Potential modulation of the intestinal microbiome by HIV and antibiotics and implications for EPEC susceptibility and severity: A pilot study

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ABBREVIATIONS & ACRONYMS

ABX	Antibiotic
ART	Antiretroviral therapy
bfpA+	Bundle-forming pilus structural gene
CCC	Comprehensive Care Clinic
CMiST	Center for Microbiome Sciences & Therapeutics
CTX	Cotrimoxazole
E. coli	*Escherichia coli*
EPEC	enteropathogenic *Escherichia coli*
ERC	Ethical Review Committee
DBS	Dried blood spot
GCP	Good Clinical Practice
GI	Gastrointestinal
HIV	Human Immunodeficiency Virus
HIV+	Human Immunodeficiency Virus Positive
HIV-	Human Immunodeficiency Virus Negative
IRB	Institutional Review Board
IMNCI	Integrated Management of Neonatal and Childhood Illnesses
KEMRI	Kenya Medical Research Institute
LEE	Locus of enterocyte effacement
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization with Time of Flight
MCA	Maternal, Newborn, Child and Adolescent Health
MCH	Maternal Child Health
MOH	Ministry of Health
MSD	Moderate to severe diarrhea
MUAC	Mid-upper arm circumference
OTU	Operational taxonomic unit
PID	Patient Identifier
PI	Principal Investigator
SD	Standard deviation
SERU	Scientific and Ethics Review Unit
UW	University of Washington
WHZ	Weight-for-height z-score

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ABSTRACT

Background Enteropathogenic *Escherichia coli* (EPEC) is a well-known cause of diarrhea in young infants and children. In Kenya, EPEC is more common in children with HIV-infection and this group has a higher risk of death upon infection[1,2]. Alterations to the gut microbiome from HIV or the antibiotic that is used as a prophylaxis may affect susceptibility to enteric pathogens such as EPEC. Aims This current pilot study has two objectives: 1) To evaluate the impact of HIV infection and antibiotic use (in the absence of EPEC infection) on the microbial architecture of stool and 2) To compare how HIV status and antibiotic treatment may impact the strain of EPEC. Methods Using rectal swabs from children with and without HIV (but all with HIV-exposure) and with and without antibiotic exposure, we will compare the gut bacterial communities in these four groups using targeted high throughput 16S rDNA V4 sequencing. Using EPEC isolates collected from children with and without HIV and with and without antibiotic exposure, isolates which were collected as part of a previous study, we will compare the likelihood of specific virulence genes using qPCR with primers for the LEE-encoded gene escV and EAE encoded gene bfpA. Analysis Bacterial diversity and prevalence of virulence genes will be compared across the four groups of children using t-tests and chi-square tests. Expected Outcomes We expect HIV HIV-infected children (without recent antibiotic exposure) and children recently exposed to antibiotics (without HIV-infection but who are similarly HIV-exposed) to have lower bacterial diversity in their gut microbiota and a higher likelihood of having the more lethal forms of EPEC. Such findings would suggest that the gut microbiota could be a potential target for therapeutics to improve enteric infection outcomes among HIV-infected and antibiotic-exposed children.

LAY SUMMARY

Diarrhea deaths are very high in children infected with HIV. In surviving HIV-infected children, diarrhea episodes occur more often and for longer than in their uninfected counterparts and can cause these children to have health problems and drop out of HIV treatment programs. The purpose of this study is to find out more about the germs that live in the guts of children exposed to HIV and antibiotics. All humans have germs that live in their guts, but there may be some germs that are more common in the gut of some children and these germs may be related to why some children get sick more often and recover less quickly than others. The goal of this study is to understand better, how antibiotics and HIV change the types of germs in children's guts with the potential to inform future treatment of sick children. We plan to enroll 150 children from Kenya in the study and collect rectal swabs from these children and to use previously collected bacterial isolates to see whether the germs that either live in or infect the gut are changed in children with and without HIV or antibiotic exposure. Rectal swabs will be processed to determine the number of each type of bacteria present in a sample and previously collect isolates will be processed to evaluate the presence of specific genes that can cause more severe disease. We expect children with HIV infection who have not taken antibiotics will have less of the good germs in their stomachs and more harmful forms of the bad germs in their guts than those who do not have HIV and have never taken antibiotics. This study may lead to new therapies that protect HIV infected children and those who have received antibiotics from diarrheal infections.

