INVESTIGATING INFECTION-MITIGATING MECHANISMS OF BREASTFEEDING AS A PATHWAY TO HEALTHY INFANT GROWTH AND DEVELOPMENT IN TANZANIA

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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May 2015

INVESTIGATING INFECTION-MITIGATING MECHANISMS OF BREASTFEEDING AS A PATHWAY TO HEALTHY INFANT GROWTH AND DEVELOPMENT IN TANZANIA

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Cornell University 2015

The UNICEF conceptual framework for child undernutrition indicates that malnutrition occurs as a result of inadequate dietary intake and disease. Exclusive breastfeeding for six months is the "gold standard" in infant feeding, yet the majority of women in sub-Saharan Africa do not practice EBF for the recommended duration. Additionally, diarrhea remains one of the leading causes of child death in developing countries, with *Cryptosporidium* as one of the most common diarrhea-causing parasites. Despite the health consequences of *Cryptosporidium* infection in childhood, little is known about the natural history of and risk factors associated with infection. The aim of this study was to determine the prevalence of *Cryptosporidium* in mothers and infants up to six months post-partum and the factors associated with infection, namely duration of exclusive breastfeeding and immune composition of breast milk.

In a prospective cohort of 125 mothers and infants we found that maternal post-partum prevalence of *Cryptosporidium* was high, yet prevalence of infant infection remained low until six months of age. Factors associated with an increased risk of infant *Cryptosporidium* infection included maternal *Cryptosporidium* infection and maternal hand washing prior to infant feeding. We also saw increases in infant *Cryptosporidium* infection that corresponded to changes in infant feeding patterns.

Using the same cohort of mothers, we collected breast milk and blood samples

to determine how concentrations of immunoglobulins and cytokines evolved over the six months post-partum period. We found that there were no differences in breast milk immune composition based on maternal HIV-status or maternal nutritional status. However, we did find that concentrations of breast milk immunoglobulins increased as the duration of exclusive breastfeeding increased and with increased breastfeeding frequency. Breast milk cytokine concentrations were associated with indicators of maternal illness, such as mastitis and fever.

Finally, we explored the relationship between infant feeding patterns and breast milk immune concentrations to determine if they were associated with infant *Cryptosporidium* infection. We found that an increased duration of exclusive breastfeeding and higher doses of breast milk immunoglobulins and cytokines were associated with a decreased risk of infant *Cryptosporidium* infection, while the feeding of certain complementary foods was associated with an increased risk of infection.

We conclude that *Cryptosporidium* is an important gastrointestinal parasite in this region of Tanzania. The research underscored the importance of appropriate infant feeding in the first six months as optimal infant feeding behaviors were associated with both an increase in breast milk immune molecules as well as a decreased risk of infant *Cryptosporidium* infection.

BIOGRAPHICAL SKETCH

Sarah Pedersen was born and raised on a farm in rural, upstate New York. She is the daughter of Richard and Laura Pedersen, owners of Pedersen Farms and both graduates of Cornell University. She received her Sc.B. from Brown University with a concentration in psychology in 2004. After graduation, Sarah worked as an admissions and financial aid counselor at Manhattan College in New York City and as a clinical research assistant in a pulmonary physiology laboratory at Beth Israel Deaconess Medical Center in Boston. Sarah received her M.S. in Food Policy and Applied Nutrition from Tufts University in 2009 and completed an internship with USAID working on a socio-economic impact survey of coffee farmers in Rwanda. Before returning to upstate New York, Sarah was an intern in the Micronutrients Unit at the World Health Organization in Geneva, Switzerland. In 2010, Sarah was selected as a National Science Foundation Fellow in Food Systems and Poverty Reduction in East Africa at Cornell University to study international nutrition.

To those who supported me unconditionally on my journey:

Richard and Laura Pedersen, Amanda Wilkinson, and Tito Pedersen

ACKNOWLEDGMENTS

I have many people to thank for guiding me on this path. First, I'd like to thank Dr. Robert Banzett for being more than just a boss, but a true mentor, teaching me the ins and outs of academic research and scientific publishing. Dr. Beatrice Rogers and Dr. Irv Rosenberg of Tufts University believed in me and supported my goal of studying the interaction between nutrition and infection. My PhD committee, Dr. Joann McDermid, Dr. Sera Young, Dr. Daryl Van Nydam, and Dr. Alice Pell, at Cornell have provided invaluable insights and guidance. The National Science Foundation Integrative Graduate Education and Research Traineeship brought me to Cornell and the principal investigators, Dr. Chris Barrett, Dr. Beth Medvecky, Dr. Alice Pell, Dr. Rebecca Nelson, Dr. Per Pinstrup-Andersen, and Dr. Alison Power, provided me with the skills to work within an interdisciplinary research team in an international context. The Cornell Statistical Consulting Unit, particularly Francoise Vermeylen, helped to guide the statistical analyses for this dissertation. Dr. Daryl Van Nydam and Janice Liotta from Cornell and Dr. David Warhurst and Dr. Aura Andreasen from the London School of Hygiene and Tropical Medicine spent countless hours training me in the methods of *Cryptosporidium* detection. Victoria Simon provided training for the Magpix Luminex technology.

Without the support of my collaborators and friends in Tanzania, this research would not have been possible. I thank my collaborators at NIMR-Mwanza and the London School of Hygiene and Tropical Medicine, Dr. Safari Kinung'hi, Mark Urassa, Dr. Denna Michael, Jim Todd, John Changalucha, and Dr. Aura Andreasen,

and especially Kolo who passed away during my time at NIMR. I'd also like to thank the nurses and clinical staff at Kisesa Health Center for their help in my day-today activities at the clinic. Gloria Majaliwa, our study nurse, was an invaluable resource, administering every questionnaire and helping me to understand the local context. Our field worker, Zabron Guke, worked endlessly to locate participants and is the reason why our study retention was so high. I'd also like to thank my friends, Tito Lumboyo, Agatha, and Ramson for making my stay in Tanzania just a little bit easier. Most of all, I'd like to thank the women and infants who participated in this study; without them, this research would have truly been impossible.

Lastly, I'd like to thank my family and close friends for accompanying me on this difficult journey. My research partner and best friend, Amanda Wilkinson, helped to design and manage the study and also kept me sane during our extended stay in Tanzania and during the writing process after returning to Cornell. My brother, Benjamin Pedersen, proofread all my manuscripts and this dissertation and through his edits made me a better writer. My parents, Richard and Laura Pedersen, supported me unconditionally throughout my time in Tanzania and at Cornell, both emotionally and financially. I feel blessed that I was able to introduce them to the beautiful and fascinating country of Tanzania. Lastly, I wouldn't be here without my dog Tito. He accompanied me to Tanzania and was my only companion for the six months I spent alone in my gated compound. Upon my return to Cornell, he sat with me everyday as I wrote this dissertation.

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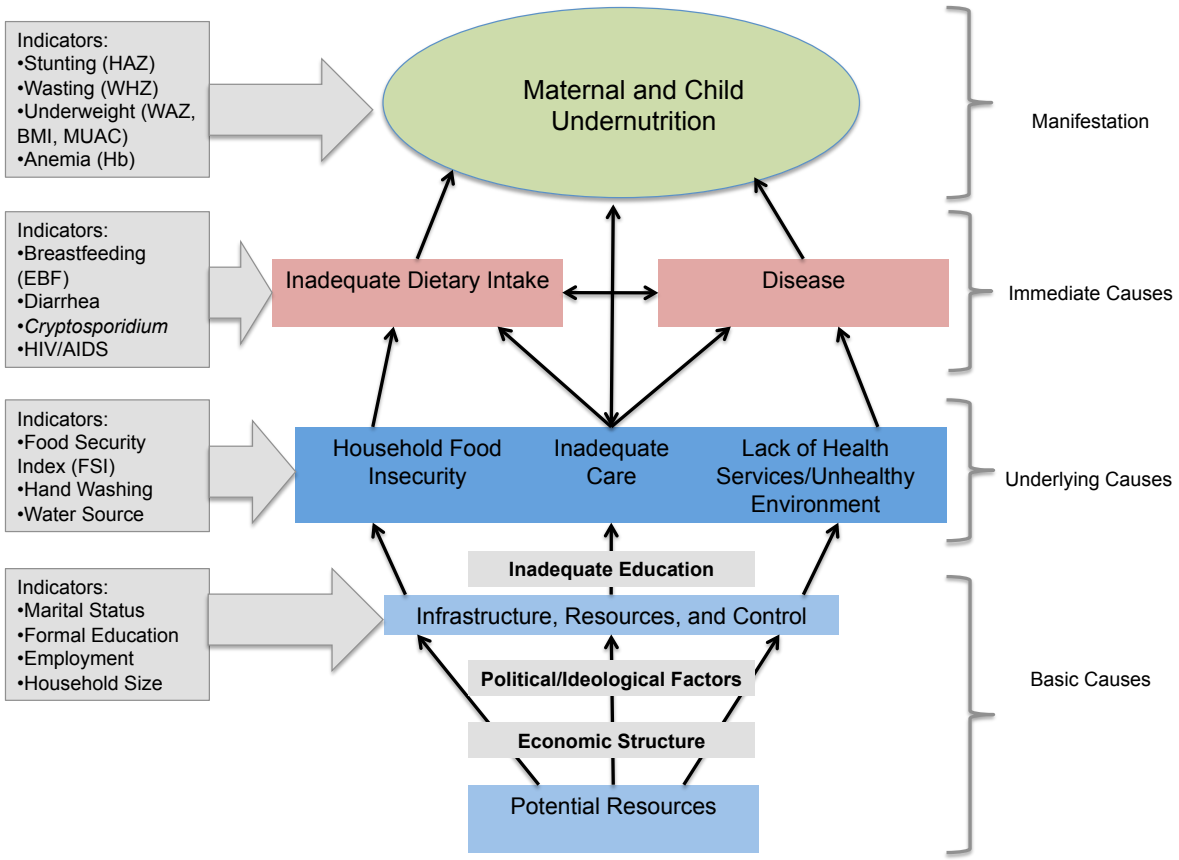
CHAPTER 1

INTRODUCTION

1.1 Introduction

Despite decades of research and interventions, maternal and child undernutrition remains a staggering health challenge in developing countries. Globally, approximately 3.5 million deaths each year are attributable to maternal and child undernutrition [1]. Poor nutrition in the period from birth to 24 months contributes to 50% of deaths in children under five, mainly due to increased mortality from infectious disease [2]. The UNICEF conceptual framework for child malnutrition depicts a synergistic relationship between infection and undernutrition on child malnutrition, death, and disability (**Figure 1.1**). Forty-five percent of all child deaths related to diarrhea occur in sub-Saharan Africa [3]. This region also has a high childhood undernutrition burden, with stunting prevalence >40% among children under five. This combination of chronic undernutrition and frequent diarrheal infection leads to poor growth, more frequent illness, and ultimately, increased risk of mortality.

Figure 1.1. UNICEF conceptual framework for child undernutrition, adapted from [4]



One of the immediate causes of child undernutrition is inadequate dietary intake. Ideally, for the first six months of life, an infant's food system is dependent on breast milk provided by his or her mother. Therefore, it is vital to understand the best method to optimize this critical period of feeding, growth, and immune development. Breastfeeding has a profound role in shaping infant health and is influential in immune system development, and nutrition of the developing infant as well as to the development of the gut and associated microbiota [5, 6]. When practiced optimally, breastfeeding is one of the most effective available methods to promote child health and survival [6]; 50%

of child deaths due to infection may be prevented through better breastfeeding practices [7]. As a result of these benefits, the WHO recommends that mothers exclusively breastfeed for the first six months of an infant's life. However, in practice very few women in African countries follow this recommendation, despite the numerous benefits associated with exclusive breastfeeding. Barriers to exclusive breastfeeding in developing countries include: inconsistent public-health messages, lack of familial support, lack of breastfeeding education and support systems, cultural beliefs, and poor maternal and/or infant health [8-10]. Suboptimal breastfeeding, especially non-excusive breastfeeding in the first 6 months of life, results in 1.4 million deaths and 10% of the disease burden in children under 5 [1].

In addition to inadequate feeding, disease is the other immediate cause of child undernutrition. Diarrhea is the second leading cause of infant mortality, and *Cryptosporidium* (**Figure 1.2**) is a major cause of diarrhea globally, particularly in young children in the developing world [11]. However, few studies have addressed the risk factors for *Cryptosporidium* infection in young infants in resource-poor areas, likely due to a cycle driven by contextual and disease-specific factors (**Figure 1.3**). For these reasons, the WHO has included *Cryptosporidium* in its Neglected Diseases Initiative [12]. Currently there is no vaccine available for *Cryptosporidium* and treatment options remain suboptimal. Nitazoxanide is an FDA-approved drug for the treatment of cryptosporidiosis, however randomized trials have indicated that this drug is not efficacious in the treatment of cryptosporidiosis in HIV-infected individuals or children under-12 months of age [13].

Cryptosporidium is a genus of protozoan parasites, with over 15 different species capable of causing infection in humans [13]. Worldwide, *Cryptosporidium hominis* and *Cryptosporidium parvum* are responsible for the majority of human infections. The effects of *Cryptosporidium* infection can range from asymptomatic to severe, chronic diarrhea, and symptoms may vary according to the infective species. Even in the absence of symptoms, *Cryptosporidium* infection can have damaging effects on the growth and development of children. Studies in Brazil and Peru contribute evidence of this dangerous relationship. Malnutrition and stunting were common in children even after asymptomatic *Cryptosporidium* infection [14]. Moreover, detrimental long-term cognitive and functional deficits were observed in a cohort of Brazilian children 4-7 years after *Cryptosporidium* infection when compared with children who did not experience *Cryptosporidium* infection [15].

Figure 1.2. Modified Ziehl-Neelsen stained fecal sample positive for *Cryptosporidium*

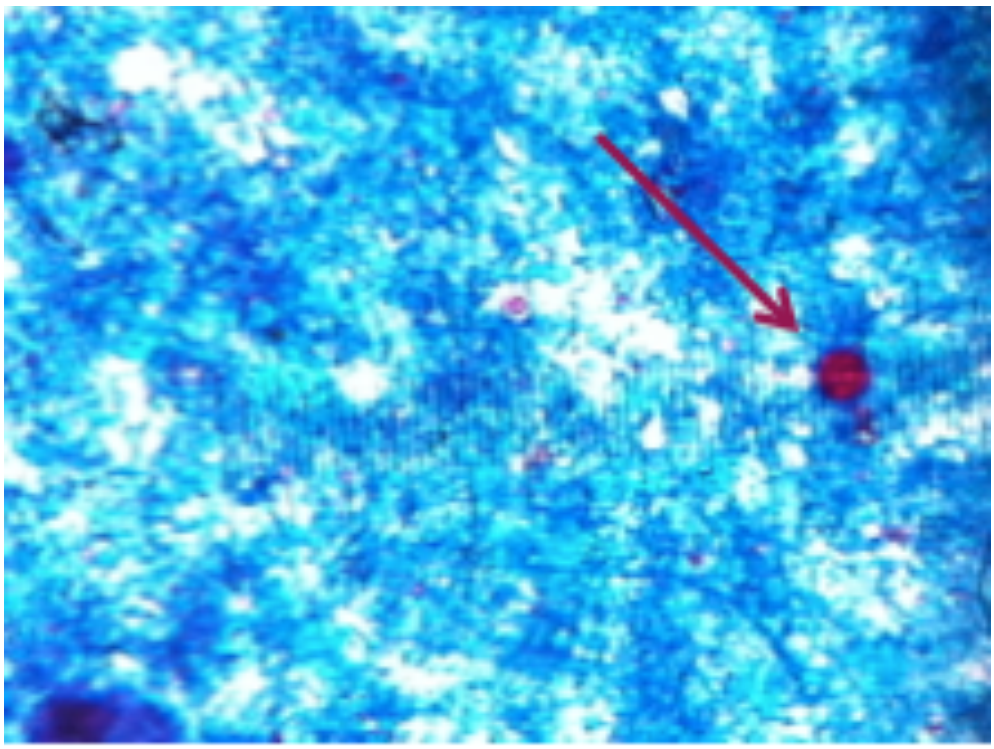
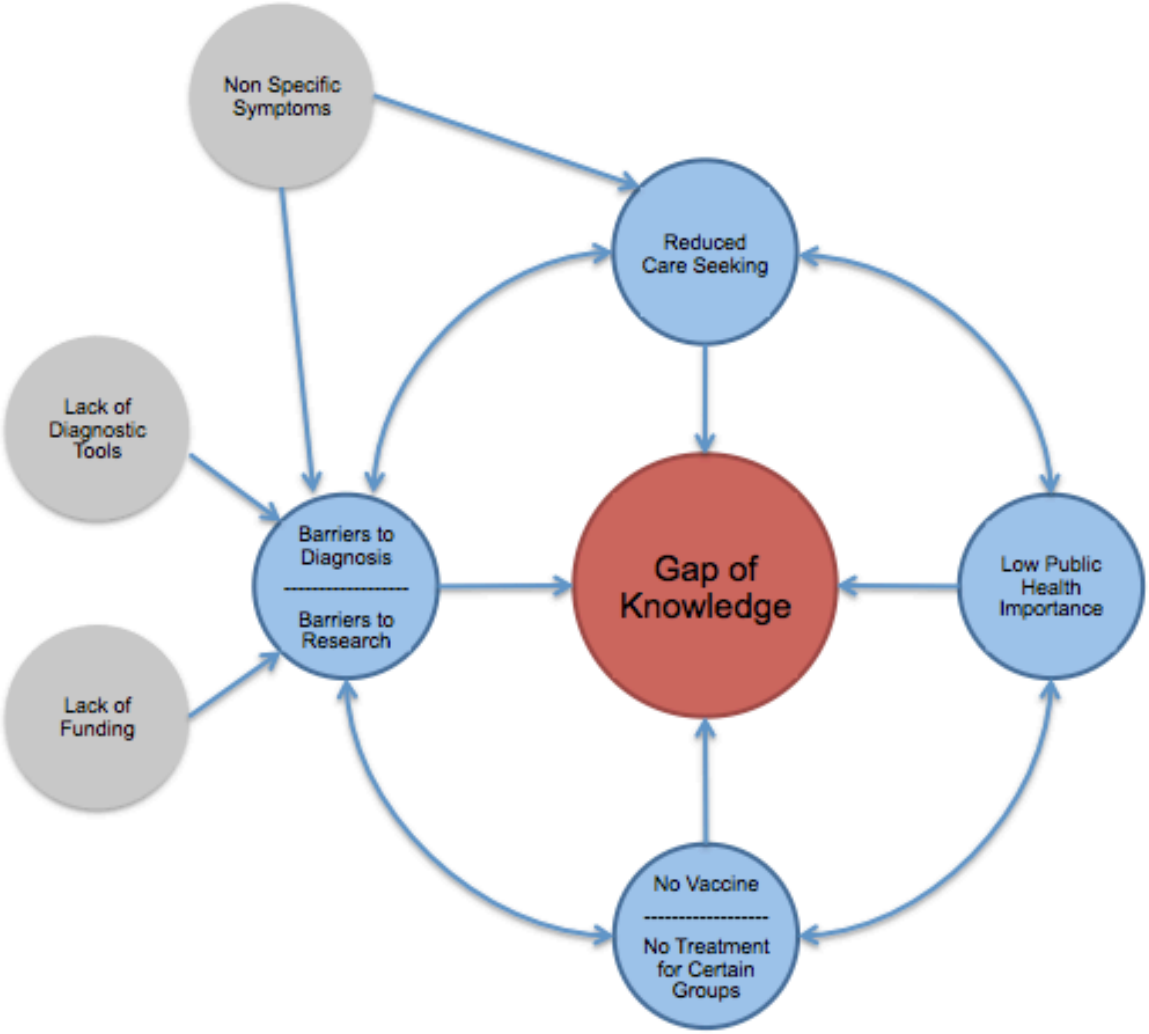


Photo credit: Sarah H. Pedersen © 2012

Figure 1.3. Cycle of the gap in knowledge of *Cryptosporidium*



1.2 The Context

Tanzania, a country situated in East Africa, is heavily impacted by childhood malnutrition. In 2010, it was estimated that over 40% of children under five were stunted, an indicator of chronic undernutrition [16]. As indicated in the UNICEF conceptual framework for malnutrition, the causes of undernutrition are related to both inadequate feeding and infection. Tanzania ranks in the top 15 countries globally in terms of annual child deaths attributed to diarrhea [3]. Additionally, over 6% of the adult population is living with HIV/AIDS, a number that is significantly greater than both global and

regional prevalence rates [17]. Prevention of mother to child transmission (PMTCT) efforts have reduced the proportion of infants born to HIV-positive mothers who themselves become infected with HIV. PMTCT interventions in Tanzania include HIV testing during antenatal care, provision of anti-retroviral therapy (ART), and infant feeding counseling. These interventions have reduced the risk of HIV transmission from 15-45% in the absence of treatment to <5% under optimal PMTCT treatment [18]. At the time of my study, Tanzania was implementing Option A of the WHO PMTCT recommendations.

Sub-optimal breastfeeding is common among Tanzanian women - only 59% of women initiate breastfeeding within one hour of birth, while only 41% of women exclusively breastfeed for six months [19]. As a result of less than adequate breastfeeding practices and a high burden of diarrhea in developing countries, more research is needed to provide further evidence to better inform intervention programmers, and policymakers alike, on strategies to decrease the burden of diarrhea and infectious disease in young infants. My research takes a holistic view of the highly interconnected system of mother and child, considering maternal health, nutrition, environment, and behaviors that have the possibility to impact morbidity and mortality in the infant.

1.3 Background

Breast milk provides the infant with a variety of soluble factors including immunoglobulins, cytokines, lactoferrin, and lysozyme, all of which confer immune benefits on the infant and influence the composition of the neonatal microbiota, playing a role in the protection of the neonatal intestine [20]. As a result of these benefits, the World Health Organization (WHO) includes two breastfeeding practices in its list of core

indicators of healthy infant and young child feeding (IYCF): early initiation of breastfeeding (within one hour of giving birth) and exclusive breastfeeding for six months [21].

Exclusive breastfeeding, as defined by WHO, means that the infant receives only breast milk (including expressed breast milk or breast milk from a wet nurse) and also allows the infant to receive oral rehydration solution (ORS), drops, syrups (vitamins, minerals, medicines), but nothing else [22]. Cross-sectional studies indicate that exclusive breastfeeding in the first six months is protective against infections, including diarrhea and acute respiratory infection, in developed and developing countries [23-29]. However, little is understood about how neonatal feeding methods correspond with the natural progression of infection due to common environmental exposures, or of pathogen-specific diarrheal incidence rates.

In developed countries, it is recommended that HIV-positive mothers use replacement feeding, however most HIV-positive mothers in developing countries do not have reliable and safe access to replacement feeding for the entire six-month duration and therefore an alternate prevention of mother-to-child transmission (PMTCT) strategy is recommended. To reduce the risk of transmitting HIV to their infants, mothers should receive antiretroviral therapy (ART) and exclusively breastfeed for six months, introducing complementary foods thereafter and continue breastfeeding for the first 12 months of life [30].

Despite the WHO recommendation that *all* women (including HIV-positive mothers) exclusively breastfeed for six months, recent studies in several developing countries have found that very few women continue exclusive breastfeeding beyond 3

months [8, 23]. During this period of partial breastfeeding, water and food are introduced thus exposing the infant to various environmental pathogens while depriving the infant of nutrient-dense breast milk containing beneficial immunoglobulins, cytokines, and other non-specific growth factors [31]. Periods of mixed feeding also disrupt the intestinal mucosal barrier leading to increased microbial translocation [32]. The underlying hypothesis of my research is that the first six months of a child's life represent a critical period in immune development and that this development is influenced by the way in which a mother feeds her child.

Following *in utero* immune protection provided by the maternal cocoon, breast milk provides an immunological bridge to protect the infant from infectious disease and also stimulates the infant's developing immune system. Breast milk immune protective factors, such as cytokines and immunoglobulins, are especially important in the context of high-risk environments, such as those encountered by infants born to HIV-infected mothers and those in developing countries where environmental contamination with pathogens is ubiquitous and access to health care is limited. Numerous studies have related exclusive breastfeeding to decreased infection, particularly gastrointestinal illness, during infancy and beyond, yet the majority of women in Tanzania fail to exclusively breastfeed for six months.

Since breastfeeding is associated with reduced diarrheal illness in infancy, and *Cryptosporidium* is one of the most common diarrhea-causing parasites in children, I designed this study to examine the relationship between infant feeding practices and early infancy *Cryptosporidium* infection. Research regarding the prevalence of *Cryptosporidium* in infant populations of Tanzania is sparse. Most estimates of

prevalence are based on samples of children with diarrhea presenting at hospitals or clinics. This method for determining prevalence likely underestimates the true prevalence of *Cryptosporidium* in the pediatric population because it excludes children with asymptomatic *Cryptosporidium* and children with diarrhea who fail to present at hospitals or clinics. This is the first study to measure the prevalence and natural history of *Cryptosporidium* infection in infants under-six months in Tanzania. A recent publication by Moyo, et al. [33] emphasized the lack of studies regarding the prevalence of enteric pathogens in Tanzania. **Table 1.1** summarizes the currently available research regarding *Cryptosporidium* prevalence in Tanzania.

Table 1.1 Prevalence of *Cryptosporidium* in Tanzania-previous research

Location, Year (Ref)	Study Design	Population	Sample Size	Detection Method (Sample Analyzed)	Prevalence
Zanzibar, 2014 [34]	Case-Control	Children 2 to 59 months	330	PCR (diarrhea/non-diarrhea)	20.0%
Dar es Salaam, 2011 [33]	Cross-Sectional	Children 0 to 60 months	270	Immunocard Stat! Rapid Assay (diarrhea)	18.9%
Kilimanjaro, 2005 [35]	Cross-Sectional	HIV-positive adults	127	IFA & PCR (active case detection)	17.0%
Dar es Salaam, 1999 [36]	Case-Control	Children 3 to 108 months	134	Kinyoun & IFA (diarrhea/non-diarrhea)	9.0%
Dar es Salaam, 1999 [36]	Cross-Sectional	HIV-positive adults	86	Kinyoun & IFA (diarrhea)	7.0%

This is one of the first studies to comprehensively study the risk factors for *Cryptosporidium* infection in sub-Saharan Africa infants. Molloy and colleagues [37] stated that there is a paucity of data relating to the risk factors for *Cryptosporidium* infection within developing regions. **Figure 1.4** outlines the risk factors related to Cryptosporidium infection in children in the context of the Ecological Model. I hypothesized that sub-optimal infant feeding would be a risk factor associated with infant *Cryptosporidium* infection. I further hypothesized that the immune composition of breast milk would have implications for infant *Cryptosporidium* infection. Both the content

(quality) of breast milk and the duration of exclusive breastfeeding may impact microbial colonization of the gut and presence of diarrheal pathogens, such as *Cryptosporidium*. Mechanisms by which exclusive breastfeeding may protect the infant from pathogens are multi-modal. Providing crucial immune components present in breast milk may be just as important to infant gut health as delaying the introduction of potentially contaminated complementary foods and beverages. However, previous cross-sectional studies have shown mixed results in terms of the protective effect of breastfeeding on *Cryptosporidium* infection in infants.

Figure 1.4 Socio-Ecological Determinants of *Cryptosporidium* infection in infants

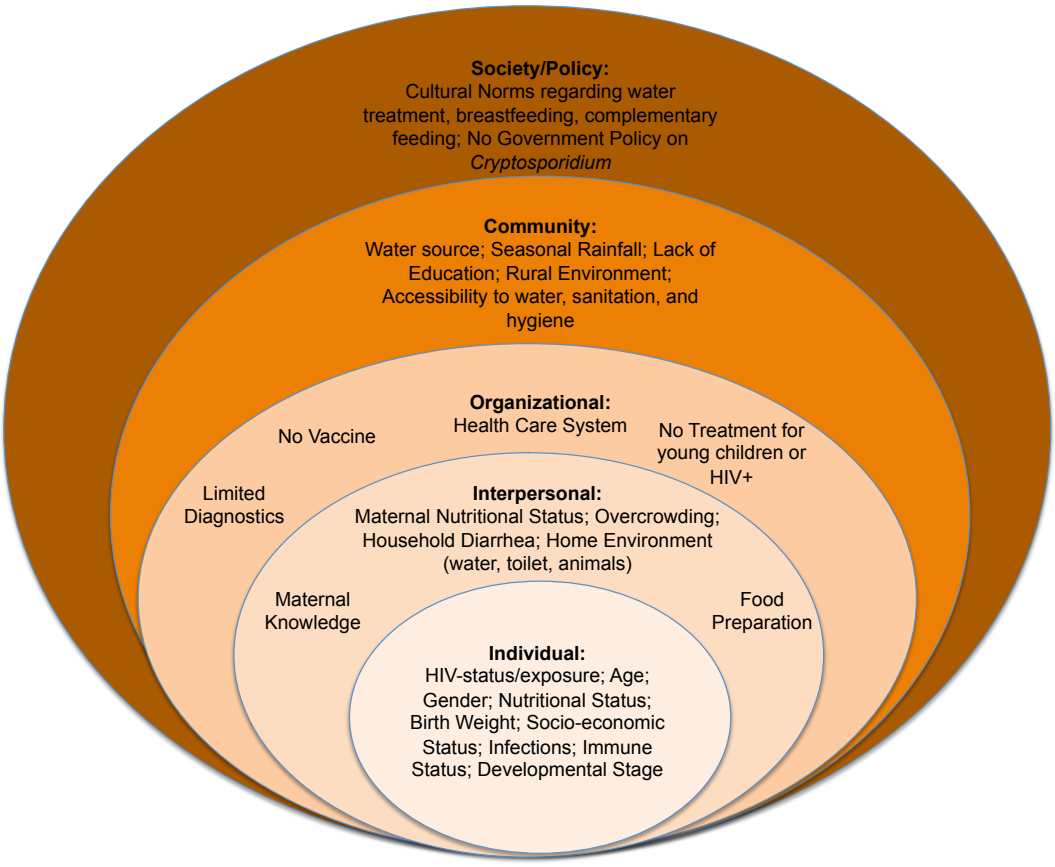


Table 1.2 summarizes the current research on the impact of breastfeeding on *Cryptosporidium* infection. A study in Zambia found that breastfeeding was associated with infant *Cryptosporidium* infection [38], several studies in developing regions have shown that breastfeeding is protective against *Cryptosporidium* [39-44], while other studies, including two from Tanzania, found no effect of breastfeeding on the presence of *Cryptosporidium* [33, 36, 45-49]. Inconsistent results regarding the relationship between breastfeeding and *Cryptosporidium* may be due to inconsistency of study design, breastfeeding definition, and method of *Cryptosporidium* detection. Many studies defined breastfeeding as simply, "ongoing breastfeeding", indicating that additional foods and water may be part of the infant's diet (36, 38, 41, 42, 44, 45, 47, 48). Additionally, most failed to consider asymptomatic *Cryptosporidium* infection and analyze only fecal samples from children presenting with diarrhea [33, 38, 41, 42-44, 47, 48]. To my knowledge, there have been no prospective, longitudinal studies examining exclusive breastfeeding duration (defined according to the WHO) and risk of *Cryptosporidium* infection in infants.

Table 1.2 Association between breastfeeding and *Cryptosporidium*-previous research

Location, Year (Ref)	Study Design	Breastfeeding	Sample Size (age)	Detection Method (Sample Analyzed)	Outcome
Bangladesh, 2013 [49]	Prospective Cohort	Duration of EBF*	226 (0 to 12 months)	PCR (active case detection)	No Association
Egypt, 2013 [43]	Cross-Sectional	Partial BF vs. No BF	322 (2 to 6 months)	Modified Ziehl-Neelsen (diarrhea)	Positive Association
Tanzania, 2011 [33]	Cross-Sectional	EBF* vs. non-EBF	280 (0 to 60 months)	Immunocard Stat! Rapid Assay (diarrhea)	No Association
Malaysia, 2011 [46]	Cross-Sectional	BF >4 months vs. BF ≤4 months	276 (2 to 15 years)	Modified Ziehl-Neelsen (active case detection)	No Association
Israel, 2008 [40]	Prospective Cohort	Predominant BF vs. No BF	238 (0 to 18 months)	ELISA (active case detection)	Positive Association
Egypt, 2005 [47]	Cross-Sectional	Currently BF vs. Not currently BF	1018 (0 to 60 months)	ELISA (diarrhea)	No Association
Iran, 2005 [48]	Cross-Sectional	BF vs. No BF	44 (6 to 18 months)	Modified Ziehl-Neelsen (diarrhea)	No Association
Uganda, 2003 [45]	Case-Control	Currently BF vs. Not currently BF	2446 (0 to 60 months)	Acid fast stain & IFA (diarrhea/non-diarrhea)	No Association
Haiti, 2002 [39]	Case-Control	EBF* vs. Non-EBF	49 (0 to 18 months)	Modified Ziehl-Neelsen (diarrhea/non-diarrhea)	Positive Association
Tanzania, 1999 [36]	Case-Control	Currently BF vs. Not currently BF	134 (3 to 108 months)	Kinyoun & IFA (diarrhea/non-diarrhea)	No Association
Zambia, 1998 [38]	Cross-Sectional	BF vs. No BF	222 (0 to 11 years)	Modified Ziehl-Neelsen (diarrhea)	Negative Association
Bangladesh, 1997 [41]	Case-Control	BF vs. No BF	272 (0 to 59 months)	Modified Kinyoun (diarrhea)	Positive Association
Mexico, 1997 [42]	Cross-Sectional	BF vs. No BF	200 (0 to 6 months)	Modified Kinyoun & IFA (diarrhea)	Positive Association
Guinea-Bissau, 1994 [44]	Case-Control	BF vs. No BF	1040 (0 to 4 years)	Modified Ziehl-Neelsen (diarrhea)	Positive Association

**EBF not defined in the publication*

Currently, there are no data on breast milk cytokines and immunoglobulins in rural Tanzanian women. My research is innovative because it is the first study to consider the composition of cytokines and immunoglobulins in breast milk of Tanzanian women. Multiple factors affect the ultimate composition of a mother's breast milk. A recent review by Agarwal, et al. [50] states that little is known about how demographic characteristics and human behaviors influence the concentrations of breast milk immune markers.

