THE IMPACT OF ENTERIC PATHOGENS AND SECRETED EXTRACELLULAR VESICLES ON AMOEBIC VIRULENCE AND OUTCOME OF INFECTION

A THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN MICROBIOLOGY AT THE UNIVERSITY OF VENDA

BY

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DECLARATION

I, Renay Ngobeni, declare that this thesis submitted to the University of Venda for the degree of PhD in Microbiology to the Department of Microbiology in the School of Mathematical and Natural Sciences and the work contained herein is my original work with exception to citations and that this work has not been submitted to any other University in part or in its entirely for the award of any degree.

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Being chosen as a recipient of the global health and enteric diseases research training program has been a huge honor to me. Without this fellowship, I would not have obtained such a high quality education and the experience in research. The main purpose of my visit in the Petri laboratory (University of Virginia) was to learn as much as I could on amebiasis research, focusing mainly on understanding the transition from colonization to invasive disease and to characterize the novel strains of amoeba in the South African population. The fellowship has also allowed me to explore additional and novel areas linked to amebiasis research, such as investigating the presence of exosomes in *E. histolytica.* I greatly appreciate the opportunity this fellowship has presented before me, I am really grateful. I consider it a great honor to have been part of the Petri lab.

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DEDICATION

I dedicate this work to my lovely daughter, Keabetswe, Boikgantsho Ngobeni. May you grow and learn something from this.

SUMMARY

Background: Diarrheal diseases have a major effect on human health, Globally; it is second only to pneumonia as a leading cause of death among children under five. They are due to a variety of infectious and non-infectious agents; including *Entamoeba spp. Entamoeba histolytica* is an invasive enteric protozoan parasite that causes amebiasis. Amebiasis is frequent in communities without clean water and poor sanitation, which include low-income South African populations in Giyani and Pretoria. In these populations, the amount of diarrhea caused by *Entamoeba histolytica* inclusive of all ages, sexes and HIV status is uncertain. Diagnosis of the parasite is usually by microscopy. However, microscopy lacks sensitivity and specificity, therefore it is not reliable. Fortunately, molecular diagnostic tests have been developed to detect different *Entamoeba* species in humans.

It is known that the parasite *E. histolytica* causes asymptomatic and symptomatic diseases. However, the transition from colonization to disease is still unclear. While parasite and host factors, as well as environmental conditions influence the infection outcome, there is currently no clear explanation of wide variation in the presentation of the disease. This could suggest that there are other factors affecting the disease outcome. A better understanding of these factors as well as their role in disease remains target objectives of modern scientists and it will definitely help in the fight against the disease. In spite of the emerging evidence that the host microbiome, parasite burden and the inflammatory response contribute to the virulence of *E. histolytica,* their roles have never been defined in developing regions such as Giyani

and Pretoria. In addition, the present study hypothesized that co-infections with *E. histolytica* and secretion of extracellular vesicles/exosomes have a significant impact on the virulence of *E. histolytica*. Little has been explored or elucidated about responses triggered by other enteropathogens/ameba interplay that could be important in the induction of tissue invasion and disease and also how *E. histolytica*/enteropathogens interplay in these infections has not been determined. Therefore, the knowledge of this interplay could help in understanding how this modifies disease manifestations by modulating pathogen virulence and the host response. The use of secretion systems is an essential biological process exploited by pathogenic microorganisms to promote survival and spread of the pathogen, which in turn exacerbate the infection. The study of extracellular vesicles (EVs) released by pathogens is a new and exciting field that may realistically contribute to a better understanding of the pathogenic process of *E. histolytica* and provide alternate control strategies.

Aim and objective of the study: The overall aim of the study was to determine the impact of enteric pathogens and secreted extracellular vesicles on amebic virulence and the outcome of infection. This aim was addressed in through a series of six primary objectives, which were:

a. To investigate the distribution and prevalence of protozoan parasites in South Africa.
b. To investigate novel species of *Entamoeba* circulating in the South African population.

c. To elucidate the impact of gut microbiota and immune response during amebic infection.
d. To determine the role of *Entamoeba histolytica* macrophage inhibitory factor (*Eh*MIF) during amebic infection.
e. To investigate the impact of co-infections on the outcome of amebiasis.
f. To determine the presence of secreted extracellular vesicles/exosomes in *Entamoeba histolytica***.**

Brief methodology and results: A modified and validated Taqman qPCR assay (with taqman probes and genus specific primers) was used for amplification and target detection. This assay was used to investigate the distribution and prevalence of protozoan parasites (*Cryptosporidium spp* and *Giardia lamblia*) in South Africa, the assay was considered superior for this project because it is more sensitive than conventional PCR and it can be used to detect multiple infection targets. This assay allows fast, accurate, and quantitative detection of a broad spectrum of enteropathogens and is well suited for surveillance or clinical purposes. A total of 484 stool samples collected from diarrheal and non-diarrheal patients from rural and urban communities of South Africa were studied*.* The overall prevalence of parasites (*Giardia lamblia* and *Cryptosporidium spp*) in rural and urban patients were found to be 49% (112/227) and 21% (54/257) respectively (p= < 0.0001). The distribution of specific pathogens in rural areas was *Cryptosporidium spp* (20%) and *Giardia lamblia* (14%). Our findings showed no significant difference in parasitic infections between gender and the age of the participants (**Chapter 3**).

The discovery of novel species is of great importance to human health. We have recently discovered stools positive for *Entamoeba* organisms by microscopy but PCR negative for known *Entamoeba* species. This led to the hypothesis that novel species of *Entamoeba* are present in the South African population. A comprehensive assay was used which included probes to identify *Entamoeba bangladeshi* from diarrheal and non-diarrheal participants. A sensitive qPCR assays and amplicon sequencing was used to detect *Entamoeba* spp, *Prevotella copri* and *Enterobacteriaceae*. Interestingly, *E. bangladeshi* was identified in the South African population. *Entamoeba* was present in 27% (*E. histolytica* 8.5% (41/484), *E. dispar* 8% (38/484), and *E. bangladeshi* 4.75% (23/484) *E. moshkovskii* was not detected in the present study. We were also able to observe changes in the host microbiome and the parasite burden associated with *E. histolytica* infections in S. African diarrhea cases versus asymptomatic controls but not with *E. bangladeshi* or *E. dispar.* In *E. histolytica* positive samples the level of both parasite and *P. copri* were lower in non-diarrheal samples (p=0.0034) (**Chapter 4**).

There is accumulating evidence that the inflammatory response contributes to injury. Little is known about the key parasite mediators of host mucosal immunopathology. This study hypothesized that migration inhibitory factor (MIF) mediates the destructive host inflammatory response seen in amebic colitis. To determine the role of *Eh*MIF during amebic infection, we used a genetic approach to test the effect of *Eh*MIF on mucosal inflammation. We found that *Eh*MIF induces IL-8 secretion from intestinal epithelial cells. Mice treated with antibodies that specifically block *Eh*MIF had reduced chemokine expression and neutrophil infiltration in the mucosa. In addition to antibody-mediated neutralization, mice infected with parasites overexpressing *Eh*MIF had increased chemokine expression, neutrophil influx and mucosal damage. We also found that the concentration of *Eh*MIF correlated with the level of intestinal inflammation in persons with intestinal amebiasis. Together, our results reveal a novel parasite mediator of mucosal inflammation and support MIF homologs as potential immunomodulatory targets (**Chapter 5**).

To investigate the impact of co-infections on the outcome of amebiasis, we analyzed the co-occurence of *E. histolytica* with other enteropathogens known to cause diarrheal infections, such as *Shigella/EIEC (*IpaH*), Campylobacter (cadf)*, *Enterotoxigenic E. coli (STh)*, Norovirus GII and Adenovirus (Hexon). The results were compared with those obtained with *E. histolytica* that were not interacted with enteropathogens and with *E. histolytica* interacted with enteropathogens. The impact of multiple infections on the outcome of the infection was compared between non-diarrheal and diarrheal stool samples. It was found that co-infections with two pathogens were associated with diarrhea compared to single infections. Moreover, Norovirus GII, *Campylobacter* (Cadf) and co-infections were associated with diarrhea in the study population. This study did not show any significant impact of pathogens co-infecting with *E. histolytica* on the outcome of amebic infection (**Chapter 6**).

The presence of secreted extracellular vesicles/Exosomes in *Entamoeba histolytica* was determined by using the Pathogenic ameba strains (HM-1:IMSS or HM-1:IMSS (Sub-strain-US) from petri's lab to purify exosomes using the commercially available kit to isolate exosomes (total exosomes isolation kit). Our study for the first time revealed that *E. histolytica* does secrete Evs. This finding increases the appreciation that all organisms are likely to secrete these EVs (**Chapter 7**). However, the impact of these EVs on the pathogenesis of *E. histolytica* needs further investigations.

Conclusion: This study has contributed significantly to our knowledge on infectious diarrhea and the diversity of *Entamoeba* species by providing new data on the rate and prevalence of *Entamoeba* diarrheal infections and their distribution in the South African population. Our study describes for the first time the presence of *E. bangladeshi* in the South African population. Furthermore**,** our results reveal a novel parasite mediator of mucosal inflammation and support MIF homologs as potential immunomodulatory targets. This study also, for the first time revealed that *E. histolytica* does secrete EVs. The results from this work will undoubtedly open an exciting research to establish a deeper understanding of the function and role of these vesicles in amebic infection. We encourage public health interventions like health education programs and improvement of sanitation and hygiene in these populations. Molecular diagnostics should be used for specific diagnostic in clinical settings.

PUBLICATIONS AND PRESENTATIONS GENERATED DURING THE STUDY

A total of four manuscripts have been generated from the present study as well as one review article. Two manuscripts have been published and two are still in preparation as well as the review paper. A total of 17 *Entamoeba* sequences have been reported in GenBank with the following Accession numbers: MF471217.1; MF471216.1; MF471215.1; MF471214.1; MF471213.1; MF471212.1; MF471211.1; MF471210.1; MF471209.1; MF471208.1; MF471207.1; MF471206.1; MF471205.1; MF471204.1; MF471203.1; MF471202.1; MF471201.1.

PUBLISHED ARTICLES:

Renay Ngobeni, Mayuresh M. Abhyankar, Nona M. Jiang, Laura A. Farr, Amidou Samie, Rashidul Haque, Shannon N. Moonah. *Entamoeba histolytica*–Encoded Homolog of Macrophage Migration Inhibitory Factor Contributes to Mucosal Inflammation during Amebic Colitis. Journal of Infectious Diseases, Volume 215, Issue 8, 15 April 2017, Pages 1294–1302.

Renay Ngobeni, Amidou Samie, Shannon Moonah, Koji Watanabe, William A Petri, Jr., Carol Gilchrist; *Entamoeba* in South Africa: correlations with the host microbiome, parasite burden and first description of *E. bangladeshi* outside of Asia, *The Journal of Infectious Diseases*, jix535, https://doi.org/10.1093/infdis/jix535.

SUBMITTED MANUSCRIPT:

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Renay Ngobeni, Carol Gilchrist, Shannon Moonah, William. A, Petri and Amidou Samie. 2018. Prevalence of selected enteropathogens among patients attending health centers in Northern South Africa: Impact of co-infections in the outcome of amebiasis.

Renay Ngobeni, Carol Gilchrist, Shannon Moonah, William. A, Petri and Amidou Samie. 2018. Exosomes-like vesicles in the context of *Entamoeba histolytica*: Preliminary observations and review of the literature.

NATIONAL AND INTERNATIONAL CONFERENCES ATTENDED AND PRESENTATIONS:

Renay Ngobeni, Samie Amidou, Shannon Moonah, William. A, Petri and Carol Gilchrist.Impact of novel *Entamoeba* species on diarrheal infections in South Africa. Infectious Diseases and Biodefense Research Day. University of Virginia, United States of America, 2016.

Renay Ngobeni, Samie Amidou, Shannon Moonah, William. A, Petri and Carol Gilchrist. The distribution of intestinal pathogens in rural and urban communities of

South Africa. American society of tropical medicine and hygiene (ASTMH) 65th Annual Meeting, Atlanta Georgia, United States of America, 2016.

Renay Ngobeni, Samie Amidou, Shannon Moonah, William. A, Petri and Carol Gilchrist. First description of *E. bangladeshi* in South Africa. International symposium on global health research in Africa at 2Ten hotel, South Africa, 2017.

Renay Ngobeni, Mayuresh M. Abhyankar, Nona M. Jiang, Amidou Samie, and **Shannon Moonah**. *Entamoeba histolytica*–Encoded Homolog of Macrophage Migration Inhibitory Factor Contributes to Mucosal Inflammation during Amebic Colitis. Molecular parasitology meeting, Woods Hole, MA, 2016.

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Renay Ngobeni, Amidou Samie, Shannon Moonah, Koji Watanabe, William A. Petri, Jr and Carol Gilchrist. *Entamoeba* in South Africa: correlations with the host microbiome, parasite burden and first description of *E. bangladeshi* outside of Asia. American society of tropical medicine and hygiene (ASTMH) 66th Annual Meeting Baltimore, Maryland, United States of America, 2017.

Renay Ngobeni, Amidou Samie, Shannon Moonah, Koji Watanabe, William A. Petri, Jr and Carol Gilchrist. *Entamoeba* in South Africa: correlations with the host microbiome, parasite burden and first description of *E. bangladeshi* outside of Asia. XIX International conference on amebiasis, Mexico, Puebla, 2018.

Renay Ngobeni, Amidou Samie, Shannon Moonah, Koji Watanabe, William A. Petri, Jr and Carol Gilchrist. *Entamoeba* in South Africa: correlations with the host microbiome, parasite burden and first description of *E. bangladeshi* outside of Asia. The 2nd International Conference on Tissue Engineering and Regenerative Medicine (ICTERM), 2017, South Africa, Vaal.

Renay Ngobeni, Carol Gilchrist, Shannon Moonah, William. A, Petri and Amidou Samie. Prevalence of selected enteropathogens among patients attending health centres in Northern South Africa**.** The South African Society of Microbiology (SASM), 2018, South Africa, Johannesburg.

Renay Ngobeni, Amidou Samie, Shannon Moonah, Koji Watanabe, William A. Petri, Jr and Carol Gilchrist. *Entamoeba* in South Africa: correlations with the host microbiome, parasite burden and first description of *E. bangladeshi* outside of Asia. 47th PARSA conference 16-18 September 2018, South Africa, Tshipise.

Other conferences and Workshops attended:

American society of tropical medicine and hygiene (ASTMH) 64th Annual Meeting, Philadelphia, United States of America.

Eukaryotic pathogen database resource, 11th annual workshop. Athens, Georgia, United States of America, 2016.

EMBO laboratory management workshop. Heidelburg, Germany, 2017.

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%	Percentage
°C	Degrees Celsius
<	Less than
≤	Less than/equal to
≥	Greater than/equal to
µ*g/ml*	Microgram per milliliter
µl	Micro liter
µM	Micromolar
ABC	ATP-binding cassette
ALA	*Amebic liver abscess*
Anti-MIF	Anti- Macrophage migration inhibitory factor
BC	Before Christ
CCR5	Chemokine receptor 5
CDC	Centers for diseases control and prevention
CHO	Chinese hamster ovary
CMV	Cytomegalovirus
COL	Colonization

Cq	Quantification cycle
Cryo-EM	Cryo-electron microscopy
CXCL1	Chemokine ligand 1
CXCL2	Chemokine ligand 2
DNA	Deoxyribonucleic acid
Dr	Doctor
DS	*Diarrheal stool*
E. bangladeshi	*Entamoeba bangladeshi*
E. dispar	*Entamoeba dispar*
E. hartmanni	*Entamoeba hartmanni*
E. histolytica	*Entamoeba histolytica*
E. moshkovskii	*Entamoeba moshkovskii*
E. poleki	*Entamoeba poleki*
E.coli	*Entamoeba coli*
E.g	Exempli gratia
EC346	*Entamoeba coli non-pathogenic strain 346*
ECM	Extracellular matrix
*Eh*CP	*Entamoeba histolytica* cysteine protease
ELISA	Enzyme-linked immunosorbent assay
Et al	Et alia (and others)
ETEC (sth)	*Enterotoxigenic coli (*heat-stable enterotoxin)
EVs	Extracellular vesicles

*Eh*MIF	*Entamoeba histolytica* Macrophage migration inhibitory factor
Fig	Figure
FACs	Fluorescence-activated cell sorting
G. lamblia	*Giardia lamblia*
Gal/galnac	Galactose/N-acetyl/Galactosamine inhibitable lectin
GPI	Glucose phosphate isomerase
H.Polygyrus	Heligmosomoides *Polygyrus*
HCV	*Hepatitis C virus*
HIV	*Human immunodeficiency virus*
HK	Hexokinase
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cells
IFA	Immunofluorescent antibody
IHA	Indirect haemagglutination
IL-10	Interleukin 10
IL-1B	Interleukin 1 beta
IL-23	Interleukin23
IL-6	Interleukin 6
IL-8	Interleukin 8
Kb	Kilobases
LDH	Lactate dehydrogenase

LPS	Lipopolysaccharide
ME	Malic enzyme
Mg	Microgram
MIF	Macrophage migration inhibitory factor
miRNA	Micro ribonucleic acid
MMP	Matrix metalloproteinase-3
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
MUC2	Mucus layer
MVBs	Multivesicular bodies
N	Number
NCBI	National Center for Biotechnology Information
Nm	Nanomolar
NO	Nitric oxide
NTA	Nanoparticle-tracking analysis
ND	Non-diarrheal
P-Copri	*Prevortella copri*
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pg	*Picogram*
PGM	Phosphoglucomutase
PhD	Doctor of philosophy

qPCR	Quantitative polymerase chain reaction
RIA	Radioimmunoassay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
S Africa	South Africa
SD	Standard deviation
SEM	Scanning electron microscopy
SF	Secretory fraction
Spp	Species
SSU rRNA	Small subunit ribosomal ribonucleic acid
T. cruzi	*Trypanosoma cruzi*
T. gondii	*Toxoplasma gondii*
T. vaginalis	*Trichomonas vaginalis*
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor beta
TLR-4	Toll-like receptor 4
TNF-α	Tumor necrosis factor- alpha
USA	United States of America
WHO	World health organization
WT	Wild type

THESIS ORGANIZATION AND OUTLINE

This thesis is organized into eight chapters:

Chapter 1 provides the study background and highlights the rationale for conducting this study. **Chapter 2** provides an overview of the discovery of *Entamoeba histolytica* and review of published literature on amebic infection to date.

The research findings are presented in five parts in **Chapters 3, 4, 5, 6 and 7** addressing each primary objective as individual sub-studies. In each of the five chapters, a brief background is provided in addition to the specific aims and objectives of the sub-study. Detailed descriptions of the methods and materials used are also provided for each sub-study as well as discussions of published literature in relation to study outcomes. Publications generated from each study are provided in the appendices.

References are provided at the end of each of the 7 chapters for ease of access.

The final conclusions drawn from the findings of the study are described in **Chapter 8**, as well as the study limitations, recommendations and future directions.

CHAPTER ONE

General Introduction and Rationale

CHAPTER 1

1.1 Introduction

Enteric infections still constitute a serious public health problem globally, especially in low and middle-income countries, particularly in areas of poor sanitation, low socio-economic conditions, inadequate water supply and poor hygiene practices (Liu *et al.* 2016). Diarrheal infections remain the second leading cause of death worldwide after pneumonia, particularly in children under the age of five (Walker *et al*. 2012). Of those who survive the diarrhea, the morbidity burden can affect their development (growth, cognitive performance and physical fitness (Walker *et al*. 2012). A number of people are thought to be affected by neglected tropical diseases caused by various eukaryotic parasites, bacteria and viruses (Fletcher *et al.* 2012). Their prevalence is highly dependent on factors such as sanitation infrastructure, social culture and personal hygiene practices. One of those infections includes amebiasis.

Amebiasis, also known as amebic dysentery, is an intestinal and extra-intestinal disease caused by a protozoan parasite *Entamoeba histolytica*. To date, *E. histolytica* is the only known species that is pathogenic to humans and causes amebiasis, a disease that ranks third among parasitic causes of death, behind only malaria and schistosomiasis on a global scale (Mortimer and Chadee 2010). However, evidence is now emerging that *Entamoeba moshkovskii,* the second member of the genus *Entamoeba* is also associated with human diseases

(Shimokawa *et al.* 2012). The infection has been reported to occur worldwide, but prevalent in tropical and developing countries. Humans are the primary host of *E. histolytica* (Serrano-Luna *et al.* 2013), it affects people of all ages, but mostly seen in children under the age of five years.

Consumption of contaminated food and water is a major cause of new infections (Petri and Singh 1999). The clinical presentation ranges from watery diarrhea, bloody diarrhea, stomach pains and liver abscess (Gilchrist *et al.* 2012), trophozoites can physically spread to extra- intestinal organs such as lungs, brain and liver (Cheepsattayakorn and Cheepsattayakorn 2014). Amebiasis infectionS range from asymptomatic to symptomatic diseases and only about 10% to 20% of the infected individuals develop symptomatic diseases (Gilchrist *et al.* 2012). It remains puzzling why only a subset of infected individuals develops invasive diseases. One of the possible explanations for this is that genetic subgroups exist within *E. histolytica* that can give rise to infection with different outcomes (Burch *et al.* 1991), but the factors that govern the transition from colonization to invasion remain unknown despite the identification of several virulence factors Gal/GalNac/cysteine proteases/etc) (Wilson *et al.* 2012). An alternative hypothesis is that, the differences in the host immune response (Guo *et al.* 2011) or the gut milieu can substantially alter the progression of the disease (Rogers *et al.* 2016).

The diagnosis of infection through microscopic examination may provide false results as the pathogenic *E. histolytica* species does not show marked morphological differences and is indistinguishable from the non-pathogenic *E. dispar* (Santos *et al.* 2010). Fortunately, molecular diagnostic tests have been developed to detect different *Entamoeba* species in humans (Bradford *et al.* 2008). Despite the presence of many efficient species discovery methods, careful selection of a combination of methods is important to demonstrate novel species' morphology and clinical associations. Molecular techniques such as cell culture, ELISA and PCR have been developed for detection and differentiation of *Entamoeba species* (Hooshyar *et al*. 2012). The polymerase chain reaction (PCR) is by now widely used in clinical settings. This technique allows a rapid amplification of target sequences and can be a highly specific and sensitive detection method. It can be used to increase sensitivity and specificity of *Entamoeba* diagnosis in different clinical isolates (Akarsu *et al.* 2011). Genes that are detected in *Entamoeb*a isolates are used for species identification and differentiation (Ramos *et al.* 2005).

People diagnosed with invasive amebiasis require prompt treatment and individuals with no symptoms should also be treated in order to control transmission and the spread of the parasite. Medications are available for the treatment of amebiasis and they depend on how severe the infection is. The antiparasitic drugs used for treating *E. histolytica* infections include, metronidazole, iodoquinol, dehydroemitine, paromomycin and diloxanide furoate (Leitsch *et al.* 2007).

1.2 Study Rationale

Enteric diseases remain a serious health problem globally. High prevalence is seen in areas of low socio-economic conditions, poor sanitation and inadequate water supply, such as Africa. Studies on the prevalence and distribution of enteric parasites are of great importance to predict the occurrence and the frequency of pathogens in a population. Diarrheal diseases are due to a variety of infectious and non-infectious agents, including protozoan parasites, viruses and bacteria. This thesis focused on *Entamoeba spp, Cryptosporidium spp, Giardia lamblia, Campylobacter (*cadF)*, Enterotoxigenic Escherichia coli (STh),* Adenovirus *(Hexon),* Norovirus *GII* and *shigella/EIEC* (ipaH). Age and geographical location may also play a role in pathogen susceptibility and exposure. A recent large study which was restricted to children <2 years in age in the Dzimauli community of Vhembe District Limpopo Province of South Africa failed to detect *E. histolytica* (Platts-Mills *et al.* 2015), whereas in an age inclusive 2009 Vhembe study of *Entamoeba histolytica* sero-prevalence found 34.2% *E. histolytica*/*E.dispar* (Samie *et al.* 2009). To determine how generalizable the Dzimauli results are, we have performed an age inclusive survey for both the pathogens common in the < 2 age group (Norovirus GII, *Shigella/EIEC* (ipaH), *Campylobacter* (cadF), Etec (*STh*) and the protozoan parasites (*Giardia*, *Entamoeba* and *Cryptosporidium*) in the rural Giyani community neighboring Dzimauli in the Vhembe District of Limpopo and in the urban community in Pretoria (catchment area of the Dr Georges Mukhari Hospital).

South Africa is one of the developing countries that has been significantly impacted by diarrheal infections, many of which are due to *Entamoeba species*. The discovery of novel species is of great importance to human health. Novel *Entamoeba species* have been identified in different endemic regions of the world, this includes work by Royer *et al.* (2012), where the new strain of *Entamoeba* from human fecal specimens was discovered and named *Entamoeba bangladeshi,* but the occurrence of novel *Entamoeba* species in South Africa has never been investigated. We have recently discovered stools positive for *amoeboid* organisms by microscopy but PCR negative for known *Entamoeba* species. This led to the hypothesis that novel species of *Entamoeba* are present in the South African population.

Recent studies have also highlighted the relationship between *Entamoeba* and the bacterial communities of the gut. For example, in a study conducted in Cameroon, the presence of *Entamoeba* was associated with expansion of members of the *Prevotella* family in farmers and fishermen from Southwest Cameroon (Morton *et al*. 2015). The human gut microbiota functions as the microbial organ within the intestine, it plays a vital role in protective functions against pathogens (O'Hara and Shanahan 2006). The imbalance of the microbiota within the host intestine can lead to disease susceptibility, which shows that the microbial community structure is a very important factor that can influence predisposition to specific diseases in certain host contexts (Ley *et al*. 2006). The present study aims to characterize the microbiome of patients with

Entamoeba infection to identify if *P. copri* is associated with the modulation of the key aspects of the host inflammatory immune response.

Amebic infection can be asymptomatic or symptomatic, but the transition from colonization to invasive diseases is still unclear. It has been suggested that both the parasite and host factors play a significant role in the outcome of *E. histolytica* infection, however this does not explain why only a subset of infected individuals develop symptomatic diseases (Gilchrist *et al.* 2016). This suggests that there are other factors to explain this transition. Therefore, this study sought to look into such factors. Protozoan parasites secrete extracellular vesicles to promote growth and induce transmission, evade the host immune system, and promote their own survival. Understanding the broader role of these vesicles in virulence will help define the genetic program that is required for invasion of the host, identify new biomarkers of disease progression, and lead to a better understanding of the parasite factors that contribute to invasive diseases. There is no published data on the presence of exosomes in *E. histolytica*. Therefore, there is an urgent need to investigate the presence of exosomes or extracellular vesicles in *E. histolytica*. This can improve our knowledge on *E. histolytica* virulence. Exosomes have been shown to modulate the function of distant cells along the gastrointestinal tract, or regulate the homeostasis of gut microbiota, through delivery of antimicrobial products(Hu *et al.* 2013). Exosomes released basolaterally into the mucosa may also regulate local innate responses to invading bacteria through microbicidal activity (Hu *et al.* 2013).

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine that is a mediator of the innate immune response (Roger *et al.* 2016; Yao *et al.* 2016) Intestinal epithelial cells express the MIF receptor (Morris *et al.* 2014). However, the effect of MIF homologs on the intestinal epithelium and mucosal inflammation during amebic infection is unknown. Therefore, this study examined the role of *E. histolytica* MIF (*Eh*MIF) in inflammation using *in vitro* approaches, mouse model and in persons with intestinal amebiasis.

1.3 Research questions

Briefly, the primary research questions for the study were as follows:

a. Do novel species of *Entamoeba* contribute to diarrhea in the South African population?
b. Does the species variation within the genus contribute to the infection outcome?
c. What is the impact of gut microbiota in the immune response during amebic infection?
d. Does co-infection contribute to the virulence of *E. histolytica*?
e. What is the role of *Entamoeba* macrophage inhibitory factor (*Eh*MIF) during amebic infection?
f. Does *Entamoeba histolytica* produce exosomes?

1.4 Aim of the study

To determine the impact of enteric pathogens and secreted extracellular vesicles on amebic virulence and the outcome of infection.

1.5 Primary objectives of the study

a. To investigate the distribution and prevalence of protozoan parasites in South Africa.

b. To investigate novel species of *Entamoeba* circulating in the South African population.

c. To elucidate the impact of gut microbiota and immune response during amebic infection.

d. To determine the role of *Eh*MIF during amebic infection.

e. To investigate the impact of co-infections on the outcome of amebiasis.

f. To determine the presence of secreted extracellular vesicles/Exosomes in *E.histolytica*.

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CHAPTER TWO

Literature review

CHAPTER 2

2.1 Discovery of *E. histolytica*

Investigations leading to the identification of *Entamoeba histolytica* as a causative agent amoebiaisis was first reported by Hippocrates (460-377 B.C). In 1875, Fedor Losch explained the case of a young farmer who was admitted to his clinic in Saint, Petersburg (Lesh 1975). The patient was suffering from chronic dysentery, Fedor Losch isolated what he believed to be a causative agent and he named it *Entamoeba coli* from a patient who was diagnosed of amebiasis for the first time in history. In 1913, in the Philippines, Walker and Sellerds recorded the cysts form of this parasite as the infective stage. While initially the name *Entamoeba histolytica* was used to describe all the morphogenically identical ameboid organisms (Lesh 1975) *Entamoeba dispar* was recorded as the non-pathogenic organism by Brumpt, 1925 although the parasitic *E. histolytica* and non-pathogenic *E. dispar* were morphologically similar and difficult to differentiate by microscopy. It was also discovered that the pathogenic *E. histolytica* can asymptomatically colonize the human host so it was controversial whether *E. dispar* constituted species separate from *E. histolytica*. The controversy was resolved when conserved molecular differences were identified in *E. dispar* (Tannich *et al.* 1989) and the redescription of the two as separate species was published by Diamond et al (Diamond and Clark, 1993).

With the application of new molecular biology- based techniques, tremendous advances have been made in the knowledge of the diagnosis, natural history and epidemiology of amebiasis (Huston *et al*. 1999).

2.2 Morphology of *Entamoeba histolytica*

Using a light microscope, two morphological distinct life stages of the parasite are identified in infected specimens, namely: cysts and the trophozoites. The cyst is considered as the infective stage of the parasite, they are round in shape with a 10-15μm in diameter (Stanley 2003). They consist of only four nuclei, which are surrounded by a rigid chitin wall, protecting the amoeba outside the human body. The four nuclei, each contains a small, centrally located karyosome and the nuclear membrane is uniformly lined with the peripheral chromatin (**Fig 2.1. A**).

The trophozoites, also known as the diagnostic stage of the parasite, are 10- 50 μm in diameter (Stanley 2003). It consists of a single nucleus with a small centered karyosome and its cytoplasm and it lacks most eukaryotic organelles such as mitochondria, golgi complex, structure cytoskeleton and the reticulum (Cavalier-Smith 1987). It was later discovered that *E. histolytica* has the mitochondria like organelles defined as mitosomes. They harbor a small number of mitochondrial marker proteins and are surrounded by a double membrane (Aguilera *et al.* 2008). The full diversity of their content and function, however, has not been fully determined (Mi-ichi *et al.* 2009). The cytoplasm contains the granular endoplasm containing abundant vacuoles. (**Fig 2.1. B**).

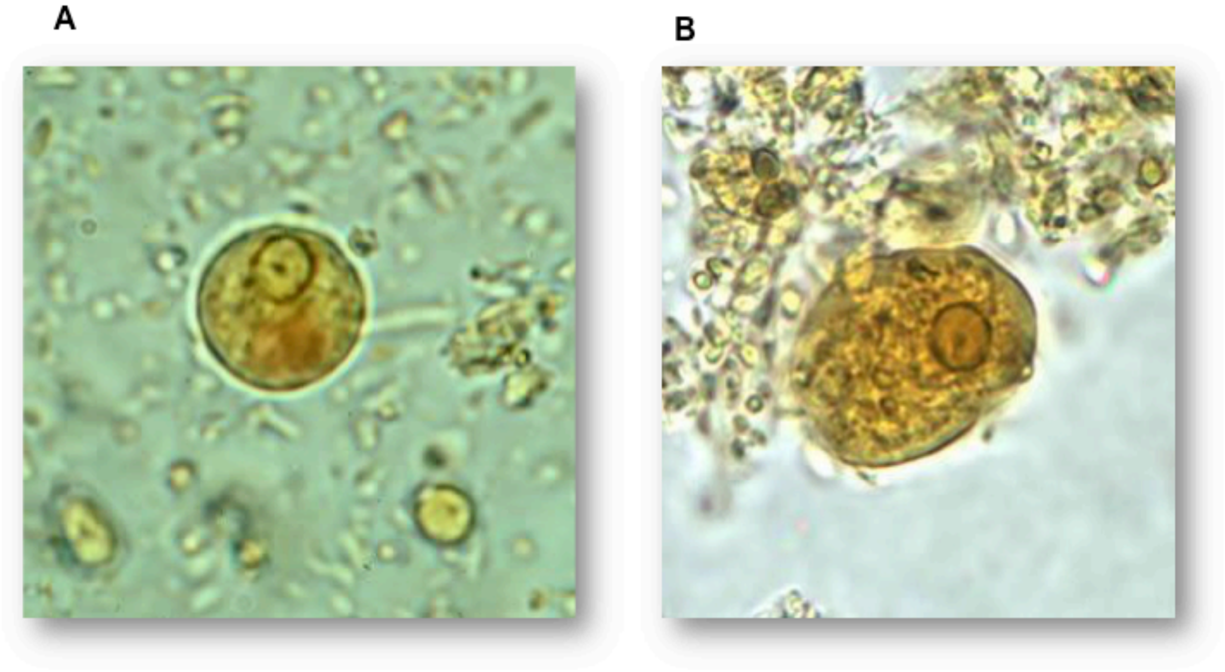

Fig 2.1: Diagrammatic representation of the morphological forms of *Entamoeba spp* as seen during light microscope. (A) Cyst form and (B) trophozoite form (Centers for Disease Control and Prevention, 2017).

2.3 Epidemiology of *E. histolytica*

Entamoeba histolytica is an enteric parasite with a global distribution, with high morbidity and mortality occurring in developing countries (Fletcher *et al.* 2012). Of the infected individuals, only 10% develop symptomatic diseases while 90% remain asymptomatic. To date it estimated that *E. histolytica* causes 34-50 million symptomatic infections worldwide, which result in 100 000 deaths every year (Wilson *et al*.2012). Until recently, scientists were not able to visually differentiate *Entamoeba* species, this led them to overestimate the prevalence of amebiasis. The true prevalence of *E. histolytica* is close to 1% worldwide (Haque *et al.* 2003). Amebic infection is prevalent in the Indian subcontinent, Africa, the Far East and areas of south and Central America (Li and Stanley 1996). It has been shown that the prevalence and presentation of both asymptomatic and symptomatic amebiasis vary with age, geographic location and with the population of individuals affected, differing between countries and areas with socio- economic conditions. The epidemiological studies have shown that low socio-economic status, poor sanitation and inadequate water supply are potential risks of *E. histolytica* infections (Guven 2003).

2.3.1 Amebiasis: Specific social and demographic characteristics

Iran:

A survey of intestinal parasite among restaurant workers was done all over the world, a study by Garedaghi and Mashaei (2011), showed that the prevalence of *E. histolytica* is 14.4% in Tabriz. It was concluded that the infected restaurant workers with or without clinical signs are risk factors for public health of the society, because contaminated food plays a major role in the occurrence of diarrheal diseases (Sheth and Dwivedi 2006).

Japan:

In East Asian countries, invasive amebic diseases caused by *Entamoeba histolytica* are increasing among HIV-infected patients and co-infection of amoeba and HIV-1 is an emerging problem in these countries. A study by

Watanabe *et al.* (2014) reported the prevalence of *E. histolytica* to be 11.3% from a total of Seventy-one asymptomatic human immunodeficiency virus-1 (HIV-1) -infected individuals who underwent colonoscopy for detection of diseases other than amebiasis. They identified Ulcerative lesions caused by *Entamoeba histolytica* by colonoscopy and biopsy in eight(8) patients. This study also showed that Stool microscopic examination was less sensitive compared to indirect fluorescent antibody assay (Watanabe *et al.* 2014).

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Turkey:

A study in the villages of Sivas region Turkey, investigated the prevalence of *E. histolytica* in the stools of children attending primary schools in the villages of Silvas using native lugol, trichome staining and monoclonal antigen detection (Malatyali *et al*. 2011). Twenty-two children (1.5%) were positive for *E. histolytica* cysts, but all the samples were antibody negative when using the ELISA test. Although not all serocovert the absence of any positive children lead the authors to postulate that direct microscopic diagnosis of amebiasis is not an efficient method and that other identical non-pathogenic amebas such as *E. dispar* may be resposible for the cyst positive samples (Malatyali *et al*. 2011). This study suggests that for the diagnosis of *E. histolytica* molecular methods are preferable for diagnosis. Immunodiagnostic ELISAs based on reliable antigens such as surface adhesins to be used for detection (Ravdin 1995) , PCR, and izoenzyme assays tests can also be used for the differentiation of *E. histolytica* and *E. dispar* (Lau *et al.* 2013).

Asia:

India is among the other countries with high rates of amebic infection. A study by Parija *et al.* (2014) showed that 15–20% of the Indian population is affected by *E. histolytica.* In Malaysia, intestinal parasitic infections, including *E. histolytica*, are more prevalent in rural areas. It was found that the overall prevalence of *Entamoeba* infection was 17.6%, where females were more prevalent than males and among the *Entamoeba* infections, *E. histolytica* was the most common with a prevalence of 75% (Ngui *et al.* 2011).

A study by Mengeloğlu *et al.* (2009), observed 44 (0.37%) of amebic cysts out of 1720 stool specimens by direct microscopy, the samples were further analysed using ELISAs to detect specific antigens of *E. histolytica* and confirmed that 59.1% of the tested individuals were *E. histolytica* positive. A study by Nath *et al.* (2015) detected three species of *Entamoeba* (*E. histolytica/E. dispar/E. moshkovskii*) in an Indian population. This study showed an overall prevalence of *Entamoeba* species to be 23.2% in North Eastern states of India. The prevalence of *E. histolytica* was shown to be 13.7% at the community level healthcare unit and hospital using DNA dot blot and species specific PCR assays which enabled them to report the true prevalence of *E. histolytica* in India (Nath *et al.* 2015).

Thus in Asia the diagnostic sensitivity and accuracy was improved by using molecular techniques in epidemiological studies. These showed that the *E. histolytica* parasite was a common parasite in Asian populations. New techniques such as real-time PCR are now being deployed in epidemiological studies(Lau *et al.* 2013). In Bangladesh, it has showed the prevalence of *E. histolytica* to be 17.5%, by real-time PCR and this infection increased with age. This study demonstrates the alarming parasitic infection in the children suffering from diarrhea in an urban slum area of Dhaka (Ahmed *et al.* 2016).

Canada:

In 2010, in Canada, 637 cases of *E. histolytica* were reported to the national enteric surveillance program and of the 637 cases reported, 29 were reported as being travel- associated (Canadian Institute for Health Information 2010).

