IMMUNOLOGICAL EFFECTS OF ASYMPTOMATIC *VIBRIO CHOLERAE*, *SALMONELLA* ENTERICA SEROVAR TYPHI AND *ENTAMOEBA HISTOLYTICA* ENTERIC PATHOGENS DURING HIV INFECTION AND EXPOSURE

By

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submitted in partial fulfilment of the requirements for the degree of

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PREFACE

This study represents original work by the author and has not been submitted in any other form to another University. Where use was made of the work of others, it has been duly acknowledged in the text.

The research described in this dissertation was carried the College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa under the supervision of Professor T Mduluza and co-supervision of Prof T Naicker during the period January 2015 to December 2017.

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DECLARATION

I, Agness Farai Nhidza declare that:

(i) The research reported in this dissertation, except where otherwise indicated is my original work.

(ii) This dissertation has not been submitted for any degree or examination at any other university.

(iii) This dissertation does not contain other person's data, pictures, graphs or other information unless specifically acknowledged as being sourced from other persons.

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Signed:  Date: **Date: 30 October 2017**

FORMAT OF THESIS

The thesis has been presented in a manuscript format comprising of submitted journal articles under peer review that have emanated from this research work.

RESEARCH APPROVALS

Ethics Approval Committee	Date	Reference number
Biomedical Research Ethics Committee (BREC)	20 April 2016	BE 409/15
Biomedical Research Ethics Committee (BREC)	20 April 2017	BE 409/15
Medical Research Council of Zimbabwe (MRCZ)	01 August 2016	MRCZ/A/2043
Medical Research Council of Zimbabwe (MRCZ)	01 August 2017	MRCZ/A/2043

DECLARATION 2: PUBLICATIONS AND MANUSCRIPTS

Part of this work has been presented in the following manuscripts, which are under review in journals and presented in a targeted journal format.

Manuscript 1

Asymptomatic *Vibrio cholerae, Salmonella typhi* and *Entamoeba histolytica* burden in pregnant women and their infants in a high HIV prevalence setting in Harare, Zimbabwe

Agness Farai Nhidza *et al.,* ***in press under review in the African Journal of Reproductive Health-AJRH***, to answer the objective

'To determine the baseline prevalence of carrier state of enteric pathogens *(Vibrio cholerae, Salmonella typhi* bacteria and *Entamoeba histolytica* protozoan) in HIV-infected pregnant women and their neonates'.

Authors: Agness Farai Nhidza, Thajasvarie Naicker, Babill Stray-Pedersen, Tawanda Chisango, Edson Sibanda, Aziah Ismail, Mildred Pepukai, Tsitsi Bandason, Curtis Makaza, Kerina Duri, Takafira Mduluza.

Author contributions

AFN conceived the idea, designed the experiments, analyzed the data and developed the final written manuscript; TM, BSP, AI and TN supervised the work throughout, provided technical guidance to AFN. AFN and CM performed the assays and analyzed the data; TJC and EPS provided technical guidance to AFN; KD coordinated collection of samples and provided technical guidance to AFN. TB and MP provided technical guidance on study design and data analysis to AFN. All authors contributed to the writing of the manuscript.

Manuscript 2

Immune response to *Entamoeba histolytica* asymptomatic infections in pregnant women and their infants in a high HIV prevalence setting (*Under Review in the Journal of Microbiology, Immunology and Infection –JMII, Manuscript number JMII-D-18-00007*)

To answer the objective;

> 'To determine the effect of single or multiple enteric infections *(Vibrio cholerae, Salmonella typhi* and *Entamoeba histolytica*) on cytokine profiles of pregnant women and their neonates in an HIV prevalent setting'.

Authors: Agness Farai Nhidza, Thajasvarie Naicker, Curtis Makaza, Babill Stray-Pedersen, Tawanda Chisango, Edson Sibanda, Aziah Ismail, Bernard Ngara, Tsitsi Bandason, Kerina Duri, Takafira Mduluza.

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Manuscript 3:

Influence of maternal characteristics during pregnancy on the infant immune responses in a high HIV prevalence setting in Harare, Zimbabwe *(Under Review in the African Journal of Reproductive Health-AJRH)*

To answer the objective;

'To determine the association between maternal characteristics that in turn affect the cytokine responses of their neonates'

Authors: Agness Farai Nhidza, Thajasvarie Naicker, Curtis Makaza, Babill Stray-Pedersen, Tawanda Chisango, Edson Sibanda, Aziah Ismail, Tsitsi Bandason, Kerina Duri, Takafira Mduluza.

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DEDICATION

This work is dedicated to all the participants who volunteered their biological samples and time for this study to be a success, my family (The Nhidzas and the Manjoros) and my son Panashe for the moral support, whose love and continued support made it possible.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACRONYMS

API	Analytical Profile Index
ART	Antiretroviral Therapy
AST	Antimicrobial Susceptibility Testing
BREC	Biomedical Research Ethics Committee
CAZ	Ceftazidime
CPD	Cefpodoxime
CPDCV	Cefpodoxime/clavulanate
CPM	Cefepime
CPMCV	Cefepime clavulanate
ELISA	Enzyme-Linked Immunosorbent Assay
ESBL	Extended Spectrum β-Lactamase
FOX	Cefoxitin
HEI	HIV-Exposed Infected
HEU	HIV-Exposed Uninfected
HIV	Human Immuno-Deficiency Virus
HUU	HIV-Unexposed Uninfected
LMP	Last Menstrual Period
MHP	Maternal High Producers
MIP	Mother-Infant Pairs
MLP	Maternal Low Producers
MRCZ	Medical Research Council of Zimbabwe
NLF	Non-lactose Fermenter
OD	Optical Density
PMTCT	Prevention of Mother to Child Transmission
RCZ	Research Council of Zimbabwe
UKZN	University of KwaZulu-Natal
UZ-CHS	University of Zimbabwe-College of Health Sciences
WHO	World Health Organisation

ABSTRACT

Background

Asymptomatic infection by enteric pathogens has been attributed to causing several outbreaks in the world. Common pathogens in this category are *Vibrio cholerae, Salmonella typhi* and *Entamoeba histolytica infection of* pregnant and lactating mothers. Exposure of the infant and the immune response during the early developmental years requires investigation. In the era of high HIV burden and the introduction of HIV prevention of mother to child transmission (PMTCT), many infants are exposed to these pathogens yet remain uninfected, however still face the challenge of high morbidity and mortality as compared to their unexposed and uninfected counterparts. The cause of such an anomaly has not been fully investigated.

Aim

The aim of this study is to investigate the burden and the immunological effect of asymptomatic enteric pathogen carriage in pregnant mothers, both HIV-infected and HIV-uninfected, and their infants in a high HIV burdened population of Harare, Zimbabwe.

Methodology

The study was a purposive cross-sectional sub-study in a birth cohort established at the University of Zimbabwe, College Of Health Sciences (UZ-CHS Birth Cohort). Participants were from the high-density suburbs of Glenview, Kuwadzana and Dzivarasekwa. These suburbs were previously affected by the 2008 cholera epidemic as well as the 2012 typhoid outbreak that hit Harare, Zimbabwe. HIV rapid testing was performed at enrolment for mothers unaware of their HIV status or those who wanted HIV re-testing for various personal reasons. Those HIV infected with documented HIV status were asked to provide the evidence and were not re-tested.

The HIV status of neonates was ascertained using nucleic acid-based testing at the National Early Infant Diagnostic (EID) department based at the National Microbiology Reference Laboratory in Harare, Zimbabwe. The test made use of the Dried Blood Spot (DBS) samples collected from the infants at various time points.

A total of 417 pregnant mothers were recruited based on their consent to provide stool samples, blood and urine for both the mother at recruitment and the infant upon delivery. A questionnaire to collect information on socio-demographic, clinical, delivery, water and sanitation, diarrheal history including antibiotic use, concurrent TB infections, ART regimen, planned baby feeding method, diet as well as nutritional data on probiotic uptake was administered to the mothers. Of the 417 mothers, only 204 mothers and 308 infants provided stool samples, which were cultured for enteric pathogens targeting *Salmonella entrica serovar* Typhi (*S. typhi*) and *Vibrio cholerae*, although in the process any non-lactose fermenter identified was considered a potential pathogen hence were further identified using an Analytical Profile Index (API; Biomerieux: France) and included in the study. The stool samples were also used for antigen ELISA targeting *Entamoeba histolytica (E. histolytica)* infection.

The microbial results were used to calculate the prevalence of *S. typhi, V. cholerae* and *E. histolytica* in the study population. Data was analysed using STATA 13 analytical software. For further analysis on immunological factors in mother-infant pairs (MIPs), only paired mothers were included based on stool as well as plasma availability and the outcome of culture and ELISA results. Multiplex immunoassay utilised a 27-plex (IL-1β, IL-1r, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17a, Eotaxin, basic PDGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1(MCAF), MIP-1α, MIP-1β , PDGF-BB, RANTES, TNF- α and VEGF) cytokine profile and a 6-plex isotyping (immunoglobulin: IgG1, IgG2, IgG3, IgG4, IgM and IgA) assay. Immunological assays were performed on the 39 MIPs purposively selected based on the culture outcomes as described, using BioRad Luminex machine according to manufacturer's instructions.

Based on the cytokine and immunoglobulin concentrations, maternal characteristics influencing infant cytokine and immunoglobulin production were analysed.

Results

The presence of *V. cholerae* nor *S. typhi* was detected but *E. histolytica* prevalence in mothers was calculated at 5.4% while in infants it was 5.6%. There was no significant difference in the prevalence of *E. histolytica* between HIV-infected and HIV-uninfected mothers (p=0.318) as well as between HIV-exposed uninfected and HIV-unexposed uninfected infants (p=0.056). *E. histolytica* infection was only noted in 1 HIV-uninfected mother-neonate pair. The resistance to 3rd generation cephalosporines in the non-lactose fermenting bacterial isolates was detected.

The cytokine groups of IL-1r, IL-4, IL-9, IL-12p70, IL-17a, G-CSF and PDGF-BB were significantly raised in *E. histolytica* infected compared to non-infected lactating mothers ($p<0.05$). In babies, *E. histolytica* carriage had no significant effect on the cytokine and immunoglobulin concentration. In this study maternal *E. histolytica* carriage was significantly (p=0.026) correlated with infant Interleukin-12p70 (IL-12p70; $p<0.026$), Fibroblast Growth Factor-basic (FGF-basic; $p<0.026$), Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF; $p<0.026$) and Tumor Necrosis Factor (TNF; $p<0.026$)-α. The odds ratio of passing high levels of these cytokines to infants is reduced by 86% if the mother is a carrier of *E. histolytica* during pregnancy (OR: 0.14). Carriage of any form of enteric infection such as Non-lactose fermenters (NLFs) including *E. histolytica* significantly increased the levels of IL-1r, IL-4, IL-9, IL-10, IL-12p70, IL17a, G-CSF, GM-CSF, IFN-γ, PDGF-BB and TNF-α cytokines ($p<0.05$).

However, no significant difference in immunoglobulin levels was noted amongst the mothers. IL-13 and G-CSF levels were significantly raised in HIV-infected compared to HIV-uninfected lactating mothers (p=0.0389 and p=0.0324, respectively).

IgA immunoglobulin levels were significantly raised among HIV-exposed uninfected than HIV-unexposed uninfected infants. It has also been observed that maternal *E. histolytica* carriers are significantly more likely to have infants with low levels of IL-12p70, FGF-basic, GM-CSF and TNF-α cytokines and high levels of IgA immunoglobulin. HIV-infected mothers are significantly more likely to have infants with low levels of IgG2 and IgA immunoglobulins.

Mothers giving birth at a mean age above 30.38 ±STD 6.09 years are more likely to have infants with low IgG4 levels (p=0.050). Maternal HIV infection influences infant IgG2 and IgA immunoglobulin levels. Also, a maternal age of >30years reduces the odds ratio of passing high IgG4 to the infant (p=0.050). Maternal HIV infection influences infant IgG2 and IgA immunoglobulin levels. Infants born to HIV positive mothers are significantly associated (p=0.049) with lower odds of having high IgG2 levels, *ie*., reduced by 76% in infants born to HIV-infected mothers compared to those born to HIV-uninfected mothers. Similarly, an infant born to a HIV-infected mother is significantly associated (p=0.035) with lower odds of having high IgA immunoglobulin levels compared to those born to HIV-uninfected mothers (OR: 0.22). Underweight/small to gestational age (SGA) babies (birth weight <2500g) were found to have significantly raised IL-7 cytokine levels compared to normal for gestational age (NGA) babies whose birth weight was >2500g (p=0.0149).

Conclusion

Asymptomatic enteric *E. histolytica* carriage is a reality in pregnant women and their neonates with the possibility of congenital transmission. We confirm that there is no *V. cholerae* carriage in Glenview, Dzivarasekwa and Kuwadzana, areas affected in the 2008 cholera epidemic in Zimbabwe. Moreover, there was no evidence of *S. typhi* carriage in these suburbs following the devastating 2012 typhoid outbreak. This study reports an expansive pro-inflammatory cytokine and

chemokine profile that is up-regulated in lactating mothers with asymptomatic enteric pathogens. HIV exposure had no effect on baby cytokine profiles. The importance of IL-7 in neonates requires further investigation as it might assist in reducing the prevalence of small for gestational age (SGA) babies.

Significance and impact of study

Our study culminates in the need to consider maternal characteristics such as age, HIV status and enteric pathogen carriage in pregnancy as these might impact on the infants' immune responses. This will assist in the decisions on disease treatment and management of pregnant women and infants. The study findings also assist in developing effective immunotherapy in *E. histolytica* and HIV exposures and infections in an effort to promote maternal and child health.

Keywords

Cytokines, Immunoglobulins, HIV-infected mothers, enteric infections, *E. histolytica*, HIV-exposed infants, mother-infant pairs.

Chapter 1 : BACKGROUND AND LITERATURE REVIEW

1.0 INTRODUCTION

Low and middle-income countries battle with diseases afflicting the poor that include enteric diseases. Such diseases include, amongst others, typhoid, dysentery and enteric fever. These diseases can be endemic to particular geographical areas. These enteric diseases have become a cause of concern for researchers. Also, the challenges posed by diseases as malaria, HIV/AIDS and tuberculosis has resulted in many health practitioners, government officials and funders diverting most of their attention to these three main areas with a reduced focus on cholera, typhoid and dysentery. Lack of a holistic approach in the fight against enteric diseases in this era of high HIV burden may result in a huge burden of opportunistic infections. At present and during the past years diarrhoeal diseases predominate in Africa, specifically in Zimbabwe, viz., the cholera outbreak of 2008, that affected 11735 cases and 484 deaths (4% death rate) was reported between August-December 2008, by the World Health Organization. Moreover, 50% of the cases came from a high-density suburb in Harare[1]. Although the cholera was widespread within Zimbabwe[2], the suburbs that were most affected were Dzivarasekwa and Kuwadzana. These same suburbs were also the most affected by the typhoid outbreaks in 2008 and 2012[3]. In this regard, there is a likelihood that the surviving cases are still carriers of the deadly *Vibrio cholerae* with the ability to unknowingly spread the pathogen. The World Health Organization (WHO) has indicated access to safe drinking water, improved sanitation, exclusive breastfeeding for the first six months of life, good personal and food hygiene and health education about how infections are spread as effective measures to prevent diarrhoea[4]. In this study, we focus on the enteric diseases in particular typhoid, cholera and dysentery affecting sub-Saharan Africa, specifically Zimbabwe. We also focus on the cytokine and antibody levels in HIV-infected pregnant women and their neonates in the presence or absence of the studied enteric pathogens.

1.1 Background on Immunology and Epidemiology of Enteric infections

The diseases considered in this study are caused by the Human immunodeficiency virus (HIV), bacteria *(S. typhi and V. cholerae)*, and a protozoan *(E. histolytica)* thus microbiologists, immunologists, clinicians and epidemiologists have all exhibited extensive interest. Diarrhoea has been found to be the second commonest cause of deaths among the under 5-year-olds [5]. Subclinical pathogen carriage and enteropathy, without diarrhoea, are common, especially in developing countries. These intestinal pathogens "…drive a cycle of gut damage, malabsorption, chronic inflammation and failed mucosal regeneration, leading to malnutrition and susceptibility to further enteric infections"[5]. The gut microbiota has been noted to impact on an individual's health status and play an important role in protective and susceptibility to infections.

1.1.1 Gut Microbiota and its role in enteric infections

Gut microbiota refers to the total microbes such as bacteria, viruses and fungi that live in a complex community[6,7] within the host gut and adapt to live with the host in a symbiotic nature.

A. Development and composition of the gut microbiota

Gut microbiota is critical in maintaining health *in utero* through postpartum to adulthood. The composition and diversity have been noted to be influenced by age and diet[8] among other factors. Infant bacterial gut colonisation starts at the time the foetus is in the lower uterus although establishment takes off during birth[6,9]. Since the development of the gut mucosal barrier starts prenatally, pre-term babies are faced with challenges of gastrointestinal (GI) abnormalities resulting in higher morbidities and mortalities compared to their full-term counterparts[9]. During the last weeks

of gestation, the developing fetus sips some of the amniotic fluid[9] in preparation for the gut mucosa role for digestion and absorption of nutrients after birth.

The type of the microbiota that invades the infant's gut depends on the mode of delivery, breastfeeding versus formula feeding and timing of introduction of solid foods at weaning stage[9]. Infancy is a critical stage in gut microbiota development and need not be disturbed, or else immune-related disorders will occur later in life. The type of the microbiota that invades the human gut early in life has a life-long effect hence it is crucial that parents introduce appropriate foods and conducive environment for the development of a healthy microbiota. Pre- and pro-biotic foods are very important in the human diet as these influence the nature of the gut microbiome[10]. It has been shown for example that the gut microbiome composition influences temperament at an early age[11]. This emphasises the need for a rich nutrition from early stages of child development such as breast milk and a balanced diet.

Normal vaginal delivered infants tend to have gut microbiota resembling maternal vaginal microbiota which is mainly populated by *Lactobacilli, Provitella* or *Sneathia*. On the other hand, caesarian section delivered babies have gut microbiota resembling the maternal skin microbiota which is mainly *Staphylococcus, Corynebacteria* and *Propionibacteria* [9]. Breast milk influences the infant immune development such as the '…IgA and IgG, antimicrobial compounds such as lysozyme and lactoferrin, immune regulatory cytokines such as TGF-β and interleukin 10 (IL-10), and lymphocytes that express gut homing markers'. These breast milk factors influence the type of bacteria that will colonise the gastro-intestinal tract (GIT).

Soon after birth, the infant gut normally has a higher demand for digestion and nutrient absorption in order to have a good maintenance of the rapid growth rate associated with the growing infants. This

demand is assisted by gut microbiota such as *Bifidobacteria* that digest human milk oligosaccharides (HMO).

Infant gut microbiota is dynamic and develops rapidly then stabilises around 2-3 years when it resembles the adult gut microbiota[12]. However, at 5 years the infant gut microbiota is often not fully established[8].

B. Adult gut microbiota

The Human Microbiome Project (2012) outlined the human gut microbiota[13]. The GIT is the highest colonised with 70% of all the human body microbes being located there. In a healthy adult, about 90% of this 70 % of the microbes originate from two major phyla which are the *Firmicutes* and *Bacteroidetes* and other phyla like *Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria* and *Cyanobacteria* are in smaller amounts[14]. The human gut microbiota is composed of bacteria, archaea, viruses, and eukaryotic microbes that reside in and on our bodies most of which cannot be explored through our routine cultures[15]. These microbes have tremendous potential to impact our physiology, both in health and in disease.

C. The gut microbiota and human immunity to infections

Microbiota contributes to metabolic functions, protect against pathogens, educate the immune system and through these basic functions directly or indirectly affect most of our physiologic functions[16]. Literature has revealed a stability in the composition of the gut microflora over time (microbiota equilibrium), which however can be affected by various confounders such as diet, diseases, host genotype *etc* [17].

Diet influences the composition and diversity of the gut microbiota, in that people consuming a 'Western diet' which is mainly protein, have more of Firmicutes and Proteobacteria as well as some

Bacteroides whilst people that consume an agrarian diet have increased amounts of Actinobacteria, mostly Prevotella[17; 18].

High fibre diets favour the commensal bacteria, providing colonisation resistance to pathogenic bacteria usually associated with diseases such as colorectal cancer and IBD[18].

The gut microbiota is in a position to assist the host in fighting infection through activities like production of antimicrobials such as bacterocins that are directed against members of the same or similar bacterial species[19], a process also known as colonisation resistance[20]. Gut microbiota also assists the host in the maturation of the host immune system and also competes for resources with the pathogens[16; 21]. A successful pathogen has to overcome the mucosal barrier. In some cases, naturally low-complexity of gut microbiota[19], and usage of antibiotics will destroy the normal gut flora and give rise to overgrowth of an opportunistic member of the intestinal microbial community[16] which then becomes pathogenic.

Normally, microbiota offers a state of resilience to the effect of enteric pathogens and other factors that can trigger an imbalance which is referred to as dysbiosis. Dysbiosis can either result in loss of beneficial organisms, loss of overall microbial diversity or excessive growth of potentially harmful organisms, which in most cases occur at the same time[18]. Microbial resilience is heavily dependent on the microbial diversity hence an increase in microbial diversity helps in maintaining microbial stability[17] which is important in controlling gut invasion by pathogenic organisms.

Host inflammatory responses, despite being able to clear invading pathogens, can create an enabling environment. For example, virulence factors produced by virulent bacteria induce host inflammation against resident microbiota while favouring the growth of the virulent bacteria[19], hence interfering

with colonisation resistance and leading to host infections[16]. Overt or indigenous pathobionts may also take advantage of the dysbiosis[19].

A variety of immune cells are found in the intestinal environment, harboured within Peyer's Patches (PP) as well as in the Small Intestine Lymphoid Tissue (SILT) and the Intestinal Mesenteric Lymph Nodes (IMLN)[16]. The intestinal epithelial cells such as enterocytes and colonocytes express pattern recognition receptors (PRR) namely both extracellular Toll-like receptors (TLR) and intracellular NOD-like receptors (NLR). These receptors are insensitive to the normal flora but are activated by an infection, injury or any other assault. They respond by initiating the host inflammatory response[16; 22; 23].

The beneficial effects of TLR assist in pathogen recognition and bacterial clearance by leukocytes[22] with consequential detrimental damage to the host. An example is the activation of TLR4 in enterocytes which inhibits enterocyte migration whilst propagating and stimulating enterocyte apoptosis-factors which promote intestinal injury and at the same time preventing intestinal repair[22]. Successful pathogens modify their transcriptional actions to counter host immune responses including immunoglobulins (Ig) such as secretory IgA (s-IgA) which binds and agglutinates the pathogen[24]. Resident microbiota can also produce by-products that facilitate the growth of pathogenic organisms such as bile salt which promote *Clostridium difficile* spore germination[19].

Intestinal dysbiosis has been linked with autoimmune and/or auto-inflammatory conditions such as Inflammatory Bowel Disease (IBD), metabolic disorders such as obesity, type 2 diabetes, allergies and neurological disorders[14]. Recently, faecal implant, also known as faecal microbial therapy has been recommended in some developed countries for the treatment of *Clostridium deficile* infections

(CDI) which resist antimicrobial drugs[18; 25–27]. Dysbiosis can result in inflammatory diseases of the gut as well as other distant organs and can sometimes have life-threatening effects[20].

Another challenge is from bacterial translocation and sometimes bacterial products or their fragments into the bloodstream that may occur as a result of mucosal insult leading to a leaky gut. The microbial products or fragments that will enter into circulation from the lumen through the disrupted luminal T-junctions include endotoxins, bacterial DNA, peptidoglycan and lipopeptides. These products of microbial translocation usually trigger inflammation and disease in distal organs such as liver cirrhosis, acute pancreatitis, chronic kidney disease and chronic heart failure[28]. Markers of microbial translocation can be used for disease diagnosis such as IBD[29].

Great progress in characterizing the structure of the microbiome recently has paved the way for ongoing and future studies on the functional interactions among the microbiota, pathogens and the host. The function of the microbiota is critical to understanding the role of the microbiota in human homeostasis and disease pathogenesis[30].

1.1.2 Human Immuno-deficiency Virus (HIV)

The HIV virus is one of the most studied virus to-date and has the primary target attack on the cells of the immune system causing their damage and exposing the host to opportunistic infection. Regardless of the infection route, the primary site of HIV-1 infection establishment had been reported to be the gut-associated lymphoid tissue (GALT) of the intestine where the HIV results in a "….significant mucosal CD4+ T lymphocyte depletion, induces an inflammatory state that propagates viral dissemination, facilitates microbial translocation and fosters establishment of one of the largest HIV reservoirs"[31]. It is from the gut that this virus damages the gut mucosal lining and get into the nearby cells and into the circulatory system and the lymph nodes. Due to the mucosal

damage, it is possible that HIV facilitates other systemic infections. Significant efforts have been made in treating HIV/AIDS through lowering HIV multiplication and infection rate by the use of anti-retroviral drugs (ARVs). However, a vaccine is not available as yet.

The ARVs have proven to be very effective in reducing viral load and improving the well-being of an HIV infected patient thereby reducing the rate of progression to AIDS development. These drugs have also been proven to prevent mother to child transmission of the virus hence the observed increased pool of HIV-exposed uninfected (HEU) neonates. Despite the lack of virus, the neonates have high morbidity and mortality rate compared to their HIV-unexposed uninfected counterparts. The study was designed to investigate the effect of HIV infection exposure and the resultant immune responses in HIV exposed but uninfected individual.

1.1.3 ***Salmonella***

Salmonellae are Gram-negative intracellular bacteria that are facultative and belong to the species *Salmonella enterica*. *Salmonella typhi* and *S. paratyphi* are host-adapted to humans and can cause enteric fever. However, a small percentage of silent, chronic carriage of the *Salmonella* bacterium, usually in the biliary tract, can persist for decades, with the sustained dissemination of the disease[32].

This chronic carriage has been estimated at 1-3% of acute infections[33]. The fact that *S. typhi* and *S. paratyphi* are human-adapted pathogens, the chronic asymptomatic carriers will act as vehicles in transmitting the pathogens, especially in the previously uninfected communities hence posing a danger to the communities. Enteric diseases as a result of *S. typhi* and *paratyphi* infection are a major public health problem in many developing countries[32]. It has also been noted by Roy and Malo, (2002), that the use of antimicrobials result in the loss of normal host flora hence favouring the

proliferation of *Salmonella* spp and also that the use of acid suppressor drugs which result in the loss of the acid barrier creates an increased susceptibility of the host to enteric pathogens[32].

