Potential Etiologies Of Pediatric Acute Diarrhea And Assessment Of Current Treatment Guidelines In Rural Western Kenya

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Abstract

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Introduction: Diarrhea is the second leading cause of death in children under 5 years of age and diarrhea case fatality rates are highest in sub-Saharan Africa where the HIV burden is concentrated and where laboratory infrastructure is lacking. Children who are HIV-infected or whose mother is infected with HIV but who are not infected (HIV-exposed uninfected [HEU]) may be more susceptible to specific high-risk enteric pathogens such as those associated with death and other lethal complications. Current syndromic algorithms for presumptive diarrhea diagnosis and treatment may not capture these high-risk pathogens and may require updating to reflect the most recent evidence in diarrhea causes and consequences.

Methods: Nested within a surveillance study of enteric and bloodstream infections in Western Kenya, we conducted sub-studies to determine the association among HIV-infection, HIV-exposure, and pathogen prevalences (chapter 1) and to determine the diagnostic performance of the World Health Organization's Integrated Management of Childhood Illness (IMCI) syndromic guidelines at classifying suspected *Shigella spp*. against the gold standard of stool culture (chapter 2).

Results: Risk of infection with specific pathogens differed depending on HIV infection and exposure. Over 1000 children with acute diarrhea were enrolled and at least one potential pathogen (bacterial or parasitic) was identified in 45.8% of the children. HIV-infected children were more likely to be infected with enteropathogenic *Escherichia coli* (EPEC)

(10.7% vs. 3.6%, prevalence ratio [PR]: 2.95, *P*=0.008). HEU children were more likely to have *Cryptosporidium species* (*spp.)* identified than HIV-unexposed children (9.9% vs. 3.3%, PR: 2.98, *P*=0.003). Associations were independent of measured confounders. WHO IMCI guidelines did not perform well in correctly classifying children who may benefit from antibiotic therapy. Among the 51 children with *Shigella*-positive stools in whom we ascertained history of bloody stool, only 7 had a reported history of bloody stool meeting the classification for antibiotic therapy by IMCI (sensitivity: 13.7% [95% CI: 5.7%-26.6%] and PPV: 9.9% [95%CI: 4.0%-19.0%]). Among the 1,006 children without microbiologic isolation of *Shigella*, 942 did not have a history of bloody stools (specificity: 93.6% [95%CI: 91.8-95.0%] and NPV: 95.5% [95%CI: 94.1% - 96.7%]).

Conclusion: EPEC and *Cryptosporidium* were more frequently detected in HIV-infected and HIV-exposed children, respectively. These data may explain recently reported increased mortality attributed to these two pathogens. Current international diarrhea guidelines do not consider suspected EPEC or *Cryptosporidium* infections as indication for treatment or intense follow-up. Guidelines such as the IMCI do however indicate treatment for suspected *Shigella* infections, but the currently used suspected *Shigella* criteria identified few children infected with *Shigella*. In the absence of laboratory diagnosis, these children may have missed the opportunity to receive potentially life-saving, and transmission-reducing, antibiotic treatment. Therefore, reductions in childhood deaths due to diarrhea in sub-Saharan Africa may require increased attention to HIV-infection in children and their caregivers as well as possible updates to current syndromic treatment and management guidelines.

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Introduction

Diarrhea is the second leading cause of death in children worldwide and in sub-Saharan Africa it accounts for approximately 12% of the deaths in children under 5[1,2]. In addition to the acute morbidity and mortality attributable to diarrhea, the enteric mucosal damage that occurs in diarrhea leads to decreased nutrient absorptive capacity, growth failure, and cognitive delay [3-9]. Infectious causes, including bacteria, parasites, and viruses, may explain between 40 and 80% of the diarrhea afflicting children and these organisms and subsequent illness have important implications for childhood survival and growth[10]. Recently published were the results from the largest childhood diarrhea etiology study to date, the Global Enteric Multicenter Study, or GEMS, a project funded by the Bill and Melinda Gates Foundation[10]. Stool was evaluated for over 40 bacterial, viral, and parasitic organisms and the majority of diarrhea cases were attributed to rotavirus, *Cryptosporidium*, *Shigella*, and enterotoxigenic *Escherichia coli*. The study also found that children experiencing a single episode of diarrhea had an almost 9-fold higher risk of death and grew less in length compared to control children. Three pathogens were independently associated with death in the 60-day follow-up period; Among infants aged (0-11months) infection with typical EPEC was associated with a 2.6-times higher risk of dying in the 60-days after a moderate-to-severe diarrhea episode (95%CI: 1.6-4.0) after adjustment for potential confounders as well as other pathogens. In this same age-group, ST-ETEC infection was independently associated with an almost 2-times higher risk death (hazard ratio [HR]: 1.99, 95%CI: 0.99-3.5). Toddlers aged 12-23 months infected with *Cryptosporidium* spp. had a 2.1-times higher risk of death than similarly-aged children without *Cryptosporidium* infection after adjustment for confounding factors (95%CI: 1.3-4.3).

A limitation of this otherwise hugely informative study was that maternal and children's HIV status was not obtained at all sites. However the seven sites were

purposefully chosen for their varied HIV-prevalence and the countries with the highest rates of diarrhea case fatality rates overlapped with the countries of highest HIV burden. Of the seven included sites, diarrhea deaths occurred most frequently in the sub-Saharan Africa sites with high HIV-prevalence despite some similar social-economic risk factors between the lower HIV-prevalence sites, such as those in South East Asia. The areas of highest diarrhea-related mortality rates correspond to the areas where the highest number of people are living with HIV and thus understanding the role that HIV plays in childhood diarrhea is vital.

In 2011, an estimated 23,000,000 adults and children were living with HIV in Sub-Saharan Africa. As HIV-infected individuals live longer, this number is likely to increase [11]. HIV-infected children experience more frequent and more severe diarrhea episodes than their uninfected counterparts[12-15]. Furthermore, diarrhea in HIV-infected children exacerbates their already compromised ability to absorb important nutrients and tolerate antiretroviral therapy, leading to a higher risk of malnutrition and subsequent stunting, diminished cognitive function, and reduced human potential. It is unknown whether HIV-infected children are simply more susceptible to all diarrhea-causing enteric infections, or whether there are specific pathogens adept at taking advantage of the T-cell depletion characteristic of HIV-infection.

There have been significant successes in prevention of mother to child transmission, such that the risk of transmission is less than 5% when appropriate guidelines are followed[16]. Therefore, as we move along in the HIV epidemic, there are fewer and fewer children infected with HIV. However, because HIV-infected individuals are living longer and healthier lives, there is an increasing number of children born to mothers who are infected with HIV or who are living with HIV-infected household members, hereafter referred to as HIV-exposed, uninfected (HEU) [16]. Despite avoiding infection with HIV, HEU children suffer from higher morbidity and mortality rates than

children who are not exposed to HIV [17-21]. This may be due to immune changes among infants born to mothers with HIV, social differences in HIV-exposed children, or exposure to more pathogens from immunocompromised caregivers [22,23]. Therefore HIV-infected and HIV-exposed children may have a unique set of diarrhea etiologies and if so, in countries like Kenya where diarrhea is diagnosed and treated syndromically rather than with laboratory tests, knowing the pathogens associated with HIV- could help clinicians better manage diarrhea in these particularly high-risk groups of kids.

It is highly likely that HIV plays a role in a child's susceptibility to the enteric pathogens with fatal consequences. Once a child presents to a health facility with diarrhea, the clinical management they receive might mean the difference between life and death. Many clinical settings lack access to microbiology facilities for diagnosis of enteric pathogens. In the absence of such laboratory diagnostic capabilities, health workers rely on clinical suspicion and syndrome-based guidelines for management of diarrhea. In 1995, the World Health Organization (WHO) developed the Integrated Management of Childhood Illness (IMCI) algorithm outlining suggested management of various childhood morbidities based on presenting signs and symptoms. The algorithm has been updated to reflect new evidence, most recently in March of 2014[24]. The IMCI illness classification system includes multiple action-based items (assessment, classification, treatment identification, treatment, and follow up) for the management of sick children aged 2 months to 5 years [25,26]. The diarrhea module of the guidelines instructs health workers to determine the number of days diarrhea has been present, to assess the child's level of dehydration, and to evaluate whether the child has dysentery. Antimicrobials are indicated for children with acute diarrhea only when accompanied by bloody stool (suspected shigellosis [intestinal lining infection by *Shigella* spp.]) or in children with suspected cholera, classified by severe dehydration, age ≥ 2 years, and living in cholera endemic areas. These conservative antimicrobial recommendations likely

stem from the difficulty in distinguishing between pathogens without microbiologic testing, suspected low prevalence of treatable pathogens, and the knowledge that injudicious use of antimicrobials diminishes the efficacy of available, affordable, first-line drugs to treat infections when indicated.[27] Although the antibiotic indications used in the diarrhea module were informed by expert opinion and observational studies, to the best of our knowledge, use of the IMCI diarrhea algorithm for antimicrobials has not been validated against microbiologic diagnoses.

Substantial gaps remain in our understanding of potential etiologies of acute diarrhea in HIV-prevalent settings such as Western Kenya, where the IMCI guidelines are relied upon for diarrhea management. The current syndromic management of diarrhea is likely missing important opportunities for treatment of pathogens that may exacerbate stunted growth and delays in development by limiting antibiotic indication to a relative small subset of children. Conversely, empiric treatment may be over-indicating antimicrobials in children without a susceptible pathogen and therefore perpetuating individual and community-wide antimicrobial resistance. By simultaneously describing the bacterial and parasitic pathogens found in pediatric diarrhea and comparing the pathogen distribution among HIV-infected, HIV-exposed/uninfected, and HIV-unexposed children, the proposed study will improve our understanding of gastrointestinal pathogens in children who are at an already elevated risk of failure to thrive and death. Further, the evaluation of current guidelines and development of alternative syndromic algorithms will shed light into the appropriateness of diarrheal management guidelines and may create impetus for consideration of additional clinical/host correlates of specific pathogens in future iterations of diarrhea management guidelines.

Chapter 1. High-Risk Enteric Pathogens Associated with HIV-Infection and HIV-Exposure in Kenyan Children with Acute Diarrhea

1.1 Background

Diarrheal disease remains a leading cause of death in children under 5 years of age [1]. Children with a recent episode of moderate-to-severe diarrhea (MSD) have an eight-times higher risk of death than similarly aged children without diarrhea [10]. Most diarrhea is due to infection; rotavirus, *Shigella species* (*spp.*), *Cryptosporidium* (*spp.),* and enterotoxigenic *Escherichia coli* (ETEC) cause most MSD in under-five children [10]. In addition to the acute morbidity and mortality attributable to diarrhea, the enteric mucosal damage that occurs in diarrhea leads to decreased nutrient absorptive capacity, growth failure, and cognitive delay [3,5-8].

HIV-infected children experience more frequent and severe diarrhea episodes and are at higher risk of malnutrition and cognitive impairment than their uninfected counterparts [12-15,28]. Specific enteric pathogens, including *Cryptosporidium spp.* and non-typhoidal Salmonella (NTS), occur more frequently and with greater severity in HIV-infected individuals [29-31]. As prevention of mother-to-child HIV transmission (PMTCT) programs expand, pediatric HIV infections are declining, however, there is a growing population of HIV-exposed uninfected (HEU) children [16]. HEU children experience greater risk of death, hospitalization and neurodevelopmental delays compared to HIV-unexposed children [18-21]. The increased morbidity and mortality observed among HEU children may be due to more frequent enteric infections, earlier weaning, reduced breast milk exposure, decreased immunologic development during infancy, poor socioeconomic status, or diminished parental caretaking capacity [22,23,28].