African Continent:

There are little data on the true prevalence and incidence of *Entamoeba histolytica* infection in Africa. This is due to the inability, historically, to differentiate *Entamoeba histolytica* from the more common, but non-pathogenic, *Entamoeba dispar*. Studies in South Africa, Kenya, Mozambique, Egypt, Sudan, and Nigeria have reported different *E. histolytica* prevalence rates. A study in South Africa in the Vhembe district on the prevalence of intestinal parasitic and bacterial pathogens in diarrheal and non- diarrheal human stools, found that 34.2% *E.histolytica/dispar* were the most common cause of diarrheal infection (Samie *et al.* 2009).

A second study was conducted in South Africa to determine the prevalence of intestinal parasites in primary school children of Mthatha, and relate this to their socio-economic status. This study found that the prevalence of *E. histolytica /dispar* was 6.8%(Nxasana *et al.* 2013).

These studies suggests that *Entamoeba* infection is still prevalent in South Africa. Recently, a study conducted in Mozambique, assessed the prevalence and molecular diversity of enteric parasites among HIV- and/or TB-infected patients at a medical reference centre in Chowke district. This study reported the overall prevalence of *E. histolytica*/*dispar* to be 6.1% of the examined specimens by real-time PCR. However in this study five stool samples that were microscopically positive for cysts of *E. histolytica*/*dispar* failed to yield detectable real-time PCR products. This confirms other studies which report that microscopy is insensitive and unreliable (Irisarri-Gutiérrez *et al.* 2017).

Amir Saeed (2011) used both microscopy and PCR methods for detection of *Entamoeba histolytica* in Sudanese patient. By microscopy 196 stool samples were reported as positive for *E. histolytica*. The PCR assay only 54% (106 of 196) as caused by *E. histolytica* were positive. As a control 50 negative stool samples examined by microscopy were subjected to a test by PCR and were found negative, suggesting that microscopy is not reliable and it is insensitive. Therefore, PCR is the most specific and sensitive method for detection and accurate identification of *Entamoeba* species in stool samples (Amir Saeed 2011).

In Kenya, faecal samples were tested for a wide range of infectious agents. Among all the infectious agents tested, *E.histolytica/dispar* was the second agent with a high prevalence of (30.1%) (Fèvre *et al.* 2017) . In Nigeria, a study of the prevalence of intestinal parasitic infection was conducted among children aged 5-16 years in primary schools in rural parts of Imo State and it was reported that *E. histolytica* (36.6%) was the highest occurring protozoan parasite in this population, this infection was found to be high (44.4%) in children aged 5-7 years (Ugochi *et al.* 2015). The presence of these parasites could be as a result of other factors such as poor toilet facilities, children playing in filthy environment as well as the geophagus habit of the children(Ugochi *et al.* 2015).

2.4 The genome of *Entamoeba histolytica*

The genome of *E. hstolytica* is about 24mb in size, with a number of 14 chromosomes and is functionally tetraploid (Clark *et al.* 2006). Both linear chromosomes and a number of plasmid-like molecules are present in the *Entamoeba* genome, however the structure and organization of *E. histolytica* chromosomes is not well understood. The G +C content is about 22.4% with the coding regions of approximately 33% (Willhoeft *et al.* 2001). The current assembly predicts that the genome of *E. histolytica* contains about 10,000 genes (Gardner *et al.* 2002), each averaging 1.7kb in size, comprising an approximately 49% of the genome (Loftus *et al.* 2005).

The organization of structural RNA genes in *E. histolytica* is unusual with the rRNA genes carried exclusively on 24kb Circular episomes (Bhattacharya *et al.*1998) that have two transcription units in an inverted repeat. It was suggested that these episomes make up about 20% of cellular DNA in total. There are many circular DNA molecules consisting of different sizes but with unknown functions (Dhar *et al.* 1995). The *E. histolytica* genome contains about 86 peptidases genes including 50 cystein, four aspartic, 10 serine and 20 metallo peptidases (Loftus *et al.* 2005; Clark *et al.* 2007).

Among the 50-cysteine proteases found in *Entamoeba* species, only Ehcp1 and Ehcp5 are found only in *E. histolytica*. The parasite also contains a large gene family of 90 transmembrane kinases, which has a role in pathogenesis of human erythrocytes (Boettner *et al.* 2008). The genome has been proven to encode for 91 Rab guanosine triphosphate (GTpases) that are involved in many aspects of vesicle trafficking, for example, Rab113 is involved in secretion of cysteine proteases (Nakada-Tsukui *et al.* 2010).

2.5 Life cycle of *E. histolytica*

Entamoeba histolytica's life cycle begins when the cyst enter into the small intestine through consumption of food or water contaminated with the parasite' cyst. When the cysts get into the small intestine they go through the excystation process to produce trophozoites, the life stage who asexually replicate by binary fission (Centers for Disease Control and Prevention 2017).

The trophozoites remain confined to the intestinal lumen of individuals who are asymptomatic carriers, passing cysts in the stools, however, in some patients the trophozoites invade the intestinal mucosa, where it causes intestinal amebiasis, or through the bloodstream, extra- intestinal sites, such as liver, brain and lungs, which can result in extra- intestinal diseases, such as liver abscess (Centers for Disease Control and Prevention, 2017). The life cycle of *E. histolytica* is shown in **fig 2.2** below.

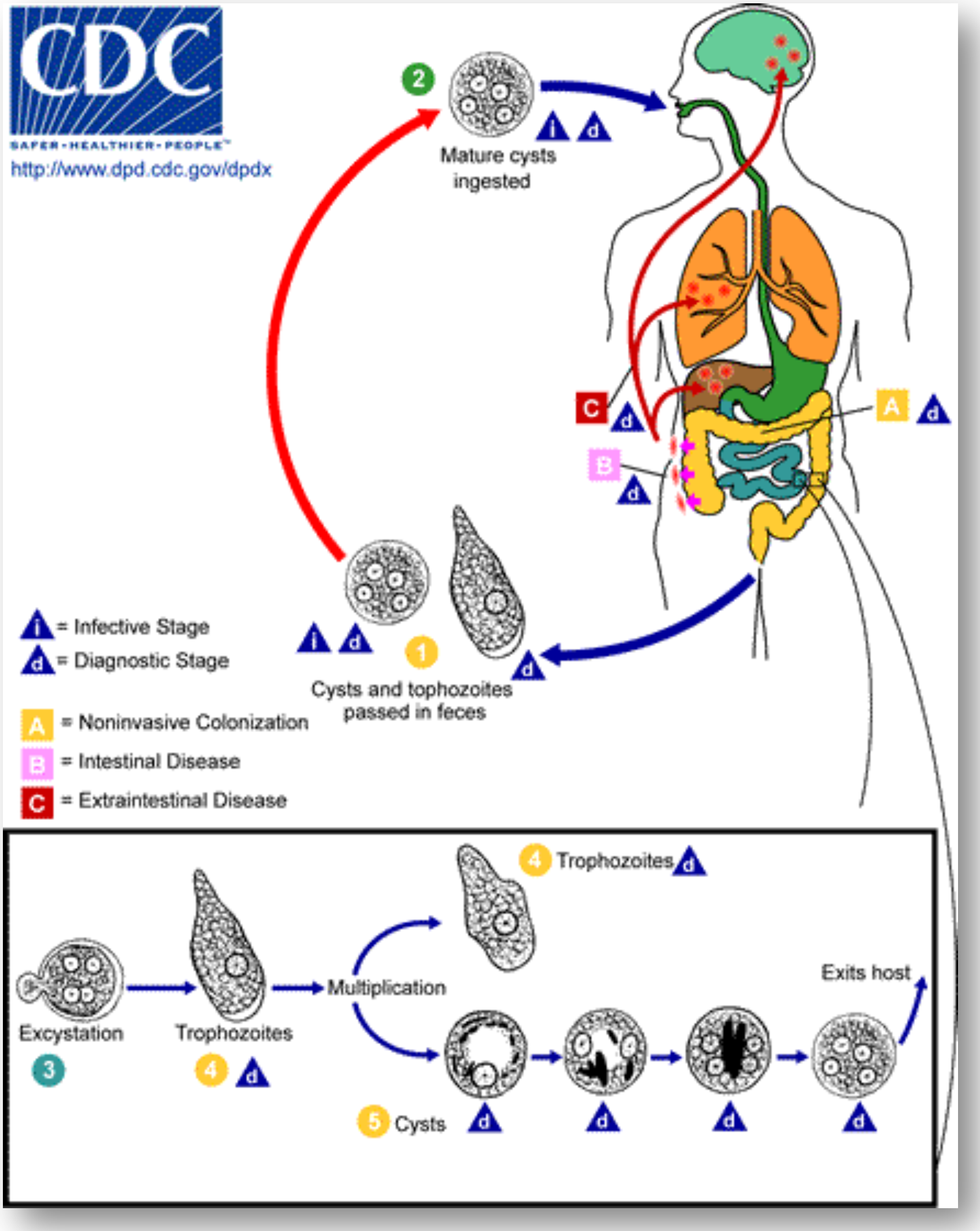

Fig 2.2: Life Cycle of *E. histolytica*. (Centers for Disease Control and Prevention, 2017)

2.6 Pathogenesis and invasion of intestinal mucosal epithelium by *E. histolytica*

The infection with *E. histolytica* is divided into two forms: luminal amebiasis (Asymptomatic) and invasive amebiasis where the trophozoites invade the intestinal mucosa to produce dysentery. The trophozoites adhere to the mucus layer (Beck *et al.* 2005). The mucus layer serves as the first line of defense against enteric pathogens, it prevents the attachment of pathogens to the intestinal wall and the intestinal bacterial flora prevents the amebic lodgment (Loftus *et al.* 2005). However, the pathogenic parasites secrete virulent enzymes that facilitate their invasion into the mucosa and sub-mucosa. Depletion of this layer allows the trophozoites to come into contact with the epithelial cells. The important factors in pathogenesis of *E. histolytica* are adherence, cytotoxicity and disruption of tissues. The proteins that play a role in this process include the Gal/GalNAc lectin, amebapore and proteases (Bracha *et al*. 2002).

Gal/GalNAc lectin is one of the common virulent proteins of *E. histolytica*, it is an important protein which plays a crucial role in killing of host cells, it acts against cells such as erythrocytes, neutrophils lymphocytes and cells of human intestinal epithelium. The process of cell killing occurs through adhesion to target cells via Gal/GalNAc lectin, the protein binds to exposed glycoproteins and kill the cells within 5- 15 minutes (Clark *et al.* 2007). Once the parasite develops contact with the host cells, it causes cytolysis, which then results in swelling and lysis of target cell.

Amoebapore is another protein that has the cytolytic effect towards human host cells (Clark *et al.* 2007). It kills metabolically active eukaryotic cells and display antibacterial activity (Leippe *et al.* 2005). Proteases are enzymes that degrade other proteins and could contribute to the pathogenesis of *E. histolytica*. The cysteine proteases appear to have multiple roles in amebic invasion. It disrupt the polymerization of MUC2, the major component of colonic mucus, making the mucus layer less permeable, blocking adherence of trophozoites to epithelial cells. Destruction of extracellular matrix (ECM) by protease may also facilitate trophozoite invasion (Huston 2004). **Fig 2.3 A and B** shows the pathogenesis and the virulence factors of *E. histolytica.*

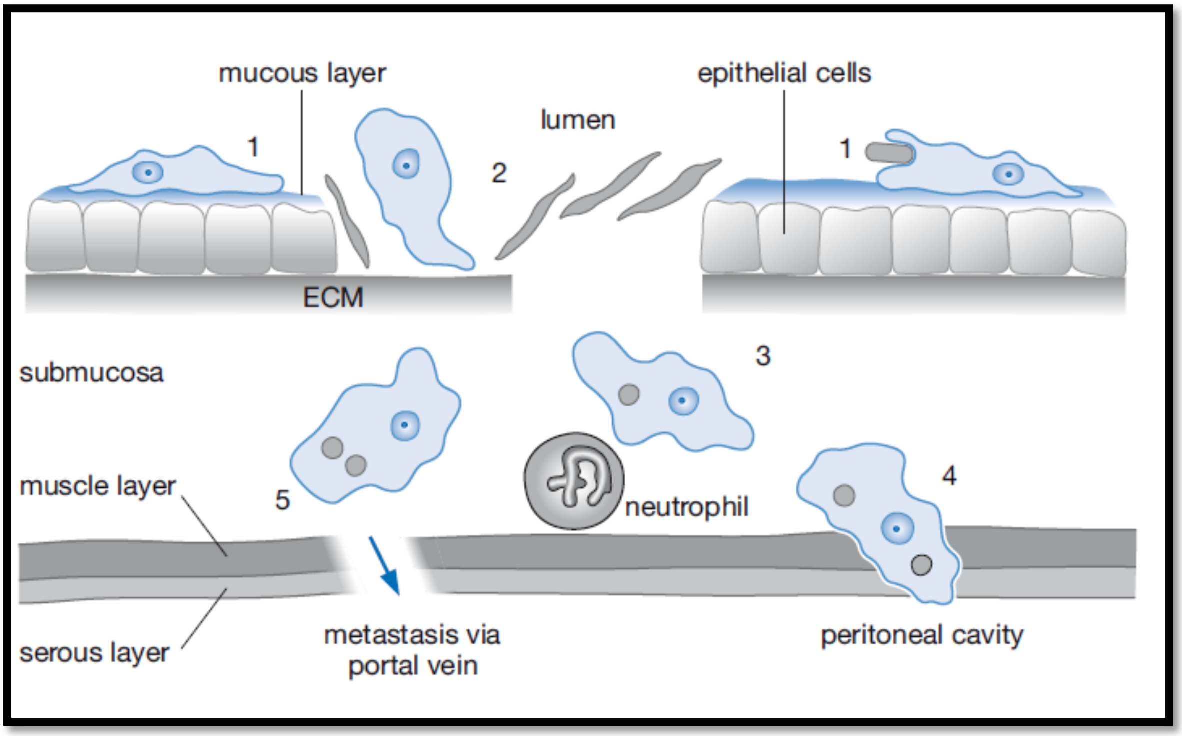

Fig 2. 3: (A): Pathogenesis of amebiasis.(figure reproduced with authors permission(Huston 2004).

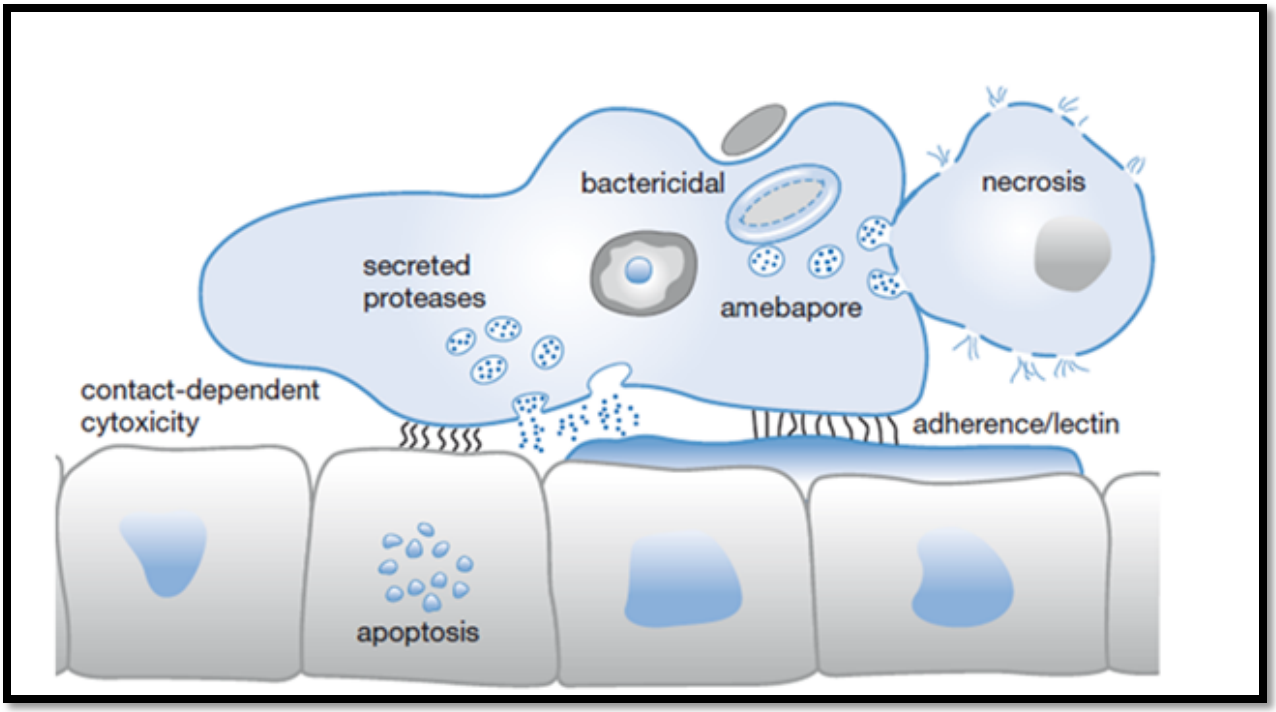

Fig 2.3: (B): Schematic presentation of virulence factors of *E.histolytica.* (figure reproduced with authors permission)(Huston 2004)

2.7 Tissue destruction by *E. histolytica*

Invasion of the intestinal mucosa by *E. histolytica* resulting in mucosal inflammation is a hallmark of amebic colitis. The mucosal inflammatory response contributes to intestinal tissue destruction with proinflammatory cytokines playing a major role. Little is known about the key parasite mediators of host immunopathology. Mammalian MIF promotes inflammatory cytokine production and few studies have implicated a crucial role for MIF in murine colitis and inflammatory bowel disease (Moonah *et al.* 2014). This study have identified a MIF homolog in the *E. histolytica* genome and discovered that, like mammalian MIF, *E. histolytica* MIF (*Eh*MIF) has proinflammatory properties. *Eh*MIF is capable of immunomodulating host macrophages by inducing IL-6 production and enhancing TNF-α secretion (Moonah *et al.* 2014). These are exciting and important findings with great implications for enhancing our understanding of host-parasite interactions during amebiasis. This will again help us to further understand and explore potentially novel mechanism of *E. histolytica*-induced mucosal inflammation and immunopathology.

The present study hypothesized that *Entamoeba histolytica* homolog of the upstream inflammatory cytokine macrophage migration inhibitory factor (MIF) mediates the destructive host inflammatory response seen in amebic colitis (**Fig 2.4**). There is currently no vaccine and only a single class of drugs for treatment of this devastating disease. Thus, an understanding of the interaction of parasite with host, particularly the immune response toward *E. histolytica,* would greatly enhance the ability to develop effective immunotherapies.

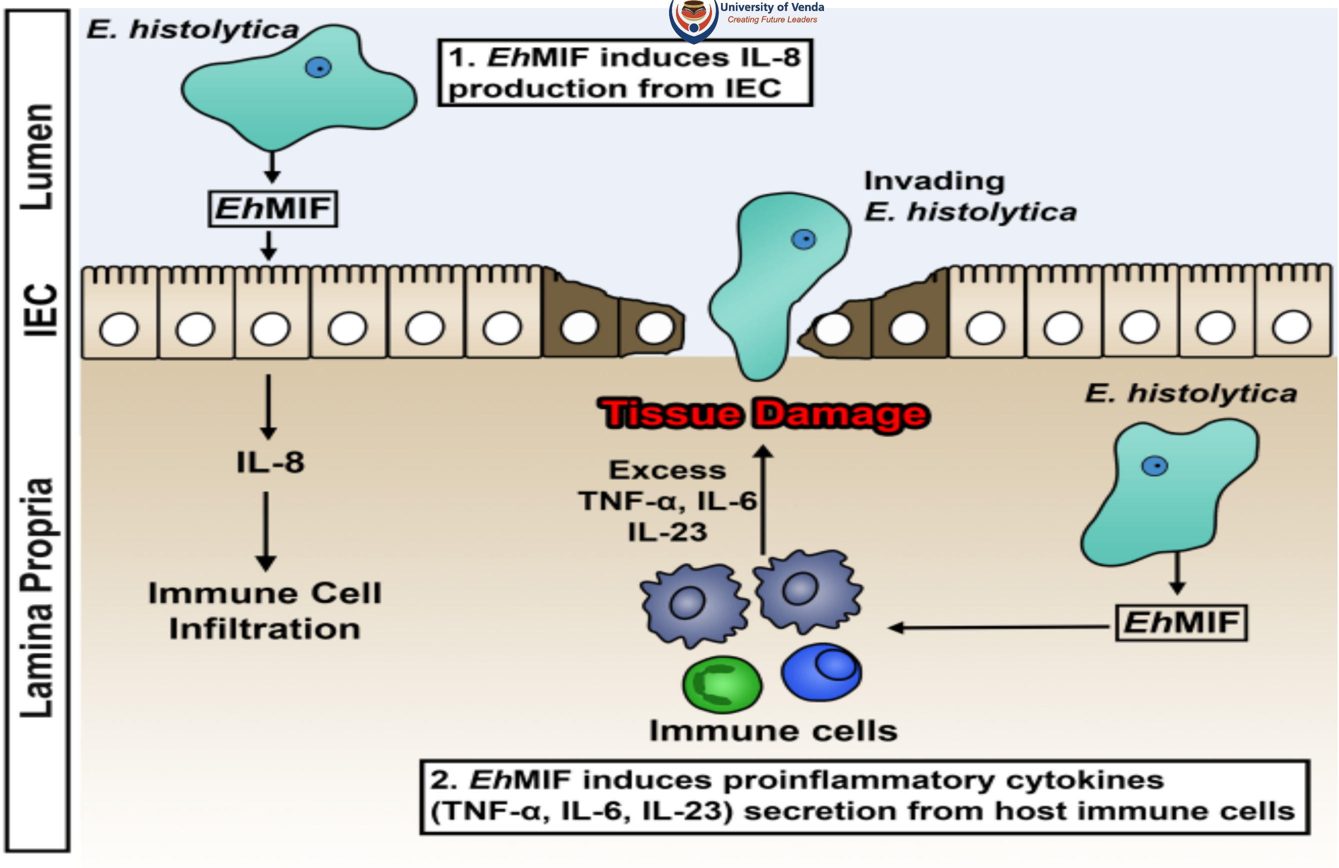

Fig 2.4: Hypothesis: *Eh*MIF is a key parasite mediator of the destructive host immune response during amebic colitis. (figure reproduced with authors permission) (Moonah *et al.* 2014)

2.8 Functions of *Eh*MIF

The proinflammatory cytokine mammalian MIF is a critical upstream mediator of the innate immune response. Proinflammatory functions include the following: (i) MIF stimulates the secretion of inflammatory mediators, such as IL-8 and IL-6; (ii) MIF enhances TNF-α production by lipopolysaccharide (LPS)-stimulated immune cells. It enhances the host response to LPS by upregulating Toll-like receptor 4 (TLR4), the receptor for LPS; and (iii) MIF counter-regulates the anti-inflammatory activities of glucocorticoids (Merk *et al.* 2011; Bai *et al.* 2012).

Glucocorticoids are locally synthesized in the intestine and play an important role in maintaining intestinal homeostasis (Atanasov *et al.* 2008; Noti, Corazza, Tuffin, *et al.* 2010). Glucocorticoids downregulate the inflammatory immune response and mediate repair of damaged mucosa. It is reasonable to assume that the glucocorticoid-overriding activity of MIF disrupts the tightly regulated mucosal immune response, creating a proinflammatory state in the gut. MIF homologs have been identified in several pathogenic protozoans including *Plasmodium*, *Toxoplasma* and *Trichomonas*. These protozoan MIF homologs have demonstrated proinflammatory activities similar to that of mammalian MIF(Sun *et al.* 2012; Sommerville *et al.* 2013; Twu *et al.* 2014)

2.9 Exosomes/ extracellular vesicles

2.9.1 The role of extracellular vesicles in infectious diseases

Extracellular vesicles are defined as small cellular particles of 30- 100nm that are conserved in all cell types, they are released by bacteria, fungi, viruses and parasites and they can be isolated from serum, blood urine, cell culture and saliva (Théry *et al.* 1999). The presence of extracellular vesicles have been described by immunologists a couple of decades ago, and by the time they were just taken as some garbage cans that are used by the cells to get rid of unwanted cellular componets, so for this reason, they remained less studied for a period of time (Théry 2011). Recently, previously unrecognized communication route was discovered, showing that these vesicles have a significant role of intercellular communication between cells, they act as messengers that transport information from distant cells (EL Andaloussi *et al.* 2013). Extracellular vesicle/exosomes produced during an infection can be pathogen or host derived (Acevedo *et al.* 2014). Intracellular and extracellular protozoan parasites secrete extracellular vesicles such as exosomes to promote growth and induce transmission, to evade the host immune system, and promote their own survival. Exosomes are produced from the multivesicular bodies (MVBs). The membrane of the MVBs bulges inward to form exosomes. During this process, proteins (e.g., receptor, cytoplasmic proteins, tetraspanin), nucleic acids (e.g., DNA, mRNA, miRNA), and lipids (e.g., cholesterol, ceramide) are packed into exosomes in a cell type-dependent manner (Zhang *et al.* 2015). MVBs fuse with the cellular membrane to release exosomes into the extracellular space. Several mechanisms have been suggested to mediate the uptake of exosomes, including exosome fusion with the cellular membrane of the recipient cell, leading to the release of the exosomal cargo into the cytoplasm. The figure below shows Biogenesis of exosomes.

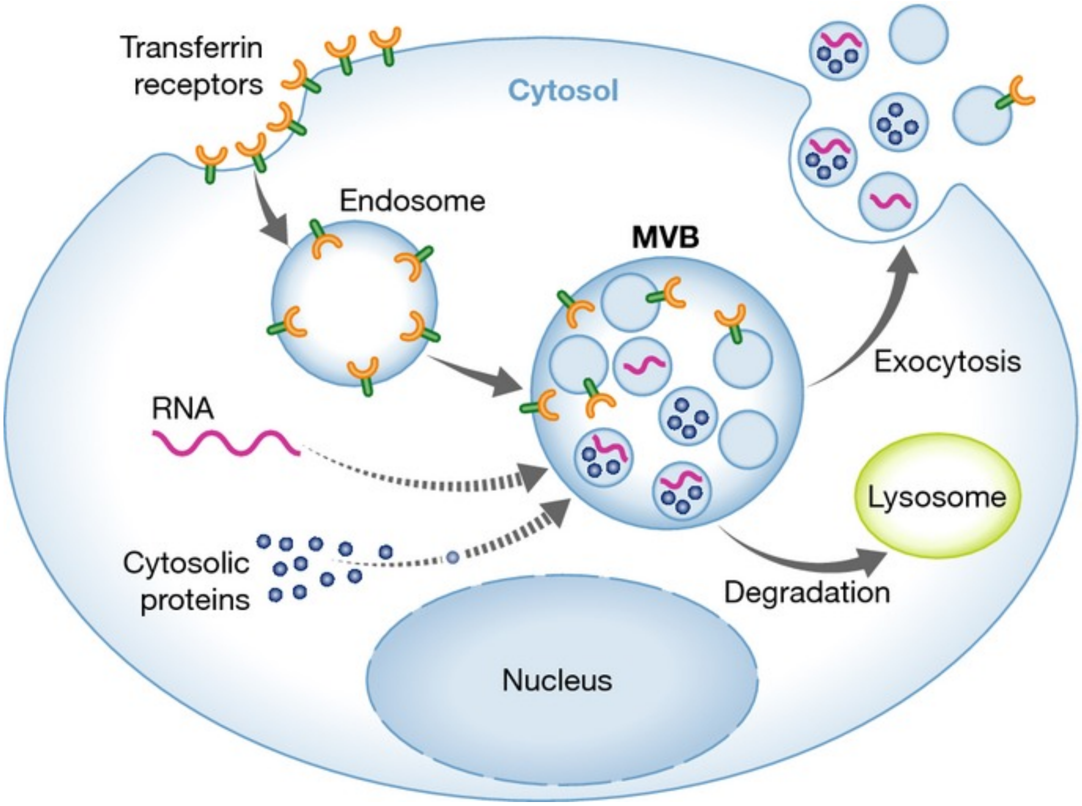

Fig 2.5: Biogenesis of exosomes (Schorey *et al.* 2015)

2.9.2 Exosomes in host-pathogen interation

Extracellular vesicles or exosomes are broadly defined as membrane-bound vesicles released from cells. The subject of exosome release in response to eukaryotic parasitic infection is a fascinating one, as exosomes are shown to be released by the eukaryotic parasites themselves as a part of their pathogenicity (Torrecilhas *et al.* 2012). These vesicles contain a diverse suite of molecules including proteins, lipids, and nucleic acids, some of which are known to have immunomodulatory properties. Vesicles can function by transmitting signals between parasites, from parasite to host, or from host to the environment for antigen presentation and other aspects of host defense.

Many parasites are able to successfully modify the activity of the host immune system using highly sophisticated mechanisms such as immune evasion, molecular exploitation and molecular piracy, parasitic infections are typically chronic and allow the long lasting persistence of the parasite and survival of the host (Damian 1997). The description of parasite extracellular vesicles (EVs) in protozoa and helminths suggests that they may play an important role in host–parasite communication. The secretion of exosomes/extracellular vesicles has been described in different parasites, viruses, fungi and bacteria (Théry *et al.* 1999).

Several protozoan parasites have been shown to release exosomes and/or microvesicles. Recently, it has been discovered that parasitic helminths produce exosomes. This was initially reported in the excretory-secretory components of the trematodes, *Echinostoma caproni* and *Fasciola hepatica*, which infect the gastrointestinal tract and liver respectively (Marcilla *et al.* 2012), and in the nematode *H. polygyrus,* which infects the small intestine (Buck *et al.* 2014). Another study by Martin-Jaular *et al.* (2011) have described exosomes in malaria using mice model plasmodium yoeli 17X-BALB/c, this study demonstrated that that exosomes derived from infected reticulocytes contained host and parasite proteins and had a role in modulating immune responses. Exosomes were isolated from plasma of mice and from reticulocyte culture by differential centrifugation and their identity was confirmed by electron microscopy, FACS and proteomic analyses. Studies on EVs and exosomes of other apicomplexa have been reported. Trypanosomes are able to produce and release different types of vesicles into the extracellular milieu that play important roles in the parasite–host interaction, enabling pathogen survival and replication within the host (Geiger *et al.* 2010). The existence of EVs (65 nm) in MVBs has been detected in *Toxoplasma* secretory organelles (Torres *et al.* 2013). Lately, the presence of miRNA in *T. gondii* exosomes has been reported (Pope and Lässer 2013). A study by Twu *et al.* (2013) have found that *T.vaginalis* produces and secretes microvesicles with physical and biochemical properties similar to mammalian exosomes. These exosomes were characterized by the presence of RNA and conserved exosomal proteins as well as parasite-specific proteins. They also demonstrated that *T. vaginalis* exosomes fuse with and deliver their contents to host cells and modulate host cell immune responses.

Trypanosoma Cruzi also induces the release of microvesicles from infected host cells, including lymphocytes and monocytes *in vitro* and erythrocytes *in vivo*. These microvesicles express surface transforming growth factor beta (TGF-β), which has been shown to facilitate eukaryotic cell invasion by the parasite and leads to maturation and continuation of the life cycle (Cestari *et al.* 2012). It has been published that *Cryptosporidium parvum* infection increases luminal release of EVs from the biliary epithelium, probably through TLR4/IKK2-mediated activation of the multivesicular body exocytic pathway (Hu *et al.* 2013). Although knowledge of the contribution of exosomes in parasitic pathogens has been reported. There is no study describing the production of exosomes by *Entamoeba histolytica*. The present study is the first to report on the production of extracellular vesicles/exosomes by *E. histolytica.* Improved understanding of how this parasite adapts to, and manipulates their host environment is important in understanding the progression of the disease. Most parasites at some stage in their life cycle rely on the ability to communicate with one another and with their hosts, but the mechanisms underpinning this communication are still coming to light.

2.9.3 Role of Extracellular vesicles/ exosomes in pathogenesis

Extracellular vesicles/ exosomes have been shown to have an impact in the pathogenesis of viruses, parasites and bacteria. These vesicles are captured by cells upon release, and the transfer of host and viral proteins could increase the infection or inhibit the immune response. For example, in human CMV, the infected cells releases the microvesicles containing soluble DC-SIGN, a C-type lectin family molecule, in complex with CMV glycoprotein B. Release of this complex appears to increase the susceptibility of recipient cells to CMV infection (Plazolles *et al.* 2011). It has also been shown in HCV-infected patients, the interaction of the cellular membrane protein CD81 with HCV envelope glycoprotein E2 and the release of this complex within microvesicles and subsequent interaction with recipient cells increase the susceptibility of recipient cells to HCV (Masciopinto *et al.* 2004).

Exosomes released from HIV-1–infected peripheral blood mononuclear cells contain CCR5 and the transfer of this chemokine receptor to target cells enhances the susceptibility to HIV infection (Rozmyslowicz *et al.* 2003) Together, these results suggest that ExMVs released during viral infections enhance the infectivity of neighboring cells, preparing the way for the soon-to-be-released viral particles (**Fig 2.6**)

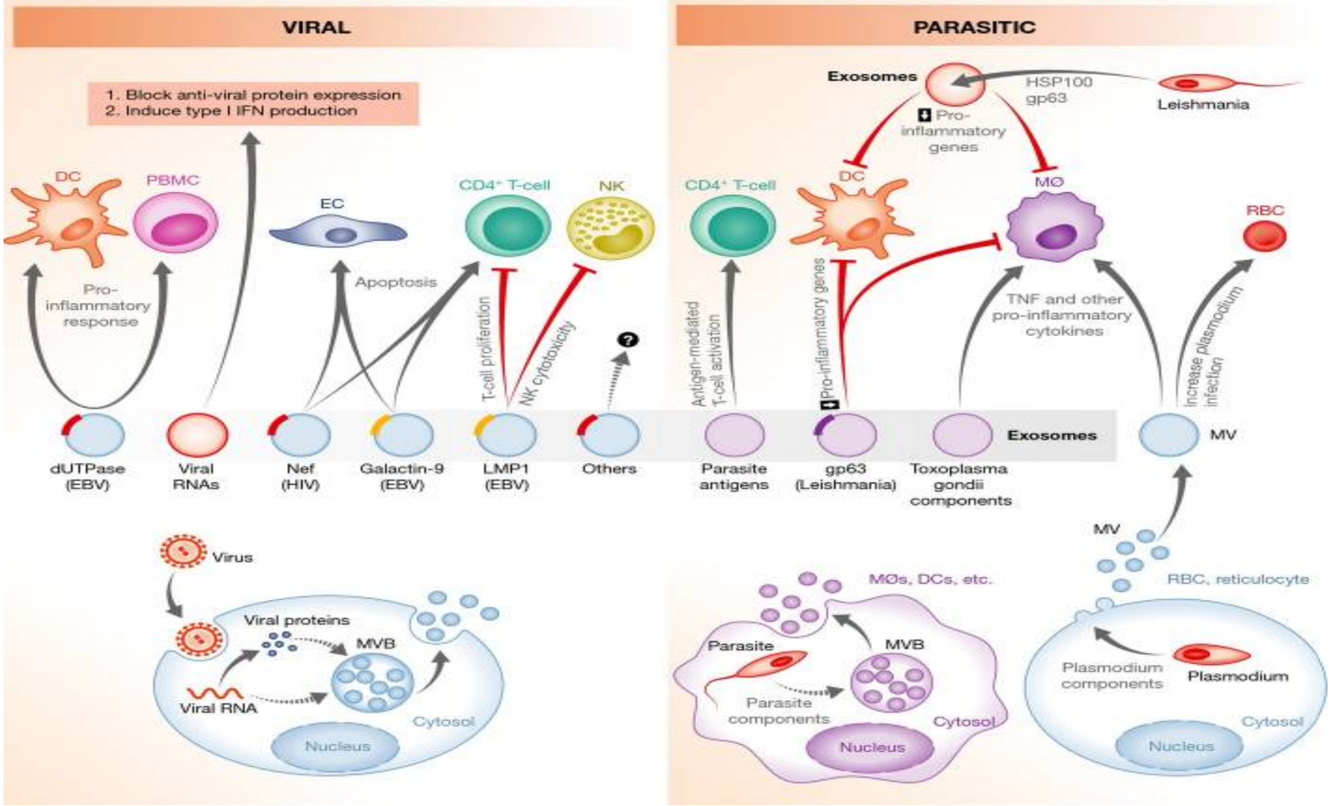

Fig 2.6: Modulation of host immunity by exosomes during a viral or parasitic infection (Schorey *et al.* 2015).

2.10 The host inflammatory response contributes to tissue damage

Invasion of the intestinal mucosa by trophozoites resulting in gut inflammation is a hallmark of amebic colitis. While immune cells represent the first line of defense, there is accumulating evidence that the immune response contributes to tissue damage seen in amebic colitis (Moonah *et al*. 2013). Similar to inflammatory bowel disease (IBD), cytokines play a major role in the pathogenesis of amebic colitis. TNF-α is a proinflammatory cytokine that plays a crucial role in gut inflammation, including the intestinal inflammation seen in amebic colitis. Higher TNF-α production was shown to correlate with *E. histolytica* diarrhea in children, and blocking TNF-α with neutralizing monoclonal antibodies was shown to reduce inflammation and intestinal damage in an amebic mouse model (Peterson *et al.*2010). Also, TNF-α was recently shown to mediate the tissue destruction seen in amebic liver abscess (Helk *et al.* 2013). Interleukin-10 (IL-10) is an anti-inflammatory cytokine and an important immunoregulator in the intestinal tract. IL-10 counteracts an exaggerated pro-inflammatory immune response by inhibiting the production of inflammatory mediators such as TNF-α. Naturally resistant B6 mice become highly susceptible to amebic colitis after disruption of the anti-inflammatory cytokine IL-10 gene, supporting the role of inflammation in *E. histolytica* pathogenesis (Hamano *et al.* 2006). There is a gap in the knowledge of key parasite mediators of host immunopathology.