Salmonellosis is a serious disease in sub-Saharan Africa and is associated with HIV infection, malaria and poor nutritional status [34]. Despite *Salmonella* entering the host via the enteric system, initially infecting PP and mesenteric lymph nodes (MLN), it normally ends up infecting systemic organs[34] hence the need to understand the association of this pathogen with the gut microbiota. The initial immune response involves the recruitment of neutrophils around the infected PP and MLN where they are associated with IFN-γ production, together with the involvement of Natural Killer (NK) cells. The IFN-γ contributes to initial defence as well as in preventing bacterial dissemination[34]. Inflammatory monocytes also populate the infected PP and MLN and produce anti-bacterial factors like IL-1β, TNF-α and iNO. On the other hand, in the event that the *Salmonella* evades the host immune system, it '…exploits TLR/NLR signalling and cell death to establish infection'[35] and 'persists in M2 macrophages during chronic infection'[35], as well as benefitting from '…host-derived ROS, RNS, and antimicrobial proteins' to successfully out-compete resident microbiota[35].

1.1.4 Vibrio cholerae

Vibrio cholerae is also a bacterium that causes enteric infections. This Gram-negative, curved rod, flagellated bacterium can cause death to the infected individual within a couple of hours due to excessive loss of water if not attended to urgently. Symptoms of cholera have been observed to start from as early as a few hours to about 5 days after infection with symptoms ranging from mild to severe. Normally 1 in 20 (5%) of infected persons show severe symptoms of infection, however, 95%

may be asymptomatic but still continue spreading the bacteria for 7 to 14 days[36]. Severe cases experience diarrhoea and vomiting which cause dehydration with possible death.

There is a paucity of current information on the host immune response to *V. cholerae*, hence further research in this area is warranted. The Cholera bacterium is non-invasive, and host protection from the bacterium is believed to be mainly conferred by secretory sIgA[37]. Children and adults respond similarly to cholera infection[38] but neonates still need their gut microbiota to develop as they have an immature gut and takes up to 2-3 years[12] or up to 5 years[8] for the gut microbiota to mature into that of an adult. The carrier state can persist for months and chronic biliary infections may persist for years with the intermittent shedding of the microbe. Sero-groups **O**1 and **O**139 are currently implicated in pathogenesis. It is not possible for an individual to develop immunity for both serotypes after recovering from an infection resulting from only one of the two cholera serotypes[38].

1.1.5 Entamoeba histolytica

One interesting possibility that might drive the interaction in the direction of acute HIV/AIDS disease is when the individual is already infected with an intestinal parasite, leaving the mucosal defences damaged and perhaps less effective in eliminating the infectious organisms. A common parasite in Africa is *Entamoeba histolytica*; which is a single cell, pathogenic, enteric protozoan parasite that causes amoebic dysentery in humans. It also causes other invasive diseases such as genito-urinary amoebiasis, liver abscess, cerebral amoebiasis and respiratory tract infections. About 90% of human infections can be asymptomatic or the symptoms may be mild; this is, therefore, the most common manifestation of *E. histolytica*[39].

Since *E. histolytica* damages the intestinal mucosa, there is a possibility that the presence of this pathogen will support HIV disease progression. Of the 10% that exhibit symptoms, 80-98% are intestinal and 2-20% are extra-intestinal[40]. The seroprevalence of *E. histolytica* can range between 5-55%[40]. There is no correlation between antibodies against the *E. histolytica* antigen and resistance of the host to the infection or host protective immunity[41].

It is possible that multiple infections of *E. histolytica, V. cholerae* and *Salmonella* could trigger a unique immune response that may lead to complications. In a surveillance study, Harris *et al*., (2009), observed co-infection with *V. cholerae* and intestinal parasites in Kolkata, India, amongst children aged 2-10 years who presented with acute diarrheal illness[42]. They demonstrated a 30% prevalence of concomitant parasitic infection with *V. cholerae* infection. It is however still not known whether an intestinal parasitic co-infection modifies the clinical manifestation of *V. cholerae* infection in humans[42]. On the other hand, Ayala-Sumuano *et. al*., (2013), noted the mixed infection of enteropathogenic bacteria and *E. histolytica* implicating an important role in the establishment of invasive disease via increasing adhesion, chemotaxis and cell damage capacity of trophozoites[43]. The fact that all these pathogens will eventually become systemic, highlights the possibility of congenital transmission.

Infection by most enteric organisms will trigger an immune response by the host that persists for years after *E. histolytica* infection, whereas the presence of IgM antibodies is short-lived and can be detected during the present or current infection[39]. IgM is mainly indicative of fresh infections whilst IgG detects chronic infections. IgG is the primary indicator of carriers as cited by Ismail *et al.*, (2000)[33].

Galactose-N-acetyl galactosamine (Gal-lectin) inhibitable lectin is the surface antigen of *E. histolytica's* and one of its major adhesion molecules[45]. Wong-Baeza et al., (2009), identified the presence of lipo-peptido-phospho-glycan (LPPG) as one of the *E. histolytica* surface antigens. LPPG is slightly different from the lipopolysaccharide (LPS) found in Gram-negative bacteria and is one of the pathogens associated molecular patterns (PAMPs) found on parasite surfaces.

The first encounter of the parasite is on the mucosal surface, the first line of defence, where pathogen recognition receptors (PRRs) such as TLRs are expressed by the host cells. Neutrophils are the first cells to express amoebicidal activity after activation by IFN-γ or TNF-α and LPS trigger the release of reactive oxygen species that kill the parasite. The intestinal epithelial cells (IEC) which provide the second line of defence, on their first encounter with the parasite, will recognise the Gal-lectin, or LPPG parasite surface antigens from the lysed trophozoites by means of TLR2 and TLR4/CD14 resulting in activation of NF-κβ[40; 46; 47]. Once NF-κβ is activated, that triggers the release of pro-inflammatory cytokines by monocytes releasing IL-8, IL-10, IL-12p40, and TNF-α. Cell-mediated IFN-γ or TNF-α production activates macrophages to destroy the parasite[40; 46].

E. histolytica DNA also activates macrophages through interaction with TLR-9[46] thus destroying the parasite. In the process of evading the host immune response, the parasite decomposes the mucin barrier using their glycosidase enzymes and accesses the IEC. This process may also benefit the parasite as well as the gut microbiota in supplying of the carbon source of energy. Studies by Nakada-Tsukui and Nozaki, (2016), have suggested that gut microbiota may influence the *E. histolytica* pathogenesis as germ-free animals were seen to be resistant to the parasite but 'introduction of single bacterial species restored amebic pathogenesis'[47].

E. histolytica is also in a position to degrade cytokines but this is not yet clear whether this act is beneficial or detrimental to the parasite. *E. histolytica* is also in a position to phagocytose or

togocytose the host cells including neutrophils, T lymphocytes and macrophages[47], putting itself in a position to survive the host immune defence mechanism, and able to chronically stay with the host.

Generally, the host immune response to pathogenic effect involves the production of cytokines such as IFN-γ, interleukins (IL), tumour necrosis factor-alpha (TNF-α) and others. The pathogens will also develop survival strategies to evade the host immune system. Despite all the effort to conquer the pathogenic invasion, the host may fail to achieve its goal.

There are several reasons including the presence of mutations on the cytokines themselves and weakened immunity due to other diseases such as HIV infection and cancer. The host natural defence failure can be assisted by the use of drugs may be successful depending on the susceptibility of the pathogen to the drugs. This susceptibility can also be due to a number of factors including mutations in the pathogen's genome sequence. Because immune responses will also be affected by concomitant infections that are known to influence immune responses, the relationship between clinical disease or carrier development in people with HIV infection is also of interest.

It is against this background, that the current prevalence of enteric pathogens in pregnancy and possible congenital transmission, as well as the type of immune response, that is more important in host carriers and continued exposure of the host to the pathogens, was carried out. Different factors influencing host susceptibility to infection by microorganisms are indicated in Figure 1-1.

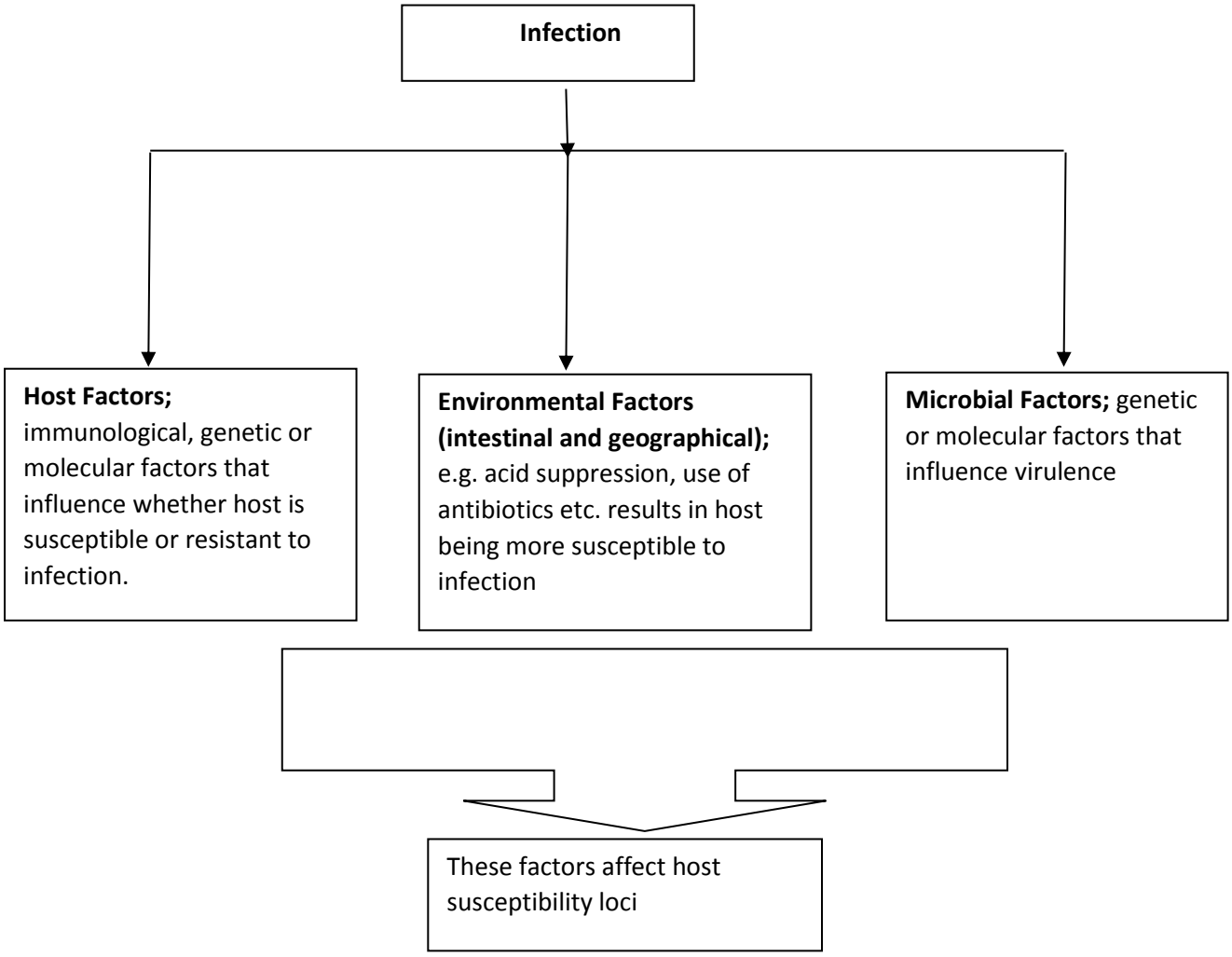

Figure 1-1: Factors influencing host susceptibility to infection by microorganisms

1.2 PROBLEM STATEMENT

Zimbabwe has for several years been devastated by episodes of diarrheal infections that can be traced to *Salmonella typhi*, *Vibrio cholerae* and *Entamoeba histolytica* as the causative organisms. The Government of Zimbabwe together with various partners are making significant efforts to make sure safe water and good sanitation are provided to the citizens and that HIV prevention of mother-to-child transmission (PMTCT) programmes coverage is increased. Unfortunately, the economic hardships being currently faced by the nation has made it difficult to provide a constant supply of treated water to the residents with a consequential high rate of water-borne infections. This has forced the residents to fetch alternative water supplies, most of which are unsafe for human consumption. As a result, waterborne disease episodes are rife and many deaths have occurred; for example the Zimbabwe 2008 cholera outbreak[1]. It is probable that a number of these surviving cases are still carriers of the cholera pathogen.

History has shown that individuals pre-exposed to these pathogens have a high likelihood of becoming asymptomatic carriers. As a result, the diseases keep spreading to other individuals usually unknowingly in a snowball effect. Logically, it is far cheaper to treat one carrier individual compared to treating the multiple diseased individuals infected by one carrier since these diseased might end up being hospitalised, accumulating medical bills, and in worst case scenario, end up with loss of lives.

In Zimbabwe, there is no or limited published data on the costing of diarrheal disease management. However, Zambia has given an indication of 3usd and 18usd general hospital costs for out-patient and in-patient respectively. Childhood diarrheal treatment cost in Zambia was estimated at 28usd and 78usd for out-patient and in-patient respectively[48].

Similar trends can also be said for Zimbabwe as these are neighbouring countries. There is, therefore, the need to identify the asymptomatic cases and treat to evade future outbreaks. Under normal circumstances, the human body has the ability to fight infections and can rectify the situation depending on the degree of infection. The immune status of an individual is also a confounder as far as the fight against infections is concerned. Infection by HIV results in an individual having a compromised immune system hence easily affected by opportunistic infections. This status is worsened during pregnancy. The pathogens can enter into the host bloodstream and there is a high possibility of them spreading to the unborn baby congenitally, hence the need for further investigation.

In a case reported by Beyhan *et al.,* (2016), it was observed that the baby that was delivered at 16 weeks and died 15 minutes after delivery, had *S. typhi* in its tissues suggestive of vertical transmission of the pathogenic organism. This indicated the need to raise awareness that organisms not typically associated with TORCH (*Toxoplasma gondii*, other, *Rubellavirus*, *Cytomegalovirus*, *Herpes Simplex virus)* infection can nevertheless cause placental infection and pregnancy loss. *S. typhi* appears to be one of the "other" in the TORCH list[49].

The neonates born to the HIV-infected women who already have a compromised immunity will possibly have a poorly developed immune system hence the high morbidity and mortality rates. The fact that the mothers will be exposed to anti-retroviral therapy (ART) might expose the neonates to these drugs with the possibility of the neonates resulting in cytokine polymorphisms and this needs to be investigated. Mutations in the cytokine genes can also be a contributory factor on the possible poor immune development on the HIV-exposed but non-infected neonates.

Much of the information on these diseases is derived mainly from clinical cases, of infected people who consulted a health service facility. However, there is also the phenomenon of local people being carriers of the pathogens, in some cases, having multiple infections with no or minimal clinical signs. These carriers will keep on infecting new victims, usually unknowingly. For good disease control, there is need therefore to identify such carriers as well as the clinical cases and investigate the factors that influence whether an individual will become ill or not and whether they will eliminate the infectious organism or become a carrier and continue to transmit the infection.

HIV-exposed uninfected (HEU) neonates have been found to have a high morbidity and mortality rate. The main cause of this has not yet been established. There is, therefore, a need to investigate the possible causes of this anomaly or challenge. Currently, little has been done in trying to link enteric pathogens carrier state in pregnancy to the immune responses of neonates in an effort to investigate the challenge faced by HEU. Prendergast (2016)[5] has indicated an increase in HEU infants in the past decade, and has shown that, 'Interest in the health outcomes of HIV-exposed, uninfected infants has grown in the past decade, with several studies suggesting that these infants have increased mortality rates, increased infectious morbidity, and impaired growth compared with HIV-unexposed infants'[50].

The information gathered through this study will help identify enteric pathogens carriers and decide on the best method to eliminate or control these waterborne infections in communities through possibly screening for the carriers and targeting them for treatment. The information from this study can also assist in the development of new and improvement of existing vaccines against enteric pathogens.

The study will also assists policy-makers on decision making such as shift to immunotherapy in an effort to reduce the risk of antimicrobial resistance which has become a world-wide challenge as a result of the use of antimicrobials for treatment of diseases emanating from microbial infections.

This study was carried out in a cohort of HIV-infected and HIV negative pregnant women and their neonates in 3 high-density suburbs of **Kuwadzana, Glenview** and **Dzivaresekwa** in Harare, Zimbabwe, which experienced high diarrheal infection episodes in the past.

1.3 RESEARCH QUESTION

What are the maternal and infant immunological responses to carrier status of enteric pathogens (*Vibrio cholerae, Salmonella typhi, Entamoeba histolytica and other*) in an HIV burdened community?

1.4 AIMS AND OBJECTIVES:

1.4.1 **Aim:** To investigate the maternal and infant cytokine and immunoglobulin response to carrier state of enteric pathogens (*Vibrio cholerae, Salmonella typhi* bacteria and *Entamoeba histolytica* protozoan) during HIV infection and exposure in a mother-child cohort in Zimbabwe.

1.4.2 **Main Objective-**To investigate the maternal and infant cytokine and immunoglobulin levels in response to carrier state of enteric pathogens (*Vibrio cholerae, Salmonella typhi* bacteria and *Entamoeba histolytica* protozoan) during HIV infection and exposure in a mother-child cohort in Zimbabwe.

1.4.3 **Specific Objectives-**

a. To determine the baseline prevalence of the carrier state of enteric pathogens *(Vibrio cholerae, Salmonella typhi* bacteria and *Entamoeba histolytica* protozoan) in HIV-infected pregnant women and their neonates.

b. To determine the cytokine name your profile and immunoglobulin name your profile profiles in the presence of single or multiple enteric infections *(Vibrio cholerae, Salmonella typhi* and *Entamoeba histolytica*) in HIV-infected pregnant women and their neonates.

c. To determine the influence of maternal characteristics on neonate cytokine and immunoglobulin profiles.

Chapter 2 : METHODOLOGY

2.1 ETHICAL REVIEW AND ETHICS

This study protocol was approved by Biomedical Research Ethics Committee (BREC – UKZN), approval code 409/15, Joint Research Ethics Committee (JREC), approval code 81/15, as well as the Medical Research Council of Zimbabwe (MRCZ), MRCZ/A/2043 and sample shipment was approved by the Research Council of Zimbabwe. During the course of the study, all study participants consented to take part in the study through responding to the questionnaire and providing samples. Confidentiality was observed through the use of anonymous numbers on samples which were generated by the lab and stripped of any participant identifiers. In the event that any participant sample was found to have pathogenic organisms, the main project PI was notified in order to link the participants to routine care.

2.2 MATERIALS

2.2.1. Selection of Participants and sampling

Any HIV positive pregnant woman who could consent was invited to participate in the main study and enrolled if eligible. Those with obstetric complications, psychiatric and other conditions were excluded. For every HIV positive pregnant mother, the 10th HIV negative mother was recruited, taking cognisance of the HIV prevalence of about 12% in this population**.** Inclusion and exclusion criteria were used to select 417 pregnant women and their neonates who stay in high-density suburbs of Harare (Dzivarasekwa, Kuwadzana and Glenview) in Zimbabwe, and their neonates, both male and female in line with UZ-CHS recruitment and sample collection program, taking only samples collected once from mothers during pregnancy and once from neonates as close to delivery as possible.

2.2.2 Study Design

The study was a purposive, cross-sectional study based on the availability of stool and plasma samples from the main Cohort study (***UZ-CHS birth cohort).***

2.2.3 Sampled Sites

The sampled sites were selected council maternity clinics in high-density suburbs of Harare (**Kuwadzana, Glenview** and **Dzivaresekwa**) in Zimbabwe where HIV-infected and uninfected pregnant mothers and their neonates were recruited.

2.2.4 Inclusion criteria

a. Pregnant woman 18 years of age and above at 28-36 weeks gestation based on the date of the last menstrual period (LMP).
b. Pregnant woman 18 years of age and above at 28-36 weeks gestation with documented HIV status or willing to be tested. It is recommended to screen for HIV for all HIV negative pregnant women at every scheduled study visit through the main study.
c. Pregnant woman 18 years of age and above at 28-36 weeks gestation planning to deliver at the study sites.
d. Pregnant woman 18 years of age and above at 28-36 weeks gestation able to give informed consent.
    i. Willing to be followed together with her baby from delivery up to two years in the main study.
    ii. Willing to provide the required specimens through the main study.

2.2.5 Exclusion Criteria

a. Pregnant women with severe obstetric complications and serious psychiatric disorders were excluded.
b. Pregnant women who lived outside of the high-density suburbs of Harare (**Kuwadzana, Glenview** and **Dzivaresekwa**) in Zimbabwe.

2.2.6 Biomedical samples collected

Stool, amniotic fluid, urine and blood (including cord blood) samples were collected through the main study for laboratory analysis of the asymptomatic carrier suspects.

2.3 METHODS

2.3.1 Demographic and Clinical Data Collection

Questionnaires were administered through the main study; UZ-CHS birth cohort. JREC reference number 81/15 and MRCZ reference number MRCZ/A/1968. The questionnaire was used to collect demographic and clinical information such as on water source, sanitation forms, any stomach discomforts, any exposures to antibiotic and antiacid treatment, history of enteric infection including TB infection, HIV status and drug regimen, delivery history, diet (fermented foods), alcohol uptake, etc from the selected population.

2.3.2 Summary of the Study Process

Out of 417 mothers who consented (218 (52.3% HIV-infected: 199(47.7%) HIV-uninfected) to take part in the main study, only 204 mothers (51% HIV-infected and 49% HIV uninfected) and 306 infants (52% HEUs and 48% HUUs) were purposively included in this study based on availability of stool sample for culture and ELISA to screen for enteric pathogens. It is these mothers and their infants whose results were used to calculate the prevalence of enteric pathogens. Of those mothers and infants, only 111 mothers could be paired based on stool sample availability.

Purposive sampling was also performed based on the culture results and HIV statuses of the 111 mother-infant pairs for further analysis on immunological aspects on the pairs. Microscopy, culture and ELISA on the stool samples were conducted in Zimbabwe at the Biomedical Research and Training Institute (BRTI) Laboratories. Sera from 39 out of 111 mother-infant pairs were taken for cytokine and antibody profiling at University of KwaZulu-Natal, Medical School, Optics and Imaging Centre, based on the microscopy, culture and ELISA results as explained earlier. Figure 2-1 illustrates the sampling process that was followed in this study.

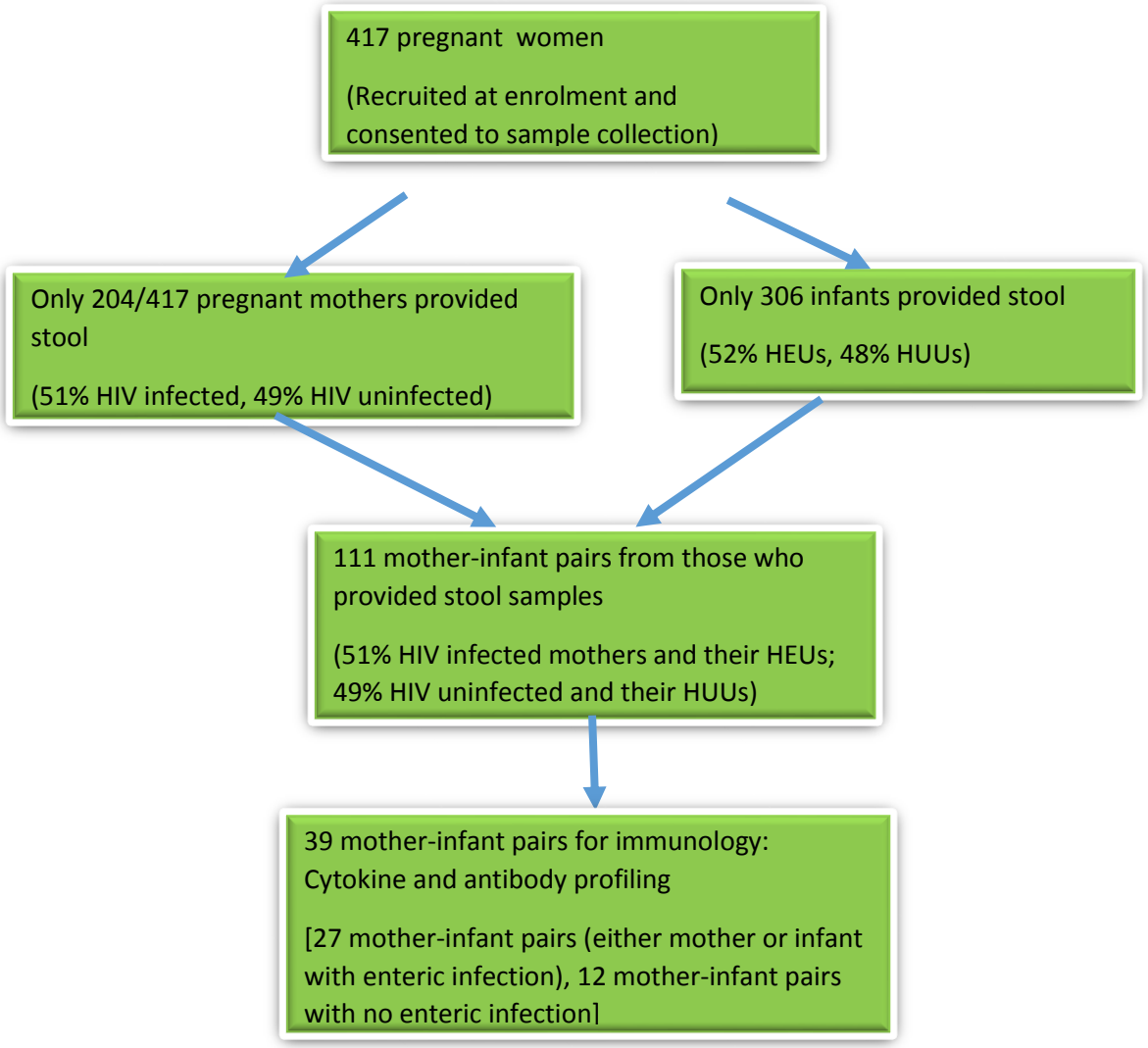

Figure 2-1: Conceptual framework of the sample selection and analysis process used in the study

Microbiome analysis and cytokine single nucleotide polymorphism (SNP) analysis were not performed due to limited funding and the complexity of the methodology that required outsourcing with a huge cost. However, when funds become available, this part of the work will be pursued later outside the scope of this thesis.

2.3.3 Laboratory Activities

2.3.3.1 Stool, whole venous blood, cord blood and urine collection, transportation, processing and storage

Stool, whole venous blood, cord blood and urine were collected, transported and processed using standard operating procedures. Briefly, the samples were transported in cooler boxes with ice packs as soon as possible from collection and processed on the same day of receipt.

2.3.3.2 Detection of Carrier Individuals

[EZ]Typhi-Paratyphi DNA dipstick/ Typhidot-c kits developed by the University of Malaysia and traditional culture methods followed by Analytical Profile Index (API) were used for *S. typhi.* The use of [EZ]Typhi-Paratyphi DNA dipstick/ Typhidot-c kits was an opportunity for validating the kits in the Zimbabwean setting. Responses from the questionnaire administered through the main study were also used to help with the identification of carriers. Culture was also employed for *V. cholerae* and *S. typhi* as well as ELISA for *E. histolytica* carrier identification.