One class of immune molecules in breast milk are the immunoglobulins, also known as antibodies, which are proteins involved in the immune response to pathogens,

such as bacteria and viruses. Immunoglobulins are classified into five major isotypes; IgA, IgD, IgE, IgG, and IgM. Isotypes of classes IgA, IgG, and IgM have been measured in human breast milk and serum [50-54]. IgA is found in higher concentrations than all other immunoglobulin isotypes in human breast milk and is a critical component of the mucosal immune response [50]. IgG has four subclasses, IgG1, IgG2, IgG3, and IgG4, and acts by binding to pathogens [50]. IgM is the second most common immunoglobulin in breast milk and is generally the first antibody produced in a primary immune response [50, 55].

Some studies show that factors such as maternal nutritional status, maternal health status, and infection may impact the concentrations of immunoglobulins present in breast milk and serum. Due to the structure of the mucosal immune system, immune responses induced in the mother's gut produce immune cells that migrate to other secretory tissues, such as the mammary glands. Therefore, it is likely that mothers exposed to microbes and pathogens present in developing countries may secrete higher concentrations of specific immune factors in their milk. A study in Botswana considered differences in immunoglobulin composition of breast milk from HIV-positive/ART-naive women versus HIV-negative women. The authors found significantly higher concentrations of total IgM, IgG, and IgA in the breast milk of HIV-positive women [51]. Researchers in Colombia showed that malnourished mothers had decreased concentrations of IgG, IgA, and IgM in serum and decreased concentrations of IgG and IgA in breast milk compared with well-nourished women [53]. Additionally, human subjects in India with cryptosporidiosis had higher concentrations of IgA and IgG in serum than subjects without *Cryptosporidium* infection, regardless of HIV status [56].

Cytokines, another class of immune molecules found in breast milk, are signaling molecules used in cellular communication. Some cytokines, such as interferon gamma (IFN-γ) and interleukin-15 (IL-15), can be measured in human breast milk and serum samples [50, 52, 57-59]. Concentrations of cytokines in breast milk vary widely between women and may be related to differences in health status, infection, environmental microbial load, caloric intake and exercise, and time since giving birth [50, 52, 57, 58].

Interferon gamma (IFN-γ) is a cytokine critical to innate and adaptive immunity against viral and intracellular bacterial infection. Greater exposure to microbes and infectious agents is shown to increase concentrations of IFN-γ in both breast milk and serum. A study of Swedish and Estonian women found that Estonian women, who have higher environmental exposure to microbes, excreted higher concentrations of IFN-γ in their milk [58]. A study of HIV-transmission in Kenyan infants showed a protective effect of IFN-γ expression in infants on vertical HIV transmission and increased concentrations of IFN-γ in infant serum 2 to 5 months after HIV infection [60, 61]. Several studies also suggest that IFN-γ is associated with the human T-cell memory response to *Cryptosporidium* [62]. Human subjects with previous *Cryptosporidium* infection produced IFN-γ when challenged with *Cryptosporidium*, while *Cryptosporidium*-naïve subjects produced no IFN-γ [63]. Overall, IFN-γ in both breast milk and serum seems to be associated with greater exposure and enhanced response to infectious disease and microbial exposure.

Interleukin-15 (IL-15) plays an important role in the innate immune response to viruses and parasites by activating natural killer cells. Among HIV-positive women in Zambia, higher concentrations of IL-15 in breast milk were associated with a decreased

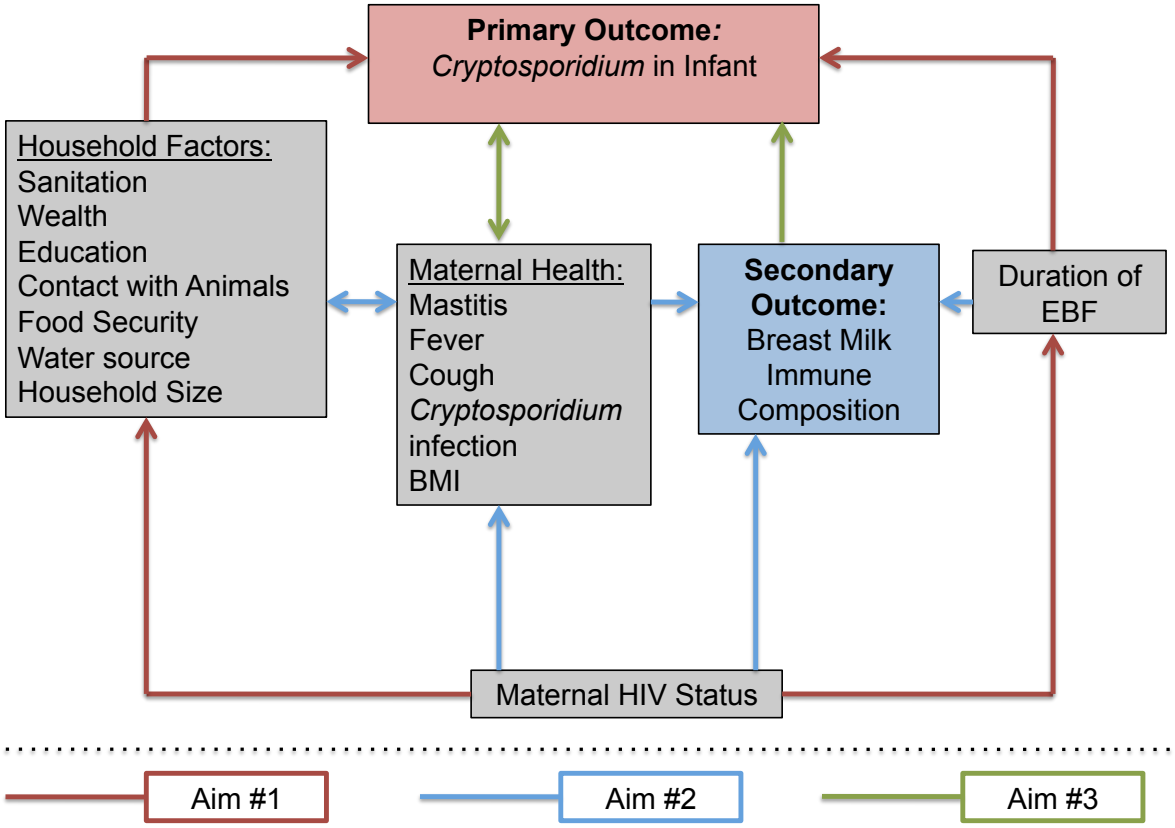
risk of maternal-to-child transmission of HIV [57]. IL-15 also appears to be an important component of the primary immune response to *Cryptosporidium* in the intestine. Dann, et al. [64] showed that within hours of *Cryptosporidium* exposure, intestinal tissues of healthy human volunteers increased expression of IL-15 and that this response is crucial to the clearance of *Cryptosporidium* infection in humans. IL-15 expression is also negatively correlated with *Cryptosporidium* parasite burden [62].

1.4 Objectives

The objective of this dissertation is to understand how exclusive breastfeeding, in the context of HIV/AIDS and a high burden of parasitological contamination, promotes healthy development of the infant gut. This dissertation also seeks to bridge the gap in knowledge between maternal breast milk immune components and infant immune response to pathogens. This research had three specific aims:

1) To determine the prevalence of *Cryptosporidium* in post-partum mothers and their infants in this setting.
2) To measure concentrations of immunoglobulins and cytokines in breast milk longitudinally, from birth to six months post-partum, and to relate these concentrations to the health and nutritional status of mothers.
3) To determine how the duration of exclusive breastfeeding and concentrations of cytokines and immunoglobulins in breast milk are related to the presence of *Cryptosporidium* in the infant's gut.

Figure 1.5 Conceptual Framework of the Research Aims



1.5 Dissertation Outline

I present the results of the research in chapters 2 through 4. The first study, which is the subject of chapter 2, reports the prevalence of *Cryptosporidium* in post-partum mothers and their infants from birth through six months. Using a cohort of 108 women attending the antenatal unit of a semi-rural public health facility in Northwestern Tanzania, I report on the longitudinal prevalence of *Cryptosporidium* and the risk factors that are associated with infection in this setting. In chapter 3, after describing the novel methods used to analyze breast milk immunoglobulins and cytokines, I report the concentrations of these immune molecules in all three biological compartments, namely,

maternal serum, breast milk, and infant serum. Chapter 3 also summarizes the associations between maternal/infant health/nutritional factors and concentrations of immunoglobulins and cytokines in breast milk. Lastly, chapter 4 incorporates the information from the previous 2 chapters to investigate how exclusive breastfeeding and concentrations of breast milk cytokines and immunoglobulins are related to *Cryptosporidium* in the infant. Finally, chapter 5 provides a discussion of the findings from all three studies. In the conclusion, I outline how my research findings could impact clinical care and public health messaging for pregnant and lactating women and their infants in the developing world.

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CHAPTER 2

CRYPTOSPORIDIUM PREVALENCE AND RISK FACTORS AMONG MOTHERS AND INFANTS 0 TO 6 MONTHS IN RURAL AND SEMI-RURAL NORTHWEST TANZANIA: A PROSPECTIVE COHORT STUDY

2.1 Abstract

Background: *Cryptosporidium* epidemiology is poorly understood, but infection is suspected of contributing to childhood malnutrition and diarrhea-related mortality worldwide.

Methods/Findings: A prospective cohort of 108 women and their infants in rural/semi-rural Tanzania were followed from delivery through six months. *Cryptosporidium* infection was determined in feces using modified Ziehl-Neelsen staining. Breastfeeding/infant feeding practices were queried and anthropometry measured. Maternal *Cryptosporidium* infection remained high throughout the study (monthly proportion=44 to 63%). Infection did not differ during lactation or by HIV-serostatus, except that a greater proportion of HIV-positive mothers were infected at Month 1. Infant *Cryptosporidium* infection remained undetected until Month 2 and uncommon through Month 3 however, by Month 6, 33% of infants were infected. There were no differences in infant infection by HIV-exposure. Overall, exclusive breastfeeding (EBF) was limited, but as the proportion of infants exclusively breastfed declined from 32% at Month 1 to 4% at Month 6, infant infection increased from 0% at Month 1 to 33% at Month 6. Maternal *Cryptosporidium* infection was associated with increased odds of infant infection (unadjusted OR=3.18, 95% CI 1.01 to 9.99), while

maternal hand washing prior to infant feeding was counterintuitively also associated with increased odds of infant infection (adjusted OR=5.02, 95% CI=1.11 to 22.78).

Conclusions: Both mothers and infants living in this setting suffer a high burden of *Cryptosporidium* infection, and the timing of first infant infection coincides with changes in breastfeeding practices. It is unknown whether this is due to breastfeeding practices reducing pathogen exposure through avoidance of contaminated food/water consumption; and/or breast milk providing important protective immune factors. Without a *Cryptosporidium* vaccine, and facing considerable diagnostic challenges and ineffective treatment in young infants, minimizing the overall environmental burden (e.g. contaminated water) and particularly, maternal *Cryptosporidium* infection burden as a means to protect against early infant infection needs prioritization.

2.2 Author Summary

Early infancy and childhood *Cryptosporidium* infection is associated with poor nutritional status, stunted growth, and cognitive deficits, yet minimal research is available regarding the burden and risk factors worldwide. Since there is no vaccine available, and because diagnostic challenges exist and treatment for children younger than one year is ineffective, prevention of early infancy infection through a better understanding of basic epidemiology is critical. This study was designed to investigate symptomatic and clinically silent infection amongst HIV-seropositive and HIV-seronegative mothers and their infants in a longitudinal cohort, and to indentify potential risk factors. Findings indicate that infants are living in a *Cryptosporidium* environment as demonstrated by the chronically high level of maternal infection throughout the 6-month post-partum period. Despite this, infant infection prevalence remains low until six months of age when it

dramatically rises. The increase in infant infection corresponds to a reduction in exclusive breastfeeding. As expected, maternal infection is associated with increased infant infection, but unexpectedly, so is maternal hand washing prior to infant feeding. Since prevention may indeed be the "best medicine" for infants, investigation of beneficial breastfeeding practices, protective correlates in breast milk, and ways to reduce the maternal and environmental *Cryptosporidium* burden are needed.

2.3 Introduction

The World Health Organization reports that the most common diarrhea-causing protozoan parasite worldwide is *Cryptosporidium* [1], and a recent, large, multi-country investigation reported *Cryptosporidium* as the second most common pathogen indentified among care-seeking African and Asian infants 0 to 11 months [2]. The significance of this infection was underscored as this study revealed that infection was associated with a greater than two-fold increase in mortality of children 12 to 23 months [2]. Despite these indications of the potential global scope and impact of *Cryptosporidium* infection, a full understanding of the epidemiology of infant and early childhood infection remains limited due to logistical and methodological difficulties in conducting such research in impoverished high-burden urban and rural settings [2].

Cryptosporidium is a pathogen transmitted via the oral-fecal route from human (*C. hominis*) and animal (predominately *C. parvum*) reservoirs. Infection risk factors include a contaminated environment with elements such as: unsafe water, poor sanitation and hygiene, and close proximity to infected livestock, while severe clinical disease risk factors include: malnutrition and compromised immunity, particularly HIV-associated immunosuppression [1,3]. Symptoms include: nausea, vomiting, voluminous and watery

diarrhea, dehydration, abdominal discomfort, anorexia, fever, fatigue, and respiratory problems [4,5], with chronic and life-threatening symptoms possible amongst immunocompromised individuals due to the increased duration and severity of illness [4]. However an unknown number of individuals experience asymptomatic *Cryptosporidium* infection [6]. This clinically silent infection may remain undetected and untreated and therefore may contribute to malnutrition, growth impairment, and long-term cognitive and functional deficits in infants and children [7,8].

The primary aim of this research was to determine the prevalence of *Cryptosporidium* in young infants living in rural and semi-rural Tanzania by identifying the timing of the first and subsequent *Cryptosporidium* events in both symptomatic and asymptomatic infections. Secondarily, I aimed to evaluate potential infant infection risk factors including: infant nutritional status, infant feeding practices, infant HIV-exposure, maternal nutritional status, maternal HIV infection, and, uniquely, maternal post-partum *Cryptosporidium* infection.

2.4 Methods

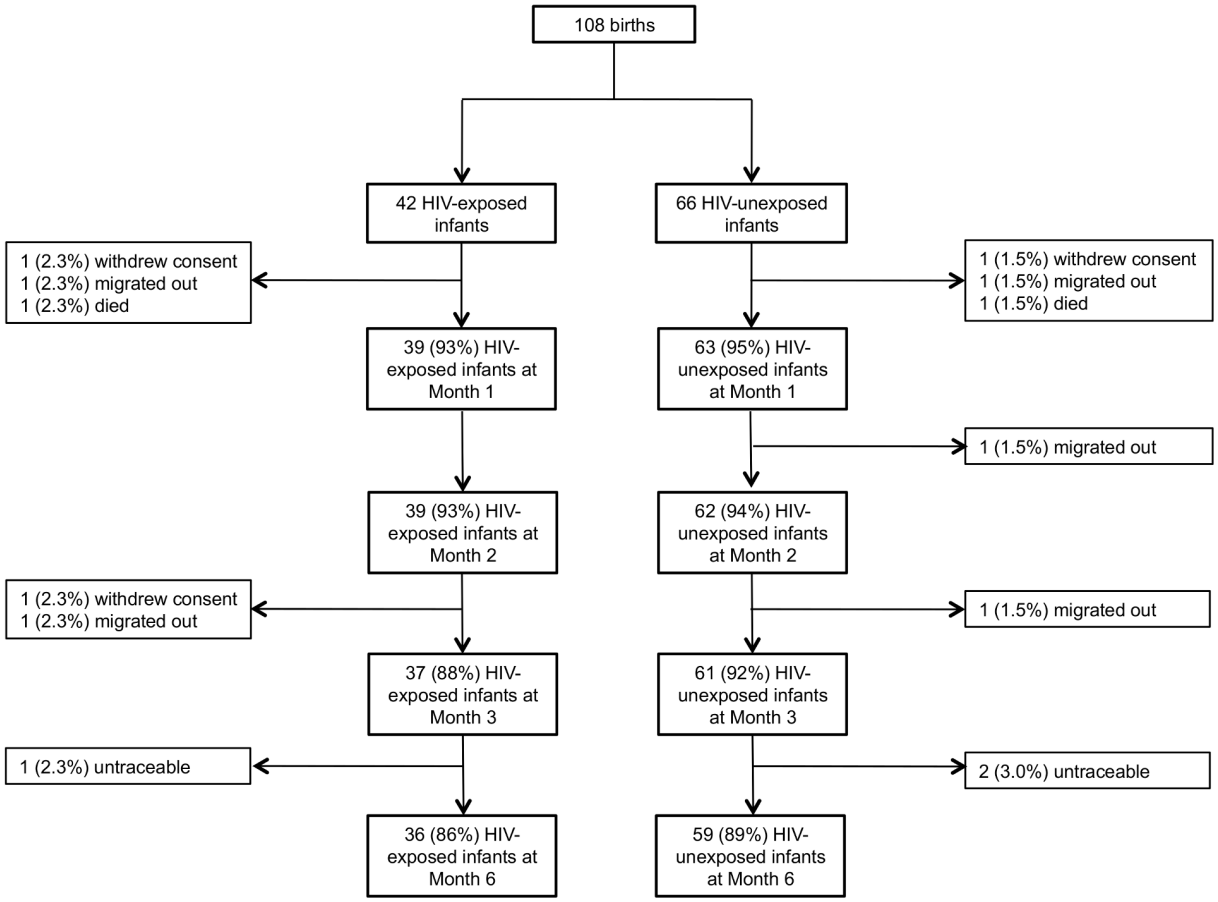
This study was a prospective birth cohort enrolling newborns and their HIV-seropositive or –negative mothers living in the rural and semi-rural areas of Kisesa Ward (population 30,000) [9] in northwestern Tanzania. Pregnant women receiving antenatal care at Kisesa Health Centre (KHC), a Tanzanian government-administered, publically accessible primary care facility were recruited from March through December, 2012, a period that included both the dry and rainy seasons (Appendix A, Appendix F, Appendix G, Appendix H). Women gave birth between April, 2012 and January, 2013 (Appendix B, Appendix I); the study follow-up appointments for mothers and infants were

conducted between May, 2012 and July, 2013 (Appendix C, Appendix J). Eligibility criteria were gestation < 37 weeks at consent, singleton birth, known maternal HIV serostatus (screening with Determine HIV-1/2 [Inverness Medical], confirmation with Uni-Gold HIV-1/2 [Trinity Biotech]), maternal ability to speak and understand the local language of Kiswahili, and stated intention to reside within the clinic catchment at delivery and through six months post-partum. The study was advertised through health workers at KHC as well as rural government-run health dispensaries in the region. All HIV-positive women were receiving anti-retroviral treatment (ART) for their own care or for prevention of mother-to-child transmission by the time of delivery. Infants born to HIV-positive women were given nevirapine daily for six weeks and tested for HIV-infection by dried blood spot DNA-PCR at the regional hospital laboratory at the Month 3 follow-up visit. The study protocol was approved by the ethics review committees of the Tanzania National Health Research Ethics Review Committee and Cornell University. Written informed consent was obtained from mothers for themselves and on behalf of their infants at enrolment with verbal assent re-confirmed at follow-up (Appendix G).

All women were encouraged to deliver at KHC unless otherwise medically advised. As many women in this region do not deliver at health clinics, and preliminary research revealed that transportation expenses were the primary barriers to accessing healthcare [10], the study provided transportation compensation and other clinical expenses typically borne by mothers for delivery and follow-up visits. For women who delivered elsewhere, including home births, mothers and infants were requested to attend a follow-up clinic visit within three days of delivery. The study flow chart is summarized

in **Figure 2.1**. If a mother-infant pair did not return for a regularly scheduled follow-up visit, a field worker traveled to their last known address to invite them to return to the clinic for a follow-up appointment.

Figure 2.1. Study profile of infant cohort participants according to infant HIV-exposure



At each follow-up, the research nurse, under supervision of the study coordinator, administered the Infant Feeding and Health Questionnaire to mothers (Appendix C, Appendix J). This questionnaire was designed to obtain data on a range of feeding, health, and environmental risk factors. Exclusive breastfeeding (EBF-WHO) was defined according to the WHO definition where "the infant receives breast milk (including expressed breast milk or breast milk from a wet nurse) and allows the infant to receive oral rehydration solution (ORS), drops, syrups (vitamins, minerals, medicines), but nothing else" [11]. Duration of EBF-WHO was defined as the time from birth until an infant first received food or liquids other than breast milk or medicines. Diarrhea was defined as loose or watery stools ≥ three times per day that represented a pattern atypical for that individual [1]. The questionnaire included: 1) infant nutrition: breastfeeding and complementary feeding practices; 2) mother-reported infant morbidity: cough, difficulty breathing, fever, convulsions, vomiting, skin rash, anorexia, unscheduled clinic/hospital visits, and episodes of diarrhea; and 3) environment: food security, using an index composed of questions relating to the mother's food consumption pattern, and sanitation and hygiene practices, such as hand-washing behavior, access to safe water, and toilet facilities. Infants exhibiting symptoms of illness were referred to the clinical officer at KHC for follow-up.

Anthropometric assessments were collected at each follow-up visit. Maternal height and weight were measured using a standard stadiometer (Health O Meter, Inc., Bridgeview, IL) to the nearest 0.2 kg and nearest 0.1 cm, respectively. Maternal mid-upper arm circumference (MUAC) and triceps skinfold thickness (TSF) were measured

to the nearest 0.1 cm and 0.5 mm, respectively. Infant weight and length were measured using a calibrated digital infant scale (Seca 334 Digital Baby Scale) to the nearest 0.01 kg and a standard infant length board to the nearest 0.1cm, respectively. Infant MUAC, TSF, and head circumference were measured to the nearest 0.1 cm, 0.5 mm, and 0.1 cm, respectively.

Active case detection was of interest so maternal and infant fecal samples were collected irrespective of self-reported intestinal symptoms at each follow-up visit. *Cryptosporidium* infection was detected using fresh stool samples that were stored in a cooler with ice packs for ≤ 5 hours before being transferred and stored at 4°C in the parasitology laboratory of the Tanzanian National Institute for Medical Research (NIMR), Mwanza Research Centre. Within 24 hours of collection, approximately 5g of stool was mixed with 5mL 10% v/v formalin and stored at 4°C until analysis. Presence of *Cryptosporidium* was confirmed using a modified Ziehl-Neelsen staining procedure (Appendix P) [12], which is estimated to have a sensitivity ranging from 32 to 79% and a specificity ranging from 89 to 100% [13-15]. After staining, slides were examined by a single technician, without knowledge of participant clinical status, using a light microscope (Olympus model CX41RF) to detect *Cryptosporidium* oocysts and estimate oocyst burden. *Cryptosporidium* infection was defined as ≥ 1 oocyst detected in stained fecal smears. A second technician re-examined a sample (10%) of the slides and inter-observer agreement was 96%.

Data were analyzed in STATA10 (STATA Corporation, Texas, USA). Means of normally distributed continuous variables were compared using Student's *t*-test and proportions of categorical variables were compared using the χ^2 test and Fisher's Exact

test. Results were considered statistically significant at $\alpha = 0.05$, two-sided. Univariate and multivariate logistic regression models were used to estimate the odds ratio (OR) and 95% confidence interval (95% CI) of *a priori* considered potential risk factors for infant *Cryptosporidium* infection (HIV-exposure, exclusive breastfeeding, maternal *Cryptosporidium* infection, and household factors, such as animal ownership, sanitation, wealth, and maternal education). This study is registered with ClinicalTrials.gov, number NCT01699841.

The sponsors (Cornell University and the National Science Foundation) were not involved in the design or oversight of the study. Members of the writing team had full access to the study data. The authors had final responsibility for the decision to submit for publication.

2.5 Results

During the study period, 108 infants were born, and of these, six infants exited the study because of death, migration, or withdrawal of consent prior to the Month 1 study visit (**Figure 2.1**) and were not included in follow-up analyses. Birth anthropometrics were statistically different between HIV-exposed and HIV-unexposed infants (**Table 2.1**). A greater proportion of HIV-exposed infants had low birth weight (LBW; defined as birth weight < 2500g) compared to HIV-unexposed infants (HIV-exposed vs HIV-unexposed = 15 vs 3%, respectively; p = 0.026). Likewise, a greater proportion of HIV-exposed infants were stunted at birth (defined as birth length < 44.7 cm) compared to HIV-unexposed infants (HIV-exposed vs HIV-unexposed = 18 vs 2%, respectively; p = 0.004). No HIV-exposed infant tested positive for HIV between birth and three months of age. Maternal and household characteristics did not differ based on HIV-status of the

mother, other than marital status, where HIV-positive women were more likely to be divorced than HIV-negative women (HIV-positive vs HIV-negative = 21 vs 0%, respectively; p = 0.002).

Table 2.1. Anthropometric characteristics of infants at birth and baseline maternal characteristics

INFANTS				
	All	HIV-exposed	HIV-unexposed	p value
Sample Size	102	39	63	
Sex				0.240
Male	52 (51%)	17 (44%)	35 (56%)	
Female	50 (49%)	22 (56%)	28 (44%)	
Birth weight (kg)				
Mean (SD)	3.2 (0.44)	3.1 (0.48)	3.3 (0.39)	0.028
Low birth weight (< 2500g)	8 (8%)	6 (15%)	2 (3%)	0.026
Birth length (cm)				
Mean (SD)	46.7 (0.22)	46.0 (0.41)	47.1 (0.22)	0.010
Stunted (< 44.7cm)	8 (8%)	7 (18%)	1 (2%)	0.003
Birth MUAC (cm)				
Mean (SD)	10.7 (0.11)	10.6 (0.19)	10.8 (0.13)	0.372
Birth head circumference (cm)				
Mean (SD)	34.5 (0.17)	34.2 (0.24)	34.7 (0.24)	0.127
Small head (< 31.5cm)	4 (4%)	1 (3%)	3 (5%)	0.578
MOTHERS				
	All	HIV-positive	HIV-negative	p value
Age (years)				
Mean (SD)	28.4 (5.9)	29.4 (6.0)	27.7 (5.8)	0.168
CD4 cell count (cells/μL)				
Median (IQR)		459 (330, 774)		
Body mass index at Month 1				
Mean (SD)	22.0 (2.6)	22.2 (2.7)	21.8 (2.5)	0.483
Underweight (< 18.5 kg/m^2)	8 (9%)	3 (8%)	5 (9%)	0.790
Parity (number of children)				
Mean (SD)	2.6 (1.7)	2.7 (1.9)	2.6 (1.6)	0.660
Water				0.342
Treats water	77 (76%)	27 (71%)	50 (79%)	
Does not treat water	24 (24%)	11 (29%)	13 (21%)	

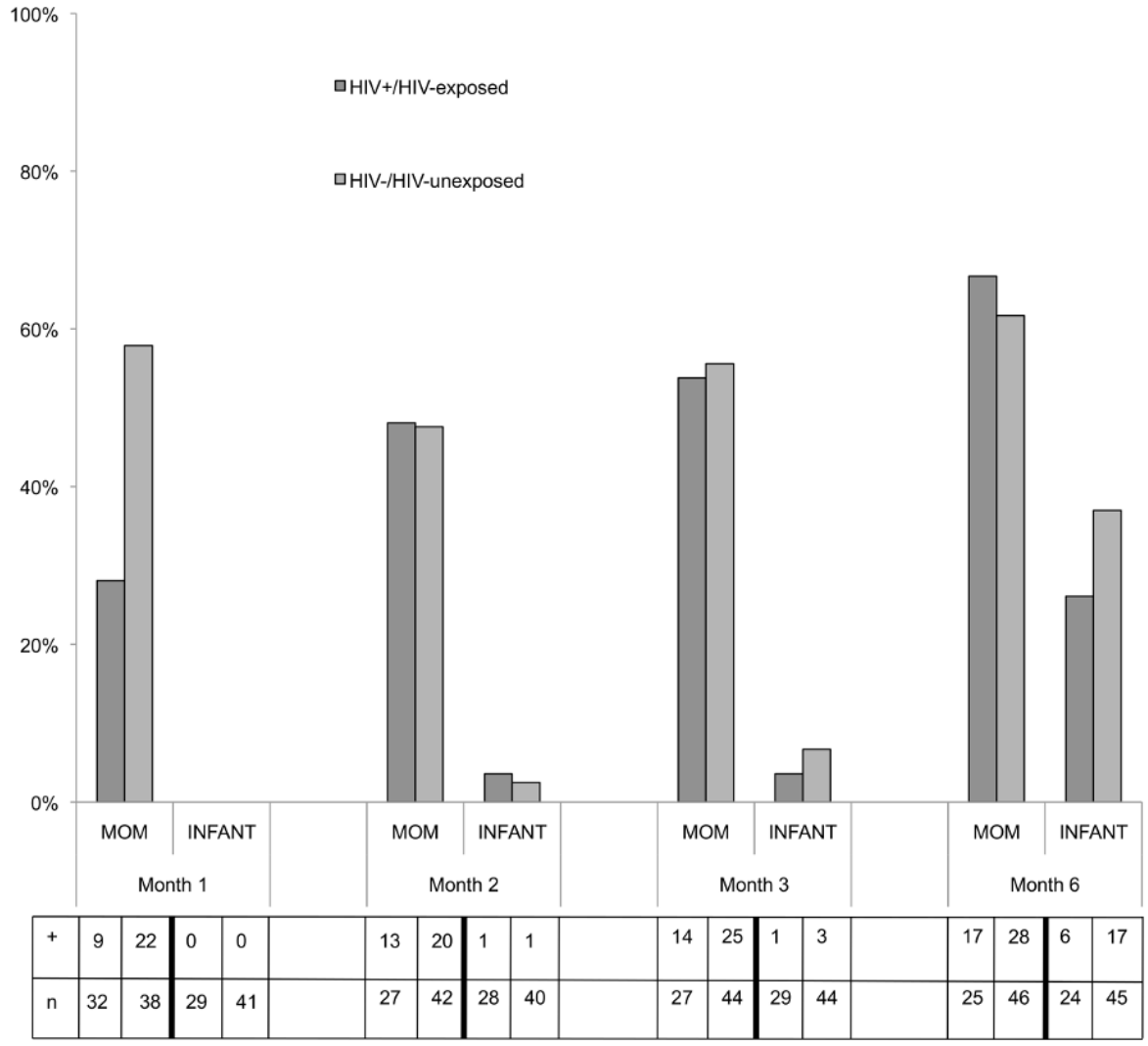
SD = standard deviation; Stunting was defined using WHO growth standards where length-for-age z-score (LAZ) < -2 (44.7 cm for infants at birth) is considered stunted. Likewise, small head was defined as a birth head circumference < 31.5 cm, which corresponds to a head circumference-for-age z-score < -2; MUAC = mid-upper arm circumference, there are currently no MUAC cut-off values for infants at birth [29]; IQR = interquartile range; Treats water = maternal report that the household takes measures to make water safe for drinking, i.e. boiling, filtration.

The proportion of all mothers (HIV+ and HIV- combined) infected with *Cryptosporidium* ranged from a low of 44% (31/70) at Month 1 to a high of 63% (45/71)

at Month 6 post-partum, and this proportion was not statistically different across time points. The majority of all mothers experienced *Cryptosporidium* infection at some point during the study follow-up period, with 82% experiencing *Cryptosporidium* infection at least once and 16% infected at every time point. Self-reported diarrhea was not related to *Cryptosporidium* infection and symptomatic infection ranged from a low of 0% at Month 3 to a high of 14% at Month 6. While the majority (60%) of mothers experienced self-recovery from *Cryptosporidium* infection between visits based on the presence/absence of oocysts in their feces, 15% of mothers who recovered later became re-infected on a subsequent visit. Maternal anthropometry (BMI) did not differ based on *Cryptosporidium* infection status. Additionally, statistically significant differences in maternal *Cryptosporidium* prevalence based on HIV-serostatus were not evident, with the exception of the Month 1 study visit ($p = 0.012$) (**Figure 2.2**).

All infants remained free from *Cryptosporidium* infection until Month 2 and infection remained uncommon through Month 3. By Month 6, the increase in infection was dramatic with 33% (23/69) of infants exhibiting evidence of infection. Infant anthropometry (HAZ, WHZ, WAZ) did not differ based on *Cryptosporidium* infection status. There were no statistically significant differences in infant *Cryptosporidium* infection based on HIV-exposure ($p = 0.284$). Prevalence of infant *Cryptosporidium* infection did not significantly differ based on whether a mother was experiencing asymptomatic versus symptomatic *Cryptosporidium* infection.

Figure 2.2. Prevalence of *Cryptosporidium* infection in mothers and infants according to HIV-status/exposure.



