

ABSTRACT

Title of Document: IMPACT OF *CRYPTOSPORIDIUM* SPP.
INTERACTION WITH CO-OCCURRING
MICROORGANISMS ON MODERATE-TO-SEVERE
DIARRHEA IN THE DEVELOPING WORLD.

Molly Carroll Reid, MPH, 2015

Directed By: Dr. Amy Sapkota, Associate Professor,
Maryland Institute for Applied Environmental
Health

Diarrheal illness is responsible for over a quarter of all deaths in children under 5 years of age in sub-Saharan Africa and South Asia. Recent findings have identified the parasite *Cryptosporidium* as a contributor to enteric disease. We examined 9,348 cases and 13,128 controls from the Global Enteric Multicenter Study to assess whether *Cryptosporidium* interacted with co-occurring pathogens based on adjusted odds of moderate-to-severe diarrhea (MSD). *Cryptosporidium* was found to interact negatively with *Shigella spp.*, with multiplicative interaction score of 0.16 (95% CI: 0.07 to 0.37, p -value=0.000), and an additive interaction score of -9.81 (95% CI: -13.61 to -6.01, p -value=0.000). *Cryptosporidium* also interacted negatively with *Aeromonas spp.*, Adenovirus, Norovirus, and Astrovirus with marginal significance. Odds of MSD for *Cryptosporidium* co-infection with *Shigella spp.*, *Aeromonas spp.*, Adenovirus, Norovirus, or Astrovirus are lower than odds of MSD with either organism alone. This may reduce the efficacy of intervention strategies targeted at *Cryptosporidium*.

IMPACT OF *CRYPTOSPORIDIUM* SPP.
INTERACTION WITH CO-OCCURRING MICROORGANISMS ON MODERATE-
TO-SEVERE DIARRHEA IN THE DEVELOPING WORLD.

By

Molly Carroll Reid

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Advisory Committee:
Professor Amy Sapkota, Chair
Professor Raul Cruz-Cano
Professor Mihai Pop

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Table of Contents

Table of Contents	iii
List of Tables	iv
Chapter 1: Introduction	1
Diarrheal Disease in the Developing World	1
Cryptosporidium as an Emerging Concern	2
Polymicrobial Enteric Infections	3
Project Rationale	4
Chapter 2: Background	5
Cryptosporidium spp. and Cryptosporidiosis	5
Species and Disease Background	5
Epidemiology	5
The Global Enteric Multi-center Study	6
Rationale	6
Study Population and Design	7
Sample Processing	8
Interaction Analysis	8
Existing Knowledge	9
Gaps in Knowledge	12
Project Objectives	12
Chapter 3: Impact of <i>Cryptosporidium spp.</i> interaction with co-occurring microorganisms on moderate-to-severe diarrhea in the developing world	13
Abstract	13
Introduction	15
Materials and Methods	17
GEMS Methods Overview	17
Statistical Analysis	18
Results	20
Discussion	22
Public Health Implications	23
Limitations	24
Conclusions	24
Table 1	26
Table 2	27
Table 3	28
Table 4	29
Chapter 4: Public Health Implications and Conclusions	30
Public Health Implications	30
Concluding Thoughts	30
Bibliography	31

List of Tables

Table 1. Equations used for multiplicative and additive interaction analysis.

Table 2. Demographic Information for GEMS Subset

Table 3. Unadjusted Incidence of Pathogen and Co-infection With *Cryptosporidium*

Table 4. Adjusted Odds Ratios of Singular and Co-infections, and Interaction Analysis

Terms

Chapter 1: Introduction

Diarrheal Disease in the Developing World

Diarrhea is the second leading cause of death in young children worldwide [1]. Diarrheal illness is responsible for 25-30% of all deaths in children under 5 years of age in sub-Saharan Africa and South Asia [2]. With time and resources, diarrhea in children is easily treatable, but in the developing world, malnutrition and healthcare access issues potentiate its harm. While the instance of diarrhea for a young individual is in itself unpleasant and life threatening, not to mention a potential economic strain on the family, the impacts of the illness do not necessarily end when the clinical symptoms subside. Diarrhea in children is well associated with long term developmental impacts including growth stunting, cognitive impairments, changes in school performance, and work life productivity [3–10]. These long term sequelae, malnutrition, and risk of enteric disease are cyclically linked, making diarrhea an extremely complex public health issue.

Enteric diseases are closely linked to environmental interactions (on a microbial scale upwards), and as such cannot easily be quantified according to a linear pathogen-to-disease relationship. Putative pathogens are not necessarily detectable in all instances of diarrhea: 27% of moderate-to-severe diarrhea (MSD) cases had no identifiable pathogen in recent findings [11]. Pathogen detection also doesn't strictly translate to disease: 72% of healthy controls in the same study had one or more pathogens detectable [11].

Asymptomatic infection is a public health concern in its own right, as infection with enteric pathogens – regardless of diarrhea outcome- is associated with environmental enteropathy: subtle changes in the intestinal composition and gut microbiota that can

impact nutrient intake and enteric illness risk long term [12,13]. These complexities limit public health understanding of the scope and burden of enteric illness.

Cryptosporidium as an Emerging Concern

Among the many microorganisms associated with diarrhea in children is *Cryptosporidium spp.* Public health awareness of the burden and impact of *Cryptosporidium* has veritably exploded in the past three years following the publication of two landmark international studies: the Global Enteric Multicenter Study (GEMS) and MAL-ED cohort study [14,15]. While it cannot be said that the protozoan is an emerging infectious disease in terms of incidence or geographic distribution, these recent findings do for the first time illuminate how broad its impact may be. Bartelt in 2013 dubbed *Cryptosporidium spp.*, among others, a “Neglected Enteric Parasite” (NEP), and called on the World Health Organization (WHO) to reconsider the protozoan for its Neglected Disease Initiatives [12]. While it is not now a WHO Neglected Tropical Disease (NTD), it is on the NTD list considered by the Public Library of Science.