2.3.3.3 Cytokine detection methods

Multiplex detection of 27 serum cytokines for mother-infant pairs (MIP) using Bio-plex Pro Assay 27-plex kit, Bio-Rad, USA, which is a magnetic-bead based multiplex immunoassay, was employed following the manufacturer's instructions and as summarised in Figure 2-2. The study measured 27 cytokines namely IL-1β, IL-1r, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17a, Eotaxin, basic PDGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1(MCAF), MIP-1α, MIP-1β , PDGF-BB, RANTES, TNF- α and VEGF and relating findings to infection status.

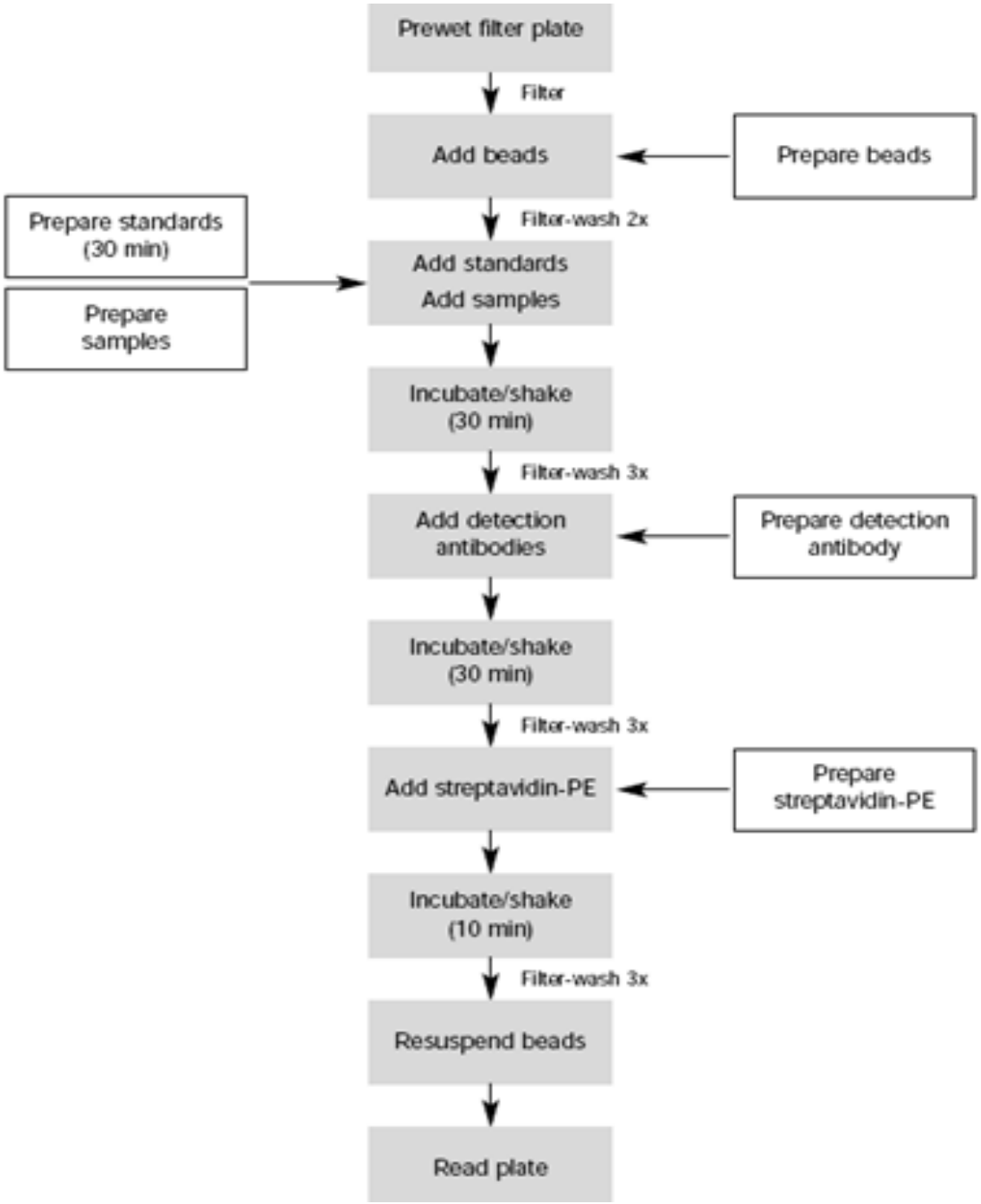

Figure 2-2: Bioplex cytokine assay workflow

2.3.3.4 Immunoglobulin detection methods

Multiplex detection of 6 serum immunoglobulins for mother-infant pairs (MIP) using 6-plex Pro Assay 6-plex kit, Bio-Rad, USA, which is a magnetic-bead based multiplex immunoassay, was employed following the manufacturer's instructions and as shown in Figure 2-3. The study measured antibodies IgG (IgG1, IgG2, IgG3 and IgG4), IgA and IgM and relating findings to infection status.

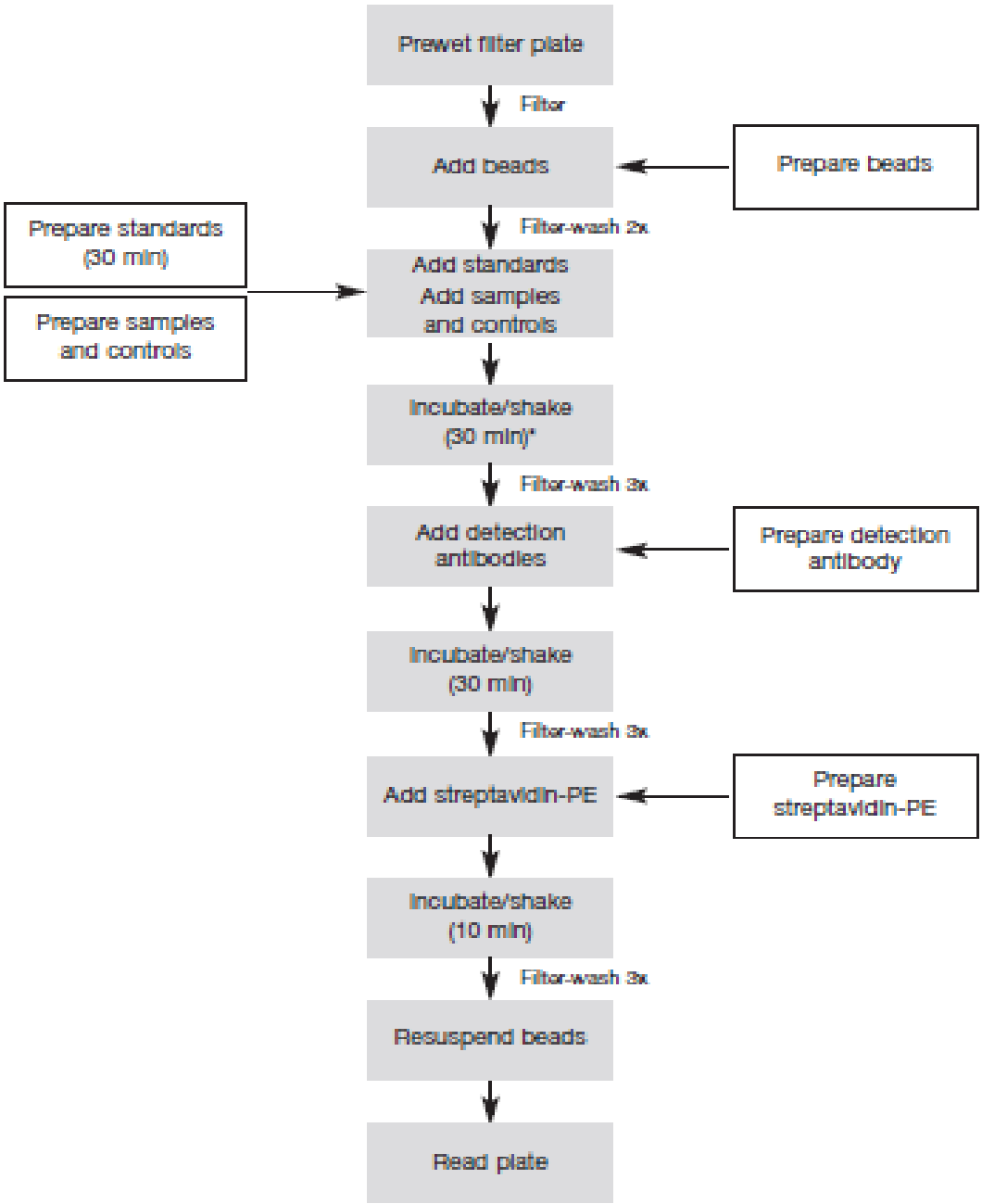

Figure 2-3: Bioplex Immunoglobulin Assay Workflow

Figure 2-4 summarises the study laboratory activities. Biomedical samples collected are indicated in blue and tests performed in yellow.

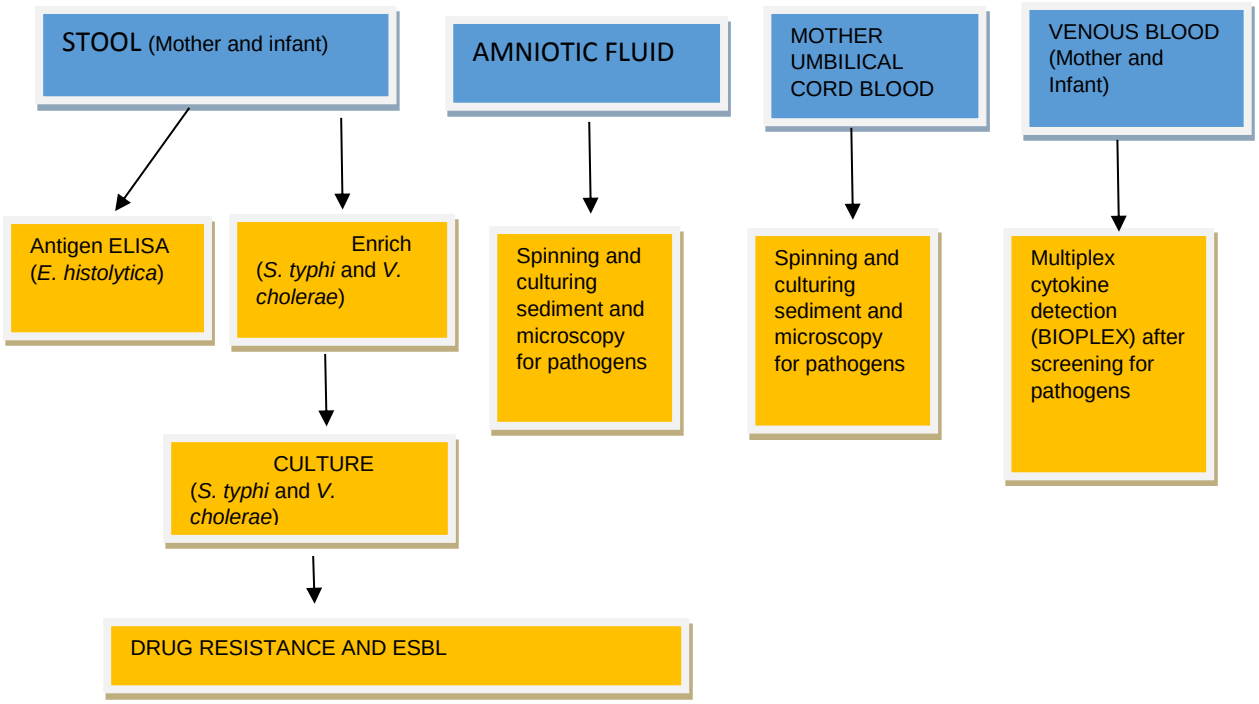

Figure 2-4: Study Laboratory activities summary flow chart.

Chapter 3 : Determination of the baseline prevalence of the carrier state of enteric pathogens (*Vibrio cholerae, Salmonella typhi* bacteria and *Entamoeba histolytica* protozoan) in HIV-infected pregnant women and their neonates.

Despite all the challenges on diarrheal outbreaks faced by Zimbabwe, there is still a paucity of information with regards to prevalence of such pathogens as *Vibrio cholerae, Salmonella typhi* bacteria and *Entamoeba histolytica* protozoan in the general population. This is even worse in pregnant women. This chapter aims to determine the baseline prevalence of carrier state of enteric pathogens *(Vibrio cholerae, Salmonella typhi* bacteria and *Entamoeba histolytica* protozoan) in HIV-infected pregnant women and their neonates and is presented in a manuscript format which has been submitted to the African Journal of Reproductive Health (AJRH) and is under review *(proof of submission is attached as appendix 8A7).* The serum samples from identified carrier individuals were then subjected to cytokine and immunoglobulin profiling to determine influence carrier state on the cytokine and immunoglobulin production in the mother-infant pairs as described in Chapter 4. The manuscript for this Chapter is as below;

Title: Asymptomatic *Vibrio cholerae, Salmonella enterica* serovar Typhi and *Entamoeba histolytica* burden in pregnant women and their infants in a high HIV prevalence setting in Harare, Zimbabwe

Sub-title: Asymptomatic enteric pathogens in pregnancy

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ABSTRACT

Background:

Despite the success of Prevention of Mother to Child Transmission (PMTCT) in Zimbabwe, higher morbidity and mortality rates in HIV-exposed and uninfected (HEUs) compared to the unexposed infants (HUUs) remains a challenge, the cause is still unknown and requires investigation.

Objectives:

This study aimed at determining the burden of asymptomatic enteric pathogens in particular *Vibrio cholerae, Salmonella enterica* serovar Typhi and *Entamoeba histolytica* in pregnant women and their infants; and possible congenital transmission.

Methods

Maternal faecal and urine, as well as infant faecal samples, were cultured for *Vibrio cholerae* and *Salmonella enterica* serovar Typhi detection. Antigen ELISA was used for *E. histolytica* detection. *Non-lactose fermenting bacterial isolates* were identified using analytical profile index using E.coli ATCC 25922 control organism and antimicrobial susceptibility done thereafter.

Results

Neither *V. cholerae* nor *S. typhi* was detected. An *E. histolytica* prevalence of 5.4% in the mothers and 5.6% in infants was observed. There was no significant difference in prevalence of *E. histolytica* between HIV-infected and HIV-uninfected mothers (p=0.318), being borderline between for HIV-exposed uninfected and HIV-unexposed uninfected infants (p=0.056). Resistance to 3rd generation cephalosporines was detected in the non-lactose fermenting bacterial isolates. One HIV-uninfected mother-neonate pair had an *E. histolytica* infection.

Conclusion

Asymptomatic carriers of *E. histolytica* exist in pregnant mothers with possible risk of congenital transmission. Our study highlights the need to screen and treat pregnant women to control congenital transmission of enteric pathogens in an effort to contribute to the regulatory strategies for the high morbidity and mortality rate in HEUs.

Keywords

Asymptomatic carriers, HIV-infected mothers, enteric pathogens, *E. histolytica, S. typhi, V. cholerae,* HIV-exposed infants.

INTRODUCTION

Worldwide consideration and available resources are focused mainly on diseases such as malaria, HIV infection and TB whilst enteric diarrheal diseases such as cholera, typhoid and dysentery get little resources. A holistic approach is required to fight enteric diseases, especially in HIV burdened; resource-limited countries, to avert opportunistic infections. Diarrhoea has always plagued Africa. In Zimbabwe, a cholera outbreak in 2008 claimed lives (4% death rate)[1; 2] and spread across 10 Provinces of Zimbabwe even affecting the neighbouring country of South Africa. In 2012, a typhoid outbreak predominantly affecting the density suburbs of Kuwadzana and Dzivarasekawa[3]. Also, since the onset of the 2016-2017 rainy season, Zimbabwe has experienced a typhoid outbreak with 1380 cases and two deaths reported[4]. According to the WHO guidelines on control of diarrheal disease, preventive measures as good hygiene practices and provision of safe water require urgent consideration[5]. Opportunistic infections are frequent in immunocompromised patients. It is perhaps plausible that when infected with an intestinal parasite, the individual develops an acute disease that lowers the mucosal defence hence is less effective in eliminating the infection. A common parasite in sub-Saharan regions and in Zimbabwe is *Entamoeba histolytica.* This parasite can also cause invasive diseases such as genito-urinary amoebiasis, liver abscess, cerebral amoebiasis and respiratory tract infections[6; 7]. Since this protozoan damages the intestinal mucosa, it is plausible that it promotes HIV/AIDS disease progression.

The typhoid fever bacterium, *Salmonella enterica* serovar Typhi, whose sole natural host and reservoir are humans[3] is a major public health concern [8]. The biology of this pathogen that makes it affect humans as the definitive host is not well understood[9] hence there is a need for more research. Apart from the faecal/urine-oral transmission route of typhoid [9], vertical transmission *in-utero* [10] is highly possible and is of great concern in many developing countries.

An irresponsible use of antibiotics causes the loss of the normal host flora with the resultant proliferation of *Salmonella* spp. It is highly possible that the abuse of acid suppressor drugs may predispose the loss of the acid barrier thereby increasing the susceptibility of the host to enteric pathogens. Host response to enteric pathogen infection varies from mild- to severe- to death. In the first group, the patient may receive treatment and heal completely clearing up all the infecting pathogens; however in the second group, the patient may heal after treatment but without clearing up all the pathogens in their systems and thirdly the patient may acquire the infection and not display any signs and symptoms such as a self-limiting diarrhoea. The latter two groups were considered asymptomatic carriers that can spread the disease or spark outbreaks of diarrhoeal disease.

Asymptomatic carriers of Typhoid are estimated at 1-3%[11]; *V cholerae* (95%)[12] and *E. histolytica* (90%) [6] In a report from Canada, 75% asymptomatic *V. cholerae* prevalence was noted[13]. Notably, there is a paucity of information on asymptomatic carriers of these pathogens in the general population in Zimbabwe. Such burden of disease remains poorly described in the Zimbabwean general population and in pregnancy yet such diseases may play an important role in the morbidity and mortality rates observed in the HIV-exposed uninfected (HEU) neonates.

The objective of this study was to determine the baseline prevalence of the carrier state of enteric pathogens carriers, in particular, *V. cholerae*, *E. histolytica* and *S. enterica* ser. Typhi in a cohort of pregnant mothers and their infants in a HIV high burdened urban setting within Zimbabwe.

MATERIALS AND METHODS

Ethics approval and consent to participate

The study protocol was approved by the Medical Research Council of Zimbabwe (Approval Code MRCZ/A/2043) and the Biomedical Research Ethics Committee of University of KwaZulu-Natal, Durban, South Africa (Approval Code BE409/15). Informed consent forms were approved by the Medical Research Council of Zimbabwe. All study participants consented to take part in the study such as responding to the questionnaire and providing samples.

Study population and sampling

This was a sub-study based on samples collected from a cohort of HIV-infected (HIV^+) and HIV-uninfected (HIV^-) pregnant women. These women resided in high-density suburbs of Harare, Zimbabwe, namely Dzivarasekwa, Glenview and Kuwadzana during the period February 2016 to February 2017. Only women 18 years of age and above at 28-36 weeks gestation based on last menstrual period (LMP), having documented HIV status or willing to be tested for HIV and who were able to give an informed consent were recruited into the study.

Study procedure

Pregnant women visiting the local clinic were invited to participate in the study, upon which they would sign an informed consent if they agree to take part in the study, including details of their infant upon delivery. A questionnaire was then administered to capture such baseline data as, demography, HIV status, ART uptake and regimen, water and sanitation, previous history of diarrheal diseases.

Biomedical Samples Collected

Stool and urine were collected from mothers at recruitment or close to delivery for culturing of *V. cholerae* and *S. typhi*. The stool was also used for *E. histolytica* microscopy and ELISA. The first voided stool or stool as close to delivery as possible was collected from infants for *V. cholerae* and *S. typhi* culture in addition to *E. histolytica* diagnosis using microscopy and ELISA. When it was difficult to collect stool, an anal swab was collected.

***Vibrio cholerae* detection in mothers and infants**

A rice grain size fresh stool samples were cultured in alkaline peptone water overnight at 37±2 ^{o}C to enhance the growth of *V. cholerae* before culturing on Thiosulphate Citrate Bile Salt (TCBS) solid media, which is also a selective media for *V. cholerae*. Yellow colonies from TCBS were considered presumptive and a gram test, oxidase test as well as string tests were further performed on the presumptive colonies.

The presumptive yellow colony isolate were gram stained to check for gram-positive curved rods which are indicative of *V. cholerae*. Oxidase test with oxidase strips was also performed using a wooden or plastic applicator to check for colonies that turn positive (deep purple) within 10 sec of testing. A string test was further done by mixing the fresh colonies with 0.5% aqueous solution of sodium deoxycholate on a clean microscope slide. Any positive samples were confirmed with Analytical Profile Index 20NE (API; Biomerieux: France).

Salmonella typhi detection in mothers and infants

A rice grain size fresh stool sample was cultured in 20ml selenite F broth for 24hr at 37±2 ºC to enhance the growth of *Salmonella* bacteria before culturing on Xylose Lysine Deoxycholate (XLD) and MacConkey agars which are also selective media for *Salmonella*. For infants, if a swab was available, one anal swab was cultured as described for the mother stool samples. Pink colonies from XLD and pale colonies on MacConkey were considered presumptive and gram tested. Any presumptive colonies were aimed for confirmation with Analytical Profile Index 20E (API; Biomerieux: France).

^{EZ}Typhi-Paratyphi DNA Dipstick kit for detection of *Salmonella typhi* in mothers and infants

Qualitative detection of *S. enterica* ser. Typhi and *S. enterica* ser. paratyphi A from mother and infant stool samples were performed using ^{EZ}Typhi-Paratyphi DNA Dipstick kit, (Universiti Sains Malaysia, Health Campus, Malaysia) following the manufacturer's instructions. The same stool sample from selenite broth culture was used for solid media culture and also used for DNA extraction required for PCR in the ^{EZ}Typhi-Paratyphi DNA Dipstick procedure. The amplicons were subjected to a lateral flow assay to determine the presence or absence of *Salmonella* species, *S. typhi* or *S. paratyphi A.*

Microscopy and Antigen ELISA detection of *E. histolytica* in mothers and infants stool samples

A wet mount technique was used, where approximately 2mg of stool sample was mixed with a drop of 0.85% sterile normal saline on a clean glass slide. Examination for trophozoites and cysts for *E. histolytica* was performed at X10 and X40 initial magnification. A qualitative detection of *E. histolytica* antigen in stool samples was performed on all samples following microscopy. ELISA detection was performed using the r-biopharm Ridascreen (C1701) AG kit (Darmstadt, Germany).

Approximately 0.1g of stool sample or an anal swab was diluted in 1ml of sample diluent buffer (diluted 1:11). A swab is estimated at 0.1g stool sample. One hundred microliters (100µl) of each sample, as well as positive and negative controls, were used in the analysis following the manufacturer's instructions. Optical Density (OD) was read at 450/620nm wavelength using the URIT-660 ELISA reader.

Other organisms isolated from culture

All non-lactose fermenters (NLFs) from culture were treated as potential pathogens and were subjected to API testing for identification, using an isolate suspension equivalent to a 0.5 McFarland standard according to the manufacturer's kit insert. After API testing, the isolates were then subjected to antibiotic susceptibility testing (AST) using the following drugs; penicillin, gentamycin, ampicillin, clindamycin, vancomycin, trimethoprim, ampicillin, cotrimoxazole, ertapenam and ceftriaxone. Organisms resistant to cefpodoxime (3rd generation cephalosporin), were considered suspect for Extended Spectrum Beta-Lactamase (ESBL) carriage and were subjected to ESBL and inducible AmpC enzyme testing using the six antibiotics which were cefpodoxime (CPD10), cefpodoxime/clavulanate (CPDCV), Cefepime (CPM30), Cefepime clavulanate (CPMCV), ceftazidime (CAZ) and Cefoxitin (FOX).

RESULTS

Demographic Data

A total of 417 participants were enrolled in the study and 52.7% (218) were HIV-infected. Of the HIV-infected participants, 91.3% (199) were on ART. The majority of the participants who were on ART, 96.2% (194), received TENOLAM E, whilst only 2.7% (5) received TENOLAM N. Nearly half of the participants (48.5%) were aged between 26 and 35 years with a mean age of 29 years (SD ±6.3). The participants' area of residence was almost equally distributed among the three high-density suburbs of Harare. The majority (96.5%) of the participants reported the use of a flush toilet located inside or outside the house. A majority (62.8%) of participants reported the use of borehole untreated water in 85.4% of the households (Table 3-1a).

Only 4.1% of the households reported always having sewage overspill in the immediate proximity of their home with 17.3% also reporting that a household member had suffered from the diarrheal disease in the last month (Table 3-1b). The majority of the participants had normal stool (79.8%) according to the Bristol stool chart. Only 10.3% were currently on antibiotic treatment at the time of recruitment (Table 3-2a). The majority of the participants (93.0%) had live births, being predominantly males (51.3%).

The mean neonate weight was 3060 grams (SD± 507) with the majority (82.3%) having a weight above 2500 grams. Underweight births were significantly higher (17.5%) in HIV-infected compared to HIV-uninfected mothers (11%) ($p=0.002$; Table 3-1b). The presence of an inside flush toilet ($p>0.042$) compared to having an outside flash toilet was significantly associated with the presence

of enteric pathogens Table 3-2a. *E. histolytica* infection was significantly higher in mothers that indicated an absence of current diarrhoea (96.6%) compared to those with current diarrhoea (p= 0.034), at the time of the study (Table 3-2b).

Type of Samples Collected from Mother and the Infant

A total of 204 mothers [HIV^- (n=100; 49.0%) and HIV^+ (n=104; 51.0%)] were included in this study based on the availability of stool samples. Only 111 (54%) mothers stratified as HIV-infected (n=54; 48.6%) and HIV-uninfected (n=57; 51.4%), could be paired to their neonates based on the availability of stool whilst 93 mothers remained unpaired. A total of 78.5% (306) infants stratified as HEUs (n=159; 52.0%) and HUUs (n=147; 48.0%), had stool samples available hence were included in the study. No HIV-exposed infected (HEI), if present, had samples available at the time of collection.

Stool Sample diagnosis for *V. cholerae, S. typhi* Detection and Other Pathogens

Neither *S. typhi* nor *V. cholerae* were detected in the cultured samples from both mothers *Table 3-3* and infants *Table 3-4*. No *Salmonella spp, S. typhi or S. paratyphi A* was detected by this method. Although neither *Salmonella enterica* serovar Typhi nor *Vibrio cholerae* was detected, some non-lactose fermenters were isolated. Using API on stool samples from mothers, a total of eight NLFs were isolated of which three were from the urine of HIV-infected mothers, two of which were identified as *Hafnia alvei* and *Proteus mirabilis*. One NLF could not be identified by API and was eliminated from further analysis.