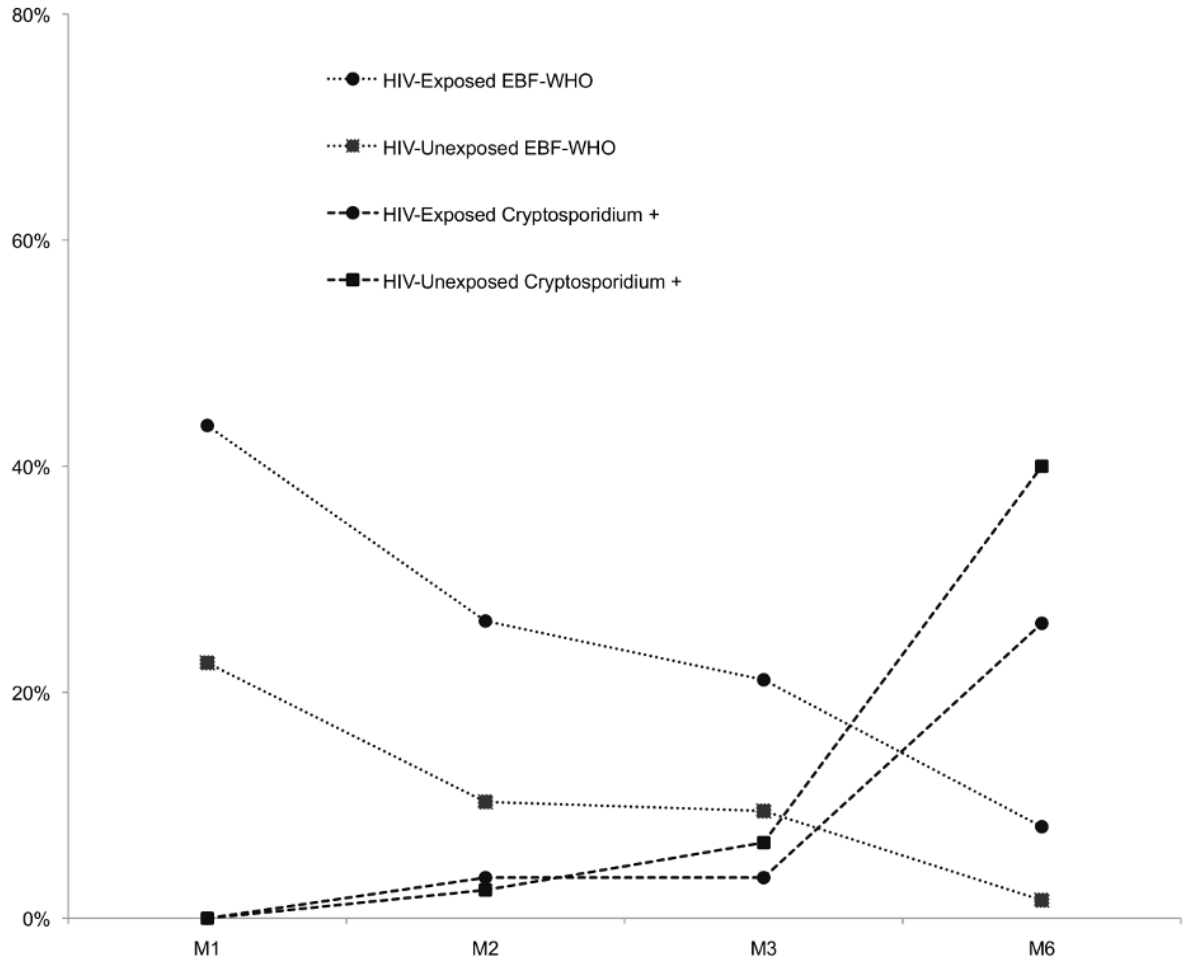
+	9	22	0	0		13	20	1	1		14	25	1	3		17	28	6	17
n	32	38	29	41		27	42	28	40		27	44	29	44		25	46	24	45

+ = number of participants with evidence of Cryptosporidium infection; n = number of fecal samples analyzed. Note: the denominator increases across the study period for some groups due to missing data resulting from a missed appointment or failure to bring a fecal sample to the follow-up appointment.

As overall EBF-WHO declined, the proportion of infant *Cryptosporidium* infection increased (**Figure 2.3**). Post-partum, there was a higher proportion of HIV-positive mothers practicing EBF-WHO compared with HIV-negative mothers and this difference was statistically significant at both Month 1 (proportion HIV-positive vs HIV-negative: 44 vs. 23%, p = 0.03) and Month 2 (proportion HIV-positive vs HIV-negative:

26 vs. 10%, p = 0.04). **Figure 2.4** depicts the survival curves of EBF by maternal HIV-status. Notably, of the four infants who continued EBF-WHO until six months, none had evidence of *Cryptosporidium* infection even though they were living in a *Cryptosporidium* environment as confirmed by evidence of maternal *Cryptosporidium* infection in all four cases. There was a pattern of lower proportion of *Cryptosporidium* infection in infants with a greater proportion of the diet consisting of breast milk (EBF-WHO vs. partial/no breastfeeding) and this was significant at Month 6 (p = 0.030) (**Figure 2.5**).

Figure 2.3. Proportion of infants exclusively breastfed (EBF) and proportion with *Cryptosporidium* infection according to HIV-exposure.



M1 = Month 1; M2 = Month 2; M3 = Month 3; M6 = Month 6; EBF-WHO = WHO definition of exclusive breastfeeding.

Figure 2.4. Kaplan-Meier survival curve of duration of EBF in weeks, by maternal HIV-status

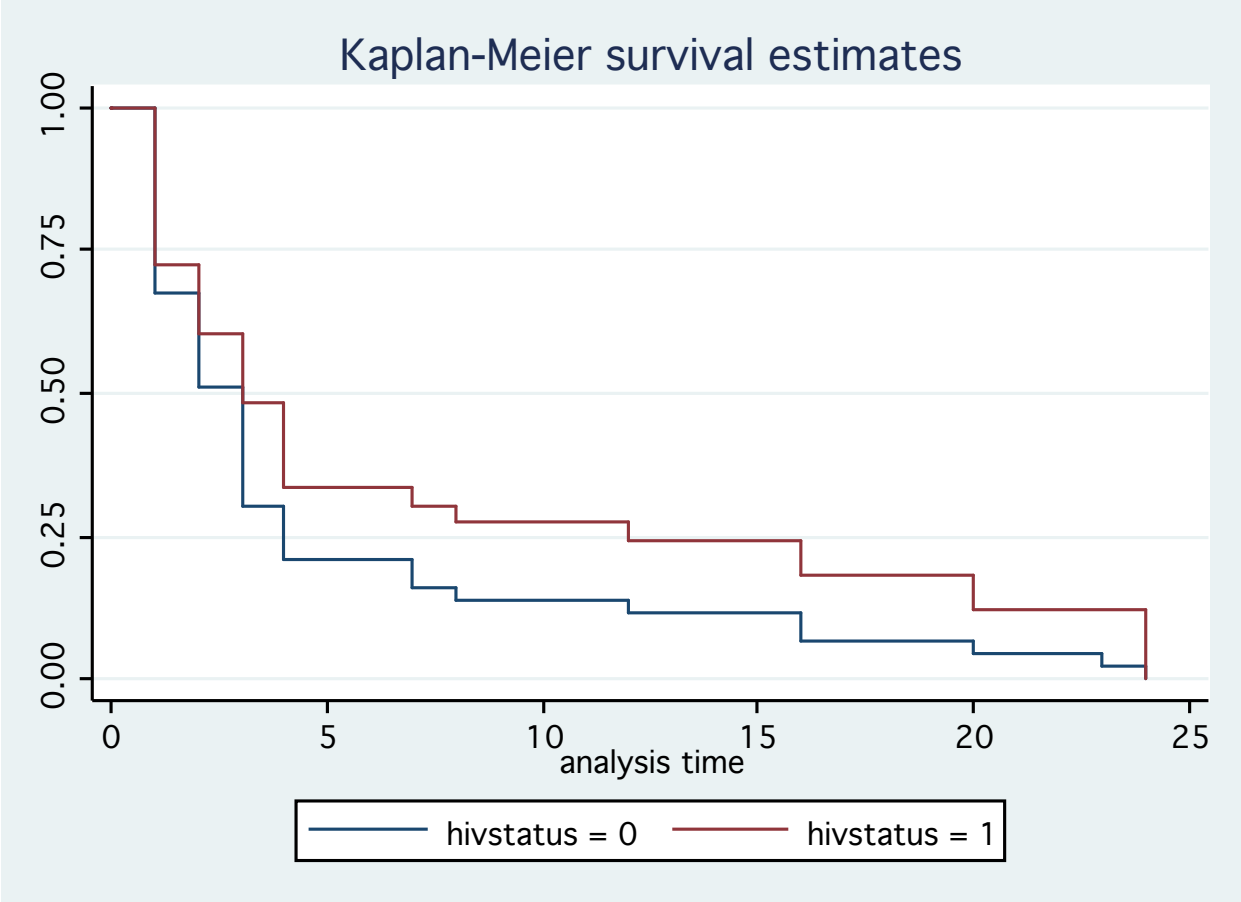
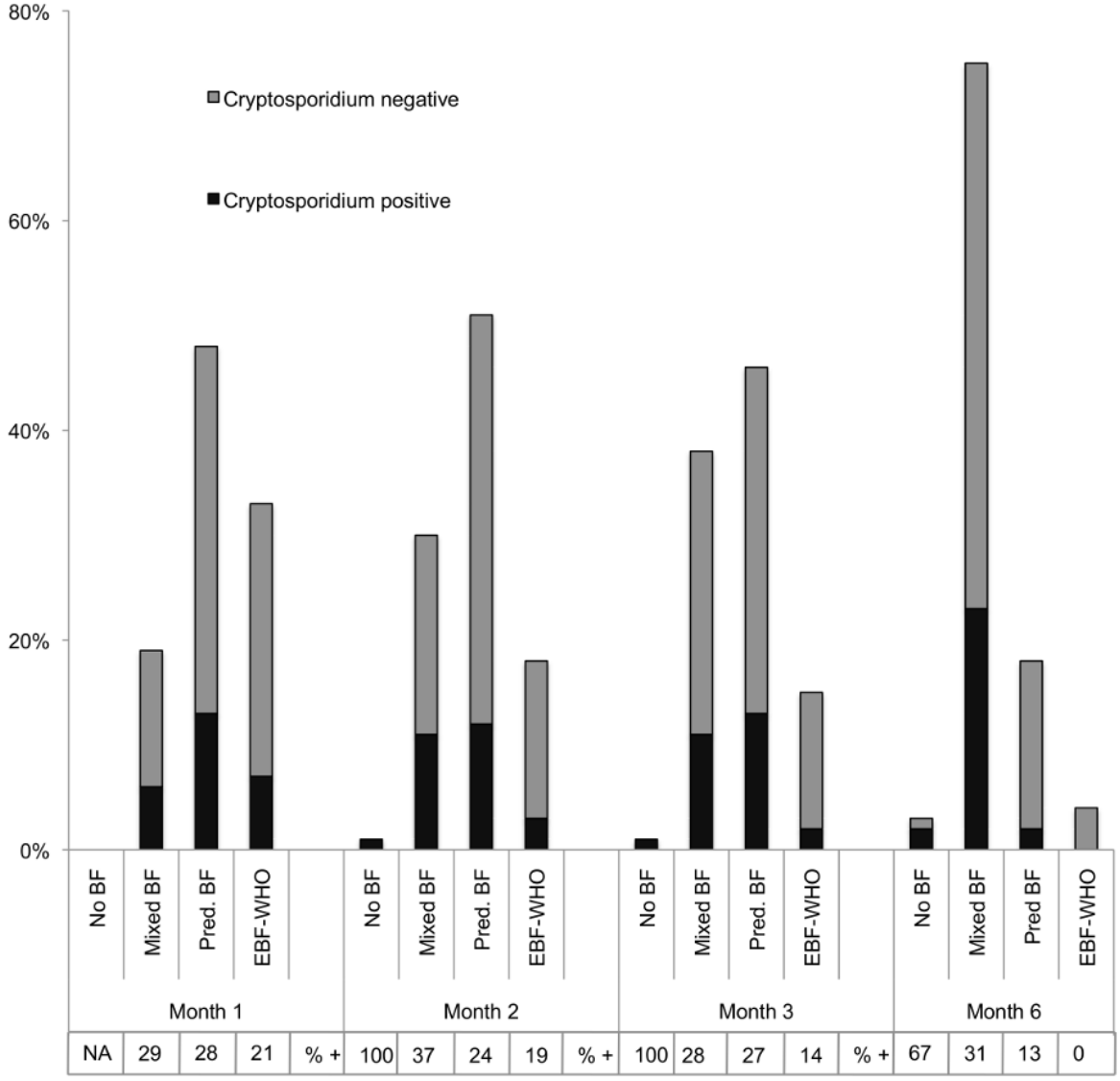


Figure 2.5. Proportion of infants infected with *Cryptosporidium* between 0 and 6 months according to status of breastfeeding practice



Cryptosporidium negative = Cryptosporidium was not detected in the feces of the infant during the study period; Cryptosporidium positive = Cryptosporidium was detected at least once during the study period; No BF (breastfeeding) = infant was not receiving any breast milk; Mixed BF = infant was receiving breast milk and other liquids and foods; Pred. BF = infant was receiving breast milk and locally prepared gripe water; EBF-WHO = WHO definition of exclusive breastfeeding.

Neither maternal nor infant *Cryptosporidium* infection was associated with reported symptoms of infection that included diarrhea, anorexia, vomiting, and in

mothers only, abdominal pain and nausea. Care-seeking behavior, operationalized as an unscheduled clinic or hospital visit, was uncommon for both mother (4%) and infant (8%) between each scheduled follow-up visit and was not associated with *Cryptosporidium* infection.

Table 2.2 summarizes the contribution of infant *Cryptosporidium* infection risk factors in this setting. In univariate analyses, only maternal *Cryptosporidium* infection at Month 1 (unadjusted OR = 3.18, 95% CI = 1.01 to 9.99) was associated with infant infection. While EBF-WHO was not significantly associated with lower odds of infant *Cryptosporidium* infection, there was a consistent trend between longer duration of EBF-WHO and lower infant infection. In the multivariate model, maternal hand washing prior to infant feeding was significantly associated with an increased likelihood of infant *Cryptosporidium* infection (adjusted OR = 5.02, 95% CI = 1.11 to 22.78). Maternal nutritional status, defined by body mass index (BMI) and MUAC, was not associated with maternal *Cryptosporidium* infection. Likewise, birth weight was not associated with infant *Cryptosporidium* infection nor was infant growth faltering up to six months a predictor of infant infection. Maternal food security index was negatively correlated with the practice of EBF-WHO at each visit; meaning that the more food secure a household, the less likely the infant was EBF-WHO. Similarly, the wealthier a household, the less likely the infant was EBF-WHO and the more educated a mother, the less likely the infant was EBF-WHO. Neither maternal nor infant *Cryptosporidium* infection were associated with season or rainfall.

Table 2.2. Risk factors for infant *Cryptosporidium* infection between birth and six months

	Infants (n)	Unadjusted OR (95% CI)	p value	Multivariate adjusted* OR (95% CI)	p value
HIV					
HIV-exposed	98	0.38 (0.14 – 1.07)	0.067	0.45 (0.10 – 1.98)	0.292
Breastfeeding					
EBF-WHO at Month 1	89	0.66 (0.23 – 1.90)	0.442		
EBF-WHO at Month 2	93	0.58 (0.15 – 2.22)	0.424		
EBF-WHO at Month 3	97	0.43 (0.09 – 2.09)	0.299		
PBF at Month 6	91	0.23 (0.05 – 1.09)	0.063	0.32 (0.05 – 2.08)	0.233
Maternal *Cryptosporidium*					
Crypto Month 1	69	3.18 (1.01 – 9.99)	0.047	3.40 (0.88 – 13.06)	0.075
Crypto Month 2	69	1.30 (0.47 – 3.63)	0.617		
Crypto Month 3	71	1.93 (0.63 – 5.89)	0.251		
Crypto Month 6	71	1.23 (0.42 – 3.58)	0.710		
Crypto any time	95	2.76 (0.58 – 13.12)	0.201		
Household Factors					
Owns Animals	98	0.80 (0.31 – 2.11)	0.654	0.75 (0.19 – 2.94)	0.676
Washes hands	98	1.72 (0.68 – 4·33)	0.249	5.02 (1.11 – 22.78)	0.036
Wealth	98	0.97 (0.31 – 3.00)	0.953	0.48 (0.09 – 2.44)	0.373
Maternal Literacy	98	1.51 (0.50 – 4.57)	0.466	0.76 (0.16 – 3.70)	0.735

**Adjusted for maternal HIV status (0 = negative; 1 = positive), PBF at Month 6 (0 = no breastfeeding or partial breastfeeding; 1 = predominant breastfeeding or exclusive breastfeeding), Maternal Cryptosporidium infection at Month 1 (0 = uninfected; 1 = infected), animal ownership (0 = no animals; 1 = owns animals), hand washing (0 = mother doesn't wash hands prior to infant feeding; 1 = mother washes hands prior to infant feeding), wealth (0 = lower 2 tertiles; 1 = top tertile), and maternal literacy (0 = mother cannot read; 1 = mother can read). OR = odds ratio; CI = confidence interval; EBF-WHO = WHO definition of exclusive breastfeeding; PBF = exclusive or predominant breastfeeding; Washes hands = mother's self report of washing hands prior to feeding infant; Wealth = index (0 – 10) calculated by summing a categorical list of household possessions and then stratified into wealth (top tertile) vs. not wealthy (lower 2 tertiles); maternal literacy = mother's self-report that she can read.*

Multivariate adjusted model: Infant Cryptosporidium *infection = β(HIV-exposed) + β(PBF at Month 6) + β(maternal* Cryptosporidium *infection at Month 1) + β(Owns Animals) + β(Washes hands) + β(Wealth) + β(Maternal literacy) + μ*

2.6 Discussion

This is the first report of maternal-infant *Cryptosporidium* infection in Sub-Saharan Africa and the prevalence of infection was high. Post-partum infection was

detected at least once in the majority of women and, for many, on multiple occasions. The *Cryptosporidium* burden in infants increased dramatically between three and six months of age, a period that corresponds to changes in breast feeding practices. My results indicate that young infants living in rural and semi-rural Tanzania are susceptible to *Cryptosporidium* infection in early infancy with approximately 1/3 of infants showing evidence of infection by six months of age.

This study confirms and extends the importance of *Cryptosporidium* infection in young infants reported in the GEMS study [2] that included both rural and urban settings. My results are comparative to the findings of a sub-sample of young Tanzanian infants in urban, hospital-based studies where 25% of infants 0 to 6 months had evidence of either *G. lamblia* or *Cryptosporidium parvum* [16], though the burden of *Cryptosporidium parvum* was not individually reported. In studies conducted in the Tanzanian capital of Dar es Salaam, only 9% of children three months to nine years and 18.9% of children 0 to 60 months had evidence of *Cryptosporidium* infection [5,16] and this may represent an urban-rural difference in young infant burden in Tanzania.

Previous studies in Tanzania of HIV-positive adults report a *Cryptosporidium* prevalence between 7 and 17% [5,6] and HIV infection has been identified as a risk factor for *Cryptosporidium* and cryptosporidiosis in some studies [6,17-20] but not others [2]. Maternal HIV infection did not appear strongly related to *Cryptosporidium* infection in my study and this may be explained in part because the majority of HIV-positive women were otherwise healthy and not severely immunocompromised based on their CD4 cell counts. Previous studies that identified HIV infection as a risk factor were primarily conducted in the pre-ARV era and greater immunosuppression may explain

differences [6,17,20]. Likewise, HIV-exposure was not a significant risk factor for *Cryptosporidium* infection in infants and this might be due in part to more optimal feeding methods in the HIV-exposed infants due to infant feeding counseling for HIV-positive mothers. While HIV infection may not be a significant risk factor for infection in this setting, it remains relevant for the clinical management of cryptosporidiosis in immunocompromised individuals given the lack of effective *Cryptosporidium* treatment other than ART's to improve HIV immunocompetency [21].

While maternal *Cryptosporidium* infection was associated with greater infant infection, previously (or even currently) infected mothers may also be providing protective passive immunity *in utero* or in breast milk. A recent study of Bangladeshi infants reported that protection from *Cryptosporidium* infection was associated with high anti-*Cryptosporidium* IgA in breast milk [22]. Despite possible passive immunity and/or risk elimination (from contaminated food/water), EBF-WHO was uncommon in my study population and was not sustained for the universally recommended duration of six months. In this study, using the WHO definition of EBF, only a third of mothers were practicing EBF-WHO at Month 1. Previous Tanzanian studies indicated much higher levels of "EBF" ranging from 49% within 3 days after birth [23], 90% at Month 1 [24], and 80% at Month 2 [25], but these large differences are likely due to the less strict non-WHO-EBF definitions and/or maternal recall methods used [24,25]. Additionally, two of these studies included HIV-positive women only and HIV maternal care includes infant feeding counseling that is typically unavailable to HIV-negative mothers in this setting [24,25]. Indeed, I found significantly higher rates of EBF-WHO in HIV-positive mothers

and this may explain why infant HIV-exposure was associated with lower infant *Cryptosporidium* infections.

Globally, knowledge of the epidemiology of *Cryptosporidium* infection in early infancy is scarce and, in Tanzania, such data are unavailable. When the lack of prevalence data is combined with barriers to diagnosis, the disease rarely features on the clinician's diagnostic radar. This leads to a cycle that likely perpetuates the underestimation of the *Cryptosporidium* burden leading to an inappropriately lower global health and research priority. This cycle reinforces ineffective clinical and public health management of *Cryptosporidium*. In my study, maternal hand washing prior to infant feeding was counterintuitively associated with infant infection, although given the wide 95% confidence interval, I recommend caution in the interpretation of this finding. Previous studies have indicated that household sanitation and hygiene, including hand washing, were related to reduced *Cryptosporidium* infection [17]. Since *Cryptosporidium* has notoriously robust survival and transmissibility [26,27], and mothers may wash their hands with contaminated water and then feed their children, my result is plausible in this setting. It may also be that the practice of hand washing is a proxy indicator for women who lived in more contaminated environments. Further research could include testing water sources and/or analysis of the species of *Cryptosporidium* in order to determine probable transmission routes of infection. Such investigations would help interpret this finding in relation to major public health messages related to hand washing in similar settings.

My study had a number of limitations. First, at each follow-up visit, only one stool sample was collected from each mother and infant. Due to the intermittent

shedding of *Cryptosporidium* oocysts, collection of a single stool sample may result in an underestimate of the true *Cryptosporidium* prevalence [28]. Additionally, my study used modified Ziehl-Neelsen staining, the most common diagnostic technique to detect the presence of *Cryptosporidium* oocysts in stool samples, however, the sensitivity and specificity of this method are not 100% leading to possible misclassification [13]. Lastly, my results may not be generalizable to other geographical settings due to urban/rural differences and geographical variation in *Cryptosporidium* contamination.

In conclusion, there is a high prevalence of infant and maternal *Cryptosporidium* infection in this setting. Public health interventions promoting EBF-WHO among all women, including HIV-negative mothers should be strengthened. Modeling the message of breast milk as an immunologically protective substance to prevent certain infectious diseases common in childhood may be effective in regions where there are high rates of vaccination coverage. Additionally, further research is needed to address efforts to minimize the maternal and environmental *Cryptosporidium* burden as a means of protecting young infants in the absence of effective vaccines, diagnostics, and treatment for early infancy cryptosporidiosis.

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CHAPTER 3

THE IMMUNOLOGICAL COMPOSITION OF MATURE BREAST MILK FROM MOTHERS OF MIXED HIV-STATUS IN RURAL NORTHWESTERN TANZANIA

3.1 Abstract

Background: Breastfeeding is the "gold standard" in infant feeding, yet the correlates of immune protection in breast milk (BM) remain unclear. In the context of rural sub-Saharan Africa, it is crucial to understand the immunological protection provided through BM to reduce infant morbidity and mortality.

Methods: A cohort of 125 women/infants in rural Tanzania was followed from birth through six months. BM and maternal/infant blood were collected to determine concentrations of immunoglobulins and cytokines and how they were associated with maternal/infant health/nutritional status.

Results: While cytokine and immunoglobulin concentrations did not differ based on maternal nutritional status or HIV status, exclusive breastfeeding (EBF) was associated with greater concentrations of IgA, IgG, and IL-12p70. There were weak/moderate correlations for immunoglobulins between maternal serum-BM and BM-infant serum, but cytokines showed a more complex, inconsistent pattern of correlation. In regression analyses, total BM immunoglobulin concentrations were positively associated with EBF and more frequent feeding, while total BM cytokines were associated with maternal and infant infection symptoms.

Conclusions: Breastfeeding practices in this cohort of rural Tanzanian mothers remain sub-optimal, yet EBF and feeding frequency were associated with increased

concentrations of certain cytokines and immunoglobulins in BM. Maternal body mass index and HIV-infection were not associated with differences in BM immunological composition. Future research should focus on a broader array of modifiable factors that may impact BM immune composition, as well as the clinical and public health relevance of BM differences on infant infectious diseases.

3.2 Introduction

The nutritional, immunological, and maternal-infant bonding benefits achieved through breastfeeding makes it the universal "gold standard" for infant feeding [1]. Breastfeeding has also repeatedly been associated with lower incidences of early childhood infectious diseases, such as pneumonia and diarrhea [2]. However, the correlates of immune protection in breast milk remain unclear. Diarrhea and pneumonia are the two largest contributors to infant morbidity and mortality in resource-limited regions [3] so the identification of immunological factors associated with infection in early infancy is needed. Consequently, a better understanding of breast milk immunology throughout the lactation period is crucial to reducing infant illness and death.

Early infancy represents a period of immunological vulnerability, as the immune system continues to mature after birth [4]. Breast milk provides an important immunological bridge between the maternal immune system and the developing infant immune system. Many immune cells and molecules in breast milk are involved in the prevention of infection and contribute to the priming and further maturation of the neonatal immune system. For example, immunoglobulin A (IgA), the most abundant

antibody in human milk, provides adaptive immune protection against certain intestinal infections by neutralizing and eliminating enteric pathogens [5]. Human milk also contains commensal microbes, cells (e.g. macrophages), molecules (e.g. cytokines, lactoferrin), and other immunoglobulins contributing to immune protection. Cytokines, through diverse signaling pathways, are key mediators of both the innate and adaptive immune response [6-8].

The nature of the immunological bridge provided by breast milk likely depends upon the specific immunological composition of a mother's milk. It has been demonstrated that breast milk immune composition is influenced by maternal factors, such as her nutritional status and health, as well as biological factors like stage of lactation. Since a mother's nutritional status can alter her own immune status [9,10], by extension, maternal nutritional status may impact breast milk immunological composition, however the limited evidence lacks consensus [11-14]. More evidence is available to support a link between maternal immune status and breast milk immune composition. A key example is the maternal entero-mammary system whereby the maternal immune response to intestinal pathogens induces the production of circulating immune cells that migrate to mucosal secretory immune tissues, including the mammary glands [15]. Additional factors, such as infant health status [16], and breastfeeding practices [17] may also influence breast milk immunology, but data are limited.

In rural sub-Saharan Africa, infants are at high risk of infectious diseases and, once infected, health consequences may be severe due to limited health care infrastructure and resources. Maternal HIV-infection remains common in this context (6.8% of pregnant women in Tanzania are infected with HIV [18]), while the success of

anti-retroviral treatment (ART) and prevention of mother-to-child transmission (PMTCT) have improved the health of both HIV-positive mothers and their infants. Public health policy recommends exclusive breastfeeding (EBF) for all mothers, irrespective of HIV status [19]. As a result, the number of infants receiving HIV-exposed breast milk is both considerable and increasing. Despite links between mucosal immunity and circulating immune cells in HIV-infection [20], evidence regarding the link between maternal HIV-infection and breast milk immune composition remains limited and inconsistent [8, 21]. Therefore, in a prospective cohort of women and infants living in rural northwestern Tanzania, this study's aims were to: 1) characterize breast milk immune composition up to six months post-partum among women with and without HIV infection; 2) investigate immunoglobulin and cytokine concentrations in different biological compartments including maternal serum, breast milk, and infant serum; and 3) compare breast milk immunoglobulin and cytokine profiles in relation to maternal and infant health and nutritional status indicators.

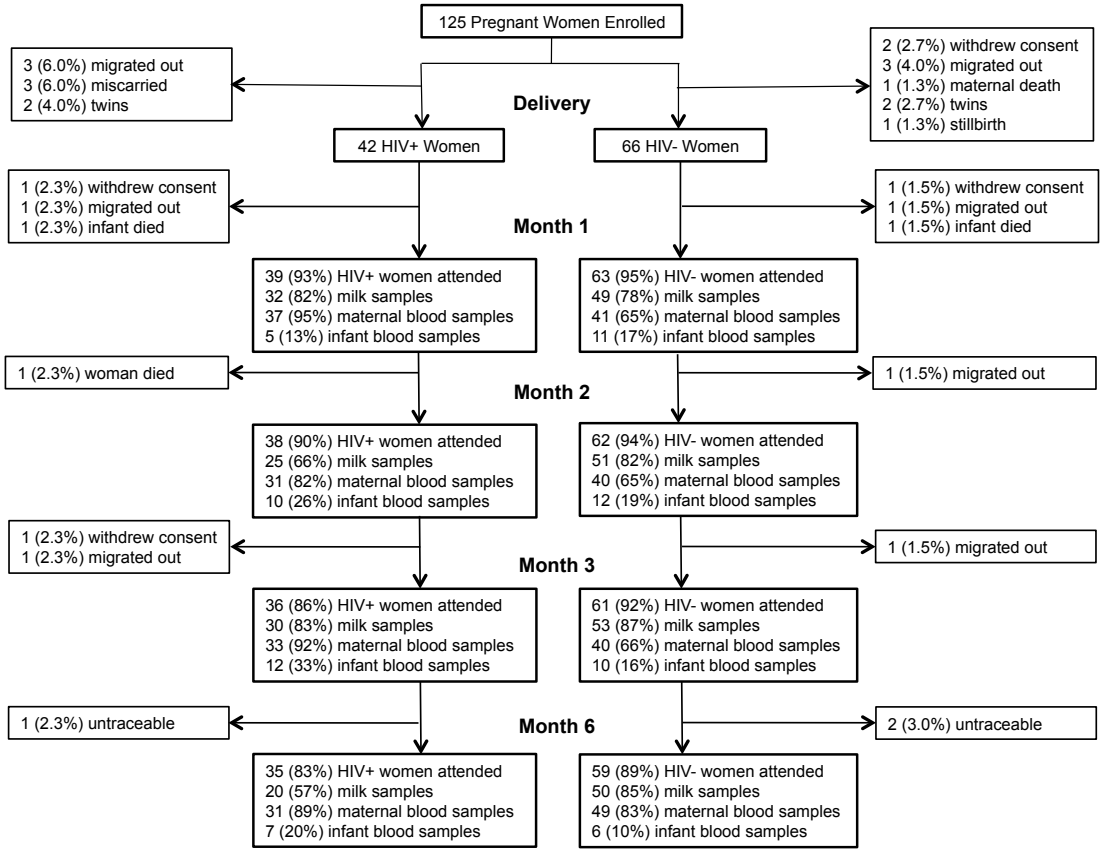
3.3 Methods

3.3.1 Study Participants

This prospective cohort included 50 HIV-positive and 75 HIV-negative pregnant women living in rural and semi-rural areas of Kisesa Ward (population 30,000) [22], Magu District, northwestern Tanzania (**Figure 3.1**). Pregnant women seeking antenatal care at Kisesa Health Centre or remote health care dispensaries, both of which are publically accessible facilities administered by the Tanzanian government, were recruited between March 27 and December 19, 2012. Prospective participants were encouraged to

attend Kisesa Health Centre for an enrollment interview (Appendix A, Appendix F, Appendix G, Appendix H). All HIV-positive pregnant women and the first three HIV-negative pregnant women attending the antenatal clinic each day were invited to participate if they met the following eligibility criteria: gestation <37 weeks at consent, singleton birth, known maternal HIV serostatus (screening with Determine™ HIV-1/2 [Inverness Medical], confirmation with Uni-Gold™ HIV-1/2 [Trinity Biotech]), maternal ability to speak and understand the local language of Kiswahili, and stated intention to reside within the clinic catchment area from delivery through six months post-partum. To maintain study retention, if a participant did not return for a scheduled follow-up study visit, a field worker traveled to her last known address to invite her to return to the clinic for a follow-up appointment. All HIV-positive women were receiving antiretroviral therapy (ART), either for their own HIV management or for PMTCT, by the time of delivery in accordance with Tanzanian national guidelines [18].

Figure 3.1. Flow of study participants



The study protocol was approved by the ethics review committees of the Tanzania National Health Research Ethics Review Committee and Cornell University (ClinicalTrials.gov, number NCT01699841). Written informed consent was obtained from mothers and on behalf of their infants at enrollment and verbal assent for mothers and infants was re-confirmed at each follow-up (Appendix G).

3.3.2 Data Collection

At each study visit, participants were administered a questionnaire on demographics, self-reported maternal and child morbidity, and infant feeding practices

(Appendix C, Appendix J). EBF was defined according to the World Health Organization (WHO) definition where "the infant receives breast milk (including expressed breast milk or breast milk from a wet nurse) and allows the infant to receive oral rehydration solution (ORS), drops, syrups (vitamins, minerals, medicines), but nothing else" [23]. EBF duration was defined as the time (in weeks) from birth until an infant first received food or liquids other than breast milk or prescribed medicines. Breastfeeding intensity was defined as the number of times a mother reported putting her infant to the breast in the previous 24 hours. Food Security was assessed using a series of five questions modified from the Household Food Insecurity Access Scale (HFIAS), previously validated in rural Tanzania [24].

3.3.3 Nutritional Status

Maternal and infant anthropometric data were collected at each study visit. Maternal height and weight were measured using a standard stadiometer (Health O Meter, Inc., Bridgeview, IL) to the nearest 0.2 kg and nearest 0.1 cm, respectively. Infant weight and length were measured using a calibrated digital infant scale (Seca 334 Digital Baby Scale) to the nearest 0.01 kg and a standard infant length board to the nearest 0.1cm, respectively. Body mass index (BMI) was calculated for mothers and weight-for-age z-score (WAZ) and height-for-age z-score (HAZ) were calculated for infants using WHO growth standards [25].

3.3.4 Breast Milk and Blood Collection and Laboratory Analysis of Immunoglobulins and Cytokines

Immunoglobulins selected for analyses were: IgA, IgG1, IgG2, IgG3, IgG4, and IgM; cytokines were: interleukin (IL)-1β, IL-2, IL-6, IL-10, IL-12p70, IL-13, IL-15,

tumor necrosis factor (TNF)-α, and interferon (IFN)-γ. **Table 3.1** summarizes the *a priori* rationale for analyte selection.

