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MOLECULAR CHARACTERIZATION OF *E.HISTOLYTICA* STRAINS AND THE IMPACT OF HOST GENETICS ON AMOEBIC INFECTION IN LIMPOPO AND GAUTENG PROVINCE, SOUTH AFRICA.

University of Venda

A DISSERTATION SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTERS DEGREE (MSc) IN MICROBIOLOGY.

BY

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SUBMITTED TO

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DECLARATION

I, Renay Ngobeni, hereby declare that the dissertation for Masters Degree in Microbiology at the University of Venda, hereby submitted by me, has not been submitted previously for the degree at this or other university, that is my own work in design and execution, and that all references contained therein have been duly acknowledged.

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ACKNOWLEDGEMENTS

First and foremost I thank God for giving me the strength and wisdom, for being a pillar of support, I wouldn't have made it without his love and protection.

I wish to express my deepest gratitude to my supervisor Prof A. Samie for the support, encouragement and unstinting patience he has shown me throughout the course of this research. His advice and guidance have been invaluable in helping me achieve the objectives we set out to accomplish.

I would also like to thank Prof Petri jr, W.A and DR David Lyerly from the University of Virginia for sponsoring us with the ELISA kits (Techlab *E.histolytica* II and *E. histolytica* serology ELISA).

I would like to express my deep appreciation to Dr. A.N. Traore for helping with ELISA experiment and analysis, without her help the objectives of this study wouldn't have been achieved.

I would like to express my gratefulness to Dr. Ramalivhana for helping with sample collection at Polokwane Provincial Hospital. In addition, I would like to thank Ms Tshepo Mafokwane for collecting relevant demographic information which have formed such a significant part of my research.

I wish to express my sincere thanks to all the Nurses at Nkomo Clinic (Giyani) for helping with stool samples collection and for helping us get the patients' cooperation. In addition, I would like to thank the people from the microbiology lab at the University of Limpopo (Medunsa campus)

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and the people from Lancet laboratories for helping us with stool samples collection from Pretoria.

I would also like to extend my sincere thanks to the group of Molecular parasitology and opportunistic infections program, for their overwhelming love, support, team work and encouragement.

Finally, I wish to extend a special note of thank you to my mother and grandmother, Gestar and Cathrine for their love, concern and support in my life and study. Their contributions towards my goal have been varied and countless and cannot be expressed in words. To my only daughter, Keabetswe Boikgantsho, your presence in my life influenced me to work hard academically.

The present study was funded by National Research Foundation.

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DEDICATION

I dedicate this work to my lovely and caring parents, Gestar Vukatimuni Mashele and Cathrine Nghamoni for their undying love, support and for the sacrifices they made for me to be where I am today. I also dedicate this work to my two siblings, promise and lethabo, may you learn something from this. Keabetswe, my daughter this is also for you.

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degrees Celsius
µl	Micro liter
µm	micrometer
AGD	Agar gel diffusion
AIDS	Acquired Immunodeficiency Syndrome
ALA	Amebic liver abscess
BC	Before Christ
bp	Base pairs
BSA	Bovine serum albumin
CBD	Chitin binding domain
CCR1	Chemokine receptor 1
CD4	Cluster of differentiation 4
CDC	Centers for diseases control and prevention.
Chit	Chitinase
Da	Daltons
Dc	Dendritic cells
DNA	Deoxyribonucleic Acid
EDTA	Ethylene diamine tetraacetate
EhSTIRP	*Entamoeba histolytica* serine threonine isoleucine-rich protein
ELISA	Enzyme-Linked Immunosorbant Assay
et al	Et alia (and others)
Fig	figure

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GPI	Glucose phosphate isomerase
HIV	Human Immunodeficiency Virus
HK	Hexokinase
HRP	Horseradish peroxidase
IFA	Immunofluorescent antibody
IgA	Immunoglobulin A
IHA	Indirect haemaglutination
IL-10	Interleukin-10
kb	Kilobase
LPPG	Lipophosphopeptidoglycan
Mb	Megabytes
ME	Malic enzyme
MHC	Major histocompatibility complex
Ml	Milliliter
MUC2	Mucus layer
NK	Natural killer cells
Nm	nanometer
NO	Nitric oxide
O.D	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PGM	Phosphoglucomutase
Phe	phenylalanine
RFLP	Restriction Fragment Length Polymorphism

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RIA	Radioimmunoassay
RNA	Ribonucleic acid
ROS	Reactive oxygen specie
Rpm	revolution per minute
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
SPSS	Statistical Package for the Social Sciences
SREHP	Serine –rich *E.histolytica* protein.
ssRNA	Single stranded ribonucleic acid
SSu- rDNA	Small subunit ribosaomal deoxyribonucleic acid
STD	Sexual transmitted diseases
TB	Tuberculosis
Th	T-heper cell
TNF	Tumor necrosis factor
tRNA	Transfer ribonucleic acid
Trp	Tryptophan
Tyr	Tyrosine
USA	United State of America
UV	Utraviolet
V	Volts
WHO	World Health Organization.

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ABSTRACT

BACKGROUND: *Entamoeba histolytica* is a protozoan parasite known to be pathogenic to human. It is the causative agent of amebiasis, which ranges from asymptomatic to symptomatic diseases. What determines the outcome of this infection is not well understood and the factors that make some infected people sick while others do not get the disease are not clearly defined. Studies have suggested that there is a genetic component that contributes to susceptibility and possibly outcomes of infection and that parasite genotypes and host responses upon exposure to a pathogen can determine a wide spectrum of illness from subclinical or mild to severe diseases. Interleukin (IL)-10, an anti-inflammatory cytokine with pleiotropic properties is involved in the progression of the disease, but the relationship between the genetic variants of IL-10 has not been extensively studied in people with amoebiasis. Therefore the present study aimed at determining the molecular characteristics of *E. histolytica* in relation to disease presentation and to determine the role that host genetics play in the susceptibility to amoebic infection. **METHOD**: This study was approved by the research and ethical committee of the University of Venda, and authorization was obtained from the Department of Health and Welfare in Polokwane. Diarrheal and non-diarrheal stool samples were collected from patients of all ages at Giyani and Pretoria. All the stool (111) samples were examined under a light microscope for the presence of *Entamoeba* cysts and trophozoites. PCR method was used to amplify the serine rich *E. histolytica* protein (SREHP) and chitinase genes using published primers.

A total of 647 Peripheral blood was collected from patients infected with HIV, who were attending different hospitals and clinics throughout the Limpopo province. Genomic DNA was extracted from buffy coat using Gen ELUTE Blood Genomic DNA kit from Sigma-Adrich. The levels of interleukin-10 in serum were measured using enzyme-linked immunosorbent assay

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(ELISA) from MABTECH. The serum samples were also used to detect antibodies against *E. histolytica* using *E. histolytica* serology ELISA kit from Techlab. The Techlab *E. histolytica* II kit was used to detect the antigen (GalNAc lectin) against *E. histolytica*. Single nucleotide polymorphisms (SNPs) at positions -1082A/G and -592C/A in the IL-10 gene promoter were analyzed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) by using MnII and RsaI restrictions enzymes respectively. **RESULTS PART 1:** From the 111 stool samples collected, 51 were positive by either PCR or microscopy and 14 samples were positive by both methods. The serine- rich *E. histolytica* protein was successfully amplified in 26 samples positive by microscopy or PCR. Out of the 26 samples (19) SREHP profiles were obtained. SREHP #2 was obtained in 5 different isolates, 4 from Pretoria and 1 from Giyani (2 symptomatic and 3 asymptomatic), based on these findings this profile is related with diarrhea, since it was also found in patients with watery stools. This profile was found to be more prevalent in Pretoria than Giyani. The chitinase gene amplified 59 samples positive by microscopy and PCR, and out of the 59 samples 22 chitinase profiles were obtained. Two interesting chitinase profiles (profile #4 and 18) were obtained in this study. Profile #4 was found in 6 different isolates, 5 from Giyani and 1 from Pretoria (3 symptomatic and 3 asymptomatic), this profile was found to corelate with diarrhea since it was found in isolates with watery stools and loose stools. However, profile # 18 was not found to corelate with diarrhea since it was only found in isolates with formed stools, in all the isolates from Giyani. All the chitinase and serine rich *E. histolytica* profiles were more prevalent in Giyani (75.1%) compared to Pretoria (24.3%). **RESULTS. PART 2:** Out of the 647 serum samples used in this study, 422 (65.2%) were positive for antibodies against *E. histolytica* by ELISA. The *E. histolytica* antigens were detected in only 3 (0.5%) samples. The seroprevalence was higher in females (66.1%) than in males

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(63.5%), with high prevalence in rural areas (66. 5%) than in urban areas (63.9%). The young (0 to 25) and old aged group (46 to 80) were more infected than people aged 26 to 45, with a prevalence rate of 68.9%, 67.6% and 64% respectively. **RESULTS PART 3:** The patients who were positive for antibodies against *E. histolytica* were found to produce high levels of IL-10 (68.1%) than the sero- negative group (34.2%) by ELISA, and the difference was significant *(P=0.016).* There was no significant difference in IL-10 expression in relation to gender and age *(P>0.005).* However, the results of this study showed that more females had high IL-10 (30.3%) than males (25.9%). The patients aged 46-80 were found to produce more IL-10 compared with the other groups (0-25 and 26-45 age groups) with percentages of 35.3%, 27.9% and 27.3% respectively. **RESULTS PART 4:** In this study we evaluated the association between the polymorphisms of the IL-10 promoter at position -592 A/C and -1082 A/C and the *E. histolytica* seroprevalence. For the IL-10 -1082A/G polymorphism, there was significant difference between the genotypes and the *E. histolytica* infection, however, the homozygous AA (68.1%) and heterozygous AG (67.5%) genotypes were associated with susceptibility to amoebiasis and the homozygous GG genotype was associated with reduced risk of amoebiasis (P=0.945). There was no significant difference between the -592 A/C polymorphism with *E. histolytica* infection. However, this study showed that AC genotype was associated with reduced risk of amoebiasis. The homozogous CC and AA genotypes were common among sero-positive patients (71.3% and 64.1 %) in comparison with the sero-negative subjects (28.7% and 37.6%) (P=0.259) respectively. There was no significant difference between IL-10 levels and the IL-10 gene promoter polymorphisms in the study. **CONCLUSIONS PART 1**: The results obtained in this study have further confirmed the genetic heterogeneity of *E. histolytica* for the SREHP and chitinase genes which might have a role in the presentation of amoebiasis (symptomatic and

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asymptomatic infections) depending on the genetic profile of the infecting strain. **PART 2:** The high seroprevalence of antibodies against *E. histolytica* suggest that the majority of the people in this study have been exposed to *E. histolytica*. Moreover, this prevalence also shows that amoebiasis is endemic in these areas. The low prevalence of antigens against *E. histolytica* in this study, suggests that amoebic liver abscess might not be common in the region. **PART 3**: IL-10 concentrations might influence the progression of the disease. This study revealed that elevated level of IL-10 is associated with reduced risk of amoebiasis, the higher the level of IL-10 in the serum the lower the infection rate. **PART 4**: This study reports for the first time that IL-10 promoter polymorphisms participate in the progression of amoebiasis. The IL-10 -1082 A/G polymorphism was more associated with amoebiasis susceptibility as compared to -592 A/C polymorphism

Keywords: *Entamoeba histolytica*, SREHP, chitinase and IL-10, genetic Polymorphisms.

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CHAPTER ONE

GENERAL INTRODUCTION AND OBJECTIVES

1.1. INTRODUCTION

It is estimated that 50 million people have invasive amoebiasis due to *E. histolytica* worldwide (Petri and Singh, 1999), a disease that has been a big health problem since the beginning of the 21st century. The organism colonizes the bowel system of the host for years without inducing clinical symptoms of the disease. The infection ranges from asymptomatic to symptomatic diseases and only about 10% of the infected individuals develop symptomatic diseases and the remaining 90% do not show symptoms (Gilchrist et al., 2012). In symptomatic patients, the trophozoites may spread to extra- intestinal organs such as lungs, brain and liver which results in extra- intestinal amoebiasis. It remains puzzling why only subset of infected individuals develop 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. One of the possible explanations for this is that genetic subgroup exist within *E. histolytica* that gives rise to infection with different outcomes. Therefore the factors that govern the transition from colonization to invasion remain to be investigated.

Entamoeba histolytica is an anerobic parasite that has been documented as the most common pathogenic parasite in human worldwide. Humans are the primary host of *E. histolytica* (Jesus' Serrano-Luna *et al.*, 2013). The infection is possible through consumption of contaminated food and water. The clinical presentation ranges from watery diarrhea, bloody diarrhea, stomach pains and liver abscess (Gilchrist *et al.*, 2012). The cysts of *Entamoeba* species are said to be morphologically identical, which makes it difficult to differentiate using a light microscopy.

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Therefore 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 (Kurt *et al*., 2008). Genes that are detected in *Entamoeba* isolates include chitinase and serine- rich *E. histolytica* protein; both the genes have tandem repeats (Ramos *et al*., 2005).

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

Little research has been documented on the impact of both the parasite and host genotypes on the outcome of amoebic infection, furthermore, few studies has been conducted on the occurrence of *E. histolytica* in the Limpopo and Gauteng province. However, there is no study that has been conducted on the association of cytokines, especially IL-10 with amoebic infection in these areas. Interleukin-10 appear to be an essential mediator in the host immune response, it can both stimulate and suppress the immune response (Kong *et al*., 2010). Several studies have shown that IL-10 may be involved in disease development and that increased IL-10 facilitates disease progression (Mocellin *et al*., 2005). It has been shown that the three promoter polymorphisms in the IL-10 gene may influence the expression of IL-10 in vitro (chand-Bhayal *et al*., 2012). Therefore the aim of the present study was to determine the molecular epidemiology of *E. histolytica* and to evaluate whether IL-10 gene promoter -1082 A/G and -592 A/C polymorphisms are associated with the development of amoebiasis in the study population.

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The findings of this study will assist in developing strategies for the prevention and control of this pathogenic parasite, which will also help in reducing the burden of the disease especially for people in developing countries.

1.2. STUDY RATIONALE

Amoebiasis a significant cause of mortality and morbidity worldwide (Petri *et al.*, 2000). Millions of people are infected with *E. histolytica* worldwide, but only 10% develop symptomatic diseases with varied outcomes (Haque *et al.*, 2002). It has long been known that not all *E. histolytica* infections lead to disease in the host, and it has been clearly indicated that one out of 4 *E. histolytica* infections progress to development of clinical symptoms (Haque *et al.*, 2006). The understanding of different aspects of *E. histolytica* and the disease it causes, makes it a very exciting and rewarding time to study amoebiasis because understanding what determines the outcome of infection and the nature of amoebic virulence is very important.

The outcome of infection may depend on several factors, among which the genetic characteristic of specific pathogen that have been identified as the significant one. A number of genes appear to influence susceptibility to infectious pathogens and explain degrees of susceptibility to infections. It has been suggested that both the parasite and host genotypes play a role in the outcome of infection (Gilchrist *et al.*, 2012). The study by Haghighi *et al.*, (2003) demonstrated that *E.histolytica* had a highly complex genetic structure, but they didn't look at the association between the parasite and outcome of infection. Therefore, the current study aims at investigating the parasite genotypes based on SREHP and chitinase gene of *E.histolytica* to study the genetic diversity of this parasite and their relation with the outcome of the disease.

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Several studies have led to an improved understanding of the host immune response in intestinal amoebiasis. However, the question remains unanswered, for example, the host –dependent immune mechanisms that might be responsible for development of severe diseases are not fully understood. Many individuals have a different genetic signature that can affect how quickly and effectively the immune system can act to buildup resistance to infections. Interleukin- 10 is an important immunoregulatory cytokine (Trifunović *et al*., 2015) that serves as a pre-determining factor for amebic invasion (Hamano *et al*., 2006). Hamano *et al*., (2006) have shown that IL-10 is required for resistance to intestinal amoebiasis and that IL-10 deficiency leads to high susceptibility to amoebic infection. However, this study didn't determine the association of IL-10 gene promoter polymorphism and severity of amoebiasis. Another study have focused on IL-10 polymorphisms associated with diseases such as cancer, pneumonia, toxoplasmic retinochonditis, malaria and T.B and no studies have been done to show any association between IL-10 promoter polymorphism and severity of amoebiasis in the Limpopo province. Host genetic susceptibility is another area that needs thorough evaluation, in order to understand the mechanisms by which the parasite disguises itself from host immune system.

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It has been suggested that human genetics variation serve as a major determinant of susceptibility to many common infectious diseases (Kong *et al*., 2010). Although IL is an important cytokine in the pathogenesis of *E. histolytica*, the role of the genetic polymorphisms on the occurrence of amoebiasis is not known. Therefore the study of parasite and host genetics in amoebic infection is valuable. A study by Kong *et al*., 2010, showed that -1082AA genotype was associated with a significantly increased risk of lymph node involvement and larger tumor size at the time diagnosis and suggested that IL-10 promoter polymorphism participate in the progression of breast cancer. So to explain to explain why other people get sick while others do not, the current

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study will focus on host genetics (IL-10) as a major determinant of susceptibility to many common infectious diseases

1.3. OBJECTIVES OF THE STUDY

1.3.1 General objectives

The main objective of this study was to determine the molecular epidemiology of *E. histolytica* and to determine the role of host genetics on amoebic infection.

1.3.2. Specific objectives

1.3.2.1. To determination of the parasite factors that could contribute to invasive diseases circulating in Giyani and Pretoria

1.3.2.2. To evaluate the relation between serum levels of IL-10 and amoebiasis.

1.3.2.3. To evaluate whether IL-10 gene promoter -1082 A/G and -592 A/C polymorphisms are associated with susceptibility to amoebiasis infection

1.4. HYPOTHESES

A. In the present study, we hypothesise that *E. histolytica* is common in different communities in the Limpopo Province.

B. Interleukin-10 gene promoter -1082 G/Aand -592 A/C polymorphisms are associated with amoebic infection. IL-10 is the major candidate gene in susceptibility to amoebic infection.

1.5. RESEARCH QUESTION: What determines the outcome of *E. histolytica* infections?

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CHAPTER TWO

LITERATURE REVIEW

2.1. HISTORICAL PERSPECTIVES OF *E. HISTOLYTICA*

The infection with *E. histolytica* was first described as a fatal disease by Hippocrates (460-377 B.C). In 1875, (Kean *et al*., 1988) Fedor Losch explained the case of a young farmer who was admitted to his clinic in St Petersburg (Kean *et al.*, 1988). The patient was suffering from chronic dysentery, Fedor Losch isolated what he believed to be a causative agent and he named it *Entamoeba coli* and the patient in Losch's hospital was the first person known to have died from amoebiasis. In 1913, in the Philippines, Walker and Sellerds recorded the cysts form of this parasite as the infective stage and it was described that *E. histolytica. E. dispar* is a close relative of *E. histolytica* and the two are morphologically similar which makes it difficult to differentiate them using microscopy. *Entamoeba histolytica* was the only known pathogenic parasite that infect humans (Fodor Losch, 1875). *Entamoeba dispar* was recorded as the non- pothogenic organism (Brumpt, 1925). 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 amoebiasis (Huston *et al.*, 1999).

2.2 GENERAL CHARACTERISTICS OF *ENTAMOEBA HISTOLYTICA*

2.2. 1. Classification of *Entamoeba histolytica*

Entamoeba histolytica is a protozoan parasite which falls in the kingdom protista under the phylum sarcomastogophora, sub-phylum sarcodina. It is defined as the protozoan parasite that lacks most eukaryotic organelles such as the mitochondria, structure cytoskeleton, golgi apparatus and endoplasmic reticulum (Clark *et al.*, 2000). It is the only parasite known to be

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pathogenic to man, once it is in the body, it colonises and invade the intestinal mucosa and every other tissue and organs, commonly the liver. Other species that infect humans are *E, dispar, E. moshkovski, E. polka, E. hartmani and E. colicyclospora, cryptosporidium, isospora* and *eimeria. Entamoeba histolytica* is the only known pathogenic specie in the genus *Entamoeba* (Fodor Losch, 1875)

2.2.2. Morphology of *Entamoeba histolytica*

Entamoeba histolytica is a protozoan parasite that lacks most eukaryotic organelles such as mitochondria, golgi complex, structure cytoskeleton and the reticulum (Clark *et al.*, 2000). However, it has the ability to colonise and invade the intestinal mucosa and the liver. This cosmopolitan parasite has a simple life- cycle consisting of two stages, the infectious cyst and a motile trophozoite. *Entamoeba. dispar* is still considered to be morphologically similar to *E.histolytica.*

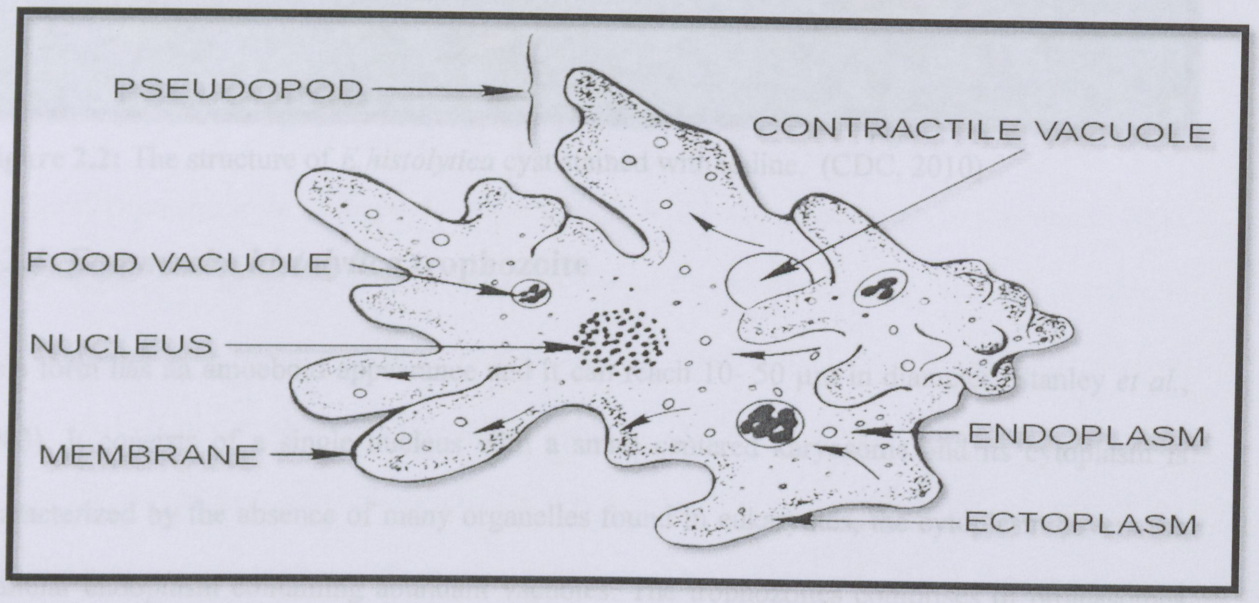

Figure 2.1: Structure of Entamoeba (Clark *et al.*, 2000).

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2.2.3. Entamoeba histolytica cyst

Entamoeba histolytica cyst, considered as the infective stage of the parasite, is round in shape with a 10-15μm in diameter (Stanley, 2003). It 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. (Figure 2.2).

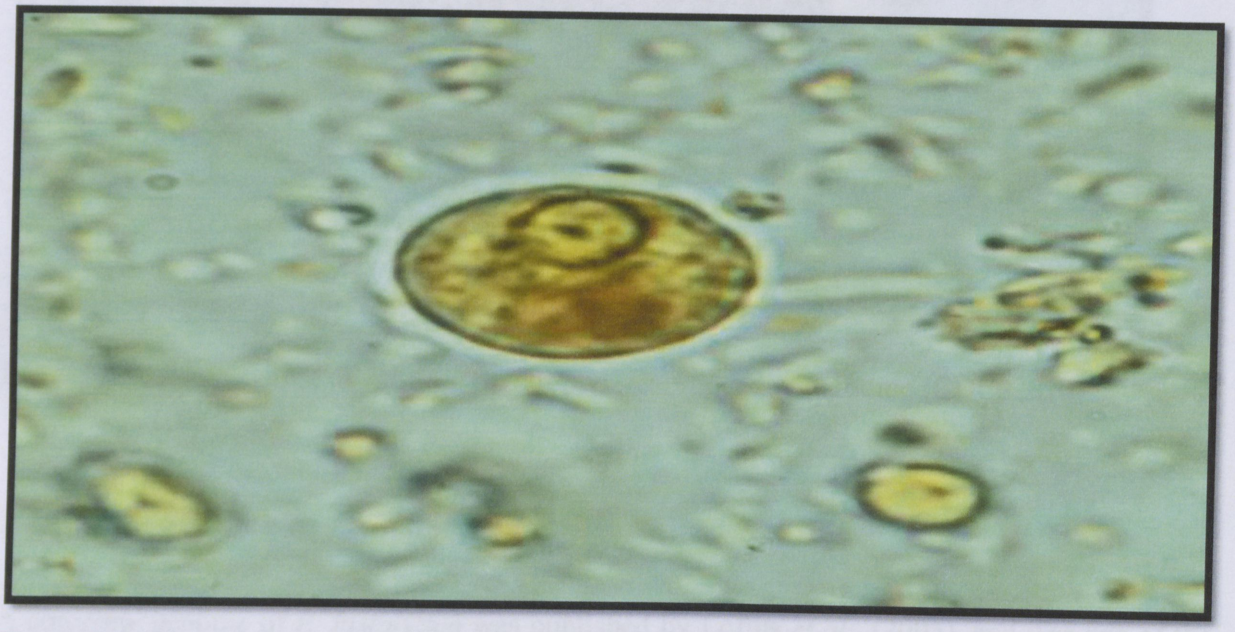

Figure 2.2: The structure of *E.histolytica* cyst stained with iodine. (CDC, 2010)

2.2.4. *Entamoeba histolytica* trophozoite

This form has an amoeboid appearance and it can reach 10- 50 μm in diameter (Stanley *et al.*, 2003). It consists of a single nucleus with a small centered karyosome and its cytoplasm is characterized by the absence of many organelles found in eukaryotes, the cytoplasm covers the granular endoplasm containing abundant vacuoles. The trophozoites comprises of proteasomes

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within their cytoplasms (figure 2.3), this proteasomes have a significant role in cell differentiation and replication (Makioka *et al.,* 2002).