Guidelines for syndromic management of diarrheal illness in low-resource settings do not differ among HIV-infected, HEU, or HIV-unexposed children [26,32]. If HIV-infected or

HEU children are more likely to be infected with pathogens independently associated with poor growth and death, differential diagnostic testing and/or empiric antibiotic/ anti-protozoal therapy may be helpful in diminishing mortality, morbidity, and transmission. We determined the prevalence of enteric pathogens among HIV-infected, HEU, and HIV-unexposed children presenting with acute diarrhea.

1.2 Methods

Population

Between November 2011 through October 2013 children aged 6 months to 15 years presenting to Kisii Provincial or Homa Bay District Hospital with acute diarrhea (defined as ≥3 loose stools within 24 hours lasting less than 14 continuous days[33]) were enrolled in an ongoing NTS surveillance study. Children were excluded if they had a diagnosis of chronic or non-infectious diarrhea, were unaccompanied by a biological parent or legal guardian, unable to provide a stool sample or rectal swab, or if the primary caregiver elected not to receive HIV counseling on behalf of the child. Study participants were recruited from both outpatient and inpatient settings. Written informed consent was obtained from primary caregivers of enrolled children and assent was obtained from children over 12 years. The University of Washington Institutional Review Board and the Kenya Medical Research Institute Ethical Review Committee approved the current study.

Data collection

Stool was collected, examined for consistency and appearance, and separated into two containers for shipment. When children could not produce stool, 3 rectal swabs were collected. Sociodemographic characteristics, possible exposures (recent antibiotic use [including cotrimoxazole (CTX)], travel history, water source and filtration, sanitation), breastfeeding and vaccination history were obtained from the primary caregiver. Study physicians measured height and weight, and assessed danger and dehydration signs

according to the WHO Integrated Management of Childhood Illness (IMCI) algorithm during a physical exam. Height for age and weight for height z-scores (HAZ & WHZ) were calculated using the 2006 and 2007 WHO reference populations for children under 5 and 5 or over, respectively and stunting and wasting defined as HAZ <-2 and WHZ <-2, respectively[34,35]. A child was classified as having a severe illness if one or more IMCI danger signs (unable to drink or breastfeed, convulsions, continuous vomiting, and/or lethargy/ unconsciousness) were identified[26]. Children were classified as having MSD if they had sunken eyes, loss of skin turgor, visible blood in stool, or required intravenous hydration or hospital admission based on diarrhea or dysentery[10].

Children were tested for HIV using antibody testing (Abbott Determine™ rapid test kit and confirmed using Uni-Gold™) or HIV DNA polymerase chain reaction (PCR) assays if <18 months. Malaria parasitemia was assessed by both rapid testing (Paracheck Pf® Orchid Biomedical Services, India) and microscopy. Maternal HIV-status was ascertained by antibody testing or by self-report if the mother reported being HIV-infected.

Stool Specimen Processing

All stool/ rectal swab specimens were shipped to the United States Army Research Unit Microbiology Hub in Kericho, Kenya, within 24-48 hours of collection. For bacterial identification, each specimen was plated on selective media as follows: BAP (blood agar plate) for hemolysis and oxidase test, Sorbitol-MacConkey agar to select for non-sorbitol fermenting (NSF) *Escherichia coli* (*E. coli*), MacConkey agar for *E. coli* colonies, Hektoen or xylose lysine deoxycholate agar for *Salmonella* and *Shigella spp.,* cefsulodin irgasan novobiocin agar for *Yersinia* spp., andcefoperazone vancomycin amphotericin (CVA) agar for *Campylobacter spp*. Suspicious colonies were further confirmed by oxidase test, catalase, gram stain, Hippurate hydrolysis test, and thiosulphate citrate bile salt- sucrose (TCBS) agar for *Vibrio* spp. Pure colonies exhibiting the proper characteristics on the various media above were further processed using

MicroScan WalkAway 40 Plus automated platform for bacterial identification and antibiotic susceptibility testing. *Salmonella*, *Shigella* and *Vibrio* spp. isolates were serologically typed using their respective commercially available typing sera in slide agglutination tests.

NSF *E. coli* isolates exhibiting different morphologies were grown separately overnight in trypticase soy broth and frozen in 25% final concentration glycerol. The frozen isolates were batch tested using multiplex PCR to identify virulent genes associated with Enterotoxigenic *E. coli* (ETEC)- heat labile enterotoxin (*elt*) and/or heat stable enterotoxin (*est*), Enteroaggregative *E. coli* (EAEC)- *aatA*; Enteroinvasive *E. coli* (EIEC)- invasion plasmid antigen H (*ipaH*); or Enterohemmorhagic *E. coli* (EHEC)- Shiga toxin 1, 2 and variants (*stx*); and Enteropathogenic *E. coli* (EPEC)- bundle forming pilus (*bfpA*) [36]. Starting in March 2013, additional gene targets for EPEC- intimin (*eae*) and for EAEC- *aaiC* were incorporated. A classification of EAEC was therefore the identification of *aatA* and/or *aaiC*. EPEC was disaggregated into two categories: typical EPEC (*bfpA* with or without *eae*) and atypical EPEC (*eae* without either *bfpA or stx).*

For parasitic identification, the stool was concentrated using the Mini Parasep® Solvent Free concentration kit (DiaSys, Berkshire, England) and then microscopy used to identify parasitic forms. Parasite testing was not performed on rectal swabs.

Statistical Analysis

Two sets of analyses were performed; the first compared risk factors and enteric pathogens between HIV-infected and HIV-uninfected children, and the second compared risk factors and pathogens between HEU and HIV-unexposed children. Children who were missing HIV-status information were excluded and children without maternal HIV status available were excluded from the second analysis.

Sociodemographic and clinical characteristics of included children were compared using Chi-square or Fisher's exact tests for categorical variables and t-tests for continuous variables. Enteric pathogens were compared using prevalence ratios (PR) and associated

P-values were estimated using relative risk (RR) regression and associated chi-square or Fisher's exact tests. For the comparison of enteric pathogens, we adjusted for multiple comparisons using the Benjamini and Hochberg method using a false discovery proportion of 0.05 [37]. For all pathogens associated with HIV-infection and/or HIV-exposure in the univariate analyses, we constructed multivariable models to account for confounding and mediating variables. Because specific enteric infections are known to be associated with age[10], age was a priori retained as a confounding variable in all multivariable models. Potential confounders, including site, year of enrollment (2011, 2012, 2013), household income, number persons/room, and drinking water source and treatment were included stepwise in multivariable models and maintained in the final model if they changed the PR for the main exposure variable of interest (HIV or HEU) by more than 10%. Variables that we considered to be plausibly on the causal pathway between HIV and enteric pathogens included breastfeeding history (exclusive breastfeeding duration, current breastfeeding), nutritional status indicators (HAZ and WHZ) and recent CTX use [38-41]. Potential mediators were individually added to age-adjusted models. Subgroup analyses were performed among children <24 months and among children with MSD because case fatality rates are highest among these groups and in to determine whether identified associations persisted in these groups. Confounder, mediator, and subgroup analyses were considered exploratory analyses and therefore an alpha of 0.05 was used to determine statistical significance.

1.3 Results

Among 1,512 children presenting with diarrhea, 1,116 met inclusion criteria, 1,099 were enrolled and 1,076 had known HIV-status and therefore were included in analysis I (HIV-infected 5.2% [N=56] and HIV-uninfected 94.8% [N=1020]). Maternal HIV status was known for 926 HIV-uninfected children, of which 105 and 821 were HEU and HIV-

unexposed, respectively. These 926 children were included in analysis II (Figure 1.1). Among the 1,076 children with known HIV-status, the median age was 22 months (interquartile range [IQR]: 11-42 months) and an IMCI danger sign, MSD, and fever were each present in about one-third of enrolled children (Table 1.1). Almost half (40%) came from extremely low-income households (<5000 Kenya shillings/month), 36.5% reported using piped water, and few had access to flush toilets (7.0%). Over a third of children were stunted or wasted (median HAZ and WHZ was -0.52 [IQR: -1.54 to 0.65] and -0.58 [IQR: -1.77 to 0.42], respectively). The median length of exclusive breastfeeding was 6 months (IQR: 4-6). HIV-prevalence was 5.2% among study children and almost half (44.6%) of the 56 HIV infected children were newly diagnosed at enrollment. Among the 31 HIV-infected children who were already diagnosed, 27 (87.1%) were reportedly enrolled in HIV care and 7 (22.6%) reported current ART use. Among the 53 HIV-infected children for whom CD4% or CD4 count was available, 19 (35.9%) were immunosuppressed based on CD4 count <350 [42]. Only 36 (34.3%) HIV-infected mothers self-reported their last CD4 count (median was 483 cells per cells/ mm^3 (IQR: 301-834)).

Bacteria and Parasite Frequency

At least one potential pathogen (bacterial or parasitic) was identified in 45.8% (493/1,076) of the children. Nearly 10% (105/1,076) had 2 organisms identified, 1.4% (15/1076) with 3, <0.5% (2/1076) with 4 , and 1 had 5 different isolates (Figure 1.2). There were no differences in frequency of bacteria isolation between the 981 children who provided whole stool samples and the 95 who provided rectal swab only (35.1% vs. 31.6%, *P*=0.50).

One or more diarrheagenic *E. colis* were identified in the stools of 262/1,076 (24.4%) children. EAEC was identified in 143 (13.3%), EPEC in 66 (6.1%) (4.0% were typical and 2.1% atypical), ETEC in 47 (4.4%), EIEC in 32 (3.0%), and EHEC in 4 (0.4%) stools. Among the 262 children with any identified diarrheagenic *E. coli,* infection with

multiple *E. coli* serotypes was common (11.1%) (Figure 1.2). Other commonly isolated bacteria included *Campylobacter spp.* (68 [6.3%]), *Shigella spp.* (49 [4.6%]), and *Salmonella spp.* (12 [1.1%]).

Almost a quarter (24.2%) of the 981 children that provided a whole stool sample had at least one parasite identified. The most frequently identified parasite was *Giardia spp.* (109 children [11.1%]), followed by *Cryptosporidium spp.* (36 [3.7%]), *Ascaris Lumbricoides* (24 [2.5%]), *Entamoeba spp.* (4 [0.4%]), and *Isospora spp.* (1 [0.10%]). Other likely non-pathogenic parasites were also identified, including *Blastocystis hominis* (73 [7.4%]), *Chilomastix spp.* (4 [0.40%]) and *Endolimax spp.* (1 [0.10%]).

HIV-infected vs. HIV-uninfected Children

Compared to HIV-uninfected children, HIV-infected children were older (mean age 50 vs. 31 months, $p<0.001$), more likely to be enrolled at the Homa Bay site (67.9% vs. 51.5%, $P=0.017$), more likely to come from low-income households (52.7% vs. 39.3%, $P=0.048$), and less likely to be accompanied by their biologic mother (85.7% vs. 93.1%, $P=0.037$) (Table 1.2a). The mean reported number of months of exclusive breastfeeding was similar between HIV-infected and. HIV-uninfected children (4.8 vs. 5.0 months, P=0.329). However, among the children under 24 months old, HIV-infected children were less likely to be breastfeeding at the time of enrollment (50% vs. 80.5%, p=0.002), and more likely to have taken CTX within the preceding week (23.2% vs. 5.4%, $P<0.001$), were more likely to be stunted (34.6% vs. 15.8%, $P=0.001$), and to have MSD (41.1% vs. 28.6%, $P=0.046$).

The prevalence of enteric bacteria and parasites by HIV-infection status are reported in Table 1.2b. In univariate analysis, HIV-infected children were nearly 3 times more likely to have typical EPEC identified in their stools compared to HIV-uninfected children (PR=2.95; $P=0.008$); this association persisted after adjusting for age (adjusted [a]PR: 3.70 [95%CI: 1.6-8.4, $P=0.002$]) and no additional measured confounders were

identified. HIV-status remained associated with typical EPEC in analyses adjusted for duration (months) of exclusive breastfeeding and age (aPR: 3.81 [1.68-8.66, *P*=0.001]), current breastfeeding and age (aPR: 3.46 [1.51-7.90, *P*=0.003]), WHZ and age (aPR: 2.9 [95%CI: 1.1-7.7, *P*=0.036]), HAZ and age (aPR: 3.9 [95%CI; 1.7-8.9, *P*=0.001]), and CTX use and age (aPR: 3.5 [95%CI: 1.5-8.0, *P*=0.004]). Among HIV-infected children in whom CD4 data were available (n=53), typical EPEC was identified more commonly among the immunosuppressed than non-immunosuppressed children (15.8% vs. 8.8%) but the difference was not significant (*P*=0.655).