Five of the NLF organisms were from the stool of HIV-uninfected mothers and were identified as *Enterobacter cloacae* (two isolates), *Acinetobacter baumannii, Rhanella aquatilis* and *Proteus*

mirabilis. Two *E. histolytica* (1%) were identified from the 204 mother stool samples by microscopy. Also, one *Giardia lamblia* and one *Schistosome* NLF were identified in the process. A total of 10 NLFs were isolated from infant stool samples of which five were from HEUs and identified as 2x *Escherichia coli 1,* 2x *Morganella morganii* and 1x *Raoultella ornithinolytica*. The other five of the 10 NLF isolated were from HUUs and identified as 2x *Escherichia coli 1,* 1x *Morganella morganii,* 1x *Escherichia fergusonii* and 1x *Citrobacter freundii*.

Antibiotic Susceptibility

Organisms from the mothers' stool indicated that *H. alvei* had a similar antimicrobial profile to the *P. mirabilis* isolates. *R. aquatilis, H. alvei* (intermediate resistance) and the two *E. cloacae* were resistant to cefpodoxime, which is an indicator of possible ESBL carriage hence were subjected to ESBL and AmpC testing. *R. aquatilis, E. cloacae* and *H. alvei* isolates did not possess ESBL carriage. However, *H. alvei* from HIV-infected mother and *E. cloacae* from HIV-uninfected mother were found to possess derepressed AmpC enzyme. While infants samples showed four isolates from HUUs and four from HEUs conferred resistance to cefpodoxime hence were tested for possibility of ESBL carriage. These four isolates from HUUs were *M. morganii, E. fergusonii, E. coli 1* and *C. freundii* and those from HEUs were *E. coli 1, R. ornithinolytica,* and 2x *M. morganii*.

Generally all the isolates were resistant to the antibiotics tested but were mainly susceptible to ertapenam, gentamycin and ceftriaxone (results not shown). None of the infant isolates carried ESBL or derepressed AmpC enzymes. Two *M. morganii* isolates (one from HEU and the other from HUU) however possessed inducible AmpC enzyme as indicated by resistance to FOX and blunting between FOX and CAZ or FOX and CPD10 forming a D zone around the CAZ disc or the CPD10 disc (results not shown).

Antigen ELISA for the detection of *E. histolytica*

Off the 204 maternal stool samples, 49% (100) HIV-uninfected and 51% (104) from HIV-infected, were tested and 11 mothers were *E. histolytica infected*. The prevalence of *E. histolytica* was 5.4% (95% CI; 2.7%-9.4%). The prevalence of *E. histolytica* among the HIV-infected was 3.9% (95% CI; 1.6%-10.9%) and among the HIV-uninfected was 7.0% (95% CI; 2.9%-13.9%). Two of the 11 *E. histolytica* infected were detected by microscopy and were included for further immunological investigations under the current study.

There was no significant difference in the *E. histolytica* prevalence between the HIV-infected and HIV-uninfected mothers (p=0.532) (Table 3-3). Majority of mother *E. histolytica* cases had normal stool type and frequency, reporting no history of diarrhoea in the past month, and no incidence of sewage overspill for more than a year as shown in Table 3-2a. Only 78.5% (306) neonate specimens were collected at enrolment and tested. Of the 306 infants tested, 17 were positive for *E. histolytica* indicating a prevalence of 5.6% (95% CI; 3.2%-8.7%). The prevalence of *E. histolytica* among HEUs group was 3.1% (95% CI; 1.0%-7.2%) and among HUUs group was 8.2% (95% CI; 4.3%-13.8%). The difference in *E. histolytica* prevalence between HEUs and HUUs was not significant (p=0.056) as shown in Table 3-4.

A total of 111 mother-infant pairs (MIP) were obtained based on the availability of stool samples. Only one (1%) *E. histolytica* infected mother who was HIV-uninfected could be linked to her *E. histolytica* infected infant.

Ten mothers out of the 111 MIP (9%) were *E. histolytica* infected while their infant pairs were *E. histolytica* uninfected. Five infants out of the 111 MIP (5%) were *E. histolytica* infected while their mother pairs were *E. histolytica* uninfected.

DISCUSSION

This study demonstrates that the prevalence of *E. histolytica* in pregnant mothers participating in the study population was 5.4%. However, the prevalence was not significantly different between HIV-uninfected and HIV-infected groups. These findings are corroborated by other studies where 6.7% *E histolytica* was reported by Guo *et al.*[14] in pregnant women in the Caribbean region. In infants, the baseline prevalence in our study was 5.6% and similarly, there was no significant difference between HEU and HUU groups. Also, *E. histolytica* prevalence was 3.9% within the HIV-infected pregnant mothers group and 7.0% within the HIV-uninfected pregnant mother group. Interestingly, we did not see a higher prevalence of the parasite in HIV-infected individuals compared to the HIV uninfected.

In most studies, *E. histolytica* prevalence in HIV-infected population has been shown to be higher than in the HIV-uninfected group[15; 16], which was not the case in our study. This could be attributed to the fact that all the HIV-infected pregnant women were urban dwellers who have access to reasonable sanitary conditions. ART uptake may have some anti-parasitic effects however this requires further investigation. In India, a prevalence of 3.7% was reported in HIV-infected individuals with diarrheal diseases[17]which is about 50% lower than what was found by Guo *et al.*,[14] and to our study. Certain ante-natal activities and hygiene lessons may have influenced the general health of the study population hence the low prevalence of *E. histolytica*.

One interesting finding was the detection of *E. histolytica* in a week old neonate, indicating the possibility of a congenital transmission. It was, however, not possible to confirm this result in the mother since her stool sample could not be collected before delivery. Also, one mother-baby pair with *E. histolytica* was identified though the neonate sample was collected at six weeks.

Despite the possibility of congenital transmission, poor hygiene within the household may have contributed to this prevalence. This implicates the possibility of *E. histolytica* contamination of the household and its immediate surrounding, nonetheless, no follow-up was done to verify this assumption. Interestingly, the majority of *E. histolytica* cases were found in a population of mothers who use flush toilets. In the resource-poor setting of Zimbabwe, detergent usage required to clean the toilets is often minimal. Notably, the majority of mother *E. histolytica* cases had normal stool type and stool frequency. This might be as a result of stabilisation of infection due to the chronic carriage of the organism where the protozoa and the host develop a mutual relationship.

The *E. histolytica* infected samples by microscopy could not be confirmed by ELISA probably because of formalin that was added to the sample which confounded the antigen-antibody reaction. Moreover, the low prevalence rate (1%) for microscopy compared to ELISA (5.4%) suggests that microscopy alone may lead to false negative diagnosis hence compromise patient management. It also indicates that the technique requires highly experienced individuals to improve on the pick-up rate, sentiments recently shared by some researchers[16; 18; 19]. However, even with highly experienced scientists, the pick rate (sensitivity) of the microscopy methods to detect *E. histolytica* is very low, at <10%[6].

No *S. enterica* ser. Typhi bacteria were detected in contrast to similar studies that reported a prevalence between 1-3% [11; 20]. The relatively small sample size may have confounded the prevalence rates. Also, it is possible that exposure from previous pathogen outbreaks may contribute to the development of a protective immunity against these diarrheal pathogens.

Our results are in concordance with Im *et al.*,[21] who reported 1077 and 1359 individuals from Guinea-Bissau and Senegal, respectively an absence of *S. typhi* after stool culture. Although in one study, *S. enterica* ser. Typhi bacteria was isolated in aborted foetus suggesting the possibility of the bacteria crossing the maternal-fetal interface[10], there is likelihood of protective immunity gained from the mothers that could have been passed onto the unborn babies. Despite an absence of *V. cholerae* detection in our study, it has been indicated that long-term convalescent carriers of *V. cholerae* are rare[22] and this could possibly explain its absence in our study. The last cholera outbreak in Zimbabwe was in 2008, hence it is possible that the carriers have since cleared their systems; totally resulting in observed 0% *V. cholerae* detection rate.

It is expected that the prevalence of pathogen carrier status in the HIV-infected pregnant mothers is high compared to their HIV-uninfected counterparts since it is a known fact that HIV-infected individuals have a compromised immune system. This is also expected in HEU infants compared to their HUU counterparts since we hypothesize that HIV-infected mothers have a compromised immunity and fail to stimulate an effective immune response of this kind to the newly born baby. The HIV-infected group were on ART, hence there is a possibility of ART playing a role in the individual's defence against these pathogenic organisms. This, however, needs to be investigated further. Organisms such as *R. ornithinolytica, E. cloacae, E. fergusonii, E. coli* and *M. morganii* that were noted in our study form part of the normal flora. *R. aquatilis* is a very rare enteric, Gram-negative rod, whose natural habitat is water. These organisms confer resistance to pathogenic organisms especially their resistance to Cotrimoxazole, a drug used as prophylaxis in HIV-infected people, and to cefpodoxime, which is a third generation cephalosporin, and this is of major concern.

The discovery of the inducible AmpC enzyme in two of the *M. morganii* isolates and derepressed AmpC in one *E. cloacae* and one *P. mirabilis* is an indicator of resistance to 3rd generation extended spectrum cephalosporins and cephamycins hence posing challenges to clinicians. Generally, some beta-lactams especially ceftriaxone have been seen to cause overproduction of AmpC enzymes through induction or derepression of inactive AmpC genes responsible for the production of the AmpC enzyme. This will cause some pathogens which initially appear sensitive to beta-lactams to suddenly become resistant[23–25]. It is really important to treat any AmpC producers as important as these may contribute to treatment failures observed in the health sector. The generalised resistance to antibiotics in the usually known gut commensals support earlier reports that gut microbiota act as a reservoir of antibiotic resistance[26–28], major concerns being of the possible transfer of this antibiotic resistance carriage to potential pathogens. There is also a possibility that these gut microflora carrying resistance genes becoming pathogenic possibly via mutations as the use of antibiotics are at an increase with some abuse being noticed where antibiotics are sometimes being prescribed for viral infections like common colds.

In a study by Al-Hulu *et al.*,[29], *R. ornithinolytica*, was isolated first time in clinical samples and was resistant to a number of antibiotics including cefotaxime[29] which is a third generation cephalosporin. *E. fergusonii*, also isolated in the present study was resistance to cefpodoxime among other tested antibiotics, despite it being normally a gut commensal it has been indicated in several studies to cause diseases in humans as well as animals, making it a possible zoonotic pathogen. It has been isolated in a number of conditions including enteric and urinary tract infections[30].

P. mirabilis was also isolated in one of the mother urine samples. This organism is part of the gut normal flora hence could have managed to cross over to the urinary tract. Other studies have however indicated the organism in some clinical gastroenteritis cases. *H. alvei* has been seen to have 'limited pathogenicity in humans that may cause clinically significant infections only in immunocompromised individuals'[31]. This could be the reason why in the current study it was isolated from a urine sample of an HIV-infected pregnant mother.

Conclusion

This study has established the prevalence of asymptomatic carriers of enteric pathogens in Zimbabwean pregnant women as 5.4% for *E. histolytica* and 0% for both *V. cholerae* and *S. typhi*. In infants, the prevalence is 5.6 % for *E histolytica* and 0% for both *V. cholerae* and *S. typhi*. Asymptomatic enteric carriers are a reality in the Zimbabwean pregnant women with a possibility of congenital transmission which might be a possible cause for the unexplained high morbidity and mortality rates in HIV-exposed and uninfected infants. There is need therefore to screen pregnant women for these pathogens to avoid transmission to the infants. Asymptomatic infections demand the need to be on the alert to control potential outbreaks. The study has also shown that there is no *V. cholerae* carriage in the vulnerable Glenview, Kuwadzana and Dzivarasekwa, Zimbabwe areas post the 2008 cholera outbreak and the 2008 and 2012 typhoid outbreaks.

Study limitations and recommendations

This was a cross-sectional sub-study and at least 3 fresh consecutive stool samples for cultures could not be obtained. There is, therefore, need to use antigen ELISA kits on stored stool samples, which could not be done in the current study due to limited funding. In addition, there is need to strengthen monitoring of urinary tract infections in pregnant women since some organisms had been isolated in urine from three of the 204 pregnant women.

AUTHOR CONTRIBUTION

AFN conceived the idea, designed the experiments, analyzed the data and developed the final written manuscript; TM, BSP, AI and TN supervised the work throughout, provided technical guidance to AFN. AFN and CM performed the assays and analyzed the data; TJC and EPS provided technical guidance to AFN; KD coordinated collection of samples and provided technical guidance to AFN. TB and MP provided technical guidance on study design and data analysis to AFN. All authors contributed to the writing of the manuscript.

CONFLICT OF INTEREST: The authors declare no conflict of interest.

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TABLES

Table 3-1: Baseline Demographic & Clinical Characteristic of Enrolled Study Patients on Education Level, Type of Water Source and Toilet of Enrolled Study Patients in Relation to HIV Status

		All		**HIV Negative**		**HIV- infected**		**p-value**
Characteristics		**N= 417**	**%**	**N= 199**	**%**	**N= 218**	**%**	
HIV Status	Positive	218	52.3	-	-	218	100	
	Negative	199	47.7	199	100	-	-	
	Completed Primary	59	14.2	32	16.1	27	12.4	
	Some Secondary	85	20.4	35	17.6	50	22.9	
	Completed Secondary	245	58.8	119	59.8	126	57.8	
	Tertiary	20	4.8	10	5.0	10	4.6	
Age (years)	≤25	141	33.8	88	44.2	53	24.3	
	26-35	204	48.9	85	42.7	119	54.6	
	≥36	66	15.8	25	12.6	41	18.8	**<0.001**
	Missing	6	1.4	1	0.5	5	2.3	
	Mean Age (STDEV)	28.8(6.3)		27.4(6.4)		30.1(5.9)		**<0.001**
Area of Residence	Location 1 (Dzivarasekwa	127	30.5	65	32.7	62	28.4	
	Location 2 (Glenview)	128	30.7	59	29.7	69	31.7	
	Location 3 (Kuwadzana)	161	38.6	74	37.2	87	39.9	0.564
	Missing	1	0.2	1	0.5	0	0.0	
Type of Toilet used	Flush Toilet (Inside)	225	54.0	106	53.3	119	54.6	
	Flush Toilet (Outside)	177	42.5	86	43.2	91	41.7	
	Other (eg Blair)	11	2.6	4	2.0	7	3.2	0.604
	Missing	4	1.0	3	1.5	1	0.5	
Water Source	Piped into dwelling	107	25.7	56	28.1	51	23.4	
	Public tap	11	2.6	2	1.0	9	4.1	
	Borehole	262	62.8	118	59.3	144	66.1	
	Protected Well	30	7.2	19	9.6	11	5.1	0.089
	Commercial Bottled	6	1.4	3	1.5	3	1.4	
	Missing	1	0.2	1	0.5	0	0.0	
Treated Water	No	356	85.4	169	84.9	187	85.8	
	Yes	58	13.9	29	14.6	29	13.3	0.828
	Missing	3	0.7	1	0.5	2	0.9	
Sewage Overspill	Never	278	66.7	126	63.3	152	69.7	0.583
	Always	17	4.1	9	4.5	8	3.7	
	Monthly	56	13.4	30	15.1	26	11.9	
	Annually	60	14.4	29	16.1	28	12.8	
	Missing	6	1.4	3	1.0	4	1.8	
Diarrhea in the last month	Never	334	80.1	157	78.9	177	81.2	
	Yes	72	17.3	35	17.6	37	17.0	0.547
	Missing	11	2.6	7	3.5	4	1.8	

Table 3-1b: Baseline Demographic and Characteristic of Enrolled Study Patients on Food Taken, Type of Stool, Gender, Birth Weight and others in Relation to HIV Status

		All		HIV Negative		HIV infected		p-value
Characteristics		**N= 417**	**%**	**N= 199**	**%**	**N= 218**	**%**	
Fermented foods	Yoghurt (Yes)	248	59.5	122	62.3	126	18.3	0.466
	Cheese (Yes)	91	21.8	51	25.6	40	14.6	0.072
	Lacto(Yes)	342	82.0	166	83.4	176	80.7	0.476
	Mahewu(Yes)	355	85.1	178	89.4	177	81.2	**0.018**
Type of Stool	Separate hard lumps(constipat	7	1.7	4	2.0	3	1.4	0.670
	Lumpy/sausage like (s constipated)	28	6.7	12	6.0	16	7.3	
	Sausage shape with cracks (nc	121	29.0	58	29.1	63	28.9	
	Smooth soft sausage/snake (n	212	50.8	106	53.3	106	48.6	
	Soft blobs with clear cut edge of fibre)	24	5.8	11	5.3	13	6.0	
	Mushy consistency with edges (inflammation)	18	4.3	7	3.5	11	5.1	
	Liquid consistency with nc pieces (inflammation)	2	0.5	0	0.0	2	0.9	
	Missing	5	1.2	1	0.5	4	1.8	
Currently on Antibiotic	Yes	43	10.3	3	1.5	40	18.4	**<0.001**
	No	372	89.2	196	98.5	176	80.7	
	Missing	2	0.5	6	3.3	2	1.0	
Currently diarrhea	Yes	14	3.4	9	4.5	5	2.3	**0.034**
	No	387	92.8	178	89.5	209	95.9	
	Missing	16	3.8	12	6.0	2	1.0	
Live Births	Yes	390	93.5	184	92.5	206	94.5	0.369
	Still to deliver	26	6.3	15	7.4	11	5.0	
	Stillbirth	1	0.2	0	0.0	1	0.5	
Neonate gender	Missing	5	1.3	2	1.3	3	1.9	0.550
	Male	159	51.3	79	52.7	80	50.0	
	Female	146	47.1	69	46.0	77	48.1	
Neonate Weight (grams)	Missing	85	21.8	36	19.6	49	21.8	**0.002**
	<2500	47	12.05	11	6.0	36	17.5	
	≥2500	321	82.3	164	89.1	157	76.2	
	Missing	22	5.6	9	4.9	13	6.3	
	Mean (SD)	3060(507)		3131(449)		3000(547)		**0.013**

Table 3-2a: Baseline demographic & Clinical Characteristic of Enrolled Study Patients on education level, type of water source and toilet in Relation to *E. histolytica* Detection.

Characteristics		**All**		***E. histolytica*- infected**		***E. histolytica*- uninfected**		**p-value**
		N= 204	**%**	**N= 11**	**%**	**N= 193**	**%**	
E. histolytica	Positive	11	5.4	11	100	-	-	-
	Negative	193	94.6	-	-	193	100	
Education Level	Some Primary	0	0.0	0	0.0	5	100	0.202
	Completed Primary	0	0.0	0	0.0	53	100	
	Some Secondary	85	20.4	3	7.7	36	92.3	
	Completed Secondary	245	58.8	8	7.5	99	92.5	
	Tertiary	-	-	-	-	-	-	
-Age (years)	≤25	62	30.4	2	18.2	60	31.1	0.405
	26-35	98	48.0	8	72.7	90	46.9	
	≥36	41	20.1	1	9.1	40	20.7	
	Missing	3	1.5	0	0.0	3	1.6	
	Mean Age (STDEV)	29.3(6.5)		31.0(5.5)		29.2(6.5)		0.374
Area of Residence	Location 1 (Dzivarasek	73	35.8	4	36.4	69	35.8	0.920
	Location 2 (Glenview)	66	32.4	3	27.3	63	32.6	
	Location 3 (Kuwadzana	65	31.9	4	36.4	61	31.6	
Type of Toilet used	Flush Toilet (Inside)	108	52.9	6	54.6	102	52.9	**0.042**
	Flush Toilet (Outside)	90	44.1	4	36.4	86	44.6	
	Other (eg Blair)	4	2.0	0	0.0	4	2.1	
	Missing	2	1.0	1	9.1	1	0.5	
Water Source	Piped into dwelling	63	30.9	1	9.1	62	32.1	0.454
	Public tap	7	3.4	1	9.1	6	3.1	
	Borehole	119	58.3	8	72.7	111	57.5	
	Protected Well	12	5.9	1	9.1	11	5.7	
	Commercial Bottled	3	1.5	0	0	3	1.6	
Treated Water	No	169	82.8	9	81.8	160	82.9	0.896
	Yes	32	15.7	2	18.2	30	15.5	
	Missing	3	1.5	0	0.0	3	1.6	
Sewage Overspill	Never	145	71.1	10	90.9	135	70.0	0.601
	Always	10	4.9	0	0.0	10	5.2	
	Monthly	23	11.3	1	9.1	22	11.4	
	Annually	25	12.3	0	0.0	25	13.0	
	Missing	1	0.5	0	0.0	1	0.5	

Table 3-2b: Baseline demographic & Clinical Characteristic of Enrolled Study Patients on Type of Stool, Diarrhoea Episodes and Others in Relation to *E. histolytica* Detection.

Characteristics		**All**		***E. histolytica* infected**		***E.histolytica* uninfected**		**p-value**
		N= 204	**%**	**N= 11**	**%**	**N= 193**	**%**	
Diarrhoea in the last month	Never	177	86.8	10	90.9	167	86.5	0.677
	Yes	27	13.2	1	9.1	26	13.5	
Type of Stool	Separate hard lumps(constipated)	1	0.5	0	0.0	1	0.5	0.183
	Lumpy/sausage like (slightly constipated)	13	6.4	0	0.0	13	6.7	
	Sausage shape with cracks (normal)	61	30.0	4	36.4	57	29.5	
	Smooth soft sausage/snake (normal)	104	51.0	6	54.6	98	50.8	
	Soft blobs with clear cut edges (lack of fibre)	11	5.4	0	0.0	11	5.7	
	Mushy consistency with ragged (inflammation)	8	3.9	0	0.0	8	4.2	
	Liquid consistency with no solid (inflammation)	2	0.9	1	9.0	1	0.5	
	Missing	4	1.9	0	0.0	4	2.1	
Stool Frequency	More than Twice daily	77	37.7	5	45.5	72	37.3	0.886
	Once daily	110	53.9	6	54.6	104	53.9	
	Once every two days	13	6.4	0	0.0	13	6.7	
	More than once every two days	1	0.5	0	0.0	1	0.5	
	Missing	3	1.5	0	0.0	3	1.6	
Currently Antibiotic	Yes	190	93.1	2	18.2	11	5.7	0.251
	No	13	6.4	9	81.8	181	93.8	
	Missing	1	0.5	0	0.0	1	0.5	
Currently on Antacid	Yes	1	0.5	0	0.0	1	0.5	0.944
	No	202	99.0	11	100.0	191	99.0	
	Missing	1	0.5	0	0.0	1	0.5	
Currently having diarrhoea	Yes	3	1.5	1	9.1	2	97.4	**0.020**
	No	197	96.6	9	81.8	188	1.0	
	Missing	4	1.9	1	9.1	3	1.6	

Table 3-3: Culture Results for the HIV infected and HIV Negative Mothers

	ALL		**HIV Nega**	**HIV infected**			**p-value**
Type	n=204	%	n=100	%	n=104	%	
Isolates (Positive)							
S. typhi	0	0.0	0	0.0	0	0.0	-
V. cholerae	0	0.0	0	0.0	0	0.0	-
E. histolytica	11	5.4	7	7.0	4	3.9	0.318
Other Pathogens							
E.cloacae	2	1.0	2	2.0	0	0.0	0.147
G.lamblia (microscopy)	1	0.5	0	0.0	1	1.0	0.326
Hafnia alvei	1	0.5	0	0.0	1	1.0	0.326
P.mirabilis	2	1.0	1	1.0	1	1.0	0.978
Rahnella Aquatilis	1	0.5	1	1.0	0	0.0	0.307
Acinetobacter baumannii	1	0.5	0	0.0	1	1.0	0.326
Schistosome (microscopy)	1	0.5	0	0.0	1	1.0	0.326

Table 3-4: Culture Results for the HIV-Exposed-Uninfected (HEUs) and HIV-Unexposed-Uninfected (HUUs) neonates

	ALL		**HUUs**		**HEUs**		**p-value**
Type	n=306	%	n=147	%	n=159	%	
Isolates (Positive)							
S. typhi	0	0.0	0	0.0	0	0.0	-
Vibrio cholerae	0	0.0	0	0.0	0	0.0	-
Entamoeba histolytica	17	5.6	12	8.2	5	3.1	0.056
Other Pathogens							
Citrobacter freundii	1	0.3	1	0.7	0	0.0	0.298
Escherichia coli	4	1.3	2	1.4	2	1.3	0.937
Escherichia fergusonii	1	0.3	1	0.7	0	0.0	0.298
Morganella morganii	3	1.0	1	0.7	2	1.3	0.608
Roultella ornithinolytica	1	0.3	0	0.0	1	0.6	0.336

Chapter 4 : Determination of the effect of single or multiple enteric infections (*Vibrio cholerae, Salmonella* typ*hi* and *Entamoeba histolytica*) on cytokine profiles of pregnant women and their neonates in an HIV prevalent setting.

Chapter 3 focused on screening pregnant women and their infants for asymptomatic enteric pathogens carriage using methodologies described in Chapter 3. Individuals screened in Chapter 3 were purposively sampled based on enteric pathogen isolation and HIV status. The individuals that were infected with enteric pathogens were matched with their neonates. Also, the neonates were matched according to enteric and HIV infection. Below is a manuscript emanating from this study and is under review (***Manuscript number JMII-D-18-00007 as shown in appendix 8A8)***

Immune response to *Entamoeba histolytica* asymptomatic infections in pregnant women and their infants in a high HIV prevalence setting. (*Under Review in the Journal of Microbiology, Immunology and Infection-JMII)*

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ABSTRACT

Background:

Asymptomatic *Entamoeba histolytica* carriage in pregnant women poses an underlying risk to congenital infection, transmission to the infant during breastfeeding and as a potential for outbreaks in areas of high HIV prevalence. This study aimed at investigating the immunological status of asymptomatic enteric pathogen carriers in mother-infant pairs and to determine factors associated with cytokine profiles amongst mother-infant pairs in a high HIV burdened community in Harare, Zimbabwe.

Methodology:

Serum samples from 39 mother-baby pairs were analysed for inflammatory cytokine and immunoglobulin profiles using BIOPLEX immunoassay technology.

Results:

IL-1r, IL-4, IL-9, IL-12p70, IL-17a, G-CSF and PDGF-BB levels were significantly raised in *E. histolytica* infected compared to non-infected lactating mothers ($p<0.05$). In babies, *E. histolytica* carriage had no significant impact on the cytokine and immunoglobulin concentrations. Carriage of any form of enteric infection such as Non-lactose fermenters (NLFs) in addition to *E. histolytica* significantly increased concentration levels of IL-1r, IL-4, IL-9, IL-10, IL-12p70, IL17a, G-CSF, GM-CSF, IFN-γ, PDGF-BB and TNF-α cytokines ($p<0.05$) but no significant differences in immunoglobulin levels among the mothers. IL-13 and G-CSF levels were significantly raised in HIV-infected compared to HIV-uninfected lactating mothers ($p=0.0389$ and $p=0.0324$ respectively). IgA immunoglobulin levels were significantly raised among HIV-exposed uninfected than HIV-unexposed uninfected infants ($p=0.0282$). IL-7 was significantly raised in low birth-weight babies (birth weight <2500g) compared to normal birth-weight babies ($p=0.0149$).