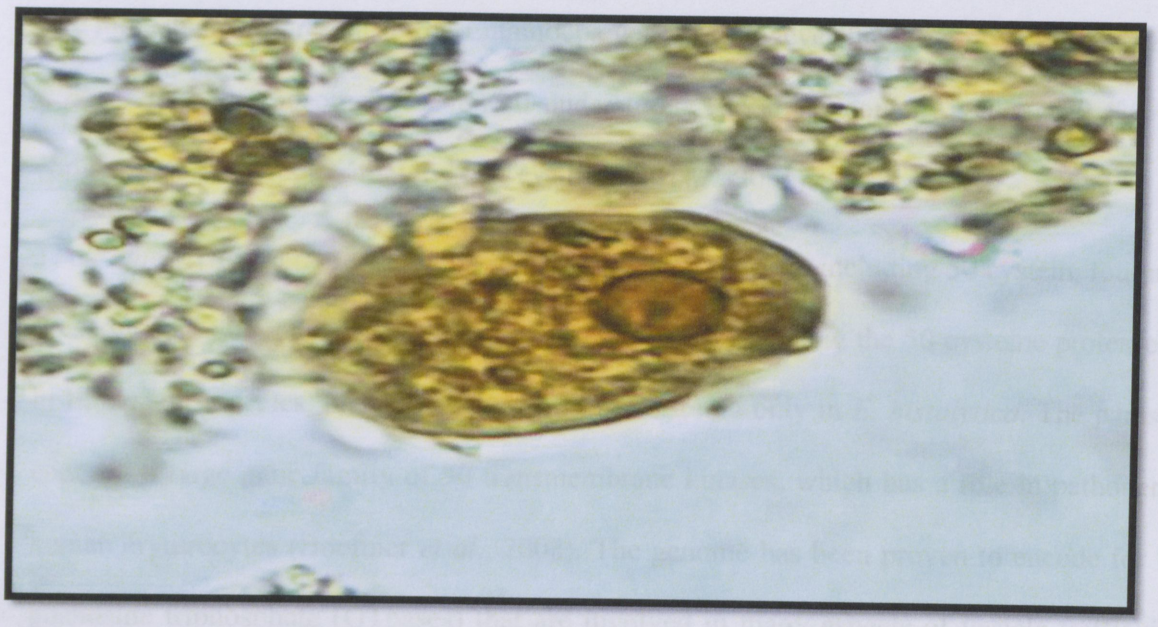

Figure 2.3: The structure of *E.histolytica* trophozoite stained with iodine (CDC, 2010).

2.2.5. The genome of *E .histolytica*

A complete genome of *E. histolytica* was published by Loftus *et al*., (2005), and was one of the first protist genome to be sequenced. The project of *E. histolytica* genome was initiated in 2000. As the number of genes in *E. histolytica* is close to thousands (10,000), it is not possible to discuss them 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 genes in *E. histolytica* have no introns, but they are contained within 25% of the putative genes of this parasite (Loftus *et al*., 2005), the gene size are surprisingly short, due to the loss of introns and the predicted length of proteins they code for. The predicted length of *E. histolytica* proteins is 389 amino acid (Clark *et al*., 2007).

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The genome of *E histolytica* is about 24mb big, 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% (Willhueft *et al*., 2001).

The *E. histolytica* genome contains about 86 peptidases genes including 50 cystein, four aspartic, 10 serine and 20 metallo peptidases (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 (Boeftner *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.

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 is also characterized by the structure and organization of tRNAs genes which are organized into varying arrays (500bp to over 1750bp) of which there are about 4500 in the genome (Clark *et al*., 2006). The arrayed genes are thought to be function because of the 42 acceptor types found in arrays. These array units encode between one and five tRNAs and a few tRNAs genes are found in more than one unit, three of these arrays also encodes the ssRNA and one is believed to be a small nuclear RNA (Clark *et al*., 2006).

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2.2.6. GENETIC DIVERSITY OF *E. HISTOLYTICA*

2.2.6.1 Serine- rich *E. histolytica* protein

Understanding the nature of amoebic virulence is important, several studies have shown that the genetic factors do influence the virulence of the infection. Among the many loci used to investigate the epidemiology of *E. histoytica*, SREHP is one of the important markers used in molecular epidemiological studies. Serine- rich protein is known as a surface protein that is involved in amoebic adhesion to host components and its antibodies are said to inhibit adhesion to host cells. Although their ligand binding properties are not clearly understood (Mortimer and Chadee, 2010). It is also known as k2 protein, that is located on the parasite surface with tandem repeats of 24 and 36 bases in length, encoding 8 and 12 amino- acid repeats (Ramos *et al*., 2005), this protein was named SREHP because of the large number of serine residues (52 of 233 amino acids) (Stanley et al., 1990)

Serine- rich *E. histolytica* protein has been investigated as a vaccine candidate that can be used to prevent amoebic liver abscesses (Kohler and Tannich, 1993). This protein has been shown to be potentially useful for determination of intra-species variation among *E. histolytica* and also in investigating the molecular epidemiology of amoebiasis. SREHP is a surface membrane protein, but it has post- translational modification including phosphorylation of serine residues and glycosylation with O- linked N- acetyl glucosamine residues, which are more commonly seen in nuclear or cytoplasmic proteins (Stanley *et al*., 1995), the function of this protein remain unclear.

The antibodies of SREHP block amoebic adherence to mammalian cells in vitro (Zhang *et al*., 1994). It was determined that the SREHP can play a role as a chemo-attractant for *E. histolytica*. The protein contain a number of epitopes and is known to be highly immunogenic, in patients

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who have amoebic liver abscess, more than 80% have antibodies that recognize the SREHP molecule (Stanley *et al*., 1995).

The outcome of the infection may depend on several factors, among which the genetic characteristics of the specific pathogen have been identified as the significant one. The genetic loci can be targeted to help in the study of the *E. histolytica* strains and their relationship with the parasite virulence and the disease outcome (Paul *et al*., 2007), SREHP has been chosen as one of these genetic markers. A study by Samie *et al*., (2008), indicated that certain SREHP profiles might be responsible for the presentation of intestinal amoebic symptoms, it was also suggested that the parasite genotype does play a role in the outcome of the infection in humans, thus linking parasite diversity and gene will help in the understanding of parasite virulence and pathogenesis (Ali *et al*., 2008)

2.2.6.2. Chitinase gene of *E. histolytica*

Entamoeba chitinase is a stage specific enzyme found to be expressed during encystation. *E. histolytica* forms chitin-walled cysts during encystation process, where the formation of the cyst wall needs chitin synthase. During excystation, a quadruplet amoebae emerge from the chitin-walled cysts by dissolving the wall, so that the chitinase may be necessary for excystation as well (Makioka *et al*., 2011). The major components of the cyst wall are chitin and chitin-binding lectins called Jacob, Jessie 3, and chitinase (Dey *et al*., 2009). The chitinase function is to break down the glycosidic bond of chitin, the major component of the cyst wall.

Amoebic chitinase consists of a series of acidic and hydrophilic repeats between the N-terminal signal and a sequence a C-half catalytic domain. *E. histolytica* chitinase gene range from 23,326 to 24,849 nucleotides, and has a molecular weight of 57309 Da (Amoeba data base). The

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chitinase gene appears to be less polymorphic compared to the SREHP gene, this gene has been used more frequently for species identification than strain differentiation (Acuna-soto *et al.*, 1993).

A chitinase was demonstrated to be present in the reptilian parasite *Entamoeba invadens*, with its presumed function related to encystation of the parasite within a cell wall, allowing resistance to environmental conditions (Dey *et al.*, 2009). *E. invadens* has been used as encystation model system for *E. histolytica,* because *E. histolytica* does not encyst well *in vitro.* Genome sequencing has established that *E. invadens* has 74% identity in genic region and 50% in inter-genic regions of *E. histolytica*, made the former an ideal material for comparative gene identification and gene model refinement study. *E. histolytica* chitinase was cloned using degenerated oligonucleotide derived consensus sequences of bacterial, fungal and insect chitinases (de la Vega *et al.*, 1997).

The *E. histolytica* chitinase has putative substrate binding and catalytic and chitin binding domains (CBD). The N-terminus has acidic and hydrophilic domains composed of multiple tandermly arrayed seven amino acid repeats. Recombinant *E. histolytica* chitinase was demonstrated to bind chitin and show endochitinase activity (Makiota *et al.*, 2011). An alignment of *E. histolytica* and *E. invadens* chitinases suggests the presence of putative CBD containing eight Cyst residues, which might form four disulphide bonds as in plant lectin. The CBD have eight positionally identical amino acids (Tyr, Phe, and Trp), which might be involved in carbohydrate binding (Ghosh *et al.*, 2000).

The *E. histolytica* chitinase is encoded by a single copy of chit gene, containing a series of degenerate heptapeptide repeats which are located between an amino terminal signal sequence and carboxyl half catalytic domain (de la Vega, 1997). The amoebic chitinase are polymorphic

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from one clinical isolate to the next, making the chit gene useful for molecular epidemiological studies (Samuelson *et al*., 1997). Chitinase gene of *E. histolytica* behaves like a single copy gene in southern blotting (Clark, 2006). Genome analysis of *E. histolytica* predicted a single chitinase while its reptilian counterpart *E. invadens* reported to have three chitinase Eichit1, Eichit2 and Eichit3 (Figure 2.4).

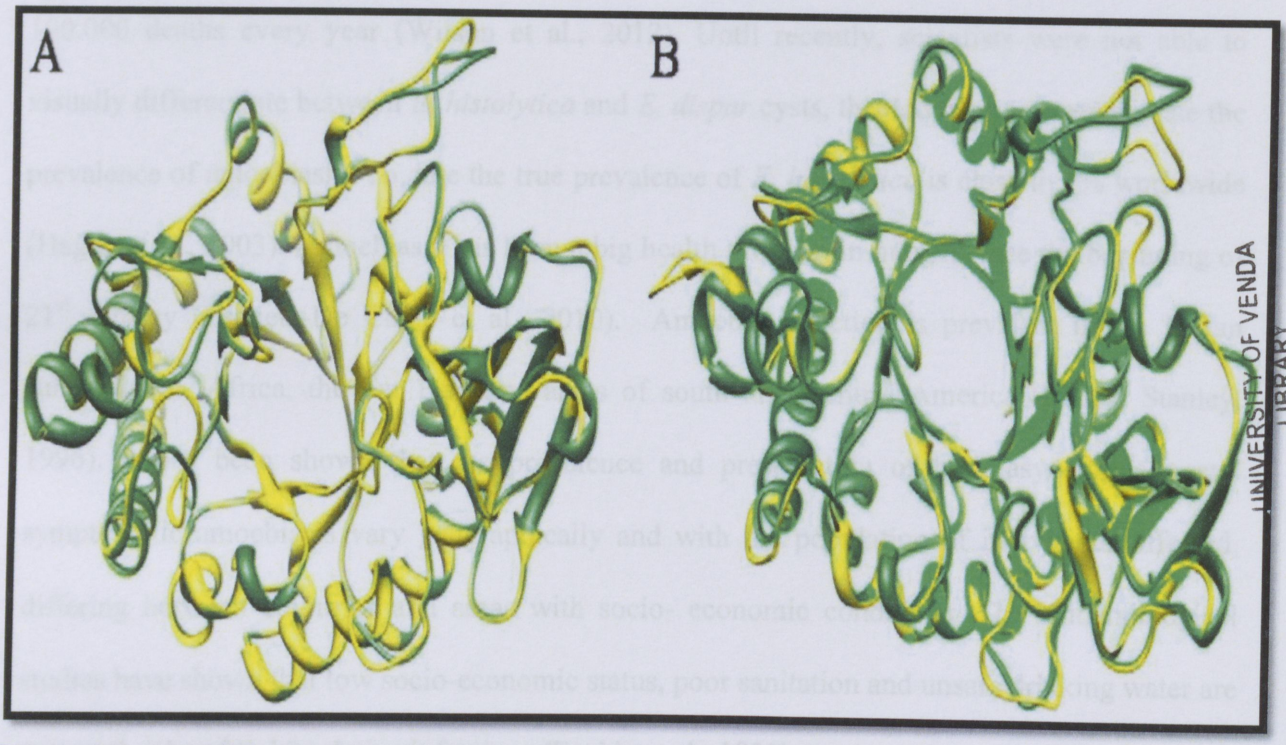

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Figure 2.4: (A) Ribbon model of EiChit2 (Green) is superimposed with EiChit1 (Yellow) and (B) EChit3 (yellow) (de la Vega, 1997).

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2.3. EPIDEMIOLOGY OF *E.HISTOLYTICA*

Entamoeba histolytica is an enteric parasite with a global distribution, but morbidity and mortality due to its infection occurs mostly in developing countries (Fletcher et al., 2012). Although the parasite can infect 500 million individuals worldwide, 90% of the infected people are said to be asymptomatic while the remaining 10% are symptomatic. To date it estimated that *E. histolytica* causes 34-50 million symptomatic infections annually, worldwide, which result in 100.000 deaths every year (Wilson et al., 2012). Until recently, scientists were not able to visually differentiate between *E. histolytica* and *E. dispar* cysts, this led them to overestimate the prevalence of amoebiasis. To date the true prevalence of *E. histolytica* is close to 1% worldwide (Hague et al., 2003). Amoebiasis has been a big health problem in human since the beginning of 21st century (Escueta-De Cadiz et al., 2010). Amoebic 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 amoebiasis vary geographically 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 unsafe drinking water are potential risks of E. histolytica infections (Fuchis et al., 1988).

Globally, it has been reported that approximately 10% of individuals who become infected with *E. histolytica* actually develop invasive diseases (Gathiram and Jackson, 1987). In countries such as china, it was found that high seroprevalence of *E.histolytica* infections occurs in homosexual men, about 73.3% of men who have sex with men were found to be *E.histolytica* seropositive (Zhou *et al*., 2013). A survey of intestinal parasite among restaurant workers was done all over the world, a study by Garedaghi and Smashaei., (2011), showed that the prevalence of

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E.histolytica is 14.4% in Tabriz, Iran, 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).

A study in some regions of sivas, investigated the prevalence of *E. histolytica* in stool samples, using native lugol, trichome staining and monoclonal antigen detection among children attending primary schools in the villages of sivas and it was proved that 22 (1.5%) were positive for *E. histolytica* cysts, but all the samples were negative when using the ELISA test (Erdogan *et al.*, 2011). In Malaysia, intestinal parasitic infections, including *E.histolytica*, are more prevalent in rural areas. In the study carried by *Ngui et al.*, (2012), in the country Malaysia, 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%. A record is available, indicating that *E. histolytica* is more common than *E.dispar* in Zonguldak, Mengeloglu *et al*, (2009), observed 44 (0.37%) of amoebic cysts out of 1720 stool specimens by direct microscopy, the samples were further analysed by ELISA method and it was found that specific antigens of *E.histolytica* was 59.1%. further studies showed that the prevalence of *E. histolytica* to be 0 to 17% in Turkey, however there are studies reporting high rates of detection between 43.2 to 77.7% (Mengeloglu *et a*l., 2009).

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 (Public health agency of Canada, 2010). Studies in parts of Africa reported prevalence rates of 22% and 21% in South Africa and Egypt respectively (Stauffer *et al.,* 2006; Ozumba, 1997) and 13.7% in Nigeria have been reported (Ogunlesi *et al.*, 2005). Another study in South Africa in the Vhembe district on the prevalence of intestinal parasitic and bacterial pathogens in diarrheal and non-

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diarrheal human stools, it was found that 34.2% was the most common cause of diarrheal infection (Samie *et al.,* 2009). In Nigeria, the study that investigated the prevalence and risk factors of *E. histolytica* infection among dysentery patients presenting at Kaduna hospital using microscopy and ELISA. Of the 189 samples collected, 14.3% were positive for *E. histolytica* which was higher than 10% recorded by WHO in 1995 in developing world (Dawah *et al*., 2010).

2.4. TRANSMISSION ROUTES OF AMOEBIASIS

The active trophozoites stages is known to exist only in the host and in fresh loose feces and the cysts survive outside the host in water, soil and on foods (American Water Works Association, 2006). When the cysts are swallowed, they cause infections by excysting in the digestive tract, the infection can be asymptomatic or can lead to symptomatic infections e.g, they lead can lead to amoebic dysentery or amoebic liver abscess (Ryan and Ray, 2004). Humans are the primary host and transmission is through the ingestion of water or food contaminated with feces containing cyst (Stanley *et al*., 2003). The infection is also confirmed to occur in homosexual men through oral-anal and oral-genital sex (Stark *et al*., 2008, yin chen *et al*., 2007). Person to person contact occur when food are contaminated by infected food handlers, this is said to be most important means of transmission (Tangku and Norhaya, 2011)

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Figure 2.5: River water contaminated with feces from sewage (www.grinningplanet.com)

Figure 2.6: Drinking of contaminated water is also a source of infection (www.tajpharm.com).

2.5. *ENTAMOEBA HISTOLYTICA* LIFE CYCLE

The genus *Entamoeba* consist of several protozoan species which colonize human, but not all of them are pathogenic, only *E. histolytica* is recognized as pathogenic amoeba, which causes amoebiasis.

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The life- cycle of this parasite is quite simple, it consists of two stages, the infective stage, known as the cyst and the diagnostic stage, known as trophozoite. The cysts are normally found in formed stools and the trophozoites can be found in diarrheal stools. The cysts and trophozoites are excreted in stools. The infection with *E. histolytica* occurs through the consumption of feacally contaminated water and food, after ingestion, the cysts get into the small intestine where they release the trophozoites through the procees called excystation, the trophozoites migrate to the large intestine and multiply by binary fission to produce the cysts (CDC, 2010). The cysts are known to survive in the external environment for days or weeks because of the protection conferred in their walls, whereas the trophozoites get destroyed rapidly when they are in the external environment and even if they are ingested, they are not capable of resisting the gastric environment in the human stomach (CDC, 2010). 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 amoebiasis, or through the bloodstream, extra- intestinal sites, such as liver, brain and lungs, which can result in extra- intestinal diseases, the life cycle of *E. histolytica* is shown in (figure 2.7) below (CDC, 2010).

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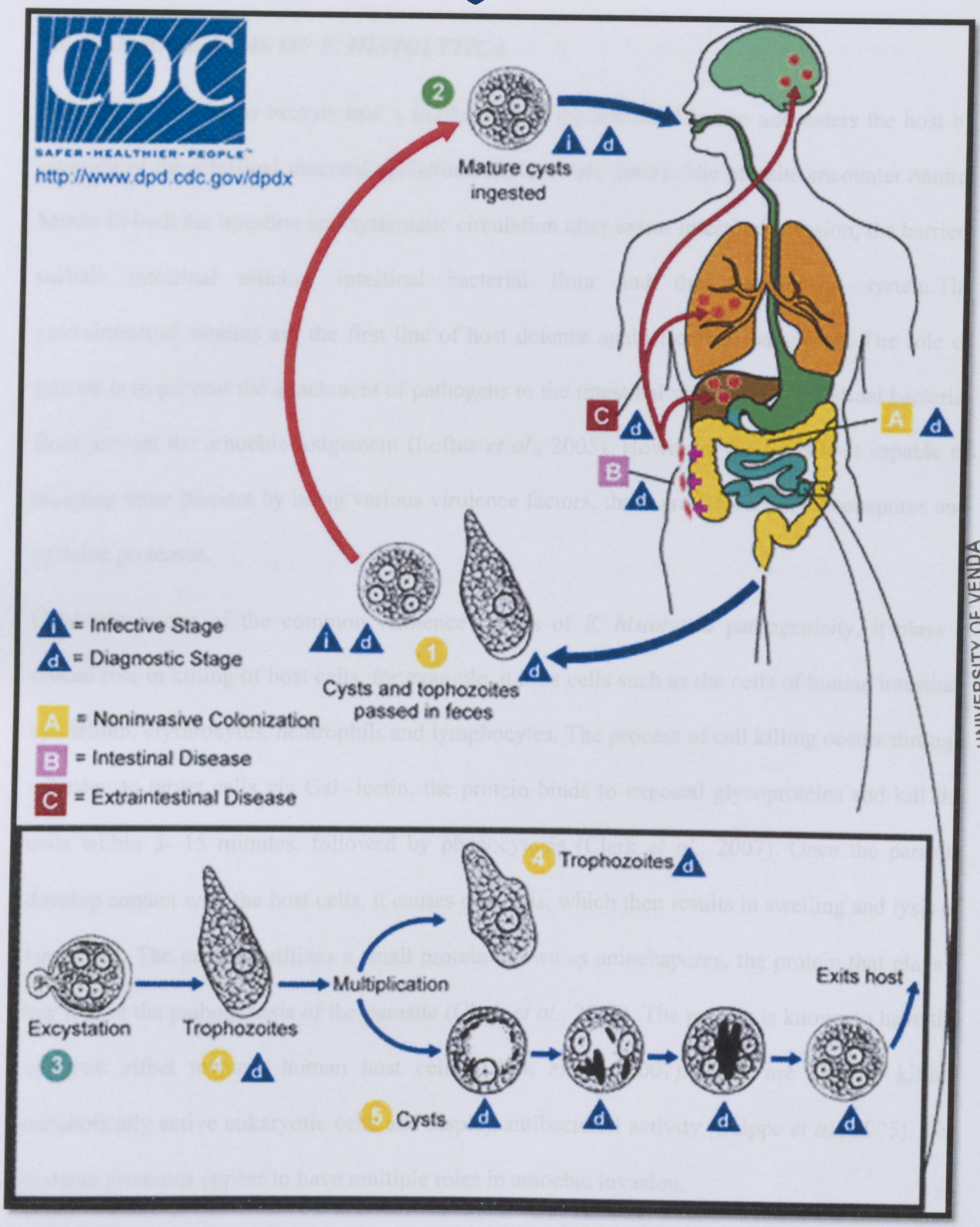

Figure 2. 7: Life Cycle of *E. histolytica*. (CDC, 2010)

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2.6. PATHOGENESIS OF *E. HISTOLYTICA*

Entamoeba histolytica excysts into a trophozoite in the intestinal lumen and enters the host by invasion of the intestinal mucosal epithelium (Beck *et al*., 2005). The parasite encounter natural barrier in both the intestine and systematic circulation after extra- intestinal invasion, the barriers include intestinal mucins, intestinal bacterial flora and the complement system.The gastrointestinal mucins are the first line of host defense against enteric pathogens. The role of mucins is to prevent the attachment of pathogens to the intestinal wall and the intestinal bacterial flora prevent the amoebic lodgement (Loftus *et al*., 2005). However, the parasite is capable of escaping these barriers by using various virulence factors, these are Gal- letin, amoebapores and cysteine proteases.

Gal-lectin is one of the common virulence factors of *E. histolytica* pathogenicity, it plays a crucial role in killing of host cells, for example, it kills cells such as the cells of human intestinal epithelium, erythrocytes, neutrophils and lymphocytes. The process of cell killing occurs through adhesion to target cells via Gal- lectin, the protein binds to exposed glycoproteins and kill the cells within 5- 15 minutes, followed by phagocytosis (Clark *et al*., 2007). Once the parasite develop contact with the host cells, it causes cytolysis, which then results in swelling and lysis of target cell. The parasite utilizes a small protein known as amoebapores, the protein that plays a key role in the pathogenesis of the parasite (Clark *et al*., 2007). The protein is known to have the cytolytic effect towards human host cells (Clark *et al*., 2007). They are able of killing metabolically active eukaryotic cells and display antibacterial activity (Leippe *et al*., 2005). The cysteine proteases appear to have multiple roles in amoebic invasion.

The parasite, *E. histolytica*, exhibit two different facets of its life in the gut. The amoebic trophozoite can remain as commensal without causing any intestinal pathology, this is seen in

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many people who become infected. However, it can destroy the colonic mucosa and invade other organs such as the liver, lungs and brain (Lejeune *et al*., 2009).