The new information on the scope of *Cryptosporidium*-associated diarrhea is particularly concerning because *Cryptosporidial* diarrhea is associated with an array of complex health effects beyond clinical diarrhea itself. While diarrhea is in general associated with developmental delay, as mentioned earlier, this is especially true for longer periods of diarrhea [3–6]. *Cryptosporidium* is strongly associated with prolonged episodes of diarrhea: it was the leading pathogen linked to prolonged symptoms in GEMS, and is associated with diarrheal episodes of greater than 7 days in a number of studies [4,6,11,16,17]. *Cryptosporidium* is linked to especially prolonged cases of diarrhea, and these are most harmful to child development. It follows that

Cryptosporidium has been thoroughly associated with developmental inhibition in children [7,17–26]. This strong link between *Cryptosporidium* and increased morbidity associated with diarrhea, in combination with the new reports of its high prevalence in children of the developing world, illustrates a daunting global health challenge. This calls for increased research into *Cryptosporidium*-induced diarrheal etiology.

Polymicrobial Enteric Infections

Along with the recent methodological advancements has been an increase in reports of polymicrobial infections in enteric illness studies. Microbial quantification techniques now available allow for the detection of a large range of pathogenic and non-pathogenic organisms, and the analytical capacity to process this information [27,28]. NEPs like *Cryptosporidium* are among the organisms whose detection is now far easier and more accurate because of these advances [29]. Polymicrobial infections in diarrhea have long been recognized, but now reflect more sensitive and inclusive diagnostics. A number of epidemiological studies have identified polymicrobial infections associated with diarrhea in study populations throughout the world [30–37]. In the GEMS, two or more putative pathogens were detected in 45% of children with MSD, and 32% of controls [11]. This finding is mirrored by more recent results from the MAL-ED cohort study, in which two or more pathogens were identified in 41.0% and 29.0% of stools for cases and controls, respectively [38]. Though the epidemiology of polymicrobial diarrhea is now better understood, the impact of co-infection on clinical outcomes remains unclear.

Project Rationale

This project explores the possible impacts of co-infection by *Cryptosporidium* with other putative enteric pathogens on diarrheal outcomes in the GEMS case-control study. Co-infecting organisms may interact to worsen disease outcomes relative to single infections, and understanding this interaction if present would help inform intervention strategies for diarrheal disease control. *Cryptosporidium* is a particularly harmful enteric pathogen, and it is as yet unclear how this morbidity is modulated.

Objective: Using GEMS data, compare microbial identity data from case and control stools to evaluate whether *Cryptosporidium* interacts with other microorganisms, and do so in a way that is replicable for future work.

Hypothesis: Children are at higher risk of moderate-to-severe diarrhea if co-infected with *Cryptosporidium* and another organism than they are if infected with either alone.

Chapter 2: Background

Cryptosporidium spp. and Cryptosporidiosis

Species and Disease Background

Cryptosporidium spp. is a ubiquitous apicomplexan monocellular protist with a global geographic distribution. An important veterinary microbe, it was first reported as a human pathogen in a 1976 case report of severe diarrhea in an immunocompromised patient [39]. It is now recognized as a significant threat to immunocompromised individuals and a major contributor to the global burden of diarrhea [40–42]. Many of the *Cryptosporidium* species are known to cause human disease, but the most prominent are *Cryptosporidium parvum* and *Cryptosporidium homini* [43]. *Cryptosporidium spp.* is environmentally hardy, resistant to chlorination, and can harbor in a number of mammalian reservoirs, including humans, in perpetuity[44]. *Cryptosporidium* may be waterborne, foodborne, transmitted via oral-fecal contact, and is possibly capable of respiratory transmission [41]. Symptoms of Cryptosporidiosis are diarrhea, dehydration, nausea, vomiting, and weight loss though most individuals with detectable *Cryptosporidium* infections are asymptomatic [44].

Epidemiology

Heterogeneous study populations and findings have long limited epidemiological understanding of the global burden of *Cryptosporidium*-associated diarrhea. A small number of studies have indicated the parasite as a potentially important cause of enteric disease in select populations, and these have recently been reaffirmed in the two goliath

international studies, GEMS and MAL-ED. In GEMS, the parasite was the second leading cause of moderate-to-severe diarrhea in infants at five of seven sites in sub-Saharan Africa and South Asia, and the overall fraction of illness attributable to *Cryptosporidium* at all sites was far higher than anticipated [11]. In MAL-ED, it was the fifth-leading cause of diarrhea in subjects in the first year of life [38]. These studies are the most comprehensive look into diarrheal disease etiology due to their global scope, unprecedented sample sizes, and use of up-to-date diagnostic microbiologic methods.

In GEMS, the mortality associated with *Cryptosporidium* in cases was 3.0%, and 4.0% for subjects aged 0-11 months and 12-23 months, respectively, with hazard ratios of 1.2 and 2.6 relative to matched no-pathogen controls, respectively [11]. As mentioned earlier, *Cryptosporidium* is also associated with prolonged diarrheal episodes [4,6,11,16,17], and long term morbidity and developmental inhibition [7,17–26]. The protozoan is connected with changes in the intestinal tract –referred to as environmental enteropathy-- which can alter nutrient intake, lumen barrier function, and risk for repeat infection [13,45–48]. These are the extent of known *Cryptosporidium* infection sequelae, but more may present as its epidemiology continues to improve.

The Global Enteric Multi-center Study

Rationale

The Global Enteric Multi-center Study is the largest case-control study to date addressing diarrheal disease etiology. It is a 3-year, prospective, age-stratified, matched case-control study of moderate-to-severe diarrhea in children under the age of five from 7 sites worldwide in sub-Saharan African and South Asia [14]. The study, designed and

implemented by the University of Maryland Medical School Center for Vaccine Development, was funded in 2006. The goals of this effort are to quantify diarrheal disease burden in low-income populations, identify key etiological agents responsible, and understand clinical outcomes of interest. In addition to these primary goals, GEMS is also intended to guide vaccine development, identify risk factors for disease, estimate economic implications of MSD, and generate a repository of clinical specimens for continuing research and collaboration.