BACKGROUND

Diarrhea deaths are disproportionately high in HIV-infected children.[3-5] In surviving HIV-infected children, diarrhea episodes are more frequent and prolonged than in their uninfected counterparts.[6,7] Diarrhea is associated with dropout from HIV treatment programs and often leads to chronic malnutrition with consequences such as reduced school performance and earning potential, as well as cognitive impairments.[8-11] HIV-infection is associated with similar sequelae, doubly burdening the HIV-infected child. [12-14]

There are specific enteric pathogens, such as enteropathogenic *Escherichia coli* (EPEC), that appear more common in HIV-infected children.[1,15,16] Also HIV-infected children are more likely to die from enteric pathogens such as EPEC than HIV-uninfected children[2]. It is not known why some children die while others do not die after EPEC infection, but one hypothesis is that the gut microbiome plays a role. [17-20] In a mouse model of EPEC, the diversity and maturity of the gut microbiota determined whether mice became infected after exposure to EPEC, implicating the gut microbial community in determining susceptibility and virulence.[21] Alterations to the gut microbiome, such as reduction in diversity of the gut microbiota, may affect susceptibility to enteric pathogens such as EPEC, either through modulating the host response, or through direct effects on the metabolic and/or genetic regulation of EPEC, making the clinical course more severe in certain populations.

HIV-infection and its associated therapies can impact the gut microbiome and this may explain differences in diarrhea sequelae between children with and without HIV. Although most studies of the microbiome and HIV are from adults[17,22], a reduced microbial diversity was found in a study comparing the gut microbiota in Haitian HIV-exposed uninfected (HEU) infants to HIV-unexposed.[23] This study also identified increases in the abundance of *Prevotella* in HEU infants, a finding which has been shown in HIV-infected adults. [23]The functional and compositional changes in the gut microbiota due to HIV-infection and its treatment may provide new or different energy sources for invading pathogenic enteric bacteria, such as EPEC, which can then express a new set of more pathogenic genes.[24] These nutrient niches, which can only be exploited by a subset of bacteria (notably *Enterobacteriaceae*), could selectively foster bacterial proliferation and create “blooms” of bacterial species otherwise present in the gut ecosystem at low densities.[24]

It is unknown whether changes in the gut microbiome observed between populations with and without HIV-infection and/or HIV-exposure is due to the virus itself or its associated therapies, such as antiretroviral therapy (ART) and cotrimoxazole (CTX) prophylaxis[25]. A study among adults demonstrated that ART use reduced gut microbiota diversity and it was determined these compositional changes were independent of those driven by HIV infection.[26] Another study showed that ART classes have differential effects on the gut microbiome.[27] To the best of our knowledge, no study to date has evaluated the role of cotrimoxazole (CTX) on the gut microbiota, although numerous studies have shown other antibiotics reduce bacterial diversity[1428].

In summary, diarrhea-causing enteric pathogens such as EPEC are more likely to cause poor outcomes in HIV-infected populations and there is evidence in animal models to suggest that this may be due to changes in the gut microbiota, either as a result of HIV-infection or antibiotics used as daily prophylaxis in HIV-infected populations.