As *E. histolytica* colonize mucin substrate, it degrades the proteins of MUC2 polymer to weaken and traverse the mucus gel (Moncada *et al*., 2005). It was shown that the cysteine proteases can effectively cleave the cysteine- rich domains of MUC2 polymer, which cause depolymerization and disruption of the mucus gel, which then allow the parasite to interact directly with the epithelial surface (Lidell *et al*., 2006). The parasite utilizes Gal-lectin, lipophosphopeptidoglycan (LPPG) and serine threonine isoleucine- rich protein (EhSTIRP) found on the surface of *E. histolytica* (Macfarlane *et al*., 2007).

Following attachment, the parasite continues to secrete cysteine proteases, which lyse and disrupt the apical surface (Lauwaet *et al*., 2003). The trophozoites can also alter the intergrity of the tight junctional complex and transfer antigens such as Gal- letin and LPPG to the apical side of enterocytes (Sharma *et al*., 2005). The cleavage of tight junctions proteins causes a disruption in the colonic epithelial barrier, this is associated with the characteristic diarrhea observed in patients having amoebic colitis.

The parasite expresses surface perioxiredoxin, which neutralize both reactive oxygen species (ROS) and nitric oxide (NO) produced by the host to prevent the parasite from envading the sub-epithelium, thus facilitating parasite invasion (Davis *et al*., 2006).

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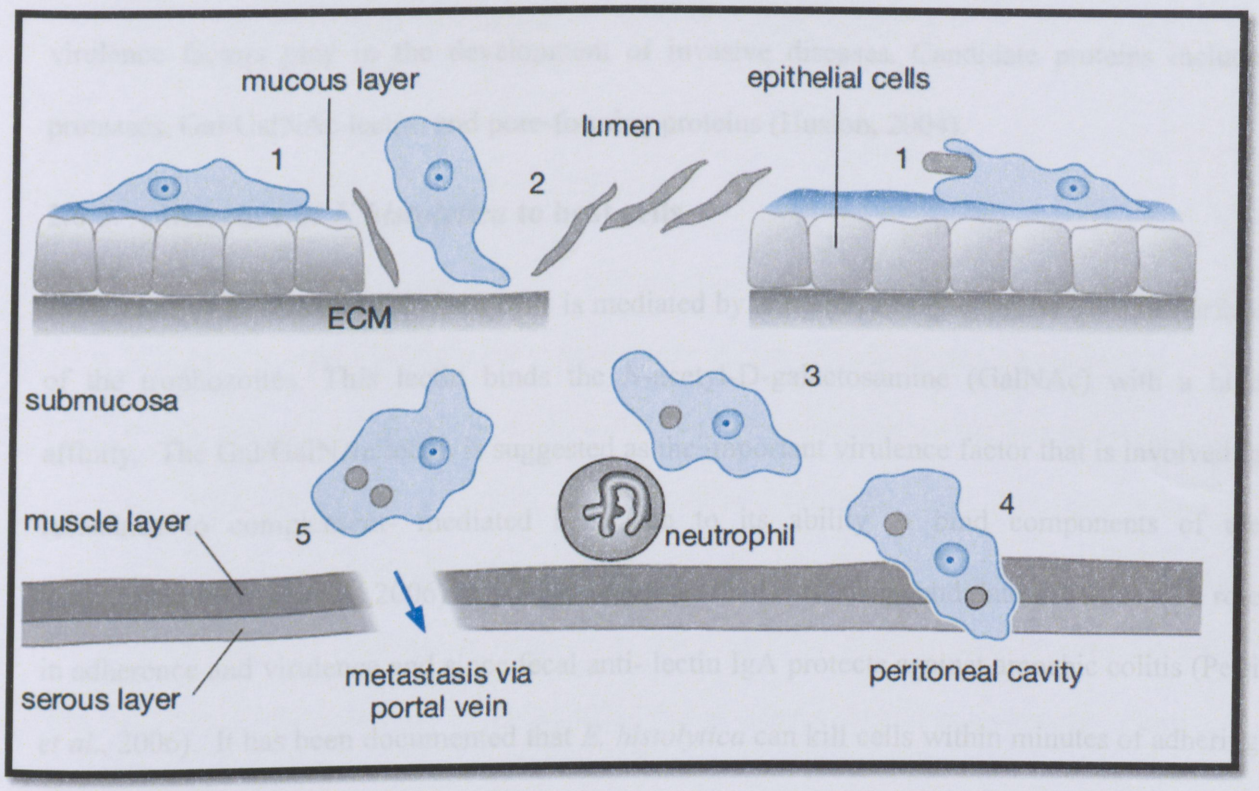

Figure 2. 8: Pathogenesis of intestinal amoebiasis (Huston, 2004).

2.6.1. Mechanisms of pathogenesis

E. histolytica is pathogenic parasite known to exhibits a wide spectrum of virulence, ranging from an avirulent commensal to virulent and destructive organism. The infection with *E. histolytica* does not always lead to invasive disease and less than 10% of the infected individuals will develop symptomatic invasive amoebiasis (Shahran *et al*., 2008). However, what determines these differences is still not clear and the exact factors responsible for the pathogenesis of *E. histolytica* are not well understood. A key feature of the pathogenesis is the ability of *E. histolytica* trophozoites to penetrate the epithelial cell layer, thereby breeching the first line of host defense (Petri *et al*., 2002). Parasite proteins play a role in the processes of adherence, cytotoxicity, and disruption of the tissues. However, it is not clear the exact role these various

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virulence factors play in the development of invasive diseases. Candidate proteins include: proteases, Gal/GalNAc lectin, and pore-forming proteins (Huston, 2004).

2.6.2. Adherence of *E.histolytica* to host cells

Adherence of *E. histolytica* to host cells is mediated by a lectin activity expressed on the surface of the trophozoites. This lectin binds the *N*-acetyl-D-galactosamine (GalNAc) with a high affinity. The Gal/GalNAc lectin is suggested as the important virulence factor that is involved in resistance to complement- mediated lysis due to its ability to bind components of the compliments (Petri *et al*., 2006). It is also suggested as the vaccine candidate because of its role in adherence and virulence and since fecal anti- lectin IgA protects against amoebic colitis (Petri *et al*., 2006). It has been documented that *E. histolytica* can kill cells within minutes of adhering to them in the presence of extracellular calcium. This killing is mediated by the Gal/GalNAc lectin in that galactose or antibodies against the protein can inhibit adherence and killing. However, the purified Gal/GalNAc lectin is not directly cytotoxic suggesting that the protein is involved in signaling the cytotoxic event.

2.6.3. Cytotoxic activity by *E. histolytica*

Cytotoxic activity of the parasite is likely to be a major contributor to the extensive tissue damage (Ralston and Petri, 2011). *E. histolytica* is cytotoxic to a variety of cell types, including neutrophils, T lymphocytes, macrophages and a variety of tissue culture lines. The parasite is able to kill and ingest host cells in a contactdependent manner and within minutes (Petri *et al*., 2006). The pore- forming peptides such as amebapore have the capability to lyse the eukaryotic cell, by poking holes in the plasma membranes of the host leading to lysis and cell death (Leippe

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et al., 2005). The amebapores are located in the food vacuole where they function to kill and ingest bacteria.

2.6.4. Tissue destruction by *E.histolytica*

Tissue destruction in the pathogenesis of *E.histolytica* is mediated by proteases, the enzymes that function to degrade other proteins; they have been shown to disrupt the polymerization of MUC2. The parasite secretes proteases such as cysteine, which breakdown the mucous layer and subsequently tissue destruction following invasion of the epithelial layer. The mucus layer functions to prevent contact between trophozoites and epithelial cells (Huston, 2004). It has been shown that inhibitors of cysteine proteases can decrease liver abscess size in experimental models. However, little is known about the roles that proteases play in pathogenesis (Huston, 2004).

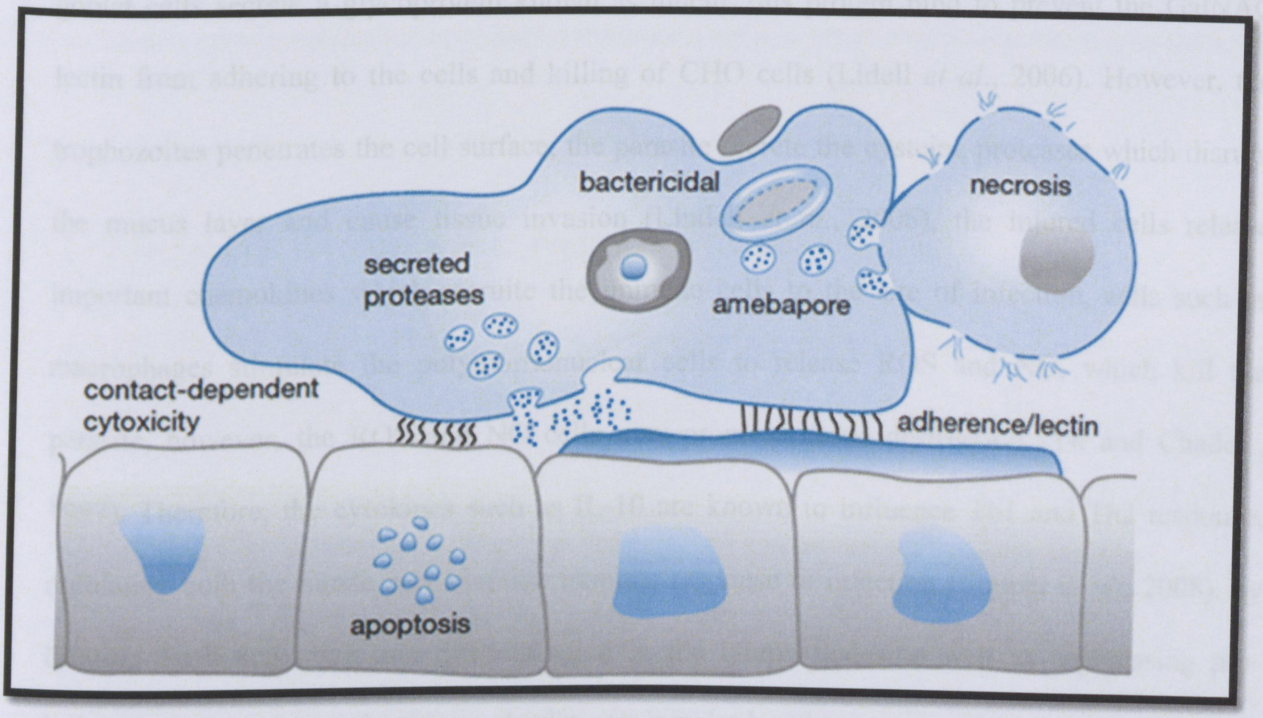

Figure 2.9: Schematic presentation of virulence factors and mechanism of pathogenesis of *E.histolytica.* (Huston, 2004)

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2.7. IMMUNE RESPONSE TO INTESTINAL AMOEBIASIS

An appropriate immune response clears pathogens without causing significant damage to the host tissue. It has been shown that tissue destruction in amoebic colitis arises from both *E. histolytica* cytolytic factors and the resultant gut inflammatory response (Shannon *et al.*, 2013). 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. Stomach acid function as the innate defense against infectious pathogens, the acid in the stomach kills the microorganisms (Shannon *et al.*, 2013). However, the cysts are particularly resistant to the acidic contents of the stomach, allowing it to attach to the epithelial cell (Lindell *et al.*, 2006). The mucus layer serves as the second line of defense against amoebiasis, it serves as a barrier that prevents the parasite from disrupting the epithelial cells (Moncada *et al.*, 2005). 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, the parasite secrete the cysteine proteases which disrupt the mucus layer and cause tissue invasion (Lindell *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, however, the ROS and NO cells may promote tissue destruction (Yu and Chadee., 1997). Therefore, the cytokines such as IL-10 are known to influence Th1 and Th2 response, regulating both the innate and adaptive immune response to infection (Couper *et al.*, 2008), by limiting Tcell activation and differentiation in the lymph nodes as well as suppressing pro-inflammatory responses in tissues, leading to impaired pathogen control and reduce immune-pathology (Couper *et al.*, 2008).

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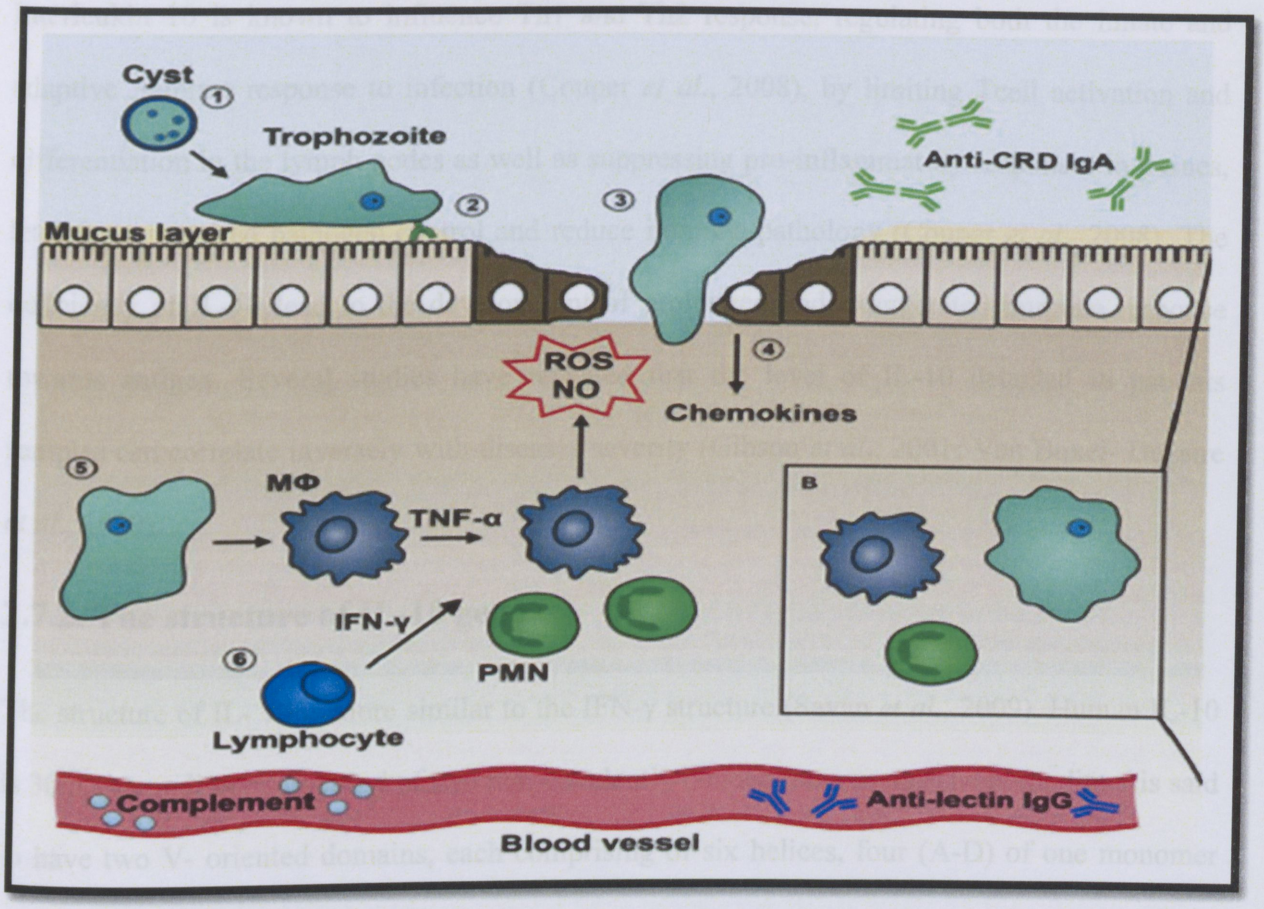

Figure 2.10: Iimmune response to intestinal amoebiasis (Shannon *et al.,* 2013).

2.7.1. Interleukin 10: The master regulator of immunity

Interleukin (IL)-10 is an important immune-regulatory cytokine produced by many cell populations (Trifunovic *et al*., 2015). Its main biological function seems to be the limitation and termination of inflammatory responses and the regulation of differentiation and proliferation of several immune cells such as T cells, B cells, natural killer cells, antigen-presenting cells, mast cells, and granulocytes (Couper *et al*., 2008)

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Interleukin 10 is known to influence Th1 and Th2 response, regulating both the innate and adaptive immune response to infection (Couper *et al*., 2008), by limiting Tcell activation and differentiation in the lymph nodes as well as suppressing pro-inflammatory responses in tissues, leading to impaired pathogen control and reduce immune-pathology (Couper *et al*., 2008). The deficiency of IL-10 lead to the development of prolonged and exaggerated immune response towards antigen. Several studies have revealed that the level of IL-10 detected in patients samples can correlate inversely with diseases severity (Gibson *et al*., 2001; Van Boxel- Dezaire *et al*., 1999).

2.7.2. The structure of IL-10 gene

The structure of IL- 10 is more similar to the IFN-γ structure (Savan *et al*., 2009). Human IL-10 is 30kD homodimer composed of two non-covalently bonded monomers. The homodimer is said to have two V- oriented domains, each comprising of six helices, four (A-D) of one monomer and two (E and F) of the other. The monomer contains two disulfide bridges that are essential for maintaining the structure and the activity of the cytokine (Windsor *et al*., 1993).

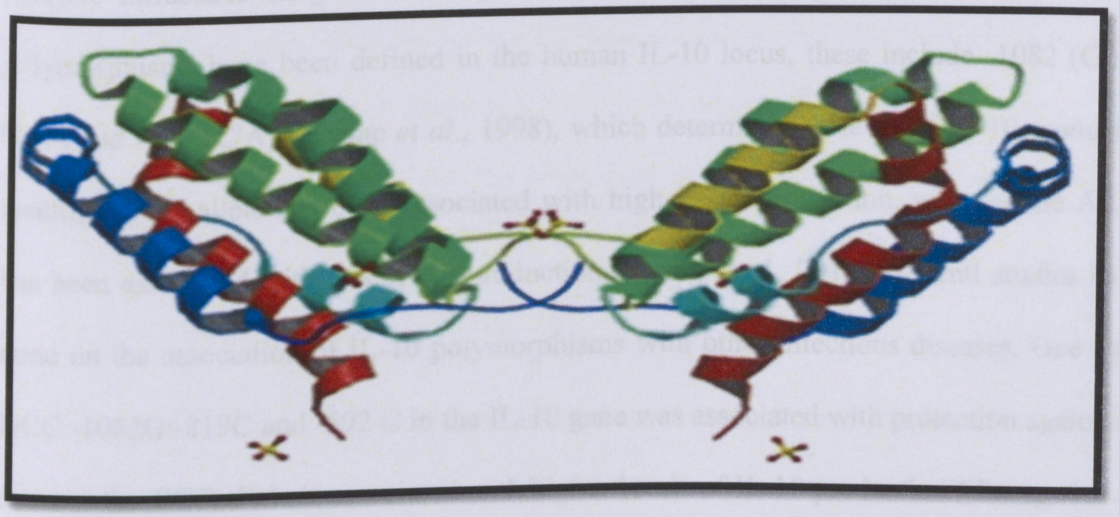

Figure 2.11: The structure of human IL-10 (Zdanav *et al*.,1996).

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2.7.3. Interleukin-10 gene

The gene encoding human IL-10 is located on chromosome1 at 1q31-1q32, on the minus strand, starting at 206,940,947 kilobases and ends at 206,945,839 kilobases. This gene is about 4.893 base pair in size. It covers a total of 5.1kb pairs and encodes for five exons (Kim *et al.*, 1992). The IL-10 gene encodes a 178 amino acid long protein which is secreted after the cleavage of 18 amino acid containing peptides.

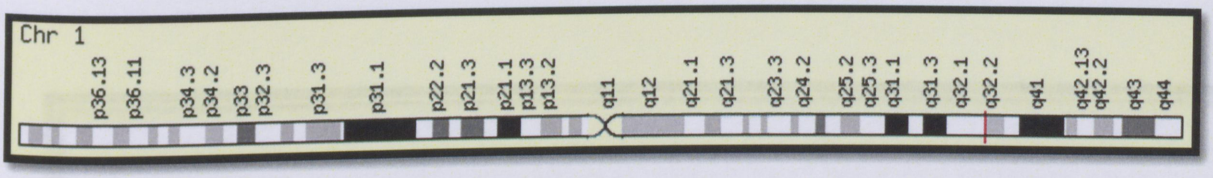

Figure 2.12: The human chromosome showing the location of IL-10 gene (taken from gene cards).

2.7.4. Interleukin 10 polymorphisms and its association with other infections

Promoter polymorphisms have been subject to the most scrutiny, particularly with regard to possible influences on gene transcription and protein production. Several single –nucleotide polymorphisms have been defined in the human IL-10 locus, these include -1082 (G/A), -819 (C/T) and -592 (C/A) (Hurme *et al.*, 1998), which determine the level of IL-10 production, the resulting GCC allele has been associated with high IL-10 production, whereas the ATA allele has been associated with low IL-10 production (Zhang *et al.*, 2012). Several studies have been done on the association of IL-10 polymorphisms with other infectious diseases. One haplotype GCC -1082G/-819C and -592 C in the IL-10 gene was associated with protection against malaria because the GCC allele is associated with higher levels of IL-10 production (Zhang *et al.*, 2012). In a study conducted by Cordeiro *et al* in (2008), it was found that the presence of 0f -1082A

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allele is associated with Toxoplasmic Retinochoroiditis. Studies have shown that there is an association between IL-10 gene polymorphism and severity of acquired pneumonia, defined as the major cause of morbidity and mortality worldwide (Gallagher *et al.*, 2003), Schaaf *et al.*, (2003) revealed that -1082G allele is associated with pneumococcal septic shock. The other associations of this gene polymorphisms were shown in breast cancer, it was shown that there is an association between the IL-10 AA and high risk of breast cancer, as this polymorphism is associated with lower production of the cytokine (Turner *et al.*, 1997).

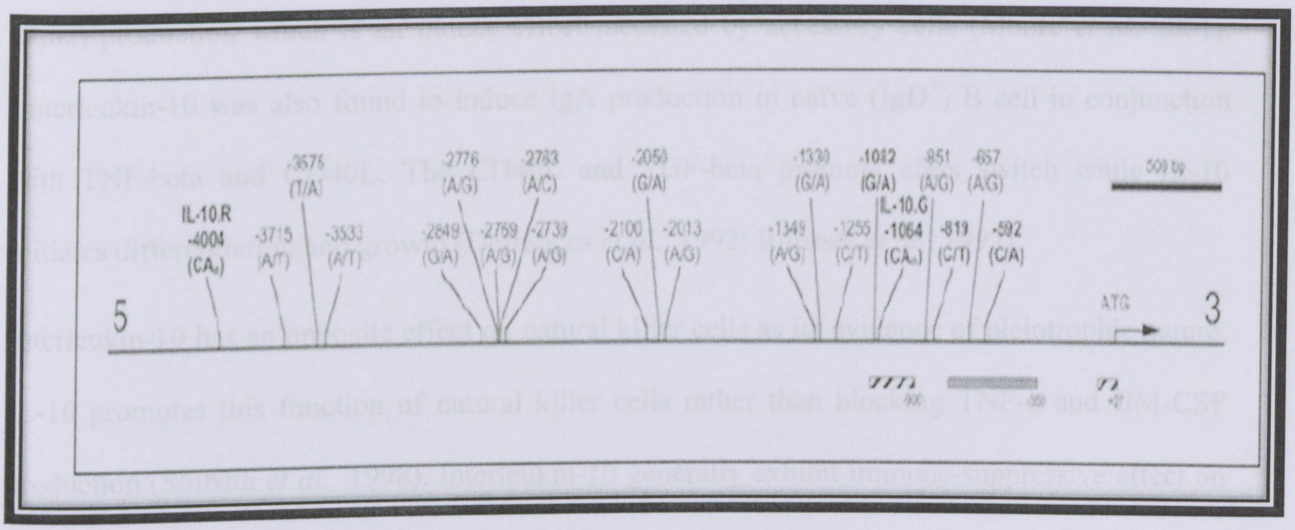

Figure 2.13: Schematic representation of IL-10 gene promoter polymorphisms. Bold letters indicates the polymorphisms used to investigate an association with disease susceptibility (Kube *et al.*, 1995)

2.7.5. Functions of interleukin-10

Interleukin 10 is a pleitropic cytokine with important immune-regulatory function whose function influence the activities many of the cell-types in the immune system and due to its multiple roles, proper function of IL-10 is required to maintain a healthy immune system and

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improper function of IL-10 can result in disease development. It was first recognized as a cytokine synthesis inhibitory factor, because of its capability to suppress macrophages, altering the production of a number of cytokines and the ability to serve as accessory cells for stimulation of T cells and Nk cells function (Moore *et al*., 2001). It can function as both immunosuppressive and immune-stimulatory cytokine in the immune system. It is also known to down-regulate the expression Th1 cytokine, MHC class 11 and co-stimulatory on macrophages. It also exhibits some stimulatory activity for proliferation and differentiation of B cells, mast cells and T cells (Ho and Moore, 1994). IL-10 was found to have an inhibition function of interferon-gama (IFN-gama) production which is an induce effect mediated by accessory cells (Moore *et al*. 2001). Iinterleukin-10 was also found to induce IgA production in naïve (IgD^+) B cell in conjunction with TNF-beta and CD40L. The CD40L and TGF-beta promote class switch while IL-10 initiates differentiation and growth (Defrances *et al*., 1992; Rousset *et al*., 1992).