Conclusion: Pro-inflammatory cytokines and chemokines were highly raised in lactating mothers with asymptomatic enteric pathogens. HIV exposure had no effect on baby cytokine profiles. Importance of IL-7 low birth-weight babies needs further investigations

Significance and impact of study

Our study highlights the need to check cytokine profiles in pregnant women and their infants to assist in decision making linked to treatment and prevention in times of pandemics and to assist in developing effective immunotherapy for reducing the observed high morbidity and mortality rates in HIV-exposed uninfected infants.

Keywords

Cytokines, Immunoglobulins, HIV-infected mothers, enteric infections, *E. histolytica*, HIV-exposed infants.

INTRODUCTION

Asymptomatic chronic intestinal carriage of parasites is very common worldwide and can confer an advantage to the host by shifting immune responses from Th1 to Th2 against bacterial infections, such as *Helicobacter pylori*, hence avoiding host tissue damages[1]. *Entamoeba histolytica* commonly colonises the human gut asymptomatically[2]. Immunity plays a very critical role in an individual's protection against infection and diseases. Several immune cells and proteins are involved in body defence mechanism in a rather complex phenomenon. Cytokines and antibodies (immunoglobulins) are some of the proteins involved in the body defence system. Immunoglobulin G (IgG) is the only antibody capable of crossing the placenta and has four sub-classes (IgG1, IgG2, IgG3 and IgG4) each of which can represent different immune mechanism for fighting infection. IgG1 has the strongest and longest half-life compared to all IgG subclasses and has strong complement binding activity[2–4]. IgG1 and IgG3 target microbial proteins, IgG2 targets microbial proteins and IgG4 'weakly responds to many antigens and possesses a blocking activity to IgG1- and IgE-mediated immune functions'[2]. In contrast to IgG2, IgG4, IgG1 and IgG3 drive inflammatory processes and antigen clearance via their affinity to C1q and binding to FcyRI, FcyRII and FcyRIII that are expressed on neutrophils, natural killer cells and tissue macrophages[2–4].

The body has to maintain a balance between fighting against pathogenic organisms and its own immune cells[5]. Maternal antibodies cater for the infant for the first three to twelve after delivery months in which case IgG will be in the infant circulatory system since it manages to get into the placenta and IgA will occur mainly in the infant gut through breast milk[6]. It is impossible to produce '…protective antibodies against all relevant infectious agents' during pregnancy without harming the fetus.

As such, it is logical that the fetus or newborn benefits both from maternal immunity as well as herd immunity, which simply stated, refers to the ability to reduce the probability of wide-spreading of an infection from an infected individual to susceptible, uninfected individuals in a population. It is hence important that women get immune to life-threatening infectious agents first before pregnancy to be able to transfer protective immunity to the baby which of course depends on the maternal accumulated immunological experience as well as herd immunity[6]. It is therefore important to vaccinate mothers in preparation for pregnancies.

Cytokine is the general term for small protein molecules that signal between cells and they are named in line with their source, for example, interleukins are produced by leukocytes, monokines are produced by myeloid cells and lymphokines are produced by lymphocytes. It is however not unusual for a cytokine to be produced by two different cell types, for example, IFN-γ which is produced by T cells as well as NK cells[3]. Cytokines can also have different effects on different cell types, for example, IFN-γ can result in activation of macrophages in an effort to destroy intracellular microbes. On the other hand, this same cytokine can activate B cells for antibody class switching. Interferons are produced by quite a variety of cells in response to viral infections[3]. In asymptomatic chronic carriage of *E. histolytica*, the cytokine Interleukin-10 (IL-10) was observed to be lower and IL-4 raised compared to amoebic liver abscess patients[2].

In pregnancy, the foetus is recognised as a foreign body[7], as a result, there is need to strike a balance between the mother's immune response and that of the growing baby to avoid rejection of the pregnancy. The immune system of the pregnant mother should, therefore, be robust enough to be able maintain the most important period in species conservation. This contradicts earlier thinking that a woman's immunity is weakest during pregnancy[6].

In pregnancy, both maternal and fetal immune systems should be strengthened in order to protect the two from environmental damage. It is, therefore, appropriate to refer to pregnancy as a unique immune condition that is modulated, but not suppressed. This unique behaviour explains why pregnant women respond differently to the presence of microorganisms or its products[8]. Successful pregnancies have been linked to T-helper 2 (Th 2) immune responses while deleterious ones to T-helper 1 (Th 1) immune responses[7; 9].

During pregnancy of immune-stimulated mothers, maternal blood and amniotic fluid cytokines IL-1, IL-6, IL-12, TNF-α and G-CSF increase transiently and appear to influence fetal immune development, a phenomenon known as fetal programming[10]. Mandal *et al.*,[11] has also noted a rapid development and increased responsiveness of Th1, Th17 and cytotoxic effector T-cell subsets by infants born to immune-stimulated mothers. Immune insult occurring during pregnancy has been linked to negative effects on the developing fetus such as increased risks of autism[12]. Increased levels of inflammatory mediators such as Macrophage Chemotactic Protein (MCP-1/CCL2), C-reactive proteins (CRP), matrix metalloproteinase (MMP)-9, Interleukins (IL)-4, IL-5 and Interferon (IFN-γ) have been linked to neurodevelopmental disorders like autism[12]. Previous studies have associated male fetal sex with up-regulated maternal pro-inflammatory cytokines particularly G-CSF, IL-12p70, IL-21, and IL-33 as well as angiogenic factors in particular PlGF and VEGF while female fetal sex was linked to an upregulation of regulatory cytokines particularly IL-5, IL-9, IL-17, and IL-25 although IL-27 increase throughout pregnancy is not affected by fetal sex. Maternal analyte concentrations are however not affected by fetal sex postpartum[13].

Cytokines are the major constituents of the placental microenvironment[14]. It is important to note that a cytokine can have dual purpose for example, IL-6 can act as both pro-inflammatory as well as regulatory. Besides involvement in inflammation and infection/ disease responses, IL-6 is implicated in metabolic, regenerative and neural process regeneration[15]. IL-6, IL-1 and TNFα cytokines are mostly elevated in inflammation hence have been targeted for therapeutic intervention[15].

IL-13 is an immunoregulatory cytokine that up-regulates CD23, MHC class II expression as well as promotion of the immunoglobulin E (IgE) isotype switching of B cells. Macrophage activity is also downregulated resulting in the inhibition of pro-inflammatory cytokines and chemokines. IL-13 takes part also in B-cell maturation and differentiation[16]. This cytokine hence can act as pro- as well as anti-inflammatory. IL-13 clusters with IL-3, IL-4, IL-5 as well as CSF2 (GM-CSF) on chromosome 5q but however positioned closer to IL-4. Granulocyte-Colony Stimulating Factor (G-CSF) also known as Colony Stimulating Factor 3 (CSF3), is a cytokine as well as a hematopoietic growth factor produced by macrophages, endothelium as well as other various immune cells[17; 18]. G-CSF has been found to stimulate production and proliferation of white blood cells. In fact, they stimulate the proliferation and differentiation of neutrophil progenitors in the bone marrow, to maintain the number of mature and functional neutrophils[17–19] and stimulate the release of mature granulocytes into the blood-stream[17]. In previous studies, G-CSF has been suggested as a reliable marker for use in neonates suffering from early- or late-onset of neonatal sepsis[17].

Pregnancy-associated upregulation of IL-13, IL-4 and IL-10 has been observed in previous studies and these might result in promotion of the production of IgG4 instead of IgG1 and IgG3 leading to a possible reduction of antibody-depended killing of viruses[20].

As a result of the plausible efforts by health experts to reduce HIV burden world-wide through mother to child prevention programs such as PMTCT, HIV-exposed uninfected children (HEUs) have greatly increased in numbers. However, the major challenge facing this group of children is the high morbidity and mortality rates compared to their HIV-unexposed counterparts (HUUs). Currently, the greatest concern of most researchers in the field of HIV and AIDS and paediatricians is to investigate the cause of such anomalies between HEUs and HUUs.

In this study, we hypothesise that asymptomatic chronic enteric infections influence the mother cytokine and immunoglobulin profiles as well as those of the infants. We investigated the relationship between mother cytokine profile and that of her neonate in relation to enteric exposure and HIV status. In one study by Abdulla *et al.*,[21] chronic amoebic infections caused prolonged inflammation resulting in auto-immune diseases.

Some studies have found higher levels of IL-1ra, IL-6, IL-8, IP-10 and MIP-1a in infants with proven infections although an overlap to those without proven infections was also noted[22] which could point towards intensive studies in this subject. Current studies have indicated a high morbidity and mortality rates in HIV-exposed uninfected neonates (HEUs) compared to their HIV-unexposed uninfected (HUUs) counterparts. The actual cause of this observation is yet to be established.

This study hypothesises the asymptomatic carriage of enteric pathogens by pregnant mothers and the influence of these pathogens on the maternal immune response in particular cytokines and antibodies, to have a bearing on the neonate cytokine and antibody production in both HEUs and HUUs.

The objective of this study was to investigate the effect of enteric pathogens carriages on the cytokine (IL-1β, IL-1r, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17a, Eotaxin, basic PDGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1(MCAF), MIP-1α, MIP-1β , PDGF-BB, RANTES, TNF- α and VEGF) and antibody production levels in mother-infant pairs (MIPs).

MATERIALS AND METHODS

Ethics approval and consent to participate

The study protocol approval was granted by the Medical Research Council of Zimbabwe, Approval Code MRCZ/A/2043 and Biomedical Research Ethics Committee (BREC) of University of KwaZulu-Natal, Durban, South Africa, Approval Code 409/15. All study participants consented to take part in the study.

Study population and sampling

This study was a sub-study based on stored plasma samples collected from a cohort of HIV-infected (HIV^+) and HIV-uninfected (HIV^-) pregnant women during the period February 2016 to February 2017. The study participants were from Dzivarasekwa, Glenview and Kuwadzana, three selected high-density suburbs in Harare, Zimbabwe, based on previous burden of pathogens of interest. Those recruited in the study were women of at least 18 years of age, within 28-36 weeks gestation in relation to last menstrual period (LMP), with known and documented HIV status or if unknown or not documented, should be willing to be tested for HIV and were able to give an informed consent.

Study procedure

The pregnant women on their routine antenatal care visits were invited to participate in the study. After agreeing, an informed consent was administered and signed by participant and the interviewer before a questionnaire administration to capture demographic data such as HIV status, ART uptake and regimen. The mothers were followed up to delivery where information on delivery mode, infant sex and weight, as well as infant HIV status, were taken and infant blood collected for sera where possible.

Those mother-baby pairs (MBP) who were able to provide stool samples had cultures and ELISA done on the samples. The MBPs that had *E. histolytica,* as well as other enteric pathogens detected, were considered for cytokine and antibody profiling.

Sample collection

Venous blood was collected on recruitment and processed for plasma and stored. Only sera for mothers and infants with *Entamoeba histolytica* infections and other microbial infections was used in this study based on the preliminary antigen *E. histolytica* ELISA and culture results. Sera from mothers without *E. histolytica* or other microbial infections were also used as controls. This was done for both HIV-infected and HIV-uninfected mothers and their infants. Infant stored sera as close to delivery as possible was used in the study and selection of the sera was based on the mother enteric infection status as well as the infant enteric statuses for both HIV-uninfected (HUU) and HIV-exposed uninfected (HEUs) neonates. All the infant samples were collected at recruitment and majority of infant samples were collected at 14 weeks and 6 weeks as indicated in *Table 4-1*. Those that seroconverted were also included despite absence of enteric pathogens (*Table 4-2*).

Table 4-1: Infant serum sample collection points

Serum point of collection	Frequency	Percentage
10 days	4	10.26
30 days	1	2.56
6 weeks	15	38.46
10 weeks	4	10.26
14 weeks	15	38.46
Total	39	100

Table 4-2: Serum sample collection point for sero-convertors and mother Viral Loads at recruitment

Infant ID	Sample collection point	Time seroconverted	Mother viral load at recruitment
Infant 1	14 weeks	6 months	43 280 copiesml^{-1}
Infant 2	14 weeks	10 weeks	<20 copiesml^{-1}

Cytokine and chemokine profiling

Bio-plex Pro Assay 27-plex kit, (Bio-Rad, USA), a magnetic-bead based multiplex immunoassay, was used to perform Cytokine profiling of 27 serum cytokines (IL-1β, IL-1r, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17a, Eotaxin, basic PDGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1(MCAF), MIP-1α, MIP-1β , PDGF-BB, RANTES, TNF- α and VEGF) for mother-baby pairs (MBP) following the manufacturer's instructions. The plate washings were performed on a Bio-Plex Pro and Bio_Plex Pro 11, wash station and incubation was performed at room temperature on a Bio-Rad Shaker Fax – 2200.

Data acquisition was performed using Bio-Plex® 200 system (Bio-Rad, California, USA) which uses Bioplex Manager Software. The data output was given as Median Florescence Intensity and concentration in $pgml^{-1}$. The software extrapolated concentrations that were outside limit of quantification (LOQ), which is the minimum level the machine could detect. Those that were below limit of detection (LOD-which is the minimum level the machine could quantify), or were not present at all, were given as out of range low (<OOR). Values above upper limit of quantification (ULOQ-which is the highest level the machine could quantify) were listed as out of range high (OOR>).

6-plex Antibody Profiling

Bio-plex Pro Assay immunoglobulin isotyping 6-plex kit, Bio-Rad, USA, was used to perform antibody profiling of 6 antibodies (IgG1, IgG2, IgG3, IgG4, IgM and IgA) for mother-baby pairs (MBP) as per protocol above.

Data Analysis

Non-parametric methods were employed to analyse both cytokine and immunoglobulin data. The medians and interquartile ranges were calculated.

RESULTS

Demographic Data

A total of 39 MBP were considered for cytokine and immunoglobulin profiling. Of the 39 mothers, 15 (38.5%) were HIV-infected and 24 (61.5%) were HIV negative. Nine (23.1%) of the 39 mothers had *E. histolytica* infection, of which 7 (17.9%) were HIV-uninfected and 2 (5.1%) were HIV-infected. Of the 39 babies, 12 (30.8%) were HIV-exposed uninfected (HEUs), 3 (7.7%) were sero-convertors and 24 (61.5%) were HIV-unexposed uninfected. Out of the 39 babies, 8 (20.5%) had *E. histolytica* infection of which 5 were HUUs and 3 were HEUs. Two of the five infected HUUs had coinfection, one with *E. fergusonii* and the other with *E. coli 1* (*E. fergusonii* and *E. coli*). The three babies who sero-converted had no identified enteric pathogens.

Sampling and and biomedical samples collected

Purposive sampling was done based on the presence of an enteric infection and HIV status. Samples with no enteric pathogens were also included as controls. A total of 39 MIP sera were analysed for cytokine and immunoglobulin profiles.

Cytokine and Immunoglobulin Profiling using Multiplex Assay

Cytokine and antibody levels in mothers during pregnancy compared to their babies after delivery

Cytokines and chemokines were generally significantly higher while the immunoglobulins were significantly lower in babies compared to their mothers ($p<0.05$). Results are not shown.

IL-2, IL-5, IL-7, IL-10, IL-12(p70), IL-13, IL-15, MCP-1 and VEGF concentrations in both mothers and infants were either OOR< or values were below LOQ. Immunoglobulins, IgG1, IgG3 and IgA concentrations were OOR> in both mothers and infants as the concentrations were higher than the ULD of the machine while some values were machine extrapolated as they were above the ULQ of the machine.

One infant who did not have enteric pathogens isolated, seroconverted at 10 weeks and sample collected at 14 weeks had OOR> IgG1 and IgG3 whereas the mother had OOR< for both IgG1 and IgG3 and had viral load (VL) at recruitment was <20 copies$^{-1.}$ This mother had all the tested cytokines and chemokines concentrations that were very low, and mostly OOR<. Another baby that seroconverted at 6 weeks that had no identified enteric pathogens and whose sample was collected at 10 weeks had OOR< immunoglobulins levels.

Cytokine and antibody responses in E. histolytica carriage.

IL-1r, IL-4, IL-9, IL-12p70, IL-17a, G-CSF and PDGF-BB were significantly raised in *E. histolytica* compared to absence of *E. histolytica* in pregnant mothers ($p<0.05$) as shown Table 4-3a and Table 4-3b. However, in babies, *E. histolytica* carriage had no significant impact on the cytokine and chemokine concentration as well as on immunoglobulin concentrations ($p>0.05$).

Asymptomatic Enteric infection, Cytokine and antibody levels in pregnant mothers and their babies at delivery.

Having any form of enteric infection carriage such as *E. histolytica* and non-lactose fermenters (NLFs) significantly increased the concentration levels for IL-1r, IL-4, IL-9, IL-10, IL-12p70, IL17a, G-CSF, GM-CSF, IFN-γ, PDGF-BB and TNF-α ($p<0.05$) as indicated in *Table 4-4*a and *Table 4-4*b.

However, there were no significant differences in terms of immunoglobulin levels under study between enteric infection carriers and non-carriers among pregnant mothers ($p>0.05$). In babies, the presence of varying enteric infections did not cause a significant difference in cytokine as well as immunoglobulin levels between enteric infection carriers and non-carriers in babies ($p>0.05$).

Cytokine and antibody levels in relation to HIV infection in pregnant mothers and their babies

IL-13 and G-CSF levels were significantly raised in HIV-infected pregnant mothers compared to HIV-uninfected pregnant mothers ($p=0.0389$ and $p=0.0324$ respectively) as shown in *Table 4-5*a and *Table 4-5*b. No significant differences were observed on immunoglobulin levels between these two groups. In neonates, there was no significant difference in cytokine levels between the HEUs and HUUs. However, IgA immunoglobulin level was significantly raised among HEUs compared to HUUs ($p=0.0282$). Baby sex in pregnancy did not have significant effect on the pregnant mother's cytokine or immunoglobulin levels ($p>0.05$).

Baby cytokine and antibody levels in relation to birth weight

IL-7 was significantly raised in low birth-weight babies (birth weight <2500g) compared to babies with >2500g birth weight ($p=0.0149$) as indicated in *Table 4-6*a and *Table 4-6*b. There was however no significant difference in immunoglobulin levels between ulow birth-weight and normal birth-weight babies.

DISCUSSION

Generally cytokines and chemokines were significantly higher in babies compared to their mothers ($p<0.05$). MIP-1α concentration was however very low in both mother and infant sera. Infants had significantly higher IgG1 and IgG3 immunoglobulins compared to their mothers. Maternal IgG is the only immunoglobulin that crosses the placenta aided by the Fc receptors so their high concentrations in infants was expected. Maternal IgG is higher during gestation and begins to decrease at birth. As the maternal IgG lowers, the infant IgG begins to rise. At around 12 weeks (3 months) after birth, the mother and infant IgG concentrations intersect with the infant IgG concentrations, thereafter, rising. Thus, our observation at 14 weeks, is in concordance with previous research[3].

We report very low concentrations of IL-2, IL-5, IL-7, IL-10, IL-12(p70), IL-13, IL-15, MCP-1 and VEGF in both mothers and infants, this could be as a measure by the maternal immune system to control and escape fetal abortion. In fact an upregulation of IL-15 in trophoblasts are associated with recurrent abortions hence IL-15 could be used as a biomarker for pregnancy loss[23]. Szarka *et al.*, (2010), has indicated that Th1 immunity in pregnancy is associated with increased IL-2/IL-4 and IFN-γ/IL-4 ratios and that high levels of MCP-I was linked to pre-eclampsia in pregnancy. Also low levels of IL-12(p70) in relation to IL-12(p40) favoured Th2 immune responses in pregnancy[24]. As such, it is critical that these cytokines are monitored to maintain their low levels during pregnancy to prevent premature termination of pregnancies.

We report that IgG4 occurred at low levels in both mothers and their infants. Literature has revealed IgG4 as a 'promiscuous antibody, which could be directly pathogenic, fulfilling a protective role, or could just be a fortuitous marker of an aberrant inflammatory response'[25].

In addition, IgG4 antibodies possess exclusive structural and functional characteristics suggesting anti-inflammatory and tolerance-inducing effects[25]. The low concentration in our study may, therefore, be as a result of the body keeping check on the immunoglobulin concentration due to its potential to be directly pathogenic hence protecting the body from potential damage by IgG4 immunoglobulin. Administering IgG4 drug in animal studies at onset of organogenesis and through delivery has been noted to elevate abortions as well as premature neonatal death. Data on humans is however scanty if not unavailable[26].

In our study, one infant who did not have enteric pathogens isolated, seroconverted at 10 weeks and sample collected at 14 weeks had OOR> IgG1 and IgG3, whereas, the mother had OOR< for both IgG1 and IgG3. The mother also had viral load (VL) at recruitment of <20 copies/ml^{-1}. Another baby that seroconverted at 6 weeks, had no identified enteric pathogens and its plasma sample was collected at 10 weeks. This baby had OOR< for the immunoglobulins tested. The mother VL at recruitment was 43 280 copies/ml^{-1}. The observed low or no immunoglobulin production in the infant could be attributed to poor immune development of the baby *in utero* due to the compromised maternal immune system.

In our study, IL-1r, IL-4, IL-9, IL-12p70, IL-17a, G-CSF and PDGF-BB were significantly raised in pregnant mothers with presence of *E. histolytica* infection compared to those with an absence of *E. histolytica* ($p<0.05$). However, in babies, *E. histolytica* carriage seemed to have no significant impact on the cytokine and chemokine concentration as well as on immunoglobulin concentrations ($p>0.05$). Both IFN-γ and IL-17 provide protection against *E. histolytica*[27]. The finding on IL-17 corroborates findings in our study. However, IFN-γ was not raised which is conflicting to what Moonah *et al.*, 2013[27] found.

This could be attributed to the fact that our study focused on asymptomatic carriage and not active disease hence speculatively, *E. histolytica* could have immune modulated the host to make sure it is not attacked by host immune responses.

On the other hand, IFN-γ is usually produced against viral infections. This could be the reason why this cytokine was not raised in *E. histolytica* infections since *E. histolytica* is a protozoan parasite. It is plausible that in the study by Moonah *et al.*, 2013, there were underlying infections that gave rise to the IFN-γ cytokine concentration. Babies seem to be protected somehow *in utero*, hence no significant rise of cytokine concentrations in infection observed in this study.

In pregnant mothers with an enteric infection carriage such as *E. histolytica* and Non-lactose fermenters (NLFs) significantly upregulated levels for IL-1r, IL-4, IL-9, IL-10, IL-12p70, IL17a, G-CSF, GM-CSF, IFN-γ, PDGF-BB and TNF-α ($p<0.05$) was noted however but non significantly different in terms of immunoglobulin levels between enteric infection carriers and non-carriers ($p>0.05$). In babies, presence of any kind of enteric infection did not cause a significant difference in cytokine as well as immunoglobulin levels between enteric infection carriers and non-carriers in babies ($p>0.05$).

The fact that IL-10, GM-CSF, IFN-γ, PDGF-BB and TNF-α were raised in *E. histolytica* infection in combination with other enteric pathogens and not in *E. histolytica* infection alone indicates that the upregulation of these cytokines is not as a result of *E. histolytica* but other underlying infections. This concurs with Bernin *et al.*, (2014), since they observed low levels of IL-10 as well as raised IL-4 concentration in asymptomatic carriers of *E. histolytica*[2].

In our study, IL-13 and G-CSF levels were significantly raised in HIV-infected pregnant mothers compared to HIV-uninfected pregnant mothers (p=0.0389 and p=0.0324 respectively). No significant differences were observed on immunoglobulin levels between these two groups. In neonates, there was no significant difference in cytokine levels between the HEUs and HUUs. However, IgA immunoglobulin level was significantly raised among HEUs compared to HUUs (p=0.0282). IgA antibodies in mucosal secretions are directed against microbial antigens[28]. It is, therefore, possible that HEUs have opportunistic infections derived congenitally or from the environment which make HEUs have higher mortality and morbidity rates compared to HUUs, however, this warrants further investigation.

IL-13 among other activities, down-regulates macrophage activity resulting in the inhibition of pro-inflammatory cytokines and chemokines. IL-13 takes part also in B-cell maturation and differentiation[7; 14]. Speculatively, the significantly high concentration of IL-13 in HIV infection may be due to it down-regulating inflammatory cytokines due to HIV infection. We hypothesize that HIV pregnant women with low levels of IL-13 and G-CSF need a booster for these cytokines as the cytokines were from "healthy" HIV-infected pregnant mothers. This, however, needs further research. Speculatively, the significant high concentration levels of IL-13 in HIV-infected pregnant mothers is for immune regulation of inflammatory cytokines in a way to protect the developing baby *in utero*.

The fact that G-CSF has been suggested as a reliable marker for use in neonates suffering from early- or late-onset of neonatal sepsis in previous studies[17] may also be true for HIV-infected pregnant

women. One of the functions of G-CSF is to stimulate production and proliferation of white blood cells[17–19].

In this regard, it sounds logical that this cytokine was raised in HIV-infected pregnant women as white blood cells will be attacked and destroyed by the HIV virus in HIV infection, hence G-CSF will be needed in high concentration to replace the depleting white blood cells.

Baby sex in pregnancy did not have significant effect on the pregnant mother's cytokine or immunoglobulin levels ($p>0.05$). Our results, however, correlates to observations by Weissenbacher *et. al.*, (2012) who could not find any correlation between baby sex and maternal cytokine production[29]. Contrary, Enninga *et. al*., (2015) observed an association between male fetal sex and pro-inflammatory cytokines specifically G-CSF, IL-12p70, IL-21, and IL-33 as well as angiogenic factors VEGF and PIGF while in female fetal sex regulatory cytokines IL-5, IL-9, IL-17, and IL-25 were raised at multiple time points[13]. Our study did not look at multiple time points and also IL-21, IL-33 and PIGF hence might have contributed to these differences. IL-7 was significantly raised in under weight babies (birth weight <2500g) compared to babies with >2500g birth weight ($p=0.01$). There was, however, no significant differences in immunoglobulin levels between underweight and normal birth weight babies. IL-7 has been described as being involved in haematopoiesis and has been clinically used for a number of malignancies and in HIV infection[30].

In conclusion, asymptomatic *E. histolytica* carriage results in up-regulation of IL-1r, IL-4, IL-9, IL-12p70, IL-17a, G-CSF and PDGF-BB and the down-regulation of IL-10 and these can be possible markers for early *E. histolytica* infections before invasive infection.

Up-regulation of IL-13 and G-CSF can also be used to monitor HIV-infected pregnant mothers. Raised IL-7 can be used as a potential biomarker for the identification of small-for-gestational-age infants and the therapeutic effects for better management of the mother-baby pair.