Study Population and Design

The design of the GEMS study is published in detail elsewhere, but in brief, GEMS set up a population census at sites meeting basic health care and laboratory criteria in Kenya, Mali, Mozambique, the Gambia, Bangladesh, India, and Pakistan [49]. Cases were identified through the local health care facility if they had three or more abnormally loose stools within 24 hours, and one or more of the following: sunken eyes, loss of normal skin turgor, a decision to initiate intravenous hydration, dysentery, or a clinical decision to hospitalize the child. One or more controls were matched from within the same community if they did not have symptoms of diarrhea with seven days of the case enrollment. At enrollment, caretakers of both cases and controls completed an interview. All subjects underwent anthropometric measurements and clinical observations, and provided stool samples. Caretakers were given a chart to track diarrhea (or lack thereof) for two weeks, which was reviewed at a 60-day follow-up visit. The follow-up visit also included physical examinations and surveys.

Sample Processing

GEMS stool samples were analyzed using a set of comprehensive microbiological assays, standardized across all study sites, as described in detail by Panchalingham, *et al* [50]. The protocols for pathogen detection in GEMS analysis were selected based on the performance and robustness of the test, cost effectiveness, and the counsel of respected experts in the field for each organism. *Enterobacteriaceae*, *Vibrio* spp., *Aeromonas* spp., *Campylobacter* spp., and *E.coli* were identified using culture based and biochemical techniques. *E.coli* was further classified using PCR. Rotavirus, adenovirus, and the three parasites: *Cryptosporidium* spp., *Giardia enterica*, and *Entamoeba histolytica*, were all identified by respective immunoassays. RNA viruses were detected by multiplex PCR.

Interaction Analysis

Co-infecting microbes can interact in a number of ways to affect disease morbidity and mortality. Biological interaction occurs when two factors, organisms in this case, behave differently in combination than they do individually. This process can be due to direct interaction or indirectly through changes in the host environment via resource use or host immune response [51]. If an outcome associated with co-infection by two pathogens (P1 and P2, say) does not occur in individuals with no infection or only P1 or only P2, the pathogens interact in some way mechanistically [52]. The epidemiological groundwork for identifying biological interaction was led by Rothman, who established the conditions of “sufficient cause” and applied them to interaction theory [53,54]. This mechanistic interaction, or what is actually occurring biologically, is measured statistically in a number of ways.

Statistical interaction does *not* necessarily indicate mechanistic interaction, but can be used to identify potential biological relationships. Two scales are often employed to study interaction: additive and multiplicative. The additive scale shows the difference between the risk of outcome with simultaneous infection and the risks with either alone. The multiplicative scale shows how risk with dual infection relates to the product of risk with single infections. While the additive scale is generally more informative for identifying mechanistic interaction, the multiplicative scale is used far more commonly due to ease of analysis (regression analysis with interaction provides measure of multiplicativity) [52]. For case-control studies, odds ratios are used to show interaction on either scale. Sometimes called Relative Excess Risk due to Interaction (RERI), this is calculated as $OR_{ii} - OR_{io} - OR_{oi} + 1$. Standard error and confidence intervals for RERI can be calculated using Hosmer Lemeshow's delta method [55]. The multiplicative scale interaction analysis is equal to the odds ratio reported from the interaction term in a regression model. For the sake of simplicity, this quotient will be referred to as "MR", and can be understood as $OR_{ii}/(OR_{io} * OR_{oi})$. Standard error for this number is the same as produced in the model. Because the two scales are different measures of interaction, it is possible to have a positive additive interaction and negative multiplicative, or vice versa.

Existing Knowledge

While there is a large collection of research regarding interacting polymicrobial infections in a number of body systems, the gastro-intestinal tract has not been a major focus of this canon. As mentioned earlier, diarrheal infections with multiple pathogens

have been observed in several studies [30–37], and were found in 45% of cases of MSD in GEMS [11]. These papers did not further delineate polymicrobial infections by organism, so it is unknown what portion of mixed infections contains *Cryptosporidium*.

In chickens, a number of in vitro studies have identified interaction between *Cryptosporidium baileyi* and certain immunosuppressant viruses of veterinary importance. Especially when pre-exposed to the viruses, *Cryptosporidium*-virus co-infections are worse than either infection alone for both morbidity and mortality [56–58]. The parasite in chickens is normally asymptomatic.

In humans, three studies have considered the interaction between *Cryptosporidium* and other enteric pathogens thus far. The first, by Bilenko, *et al* was based on a cohort of 238 Bedouin children followed from birth to 18-23 months of age [32]. Using several markers of illness to generate a severity score for diarrheal episodes, they compared severity of single infections to severity of *Giardia* with other co-occurring pathogens. *Cryptosporidium* and *Giardia* co-infections did not have higher severity scores than either pathogen alone. While their results showed no interaction, this was based on only 35 samples with *Giardia* alone, 22 samples with *Cryptosporidium* alone, and 4 samples with both co-occurring.

The second study, by Lindsay, *et al* was based on diarrhea cases reporting to a large infectious disease hospital in Kolkata, India [59]. This work related the proportions of pathogens in polymicrobial diarrhea cases to the proportions of pathogens in all diarrhea cases. If no interaction occurred between pathogens, they would expect the microbial compositions of single and mixed infections in their study population to be the same. This was not the case, and they noted several potential interactions. They focused

on *Vibrio cholerae* and rotavirus, and reported the odds ratio of observed co-occurrence of either with various other pathogens relative to what co-occurrence frequency would be expected based on single infection frequencies. For *V. cholerae*, most co-infecting pathogens exhibited a negative association, including *Cryptosporidium*, which was 2.44 times less likely to be found in *V. cholerae* positive stools than in *V. cholerae* negative stools. However, after adjusting for age, gender, season, residence, and religion, this association was only significant for females. *Cryptosporidium* was significantly positively associated with rotavirus, and after adjustments, was 1.64 times more likely to be found in rotavirus-positive stools than in rotavirus negative stools. These relationships may point to possible interaction, but could also be the result of either pair originating from the same environmental sources.