Interleukin-10 has an opposite effect on natural killer cells as its evidence of pleiotrophic nature. IL-10 promotes this function of natural killer cells rather than blocking TNF-α and GM-CSF production (Shibata *et al.,* 1998). Interleukin-10 generally exhibit immune-suppressive effect on dendritic cells (DCs). Interleukin-10 blocks an inflammatory-induced DCs maturation step that normally involves CCR1, CCR2 and CCR5 down-regulation and CCR7 up-regulation. This blockage, with retention of CCR1, CCR2 and CCR5, results in a failure of DCs to migrate to regional nodes. The result is an immobile DC that will not stimulate T cells but will bind (and clear) pro-inflammatory chemokines without responding to them (D-Amico *et al.,* 2000).

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2.8. IDENTIFICATION METHODS FOR AMOEBIASIS

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

2.8.1. 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 (*Foetedar *et al*., 2007). The disadvantage of this method is that it is insensitive, nonspecific and less reliable in identifying *Entamoeba* species. Its sensitivity is poor (60%). The stool sample is stained with iodine or trichrome for determination of the cysts or trophozoites (Foetedar *et al*., 2007)

2.8. 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 (Foetedar *et al*., 2007), it is more reliable compared to microscopic examination. This culture include 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 37°c 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 are 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.

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Overgrowth of bacteria, fungi, or other protozoans during culture is the main problem encountered (Clark and Diamond, 2002)

2.8.3. 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 amoebiasis, such as amoebic liver abscess, where stool examination is often negative. This test has been shown to be 70% sensitive for the diagnosis of amoebic 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 amoebae 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 antiamoebic 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).

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2.8.4. 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 (foedetar *et al*., 2007). Many different ELISAs has 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* 11 stool antigen detection test has been showed 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 (Norhayati and Tengku., 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).

2.8.5. Isoenzyme electrophoresis

This diagnostic test is considered the gold standard for epidemiological studies, is used for differentiation between *E. histolytica* and *E. dispar* (Jackson, 2000). The enzymes that are employed for this diagnostic tool includes, 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 (Jackson, 2000). The binding pattern of PGM and GPI, more than twenty zymodemes can be recognized, of which nine belong to *E.*

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histolytica and the rest to *E. dispar* (Clark *et al.*, 1992). A zymodeme is defined as a group of ameba strains that share the same electrophoretic pattern and mobilities for several enzymes. The disadvantages of this method is that it is difficult to perform this test, it is time consuming and relies on establishing the amebae in culture, with a large number of cells needed for the enzyme analysis

2.8.6. Molecular based Methods

Polymerase chain reaction (PCR) techniques are also considered to be adequate diagnostic tools used to detect *E.histolytica*. The PCR methods are highly specific and sensitive (Mirelman *et al.*, 1997), because there is no proof of DNA from other species cross-hybridizing with primers that are designed specifically for *E. histolytica* (Evangelopoulos *et al.*, 2000). The limitations of using PCR focuses on fecal contamination and inhibition of amplification of amoebic DNA (Evangelopoulos *et al.*, 2001). Polymerase chain reaction assays are based on priming sequences from the small subunit ribosomal RNA gene of both species of this complex, followed by restriction digest analysis Usually, the PCR technique used is based on a nested PCR assay in which the first set of primers is specific for both species and the following set of primers is specific for either organism (Evangelopoulos *et al.*, 2001).

2.9. TREATMENT OF *E. HISTOLYTICA* INFECTION

The treatment of amoebic 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 amoebiasis require prompt treatment. It is recommended that individuals with no symptoms should be treated inorder to control the transmission and spread of other

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pathogenic parasites (Heymann, 2008). Among the many available antiparasitic drugs, metronidazole is rated as the most effective treatment of amoebiasis (Leitsch *et al*., 2007).

Asymptomatic carriers should be treated with luminal amebicide, for example, iodoquinol, paromomycin, diloxonide (American academy of pediatrics, 2012). This drugs are aid to protect the patients from development of invasive diseases and to reduce the risk of transmission to others (Heymann, 2008). It has been shown that metronidazole is ineffective against the cysts.

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) (American academy of pediatrics, 2012). It is suggested that metronidazole and paromomycin should not be given at the same time, because diarrhea is known as the major side effect of paromomycin (Petri and Haque, 2010).

2.10. CONTROL AND PREVENTION

Prevention and control of *E.histolytica* infection depends on interruption of fecal- oral transmission. The amoebic 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 allow 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

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CHAPTER THREE
METHODOLOGY

3.1. ETHICAL CONSIDERATIONS

This study was approved by the research and ethics committee of the University of Venda (project number: SMNS/10/MICB/005) and the Department of Health and Welfare in Polokwane, Limpopo province, South Africa. This study was also approved by the ethical committee of the hospital where the blood samples were collected. The objectives and concepts of the study were clearly explained to the potential participants and signed consent forms were obtained before a participant was enrolled in the study. Confidentiality of the participants was kept by giving each sample a code and their information was kept confidential.

3.2. STUDY SITES

The study was conducted in Nkomo (Giyani), Polokwane in Limpopo province and Pretoria (Gauteng province), South Africa. Giyani is located at the Northern part of Limpopo (Figure 13). This area is rural with people of different religious, educational and socio-economic backgrounds, living in neighborhoods with distinctly different level of sanitation. Giyani is a place that is highly dominated by black Africans (93.93%)

Polokwane is the capital city of the Limpopo province, it is located at the Far north of Limpopo. The District Municipality covers an area of 21,706.96km^2, which constitutes 12% of the total surface area of the Limpopo Province. Most of the patients were from neighboring districts of Polokwane, namely; Capricorn, Sekhukhune and Waterberg (Figure 3.1). The place is more dominated by black Africans (74.38%) compared to the other races.

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Pretoria is a city in the northern part of Gauteng Province, South Africa. It covers an area of 1,644 km² of the total surface area of Gauteng province. The samples were collected from patients attending different private clinics in Pretoria. The place has about 41.95% of black Africans, 2,50% of coloured, 52. 45% of white and 1.93% Indians.

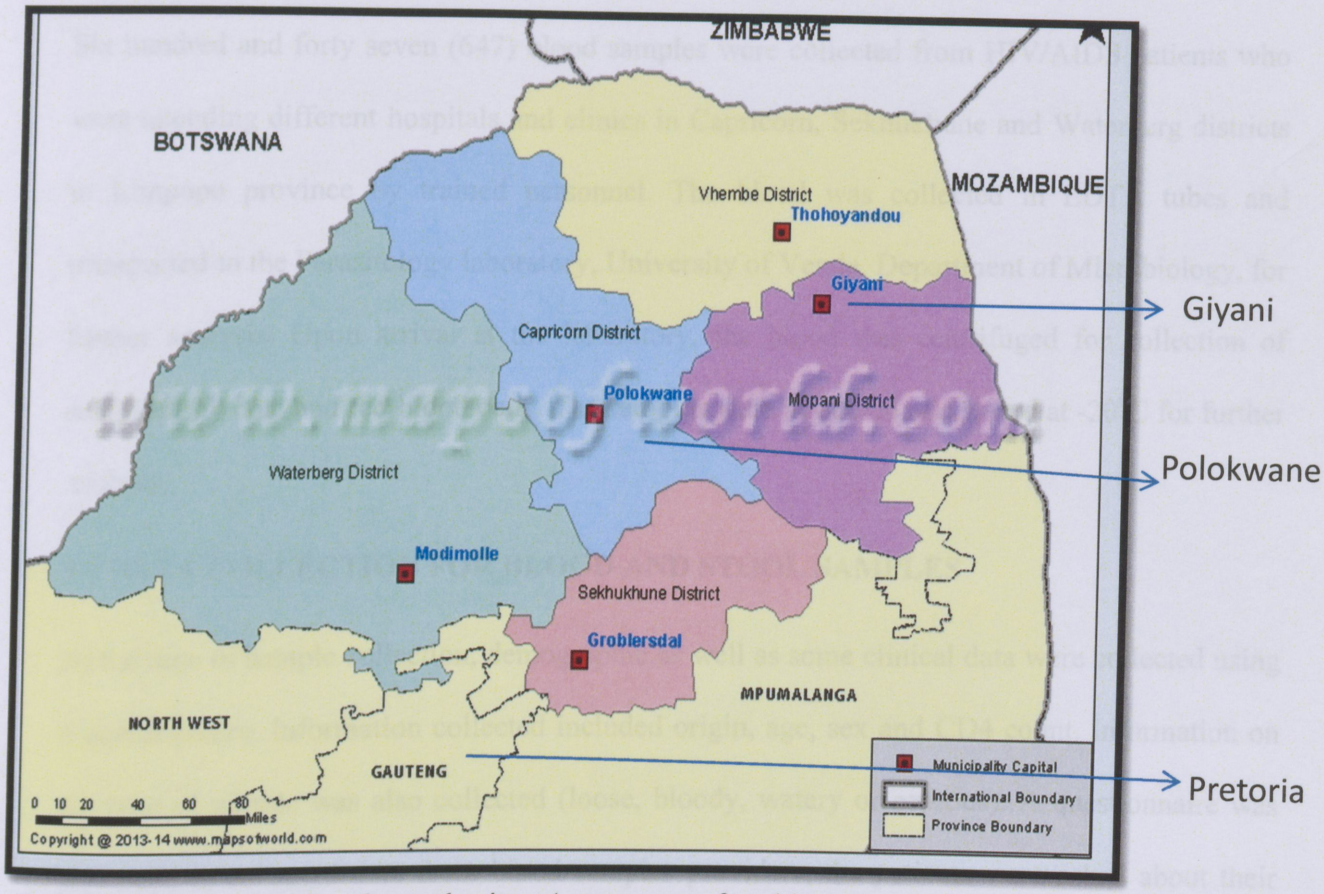

Figure 3.1: Map showing the study sites. (www.mapsofworld.com)

3.3. SAMPLE COLLECTION

A total of one hundred and eleven (111) stool samples (Both diarrheal and non-diarrheal) were collected from Giyani and Pretoria. Eighty four (84) stool samples were from Giyani and twenty seven (27) samples were from Pretoria. The samples were put in a sterile wide screw cap container and kept in cooler box with ice packs (4°C) and transported to the Parasitology

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laboratory, University of Venda Laboratory, Department of Microbiology, for further analysis. Upon arrival at the laboratory, all the samples were observed under a light microscope for the presence of *E. histolytica* cysts or trophozoites, the samples were then aliquoted and stored at -70^{0}C for further analysis.

Six hundred and forty seven (647) blood samples were collected from HIV/AIDS patients who were attending different hospitals and clinics in Capricorn, Sekhukhune and Waterberg districts in Limpopo province by trained personnel. The blood was collected in EDTA tubes and transported to the Parasitology laboratory, University of Venda, Department of Microbiology, for further analysis. Upon arrival at the laboratory, the blood was centrifuged for collection of serum, buffy coat and red blood cells. The samples were aliquoted and stored at -20^{0}C for further analysis.

3.4 DATA COLLECTION FOR BLOOD AND STOOL SAMPLES

At the time of sample collection, demographic as well as some clinical data were collected using a questionnaire. Information collected included origin, age, sex and CD4 count. Information on the type of sample was also collected (loose, bloody, watery or mucous). A questionnaire was also used to collected data from blood samples providers, the patients were asked about their origin, gender, age, CD4 counts and other medical conditions such as liver failure, kidney failure and STDs.

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3.5. SAMPLE ANALYSIS

3.5.1 PART 1: Molecular characterization of *E. histolytica* strains

3.5.1.1 Detection of *E. histolytica* cysts and trophozoites in fecal specimens by microscopy

All fecal samples were screened for *Entamoeba cysts* using microscopic examination. A portion of each unpreserved stool specimen was placed on a glass slide and iodine was added to the stool smears and covered with a coverslip and examined by a light microscopy at 40x magnification. The identification of *E.histolytica* cyst was based on morphologic characterization of the cysts and trophozoites. Fecal samples containing amoebic cells were stored at -20°C until DNA extraction was performed.

3.5.1.2. Extraction of total genomic DNA from stool samples

The genomic DNA of *E. histolytica* was purified from the stool specimens. The fecal samples were subjected to QIAamp DNA stool mini kit (QIAGEN, Hilden, Germany). The extraction procedure was performed following the manufacturer's instructions. The DNA was kept frozen at -20°c until needed for amplification analysis (Intarapuk *et al*., 2009).

3.5.1.3. Identification of *Entamoeba* species by PCR

The detection of *E. histolytica* was performed using genus- specific primers (E1 and E2) and *E. histolytica* (EH1 and EH2) primers published by Khairnar and Parija, (2007) (Table 1). In the initial PCR, the amplification was carried out in a total of 25µl reaction volume containing 12.5 µl of dreamtaq, 0.25µl of BSA, 0.6µl of each primer and 6.05µl deionised water. An amount of 5µl stool DNA was mixed with 20µl of master mix to a final volume of 25 µl. the cycling

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conditions were as follows: initial denaturation at 96°c for 2 minutes, then 35 cycles of PCR was performed with denaturation at 92°c for 60 seconds, annealing at 56°c for 60 seconds and extension at 72°c for 90 minutes and 72°c for 7 minutes. In the second round, the PCR product was used as a template with the same cycling conditions, except the annealing temperature which was changed to 60°C (Khairnar and Parija, 2007). The PCR products were separated by electrophoresis in 1.5% agarose gel at 100 V for 45 min in Tris-acetate buffer and visualized by UV-transilluminator. The EH1 and EH2 primers amplify 450bp fragment of the *E. histolytica* gene

Table 3.1: PCR primers and amplification conditions that were used in this study to detect *E. histolytica* in stool samples.

GENE	PRIMER NAME	PRIMER SEQUENCE(5'-3')	REFERENCE
Entamoeba genus	E1 E2	TAAGATGCACGAGAGAGCGAAA GTACAAAGGGCAGGGACGTA-	Khairnar and Parija, 2007.
Entamoeba histolytica	EH1 EH2	AAGCATTGTTTCTAGATCTGAG AAGAGGTCTAACCGAAATTAG	Khairnar and Parija, 2007

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3.5.1.4. Genotyping of the Serine rich *E. histolytica* protein gene by PCR

The samples that were positive by both microscopy and PCR were employed in this study. The amplification was performed in 25 µl reaction mixture containing 12.5µl of dreamtaq, 0.6µl of each primer (SREHP1 and SREHP2), 0.25µl of BSA, 6.05 µl of nuclease free water and 5µl of genomic DNA. The cycling conditions were as follows: initial denaturation at 94^0C for 5 minutes followed by 35 thermal cycles of 94^0C for 1 minute, 48^0C for 1 minute, and 72^0C for 1 minute, followed by a final extension at 72^0C for 5 minutes. The SREHP primers amplify a 550 and 700-bp fragment of SREHP gene. The primers that were be used are listed in table

Table 3.2: The target genes and the primer sequences used in PCR amplification.

GENE	PRIMER NAME	PRIMER SEQUENCE(5'-3')	PRODUCT SIZE(bp)
Serine-rich *E.histolytica* protein	SREHP1	5′-GCTAGTCCTGAAAAGCTTGAAGAAGCTG-3 (Ramos et al., 2005)	550 and 700
	SREHP2	5′-GGACTTGATGCAGCATCAAG GT-3′ (Ramos et al., 2005)	

3.5.1.5. Genotyping of *E. histolytica* based on the chitinase gene

The samples that were positive by both microscopy and PCR were employed in this study, were selected for the amplification of chitinase gene. PCR was performed in 25 µl reaction mixture containing 12.5µl of dreamtaq, 0.6µl of each primer (CEH1 and CEH2), 0.25µl of BSA, 6.05 µl

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of nuclease free water and 5µl of genomic DNA. The cycling conditions were as follows: initial denaturation at 94°C for 5 minutes followed by 35 thermal cycles of 94°C for 1 minute, 48°C for 1 minute, and 72°C for 1 minute, followed by a final extension at 72°C for 5 minutes. The primers that were used are listed in Table 3.3.

Table 3.3: The target gene and the primer sequences to be used in PCR amplification

GENE	PRIMER NAME	PRIMER SEQUENCE(5'-3')	PRODUCT SIZE(bp)
Chitinase	CEH1	-GGAACACCAGGTAAATGTATA- (Haghighi et al, 2002)	500 and 1,260bp
	CEH2	GGTATCATTTGGTCATCATTCC- (Haghighi et al, 2002)	

3.5.2. PART 2: The prevalence of *E. Histolytica* antibody and antigen in the study population

3.5.2.1. Detection of antibodies to *E. histolytica* in serum specimens by ELISA

The Techlab *E. histolytica* serology ELISA was used to detect the antibodies against intestinal amoebiasis. The test was performed according to manufacturer's instructions (Techlab Inc, Blacksburg, VA). Each patient's serum specimen was diluted at 1:50, a positive and negative control was prepared each time the test was performed. An amount of 100µl of diluted serum was transferred to each sample well, a drop (50µl)of the positive control well was added and for

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was transferred to each sample well, a drop (50µl)of the positive control well was added and for the negative control, 100µl of the diluent was used, the plate was then covered with an adhesive sheet and incubated for an hour at room temperature. After incubation the plate was washed four times with 1x wash solution and the residual liquid was removed by blotting the plate onto a dry paper towel. One drop of the conjugate was added into each well, sealed with adhesive sheet and incubated for 30 minutes at room temperature, after incubation the plate was washed as described before. Two drops of the substrate was added to each well and incubated at room temperature for ten minutes. After the incubation step, one drop of stop solution was added to each well, the stop solution converts the blue color to yellow. The absorbance was measured at 450nm on microplate ELISA reader (Versa max, microplate reader, designed in California, USA). Samples with an OD greater than 0.150 were considered positive and those that are less than 0.150 were considered negative.

3.5.2.2. Detection of *E. histolytica* antigens in serum specimens by ELISA

The Techlab *E. histolytica* test II test (Techlab, Inc, Blacksburg, VA) was employed for the antigen detection assay. According to the manufacturer's instructions, the test is intended for use on fecal specimens. However, in the present study the kit was used for detection of adhesion in serum samples, therefore some of the steps were optimized. Hundred microliters of the serum specimen was diluted with 100µl of the sample diluent. One drop of conjugate was added to each well, samples and controls were then added to wells containing conjugate, the plate was covered with adhesive sheet and incubated for 2 hours at room temperature. After the washing step, two drops of substrate reagent was added to all the test wells and incubated at room temperature for 10 minutes. After the incubation period, one drop of the stop solution was added, the stop solution changes the blue color to yellow, after two minutes, the optical density was read at

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450nm microplate ELISA reader (Versa max, microplate reader, designed in California, USA). The specimen was considered positive if the reading is greater than 0.050 and negative if it is less than 0.050. A positive test result indicates that *E.histolytica* adhesin is present in the serum sample which represent amoebic liver abscess. A negative result indicates that *E.histolytica* antigen is not present in the serum sample

3.5.3. PART 3: Detection of Il-10 concentration in serum specimens by ELISA

The concentration of IL-10 was measured in the serum samples using enzyme-linked immuno-sorbent assay (ELISA) method (MABTECH). At day 1, the plate was coated by adding 100µl of the capture antibody to each well (monoclonal antibody 9D diluted to 2µg/ml in PBS buffer) and incubated at 4-8ºC overnight. At day 2, the plate was washed two times with PBS. After the wash step, the plate was blocked with blocking buffer containing 0.05% of Tween 20 and 0.1% BSA, the plate was incubated for an hour at room temperature. After the incubation period, the plate was washed five times with buffer containing 0.05% Tween. The samples and standards were diluted in incubation buffer following manufacturer's instructions, 100µl of the samples and the standards were added in each well and incubated for 2 hours at room temperature. The plate was washed with buffer containing 0.05% Tween. Hundred microliters of the detection antibody (biotinylated monoclonal antibody 12G8) was added into each well and incubated for 1 hour at room temperature. Following the wash step, 100µl of the streptavidin- HRP was added into each well and incubate for an hour at room temperature. After washing the plate, two drops of the substrate was added into each plate well, the plate was incubated for 30 minutes at RT. One drop of stop solution was added into each well and read within 10 minutes at 450nm on microplate ELISA reader (Versa max, microplate reader, designed in California, USA).

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3.5.4. PART 4: Genotyping of the IL-10 gene promoter polymorphisms at position -592 A/C and -1082 A/G

3.5.4.1. Extraction of genomic DNA from buffy coat

Genomic DNA was extracted from 647 buffy coat specimens using Gen ELUTE Blood Genomic DNA kit (SIGMA-ALDRICH) with some slight modification. Up to 200µl of the buffy coat was added into a 2 ml tube containing 20 µl of the proteinase K solution and vortexed for 15 seconds to ensure through mixing of the enzyme. After vortexing, 200 µl of the lysis solution was added to the samples and vortexed for 15 seconds, the samples were incubated at 55ºC for 10 minutes; 200 µl of ethanol (95-100%) was added to the lysate and vortexed thoroughly. The entire contents of each tube were transferred into treated columns and centrifuged at 6500rpm for a minute. The flow-through was discarded and the column was placed into a new 2ml collection tube. An amount of 500 µl of prewash was added to the column and centrifuged for a minute. The flow-through was discarded and the column was placed in a new 2ml collection tube, 500µl of the second wash solution was added to the column and centrifuged for 3 minutes at maximum speed (12,000- 16,000rpm) to dry the column. The DNA was eluted by adding 200 µl of the elution solution to each tube. The tube was centrifuged for a minute at 6500rpm to elute the DNA. After elution the DNA was stored at 20°C for further analysis.

3. 5.4.2. Molecular analysis of Il-10 gene promoter at -592A/C and -1082A/G loci

The IL-10 -592 A/C and -1082 A/G polymorphisms in IL-10 gene were determined by polymerase chain reaction (PCR) method as described by Kong *et al* (2010). The PCR was performed with 25µl reaction mixture containing 20µl of the Dream Taq Green master PCR mix (Inqaba biotech, SA), 5µl extracted and purified template, 0.6µl of each primer (Table 3.4),

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0.25µl BSA and 6.05µl of Nuclease free water was added to make the final volume to 25µl and subjected to 35 cycles for amplification in a thermo cycler. The cycle conditions were as follows: heat denaturation 94°C for 5 minutes followed by 35 cycles at 94°C for 30 seconds, annealing at a temperature at 62°c for -592 A/C, 59°C for 819 T/C and 58°c for -1082 A/G for 45 seconds, extension at 72°C for 55 seconds and final extension at 72°C for 8 minutes. The amplified products were visualized by U.V transilluminator (Biorad) after separating them by electrophoresis on 2% agarose gel stained with ethidium bromide.

Table 3.4: Primer sequences used for IL-10 genotyping.

GENE	POLYMORPHISMS	PRIMER SEQUENCE(5'-3')	PRODUCT SIZE (bp)
IL-10 gene	-1082 A/G	F: 5'-CTCGCTGCAACCCAACTGGC-3' R: 5'-TCTTACCTATCCCTACTTCC-3' (Kong et al.,2010)	G: 106+33 A: 139
	-592 A/C	F: 5-GGTGAGCACTACCTGACTAGC-3' R: 5'-CCTAGGTCACAGTGACGTGG-3' (Kong et al.,2010)	A:236+176 C: 412

Polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) method was employed for IL-10 (-592 A/C and -1082 A/G) genotyping. The endonucleases RsaI and MnII

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were used to distinguish the IL-10 gene polymorphisms respectively. The amplified products were run on 2% ethidium bromide stained agarose gel and visualized under UV light. To confirm the genotyping results, PCR-amplified DNA was sent to Inqaba for sequencing.

3.5.4.3 Sequencing and sequence analysis

The PCR products of some amplicons were sequenced at Inqaba Technologies (Inqaba, Pretoria, South Africa). Sequences were assembled using Stande package (Staden *et al.,* 1999). This package allows room for addition and removal of amino acids from the sequence during editing. During the editing process, foreign amino acids present in the sequence were removed and replaced by appropriate amino acid in line with the reference sequence that came with the package. Thereafter, bioedit package (Hall, 1999) was used to edit the amino acid sequences which were then aligned using the Clustal W multiple aligner. The similarities and variations between various sequences were determined.After this, a similarity plot was done to determine clearly where the mutation occurred, in order to clearly determine the genotypes in the sequence.

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3. 6. STATISCAL ANALYSIS

The results were entered into an excel spread sheet and edited appropriately (Microsoft office package) and analyzed using Statistical Package for the Social Sciences (SPSS for WINDOWS version 18.0). Assuming that, the data followed a normal distribution, comparison of proportions and statistical significance was tested using the Chi-square test.

The genotype associations between the positive and negative groups were analyzed by using chi-square ($\chi 2$) analysis. A p-value of <0.05 was considered statistically significant.