JUSTIFICATION

Despite the growing body of knowledge surrounding of the role of the gut microbiome in child health, there is a paucity of information regarding the changes that occur in the microbiota during HIV infection and even less is known about how these changes may impact the susceptibility and severity of enteric infections. The present pilot study will address an important gap in the literature around the impact of HIV and cotrimaxole on the pediatric gut microbiome and in doing so, establish possible mechanisms that could explain why HIV-infected children are more prone to lethal complications of diarrhea-causing pathogens like EPEC. Additionally, the present study will see if there are unique virulence genes of EPEC isolates between children with and without HIV and with and without cotrimoxazole. Our study

has two components: 1) a cross-sectional study will gather pilot data to examine the affect HIV infection and antibiotic use has on the composition of the gut microbiome by examining the microbial diversity in stool and 2) Using previously collected and stored EPEC isolates to determine whether there are differences in virulence genes between isolates from HIV-infected and HIV-uninfected children.

OBJECTIVES

Overall objective: To determine if a reduction in bacterial diversity through antibiotics use or HIV infection is associated with severe EPEC infection we will:

Objective 1: Evaluate the impact of HIV infection and antibiotic use (in the absence of EPEC infection) on the microbial architecture of stool using 16S rRNA analysis.

Hypothesis: HIV-infected children (without recent antibiotic exposure) and children recently exposed to antibiotics (without HIV-infection but who are similarly HIV-exposed) will have lower diversity indices than age-matched children without HIV infection or antibiotics exposure.

Objective 2: Compare how HIV status and antibiotic treatment may impact the strain of EPEC.

Hypothesis: Among EPEC-infected children, those with HIV infection or antibiotic exposure will be more likely to have more lethal forms of EPEC, as characterized by presence of the bfpA+ gene on EPEC.

METHODS

STUDY DESIGN

This is a cross-sectional study involving the collection of rectal swabs from HIV-exposed Kenyan children with and without HIV-infection and with and without recent antibiotic exposure (Objective I). This study will also leverage 70 de-identified EPEC isolates collected during a study of diarrhea etiology (SERU protocol SSC #2056 and UW protocol #39952) that specifically examined differences in causes of diarrhea and bacteremia between children with and without HIV[1], for further testing of the virulence genes in EPEC isolates (Objective 2). We have divided the subsequent methods by Objective.

Objective 1: Impact of HIV infection and antibiotic use on the gut microbiome

ELIGIBILITY CRITERIA

Eligibility is defined as a child of either sex, born to an HIV-infected mother, aged between 6-12 months, who is not exclusively breastfed (due to likely differences in microbiota between those who have and have not been weaned), and who falls into one of the following categories (Figure 2):

HIV+/ABX+ (n=50): This is an HIV-infected child who is currently taking daily cotrimoxazole prophylaxis (**defined as child has taken cotrimoxazole in at least 5 of the last 7 days**) and has not taken any other antibiotic in the last 7-days

HIV+/ABX- (n=25): This is an HIV-infected child who is not currently taking daily cotrimoxazole prophylaxis (**defined as child has <u>not</u> taken cotrimoxazole in the last 7 days**) and has not taken any other antibiotic in the last 7-days

HEU/ABX+ (n=50): This is an HIV-exposed uninfected child (HEU) who is currently taking daily cotrimoxazole prophylaxis (defined as child has taken cotrimoxazole in at least 5 of the last 7 days) and has not taken any other antibiotic in the last 7-days

HEU/ABX- (n=25): This is an HIV-exposed uninfected (HEU) child who is not currently taking daily cotrimoxazole prophylaxis (defined as child has not taken cotrimoxazole in the last 7 days) and has not taken any other antibiotic in the last 7-days

Additional inclusion/exclusion criteria is represented in Table 1 below.