Subgroup analyses were performed among the 562 children aged <24 months (18 (3.2%) HIV-infected and 544 (96.8%) HIV-uninfected) demonstrated no significant differences in pathogens identified between HIV-infected and HIV-uninfected children. In subgroup analyses among 315 children with MSD (23 [7.3%] HIV-infected and 293 (93%) HIV-uninfected) HIV-infected children were more likely to have typical EPEC identified compared to HIV-uninfected children (21.7% vs. 4.1%, PR: 5.3 [95%CI: 2.0-13.7, *P*<0.001]).

HEU vs. HIV-Unexposed Children

Compared to HIV-unexposed children (n=821), HEU children (n=105) were more likely to present in Homa Bay than Kisii (87.6% vs. 48.5%, *P* <0.001) (Table 1.3a), to live in households with lower incomes (64.8% vs. 36.8%, *P*<0.001) and were more likely to report having an unprotected water source (28.6% vs. 11.1%, *P*<0.001) but more likely to treat their drinking water (87.6% vs. 71.8%, *P*<0.001). HEU and HIV-unexposed children had similar mean number of exclusive breastfeeding months (4.8 vs. 5.0, p=0.329) but among the children under 24 months, HEU children were less likely to be currently breastfeeding (50% vs. 80.5%, p=0.002). Finally, HEU children were more likely to report having taken CTX in the preceding week (18.3% vs. 4.0%, *P*<0.001) and were more likely to be stunted (24.5% vs. 15.2%, *P*=0.016).

The prevalence of bacterial and parasitic infections by HIV-exposure category are presented in Table 1.3b. *Cryptosporidium spp.* was more common in HEU children (PR: 3.0, *P*=0.003). The association between HIV-exposure and *Cryptosporidium spp.* was independent of age and site (aPR: 2.1 [95%CI: 1.0-4.5, *P*=0.046]). When we accounted for duration (months) of exclusive breastfeeding and current breastfeeding in age-adjusted models, the association remained significant (aPR: 2.70 [95%CI: 1.31-5.58, *P*=0.007]; aPR: 2.80 [95%CI: 1.32-5.92, *P*=0.007]). Similarly, the association between maternal HIV-status and *Cryptosporidium spp*. infection persisted after including WHZ, HAZ, and CTX use in age-adjusted models (WHZ-aPR: 2.8 [95%CI: 1.4-5.7, *P*=0.005]; HAZ-aPR: 2.7 [95%CI 1.3-5.6, *P*=0.008]; CTX-aPR 2.5 [95%CI: 1.2-5.4, *P* =0.020], respectively). Only 36 of the 105 HIV-infected mothers of HIV-uninfected children knew their most recent CD4 count. Although the mean CD4 count among the mothers of HEU children with *Cryptosporidium spp.* appeared to be lower (378.5 cells/mm^3) than the maternal CD4 count among those without *Cryptosporidium spp.* identified (647.9 cells/ mm^3), this difference was not statistically significant (*P*=0.300).

Cryptosporidium spp. was also more commonly identified in the stool of the 60 HEU children <24 months of age (12.2%) as compared to the 444 children <24 months who were HIV-unexposed (4.7%) (PR 2.59; [95%CI: 1.1-6.2 *P*=0.033]). Among children with MSD (35 HEU & 224 HIV-unexposed), the prevalence of *Cryptosporidium spp*. infection was also significantly higher in HEU children compared to HIV-unexposed children (PR 4.1 [95%CI: 1.4-11.7, *P*=0.008]). No other differences in pathogen distribution were observed between HEU and HIV-unexposed children.

1.4 Discussion

In this cross-sectional study of Kenyan children with acute diarrhea, HIV-infection and HIV-exposure status were both associated with specific enteric pathogens. Typical

EPEC was almost three times more common in HIV-infected compared to HIV-uninfected children and *Cryptosporidium spp.* almost three times more common in HEU compared to HIV-unexposed children. These associations were independent of measured potential confounding and mediating factors. Additionally, we found magnitude of associations to be particularly strong in the subgroup of children with MSD. This finding further supports data from the multi-site GEMS study which found typical EPEC and *Cryptosporidium* spp. infection during diarrhea episodes to be independently associated with higher case-fatality, particularly in sub-Sahahan African countries where HIV prevalence was highest [10]. If typical EPEC and *Cryptosporidium spp*. are disproportionally affecting HIV-infected and HEU children, and if HIV-testing rates in health care settings continue to increase, then health workers seeing children with acute diarrhea might consider child and maternal HIV-infection to be an indicator for more intense follow-up or empiric antibiotic/ antiprotozoal therapy.

The association between typical EPEC and HIV-infection in children with acute diarrhea suggests a possible immune-modulated mechanism of action; it could be that the attaching and effacing (A/E) lesions characteristic of EPEC exploit deficiencies in the intestinal immune system or epithelial barrier, both common pathologies in HIV-associated intestinal dysfunction [43,44]. Current Kenyan guidelines for management of diarrhea do not specify antimicrobial treatment of EPEC [45]. In other settings, the recommended treatment for EPEC is either CTX or a floroquinolone but in Kenya there are high levels of CTX resistance , limited availability of floroquinolones, and limited laboratory capacity to detect diarrheagenic *E. coli*, including EPEC [12,46,47]. We did not observe differences in the prevalence of other pathogens between HIV-infected and HIV-uninfected children, a finding consistent with reports from other studies [12,13]. However, among a subset of the GEMS study population in Western Kenya, higher prevalences of ETEC, *Cryptosporidium*, EPEC and astrovirus in HIV-infected children were reported [48].

We also observed a strong association between HIV-exposure and *Cryptosporidium spp.* infection among HIV-uninfected children. While the exact mechanism by which HIV-exposure may increase risk of *Cryptosporidium spp.* is not clear, HIV-infected persons are at greater risk for *Cryptosporidium spp*. infection and likely shed *Cryptosporidium* oocysts in greater quantities and for longer periods of time than their HIV-uninfected counterparts [49,50]. As a result, children of HIV-infected mothers may be exposed to the parasite more frequently than HIV-unexposed children. HEU children may also be less likely to acquire *Cryptosporidium*-specific antibodies from breast milk [51]. Although we did not observe differences in exclusive breastfeeding duration, we did find that among the younger children, HIV-infected and HEU children were less likely to be currently breastfed which could suggest increased exposure to enteric pathogens through contaminated feeds or fluids. Finally HEU children were more likely to have recently taken CTX than HIV-unexposed children. Recent exposure to antibiotics may result in treatment or suppression of bacterial pathogens, resulting in the preferential identification of parasitic infections such as *Cryptosporidium spp.*

Cryptosporidium spp. infections are associated with nutrient malabsorption, growth faltering, and cognitive disabilities, even in the absence of diarrhea, and these outcomes are common among HEU children [52-54]. It is plausible that some of the failure to thrive observed among HEU children may be the result of increased risk of *Cryptosporidium spp.* infection in this group. Nitazoxanide has shown limited efficacy, including among HIV-infected individuals, and could be considered in HEU children with *Cryptosporidium spp.* infection [55]. Antiretroviral treatment (ART) has also been shown to decrease susceptibility to *Cryptosporidium spp.* and earlier and/or better coverage of ART among HIV-infected mothers may be a reasonable strategy for reducing exposure to *Cryptosporidium spp.* infection in HEU children [56].

Our study had several strengths and limitations. Strengths include the large cohort with detailed characterization of bacterial and parasitic pathogens associated with diarrhea and consideration of the type 1 error rate from testing for associations among multiple pathogens. Limitations include the lack of non-diarrhea controls which limited our ability to estimate prevalence of asymptomatic carriage of each organism and subsequently estimate the proportion of diarrheal cases attributed to a given organism. However data from a large multi-country case-control study, the GEMS study, has addressed this issue among children with MSD [10]. While our study could not conclusively attribute the cause of diarrhea to the organisms identified, many of the organisms isolated are thought to be associated with poor outcomes, even in the absence of diarrhea. In addition to *Cryptosporidium spp.*, asymptomatic carriage of *Giardia*, *Campylobacter spp.,* and ETEC have been associated with poor weight and poor linear growth and thus may be important pathogens even if not causing diarrhea [57-60]. We were also not able to isolate enteric viruses in this study. In the GEMS study, rotavirus was the leading cause of MSD across all age groups and was present in 19.0% of diarrhea stool samples from children hospitalized with acute diarrhea in Western Kenya [10,48].

There were also important limitations to our assessment of HIV-exposure. We considered children presenting with an HIV-infected mother to be HIV-exposed, however we did not ascertain timing of maternal HIV infection; some HEU mothers may have acquired HIV after birth of the child. When we excluded children over 2 years of age in a subgroup analysis, leaving children most likely to have been exposed to the HIV-virus, the association between HIV-exposure and *Cryptosporidium spp.* remained. Also, we did not ascertain HIV-status of other family members and it is possible that children with HIV-infected household members, even if the mother is HIV-uninfected, may be at increased risk of *Cryptosporidium spp.* infection. Although we measured number of exclusive breastfeeding duration and current breastfeeding status, we did not ascertain the age of

weaning nor the duration of breastfeeding and thus could not evaluate how these mediating variables might impact our findings. Future studies that include surveillance stool sampling, HIV-testing, as well as detailed weaning and feeding data will help us understand why typical EPEC and *Cryptosporidium spp.* were independently associated with HIV-infection and exposure, respectively, in this study population.

Intriguingly, although we detected an association between HEU and *Cryptosporidium spp.,* we did not find an association with HIV-infection. The lack of association may be due to lack of power-- we had only 56 children with HIV, 8 of whom were not tested for parasites because they could not produce fresh stool. The cohort prevalence of *Cryptosporidium* spp. was 3.7%, a prevalence similar to studies which utilized microscopy, but lower than studies using PCR-based methods (prevalences as high as 31.3%) [61-63]. In addition, *Cryptosporidium spp.* infection is more common in children under 2 years old and the average age of HIV-infected children in our cohort was higher (4 years) [10,64]. Finally, both *Cryptosporidium spp.* infection and HIV-infection are associated with persistent diarrhea (i.e., episodes lasting more than two weeks). By excluding children with persistent diarrhea, we may have excluded HIV-infected children with *Cryptosporidium spp.* infection [13,53,62,64]. A similar study conducted in Tanzania which included HIV-infected children with both acute and persistent diarrhea reported that all *Cryptosporidium spp.* infections isolated were from patients with chronic diarrhea [61].

Despite these limitations, this study found important and novel relationships between HIV status and two enteropathogens that are significant causes of morbidity and mortality in sub-Saharan Africa. In current management guidelines for acute diarrhea, EPEC and *Cryptosporidium* are not specifically considered. For example, in the absence of laboratory-based stool testing, ascertaining HIV-infection status of the child and the mother may help clinicians determine optimal empiric treatment for acute diarrhea. If HIV-infected mothers or other HIV-infected household members are indeed exposing children

to *Cryptosporidium spp.* more frequently, then improving HIV care and treatment of mothers and the population more broadly may have indirect benefits such as reducing childhood diarrhea incidence, growth failure, and cognitive delay. Finally, this study suggests that efforts to increase coverage of water, sanitation, and hygiene (WASH) programs are particularly important in high HIV-prevalence settings.