The third study, by the same author, used direct additive and multiplicative measures of interaction [60]. The focus of this paper was *Shigella* spp., and GEMS formed the study population. They performed additional genetic testing on GEMS stools, and used levels (high or low) of the marker *ipaH* gene in order to quantify the degree of *Shigella* colonization. *Cryptosporidium* was among the many pathogens tested for interaction with *Shigella* in this analysis. No interaction was found for *Cryptosporidium*, or any pathogen. Of all pathogens studied, *Cryptosporidium* did have the smallest *p*-value for the multiplicative regression ($p=0.16$). *Shigella* did interact negatively with members of the *Lactobacillus* taxon.

Gaps in Knowledge

To our knowledge, no study has tested *Cryptosporidium* against a range of co-infecting organisms for potential interactions to date. The organism was included as a co-occurring pathogen in three studies, described above. One paper used direct additive and multiplicative interaction measures.

Project Objectives

In this study we use GEMS data to compare microbial identity data from case and control stools to evaluate whether *Cryptosporidium* interacts with other microorganisms, and create a statistical function for future analyses of this kind.

Chapter 3: Impact of *Cryptosporidium spp.* interaction with co-occurring microorganisms on moderate-to-severe diarrhea in the developing world

Abstract

Diarrheal illness is responsible for over a quarter of all deaths in children under 5 years of age in sub-Saharan Africa and South Asia. Recent findings have pointed to the parasite *Cryptosporidium* as a substantial contributor to enteric disease burden in the developing world. We assessed whether *Cryptosporidium* interacted with any other co-occurring pathogen in the Global Enteric Multicenter Study (GEMS). We examined 33 pathogens detected in stools from 9,348 cases and 13,128 controls from The Gambia, Kenya, Mali, Mozambique, India, Bangladesh, and Pakistan. Analysis for multiplicative and additive interaction was completed using R version 3.2.1, based on calculated odds of moderate-to-severe diarrhea (MSD) adjusted for site, age, sex, body mass index (BMI), antibiotic use, and stool consistency. *Cryptosporidium* was found to interact negatively with *Shigella spp.*, with multiplicative interaction score of 0.16 (95% CI: 0.07 to 0.37, p -value=0.000), and an additive interaction score of -9.81 (95% CI: -13.61 to -6.01, p -value=0.000). *Cryptosporidium* also interacted negatively on both the multiplicative and additive scales with *Aeromonas spp.*, Adenovirus 40/41, Norovirus, and Astrovirus, with marginally significant p -values of less than .15. Odds of MSD for *Cryptosporidium* co-infection with *Shigella spp.*, *Aeromonas spp.*, Adenovirus 40/41, Norovirus, or Astrovirus are lower than odds of MSD with either organism alone. Intervention strategies targeted at *Cryptosporidium* in regions with high incidence of any of these organisms, especially *Shigella spp.*, may be less effective than they would be if *Cryptosporidium* did not

interact. The ability of *Cryptosporidium* to interact with additional organisms to moderate diarrhea outcomes may impact the efficacy of targeted intervention strategies and should be further explored.

Introduction

Diarrhea is the second leading cause of death in young children worldwide [1]. Diarrheal illness is responsible for 25-30% of all deaths in children under 5 years of age in sub-Saharan Africa and South Asia [2]. With time and resources, diarrhea in children is easily treatable, but in the developing world, malnutrition and healthcare access issues potentiate its harm. Diarrhea in children is also associated with long term developmental impacts including growth stunting, cognitive impairments, changes in school performance, and work life productivity [3–10]. One of the many microorganisms associated with diarrhea in children is the parasite *Cryptosporidium spp.*, and this organism is particularly linked to these long term adverse outcomes [7,17–26].

Public health awareness of the burden and impact of *Cryptosporidium* has veritably exploded in the past three years following the publication of two landmark international studies: the Global Enteric Multicenter Study (GEMS) and the Interactions of Malnutrition & Enteric Infections: Consequences for Child Health and Development (“MAL-ED”) cohort study [14,15]. In GEMS, the parasite was the second leading cause of moderate-to-severe diarrhea in infants at five of seven sites in sub-Saharan Africa and South Asia, and the overall fraction of illness attributable to *Cryptosporidium* at all sites was far higher than anticipated [11]. In MAL-ED, it was the fifth-leading cause of diarrhea in subjects in the first year of life [38]. The new reports of the high burden of *Cryptosporidium* in children of the developing world, in combination with the strong link between *Cryptosporidium* and increased morbidity associated with diarrhea illustrate a daunting global health challenge.

Putative pathogens, such as *Cryptosporidium*, are not necessarily detectable in all instances of diarrhea: 27% of moderate-to-severe diarrhea (MSD) cases had no identifiable pathogen in recent findings [11]. Pathogen detection also doesn't strictly translate to disease: 72% of healthy controls in the same study had one or more pathogens detectable [11]. Asymptomatic infection is a public health concern in its own right, as infection with enteric pathogens – regardless of diarrhea outcome- is associated with environmental enteropathy: subtle changes in the intestinal composition and gut microbiota that can impact nutrient intake and enteric illness risk long term [12,13]. These complexities limit public health understanding of the scope and burden of enteric illness.

A number of epidemiological studies have identified individuals with two or more putative pathogens in diarrhea study populations throughout the world [30–37]. In the GEMS, two or more putative pathogens were detected in 45% of children with MSD, and 32% of controls [11]. This finding is mirrored by more recent results from the MAL-ED cohort study, in which two or more pathogens were identified in 41.0% and 29.0% of stools for cases and controls, respectively [38]. Organisms in polymicrobial infections can interact to alter disease outcomes. For example, Chonmaitree *et al.* showed that viral respiratory tract infections negatively impact clinical outcomes in acute otitis media, a common bacterial inner-ear infection [61]. However, few studies have explored whether co-infection with *Cryptosporidium* influences diarrheal disease outcomes in children. Here, we explored the possible impacts of co-infection by *Cryptosporidium* with other putative enteric pathogens on diarrheal outcomes in the GEMS case-control study.