Study limitations and recommendations

Samples were collected at just one study point and could have given a better insight if multiple data points were used.

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AUTHOR CONTRIBUTIONS

AFN conceived the idea, designed the experiments, analyzed the data and developed the final written manuscript; TM, BSP, AI and TN supervised the work throughout, provided technical guidance to AFN. AFN performed the assays and analyzed the data; TJC and EPS provided technical guidance to AFN; KD coordinated collection of samples and provided technical guidance to AFN.

CM performed sample collection and processing prior to shipment, was involved in data entry, cleaning and analysis. TB and BN provided technical guidance on study design, data cleaning and analysis to AFN. All authors contributed to the writing of the manuscript.

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CONFLICT OF INTERESTS

All the authors declare that they do not have any competing interests.

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TABLES

Table 4-3a: Cytokine Median Concentration and Interquartile Ranges during *E. histolytica* infection in Pregnant Mothers

Cytokine	**Median cytokine concentrations (pgml^{-1}) in mother**		**p-value**
name	***E. histolytica*-infected**	***E. histolytica*-uninfected**	
IL-1	3.54 (3.11-5.01)	3.045 (2.17-3.59)	0.1664
IL-1r	204.07 (161.45-222.34)	140.89 (103.33-184.58)	**0.0143**
IL-2	4.77 (1.99-8.55)	1.945 (1.54-3.98)	0.0859
IL-4	10.28 (9.72-10.94)	8.95 (6.53-10.67)	**0.0492**
IL-5	0	0.6 (0-0.6)	0.1491
IL-6	9.11 (7.47-13.96)	7.95 (4.02-10.58)	0.1991
IL-7	5.94 (1.1-12)	0 (0-3.27)	0.0762
IL-8	27.22 (24.05-52.86)	20.63 (138-49.67)	0.1336
IL-9	77.59 (73.33-96.42)	63.39 (43.44-73.83)	**0.0214**
IL-10	6.53 (4.09-21.36)	4.055 (2.4-5.25)	0.0573
IL-12 (p70)	8.87 (7.23-14.55)	6.155 (4.93-7.17)	**0.0089**
IL-13	4.14 (2.21-12.28)	3.61 (0-10.58)	0.7866
IL-15	3.22 (0-22.38)	0(0-3.72)	0.1204
IL-17A	72.3 (67.87-89.95)	53.17 (41.13-66.51)	**0.0051**

Table 4-3b:Cytokine Median Concentration and Interquartile Ranges during *E. histolytica* infection in Pregnant Mothers

Cytokine	**Median cytokine concentrations (pgml^{-1}) in mother**		**p-value**
name	***E. histolytica*-infected**	***E. histolytica*-uninfected**	
Eotaxin	26.04 (22.55-26.75)	22.484 (15.6-26.28)	0.2236
FGF basic	63.56 (61.89-87.55)	50.295 (42.61-72.01)	0.0800
G-CSF	65.4 (57.51-70.04)	54.48 (41.32-63.06)	**0.0419**
GM-CSF	109.83 (104.05-165.91)	63.77 (8.08-127.01)	0.0663
IFN-γ	212.07 (196.1-227.15)	176.355 (146.64-208.95)	0.0642
IP-10	388.79 (332.62-435.8)	405.96 (309.89-669.72)	0.7389
MCP-1	15.23 (10.62-23.27)	1.445 (0-15.48)	0.0873
MIP-1α	7.19 (2.41-8.13)	2.63 (1.68-9.22)	0.4045
MIP-1β	136.5 (131.05-185.6)	140.77 (87-230.25)	0.6892
PDGF-BB	1591.6 (1156.45-1850.04)	1041.28 (732-1434.39	**0.0455**
RANTES	2177.74 (1553.12-2369.24)	2185.455 (2074.41-2234.23)	0.9734
TNF-α	41.44 (41.17-47.48)	38.29 (31.06-43.36)	0.1131
VEGF	14.94 (8.63-16.5)	10.71 (8.07-15.33)	0.3952

Table 4-4a:Cytokine Median Concentration and Interquartile Ranges in relation to any type enteric of infection in pregnant mothers

Cytokine name	Median cytokine concentrations ($pgml^{-1}$) in mothers		p-value
	Infection absent	Infection present	
IL-1	3.045(2.1-3.58)	3.54(92.17-10.06)	0.1223
IL-1r	130.39(102.03-182.745)	204.07(159.16-229.94)	**0.0047**
IL-2	1.945(1.36-3.88)	4.77(1.85-12.62)	0.0702
IL-4	8.895(6.625-10.17)	10.28(9.37-10.99)	**0.0163**
IL-5	0.6(0-0.6)	0(0-0.6)	0.2892
IL-6	7.95(4.02-10.05)	9.11(6.77-36.42)	0.1011
IL-7	0(0-3.025)	5.94(0-13.56)	0.0524
IL-8	17.295(13.395-48.075)	32.05(24.05-55.09)	0.0569
IL-9	61.425(41.68-73.375)	77.59(72.74-98.51)	**0.0080**
IL-10	4.055(2.4-5.095)	6.53(3.64-69.85)	**0.0300**
IL-12 (p70)	6.155(5.285-7.045)	8.87(7.23-19.62)	**0.0076**
IL-13	3.43(0-10.405)	4.14(2.21-13.15)	0.4101
IL-15	0(0-2.92)	3.22(0-31.96)	0.0587
IL-17A	50.425(37.845-65.995)	72.3(64.63-111.38)	**0.0016**

Table 4-4b: Cytokine Median Concentration and Interquartile Ranges in relation to any type of enteric infection in pregnant mothers

Cytokine name	Median cytokine concentrations ($pgml^{-1}$) in mothers		p-value
	Infection absent	Infection present	
Eotaxin	22.485(15.395-26.22)	26.04(20.43-32.91)	0.1261
FGF basic	50.295(42.61-69.14)	63.56(43.31-107.6)	0.0510
G-CSF	53.87(41.105-61.875)	65.4(55.9-150.08)	**0.0192**
GM-CSF	63.77(5.26-124.215)	109.83(70.86-174.61)	**0.0336**
IFN-γ	176.355(141.315-207.375)	212.07(162.52-323.3)	**0.0378**
IP-10	400.565(309.43-578.465)	408.02(332.62-734.86)	0.5957
MCP-1	1.27(0-11.75)	15.23(0-30.75)	0.0652
MIP-1α	2.54(1.65-9.73)	6.58(2.41-8.13)	0.3029
MIP-1β	138.33(86.67-222.83)	147.15(131.05-333.38)	0.2886
PDGF-BB	940.09(713.595-1421.46)	1591.6(1156.45-1850.04)	**0.0246**
RANTES	2185.455(2075.905-2239.785)	2177.74(1553.12-2369.24)	0.8271
TNF-α	37.06(30.79-42.4)	41.44(38.98-51.89)	**0.0404**
VEGF	10.71(7.605-14.7)	14.94(8.44-54.94)	0.2545

Table 4-5a: Cytokine Median Concentration and Interquartile Ranges in relation to HIV status in pregnant Mothers

Cytokine name	**Median cytokine concentrations (pgml^{-1}) in mother**		**p-value**
	HIV-infected	**HIV-uninfected**	
IL-1	2.7(1.76-3.57)	3.495(2.5-4.565)	0.1224
IL-1r	126.83 (100.73-184.58)	156.08(112.3-221.99)	0.1939
IL-2	1.9 (1.45-3.79)	2.515(1.66-5.515)	0.2364
IL-4	9.65 (8.21-10.99)	9.155(6.895-10.515)	0.7075
IL-5	0.6(0-0.6)	0.6(0-0.6)	0.9370
IL-6	8.57(4.02-11.24)	8.77(4.095-11.765)	0.8172
IL-7	1.1(1.1-5.94)	1.835(1.1-6.55)	0.5431
IL-8	22.81(14.61-58.26)	27.705(15.685-50.275)	0.7399
IL-9	70.57(43.44-87.57)	69.82(51.54-81.425)	0.7950
IL-10	2.8(2.12-5.25)	4.4245(3.605-10.9)	0.1058
IL-12 (p70)	6.25(4-7.42)	6.92(5.79-8.93)	0.2601
IL-13	2.21(0.7-6.05)	7.355(0.7-16.385)	**0.0324**
IL-15	0	0(0-14.470)	0.0927
IL-17A	64.63(34.56-70.26)	58.14(42.17-78.25)	0.6966

Table 4-5b: Cytokine Median Concentration and Interquartile Ranges in relation to HIV status in pregnant Mothers

Cytokine name	**Median cytokine concentrations (pgml^{-1}) in mother**		**p-value**
	HIV-infected	**HIV-uninfected**	
Eotaxin	23.4(15.19-26.28)	23.405(19.795-27.040	0.6032
FGF basic	59.05(42.61-72.01)	52.025(43.135-75.025)	0.8852
G-CSF	46.85(40.89-60.69)	59.895(53.87-72.325)	**0.0389**
GM-CSF	79.62(11.22-141.25)	84.16(30.69-133.355)	0.9539
IFN-γ	179.04(114.96-205.8)	192.76(156.855-231.49)	0.1888
IP-10	435.8(343.44-829.12)	386.89(295.015-556.72)	0.1410
MCP-1	5.03(0-19.04)	2.695(0-17.515)	0.8108
MIP-1α	2.13(1.62-7.84)	4.075(1.985-9.38)	0.3555
MIP-1β	109.21(78.4-215.41)	152.58(125.005-268.385)	0.1333
PDGF-BB	1405.01(695.19-1733.99)	1107.48(859.83-1637.28)	0.7508
RANTES	2203.05(1434.56-2369.24)	2177(2088.655-2226.125)	0.9081
TNF-α	38.7(30.52-44.73)	39.66(32.965-44.46)	0.6965
VEGF	9.67(6.77-11.85)	12.765(9.1-16.4)	0.0711

Table 4-6a: Maternal Cytokine Median Concentration and Interquartile Ranges in relation to infant birth weight

Cytokine name	Median cytokine concentrations ($pgml^{-1}$) in infants		p-value
	<2500g birth weight	>2500g birth weight	
IL-1	6.52 (5.95-6.9)	5.205 (3.29-7.54)	0.3733
IL-1r	240.87 (225.11-451.25)	170.1 (110.19-291.64)	0.1559
IL-2	5.47 (3.55-8.55)	5.565 (2.26-1132)	0.9556
IL-4	7.93 (6.55-11.93)	6.74 (4.35-10)	0.3733
IL-5	30.24 (24.29-35.88)	4.4 (0-27.75)	0.0653
IL-6	25.23 (14.85-31.76)	15.735 (10.45-24.640	0.2207
IL-7	26.34 (23.84-30.76)	5.625 (0-1672)	**0.0149**
IL-8	36.94 (33.01-37.37)	32.315 (18.73-56.12)	0.9114
IL-9	70.25 (29.92-95.65)	66.585 (32.89-87.89)	0.9114
IL-10	10.74 (10.06-13.05)	8.875 (5.88-13.19)	0.4695
IL-12 (p70)	16.61 (16.26-19.55)	12.255 (8.1-23.5)	05779
IL-13	16.21 (13.36-26.12)	11.83 (3.79-15.91)	0.1815
IL-15	0(0-20.25)	0(0-12.88)	0.7618
IL-17A	59.34 (29.7-114.25)	48.535 (23.08-83.85)	0.7596

Table 4-6b: Maternal Cytokine Median Concentration and Interquartile Ranges in relation to infant birth weight

Cytokine name	Median cytokine concentrations ($pgml^{-1}$) in infants		p-value
	<2500g birth weight	>2500g birth weight	
Eotaxin	42.13 (29.76-45.01)	25.15 (20.68-37.06)	0.1066
FGF basic	85.51 (75.77-122.1)	74.9 (64.92-94.17)	0.3032
G-CSF	97.33 (95.55-129.04)	74.595 (54.28-105.8)	0.1642
GM-CSF	111.16 (68.06-238.08)	159.09 (113.38-190.2)	0.6165
IFN-γ	367.74 (325.52-420.25)	243.575 (180.8-371.75)	0.1259
IP-10	115.08 (264.34-1450.46)	575.445 (390.36-790.45)	0.4039
MCP-1	25.46 (8.85-41.02)	16.57 (6.76-26.07)	0.3160
MIP-1α	3.88 (3.07-4.14)	3.515 (2.48-6.81)	0.8674
MIP-1β	203.82 (35.38-275.25)	188.79 (131.29-222.35)	0.9114
PDGF-BB	423.89 (142.56-16.02.57)	327.1 (125.06-955)	0.6562
RANTES	2161.44 (2100.87-2476.54)	2192.09 (2060.44-2305.17)	0.6969
TNF-α	59.62 (51.34-74.59)	42.54 (32.42-67.92)	0.2538
VEGF	36.28 (31.34-94.85)	35.81(19.06-75.91)	0.5779

Chapter 5 : Determination of the association between maternal characteristics that affect the cytokine responses of their neonates

The last chapter identified maternal and infant cytokine profiles that were important during pregnancy and infection. The question that remains is how these significant cytokines and immunoglobulins affect the cytokine and immunoglobulin production in infants in the mother –infant pairs. In trying to answer the question, a manuscript entitled 'Influence of maternal characteristics during pregnancy on the infant early life immune responses in a high HIV prevalence setting in Harare, Zimbabwe' was developed and is under review in the African Jounal of Reproductive Health (AJRH). *Proof of submission is as shown in appendix 8A9*

Influence of maternal characteristics during pregnancy on the infant early life immune responses in a high HIV prevalence setting in Harare, Zimbabwe (*Under Review in the African Journal of Maternal Health-AJMH)*

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ABSTRACT

Aim

The study aimed at investigating the maternal characteristics that in turn influence the immunological status of the infant in asymptomatic enteric pathogen carriers in mother infant pairs in a high HIV burdened population in Harare, Zimbabwe.

Methodology

A Bioplex immunoassay was used to analyse serum samples from 39 mother-infant pairs (MIP) for 27 cytokines and 6 immunoglobulins. The MIP were purposively selected based on HIV infection and *E. histolytica* carriage. Logistic regression was used to identify any link between maternal demographic and clinical data with infant cytokine and immunoglobulin levels.

Results

Maternal *E. histolytica* carriers are more likely to have infants with low levels of IL-12p70, FGF-basic, GM-CSF and TNF-α cytokines (OR: 0.14; 95% CI: 0.03-0.79) and high levels of IgA immunoglobulin (OR: 8.1; 95% CI: 1.45-45.06). HIV-infected mothers are more likely to have infants with low levels of IgG2 (OR: 0.24; 95% CI: 0.06-1.00) and IgA (OR: 0.22; 95% CI: 0.05-0.90) immunoglobulins. Mothers giving birth at a mean age above 30.38 years (Standard deviation ±6.09) are more likely to have infants with low IgG4 levels (OR: 0.24; 95% CI: 0.06-1.02) albeit borderline significant (p=0.05).

Conclusion

Maternal *E. histolytica* asymptomatic carriage, age and HIV status need to be monitored to avoid their negative influence on the infant immunological development.

Significance and impact of study

The study was a culmination to the need to consider maternal characteristics such as age, HIV status, immunological status and enteric pathogen carriage in pregnancy as these might impact on their infants' immune development and responses. This will assist in the disease treatment and prevention management of pregnant women decision and might assist in developing effective immunotherapy for reducing autoimmune diseases in HIV-exposed uninfected infants.

Key words

Cytokines, Immunoglobulins, HIV-infected mothers, enteric infections, *E. histolytica*, HIV-exposed infants.

INTRODUCTION

Despite the success of HIV prevention of mother to child transmission (PMTCT) programs in Zimbabwe, there still exists an increased burden in mortality and morbidity of the HIV-exposed uninfected (HEU) compared to HIV-unexposed infants (HUUs). The frequent pattern of morbidity and mortality in HEUs correlates to diarrheal disease, pneumonia and bacterial sepsis which are the most frequent causes of childhood morbidity and mortality, however, the actual cause is still insufficiently defined [1].

Exposure of the infant to maternal environmental factors such as viral, bacterial, parasitic and other factors impact on fetal innate and adaptive immunity development. There is paucity of information with regards to the relationship between maternal characteristics and the infant immune responses. In this study we hypothesize that maternal factors such as age, ethnicity, alcohol uptake, probiotic uptake, asymptomatic carriage of enteric pathogens, HIV status as well as immunological status influences the infant's immunological response based on cytokine and immunoglobulin levels.

Infants are highly susceptible to infectious diseases possibly due to immaturity of their immune system and susceptibility to tolerogenic signals. At an early stage of life, infant antigen presenting cells and CD4 T cells were found to display reduced ability to produce cytokines as well as cytokine receptors, which may result in decreased cytotoxic effector cell function and a decreased ability to provide adequate B-cell assistance[2] affecting the high susceptibility to infections. Individuals that are asymptomatically infected with *E. histolytica* could represent an important group enabling the study of immune responses that are critical to the outcome of an infection [3].

In this study, we focus on maternal demographic and clinical factors implicated in cytokine and immunoglobulin production in infants within a HIV burdened setting.

MATERIALS AND METHODS

Ethics approval and consent to participate

The approval for this study was granted by the Medical Research Council of Zimbabwe, Approval Code MRCZ/A/2043 as well as the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal, Durban, South Africa, Approval Code 409/15. Consent to take part in the study was granted by the study participants.

Study population and sampling

This was a sub-study based on stored plasma samples collected from the University of Zimbabwe-College of Health Sciences Birth Cohort of HIV-infected (HIV^+) and HIV-uninfected (HIV^-) pregnant women. Samples were collected during the period February 2016 to February 2017. The main study was approved by the Medical Research Council of Zimbabwe, approval code MRCZ/1948 and the Joint Research Ethics Committee, approval code JREC81/15. The study participants were from Dzivarasekwa, Glenview and Kuwadzana, three high-density suburbs in Harare, Zimbabwe, based on previous burden of pathogens of interest. The mothers recruited in this study were between 18 and 41 years with mean age of 30.38± SD 6.09 years.

Study procedure

The pregnant women on their routine antenatal care visits were invited to participate in the study. After agreeing, an informed consent form was administered and signed by participant and the interviewer before a questionnaire administration to capture demographic data such as HIV status, ART uptake and regimen. The mothers were followed up to delivery where information on delivery mode, infant sex and weight, as well as infant HIV statuses, were taken and infant blood collected for sera where possible.

Those mother-infant pairs (MIP) who were able to provide stool samples had cultures and ELISA done on the samples. The MIPs that had *E. histolytica*, as well as other enteric pathogens detected, were considered for cytokine and antibody profiling

Biomedical samples collected

Stored sera for 39 MIPs were purposively selected based on *Entamoeba histolytica* infections and other microbial infections as well as maternal HIV status results based on the outcome of the screening process of this study. Controls were based on lack of enteric pathogens and also being HIV negative. Maternal prenatal samples were obtained on recruitment and for infant the sera were as close to delivery as possible. The majority of infant samples were collected at 14 weeks and 6 weeks as shown in *Table 5-1*.

Table 5-1: Summary of the serum sample collection points for infants used in the study

Serum point of collection	**Frequency**	**Percentage**
10 days	4	10.26
30 days	1	2.56
6 weeks	15	38.46
10 weeks	4	10.26
14 weeks	15	38.46
Total	39	100

Cytokine and chemokine profiling

All the 39 MIP were analysed using Bio-plex Pro Assay 27 plex kit, Bio-Rad, USA. The 27 cytokines and chemokines investigated were IL-1β, IL-1r, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17a, Eotaxin, basic PDGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1(MCAF), MIP-1α, MIP-1β , PDGF-BB, RANTES, TNF- α and VEGF. A total of 27 serum cytokines were multiplexed using this magnetic-bead based multiplex immunoassay according to the manufacturer's instructions.

The plate washings were performed on a Bio-Plex Pro and Bio_Plex Pro 11, Revision B, wash station and incubations were performed at room temperature on a Bio-Rad Shaker Fax – 2200. Bio-Plex® 200 system (Bio-Rad, California, USA) which uses Bioplex Manager Software was used for Data acquisition which gave out data output as Median Florescence Intensity and concentration in $pgml^{-1}$. In this study, only those cytokines which were analysed to have significance were further analysed to see the maternal influences on the infant immune responses linking to maternal characteristics.

Cytokines and Data Analysis

Cytokine analysis was done to observe the influence of maternal factors such as age, HIV status, *E. histolytica* carriage, tribe and area of residence on the infant immune response based on the levels of the 27 cytokines and 6 immunoglobulins tested. Data analysis was based on logistic regression to determine any associations between maternal characteristics and infant cytokine and immunoglobulin production. During analysis, MIP outliers per variable were dropped irrespective of whether the outlier is maternal or infant.

RESULTS

Demographic Data

The maternal mean age ±standard deviation (SD) was 30.38± 6.09 years and were equally distributed amongst the three residential suburbs. Twenty-four of the mothers were HIV negative and 15 were HIV positive. Of the 15 HIV positive, 14 were on Antiretroviral Therapy (ART) and the other one aged 24 years stated that the husband refused her to take medication. Out of the 14 on ART, 12 were on Telonam E, 1 on Telonam N and 1 did not indicate her ART regimen.

Sampling and sample collection

A total of 39 MBP who had cytokines and antibody profiling performed were considered for this study. Approximately 38.5% of the mothers were HIV positive and 61.5% were HIV negative based on HIV rapid tests. Of the 39 mothers, 23.1% had *E. histolytica* infection. Of the 39 babies, 12(30.8%) were HIV-exposed uninfected (HEUs), three (7.7%) were sero-converters and 24 (61.5%) were HIV-unexposed uninfected (HUUs). A total of 8 (20.5%) infants had *E. histolytica* infections of which 5 were HUUs and 3 were HEUs. No enteric pathogen was isolated in the three babies who sero-converted.

Influence of maternal *E. histolytica* carriage on infant cytokine and immunoglobulin levels

None of the maternal demographic characteristics analysed seemed to have an influence on the infant cytokine production levels ($p>0.05$). Maternal *E. histolytica* carriage seemed to significantly ($p=0.026$) influence infant Interleukin (IL)-12p70, Fibroblast Growth Factor-basic (FGF-basic), Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF) and Tumor Necrosis Factor (TNF)-α cytokines. The odds of having high levels of these cytokines in infants is reduced by 86% if the mother is a carrier of *E. histolytica* during pregnancy (OR: 0.14; 95% CI: 0.03-0.79; *Table 5-2*a and Table *5-2b*).

Immunoglobulin (Ig)A also appear to be significantly influenced (p=0.017) by maternal *E. histolytica* carriage. An infant born to a mother with *E. histolytica* carriage has 8.1 odds of having high levels of IgA greater than an infant born to a mother without *E. histolytica* carriage (OR: 8.1; 95% CI: 1.45-45.06; *Table 5-3*).

Influence of maternal HIV infection on infant cytokine and immunoglobulin production

Maternal HIV infection did not seem to influence infant cytokine production levels (Results not shown). However maternal HIV infection seems to influence infant IgG2 and IgA immunoglobulin levels. Infants born to HIV positive mothers are significantly associated (p=0.049) with lower odds of having high IgG2 levels, that is the odds of having high IgG2 immunoglobulin levels is reduced by 76% in infants born to HIV-infected mothers compared to those born to HIV-uninfected mothers (OR: 0.24; 95% CI: 0.06-1.00) . Similarly, an infant born to a HIV-infected mother is significantly associated (p=0.035) with lower odds of having high IgA immunoglobulin levels compared to the one born to HIV-uninfected mothers (OR: 0.22; 95% CI: 0.05-0.90) as shown in *Table 5-3*.

Influence of maternal age on infant cytokine and immunoglobulin production

Maternal age did not influence infant cytokine levels (results not shown) but affected the infant IgG4 immunoglobulin levels. Being born to a mother above mean age± standard deviation of 30.38± 6.09 years reduces the odds of having high IgG4 compared to those born to mothers below 30.38± 6.09 years), although on the borderline (p=0.050) as indicated in Table *5-3*.

DISCUSSION

The need to improve maternal and child well-being is one of the millennium development goals which is critical in public health since it affects the general health of the next generation. It is generally accepted that maternal immunity influences the infant immune development. This study demonstrates how maternal demographic and clinical variables influences infant cytokine and immunoglobulin levels.

This study shows that maternal *E. histolytica* carriage is a strong confounder for infant IL-12p70, FGF-basic, GM-CSF and TNF-α cytokines production levels and IgA immunoglobulin levels. There are conflicting evidences on the function of TNF-α during amoebic infections. Some studies have indicated TNF-α to have a protective effect against *E. histolytica* infection while others indicated an increased susceptibility of the host to *E. histolytica* infection in the presence of TNF-α [5].It was also observed that TNF-α blocking agents lead to healing of tissue inflammation in gastro-enteric diseases such as inflammatory bowel diseases (IBD) [4]. Also Zhang *et al.*, [5] have noted that TNF-α blocking agents reduces inflammation and intestinal damage in amebic infection, while inhibition of IL-1 reduced cytokine production but had less marked effects on inflammation and disease.

In this regard, the reduced levels of the cytokine in infants born to *E. histolytica* carriers may mean that the parasite produces the blocking agents to TNF-α release to avoid destruction by the host, and these blocking agents might be transferred to the infant in breast milk. This, however, needs further investigation.

Speculatively, the low TNF-α levels in infants born to mothers with *E. histolytica* carriage maybe also due to the immune modifications on the maternal TNF-α gene so that the TNF-α cytokine is produced in low concentration to avoid destruction. The defected cytokine genes might be genetically passed on to the infants since the cytokine genes can be inheritable[4,6].

GM-CSF is basically a hematopoietic and myelopoietic growth factor[7,8] which also promotes production of pro-inflammatory cytokines such as TNF-α, IL-6, IL-12p70, IL-23 and IL-1β as well as chemokines like CCL22, CCL24, CCL5 and CCL1 which promote leukocyte recruitment[7]. Thus we expect this cytokine to be elevated in infection. In our study, this cytokine was in low levels in infants born to *E. histolytica* carriers which might explain one of the strategies by *E. histolytica* to evade the host immune response. Possibly the parasite will modulate the host immune system leading to downregulation of GM-CSF which eventually results in less leukocytes to fight the parasite. Since the GM-CSF production will be down-regulated in the mother, we expect the infant to also have low level of the cytokine. In addition, GM-CSF has a role in the production of TNF-α and IL-12p70, Since GM-CSF was down-regulated, it is not surprising that the latter 2 cytokines were low.

FGF-basic has not been well studied in asymptomatic *E. histolytica* carrier pregnant mothers hence there is limited data in this regard. There is need for more research on the relationship between this cytokine with asymptomatically infected *E. histolytica* maternal carriers and their infants. FGF-basic has been shown, amongst other factors, to promote healing of wounds and reduction of scars although it is still not clear how this occurs at a molecular level[9]. As such, this cytokine is expected to be in high concentration during acute infections and reduced in wound healing.