Table 3.1. Immune molecule rationale for analysis

Molecule	Rationale	Breast milk-specific reference
IgA	Critical for mucosal immunity; concentration in milk associated with maternal HIV-infection and nutritional status (BMI); dominant immunoglobulin in human milk	4, 7, 11-14, 21, 26
IgG1 – IgG4	Binds to pathogens; concentrations in milk associated with maternal HIV-infection and nutritional status (BMI)	4, 11, 13, 21, 26
IgM	Concentration in milk associated with maternal HIV-infection; second most common antibody in human milk	4, 11, 13, 21, 26
IL-1β	Bio-activity reported in human milk; important inflammatory mediator	4, 26, 27, 28
IL-2	Involved in immunoglobulin and T-cell production	4, 27, 29
IL-6	Associated with mastitic milk; microbial protection against *Streptococcus pneumoniae*; inflammatory mediator	26, 30
IL-10	Associated with HIV viral load in breast milk; anti-inflammatory mediator; immunomodulation	26, 31
IL-12p70	Involved in regulation of inflammation and T-cell differentiation	4, 26, 27, 32
IL-13	Involved in immune response to enteric parasites, including *Schistosoma mansoni*	26, 33
IL-15	Associated with protection against postnatal HIV transmission; T-cell and natural killer cell proliferation	8
TNFα	Associated with HIV viral load in breast milk; pro-inflammatory cytokine; involved in the induction of acute phase response	26, 31, 34-36
IFNγ	Associated with modulation of neonatal immune development; key cytokine in regulating responses to viral, bacterial, and protozoan infections	4, 26, 33, 37

Maternal blood, infant blood, and hand-expressed breast milk samples were collected at each follow-up visit. Time of breast milk collection was noted, as was classification of fore or hind milk. Breast milk samples were stored in a polypropylene container at 4°C for ≤5 hours before transfer to the regional laboratory at the National Institute for Medical Research-Mwanza (NIMR). Breast milk samples were centrifuged and the milk fatty layer removed prior to storage at -20°C. Blood samples were collected in EDTA tubes, centrifuged, and the plasma was stored at -20°C (Appendix K, Appendix

L). Immunoglobulin and cytokine concentrations were measured using MAGPIX® instrumentation with xMAP® technology (Luminex Corporation) and MILLPLEX® MAG magnetic bead-based multianalyte panels (Cat. #HGAMMAG-301K and Cat. #HCYTOMAG-60K). Plasma assays were performed according to the manufacturer's protocol with standard curves and quality controls performed for each assay (Appendix N, Appendix O). After preliminary testing using a range of breast milk cell-free supernatant dilutions, optimal recovery of selected analytes was obtained in undiluted samples. In accordance with Groer, et al. [38], breast milk was assayed by adapting the manufacturer's protocol for plasma samples using a commercial serum matrix provided in the Millipore kit. Samples with concentrations below the limit of detection (LOD) were assigned a value corresponding to the LOD/$\sqrt{2}$ [39].

3.3.5 Statistical Analysis

Data were analyzed in STATA10 (STATA Corporation, Texas, USA). The means of normally distributed continuous variables were compared using Student's *t*-test and proportions of categorical variables were compared using the χ^2 test. Results were considered statistically significant at $\alpha = 0.05$, two-sided. Longitudinal trends were analyzed using multilevel mixed-effects linear regression. Correlation coefficients were calculated using Pearson's correlation for normally distributed variables and Spearman's correlation for non-normally distributed variables. In order to calculate total breast milk immunoglobulins for use in logistic regression, concentrations of IgA and IgM were converted from ng/ml to 10^{-7}g/ml, IgG1 and IgG2 were converted from ng/ml to μg/ml, and IgG3 and IgG4 were converted from ng/ml to 10^{-8}g/ml. The converted concentrations were summed to create a "unit-less" total immunoglobulin concentration.

Il-1β, IL-2, and IL-10 in breast milk and IL-13 in plasma were <LOD in all participants for at least one time point and were therefore excluded in the total cytokine concentration calculation, which represented the sum of the remaining cytokines (measured in pg/ml). Univariate and multivariate multiple linear regression models were used to estimate the association between maternal and infant characteristics and total breast milk cytokine and immunoglobulin concentrations.

3.4 Results

3.4.1 Participant Characteristics

Of the 125 pregnant women enrolled, 23 (18%) exited the study between enrollment prior to delivery and the first follow-up visit at Month 1 (**Figure 3.1**). Participants who exited during this period did not significantly differ from the rest of the study population based on demographic variables or HIV status at enrollment.

The majority of mothers were well-nourished: the prevalence of underweight in mothers (defined by a BMI < 18.5 kg/m^2) ranged from 9 to 15% over the six-month study period. Maternal post-partum nutritional status, based on BMI, did not differ according to maternal HIV status, and gestational age at birth was not significantly different between HIV-exposed and HIV-unexposed infants. However, as expected, HIV-exposed infants had significantly lower birth weight than HIV-unexposed infants (3.05 kg vs. 3.26 kg, $p = 0.020$) and early infancy anthropometric differences continued, as HIV-exposed infants experienced significantly lower WAZ than HIV-unexposed infants (**Table 3.2**). Infant malnutrition in the form of stunting (HAZ <-2) was common among all infants (45 to 56%), regardless of HIV-exposure, with prevalence increasing over the six-month

study period. The duration of EBF was short among all women, with the mean being only four weeks. However, on average, HIV-positive women continued EBF for three weeks longer than HIV-negative women. In early infancy, HIV-positive and HIV-negative women did not differ significantly in breastfeeding status (Month 1 EBF = 44 vs. 23%; predominant breastfeeding = 44 vs. 51%; partial breastfeeding = 13 vs. 26%; no breastfeeding = 0%, $p = 0.067$). However, from Month 2 through Month 6, HIV-positive women significantly differed in infant feeding method, where HIV-exposed infants had a greater proportion of breast milk in their diet than HIV-unexposed infants (Month 2, $p = 0.030$; Month 3, $p = 0.009$; Month 6, $p = 0.001$). Consistent differences in breastfeeding intensity or food security based on HIV-status were not apparent (**Table 3.2**).

Table 3.2. Characteristics of Mothers and Infants from birth through six months post-partum in rural Northwest Tanzania.

	WOMEN		
	All	**HIV+**	**HIV-**
Sample Size	102	39	63
Age (years)			
Mean (SD)	28.4 (5.9)	29.4 (6.0)	27.7 (5.8)
Parity (number of children)			
Mean (SD)	2.6 (1.7)	2.7 (1.9)	2.5 (1.6)
% Primiparous (n)	9% (9)	8% (3)	10% (6)
CD4 cell count (cells/μL)			
Median (IQR)		459 (330, 774)	
Mastitis			
% at Month 1 (n)	4% (4)	5% (2)	4% (2)
% at Month 2 (n)	4% (4)	3% (1)	5% (3)
% at Month 3 (n)	5% (5)	3% (1)	7% (4)
% at Month 6 (n)	8% (7)	9% (3)	7% (4)
Maternal Fever			
% at Month 1 (n)	24% (22)	21% (8)	26% (14)
% at Month 2 (n)	29% (26)	24% (8)	32% (18)
% at Month 3 (n)	29% (28)	30% (11)	29% (17)
% at Month 6 (n)	43% (40)	46% (16)	42% (24)
Maternal Cough			
% at Month 1 (n)	20% (19)	23% (9)	19% (10)
% at Month 2 (n)	17% (15)	15% (5)	18% (10)
% at Month 3 (n)	19% (18)	14% (5)	22% (13)
% at Month 6 (n)	30% (28)	40% (14)	25% (14)
Maternal BMI			
Month 1 Mean (SD)	22.0 (2.6)	22.2 (2.7)	21.8 (2.5)
Month 2 Mean (SD)	21.9 (2.6)	22.0 (2.6)	21.8 (2.6)
Month 3 Mean (SD)	21.9 (2.8)	21.9 (2.9)	21.8 (2.7)
Month 6 Mean (SD)	21.6 (2.7)	21.8 (2.7)	21.6 (2.8)
Food Security Index (FSI)			
Month 1 Mean (SD)	13.0 (2.1)	12.2 (2.5)*	13.6 (1.6)*
Month 2 Mean (SD)	12.7 (2.1)	12.3 (2.5)	13.0 (1.9)
Month 3 Mean (SD)	12.5 (2.1)	12.2 (2.2)	12.7 (2.0)
Month 6 Mean (SD)	12.1 (2.4)	11.5 (2.5)*	12.5 (2.2)*
	INFANTS		
	All	**HIV-exposed**	**HIV-unexposed**
Gestational Age at Birth (weeks)			
Mean (SD)	38.2 (3.5)	37.7 (3.0)	38.5 (3.7)
% Preterm (< 37 weeks) (n)	29% (28)	35% (13)	25% (15)
Infant Birth Weight (kg)			
Mean (SD)	3.2 (0.44)	3.1 (0.48)*	3.3 (0.39)*
% Low birth weight (< 2.5kg) (n)	8% (8)	15% (6)*	3% (2)*

	INFANTS		
	All	**HIV-exposed**	**HIV-unexposed**
Weight-for-Age Z-Score (WAZ)			
Month 1 Mean (SD)	-0.19 (0.95)	-0.50 (1.13)*	0.03 (0.73)*
Month 2 Mean (SD)	-0.13 (1.01)	-0.55 (1.17)*	0.13 (0.81)*
Month 3 Mean (SD)	-0.27 (1.12)	-0.69 (1.12)*	0.01 (1.03)*
Month 6 Mean (SD)	-0.37 (1.04)	-0.79 (1.13)*	-0.11 (0.88)*
Height-for-Age Z-Score (HAZ)			
Month 1 Mean (SD)	-1.73 (1.44)	-1.95 (1.58)	-1.58 (1.32)
Month 2 Mean (SD)	-1.78 (1.36)	-2.17 (1.43)*	-1.54 (1.26)*
Month 3 Mean (SD)	-1.87 (1.34)	-2.03 (1.31)	-1.77 (1.36)
Month 6 Mean (SD)	-2.33 (1.44)	-2.67 (1.20)	-2.12 (1.54)
Duration of Exclusive Breastfeeding (weeks)			
Mean (SD)	4.4 (6.5)	6.0 (7.5)	3.4 (5.6)
Breastfeeding Intensity (times/day)			
Month 1 Mean (SD)	11.8 (4.7)	11.8 (4.3)	11.9 (5.0)
Month 2 Mean (SD)	10.9 (4.6)	11.4 (4.7)	10.6 (4.5)
Month 3 Mean (SD)	10.7 (3.9)	11.8 (3.8)*	10.1 (3.8)*
Month 6 Mean (SD)	9.2 (4.4)	9.0 (4.5)	9.4 (4.4)
Infant Fever			
% at Month 1 (n)	26% (24)	33% (13)	20% (11)
% at Month 2 (n)	29% (26)	31% (11)	27% (15)
% at Month 3 (n)	42% (41)	45% (17)	41% (24)
% at Month 6 (n)	66% (61)	56% (20)	72% (41)

**p < 0.05, comparing HIV-positive/exposed vs. HIV-negative/unexposed.*

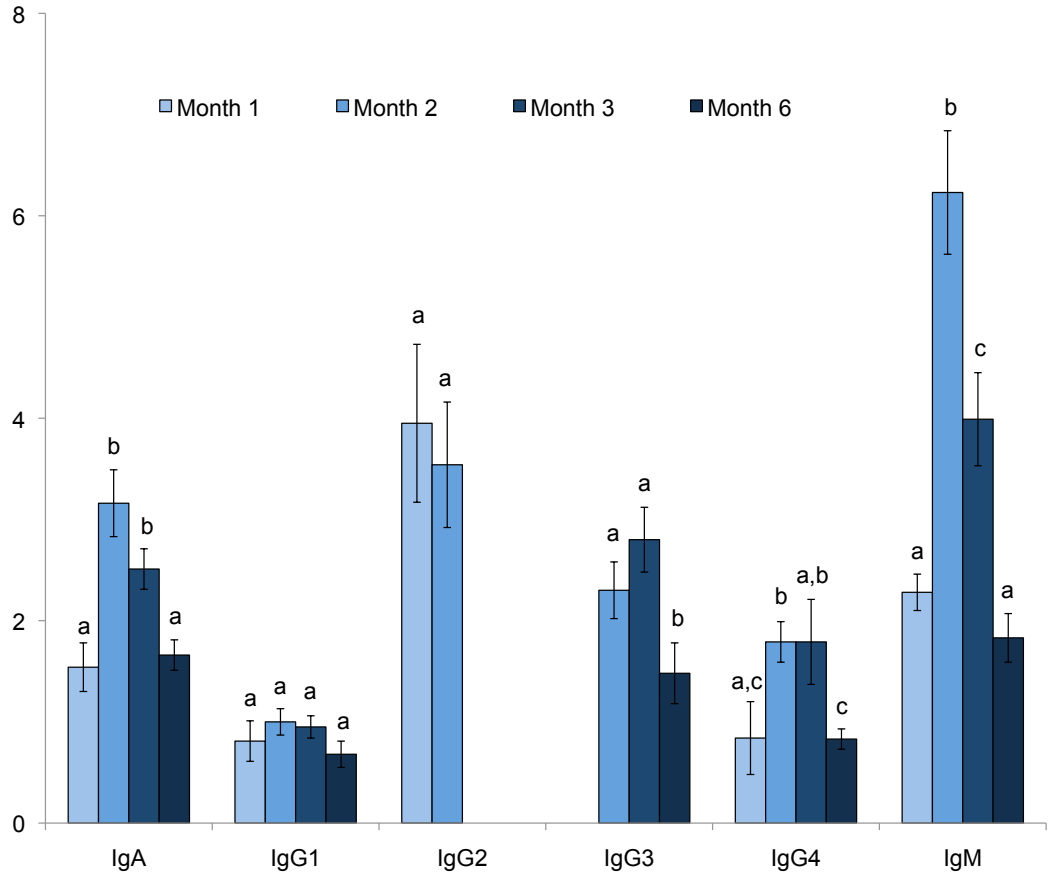
Overall, HIV-positive women were not severely immunosuppressed at enrollment: 45% of HIV-positive women had CD4 cell counts above the normal lower limit (CD4 >500 cells/μL), while only 9% of women had CD4 cell counts <200 cells/μL. Results from the questionnaire indicated that self-reported maternal morbidity did not differ based on maternal HIV-status (**Table 3.2**). The most commonly reported maternal symptoms for all women were fever (24 to 43%) and cough (17 to 30%), while other self-reported symptoms, including diarrhea, skin rash, vomiting, abdominal pain, anorexia, nausea, fatigue, and difficulty breathing were rare (data not shown). Self-reported mastitis was also rare (4 to 8%) among HIV-positive and HIV-negative women. Infant

fever was a common symptom, with the percentage increasing from 26% at Month 1 to 66% at Month 6. Other self-reported infant symptoms, including diarrhea, cough, breathing problems, convulsions, vomiting, skin rash, and anorexia did not significantly differ between HIV-exposed and HIV-unexposed infants throughout the study period (data not shown).

3.4.2 Mature Breast Milk Immunology

Breastfeeding exclusivity and intensity were associated with differential immunoglobulin concentration: the practice of EBF was associated with significantly greater breast milk concentrations of IgA (EBF = 263.4 vs. No EBF = 192.4, p = 0.048), IgG1 (EBF = 1638.4 vs. no EBF = 702.4, p = 0.003), IgG2 (EBF = 6918.4 vs. no EBF = 3299.6, p = 0.033), and IgG3 (EBF = 40.3 vs. no EBF = 17.7, p <0.001) (Appendix D). Neither maternal HIV status nor maternal malnutrition were associated with significant differences in concentrations of any immunoglobulins measured (Appendix D), although IgG1 concentration was consistently higher in breast milk of HIV-positive women at each time point (Month 1: HIV-positive = 1033.0 vs. HIV-negative = 667.8, p = 0.365; Month 2: HIV-positive = 1060.7 vs. HIV-negative = 975.0, p = 0.756; Month 3: HIV-positive = 1114.4 vs. HIV-negative = 834.2, p = 0.219; Month 6: HIV-positive = 857.9 vs. HIV-negative = 584.2, p = 0.325). Since immunoglobulin concentrations did not differ according to HIV status, all women were combined for subsequent analyses. In this combined analysis, IgA, IgG3, IgG4, and IgM varied during lactation, with an inverted U-shaped pattern apparent over the six-month study period (**Figure 3.2**).

Figure 3.2. Mean breast milk immunoglobulin concentrations from one to six months post-partum in mothers from rural northwest Tanzania.

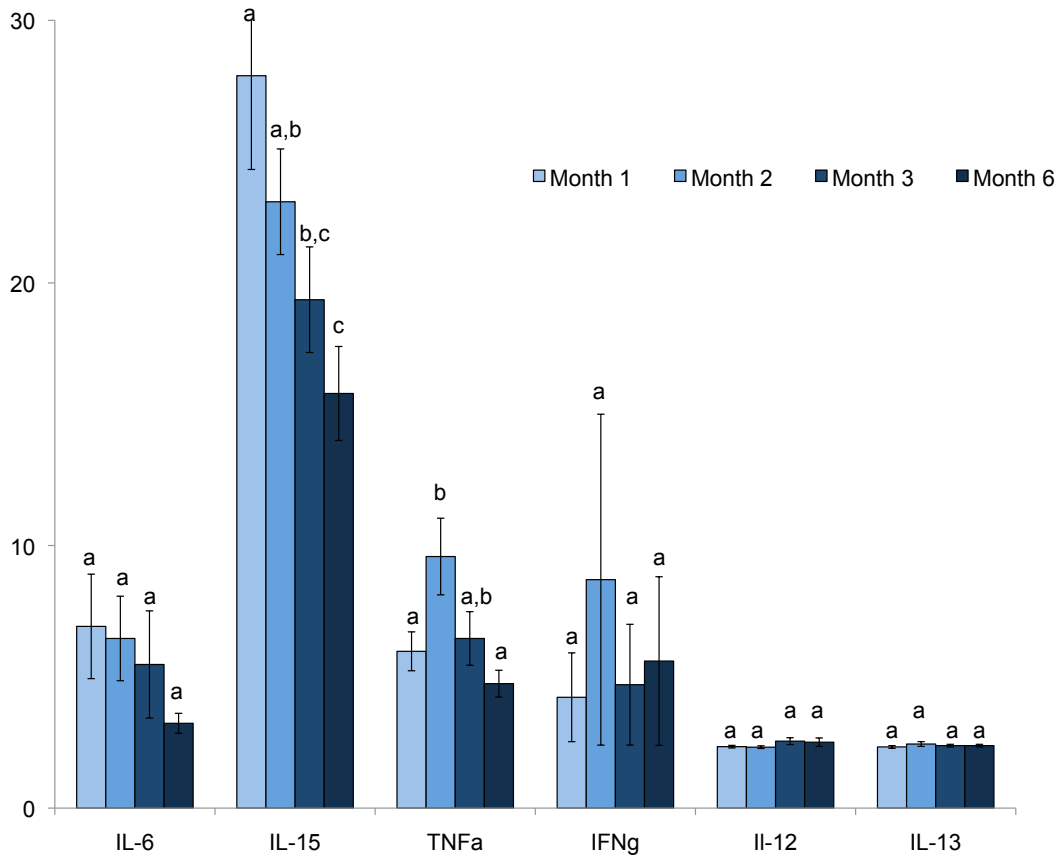


Note: Different letters above bars indicate that concentrations are significantly different, two-sided t-test, $p < 0.05$

Like immunoglobulins, breast milk cytokine concentrations varied according to breastfeeding practices, but not according to maternal HIV or nutritional status (Appendix E). Mothers who EBF at the time of breast milk collection had significantly higher concentrations of breast milk IL-12p70 at Months 2 and 3 (Month 2: EBF = 2.58 vs. no EBF <LOD, p = 0.006; Month 3: EBF = 3.31 vs. no EBF = 2.42, p = 0.017). TNFα concentration indicated an inverted U-shaped pattern during lactation, IL-6 and IL-15

concentrations exhibited a linear decrease, while IFNγ, IL-12p70, and IL-13 were static and IL-1β, IL-2, and IL-10 remained undetected (**Figure 3.3** and Appendix E).

Figure 3.3. Longitudinal patterns of mean breast milk cytokine concentrations from one to six months post-partum in mothers from rural northwest Tanzania.



Note: Different letters above bars indicate concentrations are significantly different, two-sided t-test, $p < 0.05$

Correlations ranged from negligible to strong when examining concentrations between biological compartments (e.g. maternal serum and breast milk or breast milk and infant serum) (**Table 3.3**). The data showed weak to moderate positive correlations between maternal serum and breast milk for IgA, IgG2, IgG4, IgM, and total immunoglobulins (**Table 3.3a**, left panel), while only TNFα concentrations at Month 2

were moderately correlated among cytokines (**Table 3.3b**, left panel). Breast milk and infant serum immunoglobulins were consistently correlated in early infancy through Month 2, with weak to strong positive correlations apparent for each immunoglobulin, and no consistent correlation evident thereafter (**Table 3.3a**, right panel). Cytokine correlations between breast milk and infant serum revealed a more complex correlation matrix with both positive and negative correlations evident (**Table 3.3b**, right panel).

Table 3.3. Pair-wise correlation between concentrations of immunoglobulins (a) and cytokines (b) in maternal serum vs. breast milk (left panel), and breast milk vs. infant serum (right panel).

	Maternal Serum: Breast Milk				**Breast Milk: Infant Serum**			
	Month 1	Month 2	Month 3	Month 6	Month 1	Month 2	Month 3	Month 6
a								
IgA	0.19	0.24	0.24	0.14	0.49	0.35	-0.19	-0.28
IgG1	0.13	0.15	0.11	NA	-0.18	0.20	-0.15	0.04
IgG2	0.03	0.24	NA	NA	0.24	-0.08	NA	NA
IgG3	NA	0.09	0.11	-0.03	NA	0.15	-0.12	-0.11
IgG4	0.20	0.18	0.07	0.01	0.20	0.43	-0.14	-0.01
IgM	0.09	0.34	0.14	NA	-0.01	0.41	-0.13	0.17
Total Ig	0.12	0.14	0.20	0.22	0.12	0.17	-0.15	-0.06
b								
IFNγ	-0.09	-0.12	-0.13	0.16	ND	ND	-0.08	ND
TNFα	0.10	0.34	0.16	-0.02	0.00	-0.23	0.25	-0.11
IL-6	-0.07	0.03	-0.07	-0.05	0.33	ND	-0.11	ND
IL-12p70	-0.09	ND	0.08	-0.06	ND	ND	-0.14	ND
IL-15	0.09	-0.06	0.01	0.12	-0.08	ND	ND	-0.26
Total Cyt	0.01	0.11	0.05	0.11	0.14	-0.24	0.11	-0.80

Strong Negative (-1 to -0.4)	Moderate Negative (-0.3 to -0.39)	Weak Negative (-0.2 to -0.29)	Negligible (-0.19 to 0.19)	Weak Positive (0.2 to 0.29)	Moderate Positive (0.3 to 0.39)	Strong Positive (0.4 to 1)

Note: NA = not available; ND = not detected

Table 3.4 summarizes maternal and infant health and nutrition associations with total breast milk immunoglobulins and cytokines. Total immunoglobulin concentrations were consistently positively associated with breastfeeding practices: EBF or more

frequent feeding was associated with higher total breast milk immunoglobulins throughout lactation. Gestational age at birth was inversely associated with total immunoglobulins in early infancy only. Maternal health status, including mastitis, fever, and cough, was associated with higher total breast milk cytokine concentrations in univariate and multivariate regression analyses. Interestingly, fever in infants was also significantly associated with breast milk cytokines, where the presence of fever was associated with lower total cytokine concentration. Breast milk from mothers with infants who had higher birth weight and weight-for-age also had significantly lower total cytokine concentration at Month 1 post-partum. More frequent breastfeeding episodes were associated with significantly lower total cytokines in breast milk at Month 6. Including classification of fore/hind milk or time of milk collection in the multivariate models did not affect any of the estimates (data not shown).

Table 3.4. Univariate and multivariate multiple linear regression estimators of total breast milk immunoglobulin (left panel) and cytokine (right panel) concentrations

	Total Breast Milk Immunoglobulin Concentration				Total Breast Milk Cytokine Concentration			
	Month 1	Month 2	Month 3	Month 6	Month 1	Month 2	Month 3	Month 6
Maternal Health								
HIV-Status								
Univariate	1.10	-0.53	-1.14	1.26	9.87	-15.04	-1.06	3.60
Adjusted	-1.94	-3.58	-2.27	-0.08	-6.51	-9.31	2.98	2.80
Mastitis								
Univariate	3.04	-2.86	-6.39	-1.79	65.45**	40.68	8.58	15.97
Adjusted					76.42**	6.30	7.60	13.44
Fever								
Univariate	-3.78	-2.53	-3.46	-0.58	19.78	33.50	-4.73	17.69*
Adjusted					9.18	41.40	4.34	18.55
Cough								
Univariate	-4.51	-4.62	-3.52	-1.65	9.56	-23.02	6.30	25.50**
Adjusted					9.03	-38.65	17.20	21.43*
Maternal Nutrition								
BMI								
Univariate	0.85	0.51	0.67	0.32	-0.69	3.95	2.78	0.56
Adjusted	0.71	0.47	0.64	0.31	0.50	3.02	2.48	0.57
Food Security								
Univariate	-1.17	-0.41	-0.47	-0.40	2.12	2.96	4.10	1.53
Adjusted	-1.17	-0.44	-0.35	-0.28	4.03	3.30	3.65	2.78
Infant Health								
Fever								
Univariate	1.60	1.25	5.86	0.61	19.03	8.48	-13.28	-16.59
Adjusted					3.13	16.75	-18.06	-33.03**
Infant Nutrition								
Wks of EBF								
Univariate	0.52*	0.78**	0.31	0.31***	-0.13	0.93	0.48	0.36
Adjusted	0.57**	0.85**	0.31	0.29**				
BF Intensity								
Univariate	0.61*	0.22	0.06	0.67***	-1.89	-1.89	-0.17	-2.12*
Adjusted					-0.90	-2.51	-0.08	-2.53*
Birth Weight								
Univariate	-3.19	-3.17	-4.18	-2.04	-36.78**	1.41	1.98	-4.91
Adjusted					11.39	-15.51	-9.39	11.81
WAZ								
Univariate	0.17	-0.33	-0.35	-0.71	-16.23**	4.94	5.30	5.24
Adjusted					-16.22	-6.93	4.70	3.52
HAZ								
Univariate	-0.09	-1.63	1.07	0.01	-6.69	3.12	4.54	2.09
Adjusted					-1.67	10.42	2.88	0.42
Gest. Age								
Univariate	-0.88*	0.19	-0.63	-0.31	-1.08	-0.09	1.06	-0.24
Adjusted	-0.80*	0.22	-0.66	-0.18				
Immunoglobulin Model					**Cytokine Model**			
Model p value	**0.003**	**0.041**	0.145	**0.019**	**0.007**	0.511	0.446	**0.002**

**p < 0.05; ** p < 0.01; *** p < 0.001.*

Note: HIV-status: 0 = HIV-negative or 1 = HIV-positive; Mastitis: 0 = mother's self-report that she was not experiencing mastitis or 1 = mother's self-report that she was experiencing mastitis; Fever: 0 = mother's self-report that she was not experiencing fever or 1 = mother's self-report of fever; Cough: 0 = mother's self-report of that she was not experiencing cough or 1 = mother's self-report of cough; Infant Fever: 0 = mother's self-report that her infant was not experiencing fever or 1 = mother's self-report of infant fever.

[a]*Multivariate immunoglobulin model: total breast milk immunoglobulins = β(HIV-status) + β(maternal BMI) + β(food security) + β(weeks EBF) + β(gestational age at birth) + μ*

[b]*Multivariate cytokine model: total breast milk cytokines = β(HIV-status) + β(mastitis) + β(maternal fever) + β(maternal cough) + β(maternal BMI) + β(food security) + β(infant fever) + β(breastfeeding intensity) + β(infant birth weight) + β(WAZ) + β(HAZ) + μ*

3.5 Discussion

In this prospective cohort of rural HIV-positive and HIV-negative Tanzanian mothers and their infants, neither maternal HIV status nor the broad nutritional indicator of maternal BMI were associated with breast milk immunoglobulin or cytokine concentrations. However, considering breast milk immunology over time, significant differences in immunoglobulin and certain cytokine concentrations in mature milk were evident, and indicators of maternal and infant health and breastfeeding practices were associated with total concentrations in this setting. In addition, correlations between different biological compartments were evident for immunoglobulins and cytokines in both maternal serum-breast milk and breast milk-infant serum comparisons, with a more consistent association evident for immunoglobulin compartmental correlations.

Previous studies of colostrum (birth to ~1 week post-partum) and transitional milk (<4 weeks post-partum) measuring IgA, IgG, IL-6, IL-10, and TNFα have demonstrated a dramatic decrease in breast milk concentrations between birth and four weeks, leveling off thereafter [11, 12, 34]. While significant reductions in mature breast milk immune factors were not expected, the ongoing variation in mature milk, which comprises the

longer-term immunological bridge between mother and infant, may be clinically relevant. In many regions of the developing world, infant exposure to diarrheal and respiratory pathogens is chronic and, for enteric pathogens, likely to increase as EBF is replaced with mixed feeding and infants begin to explore their environment. Given the dynamic nature of pathogen exposure, the differences in breast milk immunology demonstrated by this study, alongside changes in immunocompetence as the infant immune system matures, justify further longitudinal investigation of breast milk immunology beyond the early post-partum period.

Most breastfeeding research in the context of maternal infection has focused on determinants of HIV transmission without characterizing specific breast milk immunology. In a Zambian study that included HIV-positive and HIV-negative women, Walter, et al. [8] reported that breast milk IL-15 was lower among HIV-positive mothers whose infants became infected with HIV compared to HIV-positive/non-transmitting mothers and HIV-negative mothers. In the current study, none of the infants tested positive for HIV, and breast milk from otherwise healthy HIV-positive women on ART was not significantly different from HIV-negative women living in the same African setting. Shapiro, et al. [21] reported that total combined immunoglobulin and individual IgA, IgG, and IgM breast milk concentrations were higher in HIV-positive/ART-naïve mothers compared to HIV-negative mothers in Botswana. Together, this suggests that generalizations regarding breast milk immunology based on maternal HIV-serostatus alone are not sufficient. While maternal HIV infection was not a key determinant of breast milk immunology in this study, localized infection (maternal mastitis) at Month 1 and generalized infection symptoms (maternal cough/fever and infant fever) at Month 6

were associated with breast milk cytokine concentrations. It is unlikely that the peak in symptom reporting at six months post-partum was due to seasonal infections as the Month 6 study visit occurred throughout the year for different pairs of mothers and infants.

The relationship between maternal nutritional status and specific breast milk immunology throughout lactation remains unclear. In this study, a statistically non-significant trend towards higher total breast milk immunoglobulins and cytokines was associated with improved nutritional status (e.g. higher maternal BMI), while greater food security was associated with lower total breast milk cytokine and higher total immunoglobulin concentrations. Among Taiwanese mothers, colostrum IgA concentration in well-nourished women was twice that of malnourished women in the first two weeks post-partum [12], while well-nourished Colombian mothers had higher transitional breast milk IgA and IgG concentrations at four weeks post-partum than malnourished mothers [11]. Differences were also observed in Congolese women, but only after four weeks post-partum when IgA was lower in the breast milk of underweight mothers [13]. Similar to the current study, Bachour, et al. reported no significant differences in IgA concentration from transitional or mature milk from healthy and obese Lebanese women [14].

This is the first study to describe immune factors in all three biological compartments of the post-partum mother-infant dyad, represented by maternal serum, breast milk, and infant serum. While the correlation matrix is complex, a number of positive correlations between maternal serum and breast milk immunoglobulin concentrations throughout lactation and between breast milk and infant serum in early

infancy are apparent. Both positive and negative cytokine correlations were observed among the compartments. Investigating associations between maternal serum during pregnancy and breast milk at three weeks post-partum, Soto-Ramirez, et al. [40] observed strong inverse correlations for IgA and strong positive correlations for IL-6, IL-13, and IFNγ in healthy American women. In another American study, maternal serum and breast milk concentrations of IgA, IL-6, and TNFα appeared uncorrelated in healthy women [35]. While correlations between biological compartments are apparent in the current study, additional research is needed to confirm the complex patterns observed and the utility of using a single compartment as a proxy for concentrations in another compartment. Further investigation is required to determine the biological relevance of immune concentrations across the mother-infant dyad to infectious disease protection throughout the breastfeeding period.

Preliminary evidence from this study indicates that unraveling the determinants of breast milk immunology requires a broader consideration of potentially modifiable factors. For instance, breastfeeding practices (e.g. EBF, feeding intensity) that have rarely been considered in prior studies, had the most significantly positive association with breast milk immunoglobulin concentrations. In some of the only data available regarding breastfeeding practices and concentration of breast milk immunoglobulins, a European study of healthy, rural women reported an inverse relationship between duration of breastfeeding and breast milk IgA [17]. However, comparisons with the current study are limited because the collection of milk samples did not correspond in time with the reporting of breastfeeding practices. Furthermore, that study reports only the duration of breastfeeding rather than EBF, specifically. While Orivuori, et al. [17] speculate that

their findings may be attributed to subclinical breast tissue inflammation as a result of a short duration of breastfeeding, the current study found no association between self-reported mastitis and duration of EBF or breast milk IgA concentration.

In summary, findings from this study shed light on the complex and dynamic immunological bridge breast milk provides to HIV-exposed and HIV-unexposed infants in a rural Tanzanian setting during early infancy. While mature breast milk immunoglobulin and cytokine profiles do not appear considerably different based on maternal HIV status or maternal nutritional status, other relevant factors were identified. Generalized symptoms of maternal and infant infection, such as fever, cough, and mastitis, as well as breastfeeding practices were also associated with differential breast milk immune composition. Given the suboptimal breastfeeding practices that are common in sub-Saharan Africa, alongside high infant morbidity and mortality due to diarrheal and respiratory infections, further exploration of the public health and clinical implications of breast milk immunology in resource-poor settings is crucial.

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CHAPTER 4

BREAST MILK IMMUNE COMPOSITION AND INFANT FEEDING PRACTICES ARE ASSOCIATED WITH EARLY INFANCY *CRYPTOSPORIDIUM* INFECTION

4.1 Abstract

Background: Cryptosporidium is an important cause of infant morbidity and mortality, yet little is known about how infant feeding, including exclusive breastfeeding (EBF) and complementary foods, and breast milk immunoglobulins and cytokines are related to infection.

Methods: This prospective cohort enrolled 125 mothers/infants of mixed HIV-status/exposure and followed them from birth through six months. At each follow-up (Month 1, Month 2, Month 3, Month 6), questionnaires were administered to determine infant feeding practices, and breast milk, blood, and feces were collected. Breast milk and blood were analyzed for immunoglobulins and cytokines while feces were analyzed for *Cryptosporidium*.

Results: Cryptosporidium was prevalent among mothers at all time points and in infants at Month 6, though the majority of infections were of low intensity. Duration of EBF was negatively associated with infant *Cryptosporidium* (OR=0.95, 95% CI=0.88, 1.03), while many complementary foods were positively associated with infection. Consumption of either grains or water increased odds of infant *Cryptosporidium* (OR=7.78, 95% CI=2.15, 28.18; OR=4.62, 95% CI=1.27, 16.85, respectively). Higher breast milk concentrations of immunoglobulins and cytokines were associated with lower

odds of infant *Cryptosporidium* (OR=0.92, 95% CI=0.77, 1.09; OR=0.73, 95% CI=0.17, 3.22, respectively).