2.11 Amebic infection

It is estimated that 50 million people have invasive amebiasis due to *E. histolytica* (Petri and Singh 1999), the organism colonizes the bowel system of the host for years without inducing clinical symptoms of the disease. In symptomatic patients, the trophozoites spread through the bloodstream, affecting organs such as lungs, brain and liver, that results in amoebisis of the lungs, brain abscess and liver abscess. It remains puzzling why only subset of infected individuals develops invasive diseases. This may be due to the differences in the pathogenic capability of the strains (Burch *et al.* 1991), or due to the differences in the host immune response against the infection. It has been suggested that both the parasite and host factors play a significant role in the outcome of amebic infection (**Fig 2.7**) (Gilchrist *et al.* 2012). Therefore the factors that govern the transition from colonization to invasion remain to be answered. Amebiasis is often asymptomatic but may cause dysentery (intestinal) and invasive extra-intestinal diseases (**Fig 2.8**)

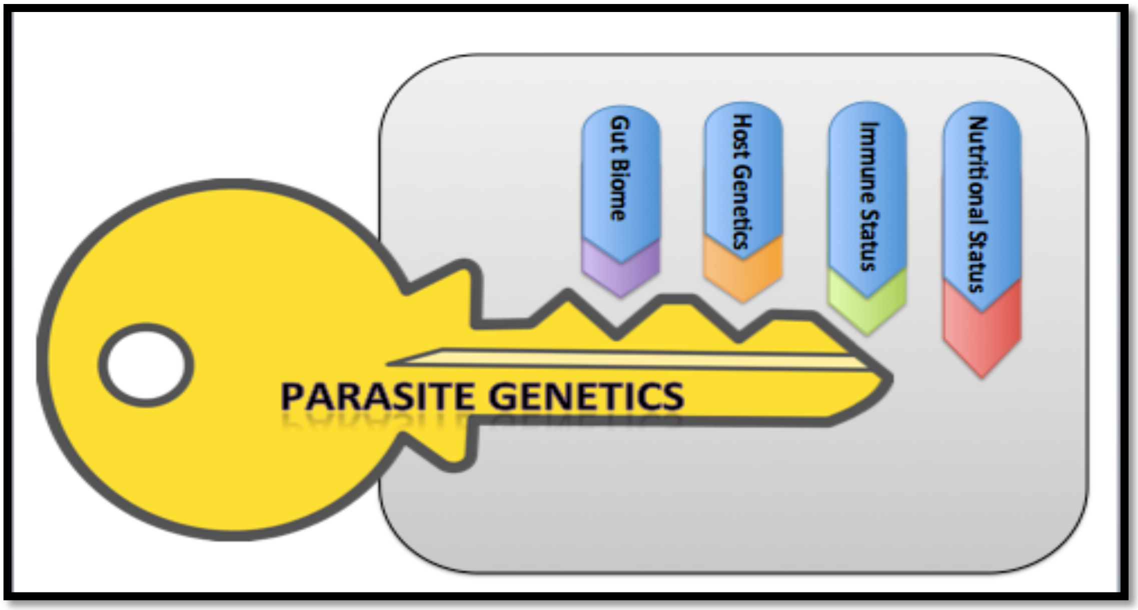

Fig 2.7: Parasite genetics represent a key that turns the lock of parasite infectivity (Used with permission from Carol Gilchrist)

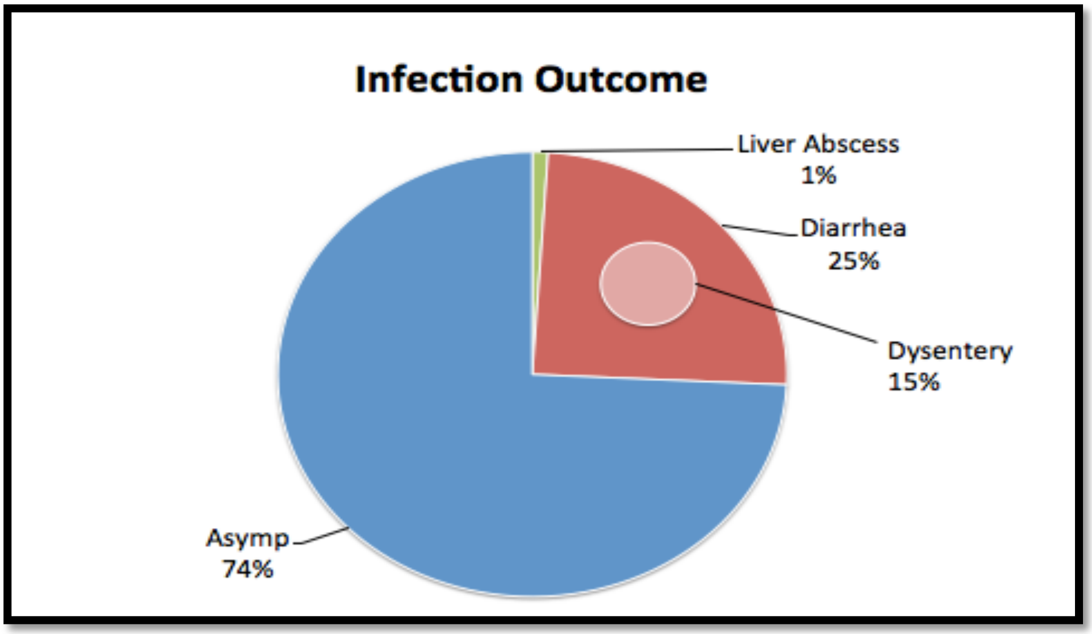

Fig 2.8: Amebiasis disease manifestations. (figure reproduced with authors permission) (Haque *et al.* 2006)

2.11.1 Intestinal amebiasis

Intestinal amebiasis is known as the most common type of amebic infection (Ximénez *et al.* 2011). The incubation period of intestinal amebiasis vary, but is generally 1 to 4 weeks. After this incubation period, the parasite invades the colonic mucosa, producing ulcerative lesions and bloody diarrhea, through lysis of the host cell tissues (Mortimer and Chadee 2010). Patients with intestinal amoebiaisis typically present with abdominal pain and tenderness, diarrhea, and bloody stools (Gilchrist *et al.* 2012). Amebic dysentery is an illness that may cause intestinal ulcers, mucus production and bleeding. Lesions are known to occur anywhere in the colon but they are frequently seen in the cecum and ascending colon and less frequently in the rectum and sigmoid (Alavi 2007).

Colonic lesion are characteristics of pathological lesions in the intestine, they vary from mucosal thickening only to flask-shaped ulcerations to necrosis of the intestinal wall (**Fig 2.9**). These intestinal lesions include: a colonic mucosa with mixed inflammatory infiltrate and congestion with presence of *E. histolytica*. Intestinal amebiasis can be characterized based on severity, this include acute and chronic amebiasis (Babić *et al.* 2016). In the acute one, the ulcers are confined to a large gut, with the lesions occuring in the mucosal layer. The lesion may be generalized (involving the whole length of the gut) or localized (involving the ileo-caecal or sigmoido-rectal regions) (Ackers and Mirelman 2006). The lesions in chronic amoebiaisis may be distributed in the mucosal and submucosal layer (**fig 2.10**). The lesions in the acute phase may be healed completely without leaving a scar while in the chronic phase; the ulcers may be healed with thickening of the intestinal granuloma.

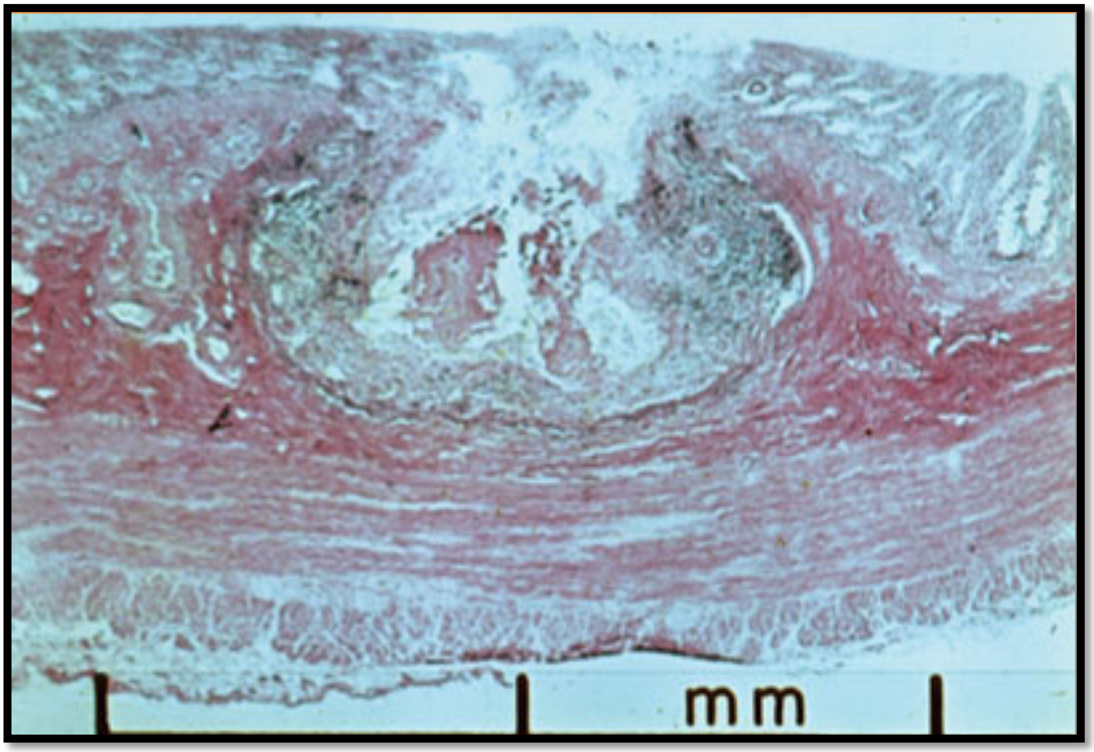

Fig 2.9: A Cross-sectional view of a colonic ulcer, "flask" shape (figure reproduced with authors permission)(Petri and Ayeh-kumi 2013)

Fig 2.10: **Colonic ulcers from a patient with amebiasis**; the ulcers measure about 1 mm in diameter. (figure reproduced with authors permission) (Petri and Ayeh-kumi 2013)

2.11.2 Extra-intestinal amebiasis

This infection occurs when the parasite spread to other organs, the trophozoite passes from the colonic lesions via the portal vein into the liver, usually into the upper and posterior portions of the right lobe. The infection begins with intrahepatic portal thrombosis and infarction; the cytolytic activity leads to liquefaction of the stromal and parenchymal structures, producing the large single abscess (Campbell *et al*.1999). The infection develops 1-3 months after the disappearance of dysentery; it might even develop in patients who haven't shown symptoms and signs of amebic dysentery. This is the most common form of extra-intestinal amebiasis in both in endemic and non-endemic areas (Campbell *et al*.1999). Invasion of the liver is characterized by the presence of nonmotile *E. histolytica* trophozoites that cause an acute inflammatory reaction. The abscess formed is filled with liquid necrotic liver, which is characterized by a yellow or yellow-green colour and later it becomes dark reddish-yellow (**fig 2.11**). The infection is predominantly seen in men (aged 18-50) than woman and it is mainly seen in about 5% of patients with symptomatic intestinal amebiasis (Wells and Arguedas 2004). It has been suggested that alcoholic hepatocellular damage in males and protective hormonal factors in women of childbearing age contributes to this sex discrepancy (Stanley 2003). However, the definitive explanation as to why adult males are more susceptible to invasive disease is unclear.

Amebic liver abscess (ALA) is a common and major health problem with high morbidity and mortality. It is common in tropical and subtropical areas, especially in India (Sharma *et al.* 2010). ALA has a highly variable presentation, which may vary depending on severity; there is an acute and sub-acute type of the infection. Most patients present with an acute illness and duration of symptoms less than two weeks, In the acute phase, the infection may present with fever, tenderness and pain in the abdominal, or sub-acutely with less frequent fever, abdominal pains and weight loss (Mathur *et al*. 2002).

Fig 2.11: Amebic liver abscess. Gross specimen of liver tissue with an abscess (white) that formed due to infection of the organ with *Entamoeba histolytica*. (Source:http://www.sciencephoto.com/media/250248/enlarge)

2.12 Immune response to amebiasis

Innate and acquired immunity play a very important for the prevention of invasive disease, however, the nature of protective immune response against *E. histolytica* remains unclear. An appropriate immune response clears pathogens without causing significant damage to the host tissues. The mucus layer serves as a line of defense against amebiasis, it serves as a barrier that prevents the parasite from disrupting the epithelial cells (Moncada *et al*. 2005). However, the parasite has developed ways to escape the host responses and destruct the host tissues. Damage of host tissues has been shown to arise from both *E. histolytica* cytolytic factors and the resultant gut inflammatory response (Moonah *et al*. 2013). The goblet cells secrete a glycoprotein known as mucin, this protein bind to prevent the GalNAC lectin from adhering to the cells and killing of CHO cells (Lidell *et al.* 2006). However, the trophozoites penetrates the cell surface and secrete cysteine proteases to disrupt the mucus layer and envade the tissue (Lidell *et al.* 2006), the injured cells release important chemokines which recruite the immune cells to the site of infection, cells such as macrophages stimulate the polymorhonuclear cells to release ROS and NO, which kill the parasite (**Fig 2.12**).

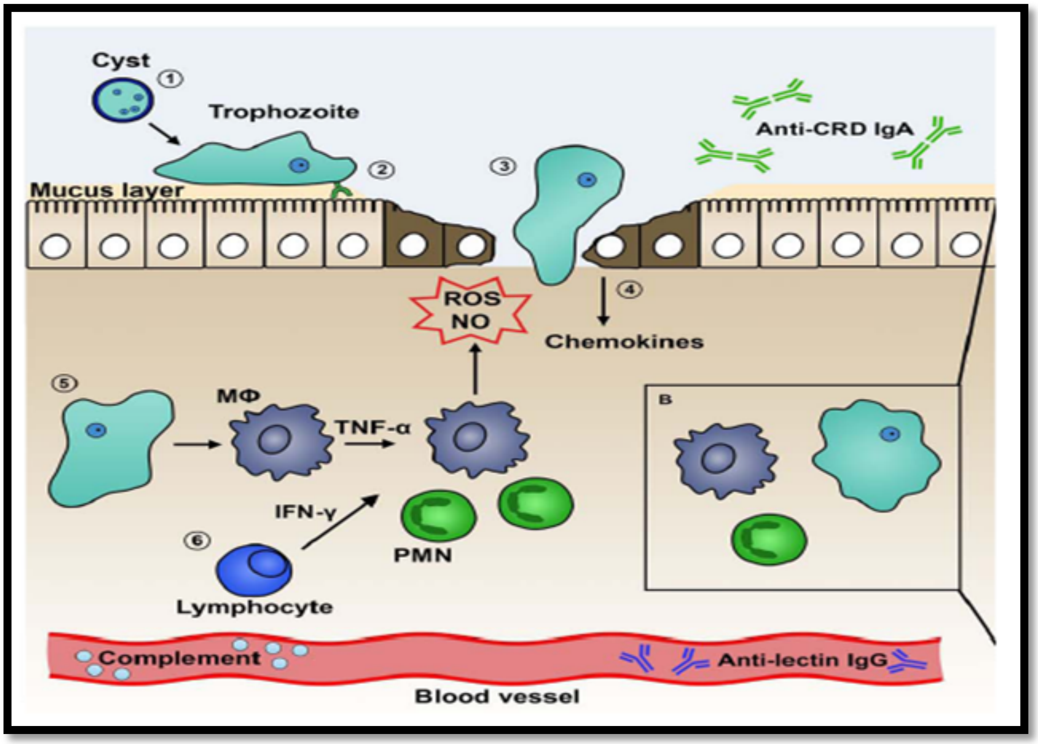

Fig 2.12: Immune response to intestinal amebiasis. (figure reproduced with authors permission) (Moonah *et al*. 2013)

2.12.1 Role of the intestinal microbiota in amebiasis

Therefore, present study sought to investigate the correlation between the host gut microbiome and The intestinal microbiota refers to the population of microorganisms found within our intestine. Microorganisms colonize the gut right after birth and the composition changes as we grow, the microbiota compositions can vary significantly from one person to the next (Fouhy *et al.* 2012), even within healthy individuals or twins in the same household (Smith *et al.* 2013). Our gut microbiota contains tens of trillions of microorganisms. These organisms form a symbiotic relationship that influences human physiology and disease progression (Sassone-Corsi and Raffatellu 2015). Some of the vital roles include modulating the metabolic phenotype, regulating epithelial development, and influencing innate immunity (Wang and Li 2015). The microbiome consist of at least 1000 different species of known bacteria with more than 3 million genes (150 times more than human genes) and provides humans with unique and specific enzymes and biochemical pathways (Qin *et al.* 2010). The metabolic microbiotic processes are known to be beneficial to the host because they help in nutrient acquisition or xenobiotic processing, including the metabolism of undigested carbohydrates and the biosynthesis of vitamins. In addition, the intestinal microbiota provides a physical barrier, preventing the entry of pathogens as well as protecting the host from these foreign pathogens (Cash *et al.* 2006). A balanced microbiota is also essential in the development of the intestinal mucosa and immune system of the host (Bouskra *et al.*2008).

Very few studies have specifically examined associations between *Entamoeba* infection and variation in the human intestinal microbiota. It is suggested that *E. histolytica* colonization may alter, or be influenced by, the ratio of bacterial phyla present in the host microbiota. In study in Northen India demonstrated that *E. histolytica* associated dysbiosis was characterized by significantly less *Clostridia, Bacteroides*, *Lactobacillus*, *Campylobacter,* and *Eubacterium* and increased *Bifidobacterium* species in stool samples as compared to healthy controls (Verma *et al.* 2012). Commensal bacteria and components of the microbiota generally exist in symbiosis with the host; however, some normal flora has the potential to cause, or increase, existing disease. Recent studies have highlighted the relationship between *Entamoeba* and the bacterial communities of the gut. The presence of *Entamoeba* is associated with expansion of members of the *Prevotella* family in farmers and fishermen from Southwest Cameroon (Morton *et al.* 2015), and the level of *P. copri* increased in diarrheal *E. histolytica* cases (Gilchrist *et al*. 2016).

the outcome of amebic infection. Several studies have noted that the bacterial microbiota may influence the virulence of individual pathogens and potentially add variability to the outcomes of parasitic protozoan infections (Marie and Petri Jr 2014; Bär *et al.* 2015).

2.12.1.1 Correlation of the microbiome with amoebiaisis

Commensal bacteria and Probiotics have been suggested to have some influence on the outcome of protozoan infections (Bär *et al.* 2015). The infection with *Entamoeba* can be asymptomatic or cause diarrhea, abdominal pain, and/or weight loss. The parasite invades the intestinal mucosa and therefore is surrounded by the mucosa-associated microbiota. It has been suggested that the interplay that occurs between the parasite, host microbiota and host immune system shapes the clinical outcome of enteric infections (Burgess and Petri 2016). The changes in the composition of the intestinal microbiota may increase resistance to parasite infection at mucosal sites, such as the intestine, by mechanisms such as decreased virulence or parasite adherence (**Fig 2.13**). A study in southwest Cameroon has shown a significant correlation of the microbiom e and *Entamoeba* infection (Morton *et al.* 2015). A study by (Gilchrist *et al.* 2016) has shown a significant association of microbiome with the outcome of *E. histolytica* infection, levels of *Prevotella copri*, a member of the *Prevotellaceae*, were found to be elevated in patients with diarrhea due *E. histolytica* (Gilchrist *et al.* 2016). The findings of these studies show that microbiota composition may play a significant role during an *E. histolytica* infection. More studies are needed to understand the nature of the connection and how it can be utilized for disease prevention.

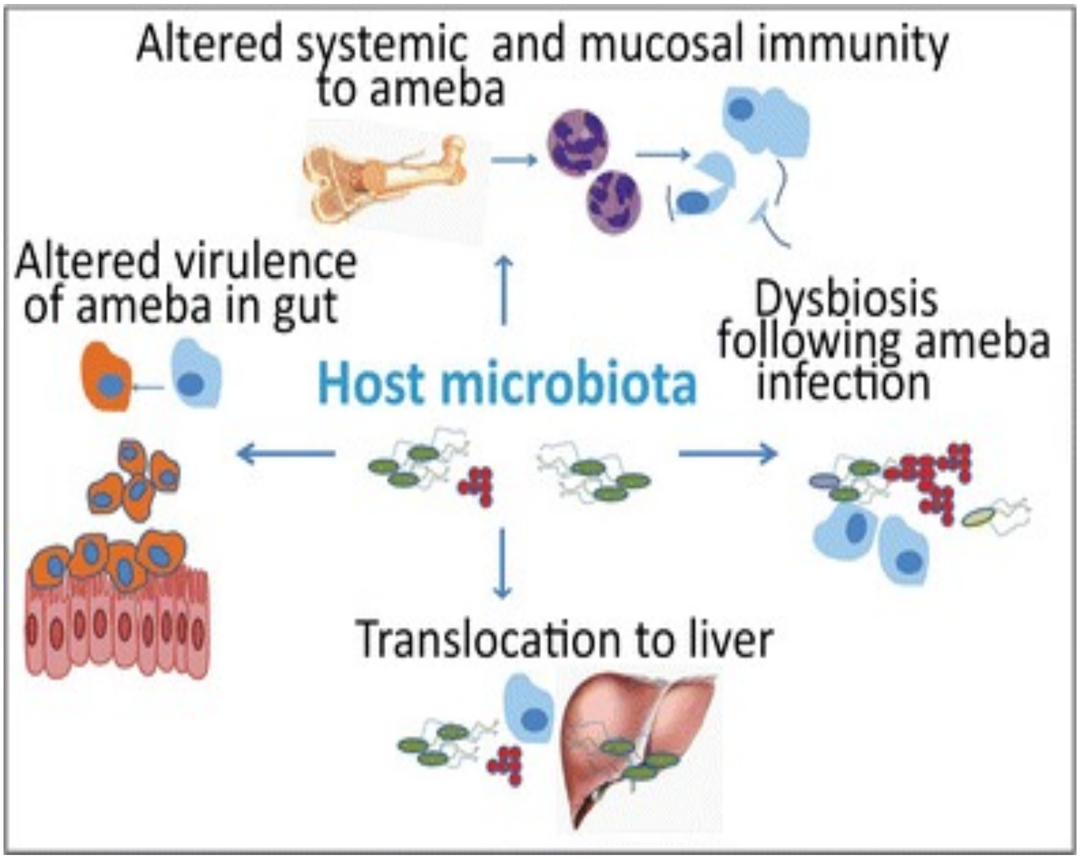

Fig 2.13: Illustration of the interactions between the host microbiota and *E. histolytica*. (figure reproduced with authors permission) (Burgess and Petri 2016).

2.12.2 Coinfections with enteropathogens and *E. histolytica* virulence

Many factors contribute to the virulence of *E. histolytica* infection. It has been suggested that both the parasite and host factors contributes to the virulence. Some studies showed that malnutrition and coinfections with enteropathogens, also influence amebic infection (Mondal *et al.* 2006; Kotloff *et al.* 2013).

Multi-infections is said to be frequent in human populations where there is poor sanitation and inadequate water supply, this infections mainly affect people in developing areas(Orlandi *et al.* 2001), especially in places where *E. histolytica* is endemic . Thus, studying *E. histolytica* in association with other pathogens may be essential in updating new approaches on this infection (Raso *et al.* 2004). Co-infection by multiple groups of enteric pathogens is demonstrated to be the norm in diarrhea cases and asymptomatic patients. It has been suggested that it is important to consider enteric pathogen communities rather than just single pathogens when assessing the severity of the disease (Serrano *et al.* 2014). What makes people prone to multiple infections is still unclear, studies suggests that people who are co-infected by multiple groups of pathogens are often considered to be in poorer health than people who have single infections (Griffiths *et al.* 2011). Co-infections can disturb or complicate the management of some infections in the human body and can also increase treatment costs, probably as a result of clinical complications due to interactions among co-infecting pathogens (Harris *et al.* 2009). Understanding the role and the impact of co-infections is a priority in global health programs for the eradication of diarrhea.

A study by Galván-Moroyoqui *et al.* (2008) showed that ameba that ingested enteropathogenic bacteria became more virulent, causing more damage to epithelial cells. Bacteria induced release of inflammatory proteins by the epithelial cells that attracted amoeba, facilitating amebic contact to the epithelial cells and higher damage. This was found by implementing an experimental system to study amebic virulence in the presence of pathogenic bacteria and its consequences on epithelial cells. Some studies propose that *E. histolytica* pathogenicity could be induced by ingestion of bacteria present in the host intestine. It has been shown that trophozoites of *E. histolytica* increased their virulence together with their adhesive properties to target cells after phagocytosis of an E. coli non-pathogenic laboratory strain (Ec346) (Mirelman 1987). In spite of the possible important role of intestinal bacteria to influence amebic behavior, little has been explored or elucidated about responses triggered by enteropathogens/ameba.

Another study in Bangladesh has shown that infection with *Shigella dysenteriae* and *Shigella flexneri* was more common in children in Dhaka with *E. histolytica* or *E. dispar* infection, potentially complicating the management of amebiasis (Petri and Singh 1999). Therefore the present study sought to investigate the role of coinfection of viruses and bacteria on the outcome of *E. histolytica* infection.

2.12.3 The impact of parasite burden in the outcome of amebic infection

Parasite burden is defined as the total number of parasites a host can habour. Although many people die each year due to amebiasis, most infections are asymptomatic. Fundamental questions on the outcome of the infection remains unresolved, such as the contributions of parasite burden on the severity of the disease. It has been hypothesized that symptomatic disease occurs when parasite numbers overwhelms host defenses (Janeway *et al.* 2001). A study by

Borges *et al.* (2013), evaluated the inflammation and immune regulation that follows *Trypanosoma cruzi* infection with low (300), intermediate (3000) or high (30 000) parasite loads, the results of this study showed that the load of parasite inoculum influenced disease outcome: the higher the number of parasites in the inoculum, the lower were the survival rates. The impact of parasite burden on amebiasis symptoms have been described by Gilchrist *et al.* (2016), this study has shown a significant association of high number of parasite with diarrhea due to *E. histolytica*. It was also shown that the parasite load during *T. cruzi* infection may also influence the activation of the immune response and disease pathology in the chronic phase of Chagas disease (Marinho *et al.* 1999) . Little has been done on impact of parasite burden in the outcome of amebic infection. The present study sought to investigate such impact; this could help in the treatment of amebiasis, by having therapeutic protocols that could control the parasite load and reduce amebic disease pathology.

2.13 Identification methods for amebiasis

It is known that the differentiation of *E. histolytica* and *E. dispar* cysts is a major diagnostic challenge encountered worldwide. Methods for diagnosis include microscopy, stool culture, antigen detection, serology and molecular assays (Fotedar *et al.* 2007).

2.13.1 Microscopical examination

This is the most common means of diagnosis used in many laboratories. Microscopic techniques that are used in a laboratory include wet preparation, concentration, and permanently stained smears for the identification of *E. histolytica* (Fotedar *et al.* 2007). The disadvantage of this method is that it is insensitive, nonspecific and less reliable in identifying *Entamoeba* species. The stool sample is stained with iodine or trichrome for determination of the cysts or trophozoites.

2.13. 2 Culture method

Stool culture is helpful especially in the cases of chronic and asymptomatic intestinal infections, excreting less number of cysts in the faeces (Fotedar *et al.* 2007); it is more reliable compared to microscopic examination. This culture includes two media, the xenic and axenic systems.

In this approach, the cysts from the microscopy positive samples are washed and inoculated into a sterile culture media. The culture media can be incubated at 37ºc and examined for growth of cells, and if any cells are present, they can be seen on the walls of the test tubes or in the debris. Locke's – egg, Robison's and monophasic TYSGM-9 are the commonly used media for cultivation (Robinson 1968). The disadvantage of this method is that none of the existing culture method is selective for *E.histolytica*, so they are not reliable for routine diagnosis. Parasite cultures are difficult, expensive, and labor-intensive to maintain in the diagnostic laboratory. Overgrowth of bacteria, fungi, or other protozoans during culture is the main problem encountered (Clark and Diamond 2002).

2.13.3 Serological method

It has been suggested that serological tests for the identification of *E. histolytica* infection may be helpful from a diagnostic perspective in industrialized nations, where infections due to *E. histolytica* are not common (Ohnishi and Murata 1997). However, in areas where infection is endemic and people have been exposed to *E. histolytica*, the inability of serological tests to distinguish past from current infection makes a definitive diagnosis difficult (Evangelopoulos *et al.* 2000).

Serological testing is an important diagnostic test for extra-intestinal amebiasis, such as amebic liver abscess. This test has been shown to be 70% sensitive for the diagnosis of amebic liver abscess. The presence of antibody usually shows the infection with *E. histolytica* since *E.dispar* is non- pathogenic and the serum antibodies to amoebae develop only during *E. histolytica* infection (Ravdin 1995). The serum antibodies to ameba are detected in up to 95% of all patients who present with liver diseases, the antibodies usually appear in the blood approximately seven days after the onset of clinical symptoms (Ravdin 1995). A number of serological test are developed for the detection of anti-amebic antibody, these include agar gel diffusion (AGD), immunofluorescent antibody

(IFA), indirect haemaglutination (IHA), radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA). The test does not give false positive results with sera that have been collected very early in the infection. ELISA and IHA can be used to measures both present and past infection (Ravdin 1995).

2.13.4 Antigen detection

Antigen detection may be useful as an adjunct to microscopic diagnosis in detecting parasites and to distinguish between pathogenic and non-pathogenic infections (Fotedar *et al.* 2007). Many different ELISAs have been developed for antigen detection in specimens. These antigen detection tests have a sensitivity approaching that of stool culture and are rapid to perform. To date, among the available kits used for antigen detection, the *E. histolytica* II stool antigen detection test has been shown to be the only test that can be used to differentiate *E. histolytica* from *E. dispar*, until recently, this is the only test that meets the world health organization recommendations for *E. histolytica*- specific diagnosis (Tengku and Norhayati 2011). There are other available antigen kits, but they are not selective for *E. histolytica*. The only limiting factor with the Tech lab kits is that is only effective on fresh or frozen stool specimens and not formalin-preserved specimens. This antigen detection test captures and detects the parasite's Gal/GalNAc lectin in stool samples. However, the disadvantage of this test is that the antigens detected are denatured by fixation of the stool sample (Haque *et al.* 1998).

2.13.5 Isoenzyme electrophoresis

This diagnostic test is considered the gold standard for epidemiological studies, is used for differentiation between *E. histolytica* and *E. dispar* (Gonin and Trudel 2003). The enzymes used include, malic enzyme (ME), phosphoglucomutase (PGM), glucose phosphate isomerase (GPI) and hexokinase (HK). Malic enzyme migrates to the same position from the origin of both species, it can also be used to confirm the species presence and HK shows double bands in either one of the two positions (Meza *et al.* 1986). The binding pattern of PGM and GPI, more than twenty zymodemes can be recognized, of which nine belong to *E. histolytica* and the rest to *E. dispar*. A zymodeme is defined as a group of amoeba strains that share the same electrophoretic pattern and mobilities for several enzymes (Sargeaunt 1987). The disadvantages of this method is that it is difficult to perform this test, it is time consuming and relies on establishing the ameba in culture, with a large number of cells needed for the enzyme analysis (Haque *et al.* 1998).

2.13.6 Molecular based Methods

12.13.6.1 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) technique is also considered to be adequate diagnostic tools used to detect *E.histolytica*. The PCR methods are highly specific and sensitive (Parija *et al.*2014), because there is no proof of DNA from other species cross hybridizing with primers that are designed specifically for *E.*

histolytica. Polymerase chain reaction assays are based on priming sequences from the small subunit ribosomal RNA gene of both species of this complex.

12.13.6.2 Quantitative polymerase chain reaction (qPCR)

Real-time PCR is a quantitative method and enables the determination of the number of parasites in various samples (Bell and Ranford-Cartwright 2002). It is a very attractive methodology for laboratory diagnosis of infectious diseases such as amebiasis because of a lack of requirement for post-PCR analysis, resulting in shorter turnaround times and minimizing the risk of amplicon contamination (Parija, 2011) Multiplex real-time polymerase chain reaction can be used for differential detection of *Entamoeba histolytica*, *Entamoeba dispar*, and *Entamoeba moshkovskii. To* date, a few real-time PCR assays for specific detection of *E. histolytica* had been published and evaluated (Qvarnstrom *et al.* 2005; Roy *et al.* 2005). Stool real-time PCR is a highly sensitive and specific technique for this infection compared with serological and microscopic methods (Singh *et al*. 2009).

2.13.7 Treatment of *E. histolytica* infection

The treatment of amebic infection is a complex issue because the patients have to be given a number of drugs to eradicate the parasite from the bowel lumen and from the tissues. People diagnosed with invasive amebiasis require prompt treatment. It is recommended that individuals with no symptoms should be treated in order to control the transmission and spread of other pathogenic parasites (Heymann 2008). Among the many available antiparasitic drugs, metronidazole is rated as the most effective treatment of amebiasis (Leitsch *et al.* 2007).

Asymptomatic carriers should be treated with luminal amebicide, for example, iodoquinol, paromomycin, diloxonide (Snyder and Moodie 2012). These drugs are aid to protect the patients from development of invasive diseases and to reduce the risk of transmission to others (Heymann 2008).

It is suggested that patients with severe intestinal tract symptoms or extra-intestinal diseases such as liver abscess, should be given metronidazole or tinidazole (Gonzales *et al*. 2009), followed by luminal amebicide (Iodoquinol or paromomycin) (Snyder and Moodie 2012).

2.13.8 Control and prevention

Prevention and control of *E. histolytica* infection depends on interruption of fecal-oral transmission. The amebic infection can be prevented and controlled by individual prophylaxis and community prophylaxis. In individual prophylaxis, water can be made safe for drinking and for food preparation by boiling, chlorination, iodination or filtration. The prevention measures include: boiling of drinking water, proper handling of food, cleaning of uncooked fruits or vegetables with boiled water or acetic acid and vinegar. In homosexual prevention can be done by avoiding sexual contact that allows fecal-oral contact. Community prophylaxis includes adequate sewage disposal and clean water supply and education about proper handling of food and sanitation (Haque *et al.* 2003).

2.14 Conclusions

From the literature above, we have identified some knowledge gaps that still need to be addressed on amebiasis, especially the diversity of *Entamoeba* species in the South African population, their prevalence in different regions of the world particularly in Giyani and Pretoria, the methods for investigating and detecting new species of *Entamoeba* as well as the factors influencing the outcome of amebic infection. In order to address these gaps, there is a need to implement new diagnostic and sensitive molecular techniques to investigate *Entamoeba* species to know their prevalence and diversity. Based on the limited data available to date, it appears that more studies are still needed to explain or clarify the factors that control the outcome of amebic infection. The information currently available on the discovery of new species of *Entamoeba* is very useful in understanding and advancing our knowledge on the diversity of *Entamoeba* species both from human and animals. However, the diversity of these species in the African continent, especially South Africa still needs to be investigated. Researchers in the field of amoebiaisis, both from developing and developed countries should collaborate to help to answer the questions on pathogenesis and the difference in disease presentation.

2.15 References

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CHAPTER THREE

Prevalence and distribution of protozoan parasites in rural and urban communities of South Africa

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Renay Ngobeni, Carol Gilchrist, Shannon Moonah, William. A, Petri and Amidou Samie 2018. Prevalence and distribution of protozoan parasites in rural and urban communities of South Africa. ***SUBMITTED*.**

CHAPTER 3

3.1 Introduction

Intestinal parasitic infections constitute a serious public health problem in low and middle-income countries globally. The risk of diarrheal disease is high in areas of poor sanitation, low socio-economic conditions, inadequate water supply and poor hygiene practices (Gelaw *et al.* 2013). Diarrheal infections are documented as the second leading cause of death worldwide, particularly in children under the age of five (Liu *et al.* 2016). Of those who survive the diarrhea, the morbidity burden can affect their development (growth, cognitive performance and physical fitness (Fischer Walker *et al.* 2012). A number of people are thought to be affected by neglected tropical diseases, of which many of the infections are due to protozoan parasites, such as *Cryptosporidium* and *Giardia* (Fletcher *et al.* 2012). Their prevalence is highly dependent on factors such as sanitation infrastructure, social culture and personal hygiene practices.

Intestinal parasites, like *Cryptosporidium* spp. and *Giardia lamblia* are major causes of diarrhea worldwide, with the highest infection in children under the age of five. Transmission of these protozoa is through consumption of contaminated water and food, zoonotic transmission may also occur especially for *Cryptosporidium spp* (Fischer Walker *et al.* 2012). The infection with these parasites cause asymptomatic and symptomatic infections, the factors that determines the outcome of the infection is still unclear. It has been suggested that both the parasite and host genotypes play a role in the outcome of the infection (Ali *et al.* 2007). Therefore, it is important to identify all the factors that play a role in disease exacerbation and protection. Participant age and geographical location however may also play a role in pathogen susceptibility and exposure. It has been shown that disease causality, risk factors, prevalence of diarrhea vary greatly depending on region/community as well as the age. The incidence of diarrheal infections varies greatly with age. Children under the age of five are most vulnerable with incidence been highest in the first two years of life which declines as the child grow older. Identifying people who are at high risk of infection is an important aspect of parasitic disease control programmes (WHO, 2005).

South Africa has been greatly impacted by diarrheal diseases as evidenced by a study by (Samie *et al.* 2010) on the Prevalence of intestinal parasitic and bacterial pathogens in Limpopo Province. This indicated that *Cryptosporidium spp* and *G. lamblia* were the most common cause of diarrhoea. A recent study of the enteric pathogens which included S. African rural children < 2 years in age detected *Cryptosporidium spp* in diarrhoeal stool with *G. lamblia* being mainly present in asymptomatic samples (Kotloff *et al.* 2013). However, information on the prevalence and distribution of these parasites is relatively scarce in Giyani and Pretoria. The present study therefore focuses on the occurrence of these parasites, particularly in Pretoria. The current study presents the prevalence and burden of intestinal infections in both rural and urban communities of South Africa.

Although a recent review of *Cryptosporidium* in Africa has been written which focused on the epidemiology and transmission dynamics much remains to be learned about the epidemiology of cryptosporidiosis and giardiasis in African countries (Mor and Tzipori 2008; Aldeyarbi *et al.* 2016). The purpose of this study was to compare the prevalence and distribution of both *Cryptosporidium* and *Giardia* in rural and urban communities in South Africa. The findings of our study will help to develop recommendations for better control of these important parasites.

3. 2 Purpose of the study

3.2.1 Research question

How prevalent are Protozoan parasites in rural and urban communities of South Africa?

3.2.2 Aim

To investigated the prevalence and distribution of protozoan parasite in rural and urban communities of South Africa.

3.2.3 Specific objectives

a. To determine the prevalence of Protozoan parasites in Giyani and Pretoria

b. To determine the distribution of Protozoan parasites in Giyani and Pretoria

3.3 Materials and Methods

3.3.1 Ethics statement

The research and ethics committee of the University of Venda granted institutional approval. The study received ethical clearance from the Department of Health and Welfare in Polokwane, Limpopo province, South Africa. We also obtained permission to collect samples from the ethics committee of participating hospitals and clinics. The objectives and concepts of the study were clearly explained in the language understood by the potential participants (English, Sepedi, Xitsonga and Tshivenda). A written, informed consent form was signed prior to study enrollment. In cases where the participant was either a non-English speaker or illiterate a witness also signed the consent form.

3.3.2 Study area and population

The tested stool samples were predominantly from urban and rural populations of moderate to low socio-economic status (Statistics South Africa, 2017). They were collected between November 2013 to June 2015 from diarrheal and non-diarrheal patients in rural Nkomo clinic, Giyani and the urban clinic within the Dr George Mukhari Hospital, Soshanguve district of Gauteng, Pretoria. The catchment area for the Dr Georges Mukhari Hospital includes Soshanguve, Ga-Rankuwa, Mabopane and parts of the Madibeng district. The Nkomo Clinic serves households within the Greater Giyani Local Municipality, Mopani district. Both adults and children of all ages were eligible for participation. A questionnaire was used to collect socio-demographic information such as the age, gender and origin of the study participants.

3.3.3 Sample collection

After the patients were given a clear explanation of the stool sample process, they received screw cap bottles into which they placed their samples. Stools were assigned as diarrheal or non-diarrheal based on the physical presentation of the sample as defined by the Bristol stool form scale (diarrhea: Types 6 and 7; non-diarrheal: Type 1 to 5) (Riegler and Esposito 2001). The bottles were labelled with unique participant identifier codes, and then placed in a cooler box and transported to the University of Venda microbiology laboratory for further processing. Upon arrival to the laboratory, samples were aliquoted in 2 ml tubes and stored frozen at -20^{o} C. The aliquoted samples were shipped to the University of Virginia (USA) Infectious Diseases Research laboratory for analysis.