Table 1. Eligibility criteria for potentially enrolled children

Inclusion criteria:	• Children aged 6-12 months, • HIV-exposed (biological mother is HIV-positive) • Vaginal delivery, • HIV-infection status of the child is known or ascertainable at the visit, • Documented or caregiver reported antibiotic use (other than the caregiver) during at least 5 of the last 7 days, • Accompanying caregiver able to provide consent on child's behalf • Willingness to have 2 rectal swabs and DBS collected from child
Exclusion criteria:	• Exclusively breastfeeding • Signs of illness (diarrhea, fever, shortness of breath, chest in-drawing), • Severe acute malnutrition defined as weight for height z-score (WHZ < -3) or MUAC < 11.5cm

SAMPLE SIZE

The per group sample size required to determine differences in mean alpha diversity (Shannon Index) between groups of children defined by HIV-infection and antibiotic status was calculated assuming an alpha level of 0.05, power of 0.80, and an equal number of children per group. Using expected mean and standard deviations of Shannon Indices from a recent study comparing stool microbiota in HIV-exposed and HIV-unexposed infants of 3.0 (SD:0.85) and 3.7 (SD:0.91),[23] we determined that 25 stool samples are needed for the HIV+/ABX- & HEU/ABX- study groups, and at least 50 stool samples are needed for the HIV+/ABX+ & HEU/ABX+ for a total of 150 children enrolled in the microbiome study (Aim 1a).

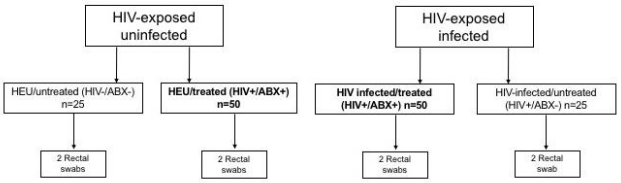
RECRUITMENT

Children will be recruited from Maternal and Child Health clinic (including vaccine and growth monitoring visits), and comprehensive care clinics (CCC) at the following sites in Homa Bay County: Ndhiwa Sub-County Hospital and Homa Bay Teaching and Referral Hospital, and in Migori County: Migori County Referral Hospital, Rongo Sub-County Hospital, Awendo Sub-County Hospital, Isebania Sub-County Hospital, and Uriri Sub-County Hospital, or will be siblings of children attending these clinics. If we are unable to recruit sufficient number of children from these healthy-visit clinics, we will recruit children from the outpatient department who are seeking care for non-gastrointestinal related conditions, so as to limit the likelihood that the gut microbiome is impacted by an acute illness. Caregivers of potentially screened children will be approached by study staff who will share a brief overview of the study objectives and ask the caregiver for permission to collect basic information about the child in order to assess whether the child would be eligible to participate. The caregiver will also be made aware that as part of the screening process, the HIV exposure status of the child (which reveals the HIV-status of the mother) will be recorded. This process is called Screening Consent and if the caregiver agrees to have the child screened, a Screening Consent form will be completed.

We will only screen children with known HIV-exposure status, which will be ascertained though the child's medical records. If the child's HIV-exposure status is unknown at the time of screening, the child will be referred to the HIV Care Clinic for evaluation and screening continued when results are available. Any mother newly diagnosed with HIV will be referred to the HIV Care Clinic at the respective study site for follow-up care and treatment.

The groupings based on antibiotic history will be made after a child screens eligible but prior to consenting and enrollment, so as to ensure we don't over-recruit in a single group. If HIV-infection status of the child is known (PCR test done within the last month) then that child will also be placed in a single group at the point between screening and enrollment. However, there will be a sub-group of children in whom HIV-infection status is not known at the time of enrollment or that the most recent PCR test was done more than a month ago (criteria for accepting recent HIV-infection test results). These children will be assumed to be HIV-uninfected and placed in one of the two HEU groups (HEU/ABX+ or HEU/ABX-), depending on his/her antibiotic history. However, once an updated HIV PCR test is made available, and the result is HIV-infected, this child may be switched into one of the two HIV+ groups. HIV-infection status will be accepted if the PCR test was done within the last month. Once a study group has reached the desired sample size (n=25 for HIV+/ABX- & HEU/CTX- study groups, and n=50 for HIV+/ABX+ & HEU/ABX+ study groups), this group will be "closed" and any participants screened and determined to be allocated to the "closed" group will be excluded. Enrollment into the other "open" groups will continue simultaneously.