Materials and Methods

GEMS Methods Overview

The design of the GEMS study is published in detail elsewhere, but in brief, GEMS set up a population census at sites meeting basic health care and laboratory criteria in six sites within Sub-Saharan Africa and South Asia [49]. Cases were identified through the local health care facility if they had three or more abnormally loose stools within 24 hours, and one or more of the following: sunken eyes, loss of normal skin turgor, a decision to initiate intravenous hydration, dysentery, or a clinical decision to hospitalize the child. One or more controls were matched from within the same community if they did not have symptoms of diarrhea within seven days of the case enrollment. All subjects underwent anthropometric measurements and clinical observations, and provided stool samples.

GEMS stool samples were analyzed using a set of comprehensive microbiological assays, standardized across all study sites, as described in detail by Panchalingham, *et al* [50]. The protocols for pathogen detection in GEMS analysis were selected based on the performance and robustness of the test, cost effectiveness, and the counsel of respected experts in the field for each organism. *Enterobacteriaceae*, *Vibrio* spp., *Aeromonas* spp., *Campylobacter* spp., and *Escherichia coli* were identified using culture based and biochemical techniques [50]. *Escherichia coli* was further classified to subgroups, enterotoxigenic *Escherichia coli* (ETEC), heat-stable (ST-) ETEC, both typical and atypical enteropathogenic *Escherichia coli* (atyp-EPEC or typ-EPEC), and enteroaggregative *Escherichia coli* (EAEC) using PCR [50]. Rotavirus, adenovirus (including serotypes 40 and 41 combined), and the three parasites: *Cryptosporidium* spp.,

Giardia enterica, and *Entamoeba histolytica*, were all identified by respective immunoassays [50]. RNA viruses were detected by multiplex PCR, including two genotypes for Norovirus (GI and GII) [50].

The *Cryptosporidium* test that was used is an enzyme-linked immunosorbent assay (ELISA) that identifies *Cryptosporidium* cyst antigens from stool specimens and is commercially available from TechLab, Inc. The assay is 98.4% sensitive and 100% specific [62], and has been validated and optimized in a number of studies [63–65].

Statistical Analysis

For this analysis, data from all GEMS participants from The Gambia, Kenya, Mali, Mozambique, India, Bangladesh, and Pakistan was used. Statistical analysis was completed using R 3.2.1 (R Foundation for Statistical Computing, Vienna, Austria)[66]. Adjusted odds ratios for each pathogen co-infection with *Cryptosporidium* and another organism were calculated using logistic regression models including the following covariates: country, study site, age in months, sex, body mass index, and whether the subject was on antibiotics when enrolled. Relative Excess Risk due to Interaction (RERI), was calculated as $OR_{ij} - OR_{io} - OR_{oi} + 1$. Standard errors and confidence intervals for RERIs were calculated using Hosmer Lemeshow's delta method [55]. The multiplicative scale interaction was derived using a logistic regression model with an interaction term containing the results of the *Cryptosporidium* assay and the additional microbe of interest. This measure of departure from multiplicativity, "MR", can be understood as $OR_{ij}/(OR_{io} * OR_{oi})$. Table 1 summarizes formulas used for this analysis and their interpretation. Pathogens whose incidence in combination with *Cryptosporidium* was fewer than 5 for either cases or controls were not included in the analyses.

A function for computation of the additive and multiplicative interaction was adapted from the Gene-Environment and Gene–Gene Interaction Research Application (GEIRA)[67]. The new function, called Co-Infection Interaction Research Application (CIIRA) incorporates multiple covariates and also reports MR and RERI, as well as incidence measures and general results of the regression model in addition to the previous GEIRA outputs.

Results

Interaction analysis was completed for a total of 22,568 subjects (9,348 cases and 13,128 controls) from The Gambia, Kenya, Mali, Mozambique, India, Bangladesh, and Pakistan. Demographic data are presented in Table 2. Multiple organisms were detected in 63.2% of all subjects, and statistically significantly different proportions in controls and cases (58.7% and 69.4% respectively, p -value= .000).

Screening co-infection by *Cryptosporidium* and 32 other pathogens of interest was conducted through the CIIRA function. Table 3 illustrates unadjusted incidences for the seventeen most common organisms by co-infection incidence in cases. 24 organisms, when paired with *Cryptosporidium*, had sufficient incidences in cases and controls to conduct multiplicative and additive analysis. This was conducted using odds ratios adjusted for country, study site, age in months, sex, body mass index, stool consistency, and antibiotic status. The 24 organisms, by incidence of co-infection in cases, were *Vibrio spp.*, *Escherichia coli*, *Giardia*, EAEC, Rotavirus, *Campylobacter spp.*, *Campylobacter jejuni*, ETEC, atyp-EPEC, typ-EPEC, ST-ETEC, *Shigella spp.*, Norovirus, Adenovirus, *Entamoeba histolytica*, Norovirus GII, *Aeromonas spp.*, *Shigella flexneri*, Sapovirus, Astrovirus, Norovirus GI, *Shigella sonnei*, Adenovirus 40/41, and *Campylobacter coli*. Results of the multiplicative and additive analysis for these organisms are presented in Table 4.

Cryptosporidium and *Shigella spp.* co-infection showed significant negative interaction on the multiplicative and additive scales, with an MR of 0.16 (95% CI: 0.07 to 0.37, p -value=0.000), and a RERI of -9.81 (95% CI: -13.61 to -6.01, p -value=0.000). Within *Shigella*, the two species, *flexneri* and *sonnei*, negatively interacted similarly with

Cryptosporidium. *Shigella flexneri* interacted with *Cryptosporidium* with an MR of 0.16 (95% CI: 0.06 to 0.58, p -value=0.004), and a RERI of -10.84 ((95% CI: -16.84 to -4.83, p -value=0.000). *Shigella sonnei* interacted with *Cryptosporidium* with an MR of 0.11 (95% CI: 0.03 to 0.43, p -value=0.001), and a RERI of -6.79 ((95% CI: -10.49 to -3.10, p -value=0.000). *Shigella spp.* was the only organism to significantly interact with *Cryptosporidium*, though four additional organisms interacted with marginal significance. *Aeromonas spp.*, Adenovirus 40/41, Norovirus, and Astrovirus all expressed negative interaction with *Cryptosporidium* on both the multiplicative and additive scales, with p -values of less than .15 (see Table 4).