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CHAPTER FOUR
RESULTS

4.1. PART 1: MOLECULAR CHARACTERIZATION OF *ENTAMOEBA HISTOLYTICA*

4.1.1. Demographic information of the study population

A total of one hundred and eleven (111) stool samples were employed in this study, of which 84 (75.7%) were from Giyani and 27 (24.3%) were from Pretoria. Of all the samples collected 50 (45.0%) were males and 61 (55.0%) were females. Most of the patients were aged between 0 to 25 (19.8%), followed by 6(5.4%) patients aged 26 to 45, only 2 patients were aged 46 to 65 (1.8%) and the age of about 81 (73.0%) participants was unknown in this study. Non –diarrheal and diarrheal stool were collected in the study, 28(25.2%) were formed, 69(62.2%) loose and 14 (12.6%) were watery stools.

Table 4.1: Demographic information of the study population.

Characteristics		Frequency	Percent (%)
Origin	Giyani	84	75.7
	Pretoria	27	24.3
Gender	Male	50	45.0
	Female	61	55.0
Age group	0 to 25 years	22	19.8
	26 to 45 years	6	5.4
	46 to 65 years	2	1.8
	Missing system	81	73.0
Stool type	Formed	28	25.2
	Loose	69	62.2
	Watery	14	12.6
Microscopy	Positive	52	46.8
	Negative	59	53.1
Total		**111**	**100.0**

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The SREHP PCR and chitinase genes were used to investigate the genetic variations among the isolates of *E. histolytica* collected from symptomatic and asymptomatic individuals Pretoria and Giyani. (Table 4.1)

4.1. 2. Detection of *Entamoeba histolytica* by microscopy and PCR

Out of 111 stool samples employed in this study, 46 (41.4%) samples were negative by both microscopy and PCR, 51(45.9%) positive by either method (microscopy or PCR) and 14 (12.6%) samples were positive by both methods (microscopy and PCR).

Table 4.2: Microscopy and PCR results.

Methods	Frequency	Percent (%)
Negative by both methods	46	41.4
Positive by either of the two methods	51	45.9
Positive by both methods	14	12.6
Total	**111**	**100.0**

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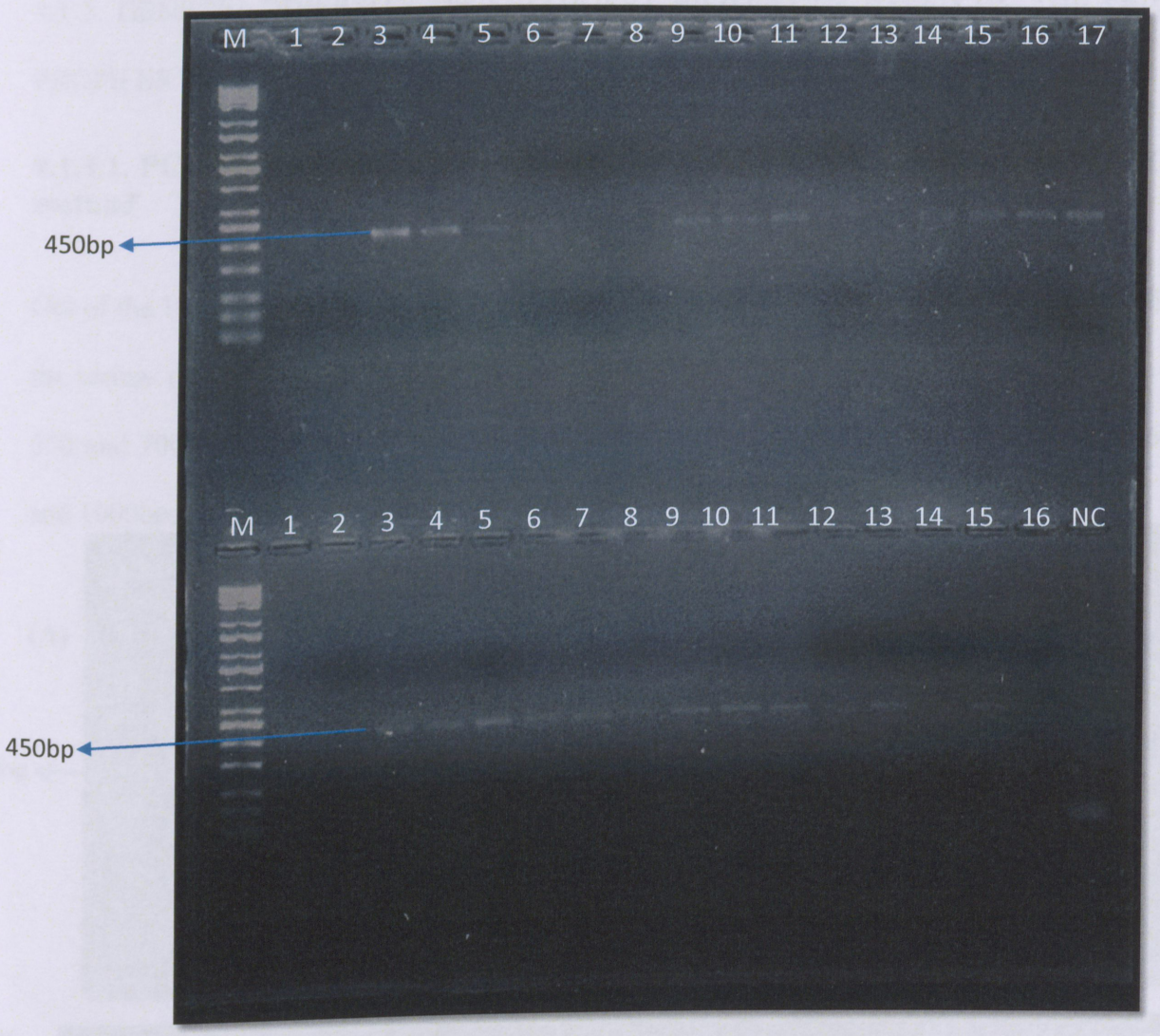

Figure 4.1: Agarose gel analysis of PCR products amplified by EH1 and EH2. M- Molecular marker (KAPA Universal Ladder), lane 1-17 DNA sample extracted from stools, NC- negative control

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4.1.3. GENETIC DIVERSITY OF *ENTAMOEBA HISTOLYTICA* BASED ON THE SREHP PROFILES

4.1.3.1. PCR amplification of the serine-rich *E. histolytica* protein (SREHP) by PCR method

Out of the 111 stool samples collected, the SREHP1 and SREHP2 primers specifically amplified the serine- rich *E.histolytica* protein gene in 19 of 111 stool DNA samples. The product size of 550 and 700bp were obtained with other additional amplicons of 150, 200, 250,300, 400, 600 and 1000bp.

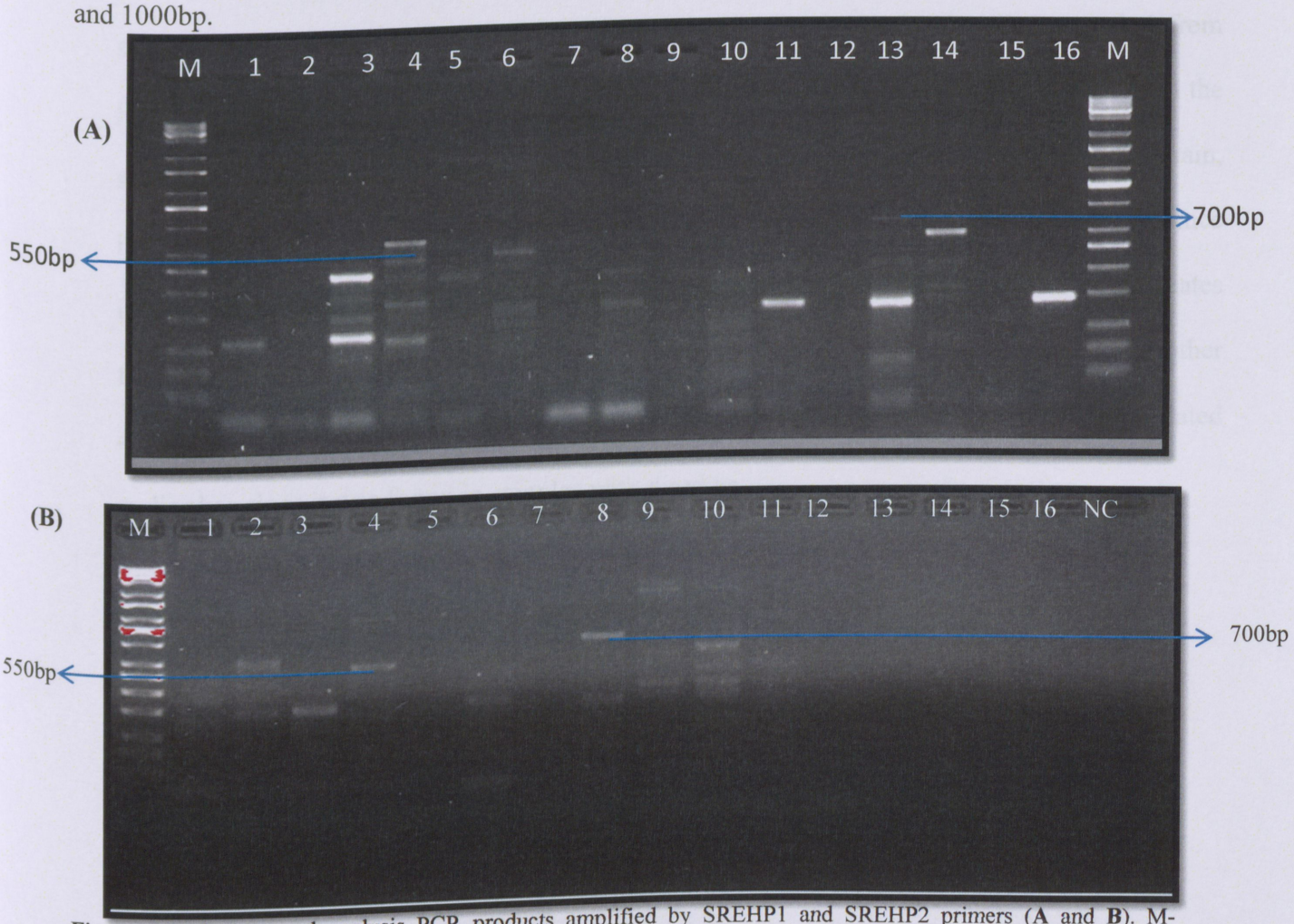

Figure 4.2: Agarose gel analysis PCR products amplified by SREHP1 and SREHP2 primers (**A** and **B**). M- Molecular marker (KAPA Universal Ladder), lane 1-16 DNA sample extracted from stools, NC- negative control. The expected band sizes were 550 and 700bp with some addition amplicons.

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4.1.3.2. Genetic diversity of *E. histolytica* in stool samples from Giyani and Pretoria.

Out of the 65 samples positive for *E. histolytica* by either PCR or microscopy, the SREHP successfully amplified from 26 samples out of the 65 samples employed in this study. The SREHP PCR reaction gave different banding patterns as represented in in table 4.3 and 4.4 below. Table 4.4 shows clearly the distribution of the SREHP genetic profiles in the study population. The band sizes varied between 210bp and 1600bp, the bands-300, 500, 400 and 600 were seen in most of the samples. This amplification revealed 19 SREHP profiles out of the 26 samples positive for the SREHP. Twenty two (22) isolates were from Giyani and 4 from Pretoria. Profile #2 was observed in 3 different isolates, interestingly, this isolates were from the same area (Giyani), these patients may possibly have been infected with the same strain, however, according to the observations of this study, this profile is not related to diarrhea, since it was found in patients with formed stools. Another profile (#18) was found mostly in isolates from Pretoria and in one isolate from Giyani, two of the isolates were symptomatic and the other 4 were asymptomatic, this profile, is more distributed in Pretoria than Giyani and it was related to diarrhea since it was found in samples with diarrhea.

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Table 4.3.: Samples that generated the Serine Rich *E. histolytica* genetic profiles

sample code	origin	gender	stool type	SREHP	SREHP profiles1
MS40	Giyani	Male	formed	210	1
MS 29	Giyani	female	formed	300	2
MS 32	Giyani	Male	Loose	300	2
MS1	Giyani	female	formed	300	2
MS44	Giyani	female	Loose	500	14
MS2	Giyani	female	Loose	600	3
MS33	Giyani	female	Loose	700	4
NS 07251	Pretoria	Male	watery	400 ^{f}	5
MS34	Giyani	female	Soft	210^{f}, 300^{f}, 400, 500	6
MS43	Giyani	Male	formed	210, 350, 500^{f} 600 ^{f} 700	7
MS42	Giyani	female	formed	300 ^{f} 500 ^{f} 800	8
MS3	Giyani	female	Loose	300^{f}, 1200	9
MS 21	Giyani	female	Loose	300, 700 ^{f}	10
MS8	Giyani	female	formed	350 ^{f} 500 ^{f}	11
MS38	Giyani	female	Loose	350 ^{f} 600	12
MS45	Giyani	Male	formed	400 ^{f}	5
NS 07994	Pretoria	Male	watery	400 ^{f}	5
NS08850	Pretoria	Male	Loose	400 ^{f}	5
NS08605	Pretoria	female	Loose	400 ^{f}	5
MS4	Giyani	Male	Loose	400, 600 ^{f} 1000	13
MS41	Giyani	female	Loose	500 ^{f} 1600 ^{f}	14
MS23	Giyani	Male	Loose	500^{f} 800	15
MS7	Giyani	Male	Loose	600 ^{f} 1200	16
MS 46	Giyani	Male	Loose	600 ^{f} 500	17
MS36	Giyani	female	Loose	600, 1600	18
MS28	Giyani	female	formed	700 ^{f}	19

Note: Major bands obtained after the amplification the SREHP gene. ^{f}Faint

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Table 4.4: Distribution of the SREHP genetic profiles in the study population

	Sample origin		Total			Sex groups			stool group	
SREHP profiles1	Giyani	Pretoria			Male	Female		Formed	Loose	Watery
1.0	1	0	1		1	0		1	0	0
2.0	4	0	4		1	3		2	2	0
3.0	1	0	1		0	1		0	1	0
4.0	1	0	1		0	1		0	1	0
5.0	1	4	5		4	1		1	2	2
6.0	1	0	1		0	1		0	1	0
7.0	1	0	1		1	0		1	0	0
8.0	1	0	1		0	1		1	0	0
9.0	1	0	1		0	1		0	1	0
10.0	1	0	1		0	1		0	1	0
11.0	1	0	1		0	1		1	0	0
12.0	1	0	1		0	1		0	1	0
13.0	1	0	1		1	0		0	1	0
14.0	1	0	1		0	1		0	1	0
15.0	1	0	1		1	0		0	1	0
16.0	1	0	1		1	0		0	1	0
17.0	1	0	1		1	0		0	1	0
18.0	1	0	1		0	1		0	1	0
19.0	1	0	1		0	1		1	0	0
Total	22	4	26		11	15		8	16	2
Total	84.6%	15.4%	100.0%		42.3%	57.7%		30.8%	61.5%	7.7%

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4.1.3.3. Molecular characterization of *E. histolytica* based on the chitinase gene

Out of the 111 stool samples collected, the CEH1 and CEH2 (Chitinase) primers specifically amplified the chitinase gene of *E, histolytica* in only 22 samples, the product size of 500 and 1,200bp were obtained with other additional amplicons of 200, 300, 400 , 600 and 1000bp.

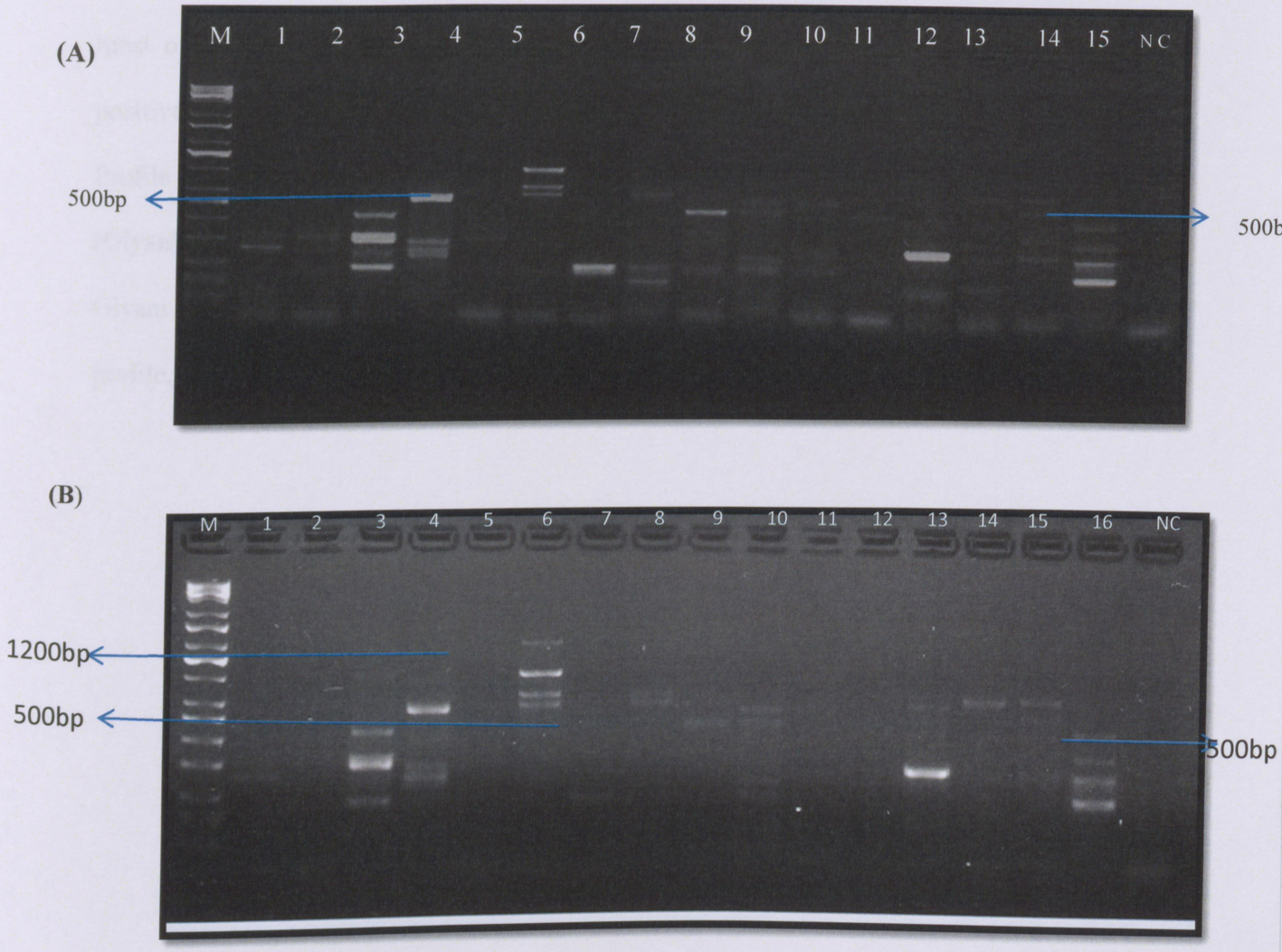

Figure 4.3: Agarose gel picture showing the PCR results of *E. histolytica* chitinase gene amplified by CEH1 and CEH2 primers (A and B). M- Molecular marker (1kp), Lane 1-16 DNA samples extracted from stools, NC- negative control

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Out of the 65 samples positive for *E. histolytica* by both PCR and microscopy, the chitinase successfully amplified 59 samples out of the 65 samples employed in this study. The chitinase PCR reaction gave different banding patterns as represented in in table 4.5 and 4.6 below. Table 4.7 shows clearly the distribution of the chitinase genetic profiles in the study population. The band sizes varied between 100bp and 1200bp, the bands-200, 250, 300 and 500, were seen in most of the samples. This amplification revealed 22 SREHP profiles out of the 59 samples positive for the chitinase. Forty seven (47) isolates were from Giyani and 12 from Pretoria. Profile #4 was observed in 6 different isolates, 5 of this isolates were from the same area (Giyani), and 1 from Pretoria. The profile is distributed in both the areas, but more distributed in Giyani. According to the observations of this study, this profile is related with diarrhea. Another profile, #18 was obtained in this study.

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Table 4.5: Summary of the samples that produced the chitinase gene profiles

Sample code	Origin	gender	stool type	chitinase	chitinase profileNo
GY 48	Giyani	female	Loose	200	1
NS 1080	Pretoria	female	Formed	300	2
MS40	Giyani	Male	Formed	350	3
HS 123	Giyani	Male	Loose	500	4
HS 121	Giyani	female	Watery	500	4
NS 09598	Pretoria	Female	Loose	500	4
GY 193	Giyani	female	Watery	500	4
GY 41	Giyani	Male	Loose	500	4
HS 48	Giyani	Male	Loose	500	4
GY 04	Giyani	female	Loose	800	5
NS08033	Pretoria	Female	Loose	100[f], 300, 400	6
NS 05219	Pretoria	Male	Loose	150[f], 500	4
MS28	Giyani	female	Formed	150, 200 [f], 600	7
NS 1039	Pretoria	female	Loose	200 [f]	1
HS 39	Giyani	female	Formed	200 [f], 250 [f]	1
GY 45	Giyani	Male	Loose	200 [f], 250 [f]	1
HS 29	Giyani	female	Loose	200 f 250 [f]	1
HS 21	Giyani	Male	Formed	200 [f],250 [f], 900 [f]	1
NS 03183	Pretoria	female	Loose	200 f 400 [f]	1
MS30	Giyani	female	Loose	200, 250 [f], 500, 600	8
NS 0170	Pretoria	Male	Loose	200, 300	9
MS 29	Giyani	female	Formed	200, 300[f], 500, 600	8
MS7	Giyani	Male	Loose	200, 300 [f], 400	10
MS34	Giyani	female	Soft	200, 300, 400	10
MS36	Giyani	female	Loose	200, 300, 400	10
NS 05951	Pretoria	female	Watery	200, 350	11
MS4	Giyani	Male	Loose	200, 350 f, 600 [f]	11
MS2	Giyani	female	Loose	200, 350, 400 [f]	11
MS5	Giyani	female	Loose	200, 350, 500	11
MS38	Giyani	female	Loose	200, 500 [f]	1
HS 38	Giyani	female	Watery	250 [f], 700 [f]	12
HS 39	Giyani	female	Watery	250, 300 [f]	12
HS 37	Giyani	Male	Formed	250, 300 [f]	12
MS37	Giyani	Male	Formed	250, 300, 400, 500 [f]	10

Note: Major bands obtained after the amplification of chitinase gene. [f]Faint.

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Table 4.6: Summary of the samples that produced the chitenase gene profiles (part 2)

sample code	origin	gender	stool type	chitinase	chitinase profileNo
NS 0597	Pretoria	Male	Watery	300 [f]	13
GY 42	Giyani	female	Loose	300 [f]	13
GY 20	Giyani	female	Loose	300 [f]	13
HS 44	Giyani	Male	Loose	300 [f]	13
MS33	Giyani	female	Loose	300 [f], 350, 600 [f]	14
NS07994	Pretoria	Male	Watery	300 [f], 400 [f]	13
NS 1085	Pretoria	Male	Loose	300 [f], 400 [f]	13
GY 25	Giyani	female	Soft	300 [f], 400 [f]	13
MS 32	Giyani	Male	Loose	300 [f], 500	15
HS 40	Giyani	Male	Formed	300 [f], 600 [f]	16
MS42	Giyani	female	Formed	300, 600	16
MS39	Giyani	female	Loose	300, 600	16
MS 21	Giyani	female	Loose	350, 600	17
MS43	Giyani	Male	Formed	400 [f]	18
MS45	Giyani	Male	Formed	400 [f]	18
GY 44	Giyani	female	Formed	400 [f], 900 [f]	18
GY 01	Giyani	Male	Formed	400 [f], 900 [f], 1200	18
MS6	Giyani	female	Formed	400, 700 [f]	18
MS8	Giyani	female	Formed	500, 400 [f]	19
MS1	Giyani	female	Formed	500, 600, 300, 350	20
MS44	Giyani	female	Loose	600 [f] 700, 1000	21
GY 02	Giyani	female	Loose	700 [f], 800 [f], 900	22
NS 03124	Pretoria	female	Loose	800 [f]	5
GY 03	Giyani	Male	Formed	800 [f], 900	22
HS 42	Giyani	female	Loose	900 [f], 1000 [f]	22

Note: Major bands obtained after the amplification of the chitinase gene. [f]Faint.