Figure 2. Comparison groups & associated sample size



Any child screened and identified to have severe acute malnutrition (defined as WHZ ≤-3 or MUAC<11.5cm) will not be eligible for study participant but will be referred to nutritionists based in the hospital or locally in the county for appropriate management.

CONSENT

After the child has been found to be eligible to be enrolled in the study, the accompanying primary caregiver will undergo informed consent in the language of the respondent's choosing (English, Kiswahili, or Luo). During consent, the purpose of the study and study procedures will be explained to the caregiver, including collection of two rectal swabs and DBS (see attached Generic Informed Consent Form). The parent or guardian (primary caregiver) must also consent to providing information on their HIV-status and sociodemographic information of the family, and sign written informed consent (or provide a witnessed thumbprint if illiterate) prior to enrollment.

ENROLLMENT

Following consent, each participant will be assigned a unique study Patient Identification Number (PID). A card detailing the PID and the contact information for the medical personnel responsible for enrollment will be given to the primary caregiver of enrolled children. The study staff will interview the primary caregiver of the child to collect information on socio-demographic characteristics, breastfeeding and vaccination history, treatment history (including anti-retroviral therapy), and HIV-related information.

Two rectal swabs and a dried blood spot will be collected from all enrolled children as described in the sample collection section. HIV-exposed children of unknown infection status or HIV-exposed children with unknown infection status who have not been tested for HIV within the last month will be tested for HIV DNA. The study will facilitate the HIV-testing and the

return of test results or abstract results from medical records. Any child newly diagnosed with HIV will be referred to the HIV Care Clinic at the respective study site for follow-up care and treatment.

SPECIMENS

Rectal Swabs

Collection

With the participant in a comfortable fetal position lying on her/his left side (left lateral position), the study staff will gently insert the swab into the anal canal until it reaches the “stopper”, or until resistance is felt. Staff will rotate the swab 3 times, then slowly remove it, and place in its storage tube. The storage tube will be labelled with the PID, date/time of collection, and study location.

Storage

Storage tubes will be placed in temporary -80°C storage and shipped on dry-ice to the KEMRI/UW storage facility in Nairobi where they will be maintained at - 80°C. The temperature of freezers at both locations are continuously monitored using an automated electronic thermometer and temperatures min and maximums recorded daily. An alarm system is also in place such that if the temperature goes below -70°C, a phone call is made to the laboratory manager.

Frozen stool in PID-labelled storage tubes will be shipped to the University of Washington for processing. Prior to shipment, all necessary exportation approvals will be sought. To the best of our knowledge, whole genome sequencing technology is not available in Kenya therefore is being conducted at the University of Washington.

Processing

Upon receipt at UW, bacterial DNA will be extracted from rectal swab samples after enzymatic lysis and bead beating using the MoBio PowerSoil DNA kit (Carlsbad, CA) according to manufacturer’s instructions. Targeted high throughput 16S rDNA V4 sequencing will be used to molecularly characterize the bacterial communities characterizing the stool. OTUs present only in very low abundances will be removed. After quality filtering using QIIME, sequences will be phylogenetically classified by comparing to a reference database such as SILVA. Laboratory personnel will not have access to information about the child other than what appears on the storage tube. Rectal swabs will be destroyed after the study is complete. Laboratory processing will occur at the laboratory of William DePaolo at the University of Washington.

Dried Blood Spots

Collection

Using a heel/finger prick, <1mL will be collected from all enrolled children for a dried blood spot, which will be used for HIV testing if the child's HIV status is unknown and viral load testing if the child is HIV-infected. Five separate dried blood spots will be placed on a single card.

Storage

The DBS will be stored in a dry cool locked box at the KEMRI/UW storage facility until ready for testing. Unused DBS will also be stored for potential future analyses, such as for assessment of antibiotic blood levels to validate self-reported antibiotic use/ non-use.