Discussion

This study considers the impact of *Cryptosporidium* interaction with other co-occurring organisms on diarrhea outcomes in children of the developing world using data from the GEMS case control study. In the participants used for this analysis, we found that *Cryptosporidium* does interact significantly with the Shigelloids, in particular *Shigella flexneri* and *sonnei* on multiplicative and additive scales. This is the first report of *Cryptosporidium* interacting with another co-occurring organism in a human case-control study.

Cryptosporidium and *Shigella spp.* showed a negative multiplicative and additive interaction. Findings from Lindsay, *et al* 2015 suggest that these organisms may interact, though these were drawn from a smaller GEMS subset and were marginally significant on the multiplicative scale with the *p*-value of 0.16 (MR not reported), and not significant on the additive scale with a RERI of -.27 (95% CI: ~~[-1.47, 0.91]~~ [60]).

The marginal significance of the *Shigella-Cryptosporidium* interaction in the Lindsay analysis agrees with our findings, and the differences in values may be explained by the different sample sizes, as well as our additional inclusion of BMI, antibiotic status, and stool consistency in our logistic regression.

This negative interaction is highly significant, though its biological explanation is not clear. It is possible that either *Cryptosporidium* or *Shigella spp.* interact antagonistically within the host environment [51,68], for example by changing intestinal binding surface or by competing for similar resources. Further analyses should investigate *Cryptosporidium-Shigella* interaction further to determine if this may have a mechanistic explanation, and consider possible biological causes.

In addition to the interaction with *Shigella spp.*, *Cryptosporidium* showed marginal interaction on both the additive and multiplicative scales with *Aeromonas spp.*, Adenovirus 40/41, Norovirus, and Astrovirus. These potential interactions also warrant further exploration for biological mechanisms and other covariates that may moderate the relationships.

Future work should consider the quantity of *Cryptosporidium* and any other pathogens included as a more accurate measure of exposure to determine whether this impacts interaction. Also important may be measures of disease morbidity, which are available for some subsets of the GEMS data. It is possible that interaction impacts factors like disease duration, type of diarrhea, wasting and a number of other outcomes. In studies of *Cryptosporidium baileyi* interaction with co-infecting viruses in chickens, pre-exposure to the virus increased severity of illness [56–58]. Potentially illuminating research may regard pre-exposure to either organism to determine if exposure order is significant in the *Cryptosporidium* co-infection interaction in humans.

Public Health Implications

While further work should be needed to assess the potential for mechanistic interaction between *Cryptosporidium* and *Shigella spp.* and the other marginally significant organisms, knowledge of these negative interactions may inform intervention strategies. As *Cryptosporidium* was so recently recognized as a major contributor to global diarrheal disease burden, efforts to reduce enteric illness are now considering *Cryptosporidium* specific interventions. In areas with high incidence of *Shigella spp.* and the other organisms, however, reducing *Cryptosporidium* alone could exacerbate the effects of these pathogens. The odds of presenting with MSD when co-infected are

smaller than the odds of presenting with MSD when singularly infected. In these cases, *Cryptosporidium* may not be an ideal candidate for targeted interventions. Knowing the negative interaction of *Cryptosporidium* with other organisms may help public health organizations determine how specific their interventions may need to be.

Limitations

This study is a brief look into interaction between co-infecting organisms in enteric disease, but our findings must be interpreted with caution. Statistical interaction does not prove mechanistic interaction, and so further investigation is required before any biological relationship is determined.

Additionally, our outcome of interest was simple MSD diagnosis, but more subtle interactions may be identified using additional measures of illness like disease severity, prolongation, or other morbidities. Similarly, our organisms were classified by presence or absence in stool, but a number of studies have found that pathogen quantity in stool is a stronger predictor of disease than simple binary detection [60,69,70]. As interaction, especially for parasites, is often a product of resource competition [51], pathogen quantity may be paramount.

Conclusions

While the statistical interaction that we observed for *Cryptosporidium* and *Shigella spp.* does not prove mechanistic interaction, the results do suggest that interaction with the parasite may moderate odds of MSD in children. Because co-infection with this pair appears to reduce odds of MSD relative to single infections, this negative interaction with *Cryptosporidium* may limit the efficacy of enteric illness

interventions targeted at the parasite specifically, especially if the region has a high incidence of shigellosis. The other potential interactions between *Cryptosporidium* and *Aeromonas spp.*, Adenovirus 40/41, Norovirus, and Astrovirus may further reduce the efficacy of *Cryptosporidium*-specific interventions. It is clear that *Cryptosporidium* is a subtly complex microbe whose role in the global burden of enteric illness is growing in importance.

Table 1

Equations used for multiplicative and additive interaction analysis.

	Equation	Notes
MR	$OR_{ii} / (OR_{io} * OR_{oi})$	MR>1 -positive multiplicative interaction MR<1 -negative multiplicative interaction
RERI	$OR_{ii} - OR_{io} - OR_{oi} + 1$	RERI>0 -positive additive interaction RERI<0 -negative additive interaction
RERI Std. Error	$\sqrt{(OR_{io}^2 * var_{io}) + (OR_{oi}^2 * var_{oi}) + (OR_{ii}^2 * var_{ii}) + (2 * OR_{io} * OR_{oi} * cov12) + (2 * OR_{io} * OR_{ii} * cov23) + (2 * OR_{oi} * OR_{ii} * cov13)}$	From Hosmer Lemeshow, 1992