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Table 4.7: Distribution of the chitinase gene profiles in the study population

	Sample origin		Total		Sex groups			stool group		
chitinase profile No	Giyani	Pretoria			Male	Female		Formed	Loose	Watery
1	6	2	8		2	6		2	6	0
2	0	1	1		0	1		1	0	0
3	1	0	1		1	0		1	0	0
4	5	2	7		4	3		0	5	2
5	1	1	2		0	2		0	2	0
6	0	1	1		0	1		0	1	0
7	1	0	1		0	1		1	0	0
8	2	0	2		0	2		1	1	0
9	0	1	1		1	0		0	1	0
10	4	0	4		2	2		1	3	0
11	3	1	4		1	3		0	3	1
12	3	0	3		1	2		1	0	2
13	4	3	7		4	3		0	5	2
14	1	0	1		0	1		0	1	0
15	1	0	1		1	0		0	1	0
16	3	0	3		1	2		2	1	0
17	1	0	1		0	1		0	1	0
18	5	0	5		3	2		5	0	0
19	1	0	1		0	1		1	0	0
2	0	1	1		0	1		1	0	0
20	1	0	1		0	1		1	0	0
21	1	0	1		0	1		0	1	0
22	3	0	3		1	2		1	2	0
Total	84	27	111		50	61		28	69	14
Total	75.7%	24.3%	100%		45%	55%		25.2%	62.2%	12.6%

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4.2. PART 2: THE PREVALENCE OF *E. HISTOLYTICA* ANTIBODY AND ANTIGEN IN SERUM SAMPLES IN THE STUDY POPULATION

4. 2.1. Demographic characteristics of the patients in the study

A total of 647 HIV positive patients who were attending different hospitals and clinics throughout the Limpopo province were considered for analysis of the sero-prevalence of *Entamoeba histolytica* and the impact of host genetics on amoebic infection. Out of these participants, 332 (51.3%) were from the Capricorn district, 169 (26%) from Sekhukhune district and 132 (20.4%) from Waterberg districts. Of these patients, 216 (33.4%) were from urban areas and 427 (66 %) from rural areas and majority of them were attending clinics than hospitals. Out of these patients 197 (30.4 %) were males and 446 (68.9 %) females. Most patients were aged between 26- 45. A high number of patients 235 (36.3%) had a CD4 count ranging between 251 – 500 cells/mm ^{3} (**Table 4.8**).

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Table 4 .8: Demographic characteristics of the patients in the study.

Characteristics		Frequency	Percent (%)
Origin	Capricorn	332	51.3
	Sekhukhune	169	26.1
	Waterberg	132	20.4
	Missing system	14	2.2
Area	Urban	216	33.4
	Rural	427	66.0
	Missing system	4	.6
Sector	Hospital	195	30.1
	Clinic	434	67.1
	Missing	18	2.8
Gender	Male	197	30.4
	Female	446	68.9
	Missing system	4	.6
Age group	0 to 25 years	61	9.4
	26 to 45 years	439	67.9
	46 to 80 years	136	21.0
	Missing system	11	1.7
CD4 count	0 to 250	192	29.7
	251 to 500	235	36.3
	501 to 1000	172	26.6
	More than 1000	33	5.1
	Missing	15	2.3
Total		647	100.

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4.2.2. Distribution of *E. histolytica* antibody in the study population

The Techlab serology Elisa (figure 4.3) detected the antibodies against *E. histolytica* in 422 (65.2%) out of 647 serum samples while the Techlab *E. histolytica* II test (figure B) detected Gal/GalNAc lectin in 3(0.5%) samples.

4. 2.3. Overall distribution of *E. histolytica* infection in the study population

The results of this study showed that seropevalence of *E. histolytica* is high is more common in the study population, about 422 (65.2%) had antibodies against *Entamoeba histolytica.* The observations of the present study also showed low seroprevalence of antigens against E. histolytica that ALA is in the study population, since only 3(0.5%) of 647 patients were found to have antigens against *E. histolytica* (Figure 4.4)

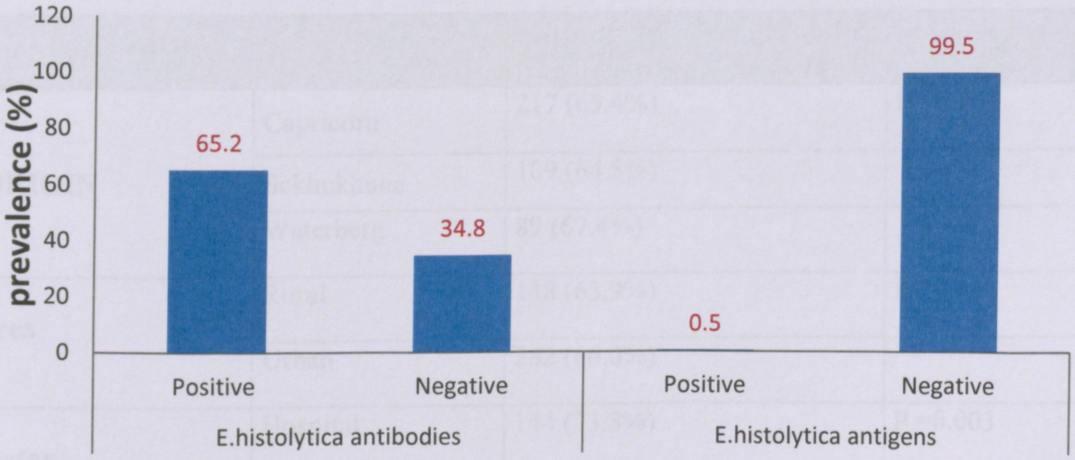

Figure.4.4: Overall distribution of *E. histolytica* infection in the study population.

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4.2.4. THE DISTRIBUTION OF *E. HISTOLYTICA* ANTIBODIES IN THE STUDY POPULATION

4.5.1. Distribution of *E. histolytica* antibodies in the study by origin, area, sector age and gender of the participants

The samples were collected from three different districts in Limpopo province. The results shows the antibodies against *E.histolytica* were high in Waterberg (67. 4 %), compared to Capricorn (65.4%) and Sekhukhune (64.5%), with high prevalence percentage in rural areas (66. 5%) than in urban areas (63.9%).

Table 4.9: Distribution of *E. histolytica* antibodies in the study by origin, area, sector age and gender of the participants.

Characteristics		*E.histolytica* antibodies (seropositive)	Statistics
ORIGIN	Capricorn	217 (65.4%)	P =0.863
	Sekhukhune	109 (64.5%)	
	Waterberg	89 (67.4%)	
Area	Rural	138 (63.9%)	P =0.588
	Urban	282 (66.0%)	
Sector	Hospital	144 (73.8%)	P =0.003
	Clinic	267 (61.5%)	
Gender	Male	125 (63.5%)	P =0.509
	Female	295 (66.1%)	
Age groups	0 -25	42 (68.9%)	P =0.609
	26-45	281 (64.0%)	
	46-80	92 (67.6%)	

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The people who were attending hospitals were more infected (73.8%) than the people in the clinics (61.5%) and the difference was significant (P<0.005). Females were more infected (66.1%) than males (63. 5%). Patients aged between 0-25 had a high prevalence of 68.9% compared to the other age groups. All the results are shown in Table 4.9 below.

4.2.5. Seroprevalence of E. histolytica in relation to CD4 count of the patients

The sero-prevalence of *E. histolytica* was much higher in patients with CD4 count more than 1000 (69.7%) than in patients with CD4 count less than 1000. The study showed that, the higher the level of CD4 count, the higher the infection. In CD4 count between 250- 500, the infection was low. However, the difference was not significant (Table 4.10).

Table 4.10: The occurrence of *E. histolytica* antibodies in relation to CD4 count and other medical conditions

Characteristics		Antibody positive	Total	Statistics
CD4 group	0 to 250	116 (60.4%)	192	χ^2=3.254, p=0.354
	251 to 500	156 (66.4%)	235	
	501 to 1000	118(68.6%)	172	
	More than 1000	23 (69.7%)	33	

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4.2.6. The prevalence of antigens against E. *Histolytica* in the study population

The Techlab *E. histolytica* II test detected antigens against *E. histolytica* in only 3 (0.5 %) samples out of the 647 serum samples analysed in this study. All the patients were females, two from Capricorn and one from Sekhukhune. All of them were from rural areas. These patients were tested for IL-10 concentration in the serum, and they all showed to produce low IL-10 level. Two of these patients were sero-positive for the antibodies against *E.histolytica*. In terms of circulating genotypes, these patients were found with AA, AG genotypes of -1082 polymorphism and one with the AC genotype of -592 polymorphism, which are all associated with low production of IL-10 and two of them had the CC genotype of -592 polymorphism. The other characteristics are represented in the table below.

Table 4.11: Characteristics of three patients positive for antigens against *E. histolytica*.

Sample	Age	gender	origin	Area	CD4 µl/ml	*E. histolytica* Antibodies	*E. histolyta* antigens	Il-10 level	-1082 A/G	-592 A/C
POI0840	64	female	Sekhukhune	Rural	338	Positive	Positive	<2000	AA	CC
POI0846	29	female	Capricorn	Rural	398	Positive	Positive	<2000	AG	CC
POI1019	49	female	Capricorn	Rural	373	Negative	Positive	<2000	AA	AC

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4. 3. PART 3: LEVELS OF INTERLEUKIN-10 IN SERO-POSITIVE AND SERO-NEGATIVE PATIENTS IN THE STUDY

4. 3.1. Detection of interleukin- 10 expression in the serum samples by ELISA method

A total of 647 serum samples tested for *E. histolytica* antibodies and antigens were also employed for detection of IL-10 expression in the serum samples by ELISA tests from MABTECH.

4. 3.2. The overall expression IL-10 in the study population

The serum samples from both sero-positive and sero-negative patients were used in this study. The sero-negative patients were used as a control group. The overall IL-10 expression between these two groups was 34.8% and 65.2%.

4.3.3. The distribution of IL-10 levels in relation to age, gender and CD4 counts of the study participants

There was no significant difference between IL-10 expression and age group, gender as well as CD4 count in the study population. The expression of IL-10 was associated with the age of the study participants and it was found that patients aged between 46-80 (35.3%) produced high IL-10 level, followed by aged group 0 to 25 (27.9%) and the least expression of this cytokine was obtained in people aged 26 to 45 with a percentage of 27.3%. Females were found to produce high levels of IL-10 (30.3%) compared to males (25.9%). The patients with CD4 greater than 1000 had high percentage of IL-10 expression than other patients with CD4 count between 0-250 (29.2%), 251- 500 (23.4%) and 500- 1000 (34.3%) (**Table 4.12**).

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Table 4.12: The distribution of IL-10 levels in relation to age, gender and CD4 counts of the study participants.

CHARACTERISTICS		IL-10 EXPRESSION		STATISTICS
		<2000	>2000	
Age group	0 to 25	44 (72.1%)	17 (27.9%)	χ^2=3.238, p=0.198
	26 to 45	319 (72.7%)	120(27.3%)	
	46 to 80	88 (64.7%)	48 (35.3%)	
Gender	Male	146 (74.1%)	51(25.9%)	χ^2=6.980, p=0.222
	Female	311(69.7%)	135 (30.3)	
CD4 group	0-250	136 (70.8%)	56 (29.2%)	χ^2=6.810, p=0.078
	251-500	180 (76.6%)	55 (23.4%)	
	501-1000	113 (65.7%)	59 (34.3%)	
	>1000	21 (63.6%)	12 (36.4%)	

4.3.4. The distribution of IL-10 levels among sero- positive and sero- negative patients in the study.

The levels of IL-10 expression did not significantly associate with sero-positive and sero-negative patients in the study population. The percentage of *E. histolytica* infection was high in patients with low IL-10 production (68.1%) and in patients with high level of IL-10, the percentage of the infection was low (58.2%). **(Table 4.13).**

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Table 4.13.: The distribution of IL-10 levels among sero- positive and sero- negative patients in the study.

	ANTIBODIES AGAISNT *E. HISTOLYTICA*		
IL-10 LEVEL	**NEGATIVE**	**POSITIVE**	**STATISTICS**
<2000	146(31.9%)	312(68.1%	χ^2=5.806, P=0.016
> 2000	79(41.8%)	110(58.2%)	
Total	225(34.8%)	422(65.2%)	

4.4. PART 4: THE IMPACT OF HOST GENETICS ON AMOEBIC INFECTION

4.4.1. Interleukin-10 detection (-592 A/C and 1082 A/G) by PCR

Out of the 647 serum samples tested for *E.histolytica* antibodies, antigens and IL-10 level, only 334 samples were employed for genetic association with amoebiasis. The PCR method was used to detect the IL-10 polymorphisms (-592A/C and -1082 A/G) in the serum samples. The expected product size of 412 and 139 were obtained in all the samples tested for IL-10 gene respectively. Figure (A and B) shows some of the agarose gel showing the positive samples of IL-10 gene promoter polymorphisms extracted from the buffy coat.

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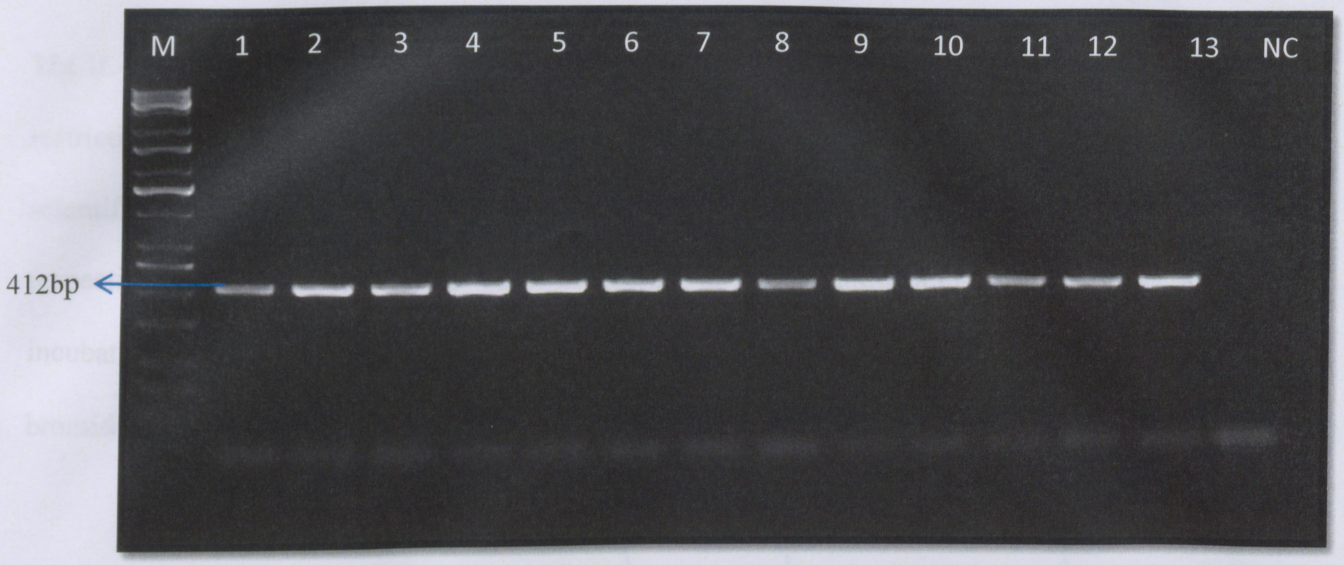

Figure 4.8: An agarose gel picture showing the results obtained from PCR in detection of IL-10 at position -592 (412bp). **M**-Molecular Marker (KAPA universal Ladder); **Lane** 1-13 test sample, **NC**-Negative Control.

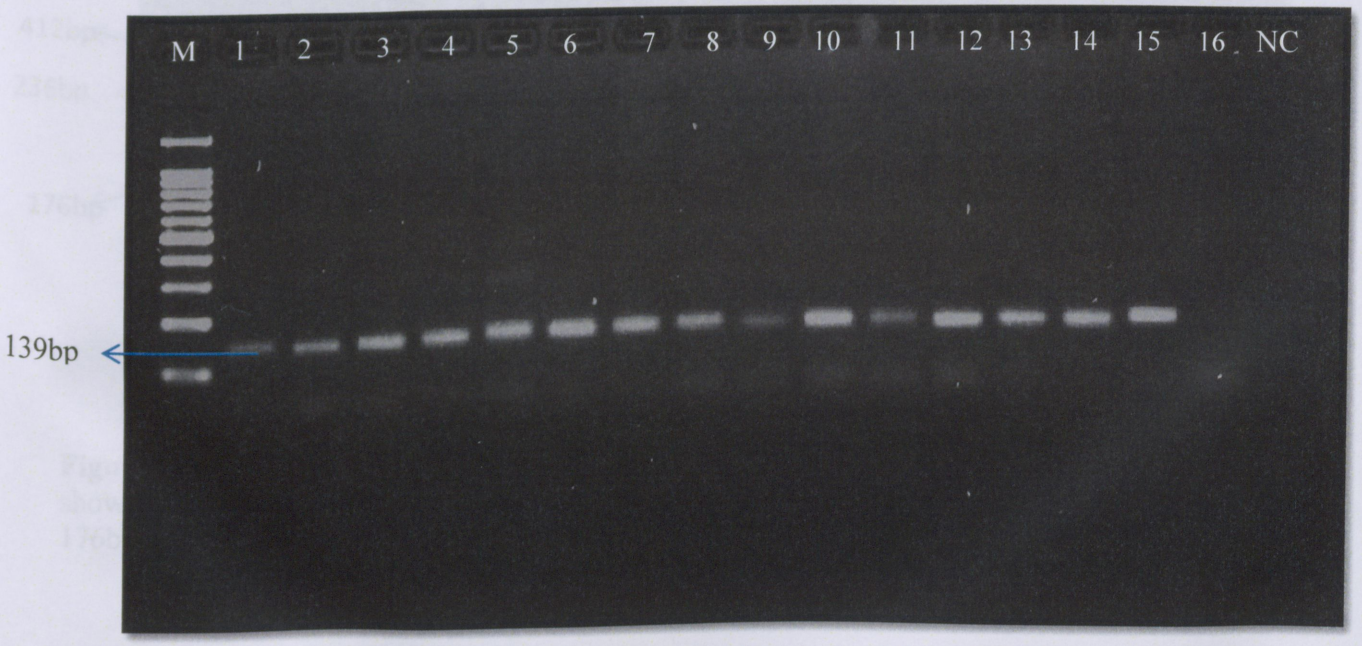

Figure 4.9: An agarose gel picture showing the results obtained from PCR in amplification of IL-10 at position -1082 (139 bp). **M**-Molecular Marker (100bp); **Lane** 1-16 DNA sample; **NC**-Negative Control.

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4.4.2. Genotyping of IL-10 -592 A/C AND -1082 A/G polymorphisms by RFLP

The IL-10 gene promoter polymorphisms at position -592A/C and -1082 A/G were genotyped by restriction fragment analysis (RFLP). The restriction endonucleases MnlI and RsaI (Thermo scientific) were used to distinguish the IL-10 gene -592A/C and -1082 A/G polymorphisms respectively. The enzymes were used according to the manufacturer's instructions. After incubating for 16 hours, the products were separated on a 2% agarose gel stained with ethidium bromide and visualized under U.V transluminator (BIO RAD).

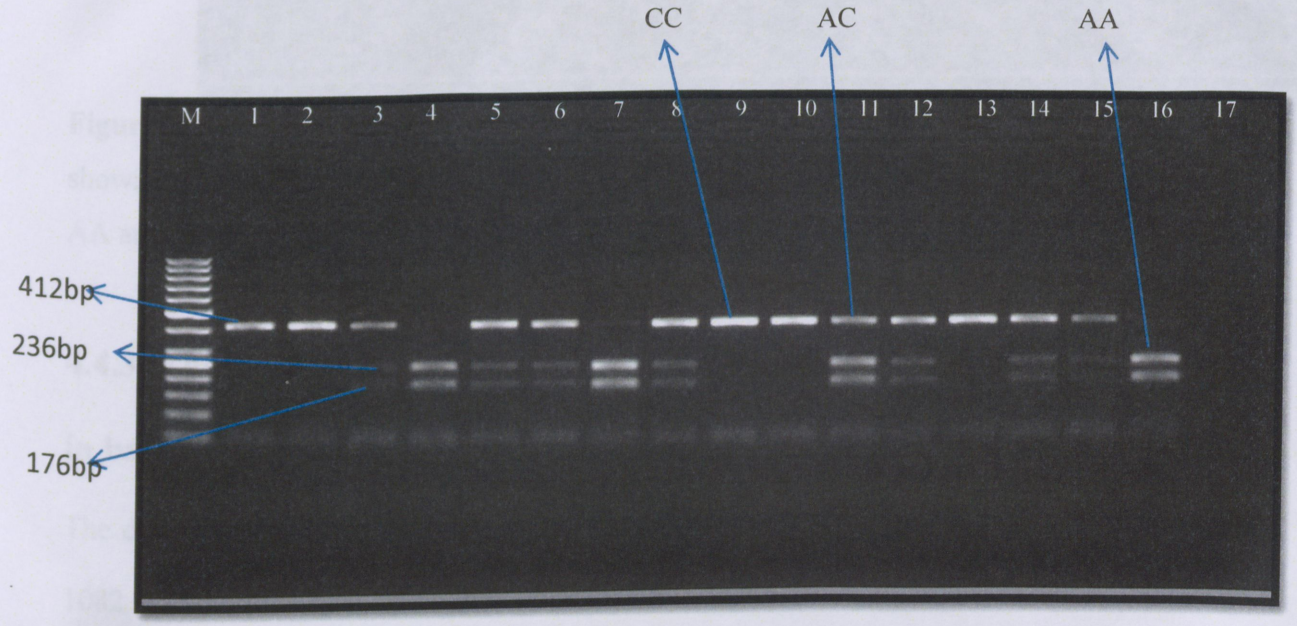

Figure 4.10: Electrophoresis pattern of the PCR-RFLP products for IL-10 polymorphisms showing the determination of IL-10 (-592) in seventeen different individuals: 412bp CC; 236, 176bp AA and 412, 236,176bp AC genotypes.

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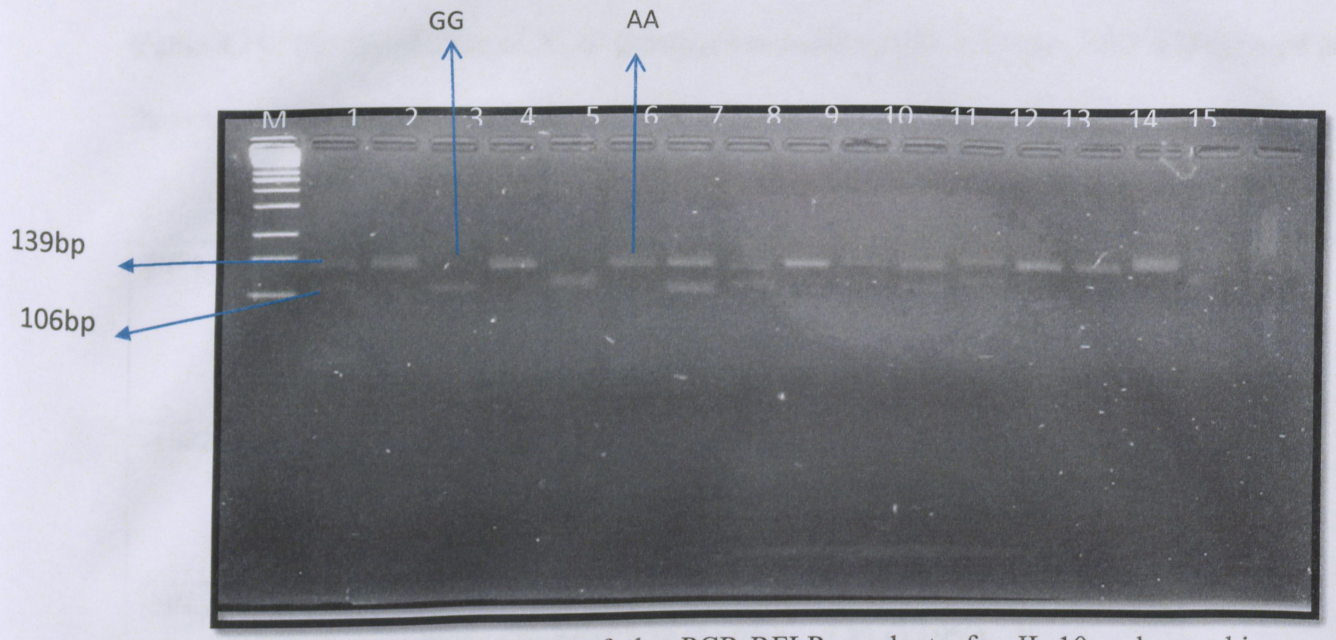

Figure 4.11: Electrophoresis pattern of the PCR-RFLP products for IL-10 polymorphisms showing the determination of IL-10 (-1082A/G) in seventeen (17) different individuals: 139bp AA and 106bp is GG.