Processing

HIV (Roche HIV-1 DNA test®, Roche Molecular Systems, Branchburg, NJ, USA) and viral load (Abbott m2000 systems or Roche Cobas Ampliprep/Cobas TaqMan) PCR testing will be done at the KEMRI/ Center for Disease Control office or the University of Nairobi Institute of Tropical and Infectious Diseases (UNITID) in accordance with the current Kenyan Ministry of Health guidelines and test kit manufacturer instructions.

DATA

Data analysis

Sequence data will be normalized using a standard number of sequence counts per sample using total sum scaling. Relative abundance of specific bacteria phylum (Proteobacteria, Bacteroidetes, Firmicutes) and family (*Enterobacteriacea*, *Prevotellaceae*, *Bacteroidaceae*, *Lactobacillaceae*, *Clostridiaceae*, *Streptoccacea*) will be compared between four groups of children (HIV+/ABX+, HIV+/ABX-, HIV-/ABX+, HIV-/ABX-) using non-parametric Mann Whitney tests. Mean Shannon and Simpson diversity indices will be compared between groups of children using unpaired t-test and linear regression adjusting for site. Principle components analysis will be used to compare similarity of microbiota within the groups. In secondary analyses, we will evaluate the role of ARV use, HIV stage (including HIV-viral load), and age of weaning and microbial diversity.

Data storage

Clinical and sociodemographic data about the child will be directly entered into REDCap electronic database, a secure, password protected, web-based application designed to support data capture for research studies hosted by the University of Washington in Seattle, Washington.

Data management

Stool and DNA samples will be labeled with a numeric patient identifier and the link between the patient identifier (PID) and the clinical characteristics of the child (such as HIV-infection and ABX-status) will be maintained in a locked file cabinet at the study site, accessible only by the local study staff. The laboratory team will not have access to the clinical characteristics of the child. After final OTU assignments are given to each sample, the corresponding PID will be linked with the clinical data by the study epidemiologist and the sample unblinded. OTU identification of particular bacteria of interest will be confirmed using quantitative PCR.

Objective 2: Impact of HIV infection and antibiotic use on gut microbiome

STUDY FROM WHICH ISOLATES WERE COLLECTED

Description

EPEC isolates were collected as part of a previous study entitled “Epidemiology and Cofactors for Non-Typhoidal Salmonella (NTS) in Kenya**”,** SSC#2056 and UW IRB: #39952**.** In this study, children under 5 years with diarrhea were recruited and bacterial culture, including the identified of EPEC was performed on stool/ rectal swabs. HIV-infection status and recent

history of antibiotic use, including cotrimoxazole status, was ascertained as part of this study as well. All data has been de-identified.

Sample Size

70 EPEC isolates were obtained from this study. With the 70 EPEC isolates we will have 80% power to detect a 2-fold higher prevalence of the *bfpA*+ in children with severe disease when the *bfpA*+ prevalence in children with less severe disease is 50% and a 3-fold higher prevalence of the *bfpA*+ gene when the comparator prevalence is 20.

Consent

Consent was obtained in this study to send pathogens isolated from stool/swabs samples to the University of Washington laboratory in the US to perform tests to determine why some children get more severe illness from enteric bacterial pathogens than others, which was the main objective of this study.

Storage

De-identified EPEC isolates are currently stored in the KEMRI/UW storage facility in Nairobi at -80°C.

CURRENT ANALYSIS OF DE-IDENTIFIED SAMPLES

Isolate Shipment

Frozen isolates in PID-labeled storage tubes will be shipped to the University of Washington for processing. We will apply for SERU approval prior to exporting any isolates from Kenya.

Processing

Upon receipt at UW, laboratory personnel will confirm that the clinical isolates are EPEC using the Matrix Assisted Laser Desorption/Ionization with Time of Flight (MALDI-TOF) and then perform qPCR with primers for the LEE-encoded gene *esc*V and EAE encoded gene *bfp*A (Appendix I). All processing will occur at the laboratory of William DePaolo at the University of Washington.