Figure 1.1 Participant Inclusion Flow for Analyses I and II

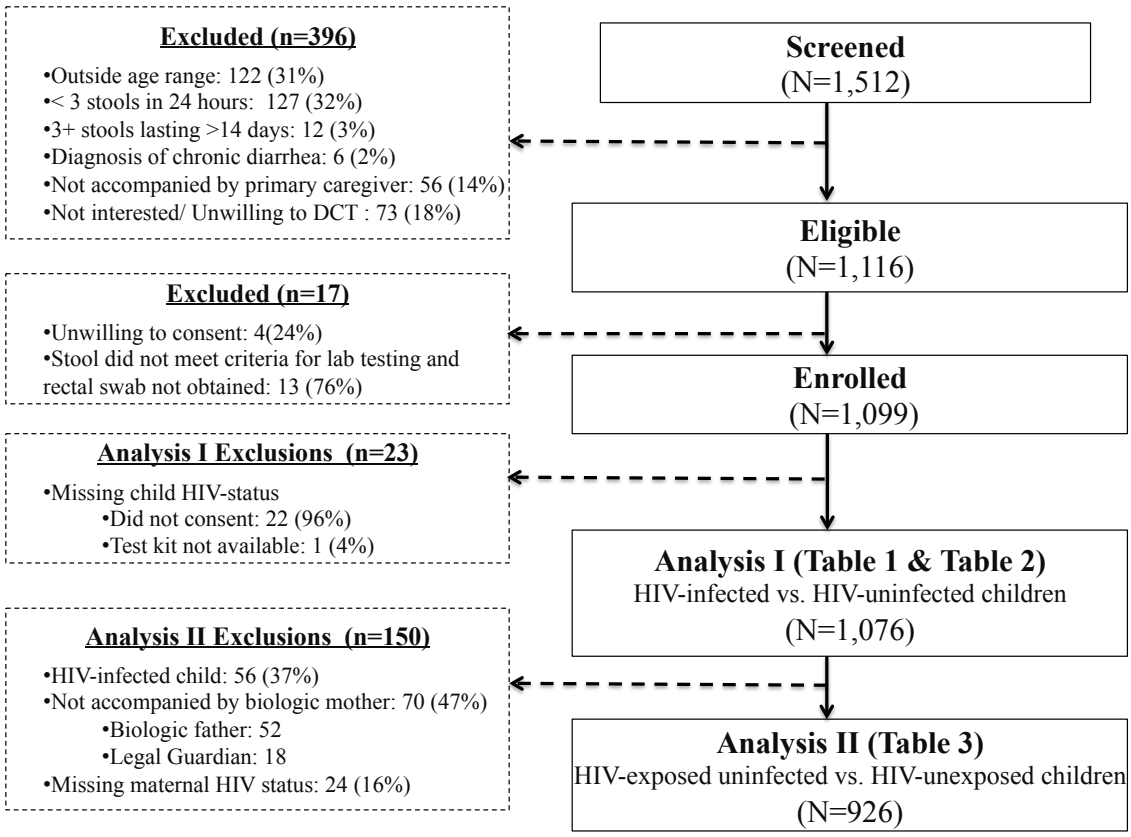


Table 1.1 Characteristics of children in Analysis I

Characteristic	Enrolled N=1,076 n (% [a]) Median (IQR)
Sociodemographic	
Site	
Kisii	513 (47.7%)
Homa Bay	563 (52.3%)
≥1 hour to get to hospital	177 (16.5%)
Child accompanied by	
Biological mother	998 (92.8%)
Biological father	53 (4.9%)
Legal guardian	25 (2.3%)
Monthly household income <5,000 Kenyan Shillings	429(40.0%)
Household owns ≥ cow	475(44.3%)
# persons/ room	2 (1-3)
Water source	
Piped in house or yard or public tap	391 (36.5%)
Protected well/ spring	456 (42.5%)
Unprotected well/spring/ surface water	149 (13.9%)
Other [b]	76 (7.1%)
Household treats drinking water	795 (73.9%)
Flush toilet	75 (7.0%)
Male	576 (53.5%)
Age	
6m-2yr	585 (54.4%)
>2yr-5yr	367 (34.1%)
>5yr	124 (11.5%)
Clinical Presentation	
1 or more IMCI General Danger Sign [c]	335 (31.3%)
Moderate to severe diarrhea [d]	315 (29.3%)
Blood observed in stool	14 (1.3%)
Mucous in stool	509 (47.3%)
Stunted (HAZ<-2)	171 (16.9%)
Wasted[e] (WHZ<-2)	202 (21.4%)
MUAC <12.5	86 (8.0%)
Malaria[f]	106 (9.9%)
Current fever (≥37.5°C)	362 (33.6%)
Clinical History	
Child ever breastfed	1086 (98.9%)
Child currently breast-feeding among children less than 2 years	452 (77.4%)
# months exclusively breastfed	6.0(4-6)
HIV positive	56 (5.2%)
New diagnosis	25
Known status	31
Immunosuppressed [g]	19 (35.9%)
HIV-exposed uninfected [h]	105 (11.3%)

[a] % among those with non-missing data
[b] Tube well or borehole (n=18), rainwater (n=55), cart with small tank (n=1), bottled water (n=2)
[c] Defined as not able to drink or breastfeed, convulsions, vomits everything, lethargic or unconscious
[d] One or more of the following; sunken eyes, loss of skin turgor, intravenous hydration administered or prescribed, visible blood in stool, or hospital admission based on diarrhea or dysentery
[e] Only calculated for children 5 years and younger
[f] Positive result on microscopy alone (n=8), on RDT alone (n=5) or both RDT and microscopy (n=93)
[g] Defined in terms of CD4% (age ≤11 months: <25%, 12 months-35 months: <20%, 36^{+} months: <15%) or, in absence of CD4 % data, in terms of CD4 count (age ≤11 months: <1500 cells/mm^3, 12 months-35 months: <750 cells/mm^3, 36^{+} months <350 cells/ mm^3)
[h] Among 926 HIV-uninfected children who were accompanied by the biological mother who was HIV-infected

Table 1.2a Demographic and clinical differences between HIV-infected and -uninfected children

Selected Factors	HIV-Infected N=56 N/mean	(%/ SD)	HIV-Uninfected N=1020 N/mean	(%/ SD)	*P*-value
Homa Bay Site	38	(67.9%)	525	(51.5%)	0.017
Age in months	49.7	(40.4)	31.3	(31.1)	<0.001
Income <5,000 KSH	29	(52.7%)	400	(39.3%)	0.048
# persons / room in house	2.4	(1.2)	2.4	(1.4)	0.73
Unprotected water source [a]	16	(29.1%)	133	(13.1%)	0.001
Household treats drinking water	46	(82.1%)	749	(73.4%)	0.15
Accompanied by biological mother	48	(85.7%)	950	(93.1%)	0.037
Child <24 months and currently breast-feeding [b]	9	(50%)	437	(80.5%)	0.002
# months exclusively breastfed [c]	4.8	(1.9)	5.0	(1.8)	0.329
Child took cotrimoxazole in last 7 days	13	(23.2%)	55	(5.4%)	<0.001
Stunted (HAZ<-2)	18	(34.6%)	153	(15.9%)	<0.001
Wasted (WHZ<-2)	12	(29.3%)	190	(21.1%)	0.212
Moderate to Severe Diarrhea	23	(41.1%)	292	(28.6%)	0.046
Rectal swab taken	8	(14.3%)	87	(8.5%)	0.140
Blood in stool	1	(1.8%)	13	(1.3%)	0.529

[a] Unprotected well, unprotected spring, or surface water
[b] Among 18 HIV-infected children and 544 HIV-uninfected children
[c] When considering only the subset of children < 24 months of age: mean # of months exclusively breastfed were 5.2 (SD: 1.8) vs. 5.1 (SD: 1.8), p=0.861

Table 1.2b Enteric pathogen differences between HIV-infected and -uninfected children

Organism Identified	HIV-Infected N=56 N	%	HIV-Uninfected N=1020 N	%	Prevalence Ratio	*P*-value
Bacteria						
Campylobacter species[a]	6	(10.7%)	62	(6.1%)	1.76	0.165
>1 *E. coli* serotype[b]	2	(11.1%)	27	(11.1%)	1.00	1.00
EAEC	6	(10.7%)	137	(13.2%)	0.81	0.560
EIEC	1	(1.8%)	31	(3.0%)	0.59	1.00
EHEC	0	(0%)	4	(0.4%)	--	1.00
EPEC-atypical[c]	2	(3.6%)	21	(2.1%)	2.74	0.178
EPEC-typical	6	(10.7%)	37	(3.6%)	2.95	0.008
ETEC	5	(8.9%)	42	(4.1%)	2.17	0.092
Salmonella species[d]	1	(1.8%)	13	(1.3%)	1.40	0.529
Shigella species[e]	1	(1.8%)	46	(4.5%)	0.40	0.508
Other bacteria[f]	1	(1.8%)	22	(2.2%)	0.83	1.00
Parasites[g]						
Giardia species	5	(10.4%)	104	(11.2%)	0.93	0.875
Cryptosporidium species	1	(2.1%)	35	(3.8%)	0.56	1.00
Entaeombea species	0	(0%)	4	(2.6%)	--	1.00
Ascaris lumbricoides	1	(2.1%)	23	(2.5%)	0.85	1.00
Blastocystis hominis	4	(8.3%)	69	(7.4%)	1.13	0.809
Other parasite[h]	1	(2.1%)	5	(0.5%)	3.89	0.261
No organism identified[i]	19	(39.6%)	456	(47.4%)	0.84	0.209

[a] HIV-infected: *Campylobacter jejuni* (n=6), HIV-uninfected: *Campylobacter jejuni* (n=39), *Campylobacter spp*. other than *jejuni* (n=23)
[b] Among children with at least one diarrheagenic *E.coli* serovar (18 HIV-infected and 244 HIV-uninfected)
[c] Among 417 patients enrolled since March 2013 who had the gene *eae* tested
[d] HIV-infected: non-typhoidal species (n=1), HIV-uninfected: *Salmonella typhi* (n=3), *Salmonella paratyphi* (n=1), *Salmonella* non-typhoidal species (n=9)
[e] HIV-infected: species not determined (n=1); HIV-uninfected: *Shigella flexneri* (n=15), *Shigella sonnei* (n=18), *Shigella dysentariae* (n=2), species not determined (n=11)
[f] HIV-infected: *Plesiomonas shigelloides (n=1)*; HIV-uninfected: *Providencia alcalifaciens*(n=6), *Providencia stuartii* (n=1), *Providencia rettgeri* (n=1) *Citrobacter freundii* (n=3), *Citrobacter amalonaticus* (n=1), *Enterobacter agglomerans* (n=2), *Enterobacter cloacae* (n=1), *Kluyvera ascorbata* (n=2), *Escherichia vulneris* (n=1), *Yersinia enterocolitica* (n=1), *Aeromonas hydrophila* (n=1), *Pseudomonas aeruginosa* (n=1), *Edwardsiella tarda* (n=1)
[g] Among 981 children in whom whole stool was collected (HIV-infected: n=48, HIV-uninfected: n=933)
[h] HIV-infected: *Chilomastix mesnili* (n=1); HIV-uninfected: *Chilomastix mesnili* (n=3), *Cystoisospora belli* (n=1), *Endolimax nana* (n=1)
[i] Among the 981 children who had both bacteria and parasite testing performed (HIV-infected: n=48, HIV-uninfected: n=933)

Table 1.3a Demographic and clinical differences between HEU and HIV-unexposed children