3.3.4 Genomic DNA purification

Genomic DNA from each patient's sample was extracted using QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's recommended procedures, using approximately 200 mg of stool samples with the modifications described in Liu *et al.* (2014). One stool sample from a healthy USA child whose stool had previously been tested and found to be negative for all *Entamoeba* species was included in each batch to monitor for the occurrence of contamination during extraction. The DNA was eluted in 200 µl of elution buffer (Qiagen) and stored at −80°C until further analysis.

3.3.5 Species identification

A diagnostic Tagman qPCR assay (with Taqman probes and species or genus specific primers) to detect the presence of *Cryptosporidium spp.* and *Giardia lamblia* in the extracted fecal genomic DNA was used for molecular detection of protozoan parasites. The following probes/ primers and cycling conditions were used for the amplification (**Table 3.1**) (Platts-Mills *et al.* 2015).

Table 3.1: Primers and probes used for species identification

Component	**Probe/primer Sequence**	**Fluorophore**	**Cycling conditions**
Cryptosporidium F Primer	GGGTTGTATTTATTAGATAAAGAACCA	TEXAS RED	1 cycle, 95 °C for 3 minutes, 40 cycles, 95 °C for 10 seconds 40 cycles 60 °C for 1 minute
Cryptosporidium R Primer	AGGCCAATACCCTACCGTCT		
Cryptosporidium Probe	GTGACATATCATTCAAGTTTCTGAC		
Giardia F Primer	GACGGCTCAGGACAACGGTT	VIC	
Giardia R Primer	TTGCCAGCGGTGTCCG		
Giardia Probe	CCCGCGGCGGTCCCTGCTAG-MGB		

3.3.6 Statistical analysis

Graphpad prism was used to perform statistical analysis, using contingency table with Chi-square test. The differences were considered significant when the p value was less than 0.05 ($P<0.05$)

3.4 Results

3.4.1 Demographic and clinical features

A total of 484 participants were recruited in this study, of which 227 (47%) were from Giyani (a rural setting) and 257 (53%) were from Pretoria (an urban setting). **Table 3.2** summarizes the demographic data of the study population.

Table 3.2: Demographic and clinical features of the study population

Characteristic		**Rural setting** (Giyani) **N=227**	**Urban setting** (Pretoria) **N= 257**
Gender (N=395)	**Male**	83 (37)	104 (40)
	Female	105 (46)	103 (40)
	Not recorded	39 (17)	50 (19)
Age groups (N=346)	**Age range, y [Mean ± SD]**	2–73 [19.2±17.71]	1-90 [41.7±22.13]
	<5 years	37 (16.3)	19 (7.4)
	6-64 years	93 (41)	154 (60)
	>65 years	6 (3)	37 (14.4)
	Not recorded	91 (40)	47 (19)
Stool type Based on Bristol stool scale (N=484)	**Diarrheal stools (Type 6 and 7)**	61 (27)	125 (49)
	Non-diarrheal stools (Type 1 to 5)	166 (73)	132 (51)

Data are presented as number (percentage), unless otherwise stated

Rural Setting: Participants with recorded ages ranged between 2–73 years, with the majority (41%) aged between 6-64 years 37% (n=83) of the participants were male and 46% (n=105) female; in 17% of cases (n=39) the gender was not recorded. Of the 227 stool samples collected, 61 (27%) were diarrheal and 166 (73%) were non-diarrheal.

Urban Setting: Participants with recorded ages ranged between 1-90 years, with the majority (60%) between 6-64 years. Participants were 40% (n=104) male and 40% (n=103) female; the gender was not recorded in 19.5% (n=50). Of the 257 stool samples collected, 125 (49%) were diarrheal and 132 (51%) were non-diarrheal.

3.4.2 The overall prevalence of protozoan parasites in the study population.

Of the 484 stool specimens collected, 34%(166/484) were positive for either *Cryptosporidium spp* or *Giardia lamblia* parasites while 66% (318/484) were negative. Of the 166 positive samples, 49% (112/227) were from the rural and 21%(54/257) from urban cohort. *Cryptosporidium* (20%) was the most prevalent parasite in the study population, followed by *Giardia* (14%) with 5% of samples containing both parasites (22/484) (**fig 3.1**).

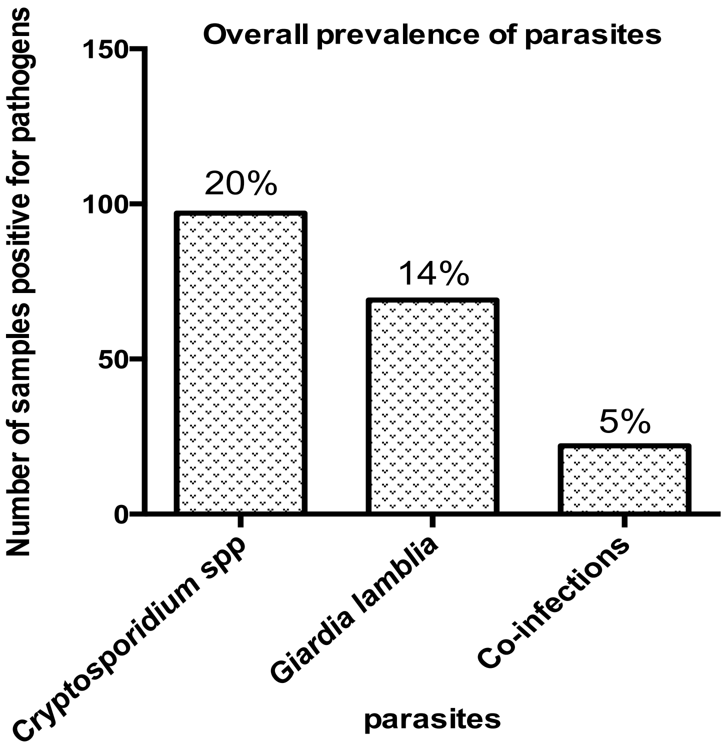

Fig 3.1: **The overall prevalence of parasites in the study population**.

3.4.3 The distribution of parasites in the study population

The distribution of parasites was also investigated by origin; both the parasites were significantly distributed in the population, as well as the co-infections (Table 3.3).

Table 3.3: The distribution of parasites by origin

Pathogen	Rural N=227	Urban N=257	P. Value	Chi-square for pathogen distribution
Cryptosporidium spp	56 (25%)	41 (16%)	0.0129	0.0040
Giardia lamblia	55 (24%)	14 (5%)	< 0.0001	
Co-infections	18 (8%)	4 (2%)	0.0008	

3.4.4 Prevalence of parasitic infections in diarrheal and non-diarrheal stools

Cryptosporidium was the most isolated organism in non-diarrheal patients; however, the difference was not statistically significant. *Giardia lamblia* was found to be more common in non-diarrheal than diarrheal patients and the difference was significant (P=0.046). There was no significant difference on the occurrence of multiple infections in the study population (p=0.0805) (**Table 3.4**)

Table. 3.4. Prevalence of parasitic infections in diarrheal and non-diarrheal samples

Pathogen	Diarrheal (n=186)	Non-diarrheal (n=298)	P. Value	Chi-square for pathogen distribution
Cryptosporidium spp	32 (18%)	65 (22%)	0.243	0.3578
Giardia lamblia	19 (10%)	50 (17%)	0.046	
Co-infections	4 (2%)	18 (6%)	0.0702	

3.4.5 Parasite burden

In this study we hypothesized that patients with high parasite burden show symptoms of the infection. Comparing the parasite burden of each parasite with diarrheal and non-diarrheal stool specimens challenged this hypothesis. Statistically significant increase in parasite load occurred in symptomatic cases due to *Cryptosporidium spp* but not *G. lamblia* (**Fig 3.2**).

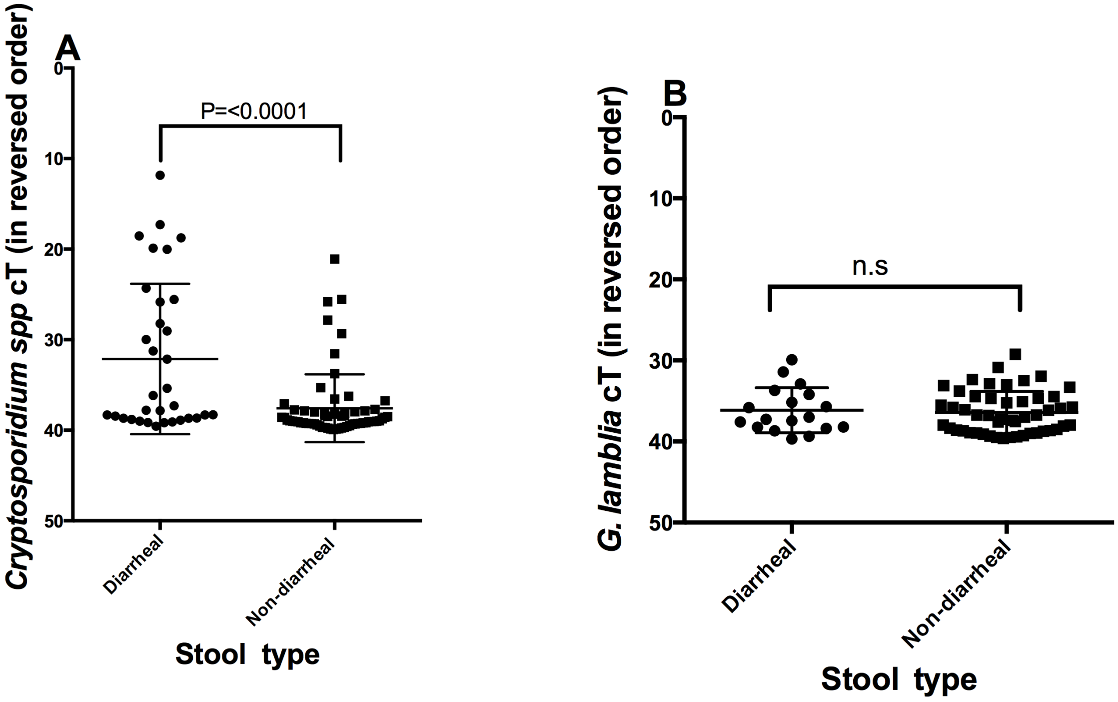

Fig 3.2: The impact of parasite burden in the outcome of the infection. In the scatter plots the *y*-axis indicates the threshold value of the qPCR assay results in samples positive for each parasite. Horizontal lines indicate the data means and vertical lines the standard deviation X axis indicates if the stool was diarrheal or non-diarrheal A) Cq values of *Cryptosporidium* positive samples B) Cq values of *Giardia* positive samples

3.4.6 Co-infections

Among the infected patients, 73% (122/167) were infected by a single intestinal parasite and 13% (22/167) had two infections.

3.5 Discussions and conclusions

The enteric protozoan parasites *Cryptosporidium* and *Giardia* are important causes of diarrheal disease (Xiao 2010; Yaoyu and Xiao 2011). Although a recent review of *Cryptosporidium* in Africa focused on the epidemiology and transmission dynamics, little is known about the epidemiology of cryptosporidiosis and giardiasis in African countries (Aldeyarbi *et al.* 2016). A number of studies have been conducted on the prevalence and distribution of protozoan parasites in South Africa (Samie *et al.* 2009; Nxasana *et al.* 2013; Platts-Mills *et al.* 2015). However, the prevalence of *Cryptosporidium spp* and *Giardia lamblia* has never been reported in Pretoria (Urban). Therefore, the present study aimed to investigate the prevalence and distribution of *Cryptosporidium spp* and *Giardia lamblia* in rural (Giyani) and urban (Pretoria) communities of South Africa.

Of the total number of samples tested, 34% were found positive for the protozoan parasites (*Cryptosporidium* and *G. lamblia*), which was higher than the one reported from Quetta Hospital, 31% by Ahsan-ul-Wadood *et al.* (2005) in Pakistan and lower than the one reported by Taheri *et al.* (2011) in Iran, 47.7%. A high prevalence was observed for *Cryptosporidium* (20%) compared to *G. lamblia* (14%). The findings of this study clearly indicate that parasites are endemic in rural and urban communities of South Africa. The findings of this study are in line with the results reported by Samie *et al.* (2009), this study reported a high prevalence of *Cryptosporidium* (25.4%), compared to *G. lamblia*

(12.8%), and it was concluded that these two parasites were among the other parasites responsible for diarrhea in South Africa, Vhembe district. Another study by Wanyiri *et al.* (2014) also showed that *Cryptosporidium* was the most prevalent enteric pathogen and was identified in 56 of 164 (34%) of HIV/AIDS patients using PCR, showing that PCR is sensitive for detection of this parasite. Additionally, a study in Kenya reported a higher prevalence of *Cryptosporidium* (10.4%) compared to *G. lamblia* (4.6%), which is in line with the resulted reported by the present study.

Giardia lamblia was more prevalent in rural (24%) setting than urban (5%), and the difference was statistically significant. Our findings correlate with (Ouattara *et al.* 2010), this study showed that high prevalence parasites were distributed throughout the Agboville area*.* These findings were in agreement with the results of studies conducted in rural Malaysia, where *Giardia lamblia* was high (11.6%). Our study also showed a much higher prevalence of *Giardia lamblia* than those previously described in the Vhembe district clinics (Samie *et al.* 2009), Kwazulu-Natal (Nxasana *et al.* 2013) and in Indian clinics (Plutzer and Karanis 2009a) . Conversely *Cryptosporidium* was lower in the urban setting (p=0.0129) which may indicate that the animals more prevalent in the rural setting could act as a substantial reservoir for this parasite and hence increase zoonotic transmission (Plutzer and Karanis 2009).

Cryptosporidium spp and *Giardia lamblia* are important causes of diarrheal illness. They are associated with moderate to severe diarrhea and increased mortality in African countries and both parasites negatively affect child growth and development (Squire and Ryan 2017). Although, there was no significant difference, our study found a higher prevalence of *Cryptosporidium* in asymptomatic patients compared to symptomatic individuals, this supports the notion that this parasite causes symptomatic and asymptomatic diarrheal diseases. A study by Asghar and Faidah (2009) in Saudi Arabia found similar results when comparing the symptomatic and asymptomatic patients, no significant differences were detected in the prevalence of various parasites, including *Cryptosporidium* (Asghar and Faidah 2009). These findings confirm the need to look for other causes associated with clinical gastrointestinal manifestations.

The prevalence of *G. lamblia* was markedly higher in non-diarrheal compared to diarrheal samples, this also supports the notion that in most cases people infected with this parasite do not show symptoms (Mbae *et al.* 2013). A study by Tellevik *et al.* (2015) has reported that *G. lamblia* was significantly more prevalent in controls (6.1%) than in cases (3.4%) in line with the findings of the present study. These findings were also in agreement with the other studies, for example a study by (Kotloff *et al.* 2013), significantly identified *Giardia* more frequently in controls than in patients with moderate-to-severe diarrhea aged 12–59 months in ten of the 14 age-site strata. Additionally, Mbae *et al.* (2013) have shown that Infections with *G. lamblia* were higher among outpatients than inpatients.

It has been suggested that the development of clinical disease depend on the parasite load (Magill *et al.* 2012). In the present study the Cq (quantitation cycle) values were used to compare the parasite load of each parasite in diarrheal and non-diarrheal samples and it was found that high parasite burden was associated with diarrhea due to *Cryptosporidium* but not *G. lamblia*. A study by Gilchrist *et al.* (2016) has reported that there is a link between parasite burden and symptomatic disease. A higher parasite burden in *Cryptosporidium* associates with diarrheal episodes in the study population.

Infections with more than one parasite were observed in the study, with a significantly higher prevalence in rural areas compared to urban areas (P=0.0008). It has been shown that people who are co-infected by multiple groups of pathogens are often considered to be in poorer health than people who have single infections (Griffiths *et al.* 2011). The observed co-infections in the study population could be explained by the fact that many species of protozoa have the same mode of transmission (Vaumourin *et al.* 2015). Co-infection with other enteric pathogens appears to also aggravate diarrhea severity (Zhang *et al.* 2016). However, further analysis in the present study had shown that co-infections could be found in both non-diarrheal and diarrheal samples. More studies are needed to understand the factors that promote multiple infections (Mbae *et al.* 2013). Furthermore, strategies to identify causative pathogens in the presence of multiple infections are needed since diagnosing a particular isolate may not rule out other potential infectious causes in diarrheal disease.

The greatest limitation of this study was the small sample size. A larger sample size would have allowed the analysis of additional demographic factors than was possible in the current data set. In other studies age, female sex, feeding practices, immunization practices, and nutritional status have been identified as diarrhea risk factors.

In conclusion, the high prevalence of protozoan parasite reported in this study, particularly in rural settings, revealed poor sanitation and environmental contamination as a public health problem among individuals in this area. The prevalence of these parasites in the study population is of concern and deserves careful consideration in the development of health policies in these regions.

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CHAPTER FOUR

Entamoeba in South Africa: correlations with the host microbiome, parasite burden and first description of *E. bangladeshi* outside of Asia

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Renay Ngobeni, Amidou Samie, Shannon Moonah, Koji Watanabe, William A Petri, Jr., Carol Gilchrist; *Entamoeba* in South Africa: correlations with the host microbiome, parasite burden and first description of *E. bangladeshi* outside of Asia, ***The Journal of Infectious Diseases*** **[Appedinx I]**

CHAPTER 4

4.1 Introduction

Entamoebas are a group of unicellular, anaerobic, parasitic organisms found in humans, non-human primates, other vertebrate and invertebrate species (Stensvold *et al.* 2011). *Entamoeba* infections in humans can result in asymptomatic carriage or a wide range of symptomatic diseases. Of the subset of individuals developing symptoms, diarrhea and dysentery are the most common manifestations. Extra-intestinal complications occur less frequently, but can be associated with high mortality (Shirley and Moonah 2016).

Species of *Entamoeba* that can infect and be found in the intestinal lumen of humans include *E. histolytica*, *E. dispar*, *E. moshkovskii*, *E. coli*, *E. polecki*, *E. hartmanni* and *E. bangladeshi* (Petri *et al.* 2012). Of these, *E. bangladeshi* is the most recent to be described, with the species name reflecting the geographic origin of the first patient it was isolated from. *E. bangladeshi* is indistinguishable by microscopy from *E. histolytica*, the prototypical pathogenic *Entamoeba* infection, but can be differentiated from other known *Entamoeba* species by immunologic and molecular techniques.

A recent study involving stool samples collected from infants (0–24 months) residing in the Vhembe district of Limpopo South Africa failed however to detect

E. histolytica by PCR (Platts-Mills *et al.* 2015). However *E. histolytica* was common (18%) in an earlier 2006 study involving participants of all ages (0 - >60 years) (Samie *et al.* 2006). The cause for this discrepancy is not fully understood. In preliminary work, samples collected from gastroenterology clinic patients of all ages between November 2013 to June 2015 in both rural Giyani (Mopani district of Limpopo) and urban Pretoria, (Soshanguve district of Gauteng), were evaluated by microscopy for the presence of ameboid organisms, and 50% of the samples were *Entamoeba* positive. These findings might be due to the presence of other morphologically identical non-*histolytica* species of *Entamoeba,* such as *E. dispar* and *E. bangladeshi,* geographical heterogeneity in the frequency of *E. histolytica* occurs in S. African populations, or that a much lower frequency of *E. histolytica* is found in the community based surveys of enteric disease than in patients requiring clinical care (Faria *et al.* 2017)

To test these hypotheses DNA was extracted from the Giyani and Pretoria samples and a multiplex qPCR assay was used to detect both *E. histolytica* and the other morphologically identical *Entamoeba* species such as E. *dispar*, *E. moshkovskii* and *E. bangladeshi*. This assay was also modified to include a general *Entamoeba* probe to capture data on the presence of novel *Entamoeba* species genetically similar to the pathogenic *E. histolytica* that may be present in South African population.

The qPCR assay data captured quantitative information; this permitted us to examine the correlation between parasite burden of these samples and the outcome of the infection. A link between parasite burden and symptomatic disease has been found in previous studies (Gilchrist *et al.*2016; J. Liu *et al.* 2016) Recent studies have also highlighted the relationship between *Entamoeba* and the bacterial communities of the gut. The presence of *Entamoeba* is associated with a decrease in members of the *P. copri* in farmers and fishermen from Southwest Cameroon (Morton *et al.* 2015) and the abundance of *P. copri* increased in diarrheal *E. histolytica* cases (Gilchrist *et al.* 2016). Hence, we also sought to quantify these bacteria in the microbiome of *Entamoeba* positive samples in our study population.

4. 2 Purpose of the study

4.2.1 Research question

a. Do novel species of *Entamoeba* contribute to diarrhea in the South African population?

b. What is the impact of gut microbiota in the immune response during amebic infection?

c. Does the species variation within the genus contribute to the outcome of amebic infection?

4.2.2 Aim

To determine the impact of *Entamoeba* species on diarrheal infections and the factors influencing the outcome of amebic infection.

4.2.3 specific objectives

a. To investigate novel species of *Entamoeba* circulating in the South African population

b. To elucidate the impact of gut microbiota, parasite burden and immune response during amebic infection.

4.3 Materials and Methods

4.3.1 Study population and sample collection

Characteristics of the study population and sample collection procedures are as described in chapter 3, section 3.3.2 and 3.3.3