E. histolytica pathogenesis involves development of a "flask shaped" ulcer [10] which the host has to heal. In chronic infections, the amoeba will have learnt to live with the host hence there is possibly remarkable reduction in host damage hence the reduced concentration of FGF-basic, which will be transferable to the infant.

The raised IgA immunoglobulin levels in infants born to *E. histolytica* carriers observed in this study correlates to findings by Nakada-Tsukui and Nozaki, who have indicated an increase in IgA transportation across the intestinal epithelium and promotion of neutrophil infiltration[11] in the presence of *E. histolytica* infection. The infants might, therefore, be exposed to this immunoglobulin during breastfeeding, and if infected due to exposure to the infected mother, will also start producing their own IgA immunoglobulins. It was shown that HIV-infected mothers are more likely to have infants with low levels of IgG2 and IgA immunoglobulins from this study. This finding is in concordance with results from Abu-Raya *et al*., [12] who, in their review, indicated the capability of HIV infection to alter the transfer of maternal immune factors to the exposed newborns and young infants. This may relate to the immune activation in HIV-infected pregnant women, associated with the production of inflammatory cytokines at the maternofetal interface associated with inflammatory responses in the newborn [12].

It has also been noted by earlier studies that the immunoglobulin IgG is the only antibody class capable of significantly crossing the placenta which is highly dependent on the (i) maternal levels of total IgG and specific antibodies, (ii) gestational age, (iii) placental integrity, (iv) IgG subclass, and (v) nature of antigen [13]. As such, since HIV infection has a negative impact on the immune cells production, it is possible that it reduces the IgG2 production in the pregnant mothers which effectively will be transported in small concentrations to the fetus and also to the newborn via breast milk.

We also observed that mothers giving birth at a mean age 30.38± 6.09 years are more likely to have infants with low IgG4 levels. This study finding is a contrast to the findings by Lamb *et al*., who found an association between higher gluten IgG4 with older age in their study on association of IgG4 with dietary factors[13]. Maybe this is because these IgG4 immunoglobulin were specific to gluten. Palmeira *et al*., found no influence of 'maternal age, weight, parity and type of delivery' on 'placental antibody transfer'[14]. Our study, however, was not looking at placental antibody transfer but on the influence of maternal characteristics on the infant immunoglobulin and cytokine levels. We, therefore, speculate that as one gets older, so do immune cells. It is well accepted that with advanced age, the immune system undergoes profound re-modelling and decline, with major impact on health and survival of the individual [15]. So with age, it is possible that maternal IgG4 was now produced in low concentrations which were also transferred in low concentrations in utero or postpartum via breast milk.

The wide confidence intervals observed in this study are more likely due to the small sample size used for this particular study, i.e, 39 mother-baby pairs. Individual immune response differences may be influenced by cytokine gene polymorphisms.

In conclusion, it is critical to encourage mothers to consider child-bearing at younger age avoid the negative influence of maternal age on infant immunoglobulin levels. Also maternal HIV status result in low levels of IgG2 and IgA in infants while *E. histolytica* carriage result in high levels if IgA in infants.

Study limitations and recommendations

The observed trends in this study between the relationships of maternal characteristics and their neonates which were not significant might indicate true possibility of these trends if sample size was increased. This study only used 39 mother-infant pairs (MIP) due to financial constraints. This resulted in a further reduction in the quantification characteristics under study, making some analysis not statistically sound, hence the need to carry out the research at a larger scale. The results, however, provide some insight in the area of study. Also, samples were collected at just one study point and could have given a better insight if multiple data collection points were used.

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CONFLICT OF INTERESTS

All the authors declare that they do not have any competing interests.

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Table 5-2a: Influence of Maternal *E. histolytica* carriage on Infant Cytokine Concentration Levels

Infant Cytokine	**Maternal *E. histolytica* carriage**		
	Odds Ratio	**P-value**	**95% Confidence Interval**
IL-1b	0.5	0.337	0.12-2.08
IL-1r	0.5	0.337	0.12-2.08
IL-2	0.83	0.798	0.21-3.38
IL-4	0.83	0.798	0.21-3.38
IL-5	0.27	0.08	0.06-1.17
IL-6	0.28	0.103	0.06-1.29
IL-7	0.7	0.636	0.16-3.1
IL-8	0.34	0.176	0.07-1.61
IL-9	0.83	0.798	0.21-3.38
IL-10	0.34	0.176	0.07-1.61
IL-12 (p70)	0.14	**0.026**	0.03-0.79
IL-13	0.5	0.337	0.12-2.08
IL-15	0.57	0.445	0.14-2.40
IL-17A	0.83	0.798	0.21-3.38

Table 5-2b: Influence of Maternal *E. histolytica* carriage on Infant Cytokine Concentration Levels

Infant Cytokine	**Maternal *E. histolytica* carriage**		
	Odds Ratio	**P-value**	**95% Confidence Interval**
Eotaxin	0.28	0.103	0.06-1.29
FGF basic	0.14	**0.026**	0.03-0.79
G-CSF	0.28	0.103	0.06-1.29
GM-CSF	0.14	**0.026**	0.03-0.79
IFN-γ	0.28	0.103	0.06-1.29
IP-10	0.5	0.337	0.12-2.08
MCP-1 mcaf	0.21	0.113	0.03-1.45
MIP-1α	0.49	0.335	0.12-2.08
MIP-1β	0.5	0.337	0.12-2.08
PDGF-BB	0.83	0.798	0.21-3.38
RANTES	0.28	0.103	0.06-1.29
TNF-α	0.14	**0.026**	0.03-0.79
VEGF	0.28	0.103	0.06-1.29

Table 5-3: Influence of Maternal *E. histolytica* carriage, HIV infection and Age on Infant Immunoglobulin Concentration Levels

Infant Immunoglobulin	**Maternal HIV Infection**			**Maternal Age**			**Maternal *E. histolytica* carriage**		
	Odds Ratio	**P-value**	**95% Confidence Interval**	**Odds Ratio**	**P-value**	**95% Confidence Interval**	**Odds Ratio**	**P-value**	**95% Confidence Interval**
IgG1	1.35	0.649	0.37-4.92	0.58	0.422	0.15-2.21	2.3	0.248	0.55-9.83
IgG2	0.24	**0.049**	0.06-1.00	1.27	0.729	0.33-4.97	2.2	0.288	0.52-9.27
IgG3	0.88	0.839	0.24-3.18	0.92	0.898	0.24-3.46	1.4	0.649	0.34-5.62
IgG4	0.4	0.183	0.10-1.55	0.24	**0.050**	0.06-1.02	2.2	0.288	0.52-9.27
IgA	0.22	**0.035**	0.05-0.90	1.1	0.898	0.29-4.12	8.1	**0.017**	1.45-45.06
IgM	0.32	0.102	0.08-1.25	1.2	0.790	0.31-4.59	2.1	0.288	0.52-9.27

CHAPTER 6: SYNTHESIS

6.1 General Overview

The final chapter of this thesis provides a comprehensive analysis of the findings, giving an indication of prevalence of asymptomatic enteric pathogens in pregnant women and their neonates, highlighting possibilities of congenital transmissions of enteric pathogens, and to understand the effect of asymptomatic enteric pathogens on the cytokine and antibody levels in the mother-infant pairs as well as the influence of maternal demographic and clinical variables on infant cytokine and immunoglobulin production.

6.2 Microbial analysis

In this study, microbial results demonstrated no prevalence of asymptomatic carriage of *S. typhi* and *V. cholerae* in both maternal and infant population. This absence may be attributed to either to the rareness of the long term convalescent *V. cholerae* carrier [62] or to the possible lack of detection of the *S. typhi* and *V. cholerae* pathogens during culture. It is worthy to mention stool used for culture was a once off collection and may have contributed to reduced sensitivity of isolation. Similar finding was also obtained by Im *et al.*, (2016) who also obtained a 0% isolation of *S. typhi* from over a thousand stool cultures from two different countries [61].

We report that *E. histolytica* asymptomatic carriage was 5.4% in the pregnant mothers and 5.6% in the infants which indicates a reality of asymptomatic *E. histolytica* carriage in pregnancy and at infancy. The possibility of congenital transmission of *E. histolytica* was also observed and this might call for the inclusion of such a pathogen on the TORCH (T = Toxoplasmosis / *Toxoplasma gondii*, O = other pathogens, R = Rubella, C = Cytomegalovirus and H = Herpes Simplex Virus) series where it could be added as the 'Other' of the 'TORCH' series. There is therefore a need to screen pregnant

women to prevent congenital transmissions of *E. histolytica* to the new-borns as well as screen new-borns for early intervention before the onset of a serious infection. Usually maternal parasitic infections are asymptomatic [114] and if total hygiene is not practised during breastfeeding, the mother can unknowingly transmit the protozoan to the infant.

6.3 Immunological Analysis

Chronic amoebiasis will result in the host developing an immune response that does not enable it to eliminate the tissue resident parasite and at the same time the parasite will be intensively immunosuppressing the host [115]. Asymptomatic carriers thus have a depressed immune reactivity to the infecting parasite [116].

Using an immunological analysis, this study reports an increased level of production of IL-5 in HEUs compared to HUUs albeit none significantly ($p=0.6973$). IL-5 together with IL-3 and GM-CSF has been verified by earlier studies to be important in the regulation of eosinophil development, although IL-5 is the most specific for eosinophil differentiation and release from bone marrow into the peripheral circulation. The three eosinophilpoetins are coded by closely linked genes on human chromosome 5q31[117].

Notably the significant upregulation of IgA in the HEUs compared to HUUs in our study may be linked to the raised IL-5 production observed in HEUs. IL-5 has been seen to be important in mucosal IgA which is mainly in the mucosal tissue where it provides defence from ingested and inhaled pathogens. IgA is also found in plasma to fight those pathogens that will have escaped the mucosal barrier [118].

Generally, HIV burden in pregnancy has been shown to significantly increase the IL-13 and G-CSF production which has been attributed to the critical need of these proteins in infection. IL-13 also has an anti-inflammatory effect. G-CSF is required for white blood cell production to fight the virus and other opportunistic infections. *E. histolytica* infections in our study was shown to stimulate the production of IL-1r, IL-4, IL-9, IL-12p70, IL-17a, G-CSF and PDGF-BB. Similarly, *Guo et al.,* (2009) report elevated levels of IL-12 and IL-17 in addition to IFN-, IL-2 and IL-10 [56], which were not significantly raised in our study.

The up-regulation of IL-9 in *E. histolytica* infections is concurrent with reports from Licona-Limon *et al.,* 2017, who have indicated its role in immune responses to parasitic infection in addition to its importance in allergic diseases like asthma and bronchial hyperactivity [120]. Chronic parasitic infections call for the host to effectively balance the immune response.

There is also growing evidence that the dominant Th2 cytokines such as IL-4 (which is usually co-produced with IL-13) and IL-10 may reduce disease severity hence enhance host survival [121]. Despite implications of Th2 responses in immunopathology, cytokines such as IL-4 and IL-13 can enhance host survival in chronic parasitic infections as well as providing protection from superinfections [120; 122]. So, the observed up-regulation of IL-4 may be due to the asymptomatic and probable chronic nature of the *E. histolytica* infection in the study population.

The increased concentration of PDGF-BB in *E. histolytica* infection may be attributed to the need to regenerate cells damaged by the parasite[123]. IL-17A is critical in neutrophils recruitment to site of infection [124] and this may explain the raised IL-17A observed in *E. histolytica* infection in our study.

Neutrophils conjugated with antigen presenting cells and will, however, be trogocytosed by *E. histolytica* as one of its defence mechanism against host immune response [47].

It is important therefore to consider these as inflammatory markers of asymptomatic *E. histolytica* infection which could be used to diagnose such infections in pregnancy and new-borns before the negative effects on pregnancy outcome and well-being of the mother and the infant. It is also important to note the possibility of *E. histolytica* congenital transmission according to the results from this study, and this will obviously influence maternal and infant immune responses negatively hence the need to continuously monitor the parasite in pregnancy to reduce congenital transmission.

Our study provides the first report of a probable *E. histolytica* congenital transmission. This, however, need to be verified at a larger scale. *E. histolytica* has surface galactose-N-acetyl-D-galactosamine inhibitable lectin (Gal-lectin) that is immunogenic and acts as a protective antigen. Gal-lectin has been indicated to induce Th1 cytokines, including IL-12, but the knowledge of the basis on which Gal-lectin provides protective immunity is scanty or unknown [45] hence there is need for further investigation.

6.4 Maternal determinants of infant immunological responses

This study examined the cytokines that were significantly raised in HIV infection, *E. histolytica* infection and infection by other enteric pathogens and their influence on the infant immune response. We report that IL-12p70 was significantly up-regulated in *E. histolytica* infection in mothers, whilst IL-13 and G-CSF were significantly up-regulated in HIV infection in pregnant mothers whilst IL-10, GM-CSF, IFN-γ and TNF-α were raised in other enteric infections. IL-13 plays a dominant role in Th2 responses [125] hence its presence in high levels in pregnancy reduces premature termination of pregnancy.

Maternal demographic and clinical variables were also analysed for their influence on the infant cytokine and immunoglobulin concentration levels but demographic factors seem to have no influence. This study has shown that maternal *E. histolytica* carriers are more likely to have infants with low levels of IL-12p70, FGF-basic, GM-CSF and TNF-α cytokines and high levels of IgA immunoglobulin.

Interestingly, this study also demonstrates a significant elevation of IL-12p70 in maternal *E. histolytica* carriers, however, infants born to these mothers were likely to have low IL-12p70 cytokine concentrations levels compared to those born to maternal non-*E. histolytica* carriers. This might implicate some *in-utero* protection of the infants from *E. histolytica* infections, however, there is need for further investigation. There is also growing evidence that infants born to mothers with parasitic infections will develop lesions in their immune systems leading to tolerance or allergy as well as potential psycho-neurological changes leading to disease [89].

HIV-infected mothers are more likely to have infants with low levels of IgG2 and IgA immunoglobulins. It is highly possible that HIV alters the maternal immunoglobulin production levels which will eventually be passed on to the infant in utero or during breastfeeding. Franca *et al.,* (2012) have realised a similar trend whereby they found out that immunoglobulin levels were lower in hyperglycemic compared to normoglycemic mothers [127].

We also report that mothers giving birth at a mean age above 30.38± 6.09 years are more likely to have infants with low IgG4 levels (p=0.05). Speculatively, since immune cells also age as one gets older, there is a high probability that giving birth at an older age will also transfer weaker immunity to the infant, resulting in a negative impact on infant's health and survival.

6.5 Conclusion

This study demonstrates an *E. histolytica* prevalence of 5.4% in mothers and 5.6% in infants with an absence of *V. cholerae* and *S. typhi* detection in the areas of Dzivarasekwa, Glenview and Kuwadzana in Harare, Zimbabwe. Notably we report a similarity in the prevalence of *E. histolytica* between HIV-infected and HIV-uninfected mothers (p=0.318). Similarity in *E. histolytica* prevalence was also noted between HIV-exposed uninfected and HIV-unexposed uninfected infants though borderline non-significant (p=0.056). Moreover, resistance to 3rd generation cephalosporines, was detected in the non-lactose fermenting bacterial isolates. Only one HIV-uninfected mother-infant pair had an *E. histolytica* infection.

IL-1r, IL-4, IL-9, IL-12p70, IL-17a, G-CSF and PDGF-BB levels were significantly raised in *E. histolytica* infected compared to non-infected lactating mothers ($p<0.05$). In babies, *E. histolytica* carriage had no significant impact on the cytokine and immunoglobulin concentration. Carriage of any form of enteric infection such as non-lactose fermenters in addition to *E. histolytica* significantly increased concentration levels of IL-1r, IL-4, IL-9, IL-10, IL-12p70, IL17a, G-CSF, GM-CSF, IFN-γ, PDGF-BB and TNF-α cytokines ($p<0.05$) but no significant differences in immunoglobulin levels among the mothers. IL-13 and G-CSF levels were significantly raised in HIV-infected compared to HIV-uninfected lactating mothers (p=0.0389 and p=0.0324 respectively). IgA immunoglobulin levels were significantly raised among HIV-exposed uninfected than HIV-unexposed uninfected infants (p=0.0282). IL-7 was significantly raised in low birth-weight babies (birth weight <2500g) compared to normal birth-weight babies (p=0.0149).

Maternal HIV infection influences infant IgG2 and IgA immunoglobulin levels. Also a maternal age of >30years reduces the odds ratio of passing high IgG4 to the infant (p=0.05). Maternal HIV infection influences infant IgG2 and IgA immunoglobulin levels. Infants born to HIV positive mothers are significantly associated (p=0.049) with lower odds of having high IgG2 levels, ie., reduced by 76% in infants born to HIV-infected mothers compared to those born to HIV-uninfected mothers. Similarly, an infant born to a HIV-infected mother is significantly associated (p=0.035) with lower odds of having high IgA immunoglobulin levels compared to those born to HIV-uninfected mothers (OR: 0.22).

In this study maternal *E. histolytica* carriage was significantly (p=0.026) correlated with infant Interleukin-12p70 (IL-12p70; p<0.026), Fibroblast Growth Factor-basic (FGF-basic; p<0.026), Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF; p<0.026) and Tumor Necrosis Factor (TNF; p<0.026)-α. The odds ratio of passing high levels of these cytokines to infants is reduced by 86% if the mother is a carrier of *E. histolytica* during pregnancy (OR: 0.14).

Finally, parasitic infections in pregnancy such as *E. histolytica* are a reality and there is real need for screening in pregnancy. The probability of congenital transmission of *E. histolytica* was noted in this study, highlighting the need to screen of infants at birth hence avoiding the negative influence of this pathogen in infants, families and the community. Amoebiasis is usually not implicated in new-borns[128], however, we advocate screening of this parasite in new-borns. Infections during pregnancy may guide vaccine production for treating pregnant mothers as well as their neonates. It is also critical to monitor maternal HIV status and *E. histolytica* carriage and encourage mothers to deliver at ages below 30 years as these eventually modulate the infant immune development.

This infant immune modulation might possibly lead to development of auto-immune diseases and a possible cause of the observed high mortality and morbidity rates in HEUs. Further research is needed to confirm this. The current lack of *E. histolytica* vaccine[46] really calls for intensive studies in immune responses towards this parasite in an effort to assist development of vaccines and cytokine therapy for this devastating protozoan parasite.

6.6 Study Limitations

The study did not look at concurrent infections (except TB of which none of the study population was on TB treatment), vaccinations, concurrent treatment regiments (except antibiotics and antacids), nutritional status (except for probiotic uptake) and hospitalisation. These factors also influence one's immune response. As such, results emanating from this study should be taken as preliminary findings to shape future research

6.7 Recommendations

Deduction from the general findings of the study recommend carrying out a larger scale study on immunology that would provide meaningful findings that can be extrapolated to the situation happening in Harare, Zimbabwe, with frequent outbreaks of *S. typhi* and *V. cholerae.* The results reported in this thesis provide important preliminary results to shape future studies. Most studies are carried out on the diseased population neglecting the most important component of asymptomatic carriers, who, if left unnoticed, will spread diseases unknowingly and in some cases result in unwarranted disease outbreaks.

Chapter 7 : Overall Study References

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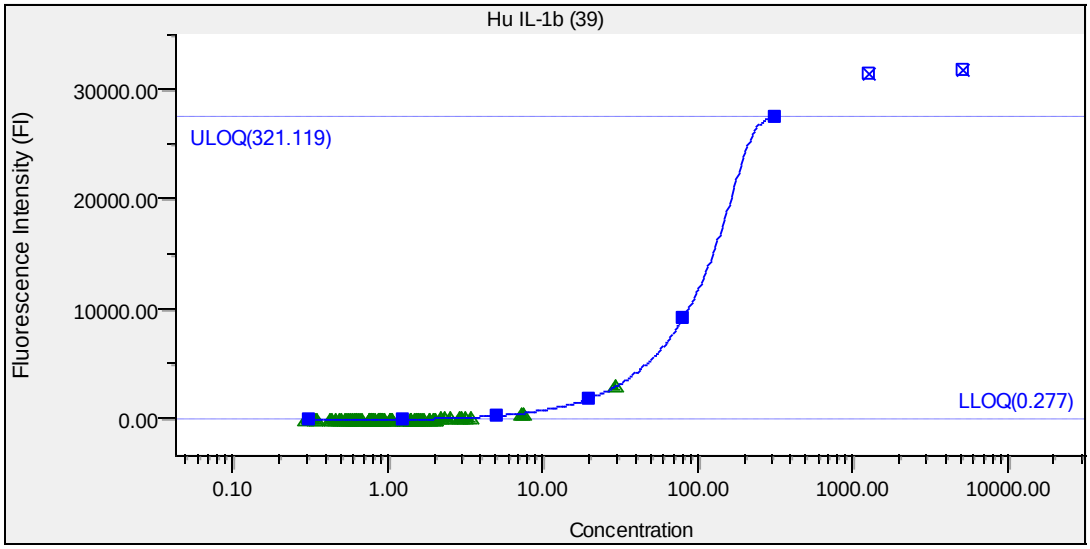
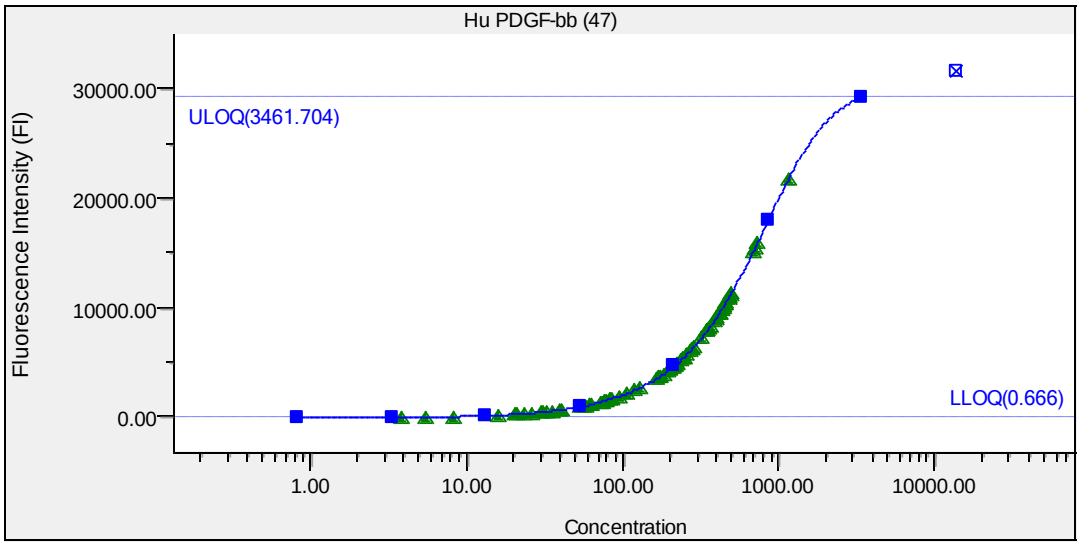
CHAPTER 8: APPENDICES

8A1: STANDARD CURVES FOR SOME OF THE CYTOKINES ANALYSED IN THE STUDY



Key: ■ **Standard** □ **Partial Outlier** ☒ **Outlier** ▲ **Unknown** ▲ **Control**

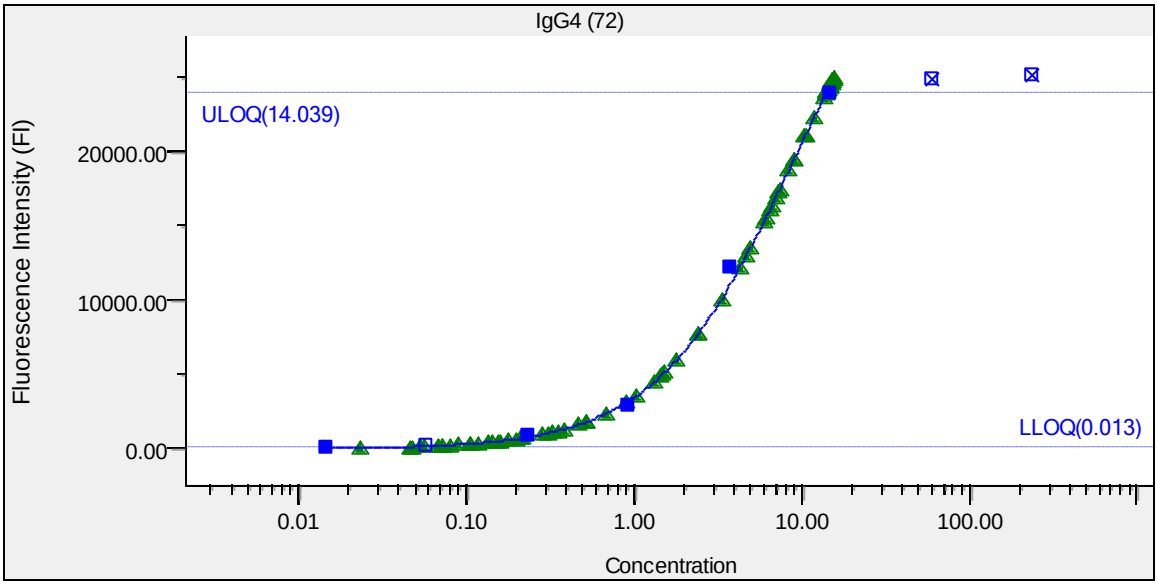
Regression Type: Logistic - 5PL
Std. Curve: FI = 9.85531 + (27759.8 - 9.85531) / ((1 + (Conc / 215.761)^-8.58709))^0.128959
FitProb. = 0.1360, ResVar. = 2.2227



■ **Standard** □ **Partial Outlier** ☒ **Outlier** ▲ **Unknown** ▲ **Control**

Regression Type: Logistic - 5PL
Std. Curve: FI = 8.20982 + (30550.6 - 8.20982) / ((1 + (Conc / 1137.73)^-2.23834))^0.494722
FitProb. = 0.7118, ResVar. = 0.3400

8A2: STANDARD CURVES FOR SOME OF THE IMMUNOGLOBULINS ANALYSED

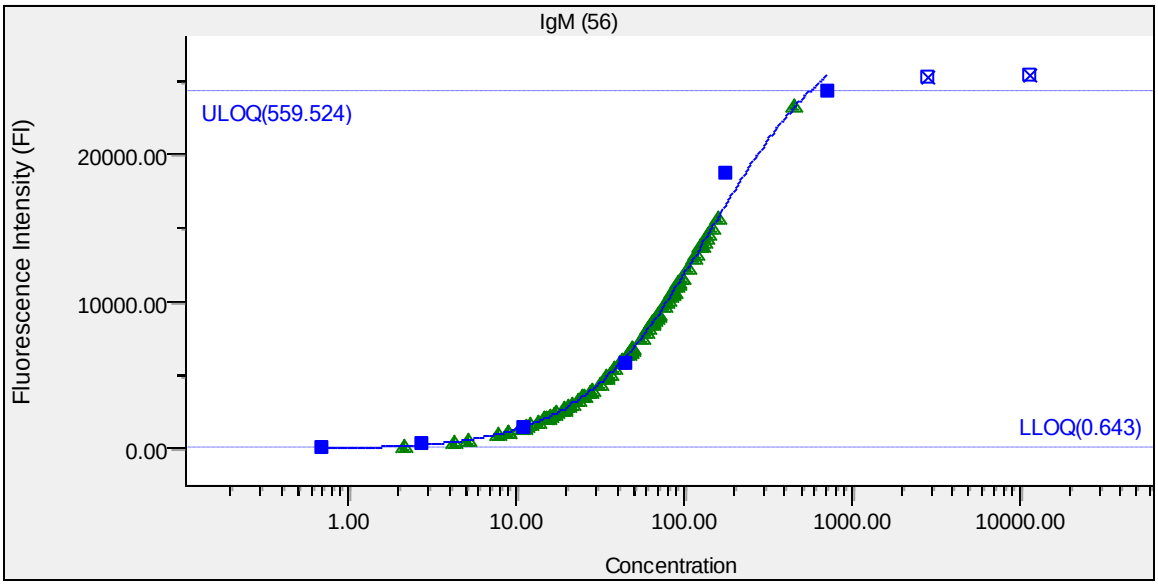


Standard Partial Outlier Outlier Unknown Control

Regression Type: Logistic - 4PL

Std. Curve: FI = 44.0269 + (38578.1 - 44.0269) / (1 + (Conc / 8.77742)^-1.06633)

FitProb. = 0.0036, ResVar. = 5.6374



Standard Partial Outlier Outlier Unknown Control

Regression Type: Logistic - 4PL

Std. Curve: FI = 71.7082 + (29356.2 - 71.7082) / (1 + (Conc / 139.011)^-1.14179)

FitProb. = 0.0042, ResVar. = 5.4690

8A3: MEDICAL RESEARCH COUNCIL OF ZIMBABWE RENEWAL OF APPROVAL

Telephone: 791792/791193
Telefax: (263) - 4 - 790715
E-mail: mrcz@mrcz.org.zw
Website: http://www.mrcz.org.zw

Medical Research Council of Zimbabwe
Josiah Tongogara / Mazoe Street
P. O. Box CY 573
Causeway
Harare

CONTINUING APPROVAL

REF: MRCZ/A/2043 **27 July, 2017**

Agness F Nhidza
8550 Kuwadzana Phase 3
Harare
Zimbabwe

Re:-Immunological Effects Of Asymptomatic Enteric Pathogens and Gut Microbiota During Infection and Exposure.