Conclusions: Appropriate infant feeding, ie. EBF and avoidance of complementary foods until six months, is strongly associated decreased risk of early infancy *Cryptosporidium*. Future research and public health strategies need to address the association between infant feeding practices and *Cryptosporidium* infection in order to reduce the burden of diarrhea in infancy.

4.2 Introduction

Diarrhea continues to be a leading cause of morbidity and mortality among infants in the developing world [1]. Almost half of child deaths attributable to diarrhea occur in sub-Saharan Africa, and the most common diarrhea-causing protozoan parasite found in children at health facilities is *Cryptosporidium* [1]. The effects of *Cryptosporidium* infection can range from asymptomatic to severe, chronic diarrhea and an increased risk of infant mortality [2]. Even in the absence of acute symptoms, *Cryptosporidium* infection can have damaging effects on the growth and development of children [3].

The detrimental health and nutritional consequences of early *Cryptosporidium* infection coupled with the lack of effective *Cryptosporidium*-treatment options for young infants and those with HIV/AIDS make it crucial to understand how to protect young infants from infection. Both the immunological content of breast milk (BM) and the duration of exclusive breastfeeding (EBF) may impact an infant's exposure and susceptibility to diarrheal pathogens, such as *Cryptosporidium***.** Breastfeeding may provide protection from intestinal pathogens through the transfer of immune molecules

and avoidance of contaminated food/water. Previous cross-sectional studies investigating the effect of breastfeeding on infant *Cryptosporidium* have found conflicting results. Studies in Mexico, Egypt, Botswana, Israel, and Haiti reported that breastfeeding infants were less likely to experience *Cryptosporidium* [4-8], yet a study in Zambia found that breastfeeding was associated with an increased risk of infection [9]. To our knowledge, there have been no prospective longitudinal studies examining the duration of EBF (as defined by the World Health Organization [WHO]) on *Cryptosporidium* among infants in sub-Saharan Africa.

Several studies also suggest an association between cytokines and immunoglobulins and both the primary immune response to *Cryptosporidium* as well as the development of partial immunity to subsequent infection [10-14]. This may result from the wide array of immune molecules - passively transferred to the infant via BM during the lactation period - conferring partial protection from enteric pathogens. Immunoglobulins are the most commonly studied BM immune molecules, yet the relationship between concentrations of BM immunoglobulins and the risk of infant *Cryptosporidium* has rarely been studied. In the only available study, BM with high concentrations of anti-*Cryptosporidium* Immunoglobulin A (IgA) was associated with a 38% reduced risk of *Cryptosporidium* infection and a 64% reduced risk of cryptosporidiosis in Bangladeshi infants [15]. Protection may occur because BM IgA coats the infective stages of *Cryptosporidium* and may prevent excystation, attachment, or invasion of host epithelial cells [15]. Cytokines are also immune molecules found in BM and they enhance and initiate immune functions that are poorly expressed in the infant [16]. To date there have been no studies

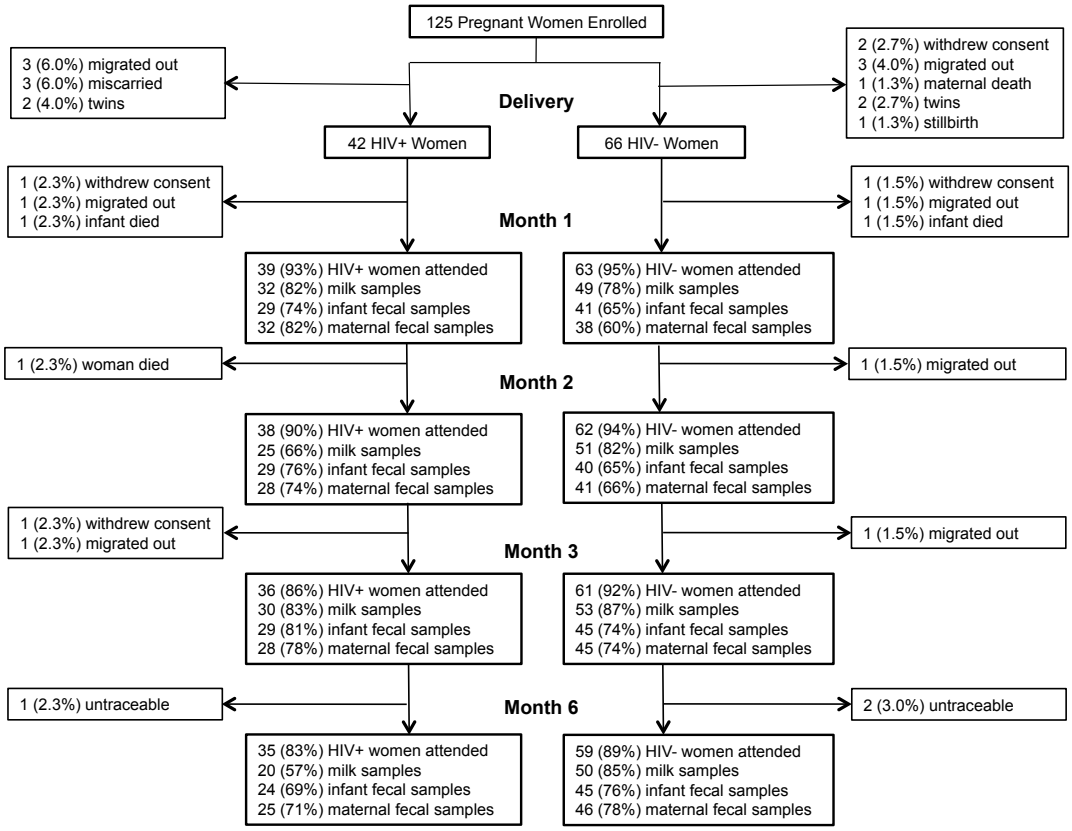
investigating the association between BM cytokine concentrations and risk of *Cryptosporidium* in infancy.

The aim of this research was to determine how early infant feeding practices and breast milk immune composition are related to early infant *Cryptosporidium* infection**.** I hypothesized that a longer duration of EBF and higher concentrations of BM cytokines and immunoglobulins would be associated with a decreased prevalence of *Cryptosporidium* in infants from birth through six months post-partum.

4.3 Methods

This prospective birth cohort enrolled 50 HIV-positive and 75 HIV-negative pregnant mothers living in rural/semi-rural areas of Kisesa (population 30,000) [17] in northwestern Tanzania. The study was based at Kisesa Health Centre (KHC), which houses an antenatal clinic (ANC), under-5 clinic, voluntary counseling and testing clinic (VCT), and an HIV/AIDS care and treatment center (CTC). **Figure 4.1** outlines the flow of study participants and sample collection. Recruitment and retention procedures have been reported in detail elsewhere [18]. The study protocol was approved by the Tanzania National Health Research Ethics Review Committee and Cornell University (ClinicalTrials.gov, number NCT01699841).

Figure 4.1. Flow of study participants



At each follow-up (Month 1 [M1], Month 2 [M2], Month 3 [M3], Month 6 [M6]), the research nurse administered a questionnaire that was designed to obtain data on a range of feeding, health, and environmental risk factors (Appendix C, Appendix J). To accurately determine infant feeding patterns at each visit, mothers were presented with a list of complementary foods and liquids and asked whether they had fed each to their infant in the previous month. EBF was defined according to the WHO definition where "the infant receives BM and allows the infant to receive oral rehydration solution, drops, vitamins, minerals, medicines, but nothing else" [19]. Duration of EBF was defined as

time from birth until an infant first received food or liquids other than BM or medicines. In order to construct a 'breastfeeding (BF) score', at each visit infants were assigned a score that corresponded to their current feeding pattern (EBF = 3; predominant BF [BM plus locally-prepared gripe water] = 2; partial BF [BM plus other foods/liquids] = 1; not breastfeeding = 0) and these scores were summed across the four visits to create a BF score that ranged from 0 to 12. Diarrhea was defined as loose or watery stools ≥3 times/day that represented a pattern atypical for that individual [1]. A food security index was constructed using a series of five questions modified from the Household Food Insecurity Access Scale that has been validated in rural Tanzania [20].

At each visit, maternal height and weight were measured using a standard stadiometer (Health O Meter, Inc., Bridgeview, IL) to the nearest 0.2kg and nearest 0.1cm, respectively. Infant weight and length were measured using a calibrated digital infant scale (Seca 334 Digital Baby Scale) to the nearest 0.01kg and a standard infant length board to the nearest 0.1cm. Weight-for-height z-scores (WHZ) and body mass index (BMI) were calculated based on WHO growth standards [21].

Maternal blood, infant blood, and hand-expressed BM samples were collected at each follow-up visit. BM samples were stored in a polypropylene container at 4°C for ≤5 hours before removal of the milk fatty layer and storage at -20°C. Blood samples were collected in EDTA tubes, centrifuged, and plasma stored at -20°C (Appendix K, Appendix L). Immunoglobulin and cytokine concentrations were measured using MAGPIX® instrumentation with xMAP® technology (Luminex Corporation) and MILLPLEX® MAG magnetic bead-based multianalyte panels (Cat. #HGAMMAG-301K and Cat. #HCYTOMAG-60K). Plasma assays were performed according to the

manufacturer's protocol with standard curves and quality controls for each assay (Appendix N, Appendix O). Undiluted BM was assayed by adapting the manufacturer's protocol for plasma samples, using a commercial serum matrix provided in the Millipore kit [22].

Active *Cryptosporidium* case detection was of interest, so maternal and infant fecal samples were collected, irrespective of symptoms, at each visit. Within 24 hours of collection, approximately 5g of fresh stool was mixed with 5mL 10% v/v formalin. Presence of *Cryptosporidium* was confirmed using a modified Ziehl-Neelsen staining procedure (Appendix P) [23]. A single technician, without knowledge of participant clinical status, examined slides; *Cryptosporidium* oocyst count was recorded, and infection was defined as ≥1 oocyst. A second technician, blinded to participant *Cryptosporidium* status, re-examined a sample (10%) of the slides and inter-observer agreement was 96%. There is currently no treatment available for *Cryptosporidium* in HIV-positive adults or children under 12 months nor are there Tanzanian National Guidelines for the treatment of *Cryptosporidium*, so infected individuals were referred to a clinical officer if they were symptomatic.

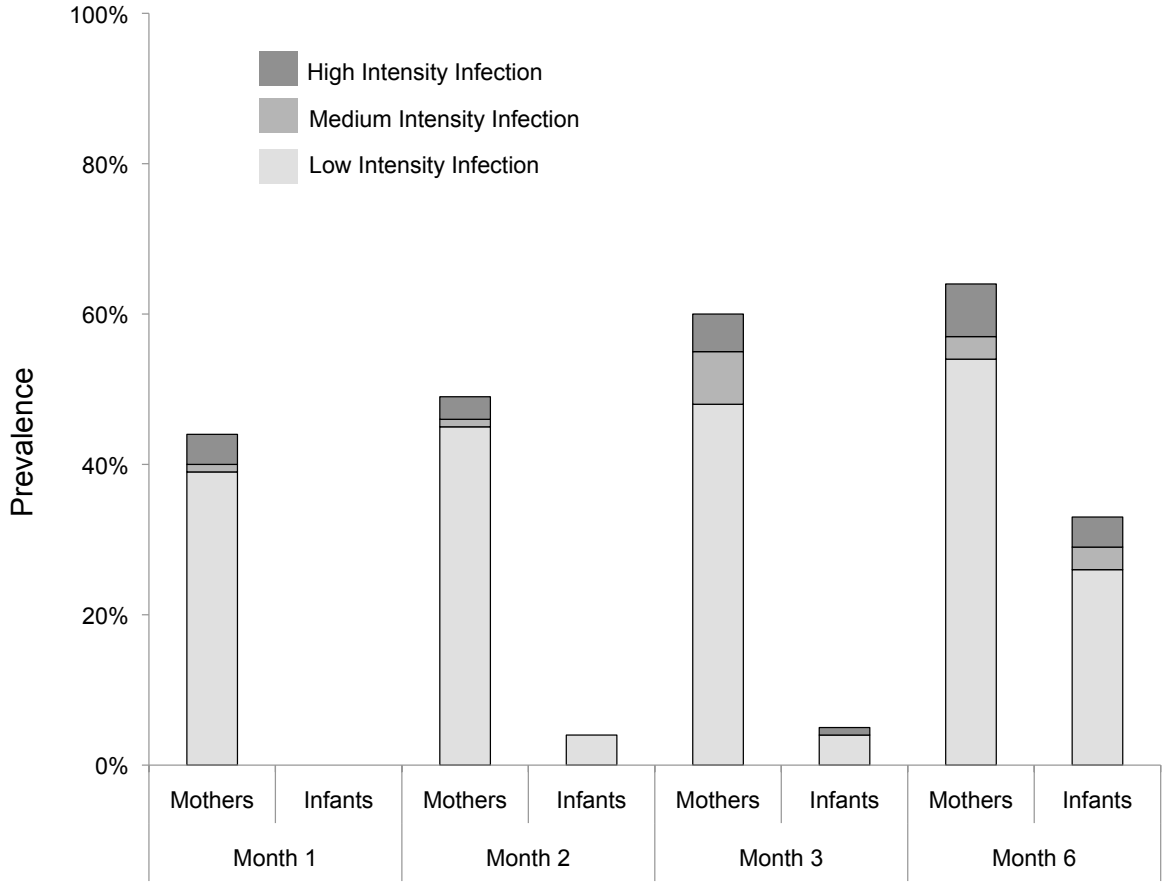
Data were analyzed in STATA10 (STATA Corporation, Texas, USA). Means of normally distributed continuous variables were compared using Student's *t*-test and proportions of categorical variables were compared using the χ^2 test. Results were considered statistically significant at $\alpha = 0.05$, using two-sided significance tests. To calculate total BM immunoglobulins, concentrations of IgA and IgM were converted from ng/ml to 10^{-7}g/ml, IgG1 and IgG2 were converted from ng/ml to µg/ml, and IgG3 and IgG4 were converted from ng/ml to 10^{-8}g/ml. The converted concentrations were

summed to create a "unit-less" total immunoglobulin concentration. Cytokine concentrations were not normally distributed, therefore concentrations were log-transformed prior to statistical analysis and results are presented as $\log_{10}$[cytokine concentration]. I constructed a breast milk cytokine and immunoglobulin index by multiplying the concentration of each analyte by the BF score at that visit; a high index of immunoglobulins/cytokines was defined as a value greater than the median, while a low index was a value less than the median. Univariate and multivariate logistic regression models were used to estimate the effect of infant feeding practices and BM immune profiles, identified *a priori,* on odds of infant *Cryptosporidium* in the first six months.

4.4 Results

Over the six-month study period, 282 infant fecal samples (39% from HIV-exposed infants) and 283 maternal fecal samples (40% from HIV-positive mothers) were analyzed for *Cryptosporidium* (**Figure 4.1**). The prevalence of *Cryptosporidium* in mothers increased from 44% at M1 to 63% at M6, with no significant differences based on maternal HIV-status. The majority of maternal infections were of low intensity throughout the study (**Figure 4.2**). Infants remained free of *Cryptosporidium* until M2, when the prevalence of infection was 4% (3/69). The first high intensity infant infection did not occur until M3, although overall prevalence of infection remained low until M6 when 33% (23/69) of infants were infected (**Figure 4.2**). The intensity of *Cryptosporidium* infection was not associated with any demographic, clinical, or anthropometric characteristics of the participants (data not shown).

Figure 4.2. Prevalence of *Cryptosporidium* infection in mothers and infants in rural northwest Tanzania from Month 1 through Month 6 post-partum.



High intensity >40 oocysts; medium intensity 20 to 40 oocysts; low intensity 1 to 19 oocysts.

Table 4.1 describes the demographic, clinical, and nutritional characteristics of the infants by *Cryptosporidium* status, with 27 (28%) infants having at least one *Cryptosporidium*-positive stool sample between M1 and M6. Infant cough (76%) and fever (79%) were the most commonly reported clinical symptoms, while the prevalence of stunting was high (55%). Participants did not differ in any characteristics based on infant *Cryptosporidium*, except for maternal underweight, where infection was more

common in infants of underweight mothers (**Table 4.1**). Although not significantly different, households with infant *Cryptosporidium* in the first six months tended to get water from public taps (44%), while households with uninfected infants tended to get water from covered wells/boreholes (45%).

Table 4.1. Socio-demographic, clinical, and nutritional characteristics of 98 mothers and infants in rural northwest Tanzania, by infant *Cryptosporidium* infection in the first six months.

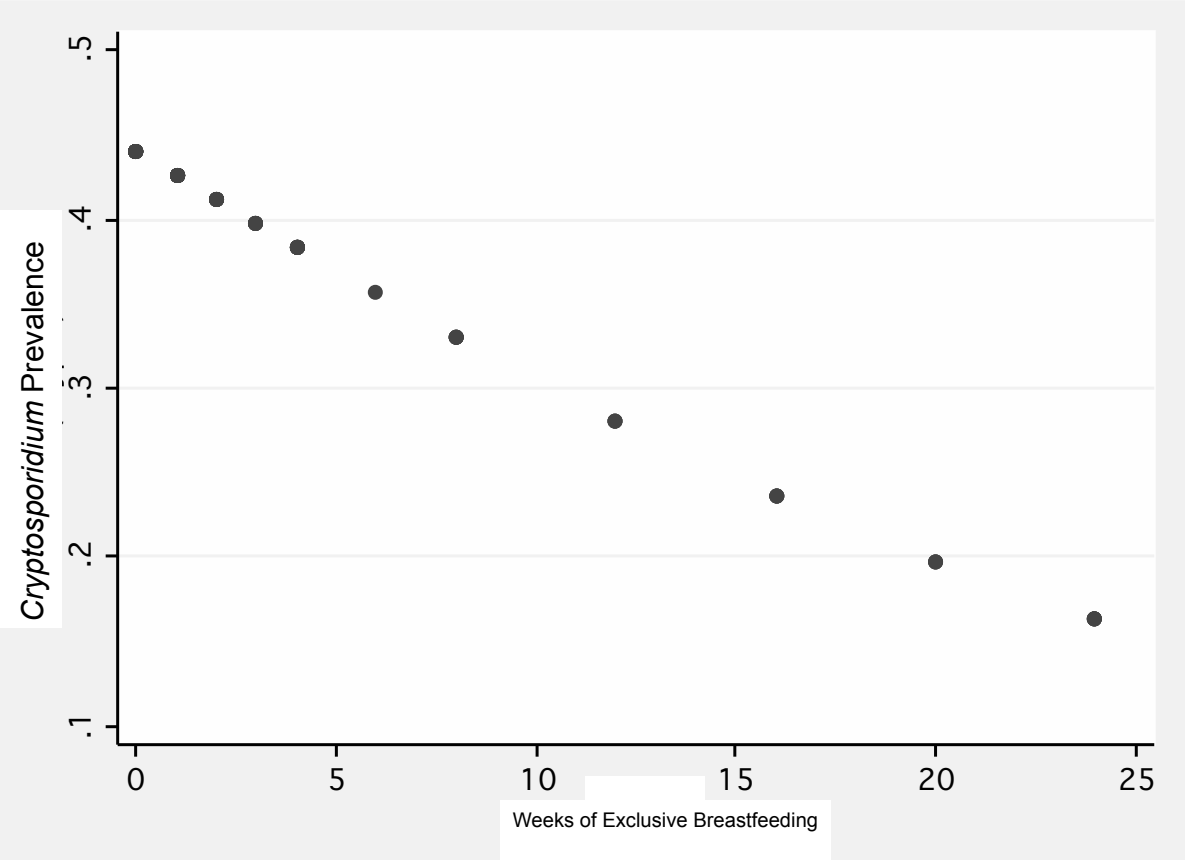
Characteristic, % (n)	Infant stool sample ever positive for *Cryptosporidium* (n = 27)	Infant stool sample always negative for *Cryptosporidium* (n = 71)	p
Socio-demographic			
Sex			0.726
Male	48% (13)	52% (37)	
Female	52% (14)	48% (34)	
Maternal Literacy			0.645
Literate	78% (21)	73% (52)	
Non-literate	22% (6)	27% (19)	
Family Size			0.667
≤ 5	67% (18)	62% (44)	
> 5	33% (9)	38% (27)	
Water Supply			0.255
Public Tap	44% (12)	28% (20)	
Open Well	26% (7)	27% (19)	
Covered Well/Borehole	30% (8)	45% (32)	
Food Security Index (FSI)			0.123
High FSI	67% (18)	49% (35)	
Low FSI	33% (9)	51% (36)	
Animals in the Household			0.368
Yes	30% (8)	39% (28)	
No	70% (19)	61% (43)	
Clinical			
Infant Diarrhea			0.526
Yes	59% (16)	52% (37)	
No	41% (11)	48% (34)	
Infant Vomiting			0.668
Yes	30% (8)	25% (18)	
No	70% (19)	75% (53)	
Infant Cough			0.565
Yes	81% (22)	76% (54)	
No	19% (5)	24% (17)	
Infant Fever			0.775
Yes	81% (22)	79% (56)	
No	19% (5)	21% (15)	
HIV-exposure			0.165
HIV-exposed	30% (8)	45% (32)	
HIV-unexposed	70% (19)	55% (39)	
Nutritional			
Low Birth Weight (< 2.5kg)			0.439
Yes	4% (1)	10% (7)	
No	96% (26)	90% (64)	
Stunted at Birth (HAZ < -2)			0.439
Yes	4% (1)	10% (7)	
No	96% (26)	90% (64)	
Maternal Underweight at Month 6 (BMI < 18.5)			0.033

Yes	22% (6)	7% (5)	
No	78% (21)	93% (66)	
Stunted at Month 6 (HAZ < -2)			0.785
Yes	52% (14)	55% (39)	
No	48% (13)	45% (32)	
Underweight at Month 6 (WAZ < -1)			0.665
Yes	19% (5)	23% (16)	
No	81% (22)	77% (55)	

Cryptosporidium-positive = infants who had at least one Cryptosporidium-positive stool sample between M1 and M6; Maternal literacy = maternal self-report that she can read; HAZ = height-for-age z-score; BMI = body mass index; WAZ = weight-for-age z-score

Infant feeding patterns differed based on maternal HIV-status, where HIV-infected mothers engaged in more optimal feeding practices than their HIV-negative counterparts. Only 28% (HIV-positive=40% vs. HIV-negative=21%, p=0.085) of mothers were still EBF at the M1 visit and this percentage decreased to just 5% (HIV-positive=10% vs. HIV-negative=2%, p <0.001) by M6. In univariate logistic regression, the odds of infant *Cryptosporidium* between M1 and M6 decreased by 21% as BM increased as a proportion of the diet based on the BF score (OR=0.79, CI=0.64, 0.99). Likewise, for every additional week of EBF, the odds of infant *Cryptosporidium* between M1 and M6 decreased by 5% (OR=0.95, CI=0.88, 1.03) (**Figure 4.3**).

Figure 4.3. Logistic regression curve of the relationship between weeks of exclusive breastfeeding and risk of infant *Cryptosporidium* infection from birth through six months.



Since the majority of infants began receiving complementary foods/liquids from an early age, the impact of specific foods on *Cryptosporidium* risk was assessed. **Table 4.2** details the proportion of infants receiving specific complementary foods at any time between birth and M6 and the association between these complementary foods and risk of *Cryptosporidium* infection. Water and grains were the most commonly fed complementary foods, and infants who received these foods had an increased risk of *Cryptosporidium* infection compared with infants who did not (Water: OR=4.62, 95%

CI=1.27, 16.85; Grains: OR=7.78, 95% CI=2.15, 28.18). Other foods associated with an increased risk of infant *Cryptosporidium* infection included: fish, legumes, roots, eggs, and honey. Notably, all infants who received formula experienced *Cryptosporidium* infection. Increased consumption of complementary foods was positively correlated with increasing food security, such that mothers with high food security were more likely to feed complementary foods associated with *Cryptosporidium*, except for eggs. The timing of the introduction of specific complementary foods did not significantly differ based on maternal HIV-status.

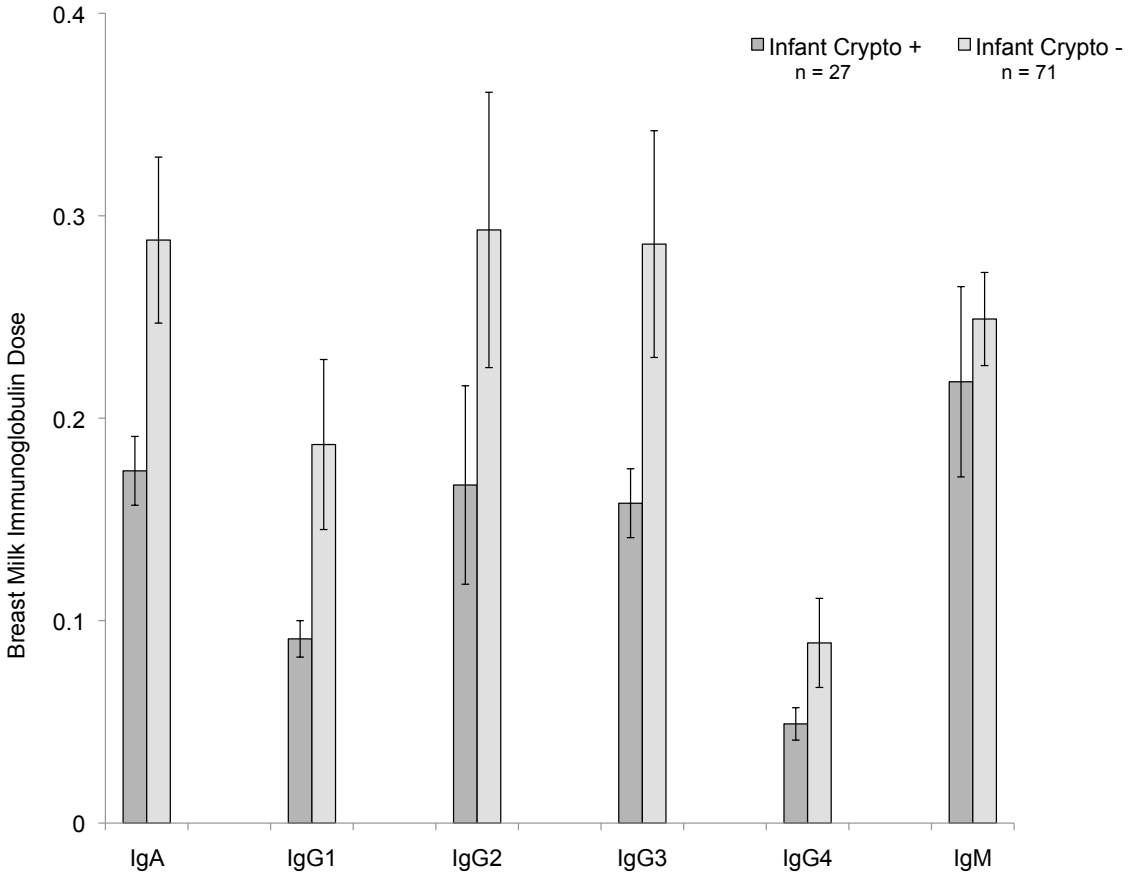
Table 4.2. Percentage of infants receiving specific complementary foods and the association between complementary foods and experience of *Cryptosporidium* infection in the first six months of life.

	Crypto+	Crypto-	p value	OR	95% CI	p value
Water	**89%**	**63%**	**0.014**	**4.62**	**1.27, 16.85**	**0.020**
Grains	**89%**	**51%**	**<0.001**	**7.78**	**2.15, 28.18**	**0.002**
Gripe Water	78%	75%	0.748	1.19	0.41, 3.41	0.748
Orange Fruit	52%	31%	0.056	2.40	0.97, 5.94	0.059
Tea	48%	46%	0.882	1.07	0.44, 2.60	0.882
Orange Vegetables	48%	32%	0.148	1.94	0.78, 4.78	0.151
Fish	**41%**	**10%**	**<0.001**	**6.29**	**2.10, 18.78**	**0.001**
Legumes	**37%**	**15%**	**0.020**	**3.21**	**1.17, 8.82**	**0.024**
Fruit Juice	26%	13%	0.113	2.41	0.80, 7.31	0.120
Roots	**26%**	**7%**	**0.011**	**4.62**	**1.32, 16.16**	**0.017**
Milk	22%	15%	0.432	1.56	0.51, 4.74	0.434
Eggs	**22%**	**4%**	**0.012**	**6.48**	**1.49, 28.16**	**0.013**
Soda	19%	15%	0.717	1.24	0.39, 3.97	0.718
Soup	19%	17%	0.850	1.12	0.35, 3.54	0.850
Leafy Greens	19%	11%	0.344	1.79	0.53, 6.05	0.349
Other Fruit	19%	7%	0.094	3.00	0.79, 11.35	0.106
Honey	**15%**	**3%**	**0.027**	**6.00**	**1.03, 34.94**	**0.046**
Sweet Water	7%	13%	0.722	0.55	0.11, 2.73	0.466
Formula	7%	0%	0.074	---	----	----
Salt/Sugar Water	4%	8%	0.670	0.42	0.05, 3.63	0.428
Meat	4%	1%	0.477	2.69	0.16, 44.64	0.489
Other Vegetables	0%	1%	1.000	---	----	----

Cryptosporidium-positive = infants who had at least one Cryptosporidium-positive stool sample between M1 and M6.

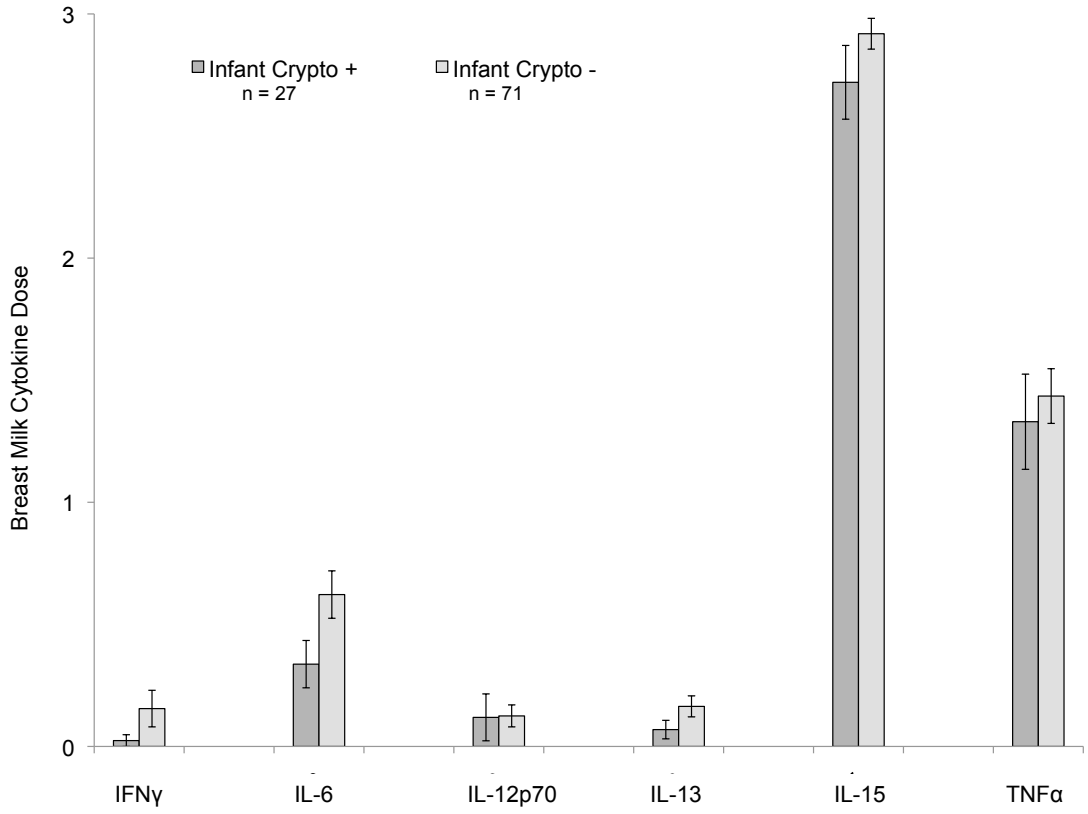
In infants who remained free from *Cryptosporidium* through six months, the immunoglobulin index at all four visits was higher compared with infants who experienced infection at least once in the six-month post-partum period (**Figure 4.4**). The same relationship was found between BM cytokine index and infant *Cryptosporidium*, where the indices of IFNγ, IL-6, IL-12p70, IL-13, IL-15, and TNFα were higher in infants who remained *Cryptosporidium*-free throughout the study (**Figure 4.5**). Infants who were exposed to a high immunoglobulin index in BM had a lower prevalence of *Cryptosporidium* between birth and six months, and this difference was statistically significant for IgA (High=21%; Low=58%, p=0.002). Likewise, a similar trend was seen for BM cytokine index, where infants who were exposed to high indices of each cytokine in BM had a lower prevalence of *Cryptosporidium*.

Figure 4.4. Mean breast milk immunoglobulin index based on infant *Cryptosporidium* infection from birth through six months.



Cryptosporidium-positive = infants who had at least one Cryptosporidium-positive stool sample between M1 and M6.

Figure 4.5. Mean breast milk cytokine index based on infant *Cryptosporidium* infection from birth through six months.



Cryptosporidium-positive = infants who had at least one Cryptosporidium-positive stool sample between M1 and M6.

In univariate logistic regression, as BF score increased, odds of infant *Cryptosporidium* infection decreased (OR=0.79 per unit increase in BF score, 95% CI=0.64, 0.99), while maternal underweight and feeding of *Cryptosporidium*-associated foods were associated with 3.77 and 2.11 higher odds of infection, respectively (**Table 4.3**). In multivariate logistic regression, only *Cryptosporidium*-associated foods remained significantly associated with higher odds of *Cryptosporidium* infection.

Table 4.3. Univariate and multivariate logistic regression odds of infant *Cryptosporidium* infection between birth and six months in rural northwest Tanzania.

