$). Comparing all the cohorts revealed the helminth-infected group with decreased *Prevotella* proportions to *Giardia*-infected ($p = 0.024$), but similar to the control group. Interestingly, the *Giardia*-helminth infected group negated these differences compared helminth-infected group ($p = 0.019$) (Figure 2). *Prevotella copri* was the major species in each cohort, *Giardia* (37%), Helminths (17%), No Parasites (22%), *Giardia*/Helminths (36%) (data not shown).

All four groups had different bacteria genera as their most abundant microbiota. *Giardia* infected children, including *Giardia*/helminth coinfection, had higher Bacteroidales, including *Prevotella* species (Figure 3).

Metagenomics of Cobalamin Biosynthesis

Vitamin B12 InterPro identifier IPR002751 biosynthesis CbiM family was used in the analysis. High *Giardia* infection had fewer quantities of cobalamin DNA sequences than the No Parasite group ($p = 0.002$) (Figure 4A). Children with *Giardia* and *Giardia*/*Ascaris* infections have decreased the proportion of vitamin B12 pathway DNA sequences, compared to children with no parasites ($p = 0.021$) (Figure 4B).

Discussion

The impact of *Giardia* on diversity was seen in this study using whole-genome sequencing. While diarrhea caused by giardiasis can reduce the brush border layer in small intestinal cells, the subjects in this cohort were all asymptomatic at the time of stool collection. *Giardia* likely has a more prominent role, since its primary site of infection and replication is in the small intestine. *Giardia* is known to cause malabsorption, steatorrhea, and diarrhea[27], with preliminary studies finding improvements in vitamin B12 serum levels after treatment for giardiasis[28, 29].

Vitamin B12 synthesis primarily occurs in anaerobes, including *Bifidobacterium* and *Lactobacillus* species[25, 30–33]. These microorganisms may promote intestinal homeostasis and may protect against inflammatory diseases[34–37]. Vitamin B12 is absorbed in the small intestines[38–40] while the majority of microbiota reside in the colon[38], although, the small intestine is not sterile and does contain a robust microbiota that influences the absorption of vitamins[41–43]. *Prevotella* is not known to make an overabundance of cobalamin, the shift to higher *Prevotella* microbiota proportions decreases the types of vitamin B12 producers[22].

Specific bacteria produce vitamin B12, and the children infected with *Giardia* may be unable to synthesize the required amounts of vitamin B12 for nutritional benefit. While proportions of vitamin B12 synthesis gene sequences are less than 3% for each group, this is an appropriate amount of vitamin B12 biosynthesis gene sequences, considering the average stool sample had over 500 species of bacteria (and 22,000,000 sequences)[44].

One limitation of this cross-sectional study is the small sample size. However, the results are consistent, and this potential limitation can be viewed in terms of the sheer number, and fidelity of, using enriched microbe DNA for whole gene sequencing, producing billions of reads for analysis.

A significant consideration that is needed is to ascertain what is occurring in these children. Our findings that the higher burden of *Giardia*, compared to lower levels, is associated with less microbial diversity and increases of *Prevotella*. Increased *Giardia* implies that there are more parasites to alter the intestinal microbiome, and thus have a higher impact on bacterial species.

It is unclear whether the parasites are impacting the intestinal microbiota or external factors such as age, diet, or sex differences altering the intestinal microbiota and making the subjects more susceptible to enabling a *Giardia* infection. Some evidence comes from a mouse model study where the mouse intestinal microbiota (enteroaggregative *Escherichia coli*), independent of *Giardia* infection, can promote inflammation [45].

Conclusions

In this study, we propose a possible link on why *Giardia* and other parasites may cause growth and developmental delays in infected children. Evidence of *Giardia*'s impact on diversity and available micronutrients is observed with the *Giardia*/helminth coinfection group, where alterations of the microbiome are seen in the helminth only group.

While the helminth-infected group did not have changes in diversity or decreased cobalamin synthesis genes, a possible explanation is that most of these helminths reside in the colon and do not alter the microenvironments as does *Giardia*.

We are currently extending these results to additional populations where *Giardia* and other intestinal parasite infections are high and are performing, confirming the biochemical analysis of the vitamin B12

pathway products in affected children.

Methods

This study aimed to determine the impact of intestinal parasites on bacterial microbiota and subsequent cobalamin metagenomics.

Study population

Samples were selected from a previously published study using qPCR in peri-urban Argentina[26]. Samples were randomized and selected as representatives of four cohorts, (1) a control group with no parasites detected by qPCR, (2) a *Giardia* only infected group, (3) a helminth, and *Giardia* coinfection group, and (4) a helminth only infected group. Helminths included in this study were either *Ascaris lumbricoides*, *Ancylostoma duodenale*, *Necator americanus*, and *Strongyloides stercoralis* (Table 1).

Multi-parallel real-time quantitative PCR

All stools were collected and immediately stored in ice and sealed in air-tight containers, frozen within three hours, and DNA extracted using MP FastDNA Spin Kits for Soil (MP Biomedicals, Irvine, CA)[26]. This collection method was found to yield adequate microbiome data for study analysis[20]. Samples were processed in Salta, Argentina, using a modified bead-beating process described previously[26, 46]. DNA was processed for qPCR in Houston, Texas, as previously described [26, 46]. The intensity of parasite DNA was calculated using reference plasmids to create a standard curve, as previously reported[26]. Argentina qPCR results were used in this study[26].