Characteristic	HEU N=105 N/Mean (% / SD)	HIV-unexposed N=821 N/mean (% / SD)	*P*-value
Homa Bay Site	92 (87.6%)	398 (48.5%)	<0.001
Age in months	25.2 (20.1)	30.3 (29.6)	0.092
Income <5,000 KSH	68 (64.8%)	301 (36.8%)	<0.001
# persons / room in house	4.9 (1.6)	4.8 (1.7)	0.6305
Unprotected water source	30 (28.6%)	91 (11.1%)	<0.001
Household treats drinking water	92 (87.6%)	589 (71.8%)	<0.001
Child <24 months and currently breast-feeding[a]	32 (53.3%)	380 (85.8%)	<0.001
# months exclusively breastfed [b]	5.4 (1.6)	5.0 (1.9)	0.096
Child took cotrimoxazole in last 7 days	19 (18.1%)	33 (4.0%)	<0.001
Stunted (HAZ<-2)	25 (24.5%)	117 (15.2%)	0.016
Wasted (WHZ<-2)	26 (26.5%)	148 (20.2%)	0.145
Moderate to Severe Diarrhea	35 (33.3%)	224 (27.3%)	0.193
Rectal swab taken	14 (13.3%)	67 (8.2%)	0.077
Blood in stool	0 (0%)	10 (1.2%)	0.614

[a] Among 60 HEU and 443 HIV-unexposed children
[b] When considering only the subset of children < 24 months of age the mean # of months exclusively breastfed were 5.3 (SD: 1.5) vs. 5.1 (SD: 1.9), p=0.235

Table 1.3b Enteric pathogen differences between HEU and HIV-unexposed children

Organism Identified	HEU N=105 N (%)	HIV-unexposed N=821 N (%)	Prevalence Ratio	*P*-value
Bacteria				
Campylobacter species	8 (7.6%)	47 (5.7%)	1.33	0.439
>1 *E. coli* serotype[a]	2 (8.0%)	21 (10.7%)	0.75	1.00
EAEC	14 (13.3%)	111 (13.5%)	0.99	0.958
EIEC	4 (3.8%)	24 (2.9%)	1.3	0.548
EHEC	0 (0%)	2 (0.24%)	--	1.00
EPEC-atypical[b]	4 (3.8%)	15 (1.8%)	1.96	0.264
EPEC-typical	3 (2.9%)	29 (3.5%)	0.81	1.00
ETEC	2 (1.9%)	37 (4.5%)	0.42	0.302
Salmonella species	0 (0%)	12 (1.5%)	--	0.380
Shigella species	2 (1.9%)	36 (4.4%)	0.43	0.302
Other bacteria	4 (3.8%)	14 (1.7%)	2.23	0.137
Parasites[c]				
Giardia species	13 (14.3%)	80 (10.6%)	1.35	0.290
Cryptosporidium species	9 (9.9%)	25 (3.3%)	2.98	0.003
Entaeombea species	0 (0%)	4 (0.5%)	--	1.00
Ascaris lumbricoides	0 (0%)	21 (2.8%)	--	0.154
Blastocystis species	8 (8.8%)	55 (7.3%)	1.21	0.608
Other parasite	2 (2.2%)	3 (0.40%)	5.52	0.092
No organism identified[d]	41 (45.1%)	377 (50.0%)	0.90	0.597

[a] Among children with at least one diarrheagenic *E.coli* serovar (25 HEU and 196 HIV-unexposed)
[b] Among 367 patients enrolled since March 2013 who had the gene *eae* tested
[c] Among 845 children in whom whole stool was collected (HEU: n=91, HIV-unexposed: n=754)
[d] Among the 845 children who had both bacteria and parasite testing performed (HEU: n=91, HIV-unexposed: n=754)

Figure 1.2 Dual Enteric Infections among 105 children (53 unique dual combinations)

	BACTERIA	*Campylobacter spp.*	*Enterobacter spp.*	EAEC	EIEC	EHEC	EPEC (atypical)	EPEC (typical)	ETEC	*Kluyvera spp.*	*Providencia spp.*	*Salmonella spp.*	*Shigella spp.*	*Yersinia spp.*	PARASITES	*Ascaris spp.*	*Blastocystis spp.*	*Chilomastix sp.*	*Cryptosporidium spp.*	*Entamoeba spp.*	*Giardia spp.*	Total
BACTERIA																						
Campylobacter jejuni				9		1		1	1								2				3	17
Campylobacter species [b]				2					1				1				3				1	8
Enterobacter agglomerans																					1	1
EAEC		11			2			6	2		1	2	1			1	4		3		4	37
EIEC				2			2		1				4			1	1		2		2	15
EHEC		1					1												1			3
EPEC (atypical)					2	1			1				1								2	7
EPEC (typical)		1		6					1				1				1				1	11
ETEC		2		2	1		1	1								2	1		1		1	12
Kluyvera ascorbata																		1				1
Providencia alcalifaciens				1												1					2	4
Providencia rettgeri																	1					1
Non-typhoidal *Salmonella*				2																		2
Shigella flexneri					1			1								1	2					5
Shigella sonnei		1			1																2	4
Shigella dysenteriae							1															1
Shigella spp. ND				1	2												1					4
Yersinia enterocolitica																					1	1
PARASITES																						
Ascaris lumbricoides				1	1				2		1		1				2			1		9
Blastocystis species		5		4	1			1	1		1		3			2		2		1	5	26
Chilomastix species										1							2					3
Cryptosporidium species				3	2	1			1												1	8
Entamoeba species																1	1				1	3
Giardia species		4	1	4	2		2	1	1		2		2	1			5		1	1		27
Total		25	1	37	15	3	7	11	12	1	5	2	14	1		9	26	3	8	3	27	

Chapter 2. Accuracy of the Integrated Management of Childhood Illness (IMCI) diarrhea module in identifying culture-confirmed *Shigella* infections. Should the IMCI algorithm be modified?

2.1 Background

Shigella is a leading cause of diarrheal disease worldwide and is associated with high mortality in young children [10,65-68]. Children with HIV or malnutrition and those with complications of infection, including encephalopathy, hyponatremia, and seizures, are at a particularly high risk of death[66,69,70]. Historically, *S. dysentariae* type 1 infections and, to a lesser extent, *S. flexneri* infections, were thought to be responsible for most *Shigella*-attributed deaths, particularly in *S. dysentariae* type 1 outbreak settings[71,72]. While *S. dysentariae* type 1 infections are often most associated with potentially fatal complications such as hemolytic-uremic syndrome, severe hyponatremia, and leukemoid reactions, other potentially lethal manifestations of *Shigella* infections do not appear to be species-specific.[66,70,73] Several large studies have reported no difference in case-fatality rates between *Shigella* species or have found higher case fatality among *S. Sonnei* and *S. flexneri* infected children , as compared to children with *S. dysentariae* type 1.[66,71]

Although several systematic reviews have suggested that antibiotics reduce clinical failure rates in young children with dysentery due to *Shigella*, most data suggesting benefit come from observational trials[74-77]. In addition, the benefit of treating *Shigella* infections without dysentery is not known. Given the risk of extra-intestinal complications and death associated with non-dysenteric species of *Shigella*, antibiotic treatment of non-bloody *Shigella* infections may be warranted[78].

In resource-limited settings where much of the burden and mortality due *Shigella* occurs, many clinical settings lack access to microbiology facilities for diagnosis of enteric pathogens. In the absence of such laboratory diagnostic capabilities, health workers rely on clinical suspicion and syndrome-based guidelines for management of diarrhea.

Included in the World Health Organization (WHO) Integrated Management of Childhood Illness (IMCI) guidelines is an algorithm outlining suggested management of diarrhea based on presenting signs and symptoms[24]. *Shigella* (particularly *S. dysentariae* type 1 infection) is the most common cause of dysentery and is associated with high complication and case fatality rates [72]. As a result, the IMCI algorithm indicates ciprofloxacin treatment for children presenting with dysentery, defined as blood in the stool. However, the use of dysentery as a diagnostic criteria for *Shigella* infection has not been validated against the 'gold standard' of culture confirmed diagnosis. In addition, the vast majority of published data on pediatric *Shigella* infections are from studies in South Asia, where clinical predictors and outcomes may be distinct from infections occurring among children in sub-Saharan Africa.

Within a large cohort of children presenting with acute diarrhea at three sites in Western Kenya, we sought to determine the diagnostic performance of the IMCI guidelines at classifying suspected *Shigella spp*. against the gold standard of stool culture. We compared the IMCI classification of suspected *Shigella* to classifications determined by sociodemographic factors, clinical history (in addition to bloody stool), clinical presentation, and stool examination. Finally, we developed a risk score for assessing likelihood of *Shigella* infection in children presenting with acute diarrhea.

2.2 Methods

Population

We enrolled children aged 6 months to 15 years presenting with the main symptom of acute diarrhea to outpatient departments at three hospitals in the Nyanza province of Western Kenya (Kisii Provincial, Homa Bay District Hospital and Migori District Hospital) as part of an ongoing diarrhea and fever surveillance study from November 2011-May 2014. Acute diarrhea was defined as 3 or more loose or watery stools in the

last 24 hours lasting less than 14 continuous days. Written informed consent was obtained from primary caregivers of enrolled children and assent collected from children 13 years and over. The University of Washington Institutional Review Board and the Kenya Medical Research Institute Ethical Review Committee approved all study procedures.

Data collection

Clinical history and sociodemographic information was collected from the accompanying caregivers and children underwent a brief physical examination which included presentation and history information specified in the WHO Integrated Management of Childhood Illness (IMCI) algorithm for children presenting with the primary symptom of diarrhea. Specific IMCI indicators included: general danger signs (not able to drink or breastfeed, vomiting, convulsions, lethargy, stiff neck), dehydration signs (sunken eyes, skin pinch goes back slowly, restlessness/irritability, drinking eagerly/thirsty) as well as IMCI-defined dysentery (asking asked whether there is blood in the stool). Height and weight were measured prior to the physical exam by nursing staff and height for age z-score (HAZ) and weight for height z-scores (WHZ) calculated using the WHO ANTHRO software with the 2006 WHO reference population. Stunting and wasting were defined as HAZ <-2 and WHZ <-2, respectively. [34] All caregivers were provided with stool collection materials and instructions for stool collection. If a child could not produce a stool within an hour, three rectal swabs were obtained.

Blood was collected from children for malaria and HIV testing (per Kenya National Guidelines). HIV status was determined using antibody testing (Abbott Determine™ rapid test kit and confirmed using Uni-Gold™) or HIV DNA polymerase chain reaction (PCR) assays if <18 months. Malaria parasitemia was assessed by both rapid testing (Paracheck Pf® Orchid Biomedical Services, India) and by microscopy. The HIV-status of consenting accompanying biological mothers was ascertained by self-report and confirmed with antibody testing if unknown or HIV negative.

Stool Specimen Processing

A portion of stool samples and rectal swabs were transferred into Cary-Blair transport medium and the remaining sample placed in a sterile shipping container for parasite determination. Samples were stored and shipped on cold packs daily to the Kenya Medical Research Institute/United States Army Research Unit Microbiology Hub-Kericho, Kenya, within 24 hours of collection. Upon arrival at the laboratory, stool was examined for gross blood and mucus and determined whether or not it appeared watery. For bacterial identification, each specimen was plated on selective media as follows: BAP (blood agar plate) for hemolysis and oxidase test, Sorbitol-MacConkey agar to select for non-sorbitol fermenting (NSF) *Escherichia coli* (*E. coli*), Hektoen or xylose lysine deoxycholate agar for *Salmonella* and *Shigella spp.,* cefsulodin irgasan novobiocin agar for *Yersinia* spp., and cefoperazone vancomycin amphotericin (CVA) agar for *Campylobacter spp*. Colonies were further confirmed by oxidase test, catalase, gram stain, Hippurate hydrolysis test, and thiosulphate citrate bile salt- sucrose (TCBS) agar for *Vibrio* spp. Pure colonies were further processed using MicroScan WalkAway 40 Plus automated platform for bacterial identification and antibiotic susceptibility testing. *Salmonella*, *Shigella* or *Vibrio* spp. isolates were serologically typed using their respective commercially available typing sera in slide agglutination tests.