4.3.2 Genomic DNA purification

The procedures for DNA extraction are as described in chapter 3, section 3.3.4

4.3.3 Multiplex qPCR Assay for detection of *Entamoeba* species

A multiplex Taqman qPCR assay with increased sensitivity was used for the amplification and detection of all *Entamoeba* species. Genus specific primers were used in combination with a 42-nucleotide probe, which should hybridize to *E. bangladeshi*, *E. dispar*, *E. histolytica* and *E. moshkovskii,* amplicons. Due to the length required to generate this probe in these A/T rich genomes, a double quencher was included in the design of the probe (**Fig 4.1**) (Biosearch Technologies Inc).

```
KX528461.1_E.histolytica           GCGGACGGCTCATTATAACAGTAATAGTTTCTTTGGTTAGTAAAATACAA
AF149906.1_E. moshkovskii          GCGGACGGCTCATTATAACAGTAATAGTTTCTTTGGTTAGTAAAGTACAA
KR025412. E. bangladeshi           GCGGACGGCTCATTATAACAGTAATAGTTTCTTTGGTTAGTAAAGTACAA
KP722600.1_E.dispar                GCGGACGGCTCATTATAACAGTAATAGTTTCTTTGGTTAGTAAAGTACAA
                                   ********************************************.*****

KX528461.1_E.histolytica           GGATAGCTTTGTGAATGATAAAGATAATACTTGAGACGATCCAGTTTGTA
AF149906.1_E. moshkovskii          GGATAGCTTTGTGAATGATAAAGATAATACTTGAGACGATCCGGTTTGTA
KR025412.1 E.bangladeshi           GGATAGCTTTGTGAATGATAAAGATAATACTTGAGACGATCCGGTTTGTA
KP722600.1_E.dispar                GGATAGCTTTGTGAATGATAAAGATAATACTTGAGACGATCCAATTTGTA
                                   ******************************************..******

KX528461.1_E.histolytica           TTAGTACAAAATGGCCAATTCATTCAATGAATTG-AGAAATGACATTCT-
AF149906.1_E. moshkovskii          TTAGTACAAGTCGGCCACTCTCTTCACGGGGAGT-GCGAATGCCATTCTG
KR025412.1 E.bangladeshi           TTAGTACAAATTGGCCATACTCTGTAAGGGGTATGAAAAATGACATTCT-
KP722600.1_E.dispar                TTAGTACAAAGTGGCCAATTTATGTAAGTAAATTGAGAAATGACATTCT-
                                   *********.  ***** :  .*  *.  ..:   . .****.******

KX528461.1_E.histolytica           AAGTGAGTTAGGATGCCACGACAATTGTAGAACACACAGTGTTTAACAAG
AF149906.1_E. moshkovskii          AATTGAATAAGGATGGTATGACAATTGTAGAGCACACAGTGTTTAACAAG
KR025412.1 E. bangladeshi          AAGTGAGTTAGGATGCCACGACAATTGTAGAACACACAGTGTTTCACAAG
KP722600.1_E.dispar                AAGTGAGTTAGGATGCCACGACAATTGTAGAACACACAGTGTTTAACAAG
                                   ** ***.*:******   * ************.************.*****

KX528461.1_E.histolytica           TAACCAATGAGAATTTCTGATCTATCAATCAGTTGGTAGTATCGAGGAC
AF149906.1_E. moshkovskii          TAACCAATGAGAATTTCTGATCTATCAATTTGTTGGTAGTATCGAGGAC
KR025412.1 E.bangladeshi           TAATCAATGAAAATTTCTGATCTATCAATCAGTTGGTAGTATCGAGGAC
KP722600.1_E.dispar                TAACCAATGAGAATTTCTGATCTATCAATCAGTTGGTAGTATCGAGGAC
                                   *** ******.****************** :******************
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Fig 4.1: Alignment of the 18S rDNA sequences of the *Entamoeba* species that are known to infect humans. A) QPCR assay design *E. histolytica* (KX528461.1) *E. dispar* (KP722600.1) *E. moshkovskii* (AF149906.1) *E. bangladeshi* (KR025412.1) Accession numbers shown in brackets. Sequences were aligned using the CLUSTAL Omega program. The target of the *Entamoeba* genus probe was highlighted in gray, the sequences of the species-specific probes and the genus specific primers are underlined. **B)** Alignment of representative S. African *E. bangladeshi* sequence Genbank MF471206 from a representative qPCR positive sample PG_24 with the Genbank *E. bangladeshi* (KR025412.1)

This probe recognizes *E. histolytica*, *E. moshkovskii E. dispar, E. bangladeshi* and *E. hartmanni* amplicons but was not similar to the rRNA region in the nonpathogenic *Entamoeba coli, E. polecki*, *Endolimax nana*, *Iodamoeba buetschlii* and *Entamoeba gingivalis*. The PCR was performed with 25-µl-reaction mixture containing Bio-Rad IQ powermix, 0.4 µM of primers and 0.2 µM for each probe. Probes and primers and reaction conditions are shown in **Table 4.1**

Table 4.1: Probes, primer and cycling conditions

Oligonucleotide name and dye	Probe/primer sequence[a]	Cycling conditions
E. histolytica probe (FAM)	TCATT+GAATGAATTGGCCATTT [b]	1. 95°C for 3 minutes 2. 95°C for 10 sec 61°C for 20 sec (40 cycles)
E. bangladeshi probe (Texas Red)	CCTTACAGAG+TATGGCCAATTT [b]	
E. dispar probe (HEX)	ACTTA+CATAAATTGGCCAACTTT [b]	
E. moshkovskii probe (Quasar 670)	CCGTGAAGAGAGTGGCCGA [b]	
Entamoeba genus probe (Quasar 705)	GGATAGCTT [I-X] TGTGAATGATAAAGATAATACTTGAGACGATCCC	
Ehd-88R	GCGGACGGCTCATTATAACA[d]	
EM-RT-F2	GTCCTCGATACTACCAAC[e]	
P. copri probe (FAM)	TGCCCACCACT+TGG	1. 95°C for 3 minutes 2. 95°C for 10 sec 60°C for 1 minute (40 cycles)
P. copri F	AAGCTTGCTTTTGATGGGCG	
P. copri R	TGATCGT+CGCCTTGG	

a) + Indicates the location of a “Locked” Nucleotide (Kumar *et al.* 1998).
[I-X] indicates the location of the internal quencher
b) Probe from Arju and Gilchrist (unpublished results)

c) Probe specific to this study

d) Primer Ehd-88R from Verweij *et al.* (2004)

e) Primer EM-RT-F2 from Lau *et al.* (2013)

4.3.4 Analysis of qPCR *Entamoeba* positive samples uncharacterized at the species level by the qPCR assay

Any sample that gave a positive signal with the *Entamoeba* probe but was negative for all four of the *Entamoeba species, E. histolytica*, *E. moshkovskii E. dispar, E. bangladeshi,* was characterized further by amplifying additional 18S regions and determining their sequences (Petri *et al.* 2012). This DNA was amplified by primers Ehd-88R and EM-RT-F2 (**Table 4.1**), using the high fidelity Phusion polymerase as described by Royer et al (98°C for 30 sec followed by 40 cycles (98°C for 20 sec; 56°C for 30 sec; 72°C for 30 sec) with a final extension of 72°C for 10 min) (Petri *et al.* 2012). A 2% agarose gel stained with 3 µl of ethidium bromide was used to separate the amplified DNA. The PCR products were extracted from the agarose gel using the Qiagen QIAquick Gel Extraction Kit and the purified amplicons were sequenced using the Sanger method (Genewiz Inc).

4.3.5 Sequence and phylogenetic analysis

After sequence results were obtained, the ABI files were downloaded and trimmed using Geneious (version 7.0.6). A phylogenetic tree was created to examine the phylogenetic relationship between novel *Entamoeba* species and the known *Entamoeba* parasites by use of the Neighbor-joining algorithm included in the Geneious Program (Biomatters) (Tamura *et al.* 2011).

4.3.6 Prevortella. copri qPCR assay

To improve the specificity of the qPCR detection of *P. copri* in clinical fecal samples, a new TaqMan assay was designed using the NCBI Reference Sequence: NR_113411.1. Optimal qPCR assay conditions were initially determined using DNA amplified from cultured *P. copri* (CB7, DSMZ; gift from D. Littman). Assay specificity was determined by purification and sequencing of the amplicons from selected fecal samples, and comparison with both the NCBI Reference Sequence: NR_113411.1 and the sequence obtained from the cultured *P. copri* DNA. The PCR was performed with 25-µl-reaction mixture containing Bio-Rad IQ powermix, 0.4 µM of primers and 0.2 µM for each probe. Probes, primers and reaction conditions are shown in **Table 4.1**. The previously described *Enterobacteriaceae* assay was used to normalize both the *P. copri* levels and as a measure of extracted bacterial DNA quality (Gilchrist *et al.* 2016). *Enterobacteriaceae* and *P. copri* negative samples were omitted from the quantitative analysis of *P. copri*

4.3.7 Statistical analysis

The Fishers exact test was used to analyze contingency tables. The D'Agostino & Pearson omnibus normality test and the nonparametric Mann–Whitney comparisons test were used to analyze and compare qualitative data. Tests were performed using GraphPad Prism version 6. The differences were considered significant if the p value was less than 0.05 ($P<0.05$).

4.4 Results

4.4.1 Demographic and clinical features

Demographic details of the study population are presented in chapter 3, table 3.2.

4.4.2 Prevalence and distribution of *Entamoeba* species by qPCR

Of the 484 samples tested by qPCR, 29% (n=140) were positive for *Entamoeba* species while 71% (n=344) were negative. The total frequency of *E. dispar* in the study population detected by qPCR was 8% (n=38), *E. histolytica* 6.4% (n=31), *E. bangladeshi* 4.5% (n=22), unknown *Entamoeba* 10% (n=49). In line with previous results, *E. moshkovskii* was not identified in the S. African populations studied (**Fig 4.2**).

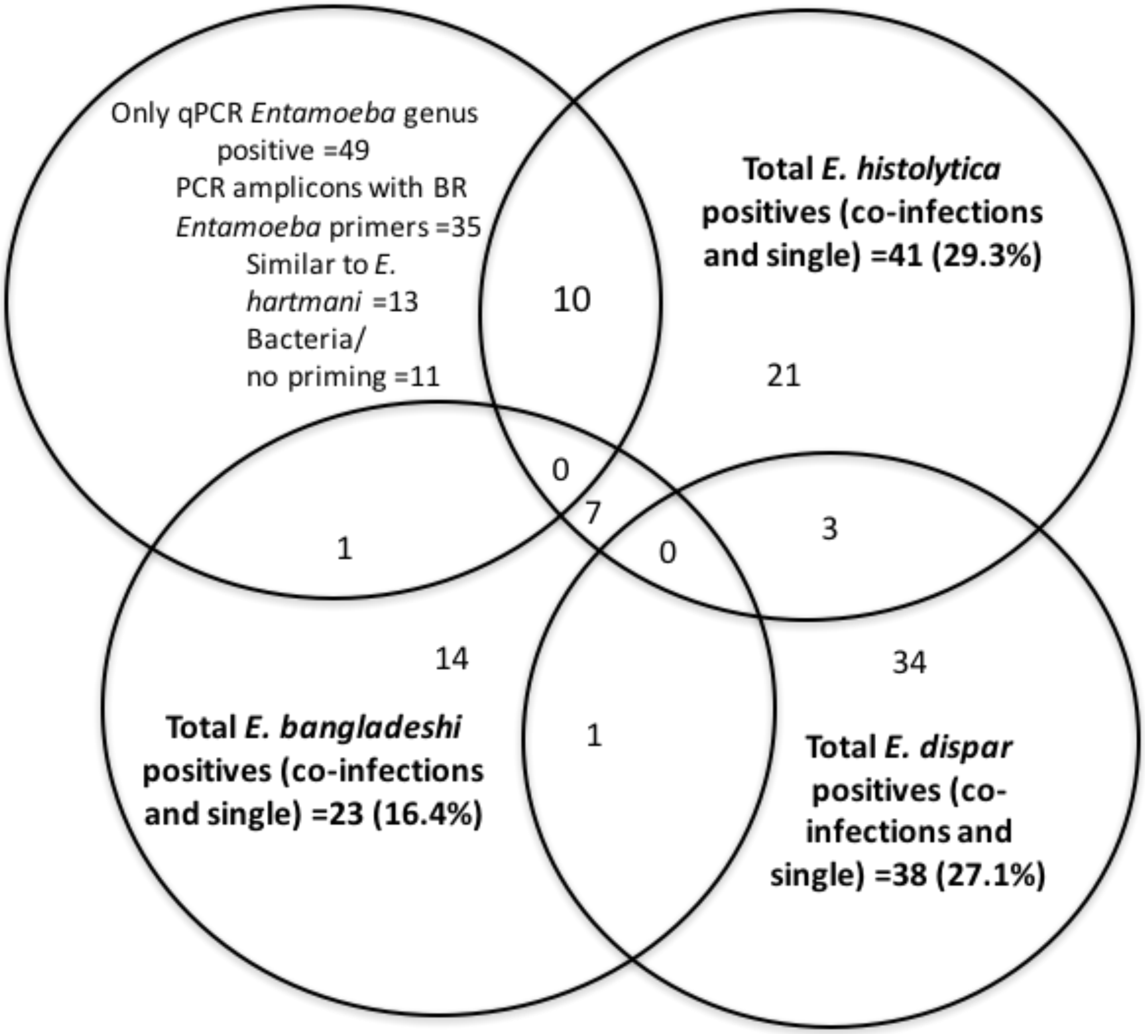

Fig 4.2: *Entamoeba* species found in South African populations surveyed. One hundred and forty *Entamoeba* positive samples were identified by qPCR. In 11 cases the infecting species could not be identified. Thirty-eight samples were positive for *E. dispar,* 41 for *E. histolytica*, 23 for *E. bangladeshi,* 0 for *E. moshkovskii.* In eleven cases co-infections of different *Entamoeba* species were observed (*E. histolytica-bangladehi*: n=7; *E. bangladeshi-dispar*: n=1; *E. histolytica-dispar*: n=3). These findings show that *Entamoeba* species are

common in this population. The presence of *E. bangladeshi* adds to the diversity of *Entamoeba* species present in the South African population.

4.4.3 Confirmatory testing

To confirm the *E. bangladeshi* qPCR assay results, we isolated and sequenced the amplicon from a select positive sample and compared the sequence from our study samples with the *E. bangladeshi* sequence deposited in the NCBI GenBank (KR025412.1). The S. African sequence was identical to that of *E. bangladeshi*.

4.4.4 Characterization of *Entamoeba* samples not identified by species-specific probes

*Entamoeb*a primers (Ehd-88R; EM-RT-F2) were used to amplify DNA fragments from the 49 samples that were qPCR positive for the broad range *Entamoeba* but negative for all the species-specific probes. The amplified DNA was separated by electrophoresis and in cases where the bands of the size predicted for the *Entamoeba* spp were identified (n=35) it was purified from the agarose using the QIAquick Gel Extraction Kit (Qiagen). The SSU rRNA gene amplicon was detected in 35 samples. Sequencing of the purified amplicon identified ten additional *E. histolytica* positive samples (n=41; adjusted frequency in Entamoeba qPCR positive samples 29.3%), and one additional *E. bangladeshi* sample (n=23; adjusted frequency 16.4%)(**Fig 4.2**). This result suggested that in these samples the parasite level had simply fallen below the detection limit of the species-specific qPCR assay. These samples were not included in the later qualitative analysis due to the degraded nature of their DNA. In 13 cases the 18S rRNA amplicon sequences were similar to those of the non-pathogenic *E. hartmanni* (all sequences were deposited in Genbank MF471201-MF471217) and in the remaining 11 cases either no useful sequence data was obtained or were similar to sequences from bacteria and had no significant similarity to any *Entamoeba* reference sequence in the NCBI database.

4.4. 5 Parasite burden in South African Samples

Other enteropathogens are common in this S. African population and in diarrheal samples co-infections can make it challenging to identify the causal organism (Platts-Mills *et al.* 2015). *Entamoeba* was no more frequent in diarrheal samples than in controls (data not shown). No differences in *Entamoeba* frequency were observed between the rural and urban populations. The cycle value at which the (baseline-corrected) amplification curve exceeds the background fluorescence (Cq) is closely related to the amount of input DNA. The Cq data provided by the qPCR assay can therefore be used to determine if the *Entamoeba* species burden was different in diarrheal and control fecal samples (Taniuchi *et al.* 2013). As the distribution of Cq values was non-Gaussian, significance was determined using the Mann-Whitney test. As expected, a significant difference in *Entamoeba* levels was observed in cases of *E. histolytica*-associated diarrhea ($p<0.01$ **Fig 4.3 A**) but the level of the non-pathogenic *E. dispar* was unchanged in control and diarrheal samples (**Fig 4.3 C**). Interestingly the level of *E. bangladeshi* was also unchanged (**Fig 4.3 B**). Again no significant differences were observed in the parasite burden in rural and urban samples.

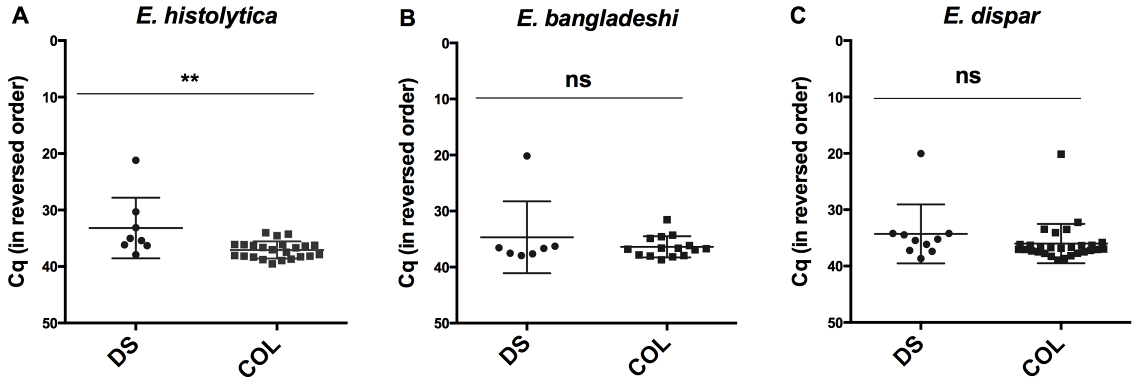

Fig 4.3: **High parasite burden was associated with diarrhea due to *E. histolytica* but not *E. bangladeshi* and *E. dispar***. In the scatter plots the *y*-axis indicates the threshold value of the qPCR assay results in samples positive for each parasite. Horizontal lines indicate the data means and vertical lines the standard deviation A) *E. histolytica* samples; B) *E. bangladeshi* samples and C) *E. dispar* samples. X-axis: Samples are categorized as from diarrheal (DS; circles) or asymptomatic colonizers (COL; squares). The Mann-Whitney test analysis was used to determine if the data is significantly different (**p≤0.01).

4.4.6 Quantity of *Prevotella copri* in *Entamoeba* positive samples

In a Bangladesh study, elevated levels of the pathobiont *P. copri* were associated with *E. histolytica*-associated diarrhea (Gilchrist *et al.*2016) The level of *P. corpi* in *Entamoeba* positive diarrheal and control samples was measured and to control for variations in fecal bacterial numbers, normalized using an *Enterobacteriaceae* bacteria reference (Gilchrist *et al.* 2016) A fecal DNA standard was used to control for any differences in amplification efficiency in the *P. corpi* and *Enterobacteriaceae* qPCR assays. Samples negative for either *P. copri* or *Enterobacteriaceae* were omitted from the quantitative analysis due to concerns about sample quality. To convert the qPCR results to bacteria concentration, DNA was extracted from a known amount of *E. coli* (ATCC 25922) and assayed. The relative level of the *P. copri* bacteria was decreased by one log in *E. histolytica* colonized samples compared to the level in *E. histolytica* diarrhea (**Fig 4.4 A**) but was unchanged in *E dispar* or *E. bangladeshi* infections in both diarrheal and non-diarrheal cases (**Fig 4.4 B, C**).

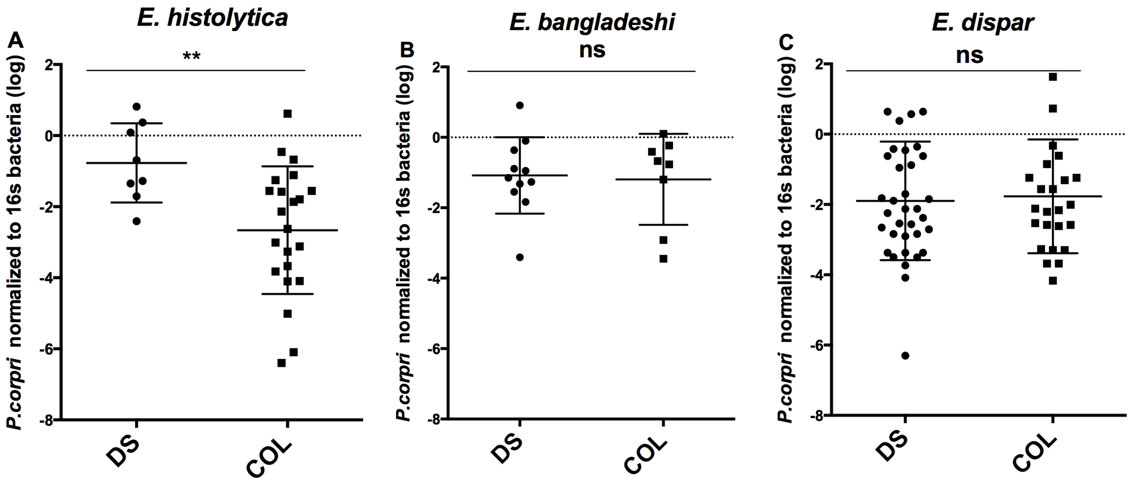

Fig 4.4: **Altered levels of *Prevotella copri* were associated with diarrhea due to *Entamoeba histolytica*.** In the scatter plots *y*-axis the Cq values were converted to bacteria numbers by use of a calibration curve and normalized to

the *Enterobacteriaceae* levels. Horizontal lines indicate the data mean. A) *E. histolytica* samples, B) *E. bangladeshi* samples and C) *E. dispar* samples. X-axis: Samples are categorized as from diarrheal (DS; circles) or asymptomatic colonizers (COL; squares). The Mann-Whitney test analysis was used to determine if the data is significantly different (**$p \leq 0.01$).

4.5 Discussion and conclusions

The present study reports an overall frequency of *Entamoeba* species in our samples collected from gastrointestinal clinics as 27% (129/484) with *E. histolytica* being present in 6.4% (31/484) of the cases. Differences in assay used as well as in age, geographic location and the fact that these samples were collected from gastrointestinal clinics make it difficult to compare these results with those obtained from previous population based studies (Platts-Mills *et al.* 2015; Liu, Oza, *et al.* 2016). A weakness in the current study was that information on individual's HIV status (expected to increase with age) was not available to researchers and that the study was not adequately powered to analyze the susceptibility to *Entamoeba* of participants stratified by age.

The Cq of the majority of our *Entamoeba* positive asymptomatic samples was ≥ 35 and would have been missed using a less sensitive assay. Assay specificity at high Cq values was confirmed by amplicon sequencing of select samples (Ajur er al, Emerging Infections & Parasitology Laboratory of icddr, b, personal communication). In the work reported here the assay included an *Entamoeba* general probe which acted as an independent control to identify any closely related novel S. African *Entamoeba* species present in these samples (an in-depth surveillance of the *Entamoeba* species in the Mopani district of South Africa had not previously been done). All assay results were analyzed to be certain the species-specific signal remained at a constant ratio to the result obtained from the broad range probe. The probe would have recognized any *Entamoeba* species similar to the pathogenic *E. histolytica, E. moshkovskii*, *E. bangladeshi* or non-pathogenic *E. dispar*. The sequences of the closely related species also blocked non-specific hybridization, allowing the higher assay cut-off of Cq of ≤ 40 and increased assay sensitivity (Lau *et al.* 2013).

A higher *E. histolytica* parasite burden in samples increases the probability that the *E. histolytica* detected is responsible for diarrheal symptoms (Taniuchi *et al.* 2013; Gilchrist *et al.* 2016). In agreement with the previous studies our results showed a statistically significant increase in *E. histolytica* parasite load occurred in South African diarrheal samples. By examining the qPCR Cq values we compared the amoeba levels of the non-pathogenic *E. dispar* in both diarrheal and control samples. The level of the non-pathogenic *Entamoeba* did not significantly change. This suggested that the diarrhea co-incident with *E. dispar* infections was due to other pathogens. The pathogenicity of the recently identified *E. bangladeshi* is still uncertain however we note that in our studies the level of *E. bangladeshi* was also the same in both diarrheal and non-diarrheal South African samples. Additional work is planned to identify if other co-infecting enteric pathogens are present in these samples.

Novel *Entamoeba* species have been identified in different geographical contexts therefore samples that were positive with the *Entamoeba* general probe but negative with the species-specific probes were characterized by amplicon sequencing. (Petri *et al.* 2012). While novel African *Entamoeb*a species were not identified, to our knowledge this study is the first to describe the presence of *E. bangladeshi* in samples collected out-width Bangladesh. This species was first described in Bangladesh in 2011, but our results suggest that *E. bangladeshi* may actually have a broad geographical range and is prevalent in both Asian and African continents (Petri *et al.* 2012). This finding also suggests that other members of the *Entamoeba* genus not identified in previous surveys may also be common in S. Africa (Nxasana *et al.* 2013; Sylvain *et al.* 2015)

In addition to the parasite burden, predisposition to diarrheal disease is thought to be influenced by the parasite environment (Singh, 2012; Marie and Petri, 2014). Moreover, it has been suggested that specific components of the microbiota might be associated with symptomatic or asymptomatic *E. histolytica* colonization (Burgess *et al.* 2014; Burgess and Petri, 2016). We examined the level of *P. copri* in the S. African samples positive for members of the *Entamoeba* species. Consistent with previous studies, the level of these bacteria was lower in asymptomatic *E. histolytica* positive samples when compared to the level in *E. histolytica* associated diarrheal samples (Gilchrist *et al.* 2016). The guts Prevotella species are anaerobic bacilli predominant in the lumen of the colon (Donaldson *et al*. 2015). Recent studies however suggest that disruption of the host mucosa can result in an increase in *Prevotella* at mucosal sites and a subsequent increase in host inflammatory responses (Scher *et al.* 2013; Larsen 2017). Additional studies are needed to determine if low *P. copri* levels could mitigate the host immune response occurring during amebic colitis. It is possible that *E. histolytica* unlike non-pathogenic *Entamoeba* disrupts the protective mucosal layer and exposes the host epithelium to the luminal microorganisms. This likely exposes the epithelium to high *P. copri* levels as well as to the *E. histolytica* parasite and results in an excessive inflammatory response with subsequent diarrhea (Pianta *et al.* 2017). In samples positive for the commensal *E. dispar* (which does not induce the inflammatory response or the coincident diarrhea) *P. copri* levels were not significantly different in either diarrheal or non-diarrheal samples(Sharma *et al*. 2005).

In summary, the presence of specific components of the microbiota appears to alter the susceptibility of the host to amebiasis. The interplay between the pathogen, host and host-microbiota maybe important in the development of symptomatic disease

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CHAPTER FIVE

The *Entamoeba* pro-inflammatory MIF homolog contributes to mucosal inflammation during colitis

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Renay Ngobeni, Mayuresh M. Abhyankar, Nona M. Jiang, Laura A. Farr, Amidou Samie, Rashidul Haque, Shannon N. Moonah. *Entamoeba histolytica*–Encoded Homolog of Macrophage Migration Inhibitory Factor Contributes to Mucosal Inflammation during Amebic Colitis **[Appendix II]**

CHAPTER 5

5.1 Introduction

Mucosal inflammation is a hallmark of amebic colitis, explaining why it is often misdiagnosed as inflammatory bowel disease (Shirley and Moonah 2016). While inflammatory cells represent a line of defense, there is a significant amount of evidence that the inflammatory response contributes to the tissue damage seen in amebic colitis (Moonah *et al*. 2013).

Trophozoites are present in the base of the ulcer. The lesion spreads laterally in the submucosa, producing a flask-shaped ulcer of amebic colitis in the human host (Ghosh *et al.*1994*)*. Neutrophils are some of the first immune cells that respond to inflammation, they recognize and destroy pathogens (Rosales *et al.* 2016). They have been shown as the first immune cells to respond to amebic invasion, they release ROS and NO to fight the parasite. These neutrophils infiltrate the intestinal tract during amebic colitis, however, excess infiltration can result in direct damage to host tissues (Moonah *et al.* 2013).

The neutrophil-derived protein, calprotectin, was in one of the top four genes that showed the greatest increase in the human colon during amebic colitis (Peterson *et al.* 2011). It has been known for decades that neutrophilic enzymes such as myeloperoxidase (MPO) generate oxygen free radicals, which kill invading pathogens. Oxygen free radicals are also responsible for collateral tissue damage during the inflammatory period (Hao *et al.* 2015). Neutrophil migration depends on chemokines produced by epithelial cells. IL-8 is a potent neutrophil chemoattractant that contributes to mucosal inflammation in various infectious and inflammatory diseases. Persons with severe forms of amebic colitis have higher colonic tissue levels of the IL-8 and neutrophils (Sierra-Puente *et al.* 2009).

It is possible that an overzealous host inflammation response occurs during amebiasis and neutralization of this response may be a crucial first step in the resolution of disease. Using the mouse-human intestinal xenograft model of *E. histolytica* infection both IL-8 inhibition and neutrophil depletion resulted in less mucosal damage (Zhang et al. 2002). Our hypothesis however is that provoking the host anti-inflammatory response is advantageous to this parasite, allowing it to spread and cause disease. *In vitro*, the *E. histolytica*-mediated destruction of colonic epithelial cells was enhanced by the addition of neutrophils (Burchard *et al*.1993). Additionally, studies using the mouse model of intestinal amebiasis showed *E. histolytica* induced the expression of cyclooxygenase-2 in epithelial cells and macrophages, and the resultant prostaglandins enhance epithelial permeability, mediating neutrophils responses (Stenson *et al.* 2001)

The host inflammatory response, as well as the production of cytokines and inflammatory mediators, followed by an influx of neutrophils, have a significant role in the pathogenesis of invasive amebiasis (Tadesse *et al*. 2008). One of the possible mediators of neutrophil influx and mucosal inflammation during the amebic infection is the Macrophage migration inhibitory factor (MIF). Human MIF is a proinflammatory cytokine that is a critical upstream mediator of the innate immune response. MIF enhances the secretion of inflammatory mediators, such as IL-8 and there is a strong association between MIF and colitis (Roger *et al.* 2016; Yao *et al.* 2016). Moreover, not only have MIF homologs been identified in the pathogenic protozoans *Plasmodium*, *Toxoplasma, Trichomonas, Leishmania* an *E. histolytica* homolog has been found (Moonah *et al.* 2014). The parasitic protozoan MIF homologs have been partially characterized and showed pro-inflammatory activities similar to that of human MIF (Sun *et al.* 2012; Sommerville *et al.* 2013; Moonah *et al.* 2014; Twu *et al.* 2014; Holowka *et al.* 2016). Intestinal epithelial cells express the MIF receptor (Uhlen *et al.* 2015). However, the effect of MIF homologs on the intestinal epithelium and mucosal inflammation during infection is unknown.

In the work described here, we examined the role of *E. histolytica* MIF (*Eh*MIF) in inflammation using *in vitro* approaches, mouse model and in persons with intestinal amebiasis. Pigs, gerbils, cats and mice are used as animal models for the study of amebiasis in the laboratory. Among these, the most commonly used model is the mouse (Spencer 2002). Mice have been shown to have very similar physiologies to humans and therefore the impact of human diseases and drugs can be tested in mice and the findings applied to humans (Spencer 2002). The use of this model has been useful for understanding many aspects of the molecular and cell biology, immunology and physiology of amebic ulcers and ALA development (Pacheco-Yépez *et al.* 2017).

5. 2 Purpose of the study

5.2.1 Research question

What is the role of *E. histolytica* MIF (*Eh*MIF) in mucosal inflammation during amebic infection?

5.2.2 Aim

To investigated the role of *E. histolytica* MIF (*Eh*MIF) in mucosal inflammation amebic during infection

5.2.3 Specific objectives

a**.** To investigate whether *Eh*MIF is secreted during infection

b**.** To investigate the effect of transport inhibitors on *Eh*MIF secretion

5.3 Materials and Method

5.3.1 Study Approval

All animal studies were performed in compliance with the federal regulations set forth in animal welfare Act, the recommendations in the Guide for Care and Use of Laboratory-Animals of the National Institute of Health, and guidelines of the University of Virginia Institutional Animal Care and Use Committee. The University of Virginia Institutional Animal Care and Use Committee approved all protocols for animal use. Use of serum and stool samples from human participants were approved by the Institutional Review Board at the University of Virginia, the research and Ethical Research Review Committees of the International Center for Diarrheal Disease Research, Bangladesh, and Research and Ethics Committee of the University of Venda, South Africa.

5.3.2 Parasites and Cell Culture

Entamoeba histolytica strain HM1:IMSS trophozoites were grown at 37°C in TYI-S-33 medium. The human intestinal epithelial cell line Caco-2 (American Type Culture Collection) was grown in Dulbecco's Modified Eagle medium (Gibco). Cell lines tested negative for *Mycoplasma* (Lonza). Coculturing of epithelial cells with ameba were done at a ratio of 10:1 host cells to parasite in M199 medium (Moonah *et al.* 2014). IL-8 in cell culture supernatant was measured by enzyme-linked immunosorbent assay (ELISA; eBioscience). For overexpression of *Eh*MIF in *E. histolytica* trophozoites, the *Eh*MIF gene with no tag was cloned into the pKT3M expression vector (Twu *et al.* 2014) and confirmed by sequencing. Parasites were transfected by a previously described technique (Maharshak *et al.* 2010). Transfectants were selected with 12 µg/ml G-418 (Gibco). *Eh*MIF protein overexpression was confirmed by immunoblot analysis using specific anti-*Eh*MIF antibodies (Sommerville *et al.* 2013). Parasites transfected only with pKT3M expression vector were used as empty vector controls. Parasite growth was measured using CyQUANT Direct Cell Proliferation Assay kit (Invitrogen) according to the manufacturer's instructions. For amebic cytotoxicity assays, *E.*

histolytica trophozoites were added to intestinal epithelial cell line monolayers in M199 as previously described (Moonah *et al.* 2014). Lactate dehydrogenase (LDH) levels in the supernatant were measured using Cyto Tox-ONE Homogeneous Membrane Integrity Assay (Promega) as directed. The maximum amount of LDH released was determined by the addition of Triton-X to intestinal epithelial cells alone. Percent cytotoxicity was calculated as: [(LDH release+E. histolytica) − (LDH – *E. histolytica*)] / [maximum LDH release]. Conditions were tested in triplicates. Each experiment was repeated at least 3 times, and representative experiments are shown.

5.3.3 Measurement of *Eh*MIF and Stool Myeloperoxidase

We developed an ELISA to measure *Eh*MIF levels, similar to a recently described method (Holowka *et al.* 2016). Corning 96-well high-protein-binding polystyrene plates were coated with 5-µg/ml rabbit polyclonal anti-*Eh*MIF (Sommerville *et al.* 2013) in phosphate-buffered saline (PBS) overnight and blocked for 1 hour with PBS containing 1% bovine serum albumin. Recombinant *Eh*MIF was used as a protein standard. Stool samples were incubated overnight at 4ºC, and then washed before the addition of biotinylated anti-*Eh*MIF at 0.25 µg/ml. After incubation and washing, avidin-conjugated horseradish peroxidase (eBioscience) was added, and detection was performed with 3,3′, 5,5′-tetramethylbenzidine ELISA detection reagent (eBioscience). The sensitivity of the ELISA was 15.6pg. There was no cross-reactivity to human MIF. *Eh*MIF concentrations were measured in de-identified diarrheal stool samples from 35 South African patients with intestinal amebiasis. ELISA (ALPCO) measured stool Myeloperoxidase (MPO) levels according to the manufacturer's instructions (Maharshak *et al.* 2010).

5.3.4 Mice

Wild-type CBA/J mice were obtained from the Jackson Laboratory. Male mice were used at 10 weeks of age.

5.3.5 Parasite Infection

Infections were carried out via intracecal inoculation of mice with *E. histolytica* trophozoites (Uhlen *et al.* 2015). A total of 5×10^5 trophozoites in 100 µl of TYI media were injected intracecally after laparotomy for antibody-mediated neutralization and *Eh*MIF overexpression studies. For antibody neutralization studies, 0.5 mg mouse anti-*Eh*MIF blocking antibodies were given by intraperitoneal injection 24 hours before and intracecally at the time of infection. Isotype antibodies given at the same dose, route, and timing were used as controls. Intracecal injection with media only was used as uninfected controls. No differences in the inflammatory markers and cytokine levels were observed among mice that did not receive intracecal injection and those injected with media only and PBS. Mice were sacrificed 24 hours postinfection. TechLab E.

histolytica–II kit was used to determine amebic antigen burden in cecal contents (Uhlen *et al.* 2015). Cecal tissue lysates were prepared as in (Flieger *et al.* 2003). C-X-C motif (CXC) chemokine ligand 1 (CXCL1), CXC chemokine ligand 2 (CXCL2), and matrix metalloproteinase-3 (MMP3) levels in cecal lysates were measured by ELISA (R&D Systems). Myeloperoxidase activity in cecal tissue was determined using the same standard protocol as used in (Loftus *et al.* 2005).

5.3.6 Human Samples

Serum sample concentrations of anti-*Eh*MIF antibodies were measured in 79 children (2–5 years old) from a well-characterized cohort in the endemic area of Mirpur within Dhaka, Bangladesh (Sommerville *et al.* 2013). After serum sample collection, monthly stool samples were routinely obtained from all children and tested for *E. histolytica* using real-time polymerase chain reaction as previously published (Huldt *et al.* 1979).

5.3.7 Secretion Assay

Preparation of *E. histolytica*–secreted fractions was modified as described in [(Yu and Chadee 1997). Briefly, 1 × 10^7 trophozoites per mL were suspended in M199 media (Gibco) and incubated at 37°C for 2 hours. Cell-free supernatant representing the secreted fraction was collected for further analysis. Proteins in the secreted fraction were not due to cell stress or cell death, as only a minor portion of cells stained positive for Trypan blue (less than 5%), and no cellular actin was found by immunoblot analysis. For inhibition assays, trophozoites were incubated with pharmacological agents brefeldin A and probenicid (Naylor *et al.* 2015).

5.3.8 Immunohistochemical Staining and Histopathological Examination

The University of Virginia Biorepository and Tissue Research Facility performed Mouse immunohistochemical staining. Staining was performed using the DAKO Autostainer Universal Staining System with specific antibody directed against *Eh*MIF at a dilution of 1:600. Mouse cecal tissue was fixed in Bouin's solution (Sigma) and stored in 70% ethanol. The University of Virginia Research Histology Core stained tissue with hematoxylin and eosin. Histological scoring for inflammatory infiltration and epithelial cell damage was performed by 2 independent blinded scorers as previously described (Kosek *et al.* 2013).

5.3.9 Mass Spectrometry

Proteins from the *E. histolytica*–secreted fraction were separated by gel electrophoresis. The section spanning 10–20 kilodaltons was then excised from the gel. The gel sample was submitted to the W. M. Keck Biomedical Mass Spectrometry Laboratory for mass spectrometry analysis.

5.3.10 Statistical analysis

Statistical differences between 2 groups were determined using the Mann–Whitney *U* test or Student *t* test. Pearson's correlation was used for correlation analysis. Survival differences were analyzed by the log-rank test. A *p* value less than .05 was considered statistically significant.

5.4. Results

5.4.1 Association of *Eh*MIF With Intestinal Inflammation in patients With Intestinal Amebiasis

Given that inflammation is a characteristic feature of amebic colitis and *Eh*MIF is expressed during human infection, we tested in humans the correlation between a marker of host inflammation (MPO) and the amebic protein *Eh*MIF: MPO is a major component of neutrophils, and the concentration of MPO in stool samples is a widely used marker of intestinal inflammation (Maharshak *et al.* 2010). We measured the concentrations of *Eh*MIF and MPO in the stools samples of persons with intestinal amebiasis and found a positive association between *Eh*MIF and intestinal inflammation by Pearson's correlation (n = 35, r = 0.41; *P* = .015 (**Fig 5.1A and 5.1 B**). We concluded that the correlation of stool *Eh*MIF with MPO was consistent with a potential role for *Eh*MIF in colonic inflammation in humans with amebiasis.

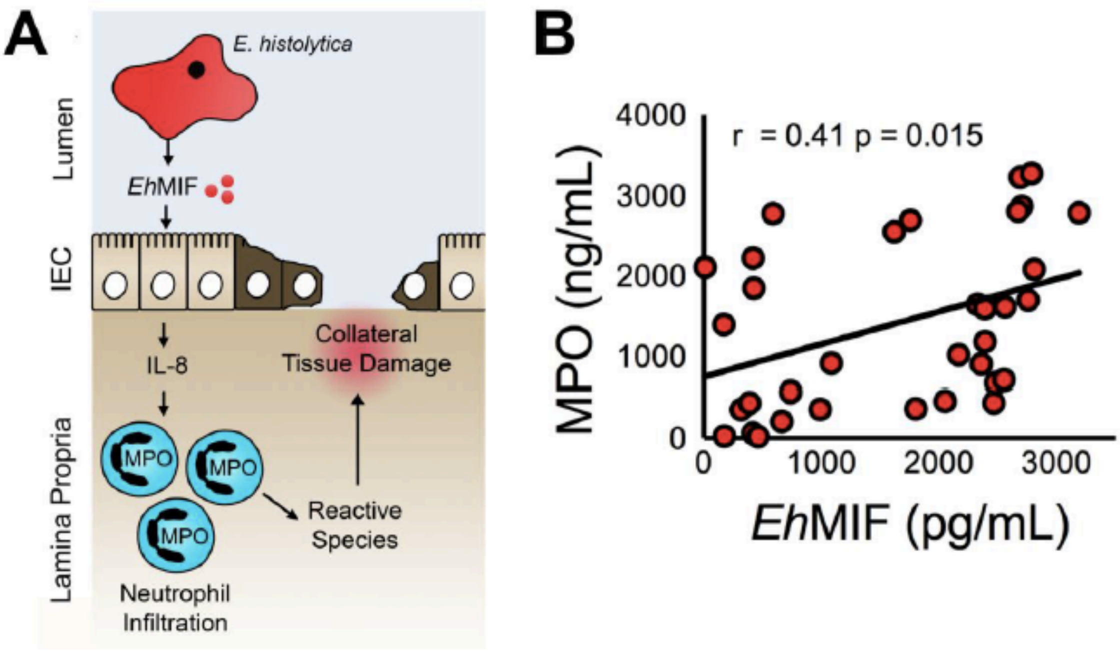