Whole-genome sequencing

DNA sequencing and library construction were performed at New England Biolabs (NEB). To optimize microbiome sequencing, all samples underwent removal of methylated DNA with NEBNext® Microbiome enrichment kits (New England Biolabs, Ipswich, MA). No DNA size selection was done. DNA Library prep was done per manufacturer protocol with 1 gm of sample DNA used and 8 cycles of PCR enrichment (NEBNext Ultra DNA Library Prep Kit for Illumina, Version 5.1, 9/17). Sequencing was performed using an Illumina NextSeq (Illumina, San Diego, CA) with paired or single ends, and 151 base pair reads.

Bioinformatics

Fastq reads were filtered for quality at a minimum Phred score of 20 (99% accuracy) and a minimum length of 50 using Cutadapt version 1.8.3[47]. Paired-end reads were interleaved using merge_fastq_reads_with_N_separator. Pl perl script included with LMAT software version 1.2.6[48]. Fastq files were converted to fasta files using seqtk software version 1.0 (<https://github.com/lh3/seqtk>). Fasta files were processed by LMAT for taxonomic classification using LMAT kFull database. LMAT output text files were filtered for LMAT defined confidence score of 1 and minimum reads of 500 using tolineage.py script. Subsequently, output files were combined using merge_metaphlan_tables.py script

Included with Metaphlan software[49]. Alpha diversity was calculated using the Phyloseq R package. Abundant different Operational Taxonomic Units (OTU) among four groups were identified using the LEfSe algorithm[50]. Metagenomics analysis was performed using Diamond v0.8.4 using blastx mode with 90% minimum identity and e-value of 10^{-5} against nr database fasta file[51]. Results from Diamond analysis were exported to Megan version 6 using daa-meganizer program[52]. GenInfo identifier to Interpro identifier mapping within Megan program was used to annotate the vitamin B12 synthesis gene[53]. STAMP software was used for statistical analysis pertaining to taxonomic and metagenomic differences[54]. Microbial attributes were derived from Livermore Metagenomics Analysis Toolkit (LMAT) taxonomic output using Megan program (Figure 5).

Statistics

qPCR results were recorded for each patient as positive or negative for each parasite and concentration of parasite DNA. All statistics were performed using Prism v. 7.0b (GraphPad, La Jolla, CA). Kruskal-Wallis test was used to compare multiple groups. Spearman rank to correlate parasite concentrations to Shannon alpha diversity. The Shannon alpha diversity is a commonly reported diversity metric that weights the numbers of species by their relative evenness data[55]. All statistical models used *p* values less than 0.05 as significant.

Abbreviations

DNA: Deoxyribonucleic Acid

GEMS: Global Enteric Multicenter Study

GI: Gastrointestinal

LMAT: Livermore Metagenomics Analysis Toolkit

OTU: Operational Taxonomic Unit

qPCR: Multi-parallel Real-time Quantitative Polymerase Chain Reaction

STH: Soil-Transmitted Helminths

Declarations

Ethics approval and consent to participate

The internal review boards approved this study of Baylor College of Medicine and Universidad Nacional de Salta Argentina.

Informed written consent was obtained from each participant or a parent/guardian. Anti-parasitic treatment, based on microscopy findings, was provided per standard of care in the region. The majority of

subjects were of preschool age and did not receive standard mass drug administration of anthelmintics. The bioethics committee approved study design and protocols of Universidad Nacional de Salta, Argentina (UNSa).

Competing interests

RM receives research funding from Romark Laboratories, LC. This association did not impact this study.

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Authors contributions

RM and AD were responsible for bioinformatics and statistical analysis. RM, RJ, and PEB performed DNA extraction, qPCR, demethylation, and library preparation. BS, EL ED, and CL were involved in the DNA sequencing sample reactions and preliminary analysis. PV, MJ, PSC, JN, AK, and ROC collected patient samples in Argentina. RM, AD, and BS wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

Figure 1

Greater than 1 fg/μl *Giardia* DNA has a significant decrease in bacterial diversity to the No Parasite group ($p = 0.0244$) (1A). There was an inverse correlation of higher than 1 fg/μl of *Giardia* DNA to decreased

Shannon alpha diversity (Spearman $r = -0.5491$, $p = 0.0244$) (1B).

Figure 2

Giardia >1 fg/ μ l group had more abundant Prevotella than No Parasite group $p = 0.037$ (2A) with Helminths group decrease Prevotella to Giardia group ($p = 0.024$) and Giardia/helminth co-infected negating these differences ($p = 0.019$) (2B).

Figure 3

Most abundant OTUs for each cohort using LDA Effect Size (LEfSe)

Figure 4

High Giardia infected children had decreased cobalamin biosynthesis genes compared to No Parasites ($p = 0.038$) (4A) with compensatory effects from helminth infections ($p = 0.021$) (4B).

Figure 5

Flow chart for bioinformatics and data processing.