NSF *E. coli* isolates exhibiting different morphologies were grown separately overnight in trypticase soy broth and frozen in 25% final concentration glycerol. The frozen isolates were batch tested using multiplex PCR to identify virulent genes associated with Enterotoxigenic *E. coli* (ETEC)- heat labile enterotoxin (*elt*) and/or heat stable enterotoxin (*est*), Enteroaggregative *E. coli* (EAEC)- *aatA*; Enteroinvasive *E. coli* (EIEC)- invasion plasmid antigen H (*ipaH*); or Enterohemmorhagic *E. coli* (EHEC)- Shiga toxin 1, 2 and variants (*stx*); and Enteropathogenic *E. coli* (EPEC)- bundle forming pilus (*bfpA*) [36]. Starting in March 2013, additional gene targets for EPEC- intimin (*eae*) and EAEC- *aaiC*

were incorporated. A classification of EAEC was therefore the identification of *aatA* and/or *aaiC*. EPEC was disaggregated into two categories: typical EPEC (*bfpA* with or without *eae*) and atypical EPEC (*eae* without either *bfpA or stx).*

Stool was concentrated using the Mini Parasep® Solvent Free concentration kit (DiaSys, Berkshire, England) and evaluated using microscopy to identify parasites. Parasite testing was not performed on rectal swabs.

Analysis

Children who were admitted to the inpatient ward outside of study hours were excluded as we did not have clinical history at the time of presentation. We also excluded children over the age of 5 as the IMCI guidelines are designed for children aged 6 months to 5 years. Among enrolled children, we described sociodemographic, clinical history, clinical presentation, macroscopic stool investigation, and enteric pathogen with frequencies and percentages or medians and interquartile ranges. We compared the likelihood of a child providing a rectal swab, as opposed to whole stool sample by age category, HIV-exposure status, HIV-infection status, wasting, stunting, and dehydration classification using Chi-square tests of equality. We determined the diagnostic accuracy (sensitivity, specificity, positive predictive value [PPV], and negative predictive value [NPV]) of dysentery at identifying children infected with *Shigella* by culture. For each diagnostic performance measure we calculated 95% confidence intervals (CI) assuming a binomial distribution.

We fit logistic regression models for predicting likelihood of prevalent *Shigella* infections by IMCI and additional clinical factors with varying resource and training requirements to determine the minimal set of predictors that improve upon the IMCI dysentery-based *Shigella* classification. For each model, we added relevant factors stepwise (starting with smallest *p*-value in univariate analyses) into a logistic regression model and variables were maintained in the model as long as they added to the

predictability of *Shigella* infection (as measured by a decreasing Akaike Information Criteria (AIC) value estimated at each model iteration). Because a prediction model's predictability, particularly as measured by R^2 or AUC, increases with increasing number of added variables, we used a measure of predictability (AIC) that increases with increasing number of parameters [79]. The following variables were considered for each model (but not all variables remained in the model based on AIC). Model 1 contained only IMCI-defined dysentery: bloody stool reported by accompanying caregiver. Model 2 added to model 1, easily collected history and presentation information: IMCI-general danger signs, IMC-dehydration signs, age, sex, history of fever, recent antibiotic use, current breastfeeding, and number months of exclusive breastfeeding. Model 3 expanded upon model 2 by adding axillary temperature, HAZ, and WHZ, measures which require slightly more time, training, and equipment (thermometer, scale, length/height board) than those in model 2. Model 4 added to model 3 variables a macroscopic evaluation of stool, which would require training in macroscopic stool evaluation and the facility for, and hygienic training in, safe collection of stool samples. Finally model 5 added HIV and malaria rapid testing (RDT) results which, while becoming more frequently available in resource-limited settings, may not be feasible for the low level health facilities in which IMCI guidelines are often used.

2.3 Results

Study Population

Among 1,913-screened children, 445 were excluded because they did not meet pre-specified analysis inclusion criteria and 907 were included in the analysis (Figure 2.1). Included children were a median of 20 months old (interquartile range [IQR]: 11-36 months), about half (53.2%) were male, and the majority (98.5%) enrolled from Kisii or Homa Bay hospitals (Table 2.1). Less than half reported a monthly household income

under 5000 Kenya shilling (~60USD) and 40.2% lived in a household with 2 or more people per room. Most (73.6%) households owned some type of livestock, 14.1% reported obtaining their drinking water from an unprotected source, such as an open well, spring, or surface water, and 73.8% reported treating their drinking water, most by adding bleach or chlorine (53.9%) or by boiling (34.4%). The majority (90%) of primary caregivers reported that the child had less than four continuous days of three or more loose stools in a 24 hour period (median: 2, IQR: 2-3 days) and that the median number of loose stools in the last 24 hours was 5 (IQR: 4-6). Blood in stool was reported by 6.8% of caregivers and bloody stool lasted median of 1 day (IQR 1-2 days). Only 7.2% of caregivers reported that the child had been hospitalized for diarrhea in the last year, although 30.0% of these recent hospitalizations were for diarrhea. At presentation, 29.9% of children had at least one general danger sign, 6.4% and 14.5% of children were classified as having some dehydration or severe dehydration, respectively, and 33.6% had a fever. The median WHZ and HAZ were -0.39 (IQR: -1.41 to 0.75) and -0.49 (IQR: -1.65 to 0.53), respectively; 10.5% were HIV-uninfected but exposed to a HIV-infected mother, and 3.8% were HIV-infected.

Stool Evaluation

Most children (91.2%) were able to provide a stool sample rather than requiring a rectal swab. Children under 2 years, who were HIV-exposed uninfected, HIV-infected, wasted, and with severe dehydration were more likely to require a rectal swab, and these children therefore did not have parasite testing by microscopy. Upon macroscopic examination of whole stool samples, 1.2% had evidence of blood, 51.2% had mucous in stool and 72.3% were recorded as watery.

Among the 1,204 enrolled children, pathogenic *E. coli* were the most commonly identified bacteria (24.0%), followed by *Campylobacter spp.* (7.1%) and *Shigella spp.* (4.9%) (Table 2). EAEC was identified in 13.5% of enrolled children, ETEC in 4.4%, and

typical EPEC in 4.1%; atypical EPEC, EIEC, and EHEC were less common, 2.8%, 2.3%, and 0.2%, respectively. Among the *Campylobacter* isolates, *C. jejuni* was the most common species (74.4%). Among *Shigella* isolates, *S. sonnei* was identified in 42.5%, *S. flexneri* in 37.3%, *S. dysenteriaea* type 1 in 3.4%, and species was not determined in 17.0%. Among the 1,098 children who were tested for parasites, *Giardia spp.* (10.3%), *Blastocystis* (8.2%), and *Cryptosporidium spp.* (4.1%) were the most commonly identified. Among the 925 children tested for both bacteria (including pathogenic *E.coli*) and parasites, 65.0% had neither a bacterial nor a parasitic pathogen identified.

Identification of more than one pathogen was common; 113 (9.4%) children had at least 2 pathogens identified, 14 (1.2%) had 3, and 2 (0.2%) had 4. Of the 59 *Shigella* isolates, 16 (27.1%) were in stools with multiple pathogens; 12 with 2 organisms, 3 with three organisms, and 1 with 4 organisms. The likelihood of identifying a co-pathogen with *Shigella* was not associated with *Shigella* species (p=0.62). EIEC was the most common co-pathogen with *Shigella*, found in 6 (37.5%) of the 16 mixed *Shigella* infections. *Campylobacter* and *Giardia* were the second most common *Shigella* co-pathogens, each found in 3 (18.8%) of the 16 mixed *Shigella* infections.

Presentation of children infected by species of Shigella

Among the 59 children with *Shigella* infections, the majority (69.5%) were identified in children in the oldest age category (24-59 months) (Table 2.3). A substantial percentage (19.0%) of *Shigella*-infected presented with an IMCI-danger sign, 5.1% with severe dehydration, and slightly less than half (45.8%) presented with current fever. Although only 3.4% had gross blood on macroscopic evaluation, a substantial percentage of stools from these children (71.2%) had a mucoid appearance and 78.0% were described as watery. There were significant differences in presentation by *Shigella* species; children infected with *S. dysentariae* type 1 were younger (*p*=0.008*)* and more

likely to present with an IMCI danger sign (p=0.02). We did not find other differences in clinical presentations or in stool appearance.

Performance of IMCI Algorithm for Shigella Identification

History of bloody stool was collected for 1,058 of 1,205 (87.8%) of children. Among the 51 children with *Shigella*-positive stools in whom we ascertained history of bloody stool, 7 had the IMCI history of bloody stool classification (sensitivity: 13.7% [95% CI: 5.7%-26.6%] and PPV: 9.9% [95%CI: 4.0%-19.0%]) (Figure 2.2). Among the 1,006 children without microbiologic isolation of *Shigella*, 942 did not have a history of bloody stools (specificity: 93.6% [95%CI: 91.8-95.0%] and NPV: 95.5% [95%CI: 94.1% - 96.7%]). Among the 8 children with *Shigella* infection in whom we did not ascertain history of bloody stool, 3 were *S. flexneri*, 3, *S. Sonnei*, and 2 did not have species determined. The AUC of IMCI suspicion of *Shigella* was 0.54 (95%CI: 0.50-0.58) (Figure 2.3, model 1). When we added additional signs and questions from the IMCI algorithm (model 2), the AUC increased to 0.69 (95%CI: 0.63-0.76). Of all considered factors in this model, age, history of fever, vomiting, and history of bloody stool were the factors responsible for improvements in the measure of prediction (AUC). The addition of more intense examination, including height and weight measurement and z-score calculation as well as axillary temperature (model 3), increased the AUC slightly to 0.72 (95%CI: 0.64-0.79), and the model that maximized AIC was age, vomiting, current fever, and HAZ. Stool visual evaluation (model 4) added slightly more predictive ability (AUC 0.75 [95%CI: 0.68-0.82], model: age, vomiting, current fever, HAZ, and mucoid stool) while HIV and malaria rapid testing did not add beyond stool visual evaluation (AUC: 0.75 [95%CI: 0.69-0.82] model: age, vomiting, current fever, HAZ, mucoid stool, and present malaria infection).

2.4 Discussion

In this study of children presenting to three Kenyan outpatient facilities for acute diarrhea, the currently used IMCI presentation-based diarrhea classification system identified few children infected with *Shigella spp*. In the absence of laboratory diagnosis, these children may have missed the opportunity to receive potentially life-saving, and transmission-reducing, antibiotic treatment. Notably, most stool from which *Shigella* was isolated had a mucoid appearance, consistent with inflammatory diarrhea. A history of bloody stools had low prognostic performance (with AUC 0.54, which is roughly similar to tossing a coin (0.5)). Sensitivity was poor (13.7%) while specificity was high (93.6%), which would result in under-treatment of true infections and infrequent over-treatment, which could be useful to preserve antibiotic regimens. In fact, if current guidelines were followed and the only indication for antibiotic therapy is a history of bloody stool, most potentially dysenteric *Shigella* infections would not be treated. The addition of other clinical indicators was able improve diagnostic performance (AUC 0.75) and suggests that IMCI guidelines might be adapted to improve outcomes among children at high risk.

Indications for antibiotic treatment for diarrhea illness are very circumscribed in international guidelines, including in the IMCI syndromic guidelines. Antibiotics are generally not useful for diarrheal disease, as infecting pathogens are frequently viral and many bacterial infections are self-limiting. In the case of some bacterial infections, such as non-typhoidal Salmonella and EHEC, antibiotic use may actually prolong pathogen carriage. For *Shigella* however, antibiotics have demonstrated efficacy in decreasing the clinical course and reducing transmission. Despite circumscribed indications, antibiotics are frequently used for diarrhea treatment in resource-limited settings with as many as 60% of children being prescribed antibiotics for diarrhea [80,81]. Treatment of viral or non-

infectious causes of diarrhea with antibiotics is a public health concern as increasing rates of antibiotic resistance supersede rates of new antibiotic development. Even if new classes of antibiotics become available, they are unlikely to be accessible and/or affordable to resource-limited settings such as Western Kenya. Reasons for the disconnect between recommended diarrhea management and clinical practice includes clinician fear of missing underlying infections that could benefit from antibiotic treatment, pressure from care-givers to receive antibiotics, and/or distrust of current guidelines [82,83]. Although speculative, clinician distrust in IMCI diarrhea algorithm may be in part, because they have not been updated since their inception in 2005, despite new data and research on pediatric enteric infections in the last decade. It is plausible that if precision of syndromic algorithms for diarrhea were improved in a way that clinicians are reassured they are not missing potentially serious bacterial infections, such as *Shigella*, we might improve both morbidity and mortality outcomes and actually reduce antibiotic use for diarrhea when non-bacterial causes are suspected.