Thank you for the application for review of Research Activity that you submitted to the Medical Research Council of Zimbabwe (MRCZ). Please be advised that the Medical Research Council of Zimbabwe has **reviewed** and **approved** your application to continue conducting the above titled study.

This approval is based on the review and approval of the following documents that were submitted to MRCZ for review:-

a) Completed MRCZ Form 102
b) Study protocol, Version 3 July, 2016
c) Maternal Informed Consent Form, version 1.1 July 2016 (English and Shona)

APPROVAL NUMBER **: MRCZ/A/2043**

This number should be used on all correspondence, consent forms and documents as appropriate.

- **TYPE OF MEETING** **: Full Board**
- **MEETING DATE** **: 27 July, 2017**
- **EFFECTIVE APPROVAL DATE** **: 1 August, 2017**
- **EXPIRATION DATE** **: 31 July, 2018**

After this date, this project may only continue upon renewal. For purposes of renewal, a progress report on a standard form obtainable from the MRCZ Offices should be submitted three months before the expiration date for continuing review.

- **SERIOUS ADVERSE EVENT REPORTING**: All serious problems having to do with subject safety must be reported to the Institutional Ethical Review Committee (IERC) as well as the MRCZ within 3 working days using standard forms obtainable from the MRCZ Offices or website.
- **MODIFICATIONS**: Prior MRCZ and IERC approval using standard forms obtainable from the MRCZ Offices is required before implementing any changes in the Protocol (including changes in the consent documents).
- **TERMINATION OF STUDY**: On termination of a study, a report has to be submitted to the MRCZ using standard forms obtainable from the MRCZ Offices or website.
- **QUESTIONS**: Please contact the MRCZ on Telephone No. (04) 791792, 791193 or by e-mail on mrcz@mrcz.org.zw

Other

- Please be reminded to send in copies of your research results for our records as well as for Health Research Database.
- You're also encouraged to submit electronic copies of your publications in peer-reviewed journals that may emanate from this study.

Yours Faithfully

.....
MRCZ SECRETARIAT
FOR CHAIRPERSON
MEDICAL RESEARCH COUNCIL OF ZIMBABWE

MEDICAL RESEARCH COUNCIL OF ZIMBABWE
2017 -08- 01
APPROVED
P.O. BOX CY 573 CAUSEWAY, HARARE

PROMOTING THE ETHICAL CONDUCT OF HEALTH RESEARCH

8A4: MEDICAL RESEARCH COUNCIL OF ZIMBABWE INITIAL APPROVAL

Telephone: 791792/791193
Telefax: (263) - 4 - 790715
E-mail: mrcz@mrcz.org.zw
Website: http://www.mrcz.org.zw

Josiah Tongogara / Mazoe Street
P. O. Box CY 573
Causeway
Harare

APPROVAL

REF: MRCZ/A/2043

01 August 2016

Agness F Nhidza
8550 Kuwadzana Phase 3
Harare
Zimbabwe

RE: - Immunological Effects Of Asymptomatic Enteric Pathogens and Gut Microbiota During Infection and Exposure.

Thank you for the application for review of Research Activity that you submitted to the Medical Research Council of Zimbabwe (MRCZ). Please be advised that the Medical Research Council of Zimbabwe has reviewed and approved your application to conduct the above titled study.

This approval is based on the review and approval of the following documents that were submitted to MRCZ for review:-

a) Full Proposal Version 3 dated July 2016
b) Informed Consent Forms (English and Shona)
c) Data Collection Tools (English and Shona)

MEDICAL RESEARCH COUNCIL OF ZIMBABWE
2017 -03- 17
RECEIVED

- APPROVAL NUMBER : MRCZ/A/2043

This number should be used on all correspondence, consent forms and documents as appropriate.

- TYPE OF MEETING : Expedited
- EFFECTIVE APPROVAL DATE : 01 August 2016
- EXPIRATION DATE : 31 July 2017

After this date, this project may only continue upon renewal. For purposes of renewal, a progress report on a standard form obtainable from the MRCZ Offices should be submitted three months before the expiration date for continuing review.

- SERIOUS ADVERSE EVENT REPORTING: All serious problems having to do with subject safety must be reported to the Institutional Ethical Review Committee (IERC) as well as the MRCZ within 3 working days using standard forms obtainable from the MRCZ Offices or website.
- MODIFICATIONS: Prior MRCZ and IERC approval using standard forms obtainable from the MRCZ Offices is required before implementing any changes in the Protocol (including changes in the consent documents).
- TERMINATION OF STUDY: On termination of a study, a report has to be submitted to the MRCZ using standard forms obtainable from the MRCZ Offices or website.
- QUESTIONS: Please contact the MRCZ on Telephone No. (04) 791792, 791193 or by e-mail on mrcz@mrcz.org.zw

Other

- Please be reminded to send in copies of your research results for our records as well as for Health Research Database.
- You're also encouraged to submit electronic copies of your publications in peer-reviewed journals that may emanate from this study.

MEDICAL RESEARCH COUNCIL OF ZIMBABWE
APPROVED
P.O. Box CY 573, CAUSEWAY, HARARE

Yours Faithfully

.....

MRCZ SECRETARIAT
FOR CHAIRPERSON
MEDICAL RESEARCH COUNCIL OF ZIMBABWE

PROMOTING THE ETHICAL CONDUCT OF HEALTH RESEARCH

8A5: BIOMEDICAL RESEARCH ETHICS COMMITTEE INITIAL APPROVAL

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

20 April 2016

Ms NA Farai
Medical Microbiology
School of Laboratory Medicine and Medical Sciences
nhidzaagness@gmail.com

Protocol: Immunological effects of asymptomatic enteric pathogens and gut microbiota in infection and exposure.
Degree: PhD
BREC reference number: BE409/15

EXPEDITED APPLICATION

The Biomedical Research Ethics Committee has considered and noted your application received on 01 September 2015.

The study was provisionally approved pending appropriate responses to queries raised. Your responses dated 14 April 2016 to queries raised on 22 March 2016 have been noted and approved by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given **full ethics approval.**

This approval is valid for one year from **20 April 2016.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be **RATIFIED** by a full Committee at its meeting taking place on **10 May 2016.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

Professor J Tsoka-Gwegweni
Chair: Biomedical Research Ethics Committee

cc supervisor: mduluza@ukzn.ac.za
cc postgrad: dudhrajhp@ukzn.ac.za

8A6: BIOMEDICAL RESEARCH ETHICS COMMITTEE RECERTIFICATION

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI
RESEARCH OFFICE
BIOMEDICAL RESEARCH ETHICS ADMINISTRATION
Westville Campus
Govan Mbeki Building
Private Bag X 54001
Durban
4000
KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604769 - Fax: 27 31 260-4609
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/ResearchEthics/BiomedicalResearchEthics.aspx

13 March 2017

Ms A F Nhidza
Medical Microbiology
School of Laboratory Medicine and Medical Sciences
nhidzaagness@gmail.com

Dear Ms Nhidza

Protocol: Immunological effects of asymptomatic enteric pathogens and gut microbiota in infection and exposure.
Degree: PhD
BREC reference number: BE409/15

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	20 April 2017
Expiration of Ethical Approval:	19 April 2018

I wish to advise you that your application for Recertification dated 11 February 2017 in relation to the above protocol has been noted and approved by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

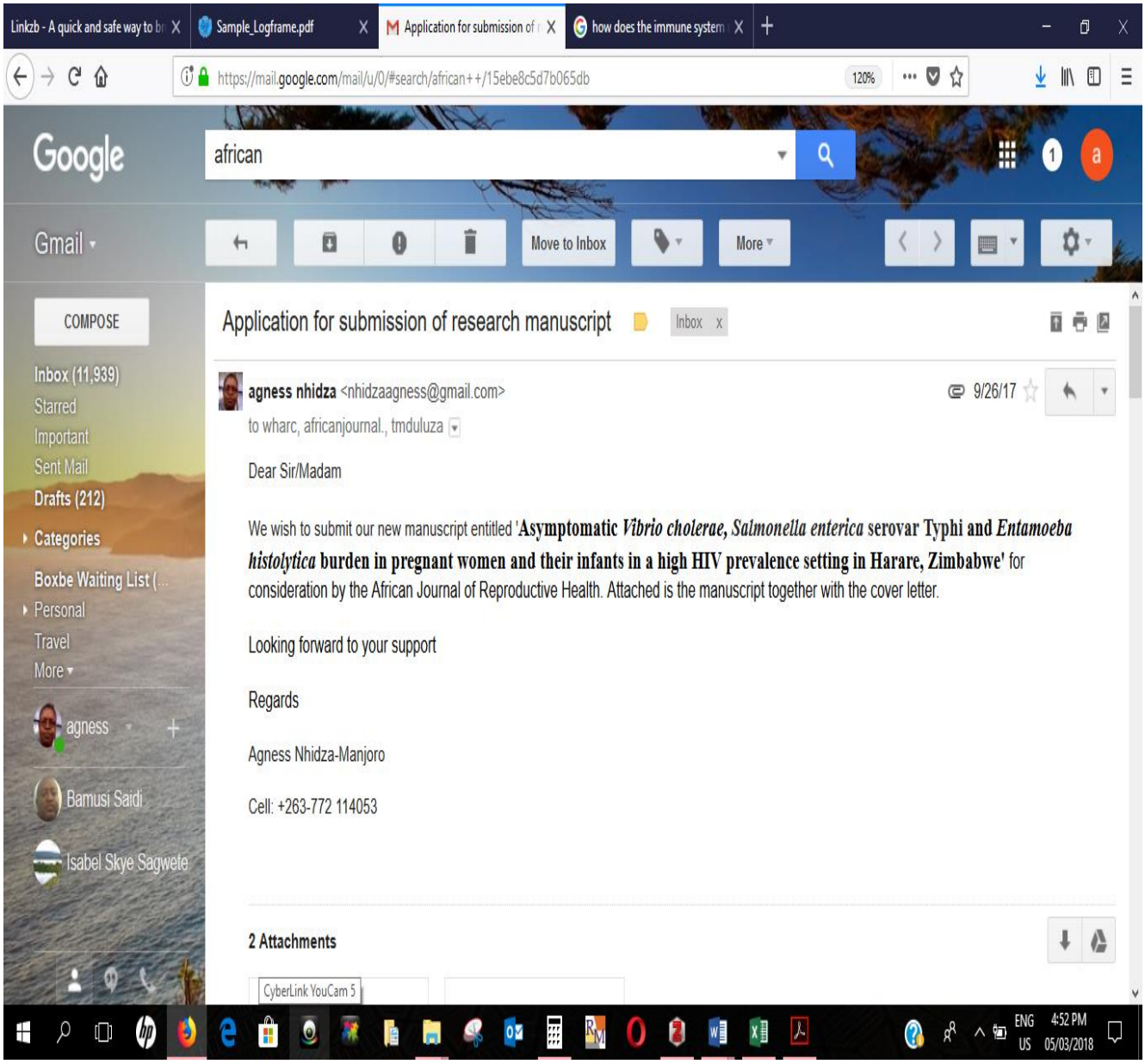
If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

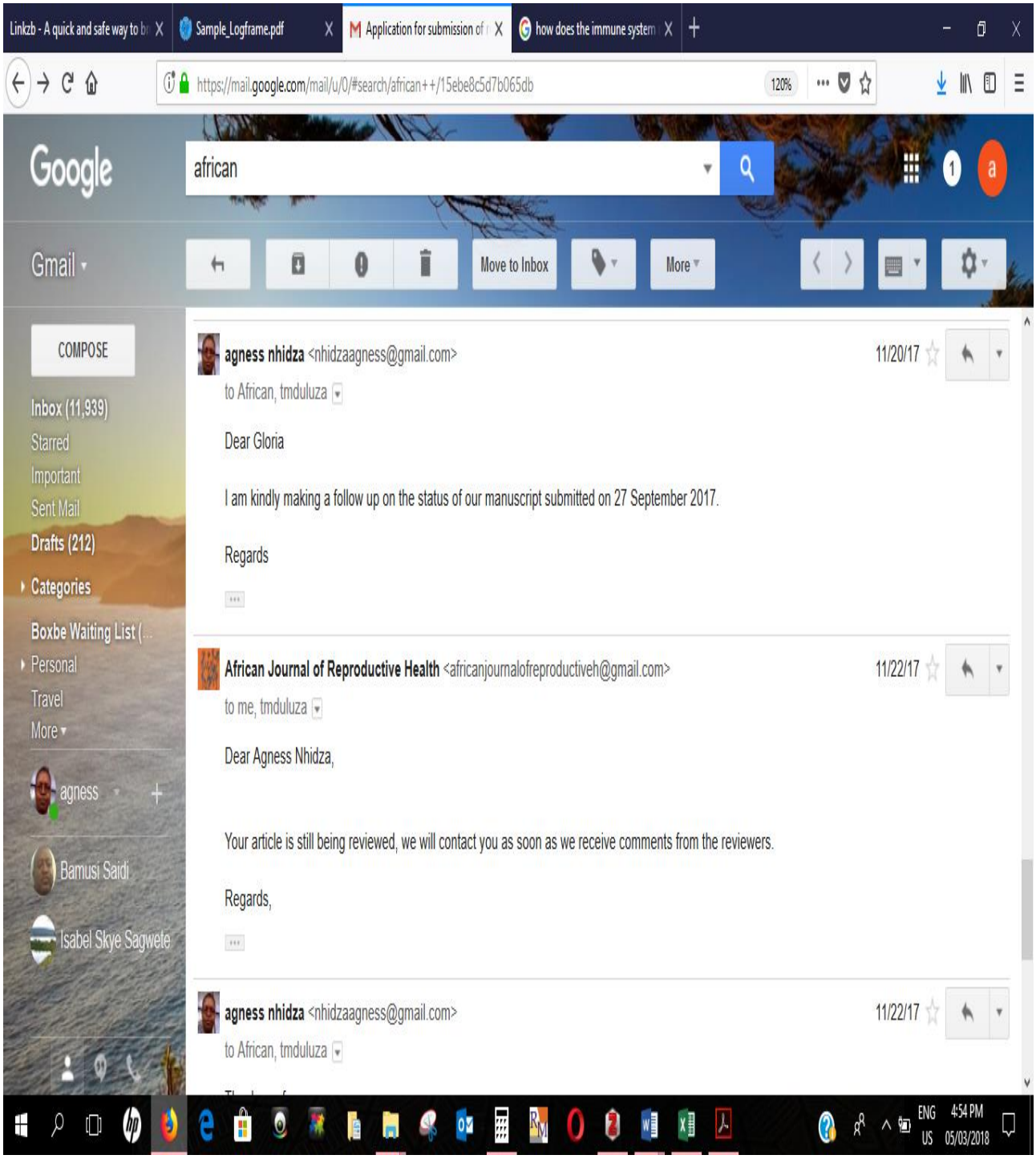
The approval will be ratified by a full Committee at a meeting to be held on 11 April 2017.

Yours sincerely

Ms A Marimuthu
Senior Administrator: Biomedical Research Ethics

8A7: PROOF OF SUBMISSION FOR MANUSCRIPT 1

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Starred
Important
Sent Mail
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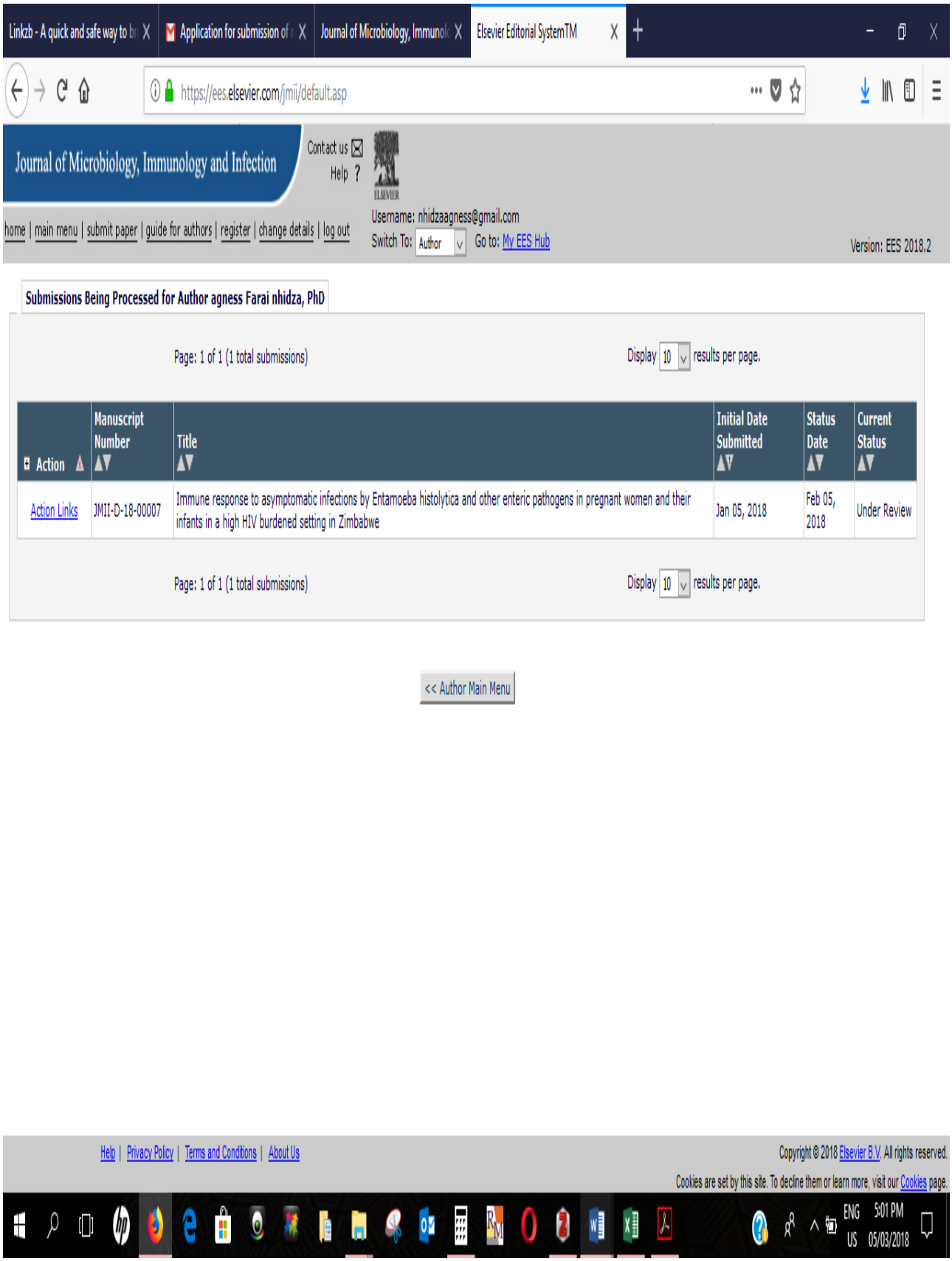
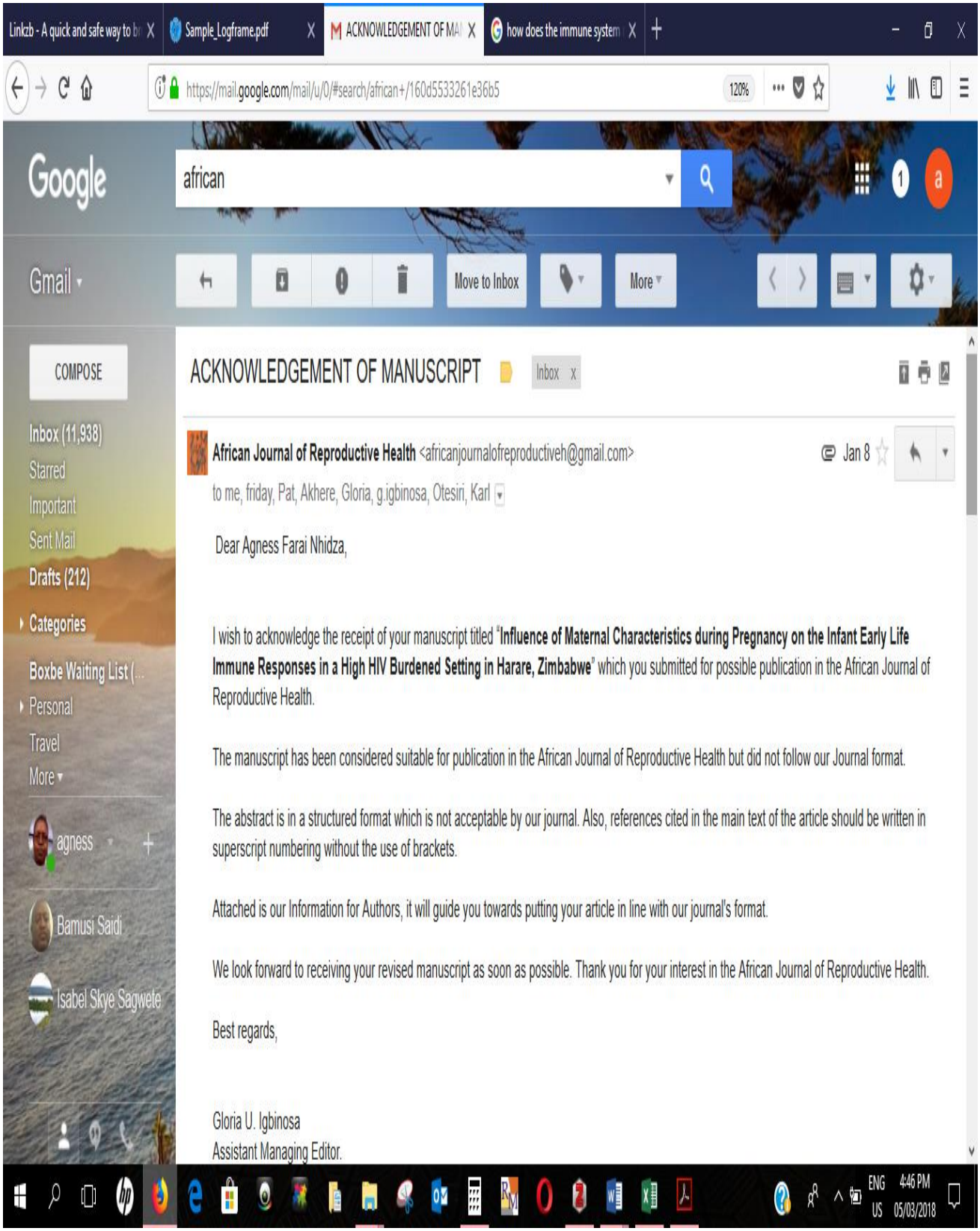
Boxbe Waiting List (...
Personal
Travel
More
agness
Bamusi Saidi
Isabel Skye Sagwete
agness nhidza <nhidzaagness@gmail.com>
11/20/17
to African, tmduluza
Dear Gloria
I am kindly making a follow up on the status of our manuscript submitted on 27 September 2017.
Regards
African Journal of Reproductive Health <africanjournalofreproductiveh@gmail.com>
11/22/17
to me, tmduluza
Dear Agness Nhidza,
Your article is still being reviewed, we will contact you as soon as we receive comments from the reviewers.
Regards,
agness nhidza <nhidzaagness@gmail.com>
11/22/17
to African, tmduluza
ENG
US
4:54 PM
05/03/2018

8A8: PROOF OF SUBMISSION FOR MANUSCRIPT 2



8A9: PROOF OF SUBMISSION FOR MANUSCRIPT 3



ACKNOWLEDGEMENT OF MANUSCRIPT

African Journal of Reproductive Health <africanjournalofreproductiveh@gmail.com> Jan 8

to me, friday, Pat, Akhere, Gloria, g.igbinosa, Otesiri, Karl

Dear Agness Farai Nhidza,

I wish to acknowledge the receipt of your manuscript titled "**Influence of Maternal Characteristics during Pregnancy on the Infant Early Life Immune Responses in a High HIV Burdened Setting in Harare, Zimbabwe**" which you submitted for possible publication in the African Journal of Reproductive Health.

The manuscript has been considered suitable for publication in the African Journal of Reproductive Health but did not follow our Journal format.

The abstract is in a structured format which is not acceptable by our journal. Also, references cited in the main text of the article should be written in superscript numbering without the use of brackets.

Attached is our Information for Authors, it will guide you towards putting your article in line with our journal's format.

We look forward to receiving your revised manuscript as soon as possible. Thank you for your interest in the African Journal of Reproductive Health.

Best regards,

Gloria U. Igbinosa
Assistant Managing Editor.