Variable	Variable Range	Variable Median	OR	95% CI
Total BM Immunoglobulins	0, 72.50	9.97		
Univariate			0.96	0.90, 1.02
Adjusted			0.96	0.88, 1.05
Total BM Cytokines	0, 4.51	3.51		
Univariate			0.35	0.07, 1.63
Adjusted			0.36	0.04, 3.01
Breastfeeding Score	3, 12	6		
Univariate			0.79*	0.64, 0.99
Adjusted			0.87	0.67, 1.14
***Cryptosporidium*-risk Foods**	0, 6	2		
Univariate			2.11***	1.48, 3.02
Adjusted			2.03***	1.38, 3.00
Maternal Underweight	0, 1	-		
Univariate			3.77*	1.04, 13.62
Adjusted			5.00	0.79, 31.65
Water Source	1, 3	-		
Univariate			0.64	0.38, 1.09
Adjusted			0.71	0.37, 1.38
Model p value			**<0.001**	

OR = odds ratio, [a]Odds Ratio per unit increase of the independent variable; CI = confidence interval; BM = breast milk; [b]Adjusted for total BM immunoglobulins, total BM cytokines, breastfeeding score, Cryptosporidium-risk foods, maternal underweight, and water source; [c]Cryptosporidium-risk foods = number of Cryptosporidium-associated foods consumed between M1 and M6; maternal underweight = body mass index ≤18.5, [d]Maternal underweight coded as: 0 = maternal BMI > 18.5 at every visit, 1 = maternal BMI ≤18.5 at any visit between M1 and M6; water source coded as 1 = public tap, 2 = open well, 3 = covered well/borehole
**p<0.05; ***p<0.001*

Multivariate logistic regression model: Infant Cryptosporidium *infection = β(Total BM Immunoglobulins) + β(Total BM Cytokines) + β(Breastfeeding Score) + β(*Cryptosporidium*-risk Foods) + β(Maternal Underweight) + β(Water Source) + μ*

4.5 Discussion

Infants in this Tanzanian cohort showed high rates of stunting alongside a high burden of infectious disease, as evidenced by the prevalence of *Cryptosporidium* in the post-partum period. In the context of undernutrition and infection, infant feeding plays

an important role. This study demonstrates that breast milk, both through passive transfer of immune molecules and avoidance of contaminated food/water through the practice of EBF, is a key factor in protecting infants from gastrointestinal illness.

Cryptosporidium remains under-recognized and under-diagnosed due to difficulties in diagnosis, limited epidemiological data, and a paucity of treatment options even if *Cryptosporidium* is diagnosed. To date, there have been few prospective longitudinal studies focused on *Cryptosporidium* infection and risk factors in young infants, despite the profound implications of early infection on growth, cognitive development, morbidity, and mortality. This study found that one of every three infants became infected with *Cryptosporidium* by six months, which is higher than previously reported Tanzanian estimates in infants and children [24, 25]. Active case detection in the current study may have contributed to higher estimates of *Cryptosporidium* infection. Previous studies were also conducted in urban environments and included older children, likely contributing to differences in prevalence estimates. Surprisingly, the current study did not find differences in *Cryptosporidium* infection in either mothers or infants based on HIV-status of the mother. This finding is likely due to the fact that HIV-positive mothers in the study were relatively immunocompetent and all were on ART. Additionally, HIV-positive mothers practiced EBF longer than their HIV-negative counterparts, which likely protected their infants.

This study identified infant feeding practice as an important factor associated with protection from *Cryptosporidium* in early infancy. According to the UNICEF conceptual framework for child undernutrition [26], inappropriate infant feeding and infectious disease lead to infant undernutrition, and in this study over half the infants were stunted

by six months. The study indicated that few mothers practiced optimal infant feeding, i.e. EBF for six months - only 5% of mothers were still EBF at six months. Notably, however, none of these infants became infected with *Cryptosporidium*. According to a Ugandan study, children are exposed to *Cryptosporidium* within a few weeks of birth, but maternal protection through breastfeeding delays symptomatic infection until at least six months [27]. Few studies have considered the impact of EBF on *Cryptosporidium* in infants, but several have identified breastfeeding as protective. An urban, Mexican study of diarrheal infants under six months found that infants who were not breastfed had a greater risk of *Cryptosporidium* infection [6]. Similarly, Israeli Bedouin infants followed from birth through 18 months had a lower risk of *Cryptosporidium* if they were fully breastfed (defined as BM, water, and tea) or partially breastfed (defined as BM and other foods) compared with non-breastfed infants [5]. Conversely, an urban Zambian study of diarrheal children up to 11 years found that breastfeeding was associated with a higher risk of *Cryptosporidium* infection [9]; however, the authors conceded that their finding was surprising and difficult to explain. Importantly, in comparison to my study, the Zambian study did not analyze EBF separately. Thus, children who were in the breastfed group were likely also receiving complementary foods and liquids.

Complementary foods and liquids are an important part of appropriate infant feeding after the first six months, yet most mothers in this study began supplementing their infants' diets far too early based on the WHO recommendation of EBF for six months. During the six month study period, most infants were consuming water and grains in addition to BM. No previous studies have linked the consumption of specific complementary foods/liquids with infant *Cryptosporidium* infection, but Mor, et al. noted

that infection with *Cryptosporidium* is often delayed until six months when complementary foods are introduced [28]. In this study, infants who became infected with *Cryptosporidium* during the first six months were more likely to have consumed both water and grains than infants who remained uninfected. Interestingly, infants who consumed tea (prepared with boiled water) were not at higher risk of *Cryptosporidium* infection, highlighting the fact that untreated water may play a role in transmission. The study also identified water source as a possible risk factor in infant *Cryptosporidium* infection, with a greater proportion of infected infants consuming water from public wells as opposed to covered wells/boreholes. As a result, health care workers should increase counseling regarding EBF to all mothers, and public health messages targeting mothers with young children should stress the importance of water treatment in the prevention of diarrhea and infection. Children who are EBF are not exposed to contaminated complementary foods; additional research is needed to determine the risks of specific complementary foods for children over six months.

In addition to EBF, the current study also found that higher BM immunoglobulin and cytokine indices were associated with a lower risk of early infant *Cryptosporidium* infection. In this study, BM immunoglobulin index was calculated using the method of infant feeding (BF score) as well as immunoglobulin concentration of the milk. Through my analyses of the BM immunoglobulin index, it became clear that both breastfeeding practices and the immunoglobulin concentration of breast milk benefitted the infant. The strongest protective association found was IgA - infants exposed to a high BM IgA index were less than half as likely to become infected with *Cryptosporidium* than infants who were exposed to a low index. Korpe, et al. [15] had similar findings in a cohort of

Bangladeshi infants. There, infants exposed to high concentrations of BM IgA had a 38% reduced risk of *Cryptosporidium* infection. In the current study, a higher BM IgG index was also associated with a decreased risk of *Cryptosporidium* infection. Though there are no studies on BM IgG and *Cryptosporidium* in infants, research shows that parasite-specific IgG is produced in response to *Cryptosporidium* infection [10], and repeat exposure to *Cryptosporidium* may promote an IgG response that imparts partial protection [28]. IgG responses to *Cryptosporidium* can last several months [29], and these circulating antibodies may be secreted in breast milk providing passive immunity to the infant.

As with immunoglobulins, a higher BM cytokine index was associated with a decreased risk of *Cryptosporidium* infection. There are no studies on BM cytokines and risk of infant *Cryptosporidium* infection, but previous research has found that IFN-γ is associated with the human T-cell memory response to *Cryptosporidium* [10-13]. Human subjects with previous *Cryptosporidium* infection produced IFN-γ when challenged with *Cryptosporidium*, while *Cryptosporidium*-naïve subjects produced no IFN-γ [30]. IL-15 also appears to be an important component of the primary immune response to *Cryptosporidium*. Dann, et al. [14] showed that within hours of *Cryptosporidium* exposure, intestinal tissues of healthy human volunteers increased expression of IL-15, and that this response was crucial to the clearance of *Cryptosporidium*. Studies also show that IL-12 is expressed during a primary immune response to *Cryptosporidium* and is associated with the development of immunity [10, 13]. Thus, BM may prime the immature infant's immune system with cytokines that are associated with protection and clearance of *Cryptosporidium.*

In conclusion, *Cryptosporidium* is a significant diarrheal pathogen in mothers and infants in this rural Tanzanian setting. Currently, there are no effective vaccines or treatments available for *Cryptosporidium* in HIV-positive individuals or young infants, and diagnosis of *Cryptosporidium* in low-resource settings is a challenge, therefore effective preventive measures are urgently needed. This study indentified BM index, which combines breastfeeding practices with concentrations of breast milk immune molecules, as protective against early *Cryptosporidium* infection. The results of this study suggest that breastfeeding protects the infant in two ways: by avoiding contaminated complementary foods and by providing passive immunity through BM. Public health efforts, similar to PMTCT counseling in Tanzania, should therefore focus on increasing the rates of EBF amongst all women while also counseling mothers regarding appropriate complementary feeding and safe water practices.

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CHAPTER 5

CONCLUSION

5.1 Conclusion

In Tanzania, diarrhea remains a major cause of infant morbidity and mortality [1]. There are a multitude of infectious agents that may cause diarrhea, including viruses (i.e. rotavirus), bacteria (i.e. *Campylobacter* spp.), and other parasites (i.e. *Giardia*); additionally, within populations in sub-Saharan Africa, multiple co-infections are common. *Cryptosporidium*, a diarrhea-causing protozoan parasite, is ubiquitous in rural northwest Tanzania; in the current study, over half of mothers in the post-partum period were infected with *Cryptosporidium* and one-third of infants were infected by six months of age. Within my study population, women and infants were also frequently diagnosed with malaria, hookworm, *Giardia,* and syphilis. *Cryptosporidium* is transmitted through contaminated water and complementary foods, therefore the practice of exclusive breastfeeding (EBF) may protect infants through avoidance of water and foods as well as through the passive transmission of immune molecules in breast milk. Few women in Tanzania practice EBF for the recommended six months, although it appears that prevention-of-mother-to-child-transmission (PMTCT) counseling has resulted in a greater proportion of HIV-positive mothers engaging in EBF.

Few prospective studies have examined the risk factors associated with infant *Cryptosporidium* infection in sub-Saharan Africa despite the fact that numerous researchers over the past 20 years have called for more prospective, longitudinal studies of *Cryptosporidium* infection in young children [2-7]. There is evidence, albeit conflicting, that breastfeeding is associated with protection from *Cryptosporidium*

infection in infancy [2, 8, 9] however most studies have not used standardized breastfeeding definitions. Moreover, only one previous study has looked at the association between breast milk immune components and the risk of *Cryptosporidium* infection in infancy [10]. As a result, the goal of this research was to investigate the magnitude of the *Cryptosporidium* burden in rural, northwest Tanzania as well as to examine risk factors associated with early infant *Cryptosporidium* infection. Specifically, my prospective cohort study had three aims: 1) to determine the prevalence of *Cryptosporidium* infection in mothers and infants during the first six-months post-partum; 2) to examine the correlates of immune molecules in breast milk; and 3) to understand the associations between exclusive breastfeeding, breast milk immune molecules, and risk of early infant *Cryptosporidium* infection.

5.2 The Burden of Cryptosporidium in Mothers and Infants in Rural, Northwest Tanzania

My first aim was to determine the magnitude of the *Cryptosporidium* burden in post-partum mothers and infants in rural, northwest Tanzania. My study revealed that a majority of mothers experienced *Cryptosporidium* infection at least once in the six month post-partum period and that prevalence did not differ over the study period. Infants, however, appeared to be protected until Month 2 and infection was rare until Month 6. By the age of six months, one-third of infants had experienced *Cryptosporidium* infection. Surprisingly, my study did not observe differences in *Cryptosporidium* prevalence based on HIV-infection in mothers or HIV-exposure in infants. I also noted that exclusive breastfeeding was associated with a decreased risk of infant *Cryptosporidium* infection, while maternal hand-washing prior to infant feeding was associated with an increased risk of infection.

My estimates of *Cryptosporidium* prevalence are much higher than previously reported, but my study design and research setting likely contributed to these differences. Previous estimates of *Cryptosporidium* in Tanzanian children have ranged from 9 to 20% [11-13], while my study found that 33% of children were infected at Month 6. None of the previous Tanzanian studies were prospective or longitudinal in nature and the studies included both children and infants. The neonatal period, particularly the first six months, represents a time of immunological immaturity and vulnerability for the infant. As a result, young infants are at greater risk of *Cryptosporidium* infection. Furthermore, researchers have found that *Cryptosporidium* prevalence peaks at an earlier age in rural settings [14]. Consequently, the inclusion of older infants and children from urban areas in previous studies likely resulted in lower prevalence estimates than those found in my study.

In addition to age differences, previous studies were also focused on children presenting with diarrhea in hospitals and health clinics. My study, in contrast, used active case detection and analyzed fecal samples from all study participants regardless of symptoms. Maternal report of diarrhea in themselves and their infants was common, yet most cases of *Cryptosporidium* in my study were asymptomatic. According to one Tanzanian study, the most common causes of diarrhea in children under-five years were diarrheagenic *Escherichia coli* (DEC), *Cryptosporidium parvum,* rotavirus, and norovirus; additionally, one of five children in the study were infected with multiple pathogens [13]. Other factors contributing to diarrhea in this population may include *Vibrio cholerae, Shigella* spp., *Salmonella* spp., *Giardia lamblia*, and hookworm.

The clinical presentation of *Cryptosporidium* infection may differ by the infecting species, however I was unable to analyze *Cryptosporidium* at the species-level in this study. Some research suggests that *C. hominis* is mainly associated with diarrhea, nausea, vomiting, and general malaise, while *C. parvum* is associated with diarrhea only [15]. Firm conclusions regarding the clinical implications of *Cryptosporidium* at the species level are difficult given the limited research at the molecular level. One study demonstrated that *C. hominis* may be associated with an increased risk of morbidity post-infection [16], while another study showed that *C. hominis* was more virulent that *C. parvum* in the developing country context [17].

Asymptomatic *Cryptosporidium* infection was common in my study population, yet may still have damaging consequences for the infant. Previous research found that after *Cryptosporidium* infection, children gained weight and height more slowly than uninfected children, regardless of symptoms or the infective species, and the largest magnitude of growth impairment was seen in children infected between 0 and 5 months [18].

Contrary to my hypothesis, the prevalence of *Cryptosporidium* in HIV-positive mothers and HIV-exposed infants did not significantly differ from HIV-negative/HIV-unexposed participants. Early *Cryptosporidium* research indicated that HIV-positive individuals were at greater risk of infection and were more likely to experience symptoms than HIV-negative individuals [19, 20]. Antiretroviral treatment (ART) for HIV-positive individuals reduces the incidence and severity of *Cryptosporidium* [20] and, in recent years, ART has become more widely available. As a result, mother-to-child transmission of HIV has decreased. Among my study population, women with HIV-infection were not

severely immunocompromised based on CD4 cell counts and all HIV-positive mothers and HIV-exposed infants were receiving treatment. Thus, increased availability and use of ART seems to have evened the playing field of susceptibility to and severity of *Cryptosporidium* in HIV-positive/HIV-exposed individuals.

My study was the first to examine the relationship between maternal *Cryptosporidium* infection and infant *Cryptosporidium* infection in the post-partum period. I found that maternal *Cryptosporidium* infection was a risk factor for infant infection. My study also showed that the majority of *Cryptosporidium* cases in this region were asymptomatic, suggesting that *Cryptosporidium* is a much larger public health problem in rural Tanzania than previous studies suggested. Lastly, I found that one of the strongest risk factors for infant *Cryptosporidium* infection was maternal hand washing prior to infant feeding. As a result, further research should focus on the impact of water source, water treatment, and hygiene as they relate to *Cryptosporidium* risk in mothers and infants. Additionally, this study provides evidence that EBF for six months is associated with protection from early infancy *Cryptosporidium* infection. Health workers should include breastfeeding education as part of antenatal counseling since PMTCT counseling for HIV-positive mothers appears to have successfully increased EBF rates among this group.

5.3 Correlates of Breast Milk Immune Protection

The second aim of the study was to characterize breast milk immune composition from Month 1 through Month 6 post-partum and to examine the factors that influence concentrations of immunoglobulins and cytokines in breast milk. My findings suggest

that neither maternal HIV-status nor maternal nutritional status influence breast milk immune composition in this setting. However, I did find that breastfeeding practices were associated with concentrations of certain immune molecules in breast milk (IgA, IgG1, IgG2, IgG3, IL-12p70). I also found weak to moderate positive correlations between maternal serum immunoglobulin concentrations and breast milk immunoglobulin concentrations. Additionally, there were positive correlations between breast milk immunoglobulin concentrations and infant serum immunoglobulin concentrations in the first two months post-partum. Lastly, there were significant associations between maternal symptoms of illness and concentrations of breast milk cytokines.

The results of this study underscore the complex and dynamic nature of breast milk immune composition over time. I observed an inverted U-shaped pattern of immunoglobulin concentrations in breast milk with concentrations rising from Month 1 to Month 2 and then falling from Month 3 to Month 6. Previous studies describe a significant decrease in breast milk immunoglobulins from birth through three months post-partum, but I did not find such patterns in our study. A recent Japanese study found that breast milk IgA concentrations peaked at Day 3 after birth and decreased through Month 3 post-partum, with the largest magnitude of decrease between birth and Month 1 [21]. Since I began milk collection at Month 1 in my study, I was not able to observe changes in breast milk as it evolved from colostrum to transitional to mature milk. Thus, the design of my study limited my conclusions to mature milk only. My analysis of breast milk cytokines indicated that concentrations of some (IL-6, IL-15) decreased over time, one (TNFα) showed an inverted U-shaped relationship, while others (IFNγ, IL-

12p70, IL-13) had no significant change over time; IL-1b, IL-2, and IL-10 were not detected in breast milk in this study.

I used a novel method of analyzing immunoglobulins and cytokines in breast milk. In preliminary testing of my breast milk protocol, I used a range of diluted breast milk supernatants in order to determine the optimal dilution for detection of immunoglobulins and cytokines in breast milk. The conclusions of preliminary testing showed that optimal recovery occurred in undiluted breast milk supernatant, thus in my laboratory analysis, I first centrifuged the breast milk, removed the fatty layer and analyzed the undiluted cell-free milk supernatant according to the manufacturer's instructions. In accordance with Groer, et al. [22], I utilized the serum matrix provided in the Millipore kit. The success of my protocol was confirmed by comparing my results to those of previous studies. Previous research has found that breast milk concentrations of IFNγ and IL-12p70 remain stable over time [23, 24] and I observed the same in my study. IL-10 is reported to have undetectable to low concentrations in breast milk [23] and this was consistent in my study where it was not detected in breast milk.

Another unique aspect of my study was the collection of samples from three biological compartments of the mother-infant dyad. Using these samples, I was able to examine correlations between the biological compartments. These analyses are useful in helping to determine whether concentrations in one biological compartment can be used as a proxy for concentrations in another compartment. Based on my results, which showed a complex correlation matrix, it is not advisable to make conclusions regarding immunoglobulin/cytokine concentrations in one compartment based on the concentrations in another compartment. Previous research indicates that extrapolating

breast milk IFNγ concentrations from maternal serum concentrations is not possible given the wide variation in concentrations as well as the lack of consistent correlations [23]. My study confirms the absence of a relationship between maternal serum IFNγ concentration and breast milk IFNγ concentration and also indicates the negligible correlation between TNFα, IL-6, IL-12p70, and IL-15 between maternal serum and breast milk.

My study was also unique in that I considered breastfeeding practices in my analysis of the factors affecting breast milk immune composition. I was surprised to find that the strongest predictors of breast milk immunoglobulin concentrations were duration of exclusive breastfeeding and frequency of breastfeeding. Previous research has often failed to consider infant feeding as a variable associated with breast milk immune composition, and instead, has focused on factors such as HIV-infection and maternal nutritional status. The results of my study indicate that there may be modifiable factors, such as infant feeding practices, that could impact immunoglobulin concentrations in breast milk. The biological relevance of such changes is unclear based on previous research, but this issue was further explored in Chapter 4.

5.4 Risk Factors Associated with Infant Cryptosporidium

Based on the results from Chapters 2 and 3, the aim of Chapter 4 was to examine how infant feeding practices and breast milk concentrations of immunoglobulins and cytokines were related to infant *Cryptosporidium* infection. I found that both infant feeding practices, namely exclusive breastfeeding, and higher concentrations of breast

milk immunoglobulins and cytokines were associated with a decreased risk of infant *Cryptosporidium* infection through six months of age.

Consumption of complementary foods was assessed and several complementary foods (water, grains, fish, legumes, roots, eggs, honey) were found to be associated with a greater risk of infant *Cryptosporidium* infection. Many of these complementary foods are commonly prepared using water (e.g. grains, fish, legumes, roots), therefore the risk associated with complementary foods may be due to contamination through unsafe water. For example, infants generally consume grains as porridge, which is commonly prepared using boiled water, but mothers often cool down the porridge with unboiled water that may result in *Cryptosporidium* exposure. Additionally, certain complementary foods may be frequently fed together (e.g. grains and honey), therefore the observed risk associated with one food (e.g. honey) may actually be due to exposure to the accompanying food (e.g. grains).

I used several novel methods to inspect the relationship between infant feeding and *Cryptosporidium* risk. First, I constructed a breastfeeding score that summarized infant feeding patterns over the six-month post-partum period. The score was calculated by assigning a value corresponding to the current infant feeding pattern (exclusive breastfeeding vs. predominant breastfeeding vs. partial breastfeeding vs. no breastfeeding) for each visit and then summing the scores over the four visits. A score of 12 indicated that the infant was exclusively breastfed for six months, while a score of 0 indicated that the infant was never breastfed. The breastfeeding score was significantly associated with a decreased risk of *Cryptosporidium* infection, demonstrating that infants

who received a greater proportion of breast milk in the diet were less likely to experience *Cryptosporidium* infection.

Using the breastfeeding score and the breast milk concentrations of immunoglobulins and cytokines, I then calculated the index of immune molecules received from breast milk. This is the first report of the effect of a broad range of breast milk immune molecules on infant *Cryptosporidium* risk. I found that higher breast milk immunoglobulin and cytokine indices were associated with a decreased odds of *Cryptosporidium* infection. This analysis allows us to make inferences regarding the biological implications of breast milk immune composition on infant infectious disease risk. In Chapter 3, I showed that breast milk concentrations of immunoglobulins and cytokines did not differ by HIV-status or maternal BMI, but that infant feeding practices and frequency were associated with higher concentrations. Chapter 4 then demonstrated that infants who are exposed to higher breast milk immunoglobulin and cytokine indices are less likely to experience *Cryptosporidium* infection. Therefore, I hypothesize that there is biological importance to increased breast milk concentrations of immune molecules.

Based on the results from both Chapter 3 and Chapter 4 there is further evidence of the benefits of exclusive breastfeeding on infant health. In Chapter 3, I reported that an increased duration of exclusive breastfeeding was associated with increased breast milk concentrations of immunoglobulins and in Chapters 2 and 4, I reported that exclusive breastfeeding was associated with a decreased risk of infant *Cryptosporidium* infection. Therefore, the act of exclusive breastfeeding synergistically improves the immunological quality of the breast milk as well as protecting infants from exposure to

contaminated liquids and foods. Based on the analyses of Chapter 4, if a mother were to increase the duration of exclusive breastfeeding from one month to just three months, the odds of infant *Cryptosporidium* infection would decrease by 40%.

Despite the numerous benefits of exclusive breastfeeding and the WHO recommendation that all women practice EBF for six months, the duration of EBF in this population was short. Previous studies have identified the following barriers to EBF in sub-Saharan Africa women: cultural beliefs and traditions, maternal work conditions, maternal anemia, lack of maternal education, poor maternal health, infant illness, HIV-infection, inadequate support from health workers, and perception that milk supply is inadequate, among others. [25-28]. Qualitative findings from the current study indicate that women enrolled in my study experienced similar barriers to maintaining EBF for six months. Barriers to EBF mentioned by study participants included: pain around the nipple, time constraints due to income-generating activities, lack of knowledge about the risks of early introduction of complementary foods, concerns about transmitting HIV to the infant, and concerns that the infant was not receiving enough milk. Many of these barriers could be overcome with increased infant feeding counseling at the clinic as well as additional community and family support for breastfeeding mothers.

5.5 Future Research Priorities

Although this research has filled some of the gaps in knowledge relating to breast milk immunology and *Cryptosporidium*-risk factors in young infants, there were some limitations. My sample size was relatively small and may not have been powerful enough to detect differences in some of my outcome variables. This study was a pilot

study intended to generate data for future, larger studies and the sample size was calculated to detect differences in TNFα in maternal serum of HIV-positive and HIV-negative women. Indeed, I was able to detect significant differences in maternal serum TNFα based on maternal HIV-status. Future studies should aim to recruit a larger sample size, especially given the difficulty in collecting infant serum samples, which was a limiting factor in my study. Based on the results of my study, I have calculated the sample size required to detect differences in breast milk immune molecules by maternal HIV-status; results are presented in **Table 5.1.** According to these sample size calculations, the current study was underpowered to find significant differences in breast milk immune molecules between HIV-positive and HIV-negative women, especially with regards to the immunoglobulins.

Table 5.1 Sample size requirements for future studies

Immune Molecule	HIV+ Mean	HIV+ SD	HIV- Mean	HIV- SD	n/group*
IgA	220.3	186.5	223.7	224.6	57867
IgG1	1016.5	1628.0	765.3	987.6	451
IgG2	3857.4	7217.2	3683.6	5963.3	22775
IgG3	23.7	32.6	21.4	23.6	2404
IgG4	13.6	27.3	12.9	20.4	18605
IgM	332.0	250.5	372.9	371.6	943
IFNγ	2.29	0.18	7.95	39.02	374
IL-6	5.09	6.54	5.83	16.83	4673
IL-12	2.32	0.30	2.50	1.02	274
IL-13	2.45	0.64	2.34	0.42	381
IL-15	24.62	26.16	19.57	15.6	286
TNFα	7.13	8.33	6.38	8.42	1958

* $\alpha = 0.05$; power = 0.8

In both Chapter 2 and Chapter 4, I noted that water-related exposures were a risk for infant *Cryptosporidium* infection; in Chapter 2, maternal hand washing was associated with an increased risk of infant *Cryptosporidium* infection, while in Chapter 4, both water source and the consumption of unboiled water were associated with infant *Cryptosporidium*. It is important for future studies to not only analyze fecal samples, but also water samples from the source and from the home in order to help determine transmission pathways. My study identified *Cryptosporidium* infection using microscopy, which is unable to differentiate between different species of the pathogen. Studies show that 90% of human *Cryptosporidium* infections are due to just two species,

namely *C. parvum* and *C. hominis* [15]. Previous research has indicated that there may be difference in the epidemiology and clinical implications based on the infective species of *Cryptosporidium*. It appears that the main reservoir for *C. parvum* infection is zoonotic, while *C. hominis* appears to infect humans exclusively [15]. In addition to water testing, genotyping of the *Cryptosporidium* species would help shed light on the mode of transmission thus allowing better planning and implementation of interventions to prevent *Cryptosporidium* infection.

Many studies have linked low CD4 cell counts of HIV-positive individuals with a higher incidence and severity of *Cryptosporidium* [20], yet none of these studies have considered CD4 cell counts of HIV-negative individuals. In order to get a more complete picture of the way in which CD4 cell counts are related to *Cryptosporidium* risk, future studies should collect CD4 cell counts for all participants, not just HIV-positive individuals.

The intrauterine environment is the first exposure the infant has to the maternal environment and to the maternal immune system. Many immune molecules are transferred to the infant transplacentally and via cord blood [29-31], thus maternal *Cryptosporidium* infection during pregnancy may impact an infant's risk of future infection. Collecting and analyzing maternal fecal samples during pregnancy may help determine if intrauterine exposure to maternal *Cryptosporidium* infection is associated with *Cryptosporidium* infection during infancy. Additionally, since infant *Cryptosporidium* prevalence rose dramatically between Month 3 and Month 6, the collection of fecal samples at monthly intervals (Month 4 and Month 5) between these visits would help our understanding of the natural history of infection in infants. During

the period between Month 3 and Month 6, infants often undergo dramatic changes in feeding and therefore this period is especially crucial in understanding the causal relationship between infant feeding practices and *Cryptosporidium* infection.

Another limitation of my study was that we only analyzed total concentrations of breast milk immunoglobulins rather than antigen-specific immunoglobulins. Breast milk IgA in the infant intestine acts to exclude antigen from circulation by preventing penetration through the gut epithelium [32]. Based on the immunology of the entero-mammary system, antigen encountered in the maternal intestine stimulates the production of antigen-specific IgA, which is then secreted in breast milk. A previous study determined that anti-*Cryptosporidium* IgA targeted the surface of *C. parvum* oocysts leading to the conclusion that *Cryptosporidium*-specific IgA in breast milk is capable of protecting the breastfeeding infant from infection [10]. Indeed, the same study showed that infants consuming higher concentrations of *Cryptosporidium*-specific IgA in breast milk were at decreased risk of becoming infected [10]. Future research should focus on *Cryptosporidium*-specific IgA in breast milk rather than simply total breast milk IgA in order to better determine how breast milk immunoglobulins are related to protection from *Cryptosporidium* infection.

Overall, the results of this prospective cohort study demonstrate that *Cryptosporidium* is an under-recognized public health problem for lactating mothers and young infants in rural Tanzania. These results indicate that infant and young child feeding practices are associated with early *Cryptosporidium* infection, and that feeding practices remain sub-optimal. Public health programs should focus on education and support for exclusive breastfeeding and appropriate complementary feeding during the

antenatal and post-partum periods. Findings of this study also inform future research priorities, namely the testing of household water samples, analysis of *Cryptosporidium*-specific breast milk antibodies, and genotyping of infective *Cryptosporidium* species in order to further refine our understanding of the epidemiology of *Cryptosporidium* infection and transmission.

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APPENDIX A

ENROLLMENT QUESTIONNAIRE

1	Mother Study ID			
2	Date of Interview	DD	MM	YYYY
3	Interview Conducted by			

Identification & Contact Information

4	ANC identification number (write the number down from the ANC card). *Place a study sticker on the ANC Card with the participant's ID number.*			
5	Date of Birth	DD	MM	YYYY
6	Age or approximate age	YY		
7	Marital Status 1=Single 2=Monogamously married or cohabiting 3=Polygamously married or cohabitating 4=Divorced or separated 5=Widow			
8	What is the name of the head of your household?			
9	What is a phone number where you can be contacted? (leave blank if none given)	# # # # # # # # # #		
10	Village			
11	Subvillage			
12	Balozi (if known)			

Education & Occupation

13	Can you read? 0 = No; 1 = Yes				
14	Can you write? 0 = No; 1 = Yes				
15	Have you had a formal education? 0 = No; 1 = Yes *If Yes, answer 15a*		15a	If yes, what is your highest education completed? 1 = Some Primary; 2 = Completed Primary; 3 = Some Secondary;	

				4 = Completed Secondary; 5 = Some University; 6 = Completed University; 7 = Still in School	
16	Has your partner had a formal education? 0 = No; 1 = Yes *If Yes, answer 16a*		16a	If yes, what is the highest level of education that he completed? 1 = Some Primary; 2 = Completed Primary; 3 = Some Secondary; 4 = Completed Secondary; 5 = Some University; 6 = Completed University; 7 = Still in School	
17	Do you perform any work that helps your household earn money? 0 = No; 1 = Yes *If Yes, answer 17a*		17a	What is the main way in which you earn money? 1 = Farming/Cultivating 2 = Tending livestock 3 = Small business (or cleaning houses) 4 = Large business 5 = Professional 6 = Other (write) ……..	
18	Does your partner perform work that helps your household earn money? 0 = No; 1 = Yes *If Yes, answer 18a*		18a	What is the main way in which he earns money? 1 = Farming/Cultivating 2 = Tending livestock	

				3 = Small business (or cleaning houses) 4 = Large business 5 = Professional 6 = Other (write) ……..	

Household

19	How many people slept in your home last night?					
20	How many children under the age of 18 slept in your home last night?					
21	How many children under the age of 5 slept in your home last night?					
22	What is the main source of drinking water for your household during the dry season?					
23	What is the main source of drinking water for your household during the rainy season?					
24	Do you do anything to the water to make it safer to drink? No = 0; Yes = 1 *If Yes, answer 24a*			24a	If yes, what do you do to make the water safer to drink?	
25	What kind of toilet facility does your household use?					
26	Is your toilet facility shared with any other households? No = 0; Yes = 1 *If Yes, answer 26a*			26a	If yes, how many other households share your toilet facility?	
27	Does your household have any of the following items: No = 0; Yes = 1					
	27a	Electricity				
	27b	Paraffin lamp				
	27c	Radio				
	27d	Television				
	27e	Mobile phone				
	27f	Iron				
	27g	Refrigerator				

	27h	Watch		
	27i	Bicycle		
	27j	Motorcycle		
	27k	Car		
	27l	Bank account		
28	What is the main material of the floor of your house?			
29	What is the main material of the walls of your house?			
30	What is the main material of the roof of your house?			
31	Does your household own any animals? No = 0; Yes = 1 *If Yes, answer 31a If No, skip to question 37*		31a	If yes, please list what type and how many of each.

Animal	#
Animal	#
Animal	#
Animal	#
Animal	#

32	Who is responsible for feeding the animals?			
33	Who is responsible for other aspects of animal care?			
34	Where are the animals kept during the day?			
35	Where are the animals kept during the night?			
36	What is the main source of drinking water for the animals?			