4.4.3. The distribution of IL-10 genotypes at position -592 A/C and -1082 A/G in based on the origin of the study participants

The distribution of the genotypes in the three districts were compared in the study. In IL-10 – 1082 polymorphism, it was found that the AA genotype is more prevalent in Sekhukhune (47.1%) compared to the other two districts, the heterozygous AG and homozygous GG genotypes were more distributed in Waterberg with percentages of 39.1%, and 35.6 % respectively, however, the difference among the three districts and genotype frequencies was not statistically significant. The overall percentage in the study population was 50.5%. (**Table 4.14**)

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Table 4.14: The distribution of IL-10 genotypes at position -592 A/C and -1082 A/G in based on the origin of the study participants

SNPs	GENOTYPE	DISTRIBUTION BY ORIGIN			TOTAL	P VALUE
		Capricorn	Sekhukhune	Waterberg		
-1082	AA	53 (34.2%)	40(47.1%)	22 (25.3%)		P=0.039
	AG	55 (35.5%)	28 (32.9%)	34 (39.1%)		
	GG	47 (30.3%)	17 (20.0%)	31 (35.6%)		
-592	AA	20 (12.9%)	11 (12.9)	7 (8.0%)		P= 0. 779
	AC	56 (36.1%)	28 (32.9%)	33 (37.9%)		
	CC	79 (51.0%)	46 (54.1%)	47 (54.0%)		

4.4.4. The distribution of IL-10 genotypes at position -592 A/C and -1082 A/G in based on gender and age of the study participants

The distribution of the genotypes was also associated with age and gender of the study participants. In the IL-10 -1082 polymorphism, the females were shown to carry high percentage of the homozygous AA (36.8%), while the males were found to carry high percentage heterozygous AG (37.3%) and homozygous GG (31.4%) genotypes compared to females. The study showed significant difference in the distribution of the IL-10 -1082 genotypes with age of the participants. The high percentage carriers of the AA genotype was patients aged between 26-45, and the high percentage of the AG genotype was found in patients aged 0-25, and the patients aged 46- 80 were found to carry 36.6% of the GG genotype.

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Table 4.15: The distribution of IL-10 genotypes at position -1082 A/G based on gender and age of the study participants

		-1082 A/G POLYMORPHISM			P VALUE
		AA GENOTYPE	AG GENOTYPE	GG GENOTYPE	
Gender	**Male**	32(31.4%)	38(37.3%)	32(31.4%)	P=0.763
	Female	84(36.8%)	79(34.6%)	65(28.5%)	
Total		116(35.2%)	117(35.5%)	97(29.4%)	
Age group	0 to 25 years	5(18.5%)	18(66.7%)	4(14.8%)	P=0.006
	26 to 45 years	85(37.1%)	77(33.6%)	67(29.3%)	
	46 to 80 years	25(35.2%)	20(28.2%)	26(36.6%)	
Total		115(35.2%)	115(35.2%)	97(29.7%)	

4.4.5. The distribution of IL-10 genotypes at position -1082 A/G in based on gender and age of the study participants

The distribution of the genotypes were also associated with age and gender of the study participants. In the IL-10 -592 polymorphism, the females were shown to carry high percentage of the homozygous AA (12.3%) and homozygous CC (53.5%), while the males were found to carry high percentage heterozygous AC (38.2%). The results of this study showed significant difference in the distribution of the IL-10 -592 genotypes with age of the participants. The patients aged 0-25 carries high percentage of the AC genotype (55.6%), the patients aged 46-80 were found with high percentage of AA (15.5%) genotype compared to other groups and the CC genotype was high in patients aged 26-45 (57.6%)

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Table 4.16: The distribution of IL-10 genotypes at position -592 A/C based on gender and age of the study participants

		-592 A/C POLYMORPHISM			
		AA GENOTYPE	AC GENOTYPE	CC GENOTYPE	P VALUE
Gender	**Male**	11(10.8%)	39 (38.2%)	52 (51.0%)	P=0.068
	Female	28 (12.3%)	78 (34.2%)	122 (53.5%)	
Total		39 (11.8%)	117 (35.5%)	174 (52.7%)	
Age group	**0 to 25 years**	4 (14.8%)	15 (55.6%)	8 (29.6%)	P=0.053
	26 to 45 years	24 (10.5%)	73 (31.9%)	132 (57.6%)	
	46 to 80 years	11 (15.5%)	26 (36.6%)	34 (47.9%)	
Total		39 (11.9%)	114 (34.9%)	174 (53.2%)	

4.4.6. Genotype frequencies for -592 A/C and -1082 A/G among sero- positive and sero-negative patients in the study population

The genotypic frequencies of IL-10 -592 A/C and -1082 A/G polymorphisms were compared between the sero-positive and sero-negative patients. There was no significant difference in genotypic frequencies of the positive patients and negative subjects. In the negative group the genotypic frequencies were 35.9% for AA genotype, 37.6 % for AC genotype and 28.7% for CC genotype. While in sero-positive patients the frequency was 64.1 % of AA genotype, 62. 4% of the A/C genotype and 71.3% of the CC genotype. In case of 1L-10 -1082 A/G polymorphism, there was a significant difference in genotypic frequencies between amoebic positive and

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negative patients. In the negative group, the genotypic frequencies were 31.9% AA genotype, 32.5% AG genotype and 34.0% GG genotype, while in amoebiasis patients, the frequency was 68.1% AA genotype, 67.5% AG genotype and 66.0 GG genotype

Table 4.17: Genotype frequencies for -592 A/C and -1082 A/G among sero- positive and sero-negative patients in the study population.

		ANTIBODIES AGAINST *E.HISTOLYTICA*		STATISTICS
SNPS	GENOTYPES	SERO-NEGATIVE	SERO-POSITIVE	
-592 A/C	AA	14 (35.9%)	25 (64.1%)	χ^2=2.703, P=0.259
	AC	44 (37.6%)	73 (62.4%)	
	CC	50 (28.7%)	124 (71.3%)	
Total		108 (32.7%)	222 (67.3%)	
-1082 A/G	AA	37 (31.9%)	79 (68.1%)	χ^2=0.113, P=0.945
	AG	38 (32.5%)	79 (67.5%)	
	GG	33 (34.0%)	64(66.0%)	
Total		108 (32.7%)	222 (67.3%)	

4. 5. SEQUESENCE ANALYISIS AND RESULTS

Some of the samples positive by PCR in detection of IL-10 gene promoter polymorphisms were sent for sequencing to confirm the RFLP results. About 28 sequences of IL-10 were analysed using staden package (Staden *et al.,* 1999) and foreign nucleotides present in the sequence were removed and replaced by appropriate amino. BIOEDIT (Hall, 1999) was used to edit the nucleotides sequences

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which were then aligned using the Clustal W multiple aligner. The IL-10 gene sequence showing the three different SNPs of IL-10 is indicated on figure. The dots indicate identical amino acids and the variations in the sequence are indicated by nucleotides. The results were compared with genotypes obtained with RFLP, and they were 100% concordant. The three polymorphisms (-592A/C, 1082 A/G and -819 T/C) of IL-10 were obtained in the sequences at different positions, 819 T/C was seen in position -668 T/C, -592 A/C was seen at position 125 T/C and -1082 A/G at position -396 A/G. The three SNPs are shown in the nucleotide sequence below (figure)

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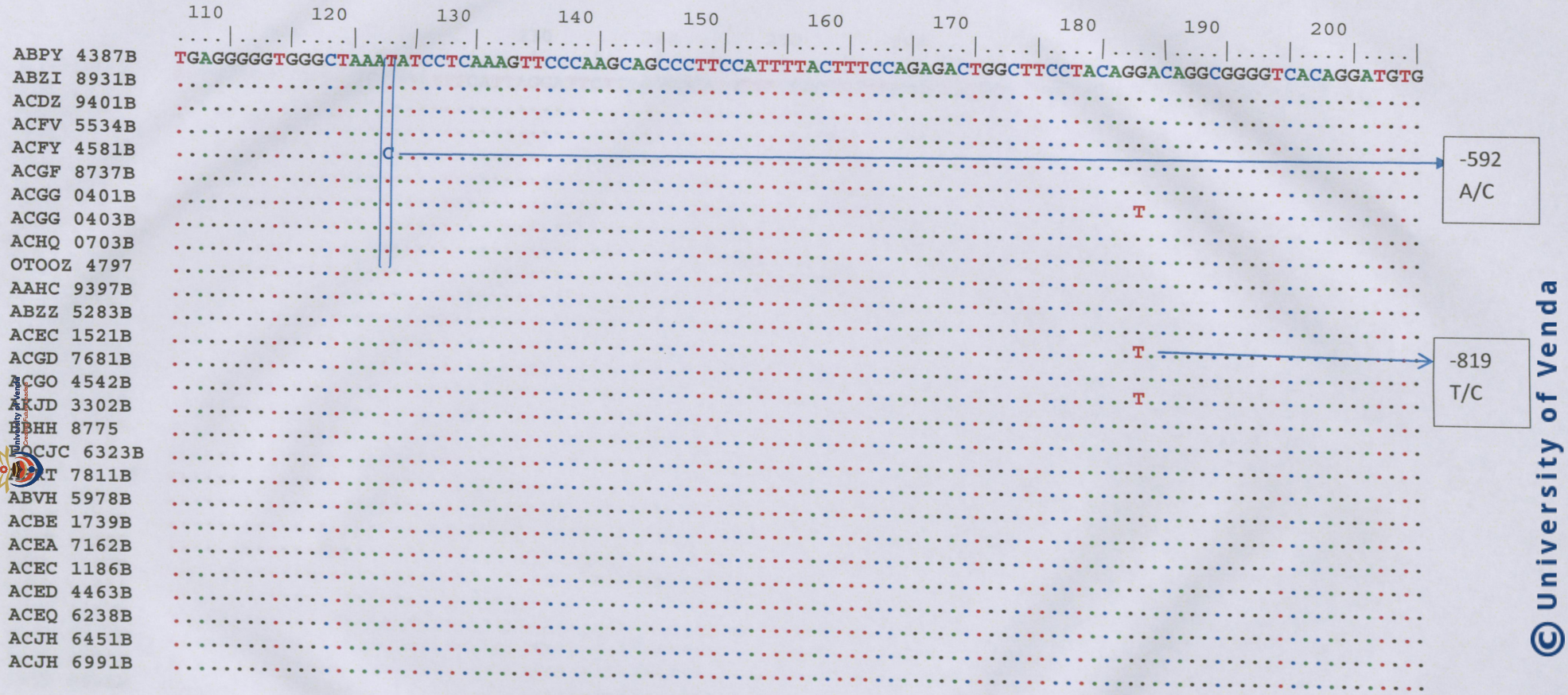

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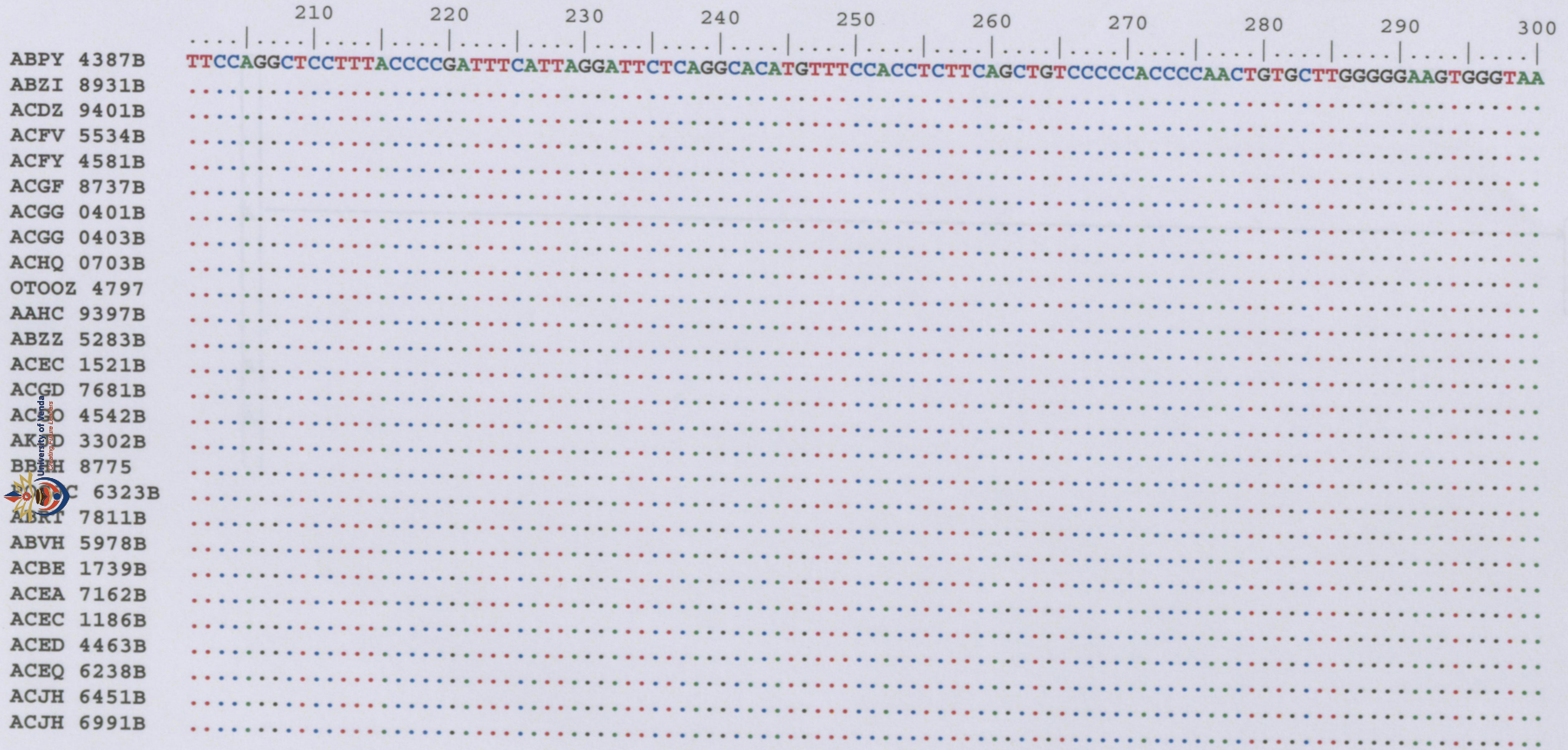

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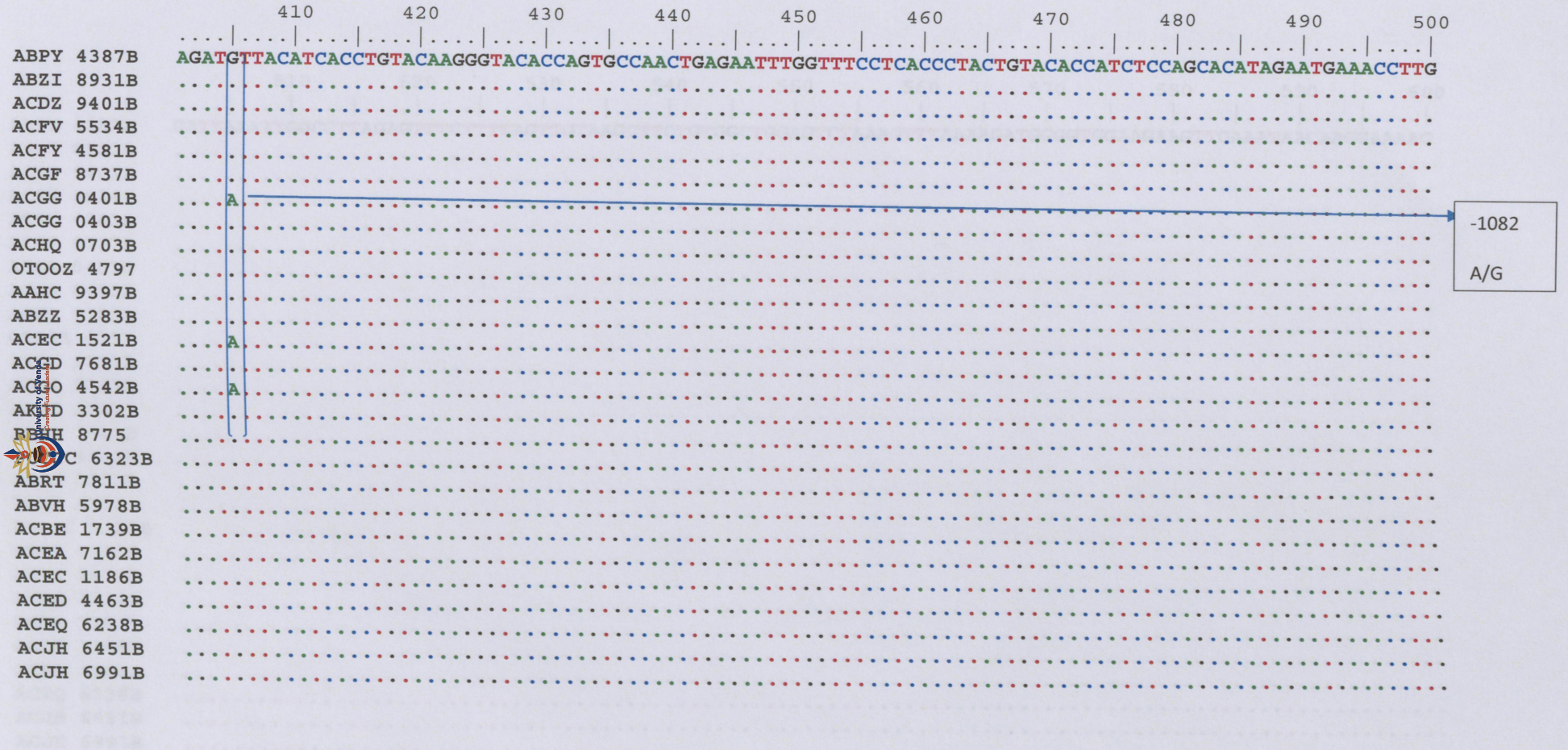

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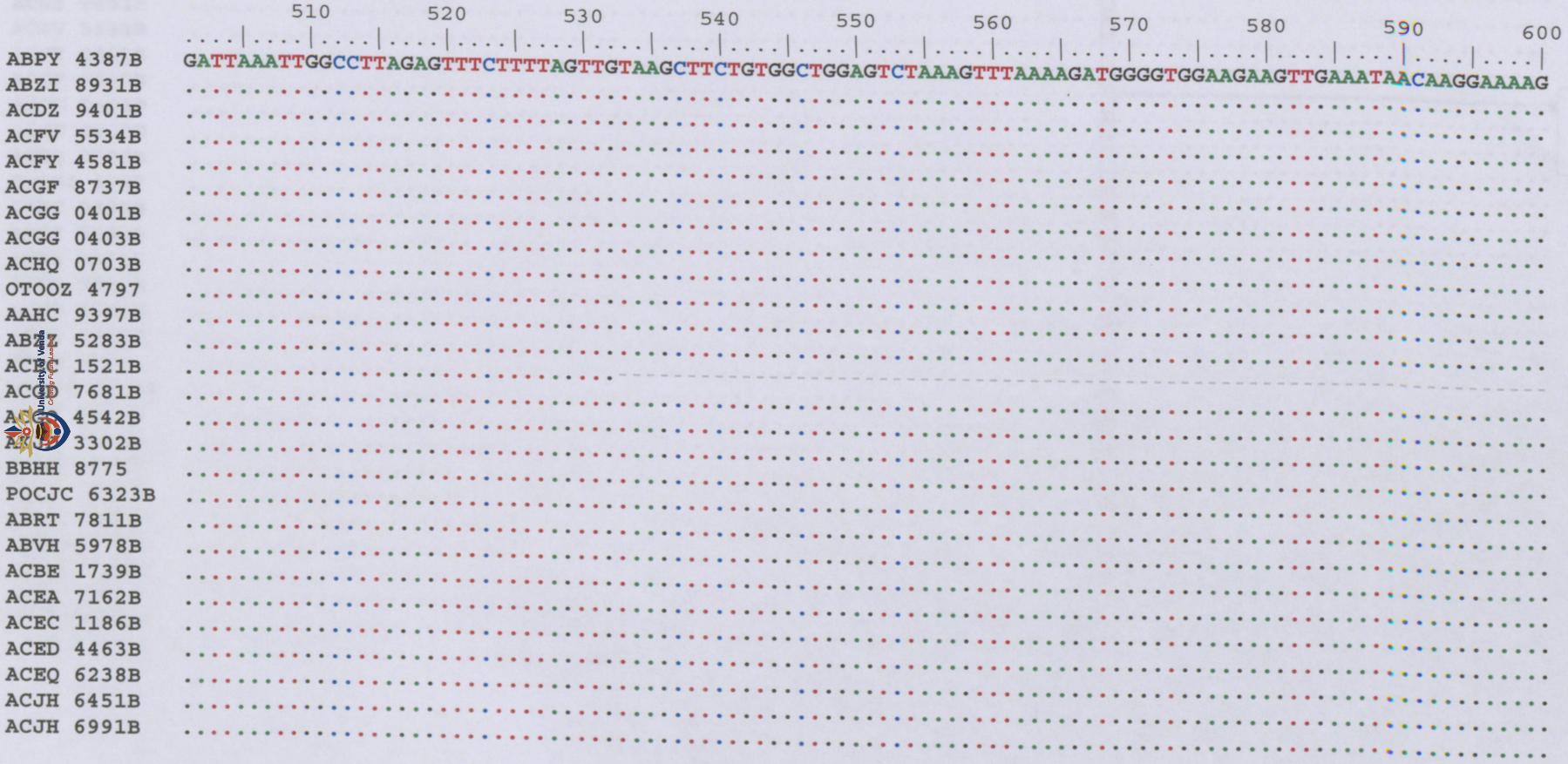

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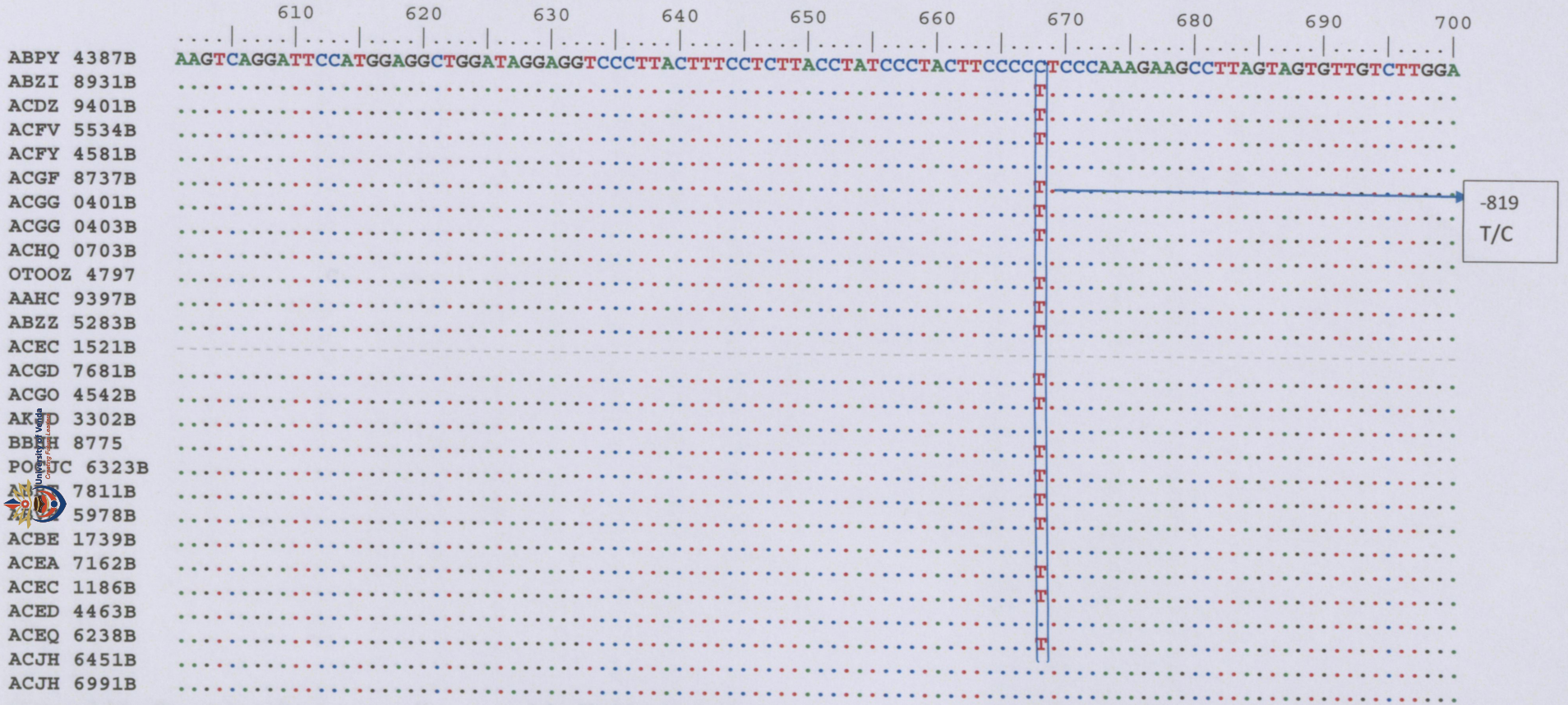

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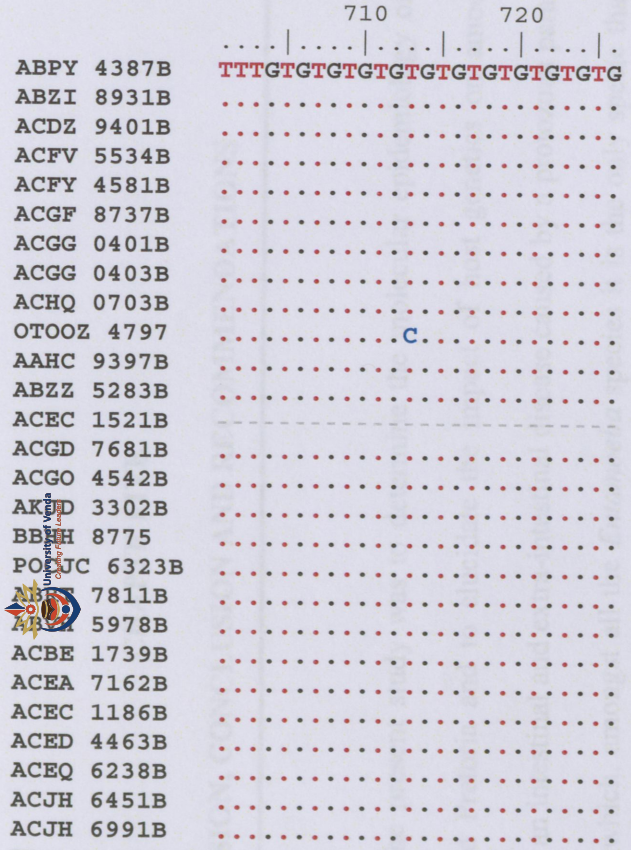

Figure 4.12: The nucleotide sequence alignment of the IL-10 gene including the three polymorphisms.