Fig 5.1: **Association between *Eh*MIF and intestinal inflammation**. *A*, Schematic of the hypothesis of how secreted *Eh*MIF promotes mucosal inflammation. *B*, Significant positive correlation between fecal *Eh*MIF levels and the MPO marker of intestinal inflammation in persons with amebiasis (*n* = 35). A *P* value < .05 was considered statistically significant.

5.4.2 *Eh*MIF Induces IL-8 Secretion From Human Intestinal Epithelial Cells

Cytokines such as IL-1β and MIF are secretory proteins that lack a signal peptide and therefore do not follow the classical endoplasmic reticulum–to-Golgi pathway of secretion. Human MIF is constitutively expressed, accumulated in the cytoplasm, and secreted by a nonclassical pathway involving an ATP-binding cassette (ABC) transporter (Weiser *et al.* 2015). Similar to human MIF, *Eh*MIF lacks a signal peptide and ABC transporters can be found in *E. histolytica* (Kissoon-Singh *et al.* 2013). We used mass spectrometry to further confirm the protein expression of *Eh*MIF. We investigated whether *Eh*MIF is secreted and found that it was present in secreted fractions by ELISA and immunoblot (**Fig 5.2A**). We further investigated the effect of transport inhibitors on *Eh*MIF secretion. We found that *Eh*MIF secretion was not inhibited by brefeldin A, an inhibitor of the classical secretory pathway. Probenicid, an inhibitor of nonclassical protein export, blocked *Eh*MIF secretion (**Fig 5.2A**). These findings suggest that *a nonclassical pathway secretes EhMIF along with other MIF homologs*. In our study, the maximum inhibition achieved was approximately 50%. This raises the possibility of other secretion pathways for *Eh*MIF. *Eh*MIF was tested for its ability to induce IL-8 using a cellular approach, given that epithelial cells are the first host cells to encounter secretory products released by enteric parasites. It has long been hypothesized that *E. histolytica*, by means of soluble mediators, stimulates chemokine production from host cells (Banerjee *et al.* 2011). The *E. histolytica* secretory protein fraction was previously shown to induce IL-8 production by Caco-2 human colonic epithelial cells (Spalinger *et al.* 2016). We were able to reproduce this finding and found that IL-8 production was inhibited by antibodies that blocked *Eh*MIF (**Fig 5.2B**). The rabbit anti-*Eh*MIF antibodies used for these experiments did not cross-react with human MIF (Supplemental **Fig 5.2A**). In addition, anti-*Eh*MIF antibodies inhibited the IL-8 secretion induced by co-culturing intestinal epithelial cells (IECs) with *E. histolytica* parasites (**Fig 5.2C**). We also determined the effect of endotoxin-free recombinant *Eh*MIF (<1 pg LPS/µg protein) on IECs. Recombinant glutathione S-transferase, an irrelevant protein, was used as a negative control and human MIF as a positive control. *Eh*MIF induced IL-8 production in a time- and dose-dependent manner, and treatment with anti-*Eh*MIF inhibited *Eh*MIF-induced IL-8 production (**Fig 5.2D**). These data indicate that *Eh*MIF was a cause of IL-8 secretion by *E. histolytica.*

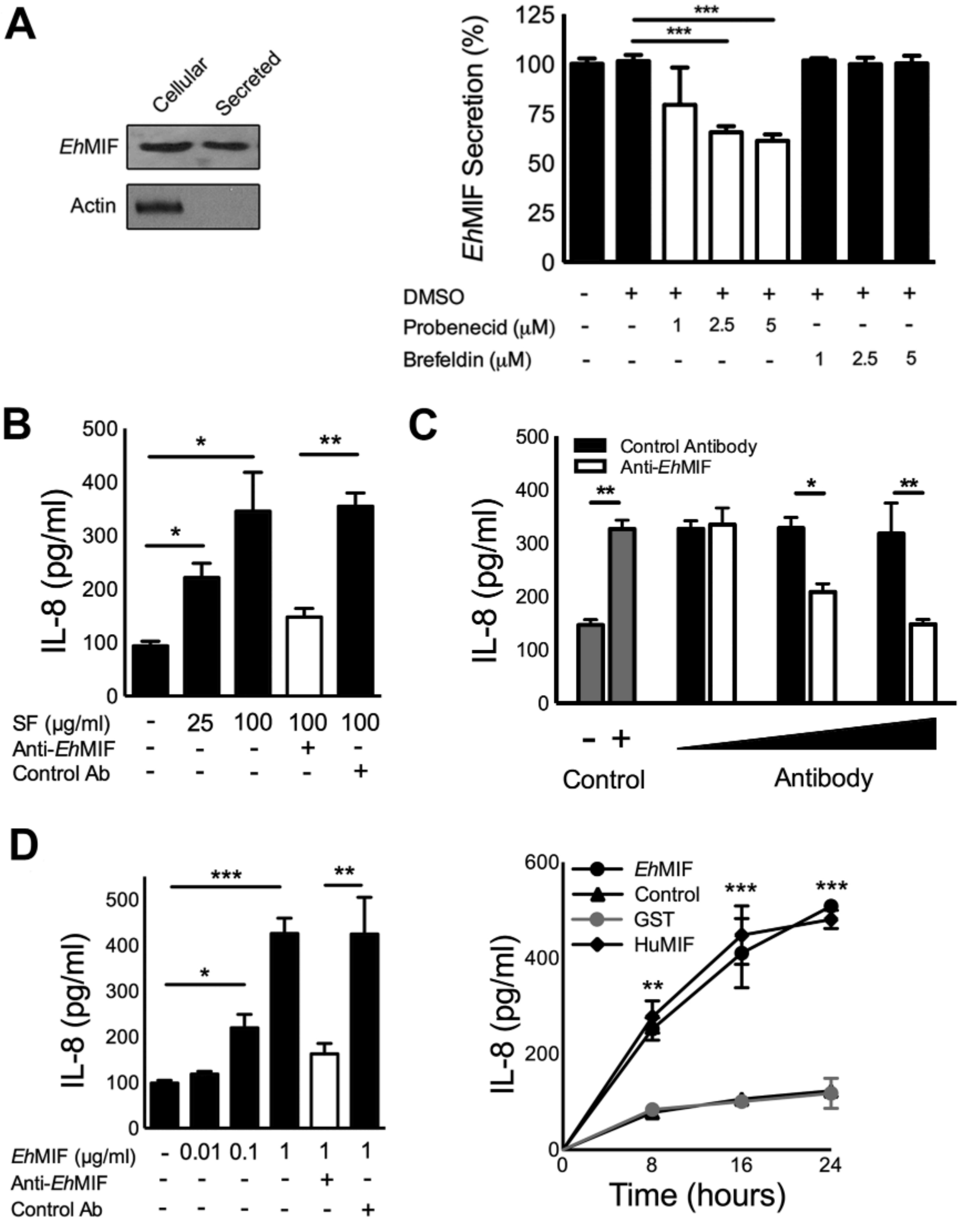

Fig 5.2. *Eh*MIF induces IL-8 production from human intestinal epithelial cells. *A*, Secretion of *Eh*MIF by amebic trophozoites. Immunoblot analyses of the cell

lysate and secreted fractions of *E. histolytica* using anti-*Eh*MIF antibodies. Actin detection serves as negative controls for cell lysis. *Eh*MIF ELISA of *E. histolytica* secreted fractions. *Eh*MIF secretion is not inhibited by the classical pathway inhibitor brefeldin A. Probenicid, an inhibitor of nonclassical protein export, blocked *Eh*MIF secretion. *B*, Anti-*Eh*MIF antibodies blocked *E. histolytica* secretory fraction-induced IL-8 production by colonic epithelial cells (Caco-2 cells). *C*, *E. histolytica* parasites cocultured with IECs in the presence of antibodies. *D*, *Eh*MIF stimulates IL-8 production in a dose- and time-dependent manner. Data represent mean and SD of triplicates from 1 experiment and are representative of 3 independent experiments. **P* < .05; ***P* < .01; ****P* < .001.

5.4.3 Anti-*Eh*MIF Antibody Treatment Reduces Mucosal Inflammation

We further investigated in an amebic mouse model the role of *Eh*MIF on chemokine secretion and mucosal inflammation. CXCL1/KC and CXCL2/MIP-2 are mouse homologs of human IL-8 and are key chemokines in neutrophil recruitment and inflammation. Host MIF was shown to induce mouse IL-8 homolog production from alveolar epithelial cells (Morf *et al.* 2013). Mice infected with *E. histolytica* had elevated levels of CXCL1 and CXCL2 (**Fig 5.3B**), in keeping with previous studies(Loftus *et al.* 2005). We found that mice treated with mouse anti-*Eh*MIF-blocking antibodies had reduced CXCL1 (**Fig 5.3B**). Neutrophil MPO activity, an indicator of neutrophil infiltration (Kosek *et al.* 2013) was significantly lower in anti-*Eh*MIF-treated mice compared with controls (**Fig**

5.3C). The reduction of neutrophil infiltration by anti-*Eh*MIF antibodies was consistent with its effect on chemokine production. These anti-*Eh*MIF antibodies did not cross-react with mouse MIF. We concluded that anti-*Eh*MIF blocked neutrophil recruitment to the gut in the mouse model of amebic colitis. In a previous study, anti-*Eh*MIF antibodies were detected in the serum samples of children living in an endemic area (Sommerville *et al.* 2013). We tested whether anti-*Eh*MIF was associated with protection from amebiasis. Children in the top 50th percentile for anti-*Eh*MIF serum immunoglobulin G had a significantly higher probability of survival free of *E. histolytica* infection, compared with children in the lower 50th percentile. This finding supports the hypothesis that anti-*Eh*MIF antibodies have a protective role.

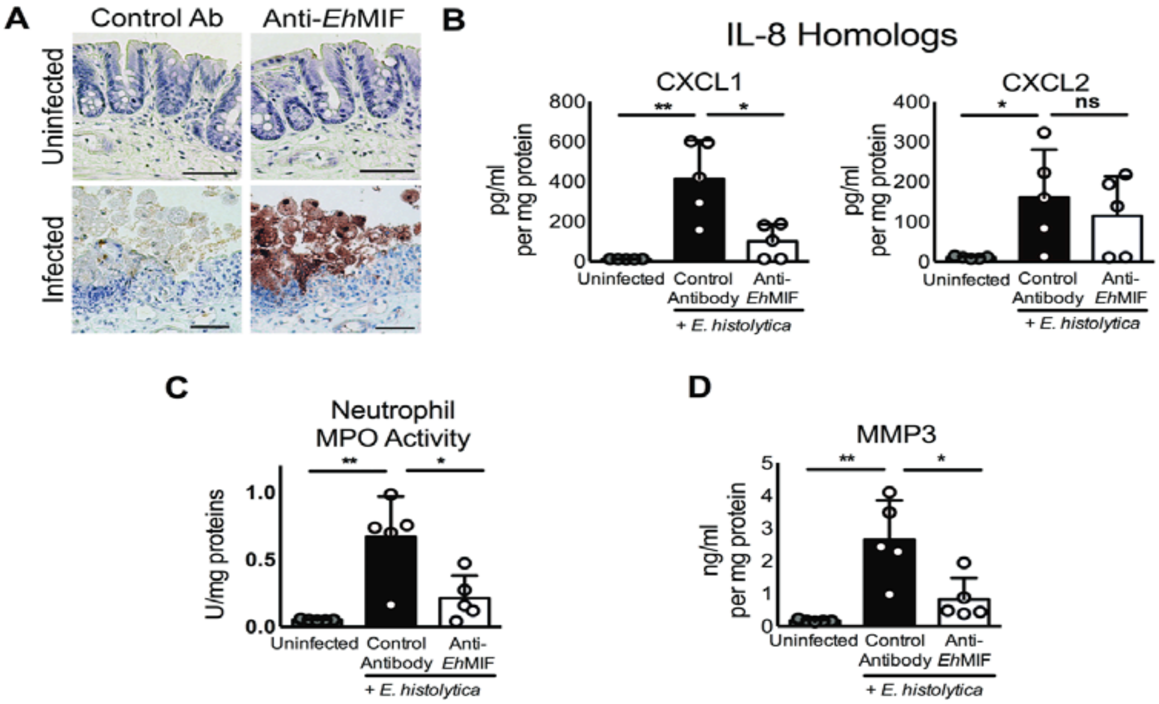

Fig 5.3. Anti-*Eh*MIF antibody treatment reduces *E. histolytica*–induced

inflammation. *A*, Immunohistochemical stain showing *Eh*MIF protein expression (brown) and interaction with host during infection. Scale bars, 50µm. *B-D*, Mice treated with anti-*Eh*MIF antibodies had reduced intestinal tissue levels of CXCL1 chemokine, neutrophil infiltration, and MMP-3. Data represent mean and SD ($n = 5$ mice per group). *$P < .05$; **$P < .01$.

Intraperitroneal

5.4.4 Overexpression of *Eh*MIF Enhances Mucosal Inflammation

In addition to antibody-mediated neutralization, we used a genetic approach to test the effect of *Eh*MIF on mucosal inflammation. We generated *E. histolytica* trophozoites that overexpress *Eh*MIF (**Fig 5.4A and 5.4B**), given that *Eh*MIF is a soluble secreted nontoxic protein, and gene overexpression can be technically accomplished in amebic strains that are adapted for virulence in the mouse model (Bai *et al.* 2012; Thibeaux *et al.* 2014). Mice infected with parasites overexpressing *Eh*MIF showed increased chemokine production, mucosal inflammation, and pathology compared with parasites transfected with the empty vector (**Fig 5.4C, 5.5 A–D**). No significant differences in parasite antigen load were observed between mice infected with parasites overexpressing *Eh*MIF and controls postchallenge. This was also true for groups given isotype antibody control or anti-*Eh*MIF antibody, indicating that the 2 groups were exposed to the same levels of *E. histolytica* antigens. In addition, parasites overexpressing *Eh*MIF did not exhibit any growth or cytotoxicity difference compared with controls (**Fig 5.4 D**). These data indicate that overexpression of *Eh*MIF increased intestinal inflammation and damage.

5.4.5 *EhMIF* and Matrix Metalloproteinases Expression

Matrix metalloproteinases (MMPs) are enzymes capable of degrading extracellular matrix proteins. MMPs are expressed in all infections with protozoan parasites (Boaventura *et al.* 2010). Matrix metalloproteinase 1 (MMP-1) and matrix metalloproteinase 3 (MMP-3) genes were among the most overexpressed genes in persons suffering from intestinal amebiasis (Hao *et al.* 2015). In the human colon explant model, MMP-3 was shown to play a central role in amebic colitis, and inhibition of MMP activity blocked colonic mucosa invasion by *E. histolytica* (Coombes *et al.* 2013). We found that mice given anti-*Eh*MIF antibodies had reduced MMP-3 mucosal levels, and parasites overexpressing *Eh*MIF generated higher MMP-3 production during infection compared with controls (**Fig 5.4 D and Figure 5.5 C**). Proinflammatory cytokines, including human MIF has shown to stimulate the expression of MMPs. However, in our hands, recombinant *Eh*MIF failed to directly induce MMP-3 production from intestinal epithelial cells and fibroblasts in vitro. These findings suggest that MMP3 elevation might be due to *Eh*MIF-induced mucosal inflammation and not a direct effect of *Eh*MIF on host cell MMP-3 secretion.

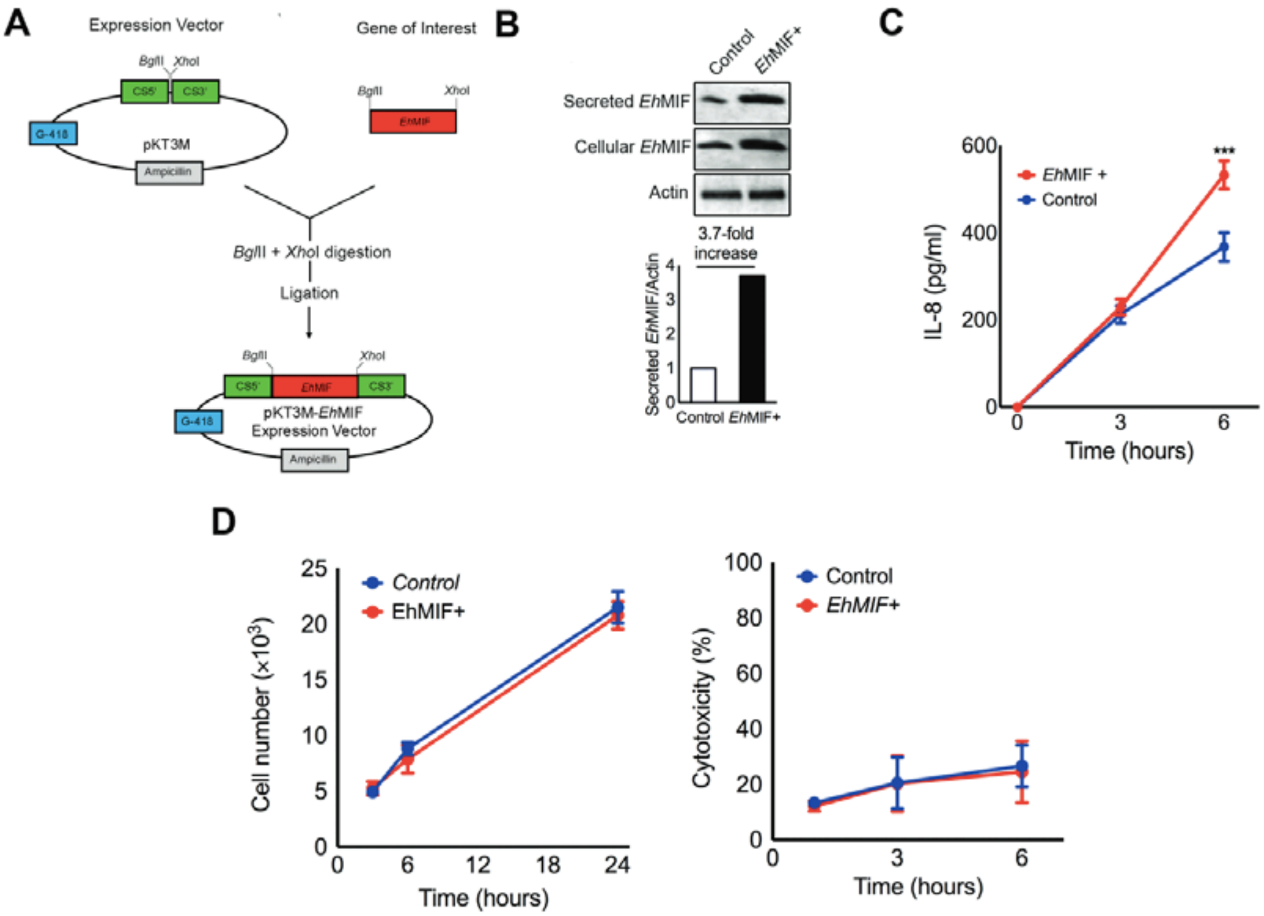

Fig 5.4. *Eh*MIF overexpression by *E. histolytica* parasites. *A*, Schematic for the preparation of the pKT3M-*Eh*MIF expression vector. *Eh*MIF gene (EHI7A_051880). CS5': CS promoter (EHI_024230). CS3': CS UTR (EHI_024230). G-418 and ampicillin resistance genes. *B*, *Eh*MIF expression assessed by immunoblot analysis. Actin was used as a loading control. Quantification of secreted *Eh*MIF bands relative to actin by densitometry. *C*, IECs cocultured with *E. histolytica* parasites overexpressing *Eh*MIF (*Eh*MIF+) compared to empty vector controls. *D*, No difference in parasite growth or parasite-induced cytotoxicity between *Eh*MIF+ parasites and WT parasite controls with empty vector. Data represent mean and SD of triplicates from 1

experiment and are representative of 3 independent experiments. A *P* value < .05 was considered statistically significant.

****P* < .001.

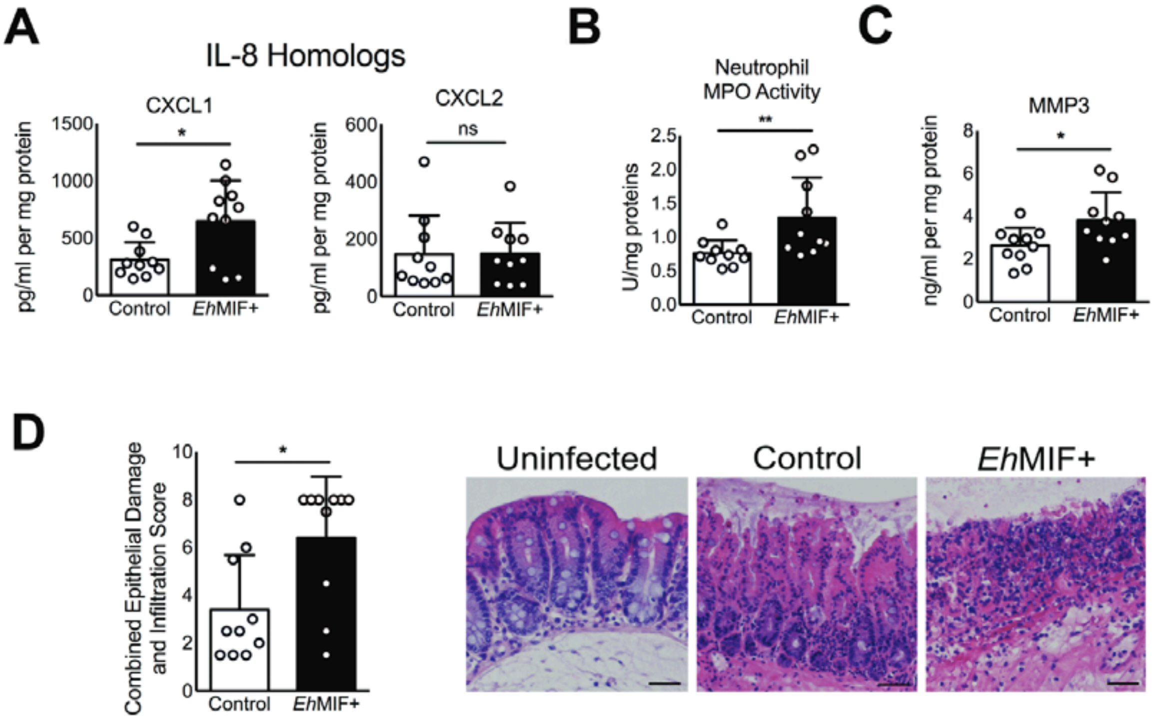

Fig 5.5. *Eh*MIF overexpression increases inflammation. *A–C*, Increased CXCL1, neutrophil influx, and MMP-3 epithelial tissue levels in mice infected with *Eh*MIF+ parasites compared with controls. *D*, Representative H&E-stained images and combined epithelial damage and infiltration scores. Scale bars, 100µm. Data represent mean and SD (*n* = 10 mice per group). **P* < .05; ***P* < .01; ****P* < .001.

5.5 Discussion and conclusions

Mucosal inflammation resulting from infection with *E. histolytica* is a hallmark of amebic colitis. In this study, we examined the role of the cytokine MIF homolog of

E. histolytica in mucosal inflammation. We found a positive correlation between *Eh*MIF levels and intestinal inflammation in infected persons. Using cellular and mouse models, we demonstrated that *Eh*MIF induces chemokine secretion from intestinal epithelial cells, resulting in neutrophil influx. These findings implicate *Eh*MIF as a causal factor of mucosal inflammation during infection.

Severe forms of amebic colitis are associated with both high mortality and morbidity. Antibiotics alone are often not enough to treat disease, and surgical removal of the inflamed colon may not prevent death (Debnath *et al.* 2012). Metronidazole is the antibiotic of choice for treating amebic colitis. In preclinical mouse models, metronidazole was shown to be very effective at killing ameba but had little effect on *E. histolytica*–induced mucosal inflammation (Moonah *et al*. 2013). Adjunctive anti-inflammatory strategies may be needed to improve the clinical outcome of amebic colitis. Neutralization of a parasite mediator of host inflammation such as *Eh*MIF may attenuate disease. However, further studies are needed to determine whether the combination of metronidazole and anti-*Eh*MIF antibodies is superior to metronidazole alone for treatment of severe amebic colitis.

Mucosal inflammation also plays a key role in other human protozoan infections. Mucosal leishmaniasis is a destructive disease caused by the protozoan parasite *Leishmania*. Neutrophil recruitment and an exaggerated inflammatory response perpetuate the disease in mucosal leishmaniasis (Boaventura *et al.* 2010). *Trichomonas vaginalis* causes the most prevalent nonviral sexually transmitted infection worldwide. Vaginitis is characterized by infiltration of the vaginal mucosa with neutrophils, which contributes to the symptoms of vaginal discharge (Twu *et al.* 2014) Toxoplasmosis is a parasitic disease, caused by *Toxoplasma gondii*, which can infect the brain, eye, and the developing foetus. The parasite first enters through the intestine and induces recruitment of neutrophils to the site of intestinal infection. This was recently shown to facilitate the spread of infection (Coombes *et al.* 2013). Similar to *E. histolytica*, these parasites encode their own MIF homolog. It is possible that these parasite MIF homologs are contributing to the mucosal influx of neutrophils during their respective infections.

A recent study found that the *Plasmodium*-encoded MIF, through its proinflammatory properties, interfered with the development of immunological memory by inducing the development of short-lived effector cells rather than memory cells. This rendered the host susceptible to reinfection by the parasite (Sun *et al.* 2012). This finding could help explain why antibodies against *Eh*MIF were associated with protection from reinfection. Additional studies, however, are needed to validate our preliminary findings.

In conclusion, we identified *Eh*MIF as a specific amebic mediator of host chemokine expression, neutrophil infiltration, and mucosal immunopathology during infection. Intestinal amebiasis remains a major global health problem, especially in children living in low-income countries. With no vaccine and only a single drug class to treat this devastating disease, *Eh*MIF may represent a promising immunotherapeutic target to prevent or attenuate amebic disease.

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CHAPTER SIX

Prevalence of selected enteropathogens among patients attending health centers in Northern South Africa: Impact of co-infections in the outcome of amebiasis

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CHAPTER 6

6.1 Introduction

Amebiasis typically occurs due to the consumption of food or water contaminated with the parasite cysts. This disease is a serious health problem in many developing countries. We anticipate that the water supply may also be contaminated with other waterborne pathogens (Pandey *et al.* 2014). Many factors contribute to the virulence of *E. histolytica* and some studies showed that in addition to the factors discussed previously malnutrition and coinfections with enteropathogens, can influence the course of an amebic infection (Kotloff *et al.* 2013). In regions where amebiasis is endemic mixed intestinal infections with *E. histolytica*/enteropathogenic bacteria are common (Orlandi *et al.* 2001).

A number of studies have been conducted to identify the responsible factors for the tissue damage inflicted by the parasite *Entamoeba histolytica* (Nakada-Tsukui and Nozaki 2016). The parasite colonizes the lumen of the colon without causing damage to the intestinal mucosa, but due to unknown circumstances becomes invasive, destroying the intestinal tissue and causing invasive diseases (Watanabe *et al.* 2014). Mixed intestinal infections with *E. histolytica* and enteropathogenic bacteria, common in endemic countries, have exacerbated manifestations of disease (Galván-Moroyoqui *et al.* 2008). Galván-Moroyoqui *et al.* (2008) implemented an experimental system to study amebic virulence in the presence of pathogenic bacteria and examined the consequences using an epithelial cell cytotoxicity assay; their results showed that amoebae that ingested enteropathogenic bacteria became more virulent, causing more damage to epithelial cells. *In vitro* experiments have shown that after phagocytosis of an *E. coli* non-pathogenic laboratory strain (Ec346), trophozoites of *E. histolytica* increased their virulence together with their adhesive properties to target cells (Padilla-Vaca *et al.* 1999)

This study hypothesized that, pathogens coinfecting with *E. histolytica* are common in the study population and cause more diarrhea than infection with *E. histolytica* only. It is also well established that co-infection with the non-pathogenic *E. dispar* is prevalent in developing countries (Ramos *et al.* 2005); co-infections with *E. histolytica* and *E. dispar* has also been shown in studies conducted in South Africa (Samie *et al.* 2009). Many amebic studies focus on individuals with infections caused by *Entamoeba* species only, ignoring the fact that many symptomatic infections may be due to a different number of pathogens within the host which in turn complicates the management or treatment of the disease (Fletcher *et al.* 2012). Little has been explored or elucidated about responses triggered by other enteropathogens and the interplay with the invading amoeba that could be important in the induction of tissue invasion and disease. Therefore, the study of co-infections could help in understanding how this modifies disease manifestations by modulating pathogen virulence and the host response.

We approached this gap by analyzing the interaction of *E. histolytica* with other enteropathogens known to cause diarrheal infections, *Shigella/EIEC, Campylobacter*, *Enterotoxigenic E. coli*, Norovirus GII and Adenovirus, *Cryptosporidium spp* and *Giardia lamblia*. The results were compared with those obtained with *E. histolytica* with enteropathogens and with *E. histolytica* interacted with enteropathogens. The impact on the outcome of the infection was compared between non-diarrheal and diarrheal stool samples. Our results will provide insights into amebic infections, as interplay with other pathogens apparently influences the intestinal environment, the behavior of cells involved and the manifestations of the disease.

6.2 Purpose of the Study

6.2.1 Research question

Does co-infection of *E. histolytica* with other enteropathogens increase diarrhea?

6.2.2 Aim

To investigate impact of co-infections with *E. histolytica and* other enteropathogens on the severity of diarrhea

6.2.3 Specific objectives

a**.** To determine the prevalence of enteropathogens in the South African population.

b**.** To investigate the impact of co-infections with *E. histolytica* on diarrhea.

6.3 Materials and Methods

6.3.1 Study population and sample collection

Characteristics of the study population and sample collection procedures are as described in chapter 3, section 3.3.2 and 3.3.3.

6.3.2 Genomic DNA purification

The procedures for DNA extraction are as described in chapter 3, section 3.3.4.

6.3.3 Pathogen detection

Three panels of multiplex real time PCRs reactions were used. All the PCR reactions were performed using the BioRad CFX96 (BioRad, Hercules, CA) for detection. The results were analyzed by use of the Bio-Rad CFX manager software. To validate the analysis, negative controls were included in the assays. The negative controls consisted of stool samples from a healthy child, negative for all the relevant targets and a no template control. A sample was considered positive for a particular target if the amplification curve crossed the threshold line Cq (quantitative cycle) lower than the cut-off value. If the amplification curve does not cross the threshold line, the sample was considered negative. The quantification cycle 35 was set as cutoff to avoid false positive results. PCR mixes and cycling conditions are as follows:

6.3.3.1 Detection of *E. histolytica*

A diagnostic Taqman qPCR assay (with Taqman probes and species or genus specific primers) was used to detect the presence of *Entamoeba histolytica* in the extracted fecal genomic DNA. The protocol was done following the method described by Ngobeni *et al.* (2017) , using the same primers and probes.

6.3.3.2 Detection of *Campylobacter spp*., Enterotoxigenic *E coli* (ETEC), Norovirus GII and *Shigella*/EIEC

The fecal DNA was also subjected to a multiplex qPCR for the detection of enteric pathogens *Campylobacter (cadF)*. Enterotoxigenic *E coli* (ETEC), Norovirus (GII) (Liu *et al.* 2014) and *Shigella/EIEC*. Twenty five microliter reaction contained Bio-Rad iQ powermix, 0.2 uM of primer and 0.4 uM of probe for ETEC (*STh*), 0.4 uM of primer and 0.2 uM of probe for Campylobacter *spp,* 0.2 uM of primer and 0.2 uM of probe for *Shigella/EIEC* and 0.4 uM of primer and 0.2 uM of probe for Adenovirus. Cycling conditions were 3 minutes at 95 °C for initial denaturation, 45 cycles of 95 °C for 10 seconds, 60 °C for 1 minute. The following probes/ primers were used for the amplification (**Table 6.1**) (Liu *et al.* 2014)

Table 6.1: Primers and probe used for detection of enteric pathogens

Target name	Oligos	Sequence (5’-3’)	Fluorophore
ETEC (*STh*)	Forward	GCTAAACCAGYAGRGTCTTCAAAA	Quasar-705-BHQ2
	Reverse	CCCGGTACARGCAGGATTACAACA	
	Probe	TGGTCCTGAAAGCATGAA	
Campy (cadf)	Forward	CTGCTAAACCATAGAAATAAAATTTCTCAC	
	Reverse	CTTTGAAGGTAATTTAGATATGGATAATCG	FAM-BHQ1
	Probe	CATTTTGACGATTTTTGGCTTGA	
Shigella/ EIEC (ipaH)	Forward	CCTTTTCCGCGTTCCTTGA	
	Reverse	CGGAATCCGGAGGTATTGC	
	Probe	CGCCTTTCCGATACCGTCTCTGCA	VIC-BHQ2
Adeno (Hexon)	Forward	GCCACRGTGGGRTTTCTCAACTT	
	Reverse	GCCGCAATGGTCTTACATGCACATC	
	Probe	TGCACCAGGCCCGGGCTCAG	Texas Red-BHQ2

6.3.3.3 Detection of Norovirus GII

Norovirus GII was tested with a real time reverse transcription-PCR assay. The PCR was performed with 25µl reaction mixture containing Agpath-ID one-step reverse transcription-PCR reagent, 0.2 uM of primer and 0.1 uM of probe. Cycling conditions were 1 cycle 50°C for 30 min, 95°C for 10 min, 40 cycles 95°C for 10 seconds, 58°C for 1 minute. The following probes/ primers were used for the amplification (**Table 6.2**) (Liu *et al.* 2014)

Table 6.2: Primers and probe used for detection of Norovirus GII

Target name	Oligos	Probe/primer Sequence	Fluorophore
Norovirus GII	Forward	CARGARBCNATGTTYAGRTGGATGA	FAM-BHQ2
	Reverse	TCGACGCCATCTTCATTCACA	
	Probe	TGGGAGGGCGATCGCAATCT	

6.3.4 Statistical analysis

The Fishers exact test was used to analyze contingency tables. The D'Agostino & Pearson omnibus normality test and the nonparametric Mann–Whitney comparisons test were used to analyze and compare qualitative data. Tests were performed using GraphPad Prism version 6. The differences were considered significant if the p value was less than 0.05.

6.4 Results

6.4.1 Demographic and clinical features

Demographic details of the study population are presented in chapter 3, table 3.2.6.4.2 Overall prevalence of pathogens in the study population

The overall prevalence of enteric pathogens in the study population was 58% (279/484). The most prevalent pathogen was Norovirus GII (14%) and the least prevalent was *ETEC (STh)* (2.1%). Co- infections were common in the study population (**Table 6.3**).

Table 6.3: Overall prevalence of pathogens in the study population

Pathogens	Samples tested (n= 484)
Norovirus GII	67 (14%)
Adenovirus	62 (13%)
Shigella/EIEC	59 (12%)
E. histolytica	41 (8.5%)
Campylobacter	40 (8.3%)
ETEC *(STh)*	10 (2.1%)
Co-infections	49 (10%)

6.4.3 Pathogen distribution in rural and urban communities

We examined if there is a difference in the frequency of pathogens in rural and urban areas. Norovirus GII*, Shigella/EIEC,* ETEC *(STh),* Adenovirus *and E. histolytica* prevalence were markedly different in the urban and rural populations. (**Table 6.4**).

Table 6.4: Pathogen distributions in rural and urban communities

Pathogens	Rural N=227	Urban N=257	Statistical significance	Chi-square for pathogen distribution
Norovirus GII	15 (7%)	52 (20%)	<0.0001	<0.0001
Shigella/EIEC	19 (8%)	40 (16%)	0.0158	
Adenovirus	50 (22%)	12 (5%)	<0.0001	
Campylobacter *spp* (CadF)	15 (7%)	25 (10%)	n.s	
ETEC (STh)	8 (4%)	2 (1%)	0.0341	
E. histolytica	25 (11%)	16 (6%)	0.0717	
Co-infections	22 (10%)	27 (11%)	n.s	

***Ns=not significant**

6.4.4 Pathogen distribution in diarrheal and non-diarrheal samples

Enteric pathogens were detected in diarrheal and non-diarrheal patients in the study population. Norovirus GII, *Campylobacter spp* and *E. histoltytica as well* as co-infections were significantly associated with diarrhea in the study population. Additionally, ETEC (*STh*), *Shigella/EIEC* and Adenovirus were found to be more common in non-diarrheal than diarrheal patients, however the difference was insignificant (**Table 6.5**).

Table 6.5: Detection rate for pathogens in diarrheal and non-diarrheal stool samples by qPCR

Pathogens	Diarrheal N=186	Non-diarrheal N=298	Statistical significance	Chi-square for pathogen distribution
Norovirus GII	45 (24%)	22 (7%)	<0.0001	<0.0001
Shigella/EIEC	24 (13%)	35 (12%)	n.s	
Adenovirus	19 (10%)	34 (11%)	n.s	
Campylobacter (CadF)	29 (16%)	11 (4%)	<0.0001	
ETEC (STh)	3 (2%)	7 (2%)	n.s	
E. histolytica	11 (6%)	30 (10%)	n.s	
Co-infections	29 (16%)	20 (7%)	0.0029	

***Ns=not significant**

6.4.5 Co-infections were common in study population

Co-infections were observed in about 10% (49/484) of the study population. Although the difference was not significant, a higher prevalence of co-infections was seen in urban (11%, 27/257) than rural areas (10%, 22/227). Moreover, among diarrheal samples, co-infections with 2 pathogens were more common in diarrheal patients than non-diarrheal (P<0.005). The most common co-infections were NorovirusGII/ *Campylobacter spp* and *Shigella/EIEC* / Norovirus GII in urban areas, and in rural areas the most occurring co-infections was Norovirus GII/ Adenovirus in diarrheal stools. Additionally, co-infections with 3 pathogens were detected more in rural areas than urban, with the following combination: *E. histolytica*/ Adenovirus / ETEC (*STh*). We also detected patients with more than 3 pathogens; this was seen in one diarrheal patient from rural areas (*E. histolytica*/ NorovirusGII / *Campylobacter (cadF)/Shigella/EIEC* (**Table 6.6**).

Table 6.6: Impact of co-infections on diarrheal and non-diarrheal samples from rural and urban areas.

	Urban (Ds=125, ND=132				Rural (Ds=61, ND=166			
	Total (%)	DS (%)	ND (%)	p.value	Total (%)	DS (%)	ND (%)	p.value
Zero pathogens	138 (54)	59 (47)	79 (60)	0.0460	121(53)	28(46)	93(56)	n.s
Pathogens (Including co-infections)	119 (46)	66 (53)	53 (40)	0.0460	106(47)	33 (54)	73(44)	ns
1 pathogen	92(36)	46(37)	46(35)	n.s	80(35)	24(39)	60(36)	n.s
Co-infections	27(11)	20(16)	7(5)	0.0073	22(10)	8 (13)	13(9)	n.s
2 pathogens	28(11)	19(15)	7(5)	0.0120	16(7)	8(13)	11(7)	n.s
3 pathogens	3 (1)	0(0)	2 (2)	n.s	1(0.4)	1(2)	0(0)	n.s
4 pathogens	0(0)	0(0)	0(0)	ns	1(0.40	1(2)	0 (0)	n.s

***ND= non-diarrheal, DS= diarrheal, n.s=not significant**

6.4.6 Pathogens Co-infecting with *E. histolytica*

Enteric pathogen co-infecting with *E.histolytica* were observed and the results were compared with those obtained with *E. histolytica* that were not interacted with enteropathogens and with *E. histolytica* interacted with enteropathogens.

The results of this study showed no impact of pathogens co-infecting with *E. histolytica* on the outcome of amebic infection (**Fig 6.3**).

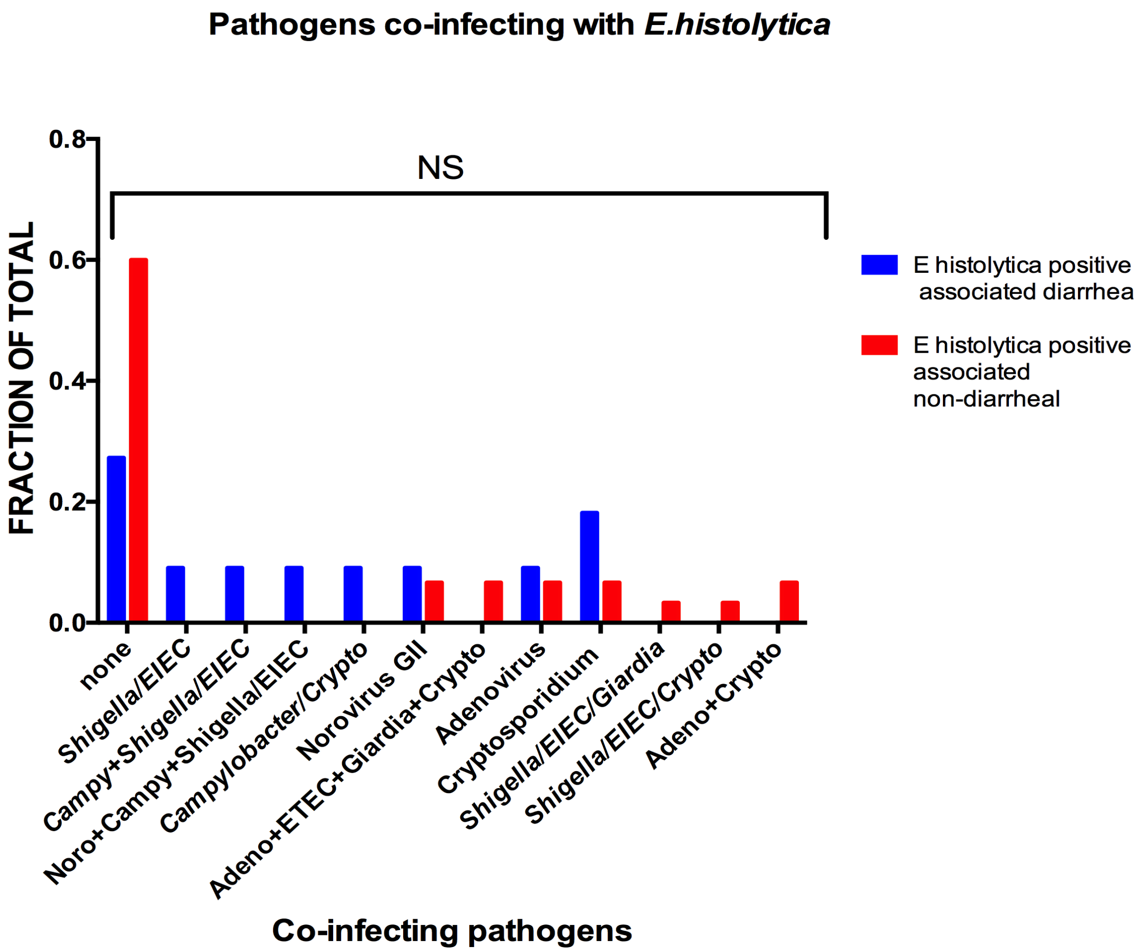

Fig 6.3: Pathogens co-infecting with *E. histolytica* in diarrheal and non-diarrheal patients. Abbreviation: Campy= *Campylobacter* spp, Noro=Norovirus GII, Adeno= Adenovirus, ETEC=Enterotoxigenic *E. coli, Crypto=Cryptosporidium*

6.5 Discussions and conclusions

Infectious diarrhea is documented as one of the most common diseases in humans, with particularly high morbidity and mortality in people from developing countries (Kotloff *et al.* 2012). It kills around 525000 of children every year, more than AIDS, malaria, and measles combined (Liu *et al.* 2012). A series of pathogens, including bacteria, parasites and viruses, may act as the etiological cause of this illness (Mandomando et al. 2007). These agents may vary according to geographical areas as well as socio-economic conditions of the analyzed population. The study of the prevalence of these agents is important to design specific control measures, vaccination strategies, and treatment regimens (Kotloff *et al.* 2012). The main purpose of this study was to examine the prevalence of selected enteropathogens among patients attending health centers in Northern South Africa and their impact in the outcome of amebiasis.

In some areas, especially developing countries, the enteropathogens causing most diarrheal infections, remain poorly investigated and characterized due to lack of most sensitive molecular techniques for identification of pathogens. The present study used Taqman qPCR assays, which enabled us to detect 6 different enteric pathogens with high prevalence of 58%. Comparing our findings with other studies, it is in accordance with other studies, a study by Fletcher *et al.* (2011), reported an overall prevalence of pathogens to be 55.7%. In Gaza strip, Abu Elamreen *et al.* (2007) and Elmanama and Abdelateef (2012) found 51.5% and 66.7% positive stools for common enteropathogens in children aged less than 5 years with acute gastroenteritis and diarrhea.

Of the 270 (58%) samples detected with enteropathogens, about 14% were Norovirus GII 13% Adenovirus, 12% *Shigella/EIEC,* 8.3% *Campylobacter spp,* *7% E. histolytica* (8.5%) and 2.1% ETEC. The findings of the study are concordant with several studies that reported the prevalence of enteropathogens, for example a study by Lee *et al.* (2017) reported a total prevalence of Norovirus GII to be 15.6 % in Korea, another study by Kabue *et al.* (2016) in South Africa, showed the prevalence of Norovirus GII to be 15.4 %. The overall pathogen detection rate of 58% in our study demonstrates the utility of multiplex PCR in epidemiologic studies and its potential to detect multiple pathogens.

The present study hypothesized that since urban environments tend to have more sophisticated and comprehensive water and sanitation infrastructure, the burden of diseases will be reduced. The pathogens were significantly different in rural and urban areas. Norovirus GII and *Shigella/EIEC*, Adenovirus, *ETEC (STh*) and *E. histolytica* were significantly different between the two study areas, with the high prevalence of Norovirus GII, *Shigella/EIEC spp* and *Campylobacter* in urban areas and the others in rural areas*.* More are people living in cities than in rural areas, increasing probability of transmission (J-Pal 2012).

Previous studies showed consistent association of *ETEC* (*STh*) in rural areas of Venda, South Africa (Platts-Mills *et al.* 2015). Geographical location or reduced virulence of pathogen circulating at the time of the study could also provide an explanation for the data's presentation. The prevalence rate of *E. histolytica* (8.5%) found in this study was not anticipated, as *E. histolytica* was not detected in the South African population (Platts-Mills *et al.* 2015). This prevalence exceeds those previously reported from other studies. In case control study of Ghanaian infants and children, *E. histolytica* was not detected in any of the stool samples tested (Krumkamp *et al.* 2015). The *E. histolytica* prevalence in our samples is in agreement with a previous study carried out among primary children in Libya in which the prevalence of 6.6% was reported (Sadaga and Kassem 2007).

Campylobacter spp are also reported as one of the most common causes of diarrhea worldwide. Every year almost 1 in 10 people fall ill and 33 million of healthy life years are lost (Lanata *et al.* 2013). The present study detected *Campylobacter spp* in 8.3%, which was lower than what Platts-Mills *et al.* (2015) reported in South Africa. Our low sample size does not preclude the fact that *Campylobacter spp* occurs at lower levels. Our findings are in contrast with the study in India where *Campylobacters* were isolated in only 2.6% samples from hospital and community (Vaishnavi *et al.* 2015). Further analysis showed that Adenovirus was markedly lower in the urban setting, which may indicate zoonotic transmission in the rural setting and the presence of a substantial animal reservoir of this virus (Ouattara *et al.* 2010).

Norovirus GII, *Campylobacter spp* and Co-infections were associated with diarrhea $p<0.05$ and none of the four pathogens tested (*Shigella/EIEC*, Adenovirus, ETEC (*STh*) and *E. histolytica* were associated with diarrhea. Thus, we found that Norovirus GII and *Campylobacter* spp as well as co-infections have a greater positive association with the diarrhea in this study than the other pathogens, a finding similar to other results reported by Li *et al.* (2016), who found a significant association of Norovirus GII with diarrhea. Previous epidemiologic studies demonstrated that Norovirus is a leading cause of diarrhea in many developing countries, and most high copy-number infections occurred among children younger than 2 years. Our findings are also in contrast with the finding in South Africa (Venda) where they documented a substantial burden of diarrhea associated with Norovirus GII infection in children under five years of age (Platts-Mills *et al.* 2015).

High rates of asymptomatic carriage of intestinal pathogens were observed in the study population. This was seen in the case of Adenovirus, *Shigella/EIEC, E. histolytica* and ETEC (*STh*). It is suggested that asymptomatic carriage may be caused by different factors including strain pathogenicity, host immunity against pathogenic factors and intestinal microbiome (Krumkamp *et al.* 2015). Co-infections pose one of the greatest challenges to global health and to our efforts to understanding the main cause of the infection and develop effective methods of enteric disease control (Susi *et al.* 2015). Populations living in rural areas of many low-income countries are exposed to both chronic and acute infection with multiple pathogens (Orlandi *et al.* 2001). Concurrent infection with more than a single pathogen might be common among people with diarrhea. Also multiple infections of diarrheal pathogens might cause more severe diarrhea compared to infection with a single pathogen, thereby complicating the treatment management procedures. This was supported by the results of the present study; we have found that among diarrheal samples, co-infections with 2 pathogens were more common in diarrheal patients than non-diarrheal (P<0.005). Similar findings were reported, where co-infections with 2 or more pathogens were found in diarrheal cases than controls (Shrivastava *et al.* 2017). Another study in China has also found that the prevalence of co-infection with two enteric pathogens was slightly higher in diarrhea cases than in asymptomatic controls (Zhang *et al.* 2016).