Pregnancy and Childbirth History

37	What was the date of your last normal menstrual period?	DD	MM	YYYY	(write XX if day or month not known, XXXX if year not known)

38	Is this your first visit to Kisesa Health Centre for antenatal care during this pregnancy? 0 = No; 1 = Yes *If No, answer 38a*		38a	If no, how many visits to Kisesa Health Centre for antenatal care during this pregnancy did you make **before** today?	
39	Are you planning on delivering your baby at Kisesa Health Centre? 0 = No; 1 = Yes *If No, answer 39a* *Encourage the mother to deliver her*		39a	If no, where do you plan on delivering your baby? 0=At my home 1=At a dispensary 2=At someone else's home (for	

	baby at Kisesa Health Centre and say that we will pay for her transportation.			example, the home of a midwife) 3=At a hospital 4=Other *(write)* ……	
40	Have you been pregnant before this pregnancy (include miscarriages and stillbirths)? 0 = No; 1 = Yes *If Yes answer 40a-c*		40a	If yes, how many children have you given birth to that were alive?	
			40b	Have you ever had a miscarriage? 0 = No; 1 = Yes	
			40c	Have you ever delivered a stillborn baby? 0 = No; 1 = Yes	

Appetite & General Health

41	During the past 7 days, my appetite (how hungry I am usually) has been: 1 = Very poor (I do not want to eat much food) 2 = Poor 3= Average (the same as usual) 4 = Good 5= Very good (I am hungry more often)	
Answer Questions 42-60 about how you have felt **during the past 7 days**, using these answers: 0=Not at all 1=A little 2=Quite a bit		
42	Did you have any trouble doing strenuous activities, like carrying a full basket from the market?	
43	Did you have trouble taking a long walk?	
44	Did you have trouble taking a short walk outside the house?	
45	Did you need to stay in bed or in a chair during the day?	
46	Did you need help with eating, dressing, washing yourself or using the toilet?	
47	Were you limited in doing your work (either your job or housework?)	
48	Have you had pain?	
49	Did you need to rest during the day?	
50	Have you had trouble sleeping at night?	

51	Have you felt weak?	
52	Have you felt nauseated (sick to your stomach)?	
53	Have you vomited?	
54	Have you been constipated (have you had trouble defecating)?	
55	Have you had diarrhea (3 or 4 loose, watery stools during one day)?	
56	Were you tired during the day?	
57	Did your pain interfere with your work?	
58	Did you worry about your health?	
59	Have you felt burdened by your health?	
60	How would you rate your overall health during the past week? 1=Very poor 2=Poor 3=A little poor 4=Average 5=Good 6=Very good 7=Excellent	

Karnofsky Performance Status Scale

61		****Study Nurse:** Based on these questions, assign the participant a "Karnofsky Performance Status" number from the chart below.	
	Able to carry on normal activity and to work; no special care needed.	100	Normal no complaints; no evidence of disease.
		90	Able to carry on normal activity; minor signs or symptoms of disease.
		80	Normal activity with effort; some signs or symptoms of disease.
	Unable to work; able to live at home and care for most personal needs; varying amount of assistance needed	70	Cares for self; unable to carry on normal activity or to do active work.
		60	Requires occasional assistance, but is able to care for most of his personal needs.
		50	Requires considerable assistance and frequent medical care.
	Unable to care for self; requires equivalent of institutional or hospital care; disease may be progressing rapidly.	40	Disabled; requires special care and assistance.
		30	Severely disabled; hospital admission is indicated although death not imminent.
		20	Very sick; hospital admission necessary; active supportive treatment necessary.
		10	Moribund; fatal processes progressing rapidly.

HIV

62	Have you ever tested positive for HIV? 0 = No; 1 = Yes *If Yes, answer 63-67 If No, take the measurements and end the interview*	

63	What date did you first test positive for HIV? (write XX if day or month not known, XXXX if year not known)	DD	MM	YYYY			
64	Are you currently enrolled in PMTCT (Prevention of Mother to Child Transmission of HIV) at Kisesa Health Centre? 0 = No; 1 = Yes						
65	Are you currently enrolled in CTC at Kisesa Health Centre? 0 = No; 1 = Yes *If Yes, answer 65a-b*		65a	If yes, what is your CTC number?			
			65b	If you have just been referred to CTC, what is your **CTC Referral** number?			
66	Are you currently taking ARV (antiretroviral) drugs? 0 = No; 1 = Yes						
67	Were you taking ARV drugs before you became pregnant with this baby? 0 = No; 1 = Yes						

Measurements and Sample Collection

68	Maternal Height	cm		Comments:
69	Maternal Weight	Kg		Comments:
70	Maternal MUAC	mm		Comments:
71	Maternal **Tricep** Skinfold Thickness	mm		Comments:
72	Fundal Height	cm		Comments:
73	Maternal Blood Sample No = 0; Yes = 1			Comments:

APPENDIX B

DELIVERY & BIRTH QUESTIONNAIRE

1	Mother Study ID			
2	Infant Study ID			
3	Date of Measurements	D	M	Y
4	Place of Measurements			
5	Measurements taken by:			

Study Nurse, Please answer the questions below based on your observations and/or the participant interview.

6	Was the baby delivered at Kisesa Health Centre? 0 = No; 1 = Yes *If No, answer 6a*			6a	If no, where was the baby delivered? 0=At the participant's home 1=At a dispensary 2=At someone else's home (for example, the home of a midwife) 3=At a hospital 4=Other *(write)* ……
7	What date was the baby delivered?	DD	MM YYYY		
8	What time was the baby delivered?	##:##			
9	Was the baby born alive (answer 'No' if it was a stillbirth)? 0 = No; 1 = Yes				
10	Did the baby die shortly after birth (within the first 3 days)? 0 = No; 1 = Yes				

Measurements and Sample Collection

11	Infant Birth Weight	g		Comments:
12	Infant Birth Length	cm		Comments:

13	Infant Head Circumference	cm		Comments
14	Infant MUAC	mm		Comments:
15	Cord Blood Sample No = 0; Yes = 1			Comments:

APPENDIX C

FEEDING AND HEALTH QUESTIONNAIRE

1	Mother Study ID			
2	Date of Interview	D	M	Y
3	Place of Interview			
4	Visit #			
5	Interview Conducted by			

General Questions

6	Have your living arrangements changed since your last visit? 0 = No; 1 = Yes *If yes, complete enrollment questionnaire again.*			
7	Infant Date of Birth	D	M	Y
8	Infant Sex 0 = Male; 1 = Female			

Feeding Questions

9	Have you ever breastfed your infant? 0 = No; 1 = Yes *If no, answer 9a*		9a	If no, why did you choose not to breastfeed? *Skip to question 18*	
10	How long after birth did you first put your infant to your breast?	Hours			
11	In the first 3 days after delivery, did you give your infant anything to drink other than breast milk? 0 = No; 1 = Yes *If Yes, answer 11a*		11a	If yes, what did you give to your infant?	
12	Since this time yesterday, did your infant consume breast milk? 0 = No; 1 = Yes *If No, answer 12a*		12a	What are the reasons you no longer breastfeed? *Skip to question 18*	
13	How many times did you breastfeed last night between sunset and sunrise?				
14	How many times did you breastfeed yesterday during the daylight hours?				
15	Have you ever felt that you could not produce enough milk for your infant? 0 = No; 1 = Yes				
16	Did your infant consume anything other than breast milk yesterday? 0 = No; 1 = Yes *If Yes, answer 16a*		16a	If yes, how many times did your infant consume anything other than breast milk yesterday?	
17	Since this time yesterday, did		17a	At what age did	Weeks

	your infant consume any of the following liquids: 0 = No; 1 = Yes *If Yes to any, answer 17a*				you first introduce liquids, other than breast milk, to your infant?	
	17b	Water				
	17c	Tea				
	17d	Fruit Juice				
	17e	Sweetened Water				
	17f	Sugar/Salt Water Solution				
	17g	Infant Formula				
	17h	Tinned/Powdered/Fresh Milk				
	17i	ORS				
	17j	Coffee				
	17k	Soda				
	17l	Broth				
	17m	Soup				
	17n	Honey				
	17o	Gripe Water for Colic				
	17p	Any Other Liquids				
18	Since this time yesterday, did your infant consume any of the following foods: No = 0; Yes = 1 *If Yes to any, answer 18a*			18a	At what age did you first introduce solid/semi-solid foods to your infant?	Weeks
	18b	Cereals/grains (porridge)				
	18c	Orange Vegetables (pumpkin, carrots, orange sweet potato)				
	18d	Roots/Tubers (potato, yam)				
	18e	Green Leafy Vegetables				
	18f	Other Vegetables (tomato)				
	18g	Orange fruits (mango, papaya, oranges)				
	18h	Other fruits (banana, apple)				
	18i	Meat (cow, lamb, goat, pig)				
	18j	Fish or Shellfish				
	18k	Eggs				
	18l	Dairy products (cheese)				
	18m	Legumes (lentils, beans)				
	18n	Oils, fats, butter				
	18o	Any other solid/semi-solid foods				
19	In the last 7 days, did your infant consume any vitamins or mineral supplements? No = 0; Yes = 1 *If*			19a	If yes, which vitamins or mineral supplements did	

	Yes, answer 19a			your infant consume?	
20	In the last 7 days, did your infant consume any medicines? No = 0; Yes = 1 *If Yes, answer 20a*		20a	If yes, what medicines did your infant consume?	

Infant Health Questions

21	Since your last visit, has your infant experienced any of the following: No = 0; Yes = 1					
	21a	Diarrhea *If Yes, answer 21b*		21b	If yes, can you describe the appearance of the diarrhea? 1 = voluminous, watery; 2 = bloody; 3 = mucus; 0 = none of these	
	21c	Cough				
	21d	Difficulty breathing, short/quick breaths				
	21e	Convulsions				
	21f	Fever				
	21g	Vomiting				
	21h	Skin rash				
	21i	Refusal to eat, drink, or consume breast milk				
22	On how days in the past month has your child had diarrhea?					
23	When your infant has diarrhea, do you offer less, more, or the same amount of breast milk or other liquids? 1 = Less; 2 = Same; 3 = More					
24	When your infant has diarrhea, do you offer ORS or other treatment? 0 = No; 1 = Yes					
25	Since your last visit, has your infant had any unscheduled clinic or hospital visits? No = 0; Yes = 1 *If Yes, answer 25a*			25a	If yes, what was the reason for the clinic/hospital visit?	
26	Since your last visit, has your infant been diagnosed with any illnesses (including malaria) by a doctor or a nurse? No = 0; Yes = 1 *If Yes, answer 26a*			26a	If yes, what was the diagnosis?	

27	Since your last visit, has your infant taken any medicines? No = 0; Yes = 1 *If Yes, answer 27a*		27a	If yes, what medicine and for what purpose?	

Maternal Health Questions

28	Have you experienced any of the following symptoms in the past month? No = 0; Yes = 1					
	28a	Diarrhea *If Yes, answer 28b*		28b	If yes, can you describe the appearance of the diarrhea? 1 = voluminous, watery; 2 = bloody; 3 = mucus; 0 = none of these	
	28c	Mastitis				
	28d	Fever				
	28e	Skin rash				
	28f	Vomiting				
	28g	Cough				
	28h	Abdominal discomfort				
	28i	Loss of appetite				
	28j	Nausea				
	28k	Fatigue				
	28l	Difficulty breathing				
29	Have you been hospitalized or had any unscheduled clinic visits in the past month? No = 0; Yes = 1 *If Yes, answer 29a*			29a	If yes, for what reasons were you hospitalized or had unscheduled clinic visits?	
30	On how many days in the past month did you have diarrhea?					
31	Have any other members of your household had diarrhea in the past month? No = 0; Yes = 1					
32	How many meals did you consume yesterday?					
33	In the past month, how many times did you go to sleep hungry?					
34	In the past month, on how many days did you consume zero meals?					
35	In the past week, on how many days did you eat meat or fish?					

36	How many times in the past month did your household have difficulty in satisfying its food needs?		

Behavior Questions

37	Do you wash your hands before eating? No = 0; Yes = 1					
38	Do you wash your hands after going to the toilet? No = 0; Yes = 1					
39	Do you wash your hands before feeding your infant? No = 0; Yes = 1					
40	Do you use soap or soda to wash your hands? No = 0; Yes = 1					
41	Do you have any adult animals in your household? No = 0; Yes = 1 *If Yes, answer 41a-c*		41a	If yes, please list the type of animal and how many of each that you own:	Animal	#
					Animal	#
					Animal	#
					Animal	#
					Animal	#
			41b	Do you care for the adult animals? No = 0; Yes = 1		
			41c	Has your infant had contact with any adult animals in the past month? No = 0; Yes = 1		
42	Do you have any young animals in your household? No = 0; Yes = 1 *If Yes, answer 42a-c*		42a	If yes, please list the type of animal and how many of each that you own:	Animal	#
					Animal	#
					Animal	#
					Animal	#
					Animal	#
			42b	Do you care for the young animals? No = 0; Yes = 1		
			42c	Has your infant had contact with any young animals in the past month? No = 0; Yes = 1		

Measurements and Sample Collection

43	Maternal Height	cm		Comments:		
44	Maternal Weight	Kg		Comments:		
45	Maternal MUAC	mm		Comments:		
46	Maternal Tricep Skinfold Thickness	mm		Comments:		
47	Maternal Blood Sample No = 0; Yes = 1			Comments:		
48	Breast Milk Sample No = 0; Yes = 1 *If Yes, answer 48a*		48a	Time of Breast Milk Collection *Record time using 24-hour clock*	Hour	Minute
49	Maternal Fecal Sample No = 0; Yes = 1 *If Yes, answer 49a*		49a	Record Appearance of Maternal Fecal Sample:		
50	Infant Length	cm		Comments:		
51	Infant Weight	g		Comments:		
52	Infant MUAC	mm		Comments:		
53	Infant Head Circumference	cm		Comments:		
54	Infant Tricep Skinfold Thickness	mm		Comments:		
55	Infant Blood Sample No = 0; Yes = 1			Comments:		
56	Infant Fecal Sample No = 0; Yes = 1 *If Yes, answer 56a*		56a	Record Appearance of Infant Fecal Sample:		

Appendix D. Mean concentrations of immunoglobulins in maternal serum, breast milk, and infant serum, by HIV-status/exposure, maternal underweight (BMI < 18.5), and exclusive breastfeeding status.

	Maternal Serum, mean (SD)						Breast Milk, mean (SD)						Infant Serum, mean (SD)					
	HIV-status		Underweight		EBF		HIV-status		Underweight		EBF		HIV-status		Underweight		EBF	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
IgA																		
Month 1	227.7 (69.0)	227.7 (76.0)	211.1 (61.8)	229.3 (73.4)	**252.0* (89.5)**	**215.8* (60.75)**	170.2 (263.0)	143.9 (200.6)	122.6 (54.4)	157.4 (236.4)	**250.0** (370.2)**	**108.9** (73.7)**	**192.6* (45.7)**	**389.4* (171.5)**	412.7 (330.6)	315.8 (154.4)		
Month 2	244.7 (84.1)	220.9 (58.1)	220.0 (51.4)	231.7 (73.4)	**270.7* (100.0)**	**223.0* (62.9)**	303.6 (238.4)	324.3 (334.0)	245.5 (109.2)	324.1 (314.1)	440.3 (445.4)	290.3 (257.4)	**289.5* (106.0)**	**603.2* (462.8)**	205.1 (31.7)	486.1 (387.2)	514.5 (498.1)	440.4 (339.5)
Month 3	62.4 (21.7)	59.6 (21.8)	52.3 (18.5)	62.2 (22.0)	68.9 (27.7)	59.8 (20.7)	230.5 (144.6)	265.8 (213.1)	232.6 (103.2)	254.6 (199.7)	330.2 (236.3)	237.6 (176.3)	491.8 (382.9)	586.2 (534.6)	856.9 (909.5)	502.5 (409.0)	318.4 (186.3)	582.8 (479.0)
Month 6	54.2 (22.0)	61.1 (24.3)	**75.6* (30.9)**	**56.1* (22.0)**	47.5 (17.0)	58.9 (23.7)	176.8 (99.8)	160.7 (150.7)	166.6 (102.1)	165.6 (140.0)	**513.1*** (595.6)**	**153.0*** (70.9)**	589.4 (469.9)	507.1 (495.6)	226.6 (63.7)	610.5 (481.8)		
IgG1																		
Month 1	905 (1066)	754 (540)	931 (676)	816 (847)	1013 (1215)	739 (565)	1033 (2315)	668 (1424)	434 (208)	849 (1907)	1167 (475)	605 (1354)	851 (559)	2478 (2902)	1055 (882)	2100 (2659)		
Month 2	**1355* (2410)**	**582* (210.9)**	586 (314)	962 (1727)	861 (1115)	936 (1731)	1061 (847)	975 (1390)	705 (437)	1038 (1267)	**1934*** (2519)**	**807*** (533)**	5236 (4203)	3467 (3276)	6184 (5397)	4080 (3677)	6505 (3856)	3433 (3451)
Month 3	318 (260)	257 (138)	237 (168)	293 (211)	221 (170)	294 (209)	1114 (1544)	834 (452)	839 (437)	972 (1134)	**1641** (2534)**	**833** (425)**	2255 (2823)	2437 (2953)	2184 (2427)	2353 (2905)	1245 (1191)	2581 (3031)
Month 6	----	----	----	----	----	----	858 (1806)	584 (684)	510 (314)	699 (1262)	**2032* (2741)**	**626* (1095)**	3979 (2899)	3109 (3316)	670 (264)	4106 (2963)		
IgG2																		
Month 1	2539 (5068)	2287 (2463)	1630 (765)	2484 (4072)	3202 (6077)	2053 (2249)	3629 (7150)	4155 (7435)	1794 (1277)	4168 (7608)	4999 (8314)	3465 (6825)	1879 (1729)	7797 (9311)	10780 (14073)	5258 (7584)		
Month 2	6503 (10522)	4556 (6879)	3324 (1487)	5609 (9144)	4453 (8495)	5554 (8805)	4086 (7285)	3212 (4491)	2118 (1762)	3669 (5890)	**6529* (9906)**	**2878* (4048)**	17893 (13374)	17671 (13374)	17052 (18311)	17844 (13061)	23769 (10057)	15523 (13602)
Month 3	548 (738)	468 (491)	515 (443)	504 (641)	352 (311)	527 (645)	----	----	----	---	----	---	8741 (12836)	13326 (14411)	15435 (20597)	10364 (13229)	8559 (14323)	11329 (13625)
Month 6	1157 (5215)	1109 (4419)	456 (863)	1213 (5013)	64 (58)	1184 (4850)	----	----	----	---	----	---	8770 (6449)	11000 (11313)	1613 (773)	11288 (8640)		
IgG3																		
Month 1	15.27 (24.83)	10.59 (7.65)	15.42 (15.38)	12.56 (18.32)	**18.95* (29.45)**	**9.96* (7.35)**	----	----	----	---	----	---	9.43 (4.87)	40.67 (46.29)	18.74 (20.41)	32.64 (43.08)		
Month 2	21.05 (29.62)	11.97 (8.07)	13.84 (11.52)	16.29 (21.78)	14.69 (11.25)	16.30 (22.35)	25.36 (28.31)	22.60 (23.44)	20.48 (13.34)	23.93 (26.15)	**42.13** (48.17)**	**19.63** (14.54)**	58.88 (64.00)	53.16 (60.82)	81.69 (96.60)	53.17 (59.34)	67.03 (65.60)	51.53 (60.64)
Month 3	2.85 (1.94)	2.53 (2.03)	2.46 (1.45)	2.71 (2.06)	2.39 (0.88)	2.72 (2.09)	26.04 (32.57)	29.32 (27.79)	28.38 (18.95)	27.88 (31.44)	**50.70** (55.30)**	**24.07** (21.05)**	29.79 (47.08)	35.42 (46.31)	39.18 (47.75)	31.67 (46.72)	14.86 (18.00)	36.24 (49.34)
Month 6	6.15 (25.92)	2.77 (4.84)	3.48 (4.94)	4.26 (17.97)	2.28 (2.91)	4.26 (17.42)	19.80 (37.03)	12.17 (19.68)	12.06 (9.27)	15.16 (28.55)	**57.97** (79.88)**	**13.14** (22.66)**	47.46 (51.33)	35.52 (56.43)	6.35 (3.56)	48.42 (54.02)		
IgG4																		
Month 1	5.60 (4.03)	5.79 (4.38)	5.40 (4.06)	5.73 (4.23)	6.60 (5.09)	5.28 (3.71)	13.46 (51.96)	5.21 (10.55)	2.55 (3.37)	9.03 (35.06)	17.23 (56.53)	4.27 (10.93)	7.81 (1.94)	19.98 (17.26)	26.42 (24.56)	14.71 (14.31)		
Month 2	9.86* (10.27)	5.99* (4.23)	5.58 (4.60)	7.88 (7.99)	7.66 (7.73)	7.65 (7.79)	15.69 (9.57)	19.29 (21.31)	19.46 (15.58)	17.82 (18.27)	**27.87* (29.34)**	**15.85* (13.85)**	33.57 (34.54)	31.32 (29.32)	60.54 (74.68)	29.53 (26.06)	**53.39* (47.09)**	**24.46* (19.12)**
Month 3	0.57 (0.78)	0.32 (0.20)	0.41 (0.39)	0.44 (0.59)	0.28 (0.00)	0.46 (0.60)	16.05 (36.04)	19.20 (42.34)	17.31 (20.33)	18.00 (42.40)	31.05 (57.76)	15.64 (35.73)	14.53 (21.00)	46.61 (91.38)	36.31 (46.53)	28.39 (66.23)	9.31 (10.65)	33.51 (70.03)
Month 6	0.97 (3.16)	0.72 (1.49)	0.89 (1.66)	0.82 (2.40)	0.28 (0.00)	0.85 (2.35)	9.32 (11.50)	7.77 (7.22)	12.93 (14.03)	7.70 (8.09)	14.91 (11.38)	7.98 (8.73)	13.81 (9.12)	13.69 (9.02)	4.16 (1.55)	15.50 (8.28)		
IgM																		
Month 1	142.8 (51.9)	193.6 (156.1)	138.9 (61.8)	172.5 (124.9)	176.6 (118.1)	166.9 (124.0)	238.6 (194.3)	221.2 (149.1)	229.3 (155.4)	227.8 (169.4)	254.0 (126.2)	214.7 (184.8)	103.7 (53.6)	178.8 (98.9)	246.7 (252.6)	142.3 (59.3)		
Month 2	195.8 (96.7)	206.9 (118.7)	194.1 (64.8)	201.6 (113.6)	221.5 (112.4)	197.0 (109.4)	539.4 (283.8)	667.7 (648.5)	554.0 (380.7)	627.9 (561.7)	677.4 (568.9)	608.8 (544.3)	232.5 (235.1)	348.0 (416.8)	433.6 (514.3)	281.7 (338.3)	**528.8* (568.3)**	**208.0* (166.7)**
Month 3	29.5 (15.9)	35.8 (51.9)	27.2 (15.4)	33.7 (41.9)	26.6 (15.2)	33.7 (41.7)	373.6 (438.6)	416.4 (428.3)	293.8 (174.3)	418.2 (461.2)	489.5 (657.0)	383.0 (383.2)	229.3 (301.0)	468.5 (778.2)	563.1 (688.8)	315.6 (570.0)	154.7 (155.7)	378.8 (619.4)
Month 6	----	----	----	----	----	----	176.3 (85.3)	186.4 (260.3)	182.3 (88.4)	182.6 (229.1)	146.0 (11.8)	184.4 (220.3)	506.5 (887.7)	528.0 (968.2)	74.2 (24.4)	596.8 (946.3)		

*p < 0.05; ** p < 0.01; *** p < 0.001

Note: Underweight = body mass index (BMI) < 18.5; EBF = exclusive breastfeeding

Appendix E. Mean concentrations of cytokines and percent of maternal serum, breast milk, and infant serum samples with detectable concentrations of cytokines, by HIV-status/exposure, maternal underweight (BMI < 18.5), and exclusive breastfeeding status.

	Maternal Serum, mean (% detectable)						Breast Milk, mean (% detectable)						Infant Serum, mean (% detectable)					
	HIV-status		Underweight		EBF		HIV-status		Underweight		EBF		HIV-status		Underweight		EBF	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
IFNγ																		
Month 1	4.36 (22%)	3.26 (17%)	3.29 (29%)	3.83 (18%)	2.90 (16%)	4.24 (21%)	2.32 (3%)	5.42 (6%)	<LOD (0%)	4.37 (5%)	7.67 (11%)	2.53 (2%)	6.23 (14%)	4.98 (30%)	<LOD (0%)	6.11 (23%)	<LOD (0%)	6.11 (23%)
Month 2	6.69 (32%)	2.60 (15%)	3.22 (43%)	4.55 (21%)	3.92 (27%)	4.51 (22%)	<LOD (0%)	12.49 (6%)	<LOD (0%)	9.41 (4%)	<LOD (0%)	10.17 (5%)	4.66 (33%)	2.72 (25%)	8.09 (100%)	3.41 (22%)	4.81 (33%)	3.48 (29%)
Month 3	6.09 (24%)	3.40 (18%)	<LOD (0%)	5.03 (24%)	<LOD (0%)	4.99 (23%)	2.32 (3%)	6.39 (8%)	<LOD (0%)	5.16 (7%)	2.63 (15%)	5.05 (4%)	<LOD (0%)	2.60 (12%)	<LOD (0%)	2.51 (9%)	<LOD (0%)	2.49 (8%)
Month 6	4.57 (18%)	2.81 (11%)	<LOD (0%)	3.70 (15%)	6.35 (25%)	3.39 (13%)	<LOD (0%)	7.43 (6%)	<LOD (0%)	6.07 (4%)	<LOD (0%)	5.76 (4%)	4.29 (50%)	42.02 (67%)	7.65 (100%)	31.75 (50%)		
IL-1β																		
Month 1	2.39 (3%)	<LOD (0%)	<LOD (0%)	2.33 (1%)	<LOD (0%)	2.35 (2%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	3.27 (14%)	<LOD (0%)	<LOD (0%)	2.81 (8%)	<LOD (0%)	2.81 (8%)
Month 2	2.51 (6%)	<LOD (0%)	<LOD (0%)	2.38 (3%)	<LOD (0%)	2.39 (3%)	2.42 (3%)	7.72 (10%)	<LOD (0%)	6.14 (8%)	**18.24*** **(13%)**	**2.92*** **(6%)**	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)
Month 3	<LOD (0%)	2.32 (3%)	<LOD (0%)	2.30 (2%)	<LOD (0%)	2.30 (2%)	2.67 (3%)	2.34 (2%)	<LOD (0%)	2.52 (3%)	<LOD (0%)	2.52 (3%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)
Month 6	2.47 (3%)	<LOD (0%)	<LOD (0%)	2.36 (1%)	<LOD (0%)	2.35 (1%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	4.17 (100%)	6.76 (33%)	<LOD (0%)	6.59 (75%)		
IL-2																		
Month 1	2.42 (8%)	<LOD (0%)	<LOD (0%)	2.35 (4%)	2.41 (8%)	2.31 (2%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	3.87 (14%)	<LOD (0%)	<LOD (0%)	3.13 (8%)	<LOD (0%)	3.13 (8%)
Month 2	2.44 (6%)	2.29 (2%)	<LOD (0%)	2.37 (5%)	<LOD (0%)	2.37 (5%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)
Month 3	<LOD (0%)	2.33 (3%)	<LOD (0%)	2.30 (2%)	<LOD (0%)	2.30 (2%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)
Month 6	2.31 (3%)	<LOD (0%)	<LOD (0%)	2.28 (1%)	<LOD (0%)	2.28 (1%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)		
IL-6																		
Month 1	<LOD (0%)	2.51 (5%)	**3.55**** **(14%)***	**2.28**** **(1%)***	<LOD (0%)	2.46 (4%)	6.24 (40%)	7.20 (23%)	2.41 (14%)	7.20 (31%)	6.01 (32%)	7.46 (29%)	4.76 (29%)	<LOD (0%)	<LOD (0%)	3.60 (15%)	4.70 (33%)	3.05 (8%)
Month 2	**3.26** **(13%)***	**<LOD** **(0%)***	<LOD (0%)	2.75 (6%)	2.68 (9%)	2.71 (5%)	5.44 (27%)	7.06 (22%)	<LOD (0%)	6.92 (26%)	11.19 (40%)	5.39 (20%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)
Month 3	2.58 (3%)	2.82 (3%)	<LOD (0%)	2.78 (3%)	<LOD (0%)	2.77 (3%)	**4.44** **(19%)***	**6.21** **(6%)***	<LOD (0%)	6.07 (12%)	5.28 (23%)	5.50 (8%)	2.77 (17%)	3.14 (12%)	4.60 (33%)	2.54 (9%)	3.78 (50%)	2.85 (8%)
Month 6	2.43 (3%)	2.38 (2%)	<LOD (0%)	2.34 (1%)	<LOD (0%)	2.41 (3%)	4.12 (14%)	2.74 (8%)	<LOD (0%)	3.37 (11%)	<LOD (0%)	3.28 (10%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)		
IL-10																		
Month 1	5.12 (49%)	8.01 (46%)	**10.81** **(86%)***	**6.23** **(44%)***	7.10 (32%)	6.20 (54%)	2.63 (3%)	<LOD (0%)	<LOD (0%)	2.42 (1%)	2.70 (4%)	<LOD (0%)	7.01 (71%)	8.89 (67%)	2.99 (67%)	9.38 (70%)	4.88 (100%)	8.94 (60%)
Month 2	5.61 (39%)	6.63 (35%)	6.37 (43%)	5.92 (35%)	5.31 (36%)	6.09 (36%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	17.37 (67%)	8.50 (75%)	35.43 (100%)	11.42 (67%)	17.37 (100%)	12.30 (57%)

Month 3	5.44 (41%)	11.21 (44%)	8.90 (50%)	8.47 (41%)	2.98 (22%)	9.30 (45%)	<LOD (0%)	3.81 (2%)	<LOD (0%)	3.34 (1%)	**8.46* (8%)***	**<LOD* (0%)***	5.32 (67%)	18.36 (62%)	13.08 (100%)	12.69 (55%)	4.43 (50%)	14.16 (67%)
Month 6	7.96 (55%)	6.72 (43%)	8.45 (62%)	7.17 (46%)	4.59 (25%)	7.37 (49%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	7.02 (100%)	14.22 (67%)	27.71 (100%)	7.25 (75%)		
IL-12p70																		
Month 1	4.54 (22%)	3.46 (15%)	4.75 (14%)	3.90 (18%)	4.98 (20%)	3.53 (17%)	2.40 (6%)	2.30 (2%)	<LOD (0%)	2.34 (4%)	2.30 (4%)	2.32 (2%)	6.19 (14%)	3.27 (20%)	<LOD (0%)	5.15 (23%)	<LOD (0%)	5.15 (23%)
Month 2	5.89 (16%)	3.08 (7%)	2.88 (15%)	4.06 (10%)	<LOD (0%)	4.26 (12%)	<LOD (0%)	2.36 (4%)	<LOD (0%)	2.33 (3%)	**2.58** (13%)****	**<LOD** (0%)****	3.59 (33%)	2.66 (25%)	7.94 (100%)	2.69 (22%)	4.16 (33%)	2.82 (29%)
Month 3	4.16 (21%)	3.35 (21%)	2.91 (20%)	3.86 (21%)	<LOD (0%)	3.93 (23%)	2.36 (3%)	2.70 (12%)	2.51 (7%)	2.56 (8%)	**3.31* (15%)**	**2.42* (7%)**	<LOD (0%)	2.63 (12%)	**3.25 (33%)***	**<LOD (0%)***	<LOD (0%)	2.51 (8%)
Month 6	6.08 (12%)	2.66 (11%)	<LOD (0%)	4.30 (13%)	**15.08* (25%)**	**3.49* (11%)**	<LOD (0%)	2.64 (6%)	3.21 (11%)	2.42 (3%)	<LOD (0%)	2.52 (4%)	5.45 (50%)	3.02 (33%)	4.54 (100%)	3.85 (25%)		
IL-13																		
Month 1	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	2.37 (4%)	<LOD (0%)	2.33 (3%)	**2.46 (7%)***	**<LOD (0%)***	3.17 (14%)	<LOD (0%)	<LOD (0%)	2.75 (8%)	<LOD (0%)	2.75 (8%)
Month 2	<LOD (0%)	2.30 (2%)	<LOD (0%)	2.29 (2%)	<LOD (0%)	2.29 (2%)	2.58 (10%)	2.36 (4%)	<LOD (0%)	2.46 (7%)	2.47 (13%)	2.43 (5%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)
Month 3	<LOD (0%)	2.37 (3%)	<LOD (0%)	2.33 (2%)	<LOD (0%)	2.33 (2%)	2.48 (11%)	2.32 (4%)	<LOD (0%)	2.41 (8%)	2.48 (15%)	2.37 (5%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)
Month 6	2.52 (3%)	<LOD (0%)	<LOD (0%)	2.38 (1%)	<LOD (0%)	2.37 (1%)	2.48 (10%)	2.32 (4%)	2.48 (11%)	2.37 (6%)	<LOD (0%)	2.38 (6%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)		
IL-15																		
Month 1	3.33 (14%)	2.41 (5%)	<LOD (0%)	2.90 (10%)	3.04 (12%)	2.76 (8%)	35.38 (97%)	22.89 (98%)	23.81 (100%)	28.25 (97%)	34.86 (100%)	24.60 (96%)	5.34 (14%)	<LOD (0%)	<LOD (0%)	3.92 (8%)	<LOD (0%)	3.92 (8%)
Month 2	3.04 (10%)	2.57 (2%)	<LOD (0%)	2.84 (6%)	<LOD (0%)	2.88 (7%)	21.37 (97%)	24.10 (100%)	17.24 (100%)	23.73 (99%)	21.76 (100%)	23.39 (98%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)
Month 3	2.81 (15%)	2.51 (5%)	2.97 (10%)	2.60 (10%)	<LOD (0%)	2.70 (11%)	21.23 (95%)	18.04 (96%)	12.99 (93%)	20.55 (96%)	19.35 (100%)	19.36 (95%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)	<LOD (0%)
Month 6	2.62 (3%)	2.32 (2%)	<LOD (0%)	2.57 (3%)	<LOD (0%)	2.46 (3%)	20.48 (90%)	13.23 (89%)	10.19 (100%)	16.22 (87%)	11.63 (100%)	16.01 (88%)	3.24 (50%)	<LOD (0%)	<LOD (0%)	2.75 (25%)		
TNFα																		
Month 1	**15.00*** (97%)**	**9.95*** (97%)**	**11.53 (86%)***	**12.43 (99%)***	**14.52* (96%)**	**11.39* (98%)**	6.86 (50%)	5.32 (52%)	4.86 (71%)	6.02 (49%)	**7.92 (68%)***	**5.00 (42%)***	21.01 (100%)	23.63 (100%)	23.45 (100%)	22.25 (100%)	20.80 (100%)	22.86 (100%)
Month 2	**14.24* (97%)**	**10.06* (97%)**	9.18 (86%)	12.19 (98%)	10.36 (91%)	12.17 (98%)	9.20 (70%)	9.79 (63%)	4.05 (37%)	10.18 (68%)	10.95 (60%)	9.26 (67%)	32.49 (100%)	25.12 (100%)	40.17 (100%)	28.36 (100%)	34.34 (100%)	27.48 (100%)
Month 3	**14.58* (94%)**	**11.42* (97%)**	12.70 (100%)	12.92 (95%)	**12.03 (78%)****	**13.01 (98%)****	7.49 (46%)	5.72 (44%)	3.20 (36%)	7.07 (47%)	10.18 (62%)	5.82 (42%)	19.59 (100%)	34.13 (100%)	**46.15* (100%)**	**22.92* (100%)**	20.88 (100%)	29.07 (100%)
Month 6	**14.43*** (97%)**	**9.12*** (97%)**	8.32 (100%)	11.75 (99%)	11.86 (100%)	11.28 (99%)	4.97 (38%)	4.61 (43%)	2.47 (11%)	4.88 (44%)	5.50 (50%)	4.70 (41%)	27.92 (100%)	24.45 (100%)	40.03 (100%)	22.29 (100%)		

*p < 0.05; ** p < 0.01; *** p < 0.001

Note: Underweight = body mass index (BMI) < 18.5; EBF = exclusive breastfeeding; LOD = limit of detection

APPENDIX F

CONSENT & RECRUITMENT PROTOCOL

PURPOSE

To ensure that study nurses obtain consent from participants according to ethical guidelines.