Table 2

Demographic Information for GEMS Study Population

	Cases n=9440	Controls n=13129
	# (%)	n (%)
Sex		
Male	5345 (57)	7478 (57)
Female	4095 (43)	5651 (43)
Age Group (months)		
0-5	1256 (13)	1534 (12)
6-11	2774 (29)	3343 (25)
12-23	3205 (34)	4382 (33)
24-35	1310 (14)	2378 (18)
36-59	895 (9)	1492 (11)
Country		
The Gambia	1029 (11)	1569 (12)
Mali	2033 (22)	2064 (16)
Mozambique	682 (7)	1296 (10)
Kenya	1476 (16)	1883 (14)
India	1568 (17)	2014 (15)
Bangladesh	1394 (15)	2465 (19)
Pakistan	1258 (13)	1838 (14)

Table 3

Unadjusted Incidence of Pathogen and Co-infection With *Cryptosporidium*

Organism	Single Infections		Co-Infected with <i>Cryptosporidium</i>	
	Cases % (#)	Controls % (#)	Cases % (#)	Controls % (#)
<i>Cryptosporidium</i>	6.4(843)	11.9(1123)	--	--
<i>Vibrio spp.</i>	53.8(5770)	64.5(9033)	7.4(696)	4.3(565)
<i>Escherichia coli</i>	32.8(3512)	32.3(4518)	4.4(419)	2.1(273)
<i>Giardia</i>	16.4(1786)	24.4(3470)	2.5(234)	2.1(270)
EAEC	17.9(1846)	19.5(2652)	2.4(220)	1.2(157)
Rotavirus	16.8(1747)	3.6(509)	1.7(161)	0.2(30)
<i>Campylobacter spp.</i>	10.8(1171)	11.1(1562)	1.6(149)	0.8(107)
<i>Campylobacter jejuni</i>	8.5(930)	8.1(1144)	1.3(127)	0.6(82)
ETEC	10.4(1066)	7.2(975)	1.4(124)	0.4(55)
Atyp-EPEC	7.0(732)	7.9(1087)	1.1(98)	0.6(72)
Typ-EPEC	6.3(652)	6.6(908)	0.8(76)	0.5(60)
ST-ETEC	6.3(644)	2.7(364)	0.8(71)	0.2(21)
<i>Shigella spp.</i>	11.0(1110)	1.6(230)	0.7(69)	0.1(19)
Norovirus	6.9(722)	6.8(940)	0.7(69)	0.4(51)
Adenovirus	3.6(385)	2.4(346)	0.5(48)	0.2(25)
<i>Entamoeba histolytica</i>	2.5(279)	2.1(299)	0.5(47)	0.2(23)
Norovirus GII	4.7(495)	3.7(511)	0.5(47)	0.2(28)
<i>Aeromonas spp.</i>	6.6(660)	4.5(620)	0.4(41)	0.2(32)
<i>Shigella flexneri</i>	7.3(728)	0.8(119)	0.4(41)	0.1(8)
Sapovirus	3.1(325)	3.3(456)	0.4(33)	0.2(24)
Astrovirus	2.2(238)	1.8(261)	0.3(28)	0.2(21)
Norovirus GI	2.5(265)	3.4(473)	0.3(26)	0.2(27)
<i>Shigella sonnei</i>	2.6(264)	0.5(71)	0.2(20)	0.1(10)
Adenovirus 40/41	10.1(235)	4.1(98)	0.9(20)	0.5(10)
<i>Campylobacter coli</i>	1.6(161)	2.2(313)	0.1(11)	0.1(18)

GI = Norovirus Genotype 1, GII = Norovirus Genotype 2, 40/41 = Adenovirus sero-group 40 or 41, ETEC = enterotoxigenic *Escherichia coli*, ST-ETEC = heat-stable enterotoxigenic *Escherichia coli*, atyp-EPEC = atypical enteropathogenic *Escherichia coli*, typ-EPEC = typical enteropathogenic *Escherichia coli*, and EAEC = enteroaggregative *Escherichia coli*

Table 4

Adjusted Odds Ratios of Singular and Co-infections, and Interaction Analysis Terms