Data analysis

We will evaluate the prevalence of *bfp*A- gene between the groups defined by HIV- and cotrimoxazole status using log-binomial regression and associated chi-square tests.

Data storage

Clinical and sociodemographic data about the child will be directly entered into REDCap electronic database, a secure, password protected, web-based application designed to support data capture for research studies hosted by the University of Washington in Seattle, Washington.

Data management

EPEC sample data will be labeled with a numeric patient identifier and the link between the patient identifier (PID) and identifying information has been destroyed. The laboratory team will not have access to the clinical characteristics of the child.

TIMELINE

We anticipate that this study will take approximately 2 years to complete (Table 1). In the first 5 months, we will apply for and obtain ethical approval from relevant institutions, develop and refine study tools, finalize SOPs, and hire appropriate staff. Following receipt of IRB approvals,

we will recruit and enroll participants over a 5-month period to enroll 100 participants. Approximately 1 month will be allocated to shipping, receiving, and archiving samples from the UW/KEMRI storage facility in Nairobi to the University of Washington and 1 month for DNA extraction. Sequencing of samples will take place over a 6-month period during which all clinical data will be cleaned and verified. Finally, laboratory and clinical data will be merged and analyzed and manuscripts prepared in the last 6 months of the study period.

Table 1. Approximate study timeline

	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6	Month 7	Month 8	Month 9	Month 10	Month 11	Month 12	Month 13	Month 14	Month 15	Month 16	Month 17	Month 18	Month 19	Month 20	Month 21	Month 22	Month 23	Month 24
Ethical approvals																								
Develop SOPs & CRFs																								
Enroll participants																								
Ship samples																								
DNA extraction																								
Sequencing																								
Data cleaning																								
Data analysis																								
Manuscript preparation																								

STUDY LIMITATIONS

This is a small pilot study that is designed to inform larger studies, including clinical trials. Therefore, the sample size is small and because of that, may not able to detect subtle differences in microbiome or EPEC virulence genes between the groups but will be able to detect clinically relevant differences that are large in magnitude. Antibiotic use in Kenya is high among this age group, which means it may be difficult to have children in the non-antibiotic exposed group of children. However, we have allotted a significant amount of time for participant recruitment of objective I allowing for adequate time to recruit children not exposed to antibiotics.

DISSEMINATION PLAN

We will conduct regular CME's at participating site hospitals; one to introduce the study, one to update the hospital staff on study progress, and one to share results of the study. Manuscripts will be prepared with the findings and submitted to open-source peer-reviewed journals. We will also plan to present the research findings at the University of Nairobi STD/HIV/SRH Collaborative Group Meeting.

ETHICAL CONSIDERATIONS

Ethical approval

The study protocol and associated documents (consent forms, stool collection CRFs, etc.) will be submitted to the IRB at the University of Washington and Kenya Medical Research Institute Scientific and Ethical Review Unit (KEMRI-SERU) for ethical approval. No human subject participation will take place until approval has been obtained from both authorities. The study will be conducted according to Good Clinical Practice (GCP), the Declaration of Helsinki, IRB and local rules and regulations of Kenya.

Risks to subjects

This study carries a minimum risk to participants. The collection of rectal swabs from participants may be uncomfortable for children. The blood collection as part of dried blood

spot collection and routine HIV testing may involve bruising and possible infection at the site of needle entry however this risk will be mitigated by using finger/heel pricks which minimize invasiveness of blood collection. There is some risk that confidential information may be disclosed; however, all clinical and laboratory providers and other staff involved in the evaluation will be provided special training on confidentiality procedures and the importance of keeping personal information private. All study providers (nurses and laboratory technicians) will be trained in confidentiality and privacy of information, consenting of patients, administration of the questionnaire, specifics of rectal swab specimen collection, and appropriate HIV testing prior to the start of the study**.** All study staff will be trained and receive certification in Good Clinical Practice standards.