Amebiasis is a global health problem, resulting in high morbidity and mortality in people from developing countries. It has been suggested that enteric pathogen co-infections play an important role in the outcome of an *E. histolytica* infection (Galván-Moroyoqui *et al.* 2008), but most research efforts have only focused on a small range of species belonging to a few pathogen groups. This study aimed to assess the impact of co-infections with four different types of enteric pathogens in people from rural and urban communities of South Africa. Mixed intestinal infections *with Entamoeba histolytica* and enteropathogenic bacteria and viruses show exacerbated manifestations of disease, and are common in endemic countries, particularly in developing areas (Galván-Moroyoqui *et al.* 2008). The present study had shown most co-infections to occur more in diarrheal patients infected with *E. histolytica* than non-diarrheal patients, however, no difference was observed. Therefore this study showed no impact of the enteropathogens co-infecting with *E. histolytica* in the outcome of the infection. Co-infection of *E. histolytica* with *Shigella/EIEC* was high in diarrheal patients compared to other pathogens. Previous studies showed that infection with *Shigella dysenteriae* and *Shigella flexneri* was more common in children in Dhaka with *E. histolytica* or *E.*

dispar infection, potentially complicating the management of amebiasis (Haque *et al.* 1997). Therefore, more studies are needed to understand the impact/role these pathogens could have in the outcome of amebic infection.

A weakness in the current study was that the study was not adequately powered, in terms of sample size and the missing data on age and gender, to analyze the susceptibility to enteropathogens among participants stratified by age and gender.

In conclusion, the present study documents a diverse range of pathogens associated with community diarrhea in both adults and children. Poor hygiene and sanitation conditions increase the transmission dynamics of diarrheal diseases in community settings. All these diarrheal agents are transmitted either through contaminated food, water, or through the fecal oral route. There are limited numbers of studies focusing on multiple etiologies particularly in Giyani and Pretoria. Further studies are needed to investigate whether these pathogens are associated with poor growth, environmental enteropathy or impaired mucosal immunity in this area. Health information about how to prevent this infection in these populations should be provided to the residents. Measures are also needed for prevention and management of these diseases.

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CHAPTER SEVEN

Exosomes-like vesicles in the context of *Entamoeba histolytica*: Preliminary observations and review of the literature

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CHAPTER 7

7.1 Introduction

Production of extracellular vesicles/exosomes is a universal feature of cellular life and has been demonstrated for organisms as diverse as Proteobacteria, Archaea, plants, and animals (Ellis and Kuehn 2010; Deatheragea and Cooksona 2012; Silverman and Reiner 2012). There are different types of this vesicles, these includes exosomes, ectosomes, and apoptotic bodies. These vesicles differ in size, density, shape and the method of isolation and purification. Secretion of exosomes has been well described in prokaryotic and eukaryotic cells (György *et al.* 2011; Silverman and Reiner 2012). Those that are produced during an infection, can be host or pathogen derived. The produced Vesicles (from pathogens) may carry virulence factors and participate in their delivery to host, promoting the spread of the pathogen which in turn exacerbate the infection.

Extracellular vesicles are key players implicated in intercellular communication without direct cellular contact. However, the exact function of circulating exosomes remained unknown for a long period of time. They were just taken as garbage cans used by the cells to get rid of unwanted cellular components (Théry 2011). Recently, several studies have shown that these vesicles play a significant role in intercellular communication between cells; they act as messengers that transport information from distant cells. They have been shown to carry antigens, proteins, lipids and RNA (mRNA and miRNA) all of which can be transferred and become functional in target cells (Ratajczak *et al.* 2006; Valadi *et al.* 2007; Skog *et al.* 2008; Iglesias *et al.* 2012).

7.1.1 Which Pathogens Secrete EVs?

While my work focuses on parasites, it is important to note all microorganisms (fungi, viruses and bacteria) secrete EVs that have been shown to carry potent virulence factors that aid in binding, invasion, cytotoxicity, release of toxins, and host immunemodulation (Kulp and Kuehn 2010). Secretion of EVs has been described for both helminths and parasitic protozoa. In helminths, their role is to export proteins and miRNA and also serve as mechanism for host manipulation (Barteneva *et al.* 2013; Marcilla *et al.* 2014). In parasitic protozoa from the kinetoplastids family, EVs released by *Leishmania spp*. have been shown to induce specific recruitment of neutrophils to the site of infection. They are also taken up by phagocytic cells, enabling the delivery of immunomodulatory proteins contributing to the creation of a permissive environment for the infection. It has also been shown in *T. cruzi* that EVs contribute to the stabilization of the C3 convertase disturbing the functioning of the complement system (Geiger *et al.* 2010; Bayer-Santos *et al.* 2013).

Exosomes from malarial infections were able to induce parasite sexual development (Nantakomol *et al.* 2011). EVs isolated from dendritic cells and primed with Toxoplasma antigens conferred protection upon immunizations being a proof-of-concept of EVs as therapeutics agents (Pope and Lässer 2013). In trichomoniasis EVs increased virulence by inducing parasite attachment to cervical epithelium, thus facilitating host cell colonization(Twu *et al.* 2013). **Fig 7.1** below shows the role of EVs in parasitic diseases.

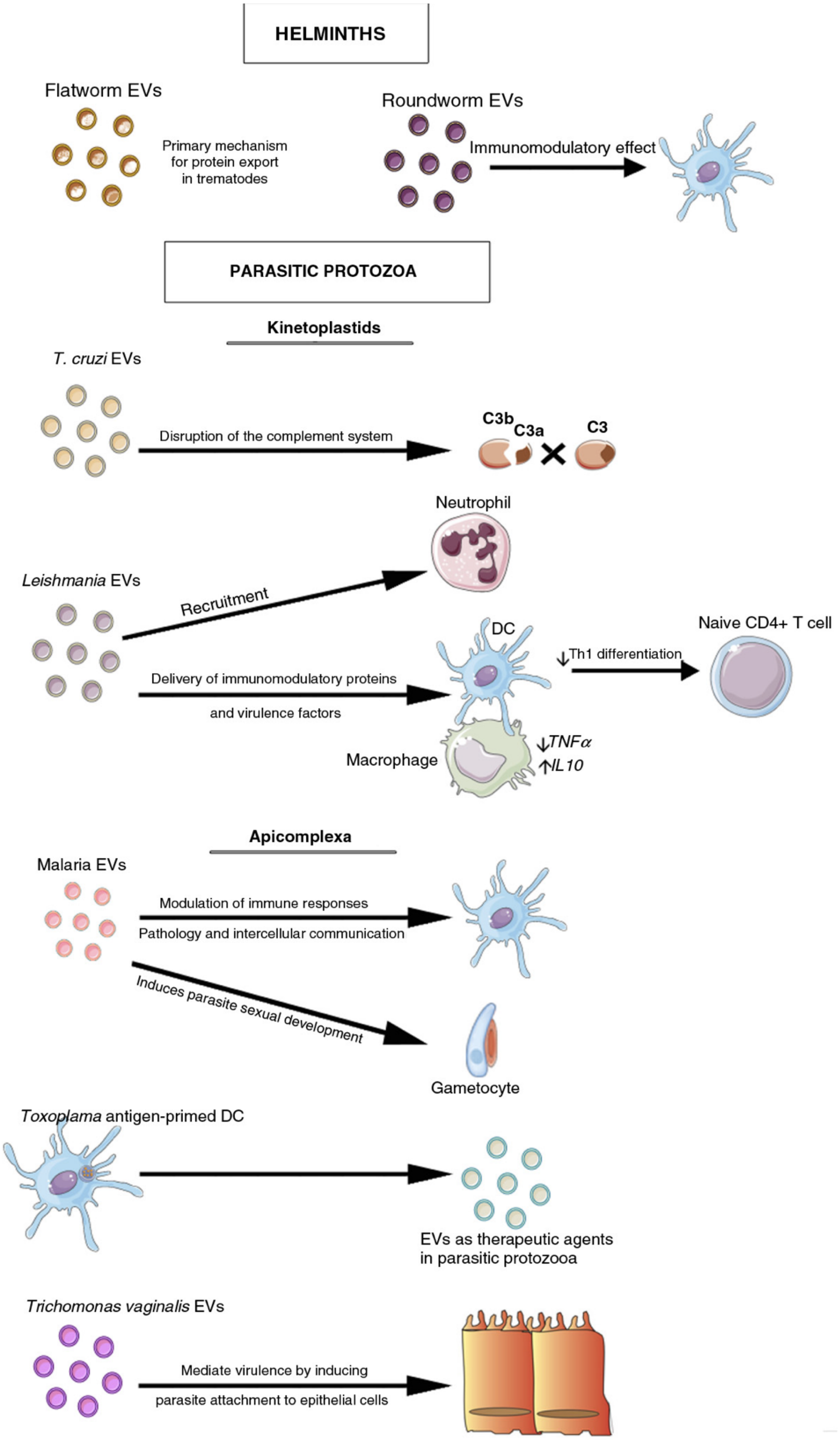

Fig 7.1: EVs in parasitic diseases (Yáñez-Mó *et al.* 2015)

Though it has become clear that extracellular vesicles (EVs) are produced by many parasites, nothing has been documented on the production of these vesicles by *E. histolytica*. The present study anticipates that findings regarding the production of EVs will add knowledge on the pathogenesis and the outcome of *E. histolytica* infection. Macrophage inhibitory migration factor (MIF), homolog has been shown to be a part of *Trichomonas* exosomal protein content. A study by (Moonah *et al.* 2014), has investigated whether MIF is secreted by the parasite *E. histolytica* and found it present in secreted fractions of this parasite (**Fig 7.2**). From this data, the present study hypothesized that *E. histolytica* does produce exosomes/EVs carrying its protein contents and deliver them to the target cells enabling the parasite to spread within the host and cause the infection.

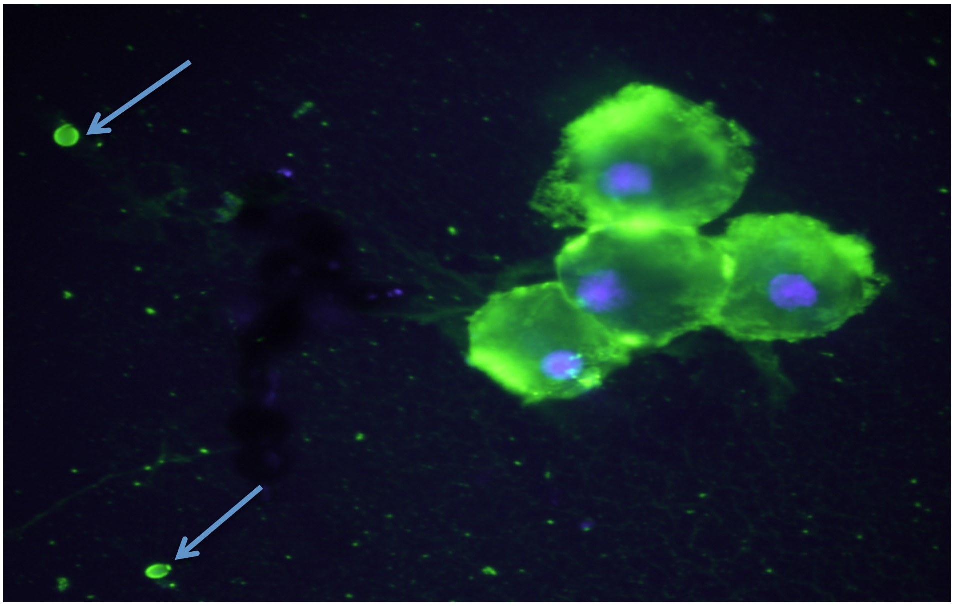

Fig 7.2: Immunofluorescence detection of *Eh*MIF in the cytoplasm of *E. histolytica* trophozoites. Fluorescence staining was achieved with Alexa Fluor

488-conjugated secondary antibodies. Nuclei were stained with DAPI. Arrows: Secreted extracellular vesicles?

This hypothesis was challenged by using the pathogenic amoeba strains (HM-1: IMSS or HM-1: IMSS (Sub-strain-US) from the Petri lab to purify the exosomes using the commercial available kit to isolate exosomes (total exosome isolation kit). Initially, the amoebas were grown in TYI complete media, in T75 flasks and placed in an anaerobic incubator at 37°C until they were confluent (70-80%).

7.1.2 Cell counting and medium selection

The automated cell counter was used to count the number of live cell in the tubes; the trypan blue dye was used to differentiate between dead and live cells. The cells were counted and transferred into four different mediums, TYI (with and without Adult bovine serum) and M199s (with and without bovine serum albumin), this was done to select the media containing the minimum of nutrients (and potential sources of artifacts) in which the ameba both grow and produce exosomes. The cells were transferred into a 48 well culture plates and incubated at different time points (19 hours, 78 hours and 120 hours). Tests were performed to optimize amebic growth media in anaerobic bag (BD; GasPak EZ) culture in conditions that would minimize the addition of contaminating mammalian exosomes.

After each time point, the numbers of cells were counted for each media. From the results of these experiments, the TYI with adult bovine serum albumin and M199s with no bovine serum albumin had a low number of cells. Therefore, we selected the serum free TYI and M199s with bovine serum albumin. The amoeba in these culture conditions were growing well at 78 hours (3 days) compared to the other time points (**Table 7.1**).

Table 7.1: ***E. histolytica*** **Growth Condition Testing**

Incubation time	TYI (without adult bovine serum albumin)	Average and standard deviation	Complete M199s (bovine serum added)	Average and standard deviation
19	0.192	0.22 ±0.05	0.218	0.25±0.03
19	0.202		0.278	
19	0.276		0.261	
78	0.807	0.87±0.07	0.37	0.34±0.04
78	0.861		0.299	
78	0.951		0.357	
120	0.319	0.37±0.05	0.187	0.16±0.02
120	0.372		0.162	
120	0.418		0.142	

7.1 3 Isolation methods

Many different methods have been described in the literature for the isolation and quantitative detection of exosomes (Théry *et al.* 2006;Taylor *et al*. 2011). However, no consensus on a 'gold standard' has been reached. Hence researchers in the field of exosomes agree that a consistent method of isolation is highly warranted to achieve a higher degree of comparability between different reports and studies. In the present study, the exosomes were isolated by selecting Confluent T75 ameba flask and transferred into 15 ml tubes for centrifugation in order to remove the cell debris/dead cells from the culture. The cells were then transferred into a 0.4 μm transwell plate. Visual inspection confirmed that ameba trophozoites (average diameter 8-10 μm) could not migrate into the lower chamber but this pore size is sufficiently large to permit exosomes to transit into the lower chamber. We used two of the selected media (TYI serum free and M199s with serum). The upper chamber contained the amebas and the media; the lower chamber contained only the media (**fig 7.3**)

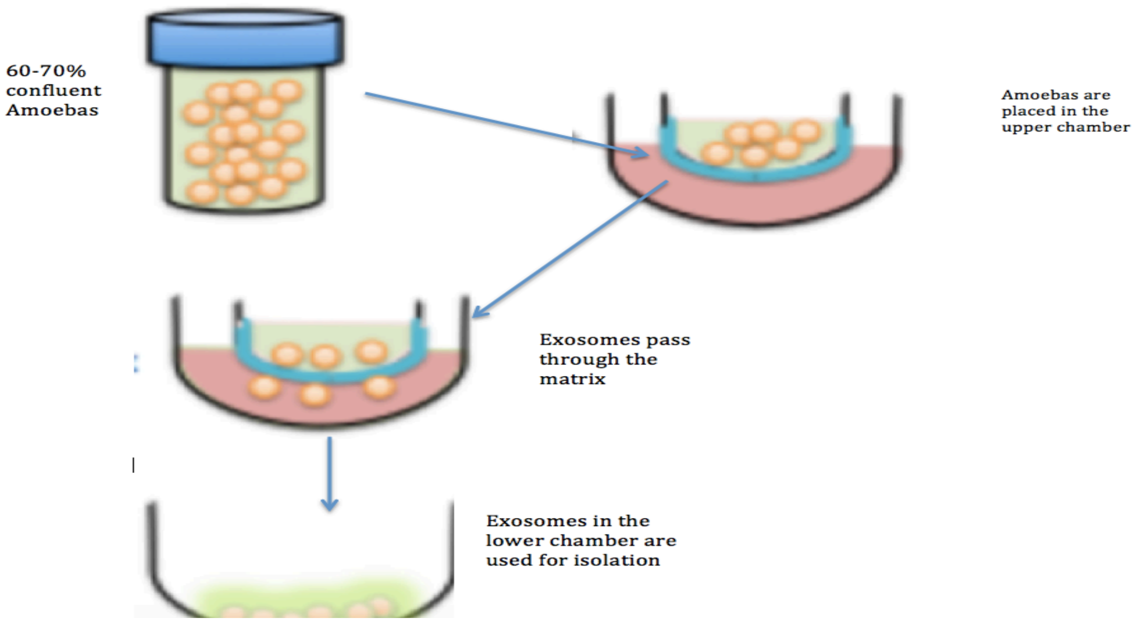

Fig 7.3: Schematic presentation of exosomes isolation assay.

7.1. 4 Exosomes purification

There is also no gold standard method for exosome purification and isolation and there are many methods for the purification of exosomes and this makes it difficult to come to a consensus on their purification and analysis. The present study reports the use of a commercially available kit (Total Exosome Isolation Reagent (from cell culture media)) from ThermoFisher scientific (Invitrogen) for exosomes purification. The exosomes in the lower chamber (**Fig 7.3**) were used for purification. The culture was first centrifuged at 200-x g for 30 minutes to remove the cell debris. The reagent was added into the cell free culture and incubated overnight at 4- 8°C. After the incubation period, the sample was centrifuged at 10000 x g for 1 hour at 4 -8°C. Exosomes are contained in the pellet, so we re-suspended the pellet with 1X PBS and stored at 4 and 8°C depending on when the sample is to be used. The figure below shows the purification process (**Fig 7.4**).

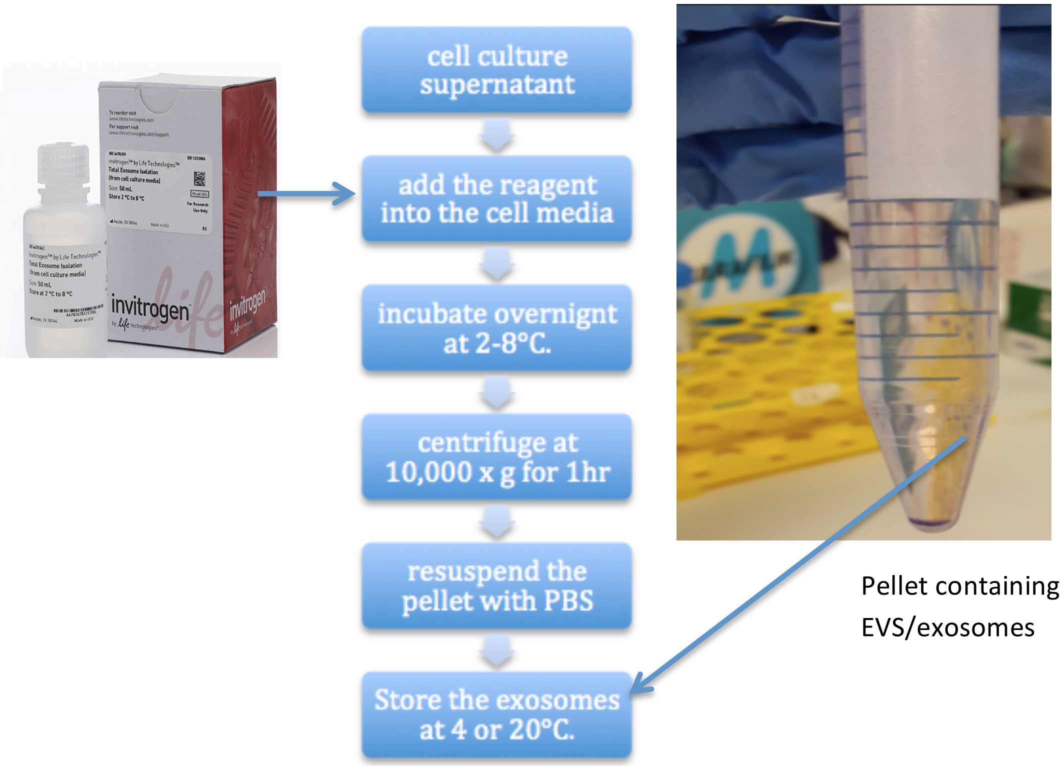

Fig 7.4: Exosomes purification process

7. 1.5 Exosomes validation

Fluorescence activated cell sorting is one of the most common method used for exosome analysis (Lässer *et al.* 2012). With this method, cells from different origins can be compared in one step. However, this is not sensitive enough to identify particles smaller than 0.5 µm (Konokhova *et al.* 2012), whereas exosomes are generally between 30-120 nm in diameter (Vlassov *et al.* 2012). Other tools for exosomes analysis include Scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Both the tools can be used for analysis of particle size and morphology of exosomes (Sokolova *et al.* 2011). However, this is not routinely used because is time-consuming and needs trained personnel. Another highly sensitive method for visualization and analysis of exosomes is nanoparticle-tracking analysis (NTA) (Carr and Warren 2012). Cryo-electron microscopy (Cryo-EM) is considered a powerful tool for imaging EVs without any added fixatives (Cheruvanky *et al.* 2007). We applied this technique for characterization of EVs, by studying the concentration and size distribution and the morphology).

A 4µl droplet of the vesicle suspension was applied to a 200-mesh R 2/2 Quantifoil holey-carbon grid. Excess of solution was removed with Whatman paper and the grid was rapidly plunged into liquid ethane and transferred under liquid nitrogen into the microscope using a side entry nitrogen-cooled Gatan 914 cryoholder. The presence of exosomes was validated by images obtained at the Molecular Electron Microscopy Core. Exosomes are characterized by cup-shaped. The results from this study (**fig 7.5 B**) were compared with the exosomes obtained from the other species such as *Trichomonas* (**fig 7.5 A)** (Twu *et al*. 2013)

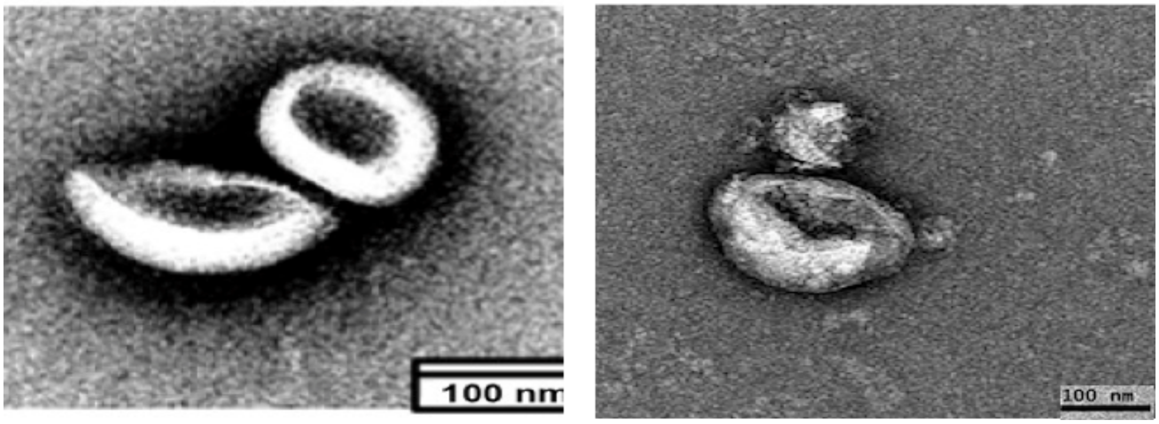

Fig 7.5: Cup-shaped exosomes. A. EVs from *Trichomonas*. **B**. EVs from the present study

These preliminary results proved that *E. histolytica* does produce/secrete EVs/exosomes. There is still more work that needs to be done to understand the role of these vesicles in *Entamoeba* infection. The EVs from parasites often contain proteins and nucleic acids, which plays a role in immunomodulation and virulence within a host. However, it is unclear which mechanism the protozoan parasite-derived EVs use to interact with target cells (Szempruch *et al.* 2016). It has been shown that EVs from *Trichomonas vaginalis* and *Trypanosoma brucei* fuse with host cells, to transfer cargo proteins and lipids. The pro-inflammatory response elicited by EVs increases the parasite burden in the host and/or disease (Twu *et al.* 2014).

7.2 Discussion and conclusions

Our study for the first time revealed that *E. histolytica* does secrete EVs. This finding increases the likelihood that all organisms are likely to secrete these EVs

(Kulp and Kuehn 2010;Yáñez-Mó *et al.* 2015). Though EVs have been shown to have important functions such as intercellular information transfer between different cell types, their biology, molecular composition and function, targeting and uptake mechanisms is still a young research field, to some extent awaiting new technological advances for the isolation and characterization of complex mixtures including very small vesicles (Apostolova and Victor 2015).

Although the secretion of EVs by the parasite *E. histolytica* is just beginning to be characterized, the research on secretion vesicles and their involvement in intra- and extracellular signaling will address whether these vesicles constitute good targets for new control strategies of amebiasis, which could be implemented as new diagnostic and treatment tools and vaccines, like in the case of *Plasmodium yoelli* (Martin-Jaular *et al.* 2011) and *Toxoplasma gondii* (Aline *et al.* 2004), parasite EVs or EVs from infected host cells has been shown to protect naıve animals from infection. Thus, EVs may serve as both biomarkers for infection as well as vaccine candidates.

In the past few years, there has been an exponential increase in the number of studies aiming at understanding the biology and secretion of exosomes, as well as other nanovesicles and microvesicles (Vlassov *et al.* 2012). This has allowed us to gain more insight and knowledge about the mechanisms of their formation, secretion, pathways in vivo and internalization into recipient cells and the biological roles of their protein and nucleic acid cargos (Yáñez-Mó *et al.* 2015). Similar studies have been done in a number of different parasites; these include the work in *Plasmodium* (Mantel and Marti 2014), *Trichomonas* (Twu *et al.* 2013), *Trypanosoma* (Souza 2017)*, Toxoplasma gondii* (Długońska and Gatkowska 2016)*, Cryptosporidium* (Valadi *et al.* 2007) *and Leishmania* (Silverman and Reiner 2012). Evidence for the presence of EVs in *E. histolytica* has however never, to our knowledge, been previously described. The data obtained and reviewed here shows that *E. histolytica* is among the other parasites that produce EVs. This is crucial step to further our understanding of exosomes, continuing development of reagents, tools, and protocols for not only their isolation but also characterization and for analysis of their protein contents. The method for exosome isolation described in this chapter provide a good starting point; however, it is clear that more advanced tools are required to study exosomes and other extracellular vesicles.

Extracellular vesicles are important players in determining the path of infection by pathogens and promote pathogen survival (Schorey and Harding 2016). EVs may also be critical to maintaining parasite infection by aiding colonization and modulating the host immune response (Twu *et al.* 2014). The ability of vesicles to transport and deliver diverse populations of molecules has been shown in different pathogens (Coakley *et al.* 2015). Future work is needed to investigate whether *E. histolytica* EVs could play a similar role. Investigation of EV contents will in fact provide valuable insights on the use of EVs to characterize infectious states. Another exciting potential use of EVs is for vaccines. We postulate that modified EVs from both the parasites and host cells can be used as vaccine systems to deliver immunogenic parasite proteins to the host cells.

In conclusion, exosomes in culture supernatants represent an ideal source of *E. histolytica* proteins for the diagnosis, treatment and evaluation of the prognosis of amebiasis. Further characterization of EV protein profiles, their mechanisms of action and their relation with different stages of disease in the host will undoubtedly provide the basis for a deeper understanding of the role of EVs in the pathogenesis of *E. histolytica.* This could lead to the development of new therapeutics to cure amebiasis. Finally, it is important to note that in order to establish EVs- based clinical approaches, a consistent set of practices for their isolation and characterization must be developed.

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CHAPTER EIGHT

General conclusions, limitations and recommendation

CHAPTER 8

8.1 Conclusions

Diarrheal diseases are a significant problem worldwide, especially in low and middle-income countries; it is documented as the leading cause of death particularly in children. Amebiasis, caused by *Entamoeba histolytica,* leads to diarrhea, colitis, and tissue destruction. There is no vaccine and only a single drug class to treat this devastating disease. The findings of the studies documented in this thesis contribute new and relevant information to the field of diarrheal diseases and amebiasis research. The following conclusions are drawn:

1. A new sensitive assay was developed, which enabled us to detect new isolates of *Entamoeba* in the South African population. This study describes for the first time, the presence of *Entamoeba bangladeshi* in South Africa.
2. An increase in the commensal bacterium *P. copri* was associated with diarrhea due to *E. histolytica.*
3. We identified EhMIF as a novel parasite mediator of host chemokine expression, neutrophil infiltration, and mucosal immunopathology during amebic infection.
4. *E histolytica* MIF may represent a promising immunotherapeutic target to prevent or attenuate amebic disease.

5. For the first time, our study has revealed that *E. histolytica* is among the other parasites that produce Extracellular vesicles. Norovirus GII, Campylobacter and co-infections were associated with diarrhea in the study population.
6. Co-infections with two pathogens were associated with diarrhea compared to single pathogen infections.

8.2 Limitations

Although this study has provided us with novel data in the field of amebiasis research, lack of clinical data on the study participants has limited the scope of our analysis. The age and sex of some study participants were not recorded, as a result, the power of our study was low, hence our findings showed no significant difference in parasitic infections between sex and the age of the participants. Finally, it was difficult to lay a good foundation for understanding the presence of EVs/exosomes in *E. histolytica* because of lack of prior research studies on *E. histolytica* extracellular vesicles/exosomes.

8.3 Recommendations and future studies

During the course of the study, a number of knowledge gaps were identified and they are the foundation of the following future directions:

1. Further studies are highly needed to understand the impact of intestinal parasites on the health of people residing in Giyani and Pretoria.

2. There is a need to determine the significance of *E. histolytica* associated changes in the microbiota.
3. Additional assays are needed to characterize *E. bangladeshi* in the South African population.
4. The initial goal of this study was to investigate novel species of *Entamoeba* in the South African population; therefore, future studies are planned on collecting more samples and continue with the investigation.
5. More studies are needed to determine whether the combination of metronidazole and anti-*Eh*MIF antibodies is superior to metronidazole alone for treatment of severe amebic colitis.
6. Future studies on EVs/exosomes should be focused on:
   a. Advancing the tools needed to study exosomes and other extracellular vesicles.
   b. Characterizing the EVs/exosome protein profiles, mechanism of action and their relation with different stages of the disease.

Appendices

Appendix I

The Journal of Infectious Diseases

MAJOR ARTICLE

Entamoeba Species in South Africa: Correlations With the Host Microbiome, Parasite Burdens, and First Description of *Entamoeba bangladeshi* Outside of Asia

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Background. Diarrhea is frequent in communities without clean water, which include low-income South African populations in Giyani and Pretoria. In these populations, the amount of diarrhea caused by *Entamoeba histolytica*, inclusive of all ages, sexes, and human immunodeficiency virus status, is uncertain. Infection with *E. histolytica* can modulate the host microbiota, and a key species indicative of this is the *Prevotella copri* pathobiont.

Methods. A cross-sectional study of patients attending gastroenterology clinics was conducted to determine the frequency and burden of 4 *Entamoeba* species and *P. copri*.

Results. *Entamoeba* species were present in 27% of patients (129/484), with *E. histolytica* detected in 8.5% (41)*, E. dispar* in 8% (38), *E. bangladeshi* in 4.75% (23), and *E. moshkovskii* in 0%. This is the first description of *E. bangladeshi* outside Bangladesh. In *E. histolytica*–positive samples, the levels of both the parasite and *P. copri* were lower in nondiarrheal samples, validating the results of a study in Bangladesh ($P = .0034$). By contrast, in *E. histolytica*–negative samples positive for either of the nonpathogenic species *E. dispar* or *E. bangladeshi*, neither *P. copri* nor *Entamoeba* levels were linked to gastrointestinal status.

Conclusions. Nonmorphologic identification of this parasite is essential. In South Africa, 3 morphologically identical *Entamoeba* were common, but only *E. histolytica* was linked to both disease and changes in the microbiota.

Keywords. *Entamoeba bangladeshi*; *Entamoeba histolytica*; *Protozoa*; microbiome; *Prevotella copri*.

Entamoeba species are a group of unicellular, anaerobic, parasitic organisms found in humans, nonhuman primates, and other vertebrate and invertebrate species [1]. *Entamoeba* infections in humans can result in asymptomatic carriage or a wide range of symptomatic diseases. Of the subset of individuals developing symptoms, diarrhea and dysentery are the most common manifestations. Extraintestinal complications occur less frequently but can be associated with high mortality [2].

Species of *Entamoeba* that can infect and be found in the intestinal lumen of humans include *Entamoeba histolytica*, *Entamoeba dispar*, *Entamoeba moshkovskii*, *Entamoeba coli*, *Entamoeba polecki*, *Entamoeba hartmanni*, and *Entamoeba bangladeshi* [3]. Of these, *E. bangladeshi* is the most recent to be described, with the species name reflecting the geographic origin of the first patient it was isolated from. *E. bangladeshi* is indistinguishable by microscopy from *E. histolytica*, the prototypical pathogenic *Entamoeba* species, but it can be differentiated from other known *Entamoeba* species by immunologic and molecular techniques.

A recent study involving stool samples collected from infants (age range, 0–24 months) residing in Vhembe District, Limpopo, South Africa, failed, however, to detect *E. histolytica* by polymerase chain reaction (PCR) analysis [4]. However *E. histolytica* was common (18%) in an earlier 2006 study involving participants of all ages (ie, from 0 to >60 years) [5]. The cause for this discrepancy is not fully understood. In preliminary work, samples collected from patients of all ages visiting a gastroenterology clinic between November 2013 and June 2015 in both rural Giyani (Mopani District, Limpopo) and urban Pretoria (Soshanguve District, Gauteng, South Africa) were evaluated by microscopy for the presence of ameboid organisms, and 50% of the samples were *Entamoeba* positive. These findings might be due to the presence of other morphologically identical non–*E. histolytica* species of *Entamoeba,* such as *E. dispar* and *E. bangladeshi,* to geographical heterogeneity in the frequency of *E. histolytica* in South African populations, or to a much lower frequency of *E. histolytica* in the community-based surveys of enteric disease than in patients requiring clinical care [3, 6, 7].

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To test these hypotheses, DNA was extracted from the Giyani and Pretoria samples, and a multiplex quantitative PCR (qPCR) assay was used to detect both *E. histolytica* and the other morphologically identical *Entamoeba* species, such as *E. dispar*, *E. moshkovskii*, and *E. bangladeshi*. This assay was also modified to include a general *Entamoeba* probe to capture data on the presence of novel *Entamoeba* species genetically similar to the pathogenic species *E. histolytica* that may be present in the South African population.

The qPCR assay data captured quantitative information, and this permitted us to examine the correlation between the parasite burden in these samples and the outcome of infection. A link between parasite burden and symptomatic disease has been found in previous studies [4, 8, 9]. Recent studies have also highlighted the relationship between *Entamoeba* species and the bacterial communities of the gut. The presence of *Entamoeba* organisms was associated with a decrease in the abundance of *Prevotella copri* in farmers and fishermen from Southwest Cameroon [10], and the abundance of *P. copri* increased in diarrheal *E. histolytica* cases [9]. Hence, we also sought to quantify this bacterium in the microbiome of *Entamoeba*-positive samples in our study population.

METHODS

Ethics Statement

The research and ethics committee of the University of Venda granted institutional approval. The study received ethical clearance from the Department of Health and Welfare, Polokwane, Limpopo Province, South Africa. We also obtained permission from the ethics committee of participating hospitals and clinics to collect samples. The objectives and concepts of the study were clearly explained in the language understood by the potential participants (ie, English, Sepedi, Xitsonga, and Tshivenda). A written, informed consent form was signed prior to study enrollment. In cases where the participant was either a non-English speaker or illiterate, a witness also signed the consent form.

Study Area and Population

The tested stool samples were predominantly from urban and rural populations of moderate-to-low socioeconomic status [11, 12]. They were collected between November 2013 and June 2015 from diarrheal and nondiarrheal patients in the rural Nkomo clinic (Giyani) and the urban clinic within the Dr George Mukhari Hospital (Soshanguve District).

The catchment area for the Dr Georges Mukhari Hospital includes Soshanguve, Ga-Rankuwa, Mabopane, and parts of Madibeng District. The Nkomo clinic serves households within the Greater Giyani Local Municipality, Mopani District. Household water and sewage access is summarized in Table 1 [11, 12].

Both adults and children of all ages were eligible for participation. A questionnaire was used to collect sociodemographic information, such as the age, sex, and origin of the study participants.

Table 1. Water and Sewage Arrangements Among Households, by Gastrointestinal Clinic and Districts

Clinic, District	Piped Water, %	Flush Toilet Connected to Sewage System, %
Dr Georges Mukhari Hospital		
Soshanguve	58.7	85.3
Ga-Rankuwa	73.6	90.1
Mabopane	62.5	85.4
Madibeng[a]	22.2	27.2
Nkomo clinic		
Greater Giyani Municipality[b]	13.4	11.9

Data are from the South African Government STATS SA Community Survey of 2016 [12].

[a]Only part of Madibeng District is considered to be in the Dr Georges Mukhari Hospital catchment area.

[b]Greater Giyani Municipality lies within Mopani District.

Sample Collection

After the patients were given a clear explanation of the stool sample collection process, they received screw-cap bottles into which they placed their samples. Stool samples were classified as diarrheal or nondiarrheal on the basis of the physical presentation of the sample, as defined by the Bristol stool form scale (diarrheal specimens, types 6 and 7; nondiarrheal specimens, types 1–5) [13]. The bottles were labelled with unique participant identifiers and then placed in a cooler box and transported to the University of Venda microbiology laboratory for further processing. Upon arrival to the laboratory, samples were aliquoted in 2-mL tubes and stored frozen at −20ºC. The aliquoted samples were shipped to the University of Virginia Infectious Diseases Research laboratory for analysis.

Genomic DNA Purification

Genomic DNA from each patient's sample was extracted using a QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's recommended procedures, using approximately 200 mg of stool samples with the modifications described by Liu et al [14]. One stool sample from a healthy US child whose stool had previously been tested and found to be negative for all *Entamoeba* species was included in each batch, to monitor for the occurrence of contamination during extraction. The DNA was eluted in 200 µL of elution buffer (Qiagen) and stored at −80°C until further analysis.

Multiplex qPCR Assay for Detection of *Entamoeba* Species

A multiplex qPCR assay was used for the amplification and detection of all *Entamoeba* species. Genus-specific primers were used in combination with a 42-nucleotide probe that should hybridize to *E. bangladeshi*, *E. dispar*, *E. histolytica*, and *E. moshkovskii* amplicons. Owing to the length required to generate this probe in these A/T-rich genomes, a double quencher was included in the design of the probe (Biosearch Technologies; Figure 1). This probe recognizes *E. histolytica*, *E. moshkovskii*, *E. dispar, E. bangladeshi*, and *E. hartmanni* amplicons but was not similar to the ribosomal RNA (rRNA) region in

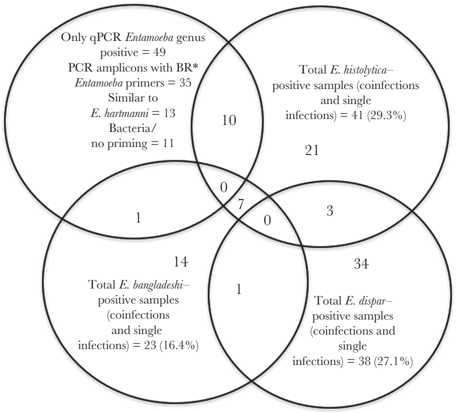

Figure 2. *Entamoeba* species found in South African populations. A total of 140 *Entamoeba*-positive samples were identified by quantitative polymerase chain reaction (qPCR) analysis. In 11 samples, the infecting species could not be identified. Thirty-eight samples were positive for *Entamoeba dispar,* 41 were positive for *Entamoeba histolytica,* 23 were positive for *Entamoeba bangladeshi,* and 0 were positive for *Entamoeba moshkovskii.* In 11 samples, coinfections with different *Entamoeba* species were observed (7 were coinfected with *E. histolytica* and *E. bangladeshi,* 1 was coinfected with *E. bangladeshi* and *E. dispar,* and 3 were coinfected with *E. histolytica* and *E. dispar*). *E. hartmanni, Entamoeba hartmanni.* *Broad range (BR).

positive for the broad range *Entamoeba* but negative for all the species-specific probes. The amplified DNA was separated by electrophoresis, and in the 35 cases where the bands of the size predicted for the *Entamoeba* species were identified, it was purified from the agarose by using the QIAquick Gel Extraction Kit (Qiagen). The SSU rRNA gene amplicon was detected in 35 samples. Sequencing of the purified amplicon identified 10 additional *E. histolytica*–positive samples (n = 41), with an adjusted frequency *Entamoeba* qPCR-positive samples of 29.3%, and 1 additional *E. bangladeshi* sample (n = 23), with an adjusted positivity frequency of 16.4% (Figure 2). This result suggested that in these samples the parasite level had simply fallen below the detection limit of the species-specific qPCR assay. These samples were not included in the later analysis. In 13 cases, the 18S rRNA amplicon sequences were similar to those of the nonpathogenic species *E. hartmanni* (all sequences were deposited in GenBank under accession numbers MF471201–MF471217), and in the remaining 11 cases either no useful sequence data were obtained or findings were similar to sequences from bacteria and had no significant similarity to any *Entamoeba* reference sequence in the NCBI database.

Parasite Burden in South African Samples

Other enteropathogens are common in this South African population, and in diarrheal samples coinfections can make it challenging to identify the causal organism [4]. *Entamoeba* were no more frequent in diarrheal samples than in controls (data not shown). No differences in *Entamoeba* frequency was observed between the rural and urban populations. The cycle value at which the (baseline-corrected) amplification curve exceeds the background fluorescence (Cq) is closely related to the amount of input DNA. The Cq data provided by the qPCR assay can therefore be used to determine whether the *Entamoeba* species burden was different in diarrheal and control fecal samples [4, 13, 17]. As the distribution of Cq values were non-Gaussian, significance was determined using the Mann-Whitney test. As expected, a significant difference in *Entamoeba* levels was observed in cases of *E. histolytica*–associated diarrhea ($P = .0072$; Figure 3A), but the level of the nonpathogenic species *E. dispar* was unchanged in control and diarrheal samples (Figure 3C). Interestingly, the level of *E. bangladeshi* was also unchanged (Figure 3B). Again, no significant differences were observed in the parasite burden in rural and urban samples.

Quantity of *P. copri* in *Entamoeba*-Positive Samples.