INSTRUCTIONS

1.1. Ask pregnant woman at the Kisesa Health Centre if she would like to be in a study on **mother and child health during pregnancy**.

1.2. Bring woman to the study room and ask if she meets study requirements:

1.2.1. Is she already in the study? (check for sticker on ANC card)

1.2.1.1. If YES, obtain her folder and use the appropriate Protocol for her visit.

1.2.1.2. If NO, continue.

1.2.2. Has she had an HIV test during this pregnancy?

1.2.2.1. If YES, or if she already knows herself to be HIV-positive, continue.

1.2.2.2. If NO, ask "will you agree to be tested for HIV?" If she does not agree to be tested for HIV, stop the interview.

1.3. Ask woman if she can read Kiswahili:

1.3.1. If YES, check the "Yes" box on the **Consent Form** and ask her to read it.

1.3.2. If NO, check the "No" box. Find a person at KHC (other than yourself) to sit in the room while you read the **Consent Form** to the woman. This will be the "Impartial Witness".

1.4. If she agrees to be in the study, have her write her name on the **Consent Form** and sign in **ALL 3 PLACES**. If she does not agree, end the interview.

1.4.1. If there is an "Impartial Witness" (because the woman being consented is illiterate), this person must write and sign ***her own name***.

1.4.2. If the woman being consented cannot write her name and signature, write on her Thumb finger with a *Sharpie* marker and have her press it down firmly in the signature lines on the Consent Form. Write her name on the form.

1.5. Write ***your name*** on the **Consent Form** and sign where it says "Signature of person witnessing consent".

1.6. Write down the woman's study number and the date in the **Log Book**. **Important** if a woman begins the consent process, but does not wish to participate, ask her the reason and write this in the **Log Book.**

1.7. Obtain the "Enrollment Interview Protocol".

APPENDIX G

Maternal and Child Health Study
Consent Form

You are being invited to take part in a research study to help us understand pregnancy, nutrition, infant feeding, and childhood infections. We are asking you to take part because you have attended the ANC clinic at Kisesa Health Centre. Please read this form carefully and ask any questions before agreeing to take part in the study. You may also request that this information be read to you.

The interview will take approximately 45 minutes to complete. If any of our tests show unusual results, we will notify Kisesa Health Centre so that they may see you to make a diagnosis and offer appropriate treatment if needed.

What the study is about: The purpose of this study is to learn how nutrition and infections may affect infant growth during pregnancy, and how infant feeding affects early childhood growth and chance of getting childhood infections.

What we will ask you to do: If you agree to be in the study, we will ask you some questions about your health and look at your medical records to gather information about your past health. We will also take 5mL of your blood and take measurements including your height, weight, mid-upper arm circumference, fundal height, and skinfold thickness two times during your antenatal care visits and four times after you deliver your baby. These appointments will last approximately 45 minutes. We will encourage you to deliver your baby at the Kisesa Health Centre as is offered to all women, and if you do, we will collect 5mL of blood from your umbilical cord and collect a small portion of your placenta after delivery. If you miss a study visit, a field worker and the Study Coordinator will visit your home address to ascertain the health status of you and your infant and whether you wish to remain enrolled in the study. After you give birth, we will ask you to provide a small sample of your breast milk at four times. We will also ask questions about how you and caregivers (i.e. family members) feed your infant.

What we will ask you to provide parental permission for: After you give birth, we will take a very small amount of blood from your child using a heel prick and measure the length, weight, arm circumference, skinfold thickness, and head circumference of your child at four times. Follow-up visits will be scheduled during your regular vaccination appointments for your child when your child is 1, 2, and 3 months old. We will also ask you to come for another appointment at the Kisesa Health Centre when your child is 6 months of age. At each of these follow-up visits we will request that you bring a small sample of fresh stool from your infant. We will also take another very small amount of blood using a heel prick from your child at these visits. These appointments will last approximately 30 minutes. If you miss an appointment, a study team member will contact you to reschedule the appointment or if you agree, we will visit your home to collect a stool sample from your infant, and ask a few questions about the general health of your baby.

Risks and benefits: We anticipate limited risks to you and your child from participating in this study. There is minimal risk of brief temporary discomfort from taking blood. We will minimize this discomfort by using special blood collection tools designed for newborns and young infants. The umbilical cord and placenta are usually discarded after delivery, and the collection of cord blood and placenta will not affect the delivery or care of you or your child. Placentas and leftover blood that is not used in this study will be stored at NIMR (National Institute of Medical Research), Mwanza. All stored samples will be marked with a number code that will not contain your name. By signing this document, you are giving us, or other researchers, permission to conduct future research using these stored samples.

This study will help us understand how a mother's health during pregnancy may change her child's health and growth. This study will also help us understand how infant feeding methods affect early infant growth and chance of childhood infections. There are no direct benefits to you or your child, but the study will pay for your transportation to Kisesa Health Centre and provide you with a small amount of food while at the clinic during your visits.

Your answers will be confidential. This paper containing your name will be kept private in a locked file at NIMR, and only authorized researchers will have access to this paper. It will be destroyed by shredding after seven years. Public reports of our findings will not include any information that will make it possible to identify you or your child.

Taking part is voluntary: Taking part in this study is completely voluntary and you will not be compensated for your participation. You may decline to answer any question or take any test as part of this study, or completely end your participation in this study without prejudice to your current or future care at Kisesa Health Centre. You may also stop your or your child's involvement at any time during any interview or examination. If you decide not to take part, it will not affect you or your family's current or future medical care.

If you have questions in the future: The researchers conducting this research study are Mr. Mark Urassa, NIMR and Professor Joann McDermid, Cornell University. Your healthcare will continue to be provided by Kisesa Health Centre. If you have questions later, you may contact Mr. Mark Urassa at: National Institute for Medical Research (NIMR), P.O. Box 1462, Mwanza. Tel: +255-282500399 /2503012, Email: malloomark@yahoo.com. If you have any questions or concerns regarding you rights as a subject in this study, you may contact the National Health Research Review Sub-committee (NatRec) at: P.O. Box 9653, Dar es Salaam, Tanzania. Tel: 255-22-2121 400, Email: ethics@nimr.or.tz. You will be given a copy of this form to keep for your records.

Statement of Consent: I have read the above information, and have received answers to any questions I asked. I consent to take part in the study.

Participant is Literate: Yes [ ] No [ ]

Your Signature _____ Date
_____

Your Name (printed)
_____

Parental Permission

In addition to agreeing to my participation, I also consent to have my infant participate in the study and have blood taken up to six times.

Your signature _____ Date
_____

Signature of person witnessing consent _____Date
_____

In addition, I give permission for my placenta and any blood that is collected from me or my child that is not used in this study to be used in future research.

Your signature _____ Date
_____

Printed name of person witnessing consent _____Date
_____

Signature of Impartial Witness (for illiterate participants)

_____ Date
_____

This consent form will be kept by the researcher for at least seven years beyond the end of the study and has been approved by the National Health Research Ethics Subcommitttee, the Cornell Institutional Review Board and the London School of Hygiene and Tropical Medicine Ethics Committee.

APPENDIX H

ENROLLMENT INTERVIEW PROTOCOL

PURPOSE

To ensure that study nurses complete questionnaires, and collect body measurements and blood appropriately during the Enrollment (first) Interview with a participant. This interview is to be completed immediately after obtaining consent from a participant (see "Consent & Recruitment Protocol").

INSTRUCTIONS

1.1. Obtain the **Enrollment Questionnaire Form** from the participant's folder. Ensure that the "Study ID" number on this form matches the number on the Consent Form.

1.2. Ask the participant all of the questions on the **Enrollment Questionnaire Form** and write down her answers.

1.3. Take all of the participant's measurements and write them on the **Enrollment Questionnaire Form** (see attached pages for instructions and measurement diagrams).

1.4. Put **STUDY STICKERS** on the large tube, and on each of the 3 small tubes.

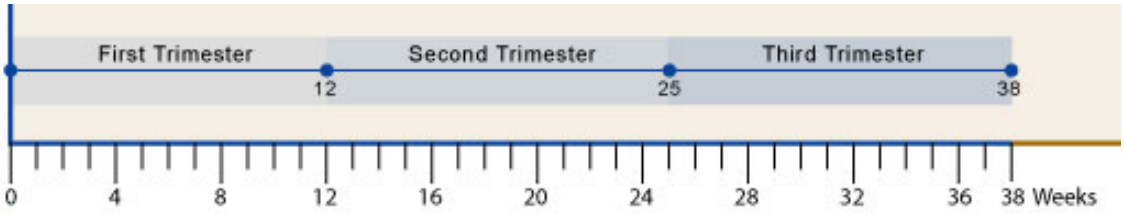
1.4.1. Take blood from the woman and put it into the large plastic tube.

1.4.2. Give the large plastic tube and the 3 small tubes to the Study Lab Technician.

1.5. Obtain the **Participant Information Card** from her folder.

1.5.1. Explain which day she should come for her next study visit and write this date and the day of the week it will be on the **Participant Information Card.** Explain that if she comes to the ANC between now and her next appointment, she does not need to see us.

1.5.2. **Important** We need to see the participant again before she gives birth, and we want this to be during the **Third Trimester** and as close to the **32nd** week as possible.



1.5.3. Staple the **Participant Information Card** to the participant's ANC Card.

1.6. Give woman her **money and food** and thank her for being in the study.

1.7. Write down the woman's study number on the **Participant List** and write today's date under the "Enrollment" Box.

APPENDIX I

DELIVERY & BIRTH PROTOCOL

PURPOSE

To ensure that study nurses collect body measurements and blood appropriately during the Delivery & Birth Follow-up with a participant.

INSTRUCTIONS

1.1. Verify mother's name and study ID number on **Participant List** and the **Participant Information Card** (if she has it). Obtain the **Delivery & Birth Questionnaire Form** from the participant's folder. Ensure that the "Study ID" number on this form matches the number on the **Participant Information Card**.

1.2. Put a **STUDY ID STICKER** on a large (8.5 mL) tube, and on 3 small (eppendorf) tubes.

1.3. After the participant has delivered her baby, obtain the woman's umbilical cord.

1.3.1. Use a syringe to take blood from the umbilical cord (just as you would if you were taking blood from a patient).

1.3.2. Give the large plastic tube and the 3 small tubes to AW or SP and they will deliver them to the Study Lab Technician.

1.4. Take all of the infant's measurements and write them on the **Delivery & Birth Questionnaire Form** (refer to **Measurement Diagrams** for instructions regarding measurements and biological sample collection).

1.5. Before the participant is discharged from Kisesa Health Centre, give her **money and food** and thank her for coming to the appointment.

1.6. Find the woman's Study ID number **Participant List** and write today's date under the "Birth" Box.

APPENDIX J

MATERNAL AND INFANT FOLLOW-UP PROTOCOL

PURPOSE

To ensure that study nurses complete questionnaires, and collect body measurements and biological samples correctly during the maternal and infant follow-up visits (completed at month 1, month 2, month 3, and month 6 post-partum) with a participant.

INSTRUCTIONS

1.1. Verify mother's name and study ID number on **Participant List**. (check for sticker on ANC card and/or infant under-5 card)

1.2. Bring woman to the study room and ask if she would still like to participate in the study:

1.2.1. If YES, obtain her folder and use the appropriate Feeding and Health Questionnaire for her visit.

1.2.2. If NO, record that the mother terminated consent on the **Study Exit Form**.

1.3. Give the woman a plastic container labeled with her study ID number and ask her to try to produce a fecal sample at any time during the visit. Record the appearance of the fecal sample on the **Feeding and Health Questionnaire.**

1.4. Instruct the woman to alert you at any time during the visit if her infant has a bowel movement.

1.5. When the infant has a bowel movement, collect the feces and place in a universal container labeled with the infant's study ID number. Record the appearance of the fecal sample on the **Feeding and Health Questionnaire.**

1.6. Ask the woman all of the questions on the **Feeding and Health Questionnaire** and write down her answers. Explain questions to the woman if she does not understand them.

1.7. Take all of the measurements from the woman and her infant and write the numbers on the **Feeding and Health Questionnaire**

1.7.1. Refer to **Measurement Diagrams** for instructions regarding measurements and biological sample collection.

1.8. Put a sticker with the woman's study ID number on a universal container.

1.9. Ask the woman to feed her infant and then manually express her breast milk into the universal container at the end of the feed. Record the time of collection on the **Feeding and Health Questionnaire.**

1.10. Put a sticker with the woman's Study Identification Number on the blood collection tube.

1.11. Take blood from the woman and put it into the plastic tube.

1.12. Put a sticker with the infant's study ID number on the microtainer

1.13. Take blood from the infant and put it in the microtainer

1.14. Check to ensure that there is a study sticker on both the woman's ANC card and the infant's under-5 card. If not, put a **sticker** on the woman's ANC (Antenatal Clinic) Card and/or the infant's under-5 card.

1.15. Place the containers of breast milk, blood, and feces in the **refrigerator**.

1.16. Give woman her **money and food** and thank her for being in the study.

1.17. Write down the woman's study number and the date in the **Log Book**.

APPENDIX K

KISESA LAB SAMPLES PROTOCOL

PURPOSE

To ensure that blood, fecal, and breast milk samples are stabilized correctly.

INSTRUCTIONS

2. **Maternal Blood Samples** will be collected by the Study Nurse into pre-sticker-labeled 8.5mL tubes. All tubes will have been labeled with Study ID stickers prior to blood being drawn in accordance with "Interview" Protocols.

   2.1. After blood collection, AW or SP will immediately transport tubes to a plastic rack in the Kisesa Health Centre Laboratory refrigerator. Three pre-sticker-labeled Eppendorf tubes will also be transported to the refrigerator and placed in a plastic container adjacent to the tube rack.

   2.2. Blood will be processed, and serum extracted by AW or SP **within 1 hour** of blood collection, according to the instructions below:

      2.2.1. Obtain the 3 Eppendorf tubes that correspond to each 8.5 mL blood tube to be processed. *Ensure that the Study ID numbers on the Eppendorf tubes match those on the blood tube*.

      2.2.2. Ensure that the 8.5 mL blood tube cap is secure, place it in the centrifuge, and spin for 15 minutes at 2500 rpm. **Important** Be sure to balance the tubes before spinning. If blood is not separated after 15 minutes, centrifuge for an additional 10 minutes.

      2.2.3. Use a 100mL pippete to collect supernatant and aliquot equally into the three Eppendorf tubes.

      2.2.4. Store the Eppendorf tubes containing serum in a tube rack in the refrigerator.

      2.2.5. Dispose of 8.5mL tube (now containing only red blood matter), in the red Biohazard bag.

      2.2.6. Write the "Study ID" number of the lab sample on the **Lab Sample Transport & Submission Form**, fill in the appropriate circles under "Type of Sample", and write your initials under "Processed". If you have any comments about the sample (for example, if a tube breaks), write them under "Comments" and write your initials.

3. **Infant Blood Samples** will be collected by the Study Nurse into pre-sticker-labeled microtainers. All microtainers will have been labeled with Study ID stickers prior to blood being drawn in accordance with "Interview" Protocols.

3.1. After blood collection, AW or SP will immediately transport tubes to a plastic container in the Kisesa Health Centre Laboratory refrigerator. *Microtainers will be processed at NIMR, Mwanza*. AW or SP will follow the instruction below:

3.2. Write the "Study ID" number of the lab sample on the Lab Sample Transport & Submission Form, fill in the appropriate circles under "Type of Sample", and write your initials under "Processed". If you have any comments about the sample (for example, if a tube breaks), write them under "Comments" and write your initials.

4. **Maternal Breast milk Samples** will be collected by the participant into into pre-sticker-labeled universal tubes.

4.1. After blood collection, AW or SP will immediately transport tubes to a plastic container in the Kisesa Health Centre Laboratory refrigerator.

4.2. Breastmilk will be processed, and serum extracted by AW or SP **within 1 hour** of collection, according to the instructions below:

4.2.1. Obtain the 3 Eppendorf tubes that correspond to each universal breast milk tube to be processed. *Ensure that the Study ID numbers on the Eppendorf tubes match those on the universal tube*.

4.2.2. Transfer breastmilk into a 10 mL tube.

4.2.3. Ensure that the 10 mL tube cap is secure, place it in the centrifuge, and spin for 15 minutes at 2500 rpm. **Important** Be sure to balance the tubes before spinning. If supernatant is not separated after 15 minutes, centrifuge for an additional 10 minutes.

4.2.4. Use a 100mL pippete to collect supernatant and aliquot equally into the three Eppendorf tubes.

4.2.5. Store the Eppendorf tubes containing breast milk supernatant in a tube rack in the refrigerator.

4.2.6. Dispose of 10 mL tube and universal tube in the red Biohazard bag.

4.2.7. Write the "Study ID" number of the lab sample on the **Lab Sample Transport & Submission Form**, fill in the appropriate circles under "Type of Sample", and write your initials under "Processed". If you have any comments about the sample (for

example, if a tube breaks), write them under “Comments” and write your initials.

5. **Maternal and Infant Fecal Samples** will be collected by the participant into pre-sticker-labeled universal tubes.

   5.1. After fecal sample collection, AW or SP will transfer approximately 5 g (5 mL) of feces into a 30 mL universal container.

   5.2. Add 5 mL 10% v/v formalin with a Pasteur pipet.

   5.3. Invert tube to mix thoroughly.

   5.4. Store the universal container with the fecal sample in the refrigerator until transport to NIMR

   5.5. Write the “Study ID” number of the lab sample on the Lab Sample Transport & Submission Form, fill in the appropriate circles under “Type of Sample”, and write your initials under “Processed”. If you have any comments about the sample (for example, if a tube breaks), write them under “Comments” and write your initials.

APPENDIX L

TRANSPORT OF LAB SAMPLES PROTOCOL

PURPOSE

To ensure that blood, fecal, and breast milk samples are transported under appropriate conditions from Kisesa Health Centre to NIMR, Mwanza.

INSTRUCTIONS

1.1. AW or SP will be responsible for the transport of all laboratory samples from Kisesa Health Centre to NIMR, Mwanza and will follow the instructions below.

1.2. At the end of the day, cross-reference the **Lab Sample Transport & Submission Form** with the blood, fecal, and breastmilk samples in the Kisesa Health Centre Laboratory refrigerator.

1.2.1. Ensure that all samples are present.

1.2.2. Any problems or irregularities with a sample should be noted under "Comments" on the **Lab Sample Transport & Submission Form**.

1.2.3. Ensure that the **Lab Sample Transport & Submission Form** is dated, and that all necessary fields are filled out.

1.3. Ensure that all tube caps are securely fashioned and load samples into the transportable refrigerator.

1.4. Initial under "Transported to NIMR" on the **Lab Sample Transport & Submission Form.**

1.5. Upon arrival at NIMR, Mwanza, place all samples into the McDermid refrigerator.

1.6. Initial under "McDermid fridge" on the **Lab Sample Transport & Submission Form.**

APPENDIX M

DATA MANAGEMENT PROTOCOL

PURPOSE

To ensure that questionnaire and sample data are entered consistently and accurately.

INSTRUCTIONS FOR QUESTIONNAIRE DATA ENTRY

5.6. At the end of each day, bring all completed questionnaire forms back to NIMR and store in a locked file cabinet.

5.7. Every Friday, double enter data into prepared Excel spreadsheet and write the date of entry and data enterer's initials on the questionnaire. Return the questionnaire to the locked file cabinet.

5.7.1. Following double entry compare documents to ensure data entry accuracy.

5.7.2. After checking for accuracy, save the data sheet on AW and SP's personal computers, SP's personal external hard drive, and AW's Research-Specific Online Drop-Box.

INSTRUCTIONS FOR SAMPLE DATA ENTRY

1.1. Enter results of sample analysis within one week of completion of analysis.

1.2. Double enter results of sample analysis into prepared Excel spreadsheet and write the date of entry and data enterer's initials on the printed sample results sheet. Return the printed sample results sheet to the locked file cabinet.

1.2.1. Following double entry compare documents to ensure data entry accuracy.

1.2.2. After checking for accuracy, save the data sheet on AW and SP's personal computers, SP's personal external hard drive, and AW's Research-Specific Online Drop-Box.

APPENDIX N

MAGPIX Human Cytokine/Chemokine Magnetic Bead Panel

Standard Operating Procedure

1. Sample Preparation: begin only after VERS has passed. All instruments should be available in Serology room.
    a. Remove from freezer box and defrost
    b. Vortex each sample for 3 seconds
    c. Centrifuge samples for 5 minutes at 3,000rpm
    d. Remove kit reagents from fridge and allow to come to room temperature
2. Preparation of Reagent for Immunoassay
    a. Diluted wash buffer = 30mL wash buffer + 270mL DI (deionized water, located in clear water bladder)
        i. Store for up to 1 month at 2-8°C
        ii. Label and date unused vials. Place in secure location in Serology fridge 2. Add to all similar sequences.
    b. Antibody-Immobilized Beads
        i. Sonicate each bead vial for 30 seconds; vortex 1 minute
        ii. Add 60µL from each vial into Mixing Bottle
        iii. Bring final volume to 3mL with Bead Diluent (for 9 beads, add 2.46mL)
        iv. Vortex mixed beads for 1 minute
        v. Store for up to 1 month at 2-8°C
    c. Prepared Quality Controls (each) = Quality Control vial + 250µL DI
        i. Invert vial 5 times
        ii. Vortex for 30 seconds
        iii. Allow to sit 5-10 minutes
        iv. Store for up to 1 month at ≤-20°C
    d. Prepared Serum Matrix = Serum Matrix bottle + 1mL Assay Buffer
        i. Invert vial 5 times
        ii. Vortex for 30 seconds
        iii. Allow to sit for 10 minutes
        iv. Store for up to 1 month at ≤-20°C
    e. Preparation of Human Cytokine Standard 6
        i. Reconstitute standard with 250µL DI
        ii. Invert vial 5 times to mix
        iii. Vortex for 10 seconds
        iv. Allow to sit for 5-10 minutes
    f. Preparation of Standards 1-5
        i. Label eppendorf tubes 1-5 and arrange in descending order
        ii. Add 200µL Assay Buffer to each of the 5 tubes
        iii. Perform serial dilution, centrifuging each tube for 20 seconds between steps
3. Immunoassay Procedure
    a. Allow all reagents to come to room temperature

b. Using the multi-channel pipet, add 200µL Assay Buffer to each well of plate
    i. Seal and mix on plate shaker for 10 minutes at room temperature (300rpm)
    ii. Gently decant assay buffer into sink
    iii. Remove residual buffer by inverting plate several times and tapping smartly onto absorbent towels
c. Add Reagents to Wells
    i. Add 25µL Assay Buffer to the Background wells
    ii. Add 25µL of each Standard or Control into the appropriate wells
    iii. Add 25µL Assay Buffer to the sample wells
d. Add 25µL samples to sample wells
e. Add 25µL Matrix Solution in the Background, Standards, and Quality Controls wells
f. Add beads to each well
    i. Vortex bead bottle for 30 seconds
    ii. Add 25µL of prepared beads to each well
    iii. Shake beads intermittently to avoid settling, suck beads in and out of pipet/resevoir to shake
g. Seal plate with plate sealer
    i. Incubate for 2 hours at room temperature with shaking
    ii. Ensure that the plate shaker is connected to a reliable power source

4. Allow Detection Antibody and Step.-Phy. Reagents to come to room temperature
5. Wash plate 2 times
    a. Rest plate on magnet for 60 seconds
    b. While still attached to magnet, gently decant into sink
        i. Lightly tap on absorbent towel several times
        ii. Remove plate from magnet
    c. Wash plate by adding 200µL Wash Buffer to each well
        i. Shake on plate shaker for 30 seconds
    d. Repeat steps a-c for 2 cycles
6. Add 25µL Detection Antibodies to each well
    a. Seal plate and incubate with agitation on plate shaker for 1 hour at room temperature
    b. Do not aspirate after incubation
7. Add 25µL Streptavidin-Phycoerythrin per well
    a. Seal the plate and incubate with agitation on plate shaker for 30 minutes at room temperature
8. Wash plate 2 times
9. Add 150µL Drive Fluid per well
    a. Seal plate and resuspend beads on plate shaker for 5 minutes at room temperature
10. Run on Luminex (100µL, 50 beads per Bead set)

Reagents

1. Luminex Drive Fluid; located in NIMR Storage
2. MAGPIX Human Cytokine/Chemokine Panel (HYCTOMAG-60K) with analytes IFNγ, IL-10, IL-12p70, IL-13, IL-15, IL-1β, IL-2, TNFα; located in NIMR Lab #4 fridge
    a. Human Cytokine/Chemokine Standard (catalog #MXH8060-2)
    b. Human Cytokine Quality Controls 1 and 2 (catalog #MXH6060-2)
    c. Set of one 96-well plate with 2 sealers
    d. Assay Buffer (catalog #L-AB)
    e. Serum Matrix (catalog #MXHSM)
    f. Bead Diluent (catalog #LBD)
    g. 10X Wash Buffer, 2 bottles (catalog #L-WB)
    h. Human Cytokine Detection Antibodies (catalog #MXH1060-3)
    i. Steptavidin-Phycoerythrin (catalog #L-SAPE10)
    j. Mixing Bottle
    k. Anti-Human IFNγ Bead (catalog #HCYIFNG-MAG)
    l. Anti-Human IL-10 Bead (catalog #HCYIL10-MAG)
    m. Anti-Human IL-12p70 Bead (catalog #HIL12P70-MAG)
    n. Anti-Human IL-13 Bead (catalog #HIL13-MAG)
    o. Anti-Human IL-15 Bead (catalog #HIL15-MAG)
    p. Anti-Human IL-1β Bead (catalog #HCYIL1B-MAG)
    q. Anti-Human IL-2 Bead (catalog #HIL2-MAG)
    r. Anti-Human IL-6 Bead (catalog #HCYIL6-MAG)
    s. Anti-Human TNFα Bead (catalog #HCYTNFA-MAG)
3. MAGPIX CALS/VERS/Fluidics reagents; located in Parthenon fridge #2 (extra reagents available once reagents below expire)
    a. Calibrator: Lot #B21718, Exp: 2/23/2013
    b. Verifier: Lot #B22330, Exp. 2/22/2013
    c. Fluidics 1: Lot #B22323, Exp. 2/18/2012
    d. Fluidics 2: Lot #B22326, Exp. 2/18/1012

Instruments/Materials

1. MAGPIX (Luminex)
2. Adjustable pipettes with tips capable of delivering 25-1000μL (Gilson)
3. Multichannel pipettes capable of delivering 25-200μL (Gilson)
4. Reagent reservoirs
5. 50μL Falcon tubes (BD)
6. Biohazard containers
7. Polypropylene microfuge tubes
8. Paper towels
9. Vortex mixer (Scientific Industries Model #G-560E)
10. Sonicator (Branson Ultrasonic Cleaner Model #B200)
11. Microcentrifuge (VWR International catalog #521-2841)
12. Thermomixer Comfort (Eppendorf #5355 000.011)

13. Exchangeable Thermoblock for MTPs and deepwell plates with lid (Eppendorf #022670565)
14. Hand held Magnetic Separation Block (EMD Millipore catalog #40-285)
15. Gloves
16. Extra plate sealers
17. Kimwipes

APPENDIX O

MAGPIX Human Immunoglobulin Magnetic Bead Panel

Standard Operating Procedure

1. Thaw samples completely. Mix well by vortexing and centrifuge prior to use in the assay to remove particles
2. Preparation of Antibody-Immobilized Beads
    a. Sonicate each antibody-bead vial for 30 seconds, vortex for 1 minute. Add 150µL from each antibody bead vial to the Mixing Bottle and bring final volume to 3.0mL with Assay Buffer (when using 6 antibody-immobilized beads, add 150µL from each of the 6 bead sets to the Mixing Bottle. Then add 2.1mL Assay Buffer). Vortex the mixed beads well. Unused portion may be stored at 2-8°C for up to 1 month
3. Preparation of Standards
    a. Resuspend MILLIPLEX MAP Human Multi-Immunoglobulin Standard in 0.4mL DI water. Vortex at high speed for 15 seconds. Place on ice for 15 minutes. This is Standard 7.
4. Preparation of Working Standards
    a. Label six polypropylene microfuge tubes Std 6, Std 5, Std 4, Std 3, Std 2, Std 1. Add 200µL of Assay Buffer to each of the six tubes. Prepare 3-fold serial dilutions by adding 100µL of the reconstituted Standard 7 to the Std 6 tube, mix well and transfer 100µL of the Std 6 to the Std 5 tube, mix well and transfer 100µL of the Std 5 to the Std 4 tube, mix well and transfer 100µL of the Std 4 to the Std 3 tube, mix well and transfer 100µL of the Std 3 to the Std 2 tube and mix well and transfer 100µL of the Std 2 to the Std 1 tube and mix well. The Standard 0 (Background) will be Assay Buffer.
5. Preparation of Positive Control
    a. Resuspend MILLIPLEX MAP Human Multi-Ig Positive Control (Catalog #PC-301) in 0.25mL DI water. Vortex at high speed for 15 seconds. Place on ice for 15 minutes.
6. Preparation of Human κ and λ Light Chain, PE
    a. To prepare 1X detection reagent, dilute anti-Human Kappa and Lambda-PE to working solution (1:100) with Assay Buffer (for full plate, use 25µL of the 100X anti-Human kappa-PE in 2.475 mL Assay Buffer).
7. Preparation of Wash Buffer
    a. Bring the 10X Wash Buffer to room temperature and mix to bring all salts into solution. Dilute 30mL of 10X Wash Buffer with 270mL DI water. Store unused portion at 2-8°C for up to 1 month.
8. Immunoassay Procedure
    a. Add 50µL of control, standard, or sample to each well.

b. Vortex the MILLIPLEX MAP Anti-Human Multi-Immunoglobulin Beads at medium speed for 15 seconds and then sonicate for 15 seconds using a sonication bath. Add 25μL of bead solution to each well.
c. Cover with plate cover and incubate 1 hour with agitation on plate shaker at room temperature.
d. Wash plate 2 times with 200μL/well of Wash Buffer, removing Wash Buffer by aspiration between each wash. To avoid washing/aspiration related bead loss, allow approximately 60 seconds between dispensing of Wash Buffer and subsequent aspiration.
e. Add 25μL per well of diluted Anti-Human κ and λ light chain, PE.
f. Cover with plate cover and incubate 1 hour with agitation on a plate shaker at room temperature.
g. Gently remove fluid aspiration (Note: do not invert plate). To avoid aspiration related bead loss, allow the plate to soak on the magnet of the plate washer for 60 seconds prior to aspiration.
h. Resuspend in 150μL/well of Sheath Fluid.
i. Proceed to reading results on an appropriate Luminex instrument.

APPENDIX P

MODIFIED ZIEHL-NEELSEN STAIN - FECAL SAMPLES

STANDARD OPERATING PROCEDURE

No.:

Date:

Prepared by: _____ (name and date)

Developed by: _____ (name and date)

MATERIALS:

1. Tongue depressors
2. Wooden applicator sticks
3. Pasteur pipets
4. Plastic screw-top universal containers 20 mL, calibrated by 5mls.
5. Formalin (40 % formaldehyde solution)
6. Distilled water
7. Microscope slides: glass, 3 x 1 in (76 x 26 mm)
8. Diamond pencil
9. Paper towels
10. 99.5% methanol
11. Stop watch
12. Strong carbol fuchsin
13. Graduated cylinder, 50 mL
14. Beaker, 500 mL
15. HCl
16. Malachite green crystals
17. Compound Binocular Light Microscope with x10, x40, and x100 (oil) objective lenses with x10 eyepieces, and preferably with calibrated ocular micrometer.

FIXING THE FECAL SAMPLES (for fresh fecal samples only):

(Preliminary assumption: Solid feces has a sg. of ~ 1, liquid feces also)

1. Transfer 5 gm feces to a 20 mL plastic universal container (if solid use a tongue depressor or wooden applicator stick) and add 5 mL 10% v/v formalin with a Pasteur pipet. Invert tube to mix thoroughly.
2. Let stand for at least one hour.

PROCEDURES:

1. Transfer 2-3 drops of fecal suspension with a Pasteur pipet or wooden applicator stick to a labeled microscope slide and spread into a thin layer (up to 2 x 3 cm) with a wooden applicator stick.
2. Allow smear to air dry completely.
3. Fix the air-dried smear in 99.5% methanol for 3 minutes.
4. Using a Pasteur pipet, apply adequate strong carbol fuchsin stain to completely cover the smear spot and let sit for 15 minutes.
5. Rinse thoroughly with distilled water.
6. Decolorize by dripping a solution of 1% v/v conc. HCl in 94% methanol on the slide for 15 seconds.
7. Rinse thoroughly in distilled water.
8. Counterstain with 0.4% (w/v) malachite green in distilled water for 30 seconds.
9. Rinse thoroughly in distilled water.
10. Allow to air dry completely.
11. Scan the smear using the x10 objective lens and confirm the presence of oocysts under x40 objective lens and/or x100 oil immersion lens.