IMCI training and guidelines as a whole have likely improved the quality of care in many resource-limited settings by improving the ability of health workers to correctly classify illnesses, accurately prescribe medications, increase vaccination coverage, and counsel families on care and nutrition [84]. Previous studies evaluating the performance of non-physician providers using the IMCI diarrhea algorithm compared to the "gold standard" of diagnosis by fully trained pediatricians based on their clinical examination and expertise [85-89]. These studies found that in general, determinations of dysentery, persistent diarrhea, and diarrhea with dehydration were similar to those based on more highly trained clinical expertise. However, these studies, which demonstrate capability of non-physician clinicians to adhere to guidelines, do not assess whether the guidelines are accurate in identifying children who need specific treatment.

Although not explicitly evaluating the IMCI diarrhea algorithm, other studies have assessed the predictive value of bloody stool for identification of shigellosis. A large diarrhea surveillance study in seven Asian countries found that among 2,925 culture-confirmed shigellosis episodes among children aged <60 months old, 790 reported visible blood in the stool (sensitivity 27%). In this study the PPV and NPV of bloody stool for shigellosis diagnosis was 17% and 96%, respectively.[65] Among 792 Bangladeshi children <15 years old diarrhea admissions in which *Shigella* was isolated and stool characteristics documented, 332 had grossly bloody stool (sensitivity 42%) and *S. dysentariae* type 1 infections were more than twice as likely to be characterized by bloody stool than non *S. dysenteriae* type 1 infections (p<0.001) [66]. That *S. dysentariae* type 1, compared to other species, is more frequently associated with fecal blood, has been widely demonstrated [66,71,90]. The lack of sensitivity of history of bloody stool for *Shigella* identification in our study might be explained by the relative infrequency of *S. dysentariae* type 1 infection compared to *S. sonnei and S. flexneri* in our study population. The focus on presumptive treatment of *S. dysentariae* type 1 infections by the guidelines is likely missing potentially fatal *S. flexneri* and *S. sonnei* infections.

We found that identification of *Shigella* infections could be improved substantially by the addition of clinical features and macroscopic stool evaluation, particularly presence of mucous in the stool. However, given macroscopic evaluation of stool requires substantially more training and resources than those that are often available at outpatient facilities in resource-limited settings, we propose that model 3 (age, vomiting, current fever, and HAZ), which had an AUC of 0.72, a substantial improvement from the AUC of 0.54 from history of bloody stool alone, by validated in additional studies as a potential set of clinical predictors of *Shigella* infection. It is well established that *Shigella* infections are more common in older age groups of children and that current fever is positively associated with *Shigella* infection [65-67]. In this multivariable prediction model, excessive

vomiting was inversely associated with *Shigella* infection, suggesting that presentation of vomiting might be suggestive of another type of enteric infection, such as rotavirus.

Additional high-risk bacteria, specifically enterotoxigenic *Escherichia coli* (ETEC) and enteropathogenic *Escherichia coli* (EPEC), are increasingly recognized to be associated with severe morbidity and mortality[10,91]. It was estimated that 15% and 8% of diarrheal episodes requiring hospitalization and 11% and 6% of diarrhea-related deaths in children under age 5 were attributed to EPEC and ETEC in 2011 [91]. Together with *Shigella* infections, these three bacteria account for almost 30% of diarrhea-related hospitalizations and over 20% of diarrhea-related deaths in children under age 5. As with *Shigella*, antimicrobial use for EPEC and ETEC gastroenteritis decreases the duration of disease and the fecal pathogen excretion [47,92,93]. Further research is needed to determine how to best identify EPEC and ETEC infections (or suspected infections) in resource-constrained environments. Treatment with ciprofloxacin, the recommended antimicrobial agent for suspected shigellosis in the IMCI diarrhea algorithm, may substantially reduce morbidity and mortality related to EPEC and ETEC.

There were important limitations to this study that need to be improved upon in future work. First, the total number of *Shigella* infections were small, which limited our ability to understand clinical differences between species of *Shigella* infections. We were further limited by the fact that we did not have species information for 10 of the *Shigella* isolates found. This study was cross-sectional, therefore we could not determine the outcomes of *Shigella*-infected children. It would have been valuable to evaluate whether there was a difference in outcome between children with and without lab-confirmed *Shigella* among those with a history of bloody stool who were subsequently treated with ciprofloxacin. A study among Bangladeshi children <15 years old hospitalized with *Shigella* found that younger age, less frequent stool before admission, decreased WAZ,

and convulsions were independently associated with death; however, bloody stool was not predictive of poor outcome [66].

Despite these limitations, our study is strengthened by the systematic and detailed clinical characterization of the children, enabling a comparison of various sets of factors predictive of *Shigella* infections. To our knowledge, this is the first specific assessment of the IMCI diarrhea module for suspected *Shigella* infection identification and will hopefully provoke scientific discussion around the use of antibiotics for *Shigella* and other enteric bacterial pathogens that are emerging as important causes of morbidity and mortality. Improvements in the precision of diarrhea-recommendations for bacterial pathogens such as *Shigella* may result in stricter following of the guidelines which in turn, could result in more targeted antibiotic use in the context of pediatric diarrhea. Additionally, we propose that additional studies explicitly evaluate the utility of including additional clinical features in determining suspected shigellosis such as those identified in this study.

Figure 2.1 Number of screened, enrolled, and included participants

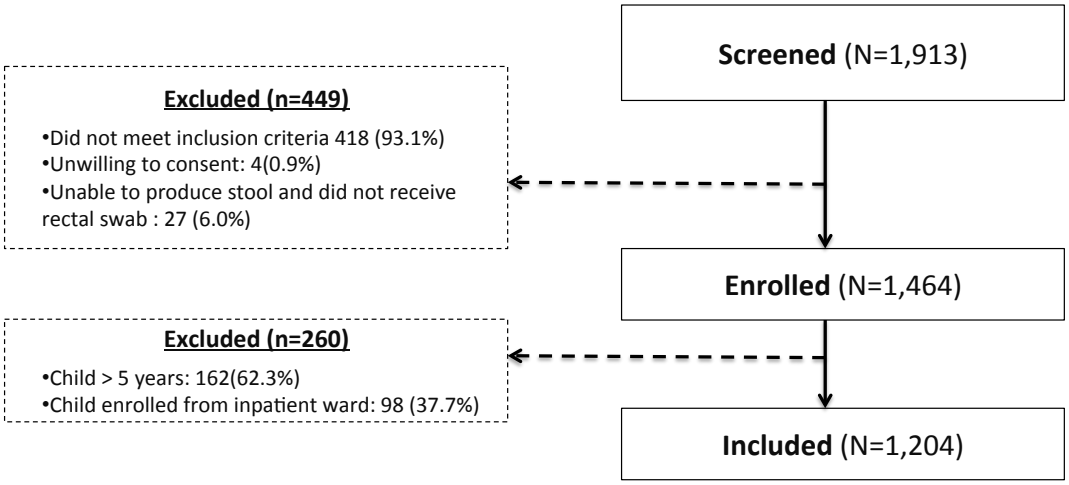


Table 2.1 Characteristics of enrolled children (N=1,204)

Characteristic	N[a]	n (%) Median (IQR)
Sociodemographic		
Male		487 (53.2%)
Median Age (months)		20 (11-36)
Site		
Kisii		560 (46.5%)
Homa Bay		626 (52.0%)
Migori		18(1.5%)
Monthly household income <5,000 Kenyan Shillings	1202	471(39.2%)
Crowding[b]	1201	483(40.2%)
Livestock[c] ownership		885(73.6%)
Unprotected water source [d]		170(14.1%)
Household treats drinking water		888(73.8%)
Clinical History[e]		
Bloody stool[f]	1,058	72 (6.8%)
History of fever within last 48 hours		532 (43.5%)
Antibiotic used in last 7-days		166 (13.8%)
Median # months exclusively breastfed	1,195	6(4-6)
Currently breastfeeding (among ≤24 months[g])	703	522 (74.3%)
Clinical Presentation		
Presenting with any IMCI danger signs:	1,194	357 (29.9%)
Unable to drink or breastfeed		49 (4.1%)
Excessive vomiting		312 (27.8%)
Convulsions		5 (0.4%)
Lethargy/Unconscious		24 (2.0%)
Presenting with any dehydration signs		391 (32.5%)
Restless/ Irritable		206 (17.1%)
Sunken eyes		235(19.5%)
Drinks eagerly, thirsty		146 (12.1%)
Skin pinch goes back slowly		77 (6.4%)
Sunken fontanelle		86 (7.1%)
IMCI Dehydration Classifications		
Severe[h]		77 (6.4%)
Some[i]		175 (14.5%)
None		952(79.1%)
Stunted (HAZ<-2)	1,145	170 (14.9%)
Wasted (WHZ<-2)	1,191	227 (19.1%)
Axillary temperature ≥37.5°C at presentation		404 (33.6%)
HIV-exposed uninfected[j]	1,087	114 (10.5%)
HIV-infected	1,181	45 (3.8%)
HIV-associated immunosuppression[k]	42	15 (35.7%)
Stool Examination[l]		
Blood observed in stool		14 (1.3%)
Mucous observed in stool		621 (56.6%)
Watery		794(72.3%)

[a] Reported if different from total (N=1204)
[b] ≥2 people per room living in house
[c] Ownership of cows, goats, or chickens
[d] Unprotected well/spring/ surface water
[e] Reported by accompanying caregiver
[f] Question added to CRF in March 2012 therefore missing for first 146 patients.
[g] Among 703 children who were ≤24 months of age
[h] 2 or more of the following signs: Lethargic or unconscious, sunken eyes, not able to drink or drinking poorly, skin pinch goes back very slowly
[I] Two or more of the following signs: restless/irritable, sunken eyes, drinks eagerly/thirsty, skin pinch goes back slowly
[J] Among children known to be HIV-infected, who were accompanied by their biological mother, and whose HIV status was known (by antibody test or self-report if positive).
[k] Defined in terms of CD4% (age ≤11 months: <25%, 12 months-35 months: <20%, 36^{+} months: <15%) or, in absence of CD4 % data, in terms of CD4 count (age ≤11 months: <1500 cells/mm^3, 12 months-35 months: <750 cells/mm^3, 36^{+} months <350 cells/ mm^3)
[l] Performed by lab technician upon receipt of whole stools samples (could not be done on rectal swabs) n=1,098

Table 2.2 Prevalence of enteric pathogens among enrolled children

Organism Identified	N	%
Bacteria (N=1,204)		
No bacteria [a]	601	(65.0%)
Any pathogenic Escherichia coli[b]	222	(24.0%)
EAEC	125	(13.5%)
EHEC	2	(0.2%)
EIEC	24	(2.3%)
EPEC (atypical) [c]	17	(2.8%)
EPEC (typical)	38	(4.1%)
ETEC	41	(4.4%)
Campylobacter spp. [d]	86	(7.1%)
Shigella spp. [e]	59	(4.9%)
Salmonella spp. [f]	11	(0.9%)
Aeromonas hyrophila	2	(0.2%)
Plesiomonas shigellosides	1	(0.1%)
Parasites (N=1098[g])		
Giardia spp.	113	(10.3%)
Cryptosporidium spp.	49	(4.5%)
Helminths [h]	34	(3.1%)
Entamoeba histolytica/dispar	5	(0.5%)
Isospora spp.	1	(0.1%)
No bacteria or parasite (N=828[i])	434	(52.4%)