	Cryptosporidium Adjusted ORs	Organism 2 Adjusted ORs	Co-infection Adjusted ORs	Multiplicative Interaction Adjusted MR	Additive Interaction Adjusted RERI		
Organism 2	OR _{io} (95% CI)	OR _{oi} (95% CI)	OR _{ii} (95% CI)	MR (95% CI)	Pr(> z)	RERI (95% CI)	Pr(> z)
<i>Aeromonas spp.</i>	1.44 (1.21 - 1.71)	2.24 (1.82 - 2.75)	1.55 (0.63 - 3.80)	0.48 (0.19 - 1.21)	0.121	-1.13 (-2.64 - 0.38)	0.141
<i>Campylobacter spp.</i>	1.36 (1.13 - 1.63)	1.23 (1.06 - 1.43)	1.94 (1.22 - 3.09)	1.16 (0.70 - 1.94)	0.567	0.35 (-0.62 - 1.33)	0.477
<i>Campylobacter jejuni</i>	1.37 (1.14 - 1.63)	1.39 (1.17 - 1.65)	2.15 (1.27 - 3.63)	1.13 (0.64 - 2.01)	0.666	0.40 (-0.80 - 1.59)	0.517
<i>Campylobacter coli</i>	1.38 (1.16 - 1.64)	0.81 (0.58 - 1.15)	1.47 (0.37 - 5.92)	1.31 (0.31 - 5.53)	0.711	0.28 (-1.81 - 2.37)	0.793
<i>Shigella spp.</i>	1.55 (1.30 - 1.84)	12.44 (9.91 - 15.61)	3.17 (1.48 - 6.82)	0.16 (0.07 - 0.37)	0.000	-9.81 (-13.61 - -6.01)	0.000
<i>Shigella flexneri</i>	1.49 (1.25 - 1.77)	14.16 (10.57 - 18.98)	3.81 (1.24 - 11.67)	0.18 (0.06 - 0.58)	0.004	-10.84 (-16.84 - -4.83)	0.000
<i>Shigella sonnei</i>	1.43 (1.20 - 1.69)	7.60 (4.90 - 11.81)	1.24 (0.36 - 4.24)	0.11 (0.03 - 0.43)	0.001	-6.79 (-10.49 - -3.10)	0.000
<i>Vibrio spp.</i>	1.30 (0.96 - 1.75)	0.48 (0.43 - 0.54)	0.71 (0.57 - 0.88)	1.13 (0.78 - 1.62)	0.520	-0.07 (-0.53 - 0.38)	0.752
<i>Escherichia coli</i>	1.38 (1.12 - 1.69)	1.00 (0.90 - 1.10)	1.40 (1.05 - 1.88)	1.03 (0.72 - 1.47)	0.890	0.03 (-0.51 - 0.58)	0.904
ETEC	1.38 (1.15 - 1.66)	1.06 (0.89 - 1.26)	1.29 (0.73 - 2.27)	0.88 (0.48 - 1.63)	0.688	-0.15 (-0.96 - 0.66)	0.714
ST-ETEC	1.38 (1.16 - 1.65)	1.44 (1.13 - 1.84)	1.60 (0.69 - 3.74)	0.80 (0.33 - 1.97)	0.634	-0.22 (-1.66 - 1.22)	0.764
Typ-EPEC	1.34 (1.12 - 1.60)	1.04 (0.86 - 1.25)	1.95 (1.00 - 3.79)	1.40 (0.69 - 2.86)	0.348	0.57 (-0.77 - 1.92)	0.403
Atyp-EPEC	1.33 (1.11 - 1.60)	1.02 (0.85 - 1.22)	1.91 (1.04 - 3.49)	1.40 (0.73 - 2.69)	0.313	0.55 (-0.66 - 1.76)	0.371
EAEC	1.37 (1.13 - 1.66)	0.91 (0.80 - 1.02)	1.22 (0.84 - 1.79)	0.99 (0.64 - 1.52)	0.946	-0.05 (-0.62 - 0.52)	0.856
<i>Entamoeba histolytica</i>	1.36 (1.14 - 1.61)	1.34 (0.96 - 1.88)	3.14 (1.27 - 7.73)	1.72 (0.65 - 4.56)	0.272	1.44 (-1.45 - 4.33)	0.328
<i>Giardia</i>	1.37 (1.13 - 1.67)	0.83 (0.74 - 0.94)	1.21 (0.87 - 1.69)	1.06 (0.71 - 1.58)	0.771	0.01 (-0.51 - 0.53)	0.980
Rotavirus	1.46 (1.23 - 1.74)	3.57 (2.92 - 4.36)	4.84 (2.22 - 10.52)	0.93 (0.41 - 2.10)	0.857	0.81 (-3.06 - 4.67)	0.682
Adenovirus	1.39 (1.17 - 1.65)	1.26 (0.95 - 1.68)	1.60 (0.69 - 3.71)	0.91 (0.37 - 2.25)	0.842	-0.05 (-1.48 - 1.37)	0.944
Adenovirus 40/41	1.87 (1.23 - 2.86)	2.57 (1.39 - 4.77)	1.15 (0.29 - 4.57)	0.24 (0.05 - 1.05)	0.058	-2.29 (-4.89 - 0.31)	0.084
Norovirus	1.44 (1.21 - 1.72)	1.11 (0.92 - 1.34)	0.94 (0.50 - 1.77)	0.59 (0.30 - 1.16)	0.125	-0.61 (-1.31 - 0.09)	0.086
Norovirus GI	1.41 (1.19 - 1.19)	0.98 (0.75 - 1.30)	0.79 (0.31 - 2.00)	0.57 (0.21 - 1.52)	0.260	-0.60 (-1.43 - 0.23)	0.154
Norovirus GII	1.42 (1.20 - 1.69)	1.35 (1.07 - 1.71)	1.10 (0.48 - 2.56)	0.57 (0.24 - 1.39)	0.218	-0.67 (-1.70 - 0.35)	0.199
Sapovirus	1.40 (1.18 - 1.67)	1.03 (0.80 - 1.34)	1.11 (0.47 - 2.63)	0.77 (0.31 - 1.91)	0.570	-0.32 (-1.36 - 0.71)	0.540
Astrovirus	1.42 (1.19 - 1.68)	1.23 (0.89 - 1.70)	0.77 (0.29 - 2.03)	0.44 (0.16 - 1.24)	0.121	-0.88 (-1.77 - 0.02)	0.054

GI = Norovirus Genotype 1, GII = Norovirus Genotype 2, 40/41 = Adenovirus sero-group 40 or 41, ETEC = enterotoxigenic *Escherichia coli*, ST-ETEC = heat-stable enterotoxigenic *Escherichia coli*, atyp-EPEC = atypical enteropathogenic *Escherichia coli*, typ-EPEC = typical enteropathogenic *Escherichia coli*, and EAEC = enteroaggregative *Escherichia coli*

ORs adjusted for age, country, site, body mass index, stool consistency, and antibiotic status.

MR is OR_{ii}/(OR_{io} x OR_{oi}), >1 is positive interaction, <1 is negative interaction.

RERI is OR_{ii} - OR_{io} - OR_{oi} +1, >0 is positive interaction, <0 is negative interaction.

Significant results bolded.

Chapter 4: Public Health Implications and Conclusions

Public Health Implications

While further work should continue to assess the potential antagonism between *Cryptosporidium* and *Shigella spp.*, this information may inform intervention strategies now developing. As *Cryptosporidium* was so recently recognized as a major contributor to global diarrheal disease burden, efforts to reduce enteric illness are now considering *Cryptosporidium* specific interventions. In areas with high incidence of *Shigella spp.*, however, reducing *Cryptosporidium* singularly could exacerbate the effects of these pathogens. Knowing the negative interaction of *Cryptosporidium* with other organisms may help public health organizations determine how specific their interventions may need to be.

Concluding Thoughts

Future work should consider the quantity of *Cryptosporidium* and any other pathogens included as a more accurate measure of exposure to determine whether this impacts interaction. Also important may be measures of disease morbidity, which are available for some subsets of the GEMS data. It is possible that interaction impacts factors like disease duration, type of diarrhea, wasting and a number of other outcomes. As all significant interactions were negative, the possibility of antagonistic interaction should also be further explored within the GEMS data and in other studies.

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