Adequacy of protection against risks

All study staff will be trained on proper infection prevention procedures and techniques for blood spot collection and rectal swabs to minimize any chances of bruising or infection. Participant files will be accessible only to site staff and investigators and will be stored in a locked file cabinet in a locked office. Any adverse events directly attributable to participation in the study will be managed by the appropriate clinic site, and if necessary, hospitalization. The costs of this care will be borne by the study. The clinical sites involved in this study are located within referral Hospital settings, all of which can provide emergency care if necessary.

Confidentiality

The consent form and the enrollment log will be the only documents containing personally identifying information and will be maintained under lock and key at the study sites. Case report form will contain no individually identifiable information. Databases will not include patient identifiers and will be encrypted and password protected. Study staff will keep signed consent forms and completed study questionnaires in a locked study cabinet. Each participant will be assigned a unique identification number at the time of enrollment. Case report forms, and laboratory samples will be identified with this number. The final study data will only have the participant's unique identification number with no link to their name or other personally identifying information. Once the study has been completed and data analyzed and disseminated, the IRB and bioethics committees will be informed of closure, and links and consent forms will be destroyed (within 3 years of study completion). All study staff involved will receive training and certification on standards of Good Clinical Practice before being allowed to work in this study.

Potential benefits of the proposed research to the subjects and others

Direct benefits: Participants will receive a reimbursement of 400 Kenyan Shillings ($4) for their time and transportation home after study participation. Additionally, if an HEU child has an unknown HIV-infection status, the study will facilitate HIV testing and if found to be HIV-infected, will be referred to the respective HIV care clinic for counselling and follow-up.

Indirect benefits: It is not known if the provision of antimicrobials and infection with HIV decrease the diversity of gut bacteria, and predispose to severe EPEC infection. This study will shed new light on diarrheal pathogen of global and regional importance, and may lead to novel interventions that protect HIV infected children and those who have received antibiotics from EPEC infections.

Importance of the knowledge to be gained

This study has the potential to benefit thousands of children in low-resource settings who become infected with EPEC in their first few years of life and are also either HIV infected or are receive antibiotic management. The results of this study will inform the design of clinical trials which may lead to the development of novel interventions that protect HIV infected children and those who have received antibiotics from EPEC infections. Additionally, if we find differences in the microbiome composition between the four study groups, this would suggest

the microbiome could be a potential target for therapeutics to improve enteric infection outcomes among HIV-infected and antibiotic exposed children.

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Appendix I

Upon receipt at UW, EPEC isolates will be re-streaked onto blood agar plates and grown for 24-48 hours. Five colonies per plate will be selected based upon the following morphology: grey white moist, glistening, opaque, circular, convex colonies with entire edge. The selected colonies are smeared onto the metal MALDI-TOF plate and run through the instrument. Strains in which no less than 4 out of the 5 colonies are identified as EPEC, will be grown up in liquid culture of blood broth. After 24 hours, the cultures are centrifuged and the bacterial pellets are mixed with garnet beads and then sheared using a bead beater. The resulting supernatants will contain bacterial genomic material that is then PCR-amplified for escV and bfpA genes (Table 2).

Table 2. PCR conditions and primers used for EPEC identification

Genes	Primers	PCR conditions	References
escV	5'-AGTGCTCGTTTTTCCCTTGA-3' 5'-AGCGAAGAACTTTTGCCTCA-3'	Amplification: 1 cycle at 95°C, 10 min & 40 cycles, 95°C, 15s & 60°C for 1 min. Melt curve analysis 95°C for 15s, followed by cooling to 60°C and slowly heating to 95°C	Leverton, L. Q (2005). Infection and Immunity, 73(2), 1034–1043.[29]
bfpA	5'-TGATTGAATCTGCAATGGTG-3' 5'-AGCATTCTGCGACTTATTGG-3'		