[a] Among the 925 children whose stool was cultured and PCR tested for pathogenic *Escherichia coli (E. coli)*
[b] Tested in the first 925 samples
[c] Among those in whom eae gene was tested (n=318)
[d] *Campylobacter jejuni* (n=64), *Campylobacter spp*. other than *jejuni* (n=22)
[e] *Shigella flexneri* (n=22), *Shigella sonnei* (n=25), *Shigella dysentariae* type 1 (n=2), species not determined (n=10)
[f] Non-typhoidal species (n=10), *Salmonella typhi* (n=1)
[g] Testable only among those who provided a whole stool sample (as opposed to rectal swab)
[h] Only able to detect *Ascaris lumbricoides* infections
[i] Among those who were able to provide a stool sample (rather than rectal swab) and who were tested for all bacteria (including pathogenic *E.coli*)

Table 2.3 Characteristics of children with *Shigella* infections by *Shigella species*

Characteristic	*Shigella spp.* (N=59)	*S. dysentariae type 1* (N=2)	*S. flexneri* (N=22)	*S. sonnei* (N=25)	*Shigella* Species ND (N=10)	*p*-value[a]
Age						
6-11 months	7 (11.9%)	1 (50.0%)	1 (4.6%)	3 (12.0%)	2 (20.0%)	
12-23 months	11 (18.6%)	1 (50.0%)	5 (22.7%)	1 (4.0%)	4 (40.0%)	0.008
24-59 months	41 (69.5%)	0 --	16 (72.7%)	21 (84.0%)	4 (40.0%)	
Clinical Presentation						
Any IMCI Danger Sign	11 (19.0%)	1 (50.0%)	2 (9.1%)	3[b] (12.5%)	5 (50.0%)	0.02
Severe dehydration	3 (5.1%)	0 --	1 (4.6%)	0 --	2 (20.0%)	0.10
Current fever (axillary temperature ≥37.5°C)	27 (45.8%)	1 (50.0%)	11 (50.0%)	11 (44.0%)	4 (40.0%)	0.94
Stunted (HAZ<-2)	5 (8.9%)	1[c] (100%)	2 (9.5%)	1 (4.0%)	1[d] (11.1%)	0.75
Wasted (WHZ<-2)	8 (14.3%)	0 --	3 (15.0%)	3 (12.0%)	2 (20.0%)	0.90
Macroscopic Stool Evaluation						
Blood	2 (3.4%)	0 --	1 (4.6%)	1 (4.0%)	0 --	>0.99
Mucoid	42 (71.2%)	1 (50.0%)	15 (68.2%)	18 (72.0%)	8 (80.0%)	0.84
Watery	46 (78.0%)	2 (100.0%)	16 (72.7%)	19 (76.0%)	9 (90.0%)	0.79

[a] Comparison across *Shigella spp.* using Fisher Exact test
[b] One missing IMCI classification therefore % out of 24
[c] One missing HFA-Z therefore % out of 1
[d] One missing HFA-Z therefore % out of 9

Figure 2.2 Comparison of IMCI classification of suspected shigellosis compared to identification of *Shigella spp.* by bacterial culture.

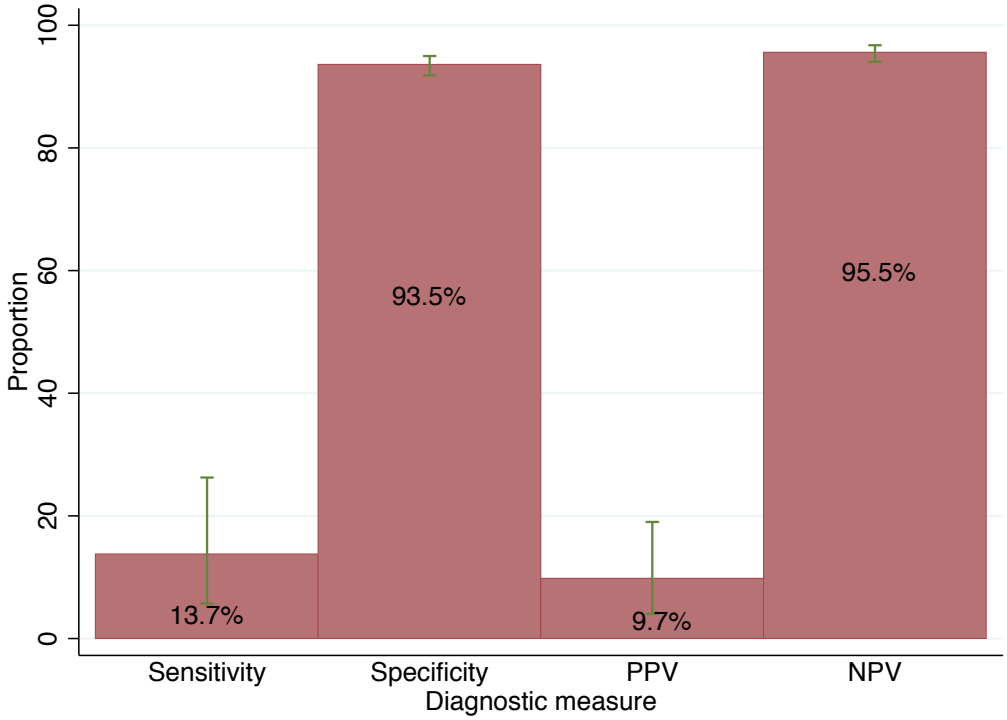
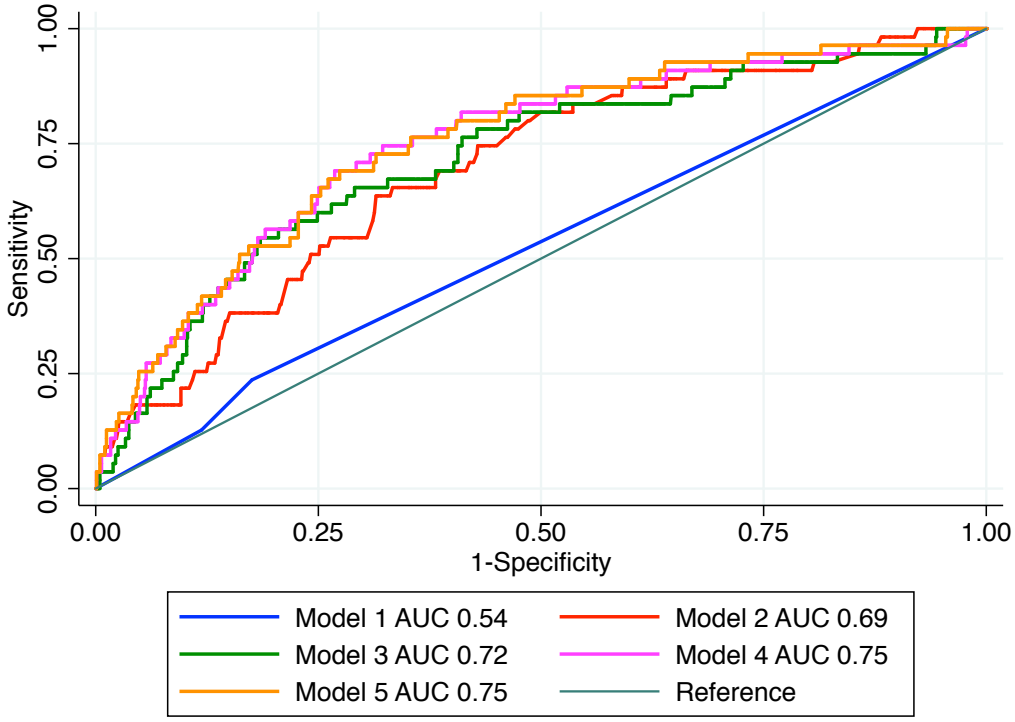


Figure 2.3 Comparison of IMCI classification of suspected *Shigella* infection by asking caregiver whether the child has had bloody stools (yes/no) (Model 1) to classifications that consider additional factors ordered from least resource-demanding to most (model 2-5). Model 2: Model 1 plus additional easily collected clinical history (IMCI danger and dehydration signs, breastfeeding history, age, sex). Model 3: Model 2 factors plus additional information collected from physical exam (axillary temperature, weight-for-height z, height for age z. Model 4: Model 3 plus macroscopic stool evaluation (blood, mucous, watery). Model 5: Model 4 plus rapid HIV and malaria blood test results.



Conclusion

The analyses presented in this dissertation demonstrate that bacterial and parasitic pathogens are frequently isolated from the stool of children presenting with acute diarrhea. Many children in sub-Saharan Africa live in environments where they are frequently exposed to enteric pathogens and the transmission cycle of these pathogens is perpetuated through animal, human-to-human, and water-borne transmission mechanisms. Children exposed to these enteric pathogens experience a wide spectrum of disease states that range from no symptoms to acute diarrhea, dehydration and death. The reasons for differences in susceptibility and consequences of bacterial and parasitic pathogens likely involve an interplay among the quantity and frequency of pathogen exposure, the robustness and quality of a child's immune response, virulence-determining features of a given pathogen, and clinical management factors that become relevant after a child presents to a health facility with symptoms. This dissertation illustrates how host, pathogen, exposure and management factors are all relevant to pediatric diarrhea is sub-Saharan Africa.

As described in chapter 1, HIV-infection in children is associated with identification of typical EPEC, a T-cell modulated pathogen[94]. Suppression of T-cells is characteristic of HIV-infection and thus the association between HIV and EPEC serves as an example of the relation between host immunity and pathogen. Given HIV is a well-established risk factor for morbidity and mortality, and EPEC was associated with death in GEMS, children presenting with diarrhea and HIV may require empiric antibiotic treatment with a locally-susceptible antibiotic and/or more intense follow up after discharge. Illustrative of the potential interplay among host, pathogen, *and* exposure factors, we also found in chapter 1 that among HIV-uninfected children, having an HIV-infected mother was associated with

prevalent *Cryptosporidium* infection. Possible explanations for this association include increased child susceptibility due to HIV-exposure related immunosuppression, increased exposure to *Cryptosporidium* from HIV-infected mothers, who are more susceptible to *Cryptosporidium* themselves, and/or increased susceptibility to the infection from other transmitting HIV-infected household members.

This dissertation also demonstrates that management factors likely play a large role in the consequences of enteric infections. As new, higher quality data (such as from GEMS) become available, it is important to review the relevance of international clinical management guidelines. We found that the current IMCI guidelines for diarrhea management do not target the pathogens deemed independently associated with death in GEMS. Further we found that the guidelines are likely missing important opportunities to treat clinically-relevant *Shigella* infections. Although conservative treatment recommendations are important to limit antibiotic misuse, particularly in resource limited settings where antibiotics are limited, costly, and where individual and community antibiotic resistance is high, antibiotic treatment of diarrhea has potential benefits both for improving the clinical course of infection and for reducing transmission. As a scientific community we need to discuss the cost-benefits of antibiotic use in the context of diarrhea, particularly as the roll-out of rotavirus vaccination reduces the proportion of diarrhea attributed to viruses.

In conclusion, there are an estimated 800,000 children dying annually from diarrhea and in sub-Saharan Africa a large proportion of these children may be *infected* or *affected* by HIV[1]. Half of the HIV-infections identified in this study were newly diagnosed suggesting there is substantial room for improvement in getting HIV-infected individuals in care and treatment. It is plausible that boosting the immune system of children and adults who are HIV infected, through anti-retroviral treatment, will lead to valuable gains in reducing the number of children who die from diarrhea. In parallel with reducing HIV

transmission and treating current HIV-infections with appropriate antiretroviral therapies, management guidelines should be systematically re-evaluated based on the most up to date evidence. Research into the consequences of treated and untreated enteric infections, as well as in the development of new interventions should continue to be a research priority in order to reduce the number of children dying from diarrhea.

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Vita

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