In a Bangladesh study, elevated levels of the pathobiont *P. copri* were associated with *E. histolytica*–associated diarrhea [9, 18]. The level of *P. copri* in *Entamoeba*-positive diarrheal and control samples was measured and, to control for variations in fecal bacterial numbers, was normalized using an Enterobacteriaceae bacterial reference [9, 19]. A fecal DNA standard was used to control for any differences in amplification efficiency in the *P. copri* and Enterobacteriaceae qPCR assays. Samples negative for either *P. copri* or Enterobacteriaceae were omitted from the quantitative analysis towing to concerns about sample quality (Supplementary Table 1). To convert the qPCR results to bacteria concentrations, DNA was extracted from a known amount of *E. coli* (ATCC 25922) and assayed. The relative level of *P. copri* was 1 log lower in *E. histolytica*–colonized samples as compared to the level in *E. histolytica* diarrheal samples (Figure 4A) but was unchanged in *E. dispar* or *E. bangladeshi* infections when diarrheal and nondiarrheal cases were compared (Figure 4B and 4C).

DISCUSSION

The present study reports an overall frequency of *Entamoeba* species in our samples collected from gastrointestinal clinics as 27% (129/484), with *E. histolytica* being present in 6.4% of the cases (31/484). Differences in the assay used, as well as in age, geographic location, and the fact that these samples were collected from gastrointestinal clinics, make it difficult to compare these results to those obtained from previous population-based studies [4, 8]. A weakness in the current study was that information on human immunodeficiency virus status (expected to increase with age) was not available. In addition, the study was not adequately powered to analyze the susceptibility to *Entamoeba* among participants stratified by age [20, 21].

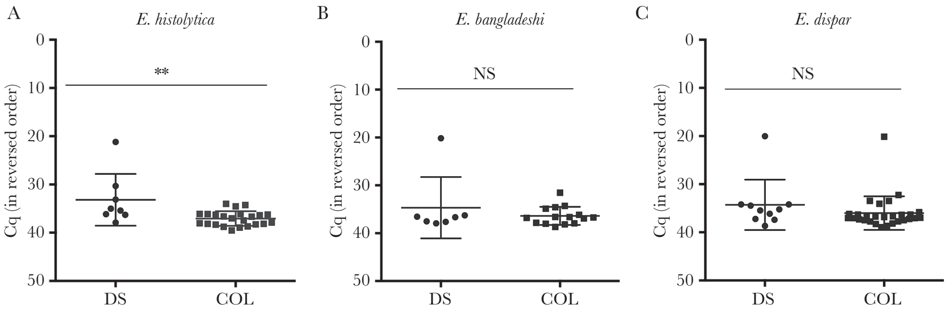

Figure 3. High parasite burden was associated with diarrhea due to *Entamoeba histolytica* (*A*) but not *Entamoeba bangladeshi* (*B*) and *Entamoeba dispar* (*C*). The *y*-axes indicate threshold values of quantitative polymerase chain reaction analyses positive for each parasite. Horizontal lines indicate mean values, and vertical lines indicate standard deviations. **$P \leq .0072$, by the Mann-Whitney test. Cq, cycle value at which the (baseline-corrected) amplification curve exceeds the background fluorescence; NS, not significant.

The Cq of the majority of our *Entamoeba*-positive asymptomatic samples was ≥35 and would have been missed by a less sensitive assay. Assay specificity at high Cq values was confirmed by amplicon sequencing of select samples (data not shown). In the work reported here, the assay included an *Entamoeba* general probe that acted as an independent control to identify any potential closely related novel South African *Entamoeba* species present in these samples (an in-depth surveillance of the *Entamoeba* species in the Mopani district of South Africa had not previously been done). All assay results were analyzed to be certain that the species-specific signal remained at a constant ratio to the result obtained from the broad range probe. The probe would have recognized any *Entamoeba* species similar to the pathogenic species *E. histolytica, E. moshkovskii*, and *E. bangladeshi* or the nonpathogenic species *E. dispar* (Figure 1A). The sequences of the closely related species also blocked nonspecific hybridization, allowing the higher assay Cq cutoff of ≤40 and increased assay sensitivity (Figure 1A) [22].

A higher *E. histolytica* parasite burden in samples increases the probability that the *E. histolytica* strain detected is responsible for diarrheal symptoms [9, 17]. In agreement with the previous studies, our results showed a statistically significant increase in the *E. histolytica* parasite load in South African diarrheal samples. The level of the nonpathogenic species *E. dispar* did not significantly change. This suggested that diarrhea coincident with *E. dispar* infections was due to other pathogens. The

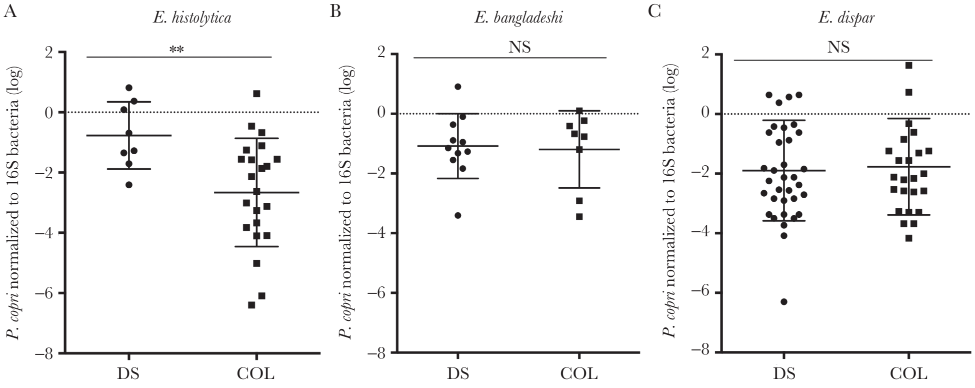

Figure 4. Increased levels of *Prevotella copri* were associated with diarrhea due to *Entamoeba histolytica* (*A*) but not *Entamoeba bangladeshi* (*B*) and *Entamoeba dispar* (*C*). In the *y*-axes, the cycle values at which the (baseline-corrected) amplification curve exceed the background fluorescence were converted to bacteria numbers by use of a calibration curve and normalized to the Enterobacteriaceae levels. Horizontal lines indicate mean values and vertical lines indicate the standard deviations. **$P = .0034$, by the Mann-Whitney test. COL, asymptomatic colonizer samples; DS, diarrheal samples; NS, not significant.

pathogenicity of the recently identified *E. bangladeshi* is still uncertain, but the level of *E. bangladeshi* was also the same in both diarrheal and nondiarrheal South African samples. Additional work is planned to identify whether other coinfecting enteric pathogens are present in these samples.

Novel *Entamoeba* species have been identified in different geographical contexts. Therefore, samples that were positive with the *Entamoeba* general probe but negative with the species-specific probes were characterized by amplicon sequencing (Figure 3) [1, 3, 23, 24]. While novel South African *Entamoeba* species were not identified, to our knowledge this study is the first to describe the presence of *E. bangladeshi* in samples collected outside Bangladesh. This species was first described in Bangladesh in 2011, but our results suggest that *E. bangladeshi* may actually have a broad geographical range and is prevalent in both Asian and African continents [3]. This finding also suggests that other members of the *Entamoeba* genus not identified in previous surveys may also be common in South Africa [5, 25–28]

In addition to the parasite burden, predisposition to diarrheal disease is thought to be influenced by the parasite environment [29–32]. Moreover, it has been suggested that specific components of the microbiota might be associated with symptomatic or asymptomatic *E. histolytica* colonization [33–35]. We examined the level of *P. copri* in the South African samples positive for *Entamoeba* species. Consistent with previous studies, the level of this bacterium was lower in asymptomatic *E. histolytica*–positive samples when compared to the level in *E. histolytica*–associated diarrheal samples [9]. Future work is needed to determine the significance of *E. histolytica*–associated changes in the microbiota. The gut *Prevotella* species are anaerobic bacilli predominant in the lumen of the colon [36]. Recent studies, however, suggest that disruption of the host mucosa can result in an increase in *Prevotella* species at mucosal sites and a subsequent increase in host inflammatory responses [18, 37]. Additional studies are needed to determine whether low *P. copri* levels could mitigate the host immune response occurring during amoebic colitis. It is possible that *E. histolytica*, unlike nonpathogenic *Entamoeba* species, disrupts the protective mucosal layer and exposes the host epithelium to the luminal microorganisms. This could expose the epithelium to high *P. copri* levels, as well as to *E. histolytica*, and result in an excessive inflammatory response with subsequent diarrhea [38–40]. In samples positive for the commensal *E. dispar* (which is not known to induce an inflammatory response or diarrhea), *P. copri* levels were not significantly different in either diarrheal or nondiarrheal samples [39].

In summary, an increase in the commensal bacterium *P. copri* was associated with diarrhea due to *E. histolytica*. The interplay between the pathogen, host, and host microbiota may be of importance in the development of symptomatic disease.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Appendix II

The Journal of Infectious Diseases
MAJOR ARTICLE
IDSA Infectious Diseases Society of America
hivma hiv medicine association
OXFORD

Entamoeba histolytica–Encoded Homolog of Macrophage Migration Inhibitory Factor Contributes to Mucosal Inflammation during Amebic Colitis

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Understanding the mechanisms by which *Entamoeba histolytica* drives gut inflammation is critical for the development of improved preventive and therapeutic strategies. *E. histolytica* encodes a homolog of the human cytokine macrophage migration inhibitory factor (MIF). Here, we investigated the role of *E. histolytica* MIF (*Eh*MIF) during infection. We found that the concentration of fecal *Eh*MIF correlated with the level of intestinal inflammation in persons with intestinal amebiasis. Mice treated with antibodies that specifically block *Eh*MIF had reduced chemokine expression and neutrophil infiltration in the mucosa. In addition to antibody-mediated neutralization, we used a genetic approach to test the effect of *Eh*MIF on mucosal inflammation. Mice infected with parasites overexpressing *Eh*MIF had increased chemokine expression, neutrophil influx, and mucosal damage. Together, these results uncover a specific parasite protein that increases mucosal inflammation, expands our knowledge of host–parasite interaction during amebic colitis, and highlights a potential immunomodulatory target.

Keywords. Chemokines; colitis; diarrhea; *Entamoeba histolytica*; host–parasite interaction; macrophage migration inhibitory factor; mucosal inflammation; neutrophils.

The mucosal surfaces of the nasal, intestinal, respiratory, and genitourinary tracts are the points of first contact for many protozoan parasites. Mucosal inflammation triggered by the interaction with these parasites plays a key role in human disease. However, the mechanisms by which parasites induce mucosal inflammation are incompletely understood.

Globally, diarrheal disease is second only to pneumonia as a leading cause of death in children under 5 years of age [1]. *Entamoeba histolytica* is a protozoan parasite that causes colitis, a leading cause of severe diarrhea in low-income countries. [2, 3]. *E. histolytica* infection is also a concern among returning travelers with infectious gastrointestinal disease: *E. histolytica* infection occurs at an estimated rate of 14 per 1000 returned unwell travelers [4]. Fulminant amebic colitis is an uncommon but life-threatening complication and is associated with high mortality and morbidity despite antimicrobial therapy, with case fatality rates ranging from 40% to 89% [5]. There is neither an effective vaccine nor have there been advancements in therapies for amebic colitis for over half a century, following the introduction of the nitroimidazole agents [6].

Mucosal inflammation is a hallmark of amebic colitis, explaining why it is often misdiagnosed as inflammatory bowel disease [5]. While inflammatory cells represent a line of defense [7], there is significant evidence that the inflammatory response contributes to the tissue damage seen in amebic colitis [8]. During amebic colitis, neutrophils infiltrate the intestinal tract [9]. It has been known for decades that neutrophilic enzymes such as myeloperoxidase (MPO) generate oxygen-free radicals, which kill invading pathogens. Oxygen-free radicals are also responsible for collateral tissue damage during the inflammatory period [10]. There is a direct positive correlation between MPO activity in the colon and the extent of intestinal tract epithelial damage [10].

Neutrophil migration depends on chemokines produced by epithelial cells. Interleukin-8 (IL-8) is a potent neutrophil chemoattractant that contributes to mucosal inflammation in various infectious and inflammatory diseases. Persons with severe forms of amebic colitis have higher colonic tissue levels of IL-8 and neutrophils [11, 12]. Both IL-8 inhibition and neutrophil depletion resulted in less mucosal damage during *E. histolytica* infection in a mouse-human intestinal xenograft model [13, 14]. Neutralization of a crucial parasite mediator of host immunopathology may prevent or attenuate disease. However, key parasite mediators of mucosal neutrophil influx during amebic infection remain incompletely understood.

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine that is a critical upstream mediator of the innate immune response. MIF enhances the secretion of

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inflammatory mediators, and there is a strong association between MIF and colitis [15–19]. *E. histolytica* encodes a homolog of the cytokine MIF. However, the effect of the *E. histolytica*–encoded MIF homolog on mucosal inflammation during infection is unknown. In the present study, we examined the role of *E. histolytica* MIF (*Eh*MIF) during infection using in vitro approaches, mouse model, and in persons with intestinal amebiasis. Taken together, the results of our study suggest that *Eh*MIF is a key contributor of parasite-induced mucosal inflammation.

METHODS

Study Approval

All animal studies were performed in compliance with the federal regulations set forth in the Animal Welfare Act, the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and the guidelines of the University of Virginia Institutional Animal Care and Use Committee. All protocols for animal use were approved by the University of Virginia Institutional Animal Care and Use Committee. Use of serum and stool samples from human participants were approved by the Institutional Review Board at the University of Virginia, the Research and Ethical Research Review Committees of the International Center for Diarrheal Disease Research, Bangladesh, and the Research and Ethics Committee of the University of Venda, South Africa.

Parasites and Cell Culture

Entamoeba histolytica strain HM1:IMSS trophozoites were grown at 37°C in TYI-S-33 medium. The human intestinal epithelial cell line Caco-2 (American Type Culture Collection) was grown in Dulbecco's Modified Eagle medium (Gibco). Cell lines tested negative for *Mycoplasma* (Lonza). Coculturing of epithelial cells with ameba were done at a ratio of 10:1 host cells to parasite in M199 medium [20]. IL-8 in cell culture supernatant was measured by enzyme-linked immunosorbent assay (ELISA; eBioscience). For overexpression of *Eh*MIF in *E. histolytica* trophozoites, the *Eh*MIF gene with no tag was cloned into the pKT3M expression vector [21] and confirmed by sequencing. Parasites were transfected by a previously described technique [22]. Transfectants were selected with 12 µg/ml G-418 (Gibco). *Eh*MIF protein overexpression was confirmed by immunoblot analysis using specific anti-*Eh*MIF antibodies [23]. Parasites transfected only with pKT3M expression vector were used as empty vector controls. Parasite growth was measured using CyQUANT Direct Cell Proliferation Assay kit (Invitrogen) according to the manufacturer's instructions. For amebic cytotoxicity assays, *E. histolytica* trophozoites were added to intestinal epithelial cell line monolayers in M199 as previously described [20]. Lactate dehydrogenase (LDH) levels in the supernatant were measured using Cyto Tox-ONE Homogeneous Membrane Integrity Assay (Promega) as directed. The maximum amount of LDH released was determined by the addition of Triton-X to intestinal epithelial cells alone. Percent cytotoxicity was calculated as: [(LDH release + E. histolytica) – (LDH – *E. histolytica*)] / [maximum LDH release]. Conditions were tested in triplicates. Each experiment was repeated at least 3 times, and representative experiments are shown.

Measurement of *Eh*MIF and Stool Myeloperoxidase

We developed an ELISA to measure *Eh*MIF levels, similar to a recently described method [24]. Corning 96-well high-protein-binding polystyrene plates were coated with 5 µg/ml rabbit polyclonal anti-*Eh*MIF [23] in phosphate-buffered saline (PBS) overnight and blocked for 1 hour with PBS containing 1% bovine serum albumin. Recombinant *Eh*MIF was used as a protein standard. Stool samples were incubated overnight at 4°C, then washed before the addition of biotinylated anti-*Eh*MIF at 0.25 µg/ml. After incubation and washing, avidin-conjugated horseradish peroxidase (eBioscience) was added, and detection was performed with 3,3′,5,5′-tetramethylbenzidine ELISA detection reagent (eBioscience). The sensitivity of the ELISA was 15.6 pg. There was no cross-reactivity to human MIF. *Eh*MIF concentrations were measured in deidentified diarrheal stool samples from 35 South African patients with intestinal amebiasis. Stool Myeloperoxidase (MPO) levels were measured by ELISA (ALPCO) according to the manufacturer's instructions [25].

Mice

Wild-type CBA/J mice were obtained from the Jackson Laboratory. Male mice were used at 10 weeks of age.

Parasite Infection

Infections were carried out via intracecal inoculation of mice with *E. histolytica* trophozoites [26]. A total of 5 × 10^5 trophozoites in 100 µl of TYI media were injected intracecally after laparotomy for antibody-mediated neutralization and *Eh*MIF overexpression studies. For antibody neutralization studies, 0.5 mg mouse anti-*Eh*MIF blocking antibodies were given by intraperitoneal injection 24 hours before and intracecally at the time of infection. Isotype antibodies given at the same dose, route, and timing were used as controls. Intracecal injection with media only was used as uninfected controls. No differences in the inflammatory markers and cytokine levels were observed among mice that did not received intracecal injection and those injected with media only and PBS. Mice were sacrificed 24 hours postinfection. TechLab E. histolytica–II kit was used to determine amebic antigen burden in cecal contents [26]. Cecal tissue lysates were prepared as in [27]. C-X-C motif (CXC) chemokine ligand 1 (CXCL1), CXC chemokine ligand 2 (CXCL2), and matrix metalloproteinase-3 (MMP3) levels in cecal lysates were measured by ELISA (R&D Systems). Myeloperoxidase activity in cecal tissue was determined using the same standard protocol as used in [28].

Human Samples

Serum sample concentrations of anti-*Eh*MIF antibodies were measured in 79 children (2–5 years old) from a well-characterized cohort in the endemic area of Mirpur within Dhaka, Bangladesh [23]. After serum sample collection, monthly stool samples were routinely obtained from all children and tested for *E. histolytica* using real-time polymerase chain reaction as previously published [29].

Secretion Assay

Preparation of *E. histolytica*–secreted fractions were modified as described in [30]. Briefly, 1 × 10^7 trophozoites per mL were suspended in M199 media (Gibco) and incubated at 37°C for 2 hours. Cell-free supernatant representing the secreted fraction was collected for further analysis. Proteins in the secreted fraction was not due to cell stress or cell death, as only a minor portion of cells stained positive for Trypan blue (less than 5%), and no cellular actin was found by immunoblot analysis. For inhibition assays, trophozoites were incubated with pharmacological agents brefeldin A and probenicid [31].

Immunohistochemical Staining and Histopathological Examination

Mouse immunohistochemical staining was performed by the University of Virginia Biorepository and Tissue Research Facility. Staining was performed using the DAKO Autostainer Universal Staining System with specific antibody directed against *Eh*MIF at a dilution of 1:600. Mouse cecal tissue was fixed in Bouin's solution (Sigma) and stored in 70% ethanol. Tissue was stained with hematoxylin and eosin by the University of Virginia Research Histology Core. Histological scoring for inflammatory infiltration and epithelial cell damage was performed by 2 independent blinded scorers as previously described [32].

Mass Spectrometry

Proteins from the *E. histolytica*–secreted fraction were separated by gel electrophoresis. The section spanning 10–20 kilodaltons was then excised from the gel. The gel sample was submitted to the W. M. Keck Biomedical Mass Spectrometry Laboratory for mass spectrometry analysis.

Statistics

Statistical differences between 2 groups were determined using the Mann–Whitney *U* test or Student *t* test. Pearson's correlation was used for correlation analysis. Survival differences were analyzed by the log-rank test. A *P* value less than .05 was considered statistically significant.

RESULTS

Association of *Eh*MIF With Intestinal Inflammation in Persons With Intestinal Amebiasis

Given that inflammation is a characteristic feature of amebic colitis and *Eh*MIF is expressed during human infection, we tested in humans for a proinflammatory effect of *Eh*MIF: MPO is a major component of neutrophils, and the concentration of MPO in stool samples is a widely used marker of intestinal inflammation [25, 33]. We measured the concentrations of *Eh*MIF and MPO in the stools samples of persons with intestinal amebiasis and found a positive association between *Eh*MIF and intestinal inflammation by Pearson's correlation (n = 35, r = 0.41; *P* = .015 (Figure 1A and 1B). We concluded that the correlation of stool *Eh*MIF with MPO was consistent with a potential role for *Eh*MIF in colonic inflammation in humans with amebiasis.

*Eh*MIF Induces IL-8 Secretion From Human Intestinal Epithelial Cells

Cytokines such as IL-1β and MIF are secretory proteins that lack a signal peptide and therefore do not follow the classical

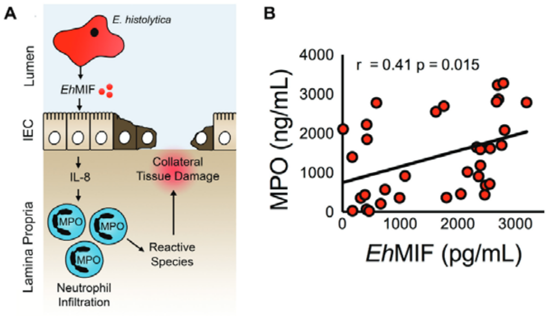

Figure 1. Association between *Eh*MIF and intestinal inflammation. *A*, Schematic of the hypothesis of how secreted *Eh*MIF promotes mucosal inflammation. *B*, Significant positive correlation between fecal *Eh*MIF levels and the MPO marker of intestinal inflammation in persons with amebiasis (*n* = 35). A *P* value < .05 was considered statistically significant. Abbreviations: *Eh*MIF, *Entamoeba histolytica* macrophage migration inhibitory factor; MPO, myeloperoxidase.

endoplasmic reticulum–to-Golgi pathway of secretion. Human MIF is constitutively expressed, accumulated in the cytoplasm, and secreted by a nonclassical pathway involving an ATP-binding cassette (ABC) transporter [31, 34]. Similar to human MIF, *Eh*MIF lacks a signal peptide and ABC transporters can be found in *E. histolytica* [35]. We used mass spectrometry to further confirm the protein expression of *Eh*MIF (Supplemental Figure 1). We investigated whether *Eh*MIF is secreted and found it present in secreted fractions by ELISA and immunoblot (Figure 2A). We further investigated the effect of transport inhibitors on *Eh*MIF secretion. We found that *Eh*MIF secretion was not inhibited by brefeldin A, an inhibitor of the classical secretory pathway. Probenicid, an inhibitor of nonclassical protein export, blocked *Eh*MIF secretion (Figure 2A). These findings suggest that *Eh*MIF shared with other MIF homologs secretion by a nonclassical pathway. In our study, the maximum inhibition achieved was approximately 50%. This raises the possibility of other secretion pathways for *Eh*MIF.

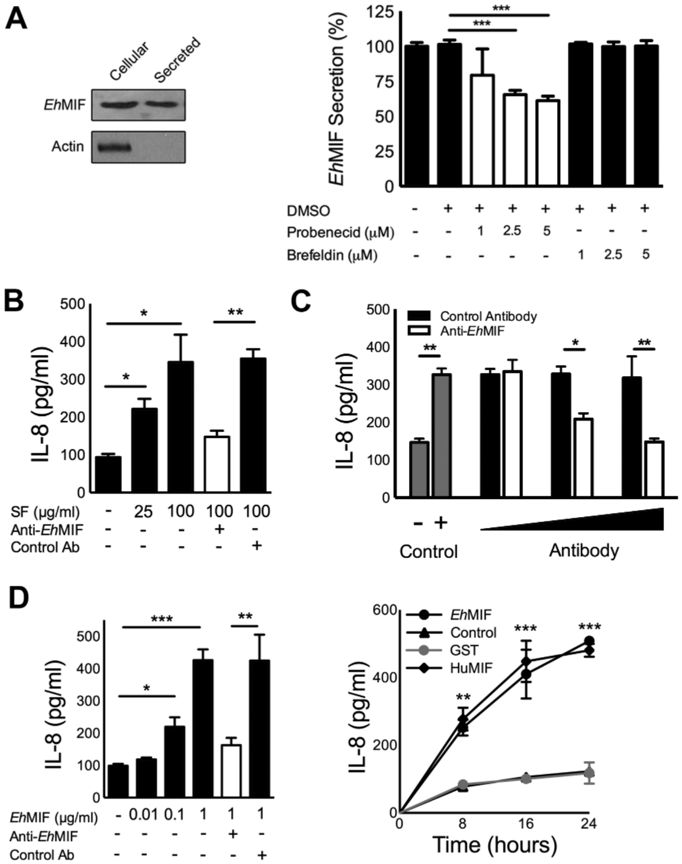

Figure 2. *Eh*MIF induces IL-8 production from human intestinal epithelial cells. *A*, Secretion of *Eh*MIF by amebic trophozoites. Immunoblot analyses of the cell lysate and secreted fractions of *E. histolytica* using anti-*Eh*MIF antibodies. Actin detection serves as negative controls for cell lysis. *Eh*MIF ELISA of *E. histolytica* secreted fractions. *Eh*MIF secretion is not inhibited by the classical pathway inhibitor brefeldin A. Probenicid, an inhibitor of nonclassical protein export, blocked *Eh*MIF secretion. *B*, Anti-*Eh*MIF antibodies blocked *E. histolytica* secretory fraction-induced IL-8 production by colonic epithelial cells (Caco-2 cells). *C*, *E. histolytica* parasites cocultured with IECs in the presence of antibodies. *D*, *Eh*MIF stimulates IL-8 production in a dose- and time-dependent manner. Data represent mean and SD of triplicates from 1 experiment and are representative of 3 independent experiments. *$P < .05$; **$P < .01$; ***$P < .001$. Abbreviations: *Eh*MIF, *Entamoeba histolytica* macrophage migration inhibitory factor; ELISA, enzyme-linked immunosorbent assay; IECs, intestinal epithelial cells; IL-8, interleukin-8; SF, secretory fraction.

*Eh*MIF was tested for its ability to induce IL-8 using a cellular approach, given that epithelial cells are the first host cells to encounter secretory products released by enteric parasites. It has long been hypothesized that *E. histolytica*, by means of soluble mediators, stimulates chemokine production from host cells [36]. The *E. histolytica* secretory protein fraction was previously shown to induce IL-8 production by Caco-2 human colonic epithelial cells [37]. We were able to reproduce this finding and found that IL-8 production was inhibited by antibodies that blocked *Eh*MIF (Figure 2B). The rabbit anti-*Eh*MIF antibodies used for these experiments did not cross-react with human MIF (Supplemental Figure 2A). In addition, anti-*Eh*MIF antibodies inhibited the IL-8 secretion induced by coculturing intestinal epithelial cells (IECs) with *E. histolytica* parasites (Figure 2C). We also determined the effect of endotoxin-free recombinant *Eh*MIF (<1 pg LPS/μg protein) on IECs. Recombinant glutathione S-transferase, an irrelevant protein, was used as a negative control and human MIF as a positive control. *Eh*MIF induced IL-8 production in a time- and dose-dependent manner, and treatment with anti-*Eh*MIF inhibited *Eh*MIF-induced IL-8 production (Figure 2D). These data indicate that *Eh*MIF was a cause of IL-8 secretion by *E. histolytica*.

Anti-*Eh*MIF Antibody Treatment Reduces Mucosal Inflammation

We further investigated in an amebic mouse model the role of *Eh*MIF on chemokine secretion and mucosal inflammation. CXCL1/KC and CXCL2/MIP-2 are mouse homologs of human IL-8 and are key chemokines in neutrophil recruitment and inflammation. Host MIF was shown to induce mouse IL-8 homolog production from alveolar epithelial cells [38, 39]. Mice infected with *E. histolytica* had elevated levels of CXCL1 and CXCL2 (Figure 3B), in keeping with previous studies [28]. We found that mice treated with mouse anti-*Eh*MIF-blocking antibodies had reduced CXCL1 (Figure 3B). Neutrophil MPO activity, an indicator of neutrophil infiltration [6, 32, 40], was significantly lower in anti-*Eh*MIF-treated mice compared with controls (Figure 3C). The reduction of neutrophil infiltration by anti-*Eh*MIF antibodies was consistent with its effect on chemokine production. These anti-*Eh*MIF antibodies did not cross-react with mouse MIF (Supplemental Figure 2B). We concluded that anti-*Eh*MIF blocked neutrophil recruitment to the gut in the mouse model of amebic colitis. In a previous study, anti-*Eh*MIF antibodies were detected in the serum samples of children living in an endemic area [23]. We tested whether anti-*Eh*MIF was associated with protection from amebiasis. Children in the top 50th percentile for anti-*Eh*MIF serum immunoglobulin G had a significantly higher probability of survival free of *E. histolytica* infection, compared with children in the lower 50th percentile (Supplemental Figure 3). This finding supports the hypothesis that anti-*Eh*MIF antibodies have a protective role.

Overexpression of *Eh*MIF Enhances Mucosal Inflammation

In addition to antibody-mediated neutralization, we used a genetic approach to test the effect of *Eh*MIF on mucosal

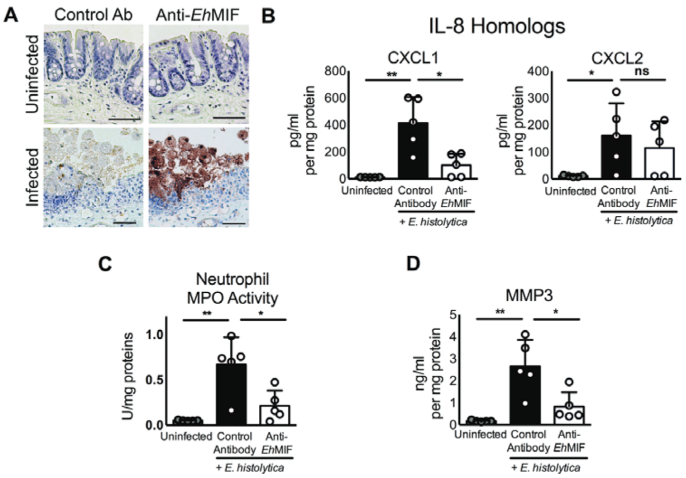

Figure 3. Anti-*Eh*MIF antibody treatment reduces *E. histolytica*–induced inflammation. *A*, Immunohistochemical stain showing *Eh*MIF protein expression (brown) and interaction with host during infection. Scale bars, 50μm. *B–D*, Mice treated with anti-*Eh*MIF antibodies had reduced intestinal tissue levels of CXCL1 chemokine, neutrophil infiltration, and MMP-3. Data represent mean and SD (*n* = 5 mice per group). **P* < .05; ***P* < .01. Abbreviations: CXCL1, C-X-C motif chemokine ligand 1; *Eh*MIF, *Entamoeba histolytica* macrophage migration inhibitory factor; MMP-3, matrix metalloproteinase 3; ns, not significant.

inflammation. We generated *E. histolytica* trophozoites that overexpress *Eh*MIF (Figure 4A and 4B), given that *Eh*MIF is a soluble secreted nontoxic protein, and gene overexpression can be technically accomplished in amebic strains that are adapted for virulence in the mouse model [22, 41, 42]. Mice infected with parasites overexpressing *Eh*MIF showed increased chemokine production, mucosal inflammation, and pathology compared with parasites transfected with the empty vector (Figure 4C, 5A–D). No significant differences in parasite antigen load were observed between mice infected with parasites overexpressing *Eh*MIF and controls postchallenge. This was also true for groups given isotype antibody control or anti-*Eh*MIF antibody, indicating that the 2 groups were exposed to the same levels of *E. histolytica* antigens. In addition, parasites overexpressing *Eh*MIF did not exhibit any growth or cytotoxicity difference compared with controls (Figure 4D). These data indicate that overexpression of *Eh*MIF increased intestinal inflammation and damage.

*Eh*MIF and Matrix Metalloproteinases Expression

Matrix metalloproteinases (MMPs) are enzymes capable of degrading extracellular matrix proteins. MMPs are expressed in all infections with protozoan parasites [43]. Matrix metalloproteinase 1 (MMP-1) and matrix metalloproteinase 3 (MMP-3) genes were among the most overexpressed genes in persons suffering from intestinal amebiasis [9]. In the human colon explant model, MMP-3 was shown to play a central role in amebic colitis, and inhibition of MMP activity blocked colonic mucosa invasion by *E. histolytica* [44]. We found that mice given anti-*Eh*MIF antibodies had reduced MMP-3 mucosal levels, and parasites overexpressing *Eh*MIF generated higher MMP-3 production during infection compared with controls (Figure 4D and Figure 5C). Proinflammatory cytokines, including human MIF have shown to stimulate the expression of MMPs [45]. However, in our hands, recombinant *Eh*MIF failed to directly induce MMP-3 production from intestinal epithelial cells and fibroblasts in vitro. These findings suggest that MMP3 elevation might be due to *Eh*MIF-induced mucosal inflammation and not a direct effect of *Eh*MIF on host cell MMP-3 secretion.

DISCUSSION

Mucosal inflammation resulting from infection with *E. histolytica* is a hallmark of amebic colitis. In this study, we examined the role of the cytokine MIF homolog of *E. histolytica* in mucosal inflammation. We found a positive correlation

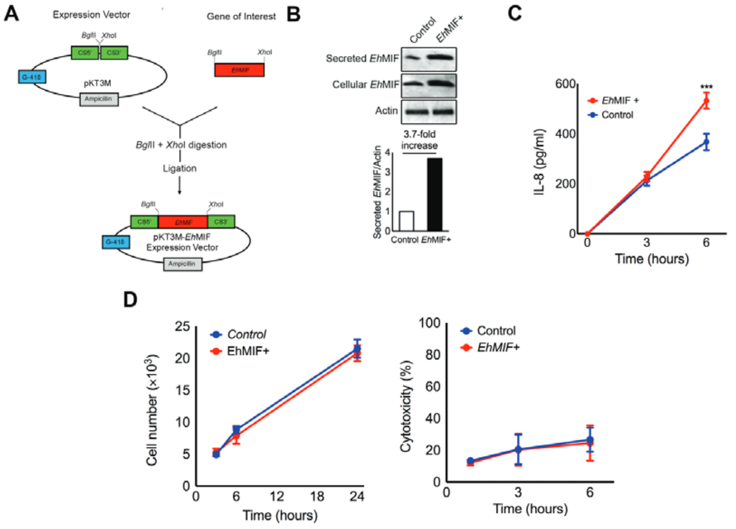

Figure 4. *Eh*MIF overexpression by *E. histolytica* parasites. *A*, Schematic for the preparation of the pKT3M-*Eh*MIF expression vector. *Eh*MIF gene (EHI7A_051880). CS5': CS promoter (EHI_024230). CS3': CS UTR (EHI_024230). G-418 and ampicillin resistance genes. *B*, *Eh*MIF expression assessed by immunoblot analysis. Actin was used as a loading control. Quantification of secreted *Eh*MIF bands relative to actin by densitometry. *C*, IECs cocultured with *E. histolytica* parasites overexpressing *Eh*MIF (*Eh*MIF⁺) compared to empty vector controls. *D*, No difference in parasite growth or parasite-induced cytotoxicity between *Eh*MIF⁺ parasites and WT parasite controls with empty vector. Data represent mean and SD of triplicates from 1 experiment and are representative of 3 independent experiments. A *P* value < .05 was considered statistically significant. ***$P < .001$. Abbreviations: *Eh*MIF, *Entamoeba histolytica* macrophage migration inhibitory factor; IECs, intestinal epithelial cells; WT, wild-type.

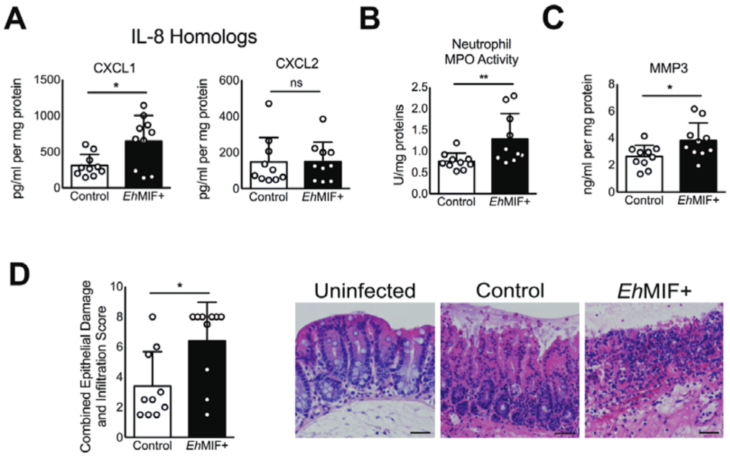

Figure 5. *Eh*MIF overexpression increases inflammation. *A–C*, Increased CXCL1, neutrophil influx, and MMP-3 tissue levels in mice infected with *Eh*MIF$^+$ parasites compared with controls. *D*, Representative H&E-stained images and combined epithelial damage and infiltration scores. Scale bars, 100µm. Data represent mean and SD (*n* = 10 mice per group). *$P < .05$; **$P < .01$; ***$P < .001$. Abbreviations: CXCL1, C-X-C motif chemokine ligand 1; *Eh*MIF, *Entamoeba histolytica* macrophage migration inhibitory factor; H&E, hematoxylin and eosin; MMP-3, matrix metalloproteinase 3; ns, not significant

between *Eh*MIF levels and intestinal inflammation in infected persons. Using cellular and mouse models, we demonstrated that *Eh*MIF induces chemokine secretion from intestinal epithelial cells, resulting in neutrophil influx. These findings implicate *Eh*MIF as a causal factor of mucosal inflammation during infection.

Severe forms of amebic colitis are associated with both high mortality and morbidity. Antibiotics alone are often not enough to treat disease, and surgical removal of the inflamed colon may not prevent death [5]. Metronidazole is the antibiotic of choice for treating amebic colitis. In preclinical mouse models, metronidazole was shown to be very effective at killing ameba but had little effect on *E. histolytica*–induced mucosal inflammation [6, 46]. Adjunctive anti-inflammatory strategies may be needed to improve the clinical outcome of amebic colitis. Neutralization of a parasite mediator of host inflammation such as *Eh*MIF may attenuate disease. However, further studies are needed to determine whether the combination of metronidazole and anti-*Eh*MIF antibodies is superior to metronidazole alone for treatment of severe amebic colitis.

Mucosal inflammation also plays a key role in other human protozoan infections. Mucosal leishmaniasis is a destructive disease caused by the protozoan parasite *Leishmania*. Neutrophil recruitment and an exaggerated inflammatory response perpetuates the disease in mucosal leishmaniasis [47].*Trichomonas vaginalis* causes the most prevalent nonviral sexually transmitted infection worldwide. Vaginitis is characterized by infiltration of the vaginal mucosa with neutrophils, which contributes to the symptoms of vaginal discharge [48]. Toxoplasmosis is a parasitic disease, caused by *Toxoplasma gondii*, which can infect the brain, eye, and the developing fetus. The parasite first enters through the intestine and induces recruitment of neutrophils to the site of intestinal infection that was recently shown to facilitate the spread of infection [49]. Similar to *E. histolytica*, these parasites encode their own MIF homolog. It is possible that these parasite MIF homologs are contributing to the mucosal influx of neutrophils during their respective infections.

A recent study found that the *Plasmodium*-encoded MIF, through its proinflammatory properties, interfered with the development of immunological memory by inducing the development of short-lived effector cells rather than memory cells. This rendered the host susceptible to reinfection by the parasite [50]. This finding could help explain why antibodies against *Eh*MIF were associated with protection from reinfection. Additional studies, however, are needed to validate our preliminary findings.

In conclusion, we identified *Eh*MIF as a specific amebic mediator of host chemokine expression, neutrophil infiltration, and mucosal immunopathology during infection. Intestinal amebiasis remains a major global health problem, especially in children living in low-income countries. With no vaccine and only a single drug class to treat this devastating disease, *Eh*MIF may represent a promising immunotherapeutic target to prevent or attenuate amebic disease.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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