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CHAPTR FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

The main objective of the present study was to determine the molecular epidemiology of *E. histolytica* in Giyani and Pretoria and to elucidate the impact of host genetics on amoebic infection. Amoebiasis, is an intestinal and extra-intestinal disease caused by a protozoan parasite known as *E. histolytica*, which, amongst all the *Entamoeba* species it is the only specie that is considered to be pathogenic to human. The infection has been reported to occur worldwide, but prevalent in tropical and developing countries. The disease affect major organs such as the colon and liver, with different symptoms or sometimes asymptomatic (Haque *et al*., 2003). However, the question on what causes the difference between disease presentations is still unanswered. It has been suggested that both the parasite and host genetics play a role in the outcome of amoebic infection.

Very few studies have been documented on the molecular epidemiology of *E. histolytica* and the impact of host genetics on disease outcome in the developing countries, where there is a high prevalence of parasitic infections. Therefore there was a high necessity to study the prevalence and the impact of host genetics on the outcome of amebic infection, especially in developing areas together with the association of cytokine level, host and parasite genotypes with amoebiasis.

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5.1. PART 1: MOLECULAR CHARACTERIZATION OF *E. HISTOLYTICA* STRAINS BASED ON SREHP AND CHITINASE GENES

5.1.1. Detection of *E. histolytica* by microscopy and PCR

The traditional diagnosis of *E.histolytica* is based on microscopy, but it appears to be impossible to distinguish between *E.histolytica* and *E.dispar* (Blessman *et al*., 2002). In the present study, 111 samples were examined for the presence of *Entamoeba* cyst in the stools, microscopy detected 46 samples negative and 65 positive, however some samples contained the other parasitic organisms such as *E.nana*, *E. coli, Giardia* and *cryptosporidium*, the presence of this organisms proved that indeed microscopy is not specific and it is not reliable. The results of our study correlates with the result obtained by Intarapuk *et al*., (2009), where they showed some mixed infections by microscopy. Due to the indistinguishable morphology between *Entamoeba histolytica* and *dispar*, the PCR based method was used to detect specifically *E. histolytica*. The results of this study showed that *E.hististolytica* is prevalent in the study population. The samples obtained negative by microscopy and PCR were excluded in the study, and the positive samples by both methods were used for genetic diversity.

5.1.2. Genetic diversity of *Entamoeba histolytica* based on the SREHP profiles

The polymorphic SREHP gene was used to study the molecular epidemiology and the diversity of *E.histolytica* strains in Giyani and Pretoria, South Africa. The SREHP gene successfully amplified 19 isolates out of 65 positive samples included in this study. Some of the isolates successfully amplified for the 700bp SREHP gene, while the other isolates amplified additional amplicons of approximately 300, 400,500, 600, 800 and 1200bp. The band sizes obtained in this study do not differ with the ones obtained recently by Ali et al., (2014) and Intarapuk et al.,

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(2009). In the present study, nineteen (19) profiles were obtained in samples from Giyani and Pretoria (Table 4.2), the obtained profiles indicates a wide variability of the SREHP gene in different areas of the study population. This study showed a high number SREHP profiles compared to those obtained by (Ali *et al.,* 2014), these profiles are also different from those obtained by (Haghighi *et al*.,2003 Ramos *et al*., 2005 and Samie *et al*., 2008, ,). The profiles of SREHP obtained in this study were associated with gender, origin and stool consistency.

The results of this study showed that three asymptomatic patients from Giyani are infected with the same profile (profile no 2). This profile was not associated with with diarrhea suggesting that the strain might not be virulent or associated with invasive amoebiasis. Another finding of this study was the occurrence of five different isolates, one symptomatic isolate from Giyani, two asymptomatic and one symptomatic isolates from Pretoria which were infected with the same profile (profile no 5), it was also found that the profile was more prevalent in Pretoria than in Giyani, with high distribution in males than females. This profile was also associated with stool consistency, and it was found in isolates with watery and loose stools, showing that this profile may cause diarrhea. These findings corroborated previous findings in a study carried out by Fu et al. (2010), which concluded that all isolates from different mental institutions in Japan were derived from a single source of *E. histolytica* source. Another study by Ali et al., (2014), obtained similar results, they obtained the same profile numbers from the four identical isolates.

Clustering of profiles from the same region (Giyani) was also obtained in the present study and no clustering was obtained from Pretoria isolates. The patients from Pretoria were infected with only one profile, which is profile no 2. The results obtained in the present study indicate that

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there is a possibility of the existence of the same strain infecting individuals from the same region. Due to the small sample size used in this study (65) the profile number were low compared to the profile numbers obtained by Haghighi *et al.,* (2003), where 22 profile were obtained from 74 positive isolates. Our study reported a small number of profiles compared to the results obtained by samie *et al*., in (2008), this study showed 26 profiles from 38. Another study by Ayeh-Kumi et al. (2001) showed a high number of profiles (25 profiles from 42 positive stool isolates). The results of this study suggests that the SREHP might have a role in the presentation of amebiasis (symptomatic and asymptomatic infections) depending on the infecting profile.

5.1.3. Genetic diversity of *Entamoeba histolytica* based on the chitinase profiles

The chitinase gene was selected to study the genetic diversity and molecular epidemiology of *E.histolytica* in the study population. In general, little is known about the extent of intestinal parasitic infections in Limpopo and Gauteng province, especially in Giyani and Pretoria, and no reports have been published on the genetic diversity of *E. histolytica* based on chitinase in Limpopo and Pretoria. Therefore, we are reporting for the first time the diversity of this parasite based on chitinase in this two areas

The chitinase gene successfully amplified 22 isolates from 65 positive isolates included in the study. The target bands of 500 and 1200bp were obtained in a number of isolates in this study (Haghighi *et al*., 2002). Chitinase gene has tandem repeats ranging from 84- 254 nucleotides (Ramos *et al*., 2005). This observation supports the results obtained in the current study, this study has shown some additional amplicons of approximately 200, 300, 400, 500, 600, 800, 900 and 1000bp were also obtained in some samples in the study. The obtained bands in this study are similar with the bands obtained by Intarapuk et al., 2009)

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Twenty two (22) profiles of chitinase were obtained in the samples from Giyani and Pretoria (Table 4.3). This study showed a high number SREHP profiles compared to those obtained by (Intarapuk et al., 2009, Haghighi et al., 2002) .The high number of the chitinase profiles observed in this study is an indicative that *E. histolytica* is prevalent in the study area. The profiles were associated with gender, origin and stool consistency of the study participants. High diversity of the chitinase profiles were obtained mostly in Giyani than Pretoria.

Interestingly, the results of this study showed that five asymptomatic patients (formed stools) from Giyani were infected with the same profile (profile no 18), of this five patients 3 were males and 2 were females. This indicates that this profile is circulating in the communities of Giyani, however our study did not associate it with diarrhea. This shows that the presentation of the infection depend on the infective profile. Another interesting finding of this study, is the occurrence of the same profile in different isolates of different gender from different geographic areas. Six different isolates, one asymptomatic isolate from Pretoria, two symptomatic (watery stool) and three asymptomatic (formed) isolates from Giyani. Of these six isolates four were females and two males. Looking at the distribution of this profile by gender it shows that more females are infected than males.

The observed results shows that this profile is associated with diarrhea, therefore it is the cause of amoebiasis in the study population. The results of this study reinforces that the polymorphic loci (chitinase) could serve as a tool to determine the diversity of *E. histolytica* and to finger printing individual isolates (Zaki and Clark, 2001). The findings of the same profiles affecting

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individuals in the same area was also demonstrated in a study conducted by Ali et al., 2014). Clustering of profiles was observed in both study sites, indicating that some patients were infected with more than one profile. Concerning the geographical distribution, the most occurring profiles, 18 and 4, are more distributed in Giyani compared to Pretoria, because this area only one isolates was infected with profile 4 and in profile 18, none was from Giyani. According to the observations of this study, profile 18 is more common in Giyani.

5.2. PART 2: THE PREVALENCE OF *E.HISTOLYTICA* IN THE STUDY POPULATION

5.2.1. Prevalence of *E. Histolytica* antibodies and antigens in the study population

The prevalence of intestinal amoebiasis varies from origin to origin or from country to country. Intestinal amoebiasis was found in 422 participants out 647 recruits, with a percentage prevalence of 65.2% using *E. histolytica* serology ELISA kit from Techlab in this study. The high seroprevalence of *E. histolytica* infection in the study indicates that the parasite is commonly found in these areas. It has been suggested that the presence of antibodies against *E.histolytica* shows present or past infection with *E.histolytica.* (Ravdin *et al*., 2003). The prevalence of amoebiasis in this study was higher compared to the study conducted in 2010 by Samie *et al*, in Limpopo province. Similar study have been conducted in patients from Dhaka, Bangladesh, the study detected intestinal amoebiasis in 26 of 50 (52%) using Techlab *E.histolytica* II kit. A study in Nasarawa state in Nigeria had reported 40.0% prevalence of intestinal amoebiasis in school age children in Lafia (Reuben *et al*., 2013). A study by Zhou *et al*., (2013), showed a prevalence of 41.1% of *E. histolytica* infection among MSM community

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from China, showing that the infection is also transmitted through sexual contact in men who have sex with men.

The present study also showed that females (66.1%) are more affected than males (63.5%). The infection pattern between females and males were not significantly different. Some observations has explained the reasons of this differences of prevalence between males and females, it has been suggested that this may be due to that most families favor female housemaids over males for cultural, religious and traditional reasons (Nada, 2005). Similar results on distribution of *E. histolytica* by gender was observed in a study by samie *et al*., (2010), this study showed that *E.histolytica* infection was higher in females than in males, showing that females are more likely than males to carry asymptomatic infections rather than developing invasive diseases. The findings of this study is also in contrast with several studies that found *E.histolytica* to be higher in females that in males, such prevalences have been reported in Saudi Arabia with a prevalence rate that varied between 55.7 % in females and 41.4% in males (Abdul- Hafez *et al*., 1987), another study in Abha found the females to have a high prevalence of 46.5% (Al- Madani and Mahfouz, 1995). A study by Jamila, (2014), also found similar results of higher *E.histolytica* infection in females (48.7%) and in males (47.8%).

Although all age groups were seropositive for *E.histolytica* antibodies in the present study, the patients aged between 0- 25 showed a very high prevalence (68.9%), followed by the older age at between 46-80 years with prevalence percentage of (67.6%) and the least percentage was observed in patients aged between 26-45 (64.0%). Several studies have found similar results, a study in Jeddah on the factors associated with high prevalence of *Entamoeba histolytica /dispar* infection among children, has shown a high prevalence of *E.histolytica* infection among children

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aged 1 month- 6years with a prevalence of (60.8%). However, their study only focused on children with a sample size of 300, hence the low prevalence of 60.8 % (Jamila, 2014) compared to the high prevalence of 68.9% in this study. The results of the present study also showed a high prevalence of *E. histolytica* in the people aged 46-80, the results of our study are in agreement with results that Samie *et al*., (2010) reported in Limpopo province, this study has shown that people aged between 50-59 had a high prevalence of 69.2 % and the least percentage was found in patients aged 20-29 (23.6%). A study by Tasawar *et al*., (2010), reported that *E. histolytica* infection was detected The prevalence of amoebiasis was highest in age group of 33 to 48 years (16.67%).

The prevalence of *E. histolytica* is said to be high in areas with poor sanitation, inadequate water supply and low socioeconomic, especially in developing countries. These observations are supported by the results of the present study. Our study found that the patients in rural areas were more seropositive (66.0%) than the patients in urban areas (63.9%). The high rates in rural areas may be influenced by improper hygiene and agricultural backgrounds (Dina *et al*., 2011). Furthermore, the high prevalence was also in patients attending hospitals than those who were attending clinics in this study. The results of the present study are in contrast with the results found in rural Southern India, the overall prevalence of intestinal infections was 97.4%/ month (Kang *et al*., 1998), another study by (Dina *et al*., 2011), showed a high prevalence rate of 73.5% in rural communities of Sierra Leone. Concerning relation between *E. histolytica* infection and CD4 count and other medical conditions of the patients recruited in this study, it was found that patients with CD4 count more 1000 had high prevalence (69.7%) rate of infection, followed by a prevalence rate of 68.6% in patients with CD4 count between 501- 1000, and the least

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prevalence of 60.4% was observed in patients with CD4 count between 0-250. The results of this study showed no significant difference between *E.histolytica* and CD4 count. Other three individuals reported to have 'other medical conditions' such as kidney and liver damage. These patients were found seropositive for *E.histolytica*, showing that the patients in this study had mixed infections, there is a possibility that the patients presented with liver damage can encounter amoebic liver abscess in fiuture

5. 2.2. Prevalence of antigens against *E.histolytica* in the study population

Amoebic liver abscess is the most common extra-intestinal manifestation of amoebiasis. It has been described as a tropical disease, a disease that is less prevalent in temperature climates, it is known as a summertime disease (Hoque *et al*., 2014 and MecGarr *et al*., 2003). However, its epidemiology and socio-demographic determinants are not well described in the literature. In this study, there were only three (0.5%) patients who had amoebic liver abscess. The observations of the present study are in agreement with the results obtained in a study by MecGarr *et al*., (2003) where only two patients were found with proven amoebic colitis. The low seroprevalence (0.5%) of ALA recorded in the present study is an indicative that this infection is uncommon in the study population. The study by Lie and Stanley, (1996), reported similar prevalence of 0.5% in travellers from Europe who develop ALA about 8 - 20 weeks after leaving an endemic area.

It has been documented that amoebic liver abscess evolves to fulminant necrotizing rupture or colitis in approximately 0.5% of cases (Aristizábal *et al*., 1991), the mortality rate due to amoebic liver abscess has fallen to 1-3% in the last century following appropriate or effective treatment (Stanley, 2003). A study by zeehaida *et al*., (2008) explained that diagnosis of ALA is sometimes difficult since its clinical manifestations are highly different. Although the Techlab *E.histolytica* II kit is meant for detection of antigens against *E.histolytica* in stools samples, our

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study have shown that this kit can also be used with serum samples because it is useful for detection of ALA. However, some studies have found the kit not useful for ALA detection (Zeehaida *et al*., (2008). A high prevalence of antigen against *E.histolytica* was obtained in 22 (96%) of 23 amoebic liver abscess using Techlab *E.histlytica* II kit (Haque *et al*., (2000).

5.3. PART 3: LEVELS OF INTERLEUKIN-10 IN SERO-POSITIVE AND SERO-NEGATIVE PATIENTS IN THE STUDY

Cytokines appear to be an important mediator of host immune response. They can be used as markers to determine the immune mechanisms underlying the outcome of amoebic infection (Bernin *et al*., 2014). Despite the high prevalence of intestinal amoebiasis, the host innate immune response as it relates to clinical outcomes of amoebic infection remains poorly characterized in humans, especially in the Limpopo province. Therefore, this study attempted for the first time to integrally evaluate the effect of IL-10 expression with the disease outcome in Limpopo province.

Out of the 467 serum samples tested for IL-10 levels, 225 were *E. histolytica* negative and 422 were *E. histolytica* positive by Techlab *E. histolytica* II ELISA in this study. The overall IL-10 expression between negative and positive patients was 34.8% and 65.2% respectively. The results of this study shows that *E. histolytica* positive patients produces more IL-10 than negative patients. Very few studies have associated the levels of IL-10 production with amebiasis. A study by Bernin *et al*., 2014, have shown that IL-10 serum levels were significantly increased in ALA patients , indicating that invasion of the liver tissue by *E. histolytica* elicit a anti-inflammatory immune response. The results of this study are in great agreement with the results obtained in the present study where elevated levels of Il-10 were increased only in *E. histolytica* positive

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patients and not in the negative group. The current study also showed that patients with high levels of IL-10 are likely to have low burden of amoebiasis compared to patients who produces low levels of IL-10 in their serum. The elevated levels of IL-10 shows that the patient is already in inflammation and the body is trying to bring the inflammation down. The findings of this study are in accordance with the results obtained by Bernin *et al.*, (2014), which suggested a protective role of IL-10 in ALA patients. This findings suggests that elevated levels of IL-10 in the serum play a significant role in the development of amoebiasis. The results of this study also showed that it is better to have high levels than low levels of IL-10 and that IL-10 can serve as a very important prognostic determinant (Florentino *et al.*, (1989). It has been suggested that IL-10 has several antiatherogenic effects, such as inhibition of adhesion of LDL proteins, activated monocytes to endothelium and down regulation of fibrinogen biosynthesis (Tedgui *et al.*, 2001) It modulates pro-inflammatory cytokines, such as TNF-α; IL-1, and IL-8, apoptosis of inflammatory cells and suppression of macrophage activation (Oberholzer *et al.*, 2002).

5.4. PART 4: THE IMPACT OF HOST GENETICS ON AMOEBIC INFECTION

5.4.1. Association of Interleukin-10 gene polymorphisms with amoebic infection

It has been suggested that human genetics does influence the outcome of amoebic infection, as for the present study we believe that development of a disease is multifactorial and it involves a combination of factors (Serrano-Luna *et al.*, 2013). Recent data have suggested that polymorphic variations in the promoter sequences of IL-10 gene may influence the gene expression and constantly play a role in susceptibility and clinical course of infections (Gibson *et al.*, 2001). Interleukin-10 has been chosen as one of the important inflammatory cytokine that is involved in regulation of the immune response and disease progression (Abodlrahim- zadeh, 2005). The goal

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of this study was to evaluate whether IL-10 gene promoter -592 A/C and -1082 A/G are associated with amoebic infection in the study population.

To the best of our knowledge this is the first study to associate IL-10 gene promoter polymorphisms with amoebiasis. Most of the studies have focused on the impact of these polymorphism with other diseases such as cancer (Kong *et al*., 2010, Howell and Rose- Zerilli., 2007), hepatitis (Helal., 2014), acute coronary syndrome (Srinkanth Babu *et al*., 2012), human papillomavirus cervical lesion development(Torres-Poveda *et al*., 2012) and no associations have been done on amoebiasis. In this study we evaluated the association between the polymorphisms of the IL-10 promoter and amebic infection. Our data did not show significant differences in genotypes between amoebic positive and negative patients. It has been reported that 50-75% of the variation in IL-10 is genetically controlled (Turner *et al*., 1997). IL-10 -1082 AA and AG genotypes are associated with low IL-10 production and GG is associated with high level of IL-10 production (Srinkanth Babu *et al*., 2012). Concerning the association of these genotypes with susceptibility to amoebiasis, this study showed that the -1082 AA genotype was higher in patients with amoebiasis (68%) compared to negative subjects (31.9%), and -1082 AG genotype was also found to be high in patients with amoebiasis (67.5%) than the negative patients (32.5%), these findings supports the results obtained with the IL-10 expression and levels in relation to disease presentation in this study, where it was shown that high IL-10 levels reduces or help to fight amoebic infection. In IL-10 polymorphisms, the homozygous AA and heterozygous AG genotypes were associated with susceptibility to amoebiasis. Therefore the patients with such genotypes tend to be more susceptible to amoebiasis.

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Interestingly, this study also found that IL-10 -1082 GG genotype which is associated with high IL-10 production, was associated with reduced risk to amoebiasis because the patients with this genotype (-1082 GG) were found to have low prevalence (66%) of *E.histolytica* infection Therefore the patients with the GG genotype of -1082 are not highly susceptible to amoebiasis. The results of other studies shows that low production of IL-10,AA and AG genotypes of -1082 reduce susceptibility to infections and are also associated with fatal outcomes in other diseases such as meningococcal (Anne *et al*., 1995), but the observations of the present study states otherwise. A study by Kong *et al*.,(2010) reported that -1082AA genotype is associated with increased risk of lymph node, which also, in the present study the AA genotype of -1082 was associated with increased risk of amoebiasis. Similar results were also obtained in a study by Howell and Rose- Zerilli., (2007), where IL-10 -1082 AA genotype was associated with susceptibility to disease. However, this results are not consistent with the study conducted in Australia, in which the IL-10-1082 AA genotype was associated with reduced risk of infection (Smith *et al*., 2004)

The results of this study shows that IL-10 genotypes might vary among different ethnic population. The results of the -592A/C polymorphism were also not associated significantly with amoebiasis in the study population. The homozogous CC and AA were high in amoebic patients (71.3% and 64.1 %) in comparison with the negative subjects (28.7% and 37.6%) respectively. The heterozagous AC -592 was associated with reduced risk of amoebiasis. This study revealed that -1082 AA, AG and GG or -592 AC are associated with amoebisis development. Our study suggests that -1082 GG and -592 AC may be related to protection against amoebic infection. A study by Howell and Rose- Zerilli., (2007), showed similar findings suggesting that -592 and 1082 alleles may be protective against inflammatory immune response

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5.4.2. Sequence analysis

Mostly, Sequencing is performed to confirm the results obtained from genotyping by restriction fragment length polymorphism. It is also done to identify sequence differences and variations such as point mutation and single nucleotide polymorphisms (Ye *et al*., 2011). In this study, about 30 samples found positive by PCR were sequenced in order to confirm the RFLP or genotyping results. Of the 30 samples sequenced, 28 samples came out with good sequences, and the two samples had bad sequences, so as a result they were excluded from the analysisSeveral variations and similarities representing the three (3) SNPs of IL-10 (-1082 A/, -592 A/C and -819 T/C) were obtained in the sequences. The variations were analysed into heterozygous and homozygous genotypes of IL-10 polymorphisms. The genotypes obtained by sequencing were then compared with the genotypes obtained by restriction analysis or RLFP, and the results were 100% consistent (figure 4.14)

5.5. OVERALL CONCLUSION AND RECOMMENDATIONS

5.5.1 Conclusions

The present results demonstrated a higher seroprevalence of intestinal than extra- intestinal amoebiasis. This study found that the patients have been in contact with the parasite and has respondend to it. About 65% individuals living in Polokwane in Limpopo province have been in contact with *E.histolytica* parasite. This findings shows that intestinal amoebisis is very common in the study population, while amoebic liver abscess has been found to be uncommon (0.5%) in the study population by ELISA. Therefore, the results of this study showed that the *E.histolytica* serology ELISA is useful for detection of intestinal amoebiasis. This study also shows that the

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Techlab *E.histolytica* II ELISA (designed for stools) can also be used for detection of ALA in the serum.

High levels of IL-10 expression in the serum demonstrates that there was already an inflammation and the body is trying to bring the inflammation down. Elevated levels of IL-10 in *E. histolytica* positive patients serve as a protection against the parasite. The higher the level of IL-10 in the serum the lower the infection. The statistical agreement between IL-10 levels and the IL-10 genotypes was poor. There was no significant correlation between IL-10 levels and IL-10 genotypes in the study.

This study report for the first time that IL-10 promoter polymorphisms participate in the progression of amoebiasis. IL-10 -1082 A/G polymorphism was significantly associated with amoebiasis, the homozygous AA and heterozygous AG of -1082 polymorphisms were associated with associated with susceptibility to amoebiasis, while the homozygous GG of -1082 polymorphism and the heterozygous AC of -592 were associated with reduced risk of amoebiasis development. Therefore, this study revealed that -1082 AC, AG and GG or -592 AC are associated with amoebiasis development and that these genotypes; GG and AC help the body to reduce amebiasis in the body.

5.5.2. Recommendations

The occurrence of *E. histolytica* in this study is of public health significance and if appropriate care is not taken could result in epidemic situation. Amoebiasis can affect anyone, however the disease mostly occurs in young and middle aged adults. The observation that this parasite affects young and old people in this study opens a new dimension as to the source of infection in old age and young group in the African setting. This study also observed that the infection is more

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prevalent in rural areas than urban. It is therefore recommended that the old aged, and the young group, as well as all the people in rural areas be educated properly on hygiene more especially on personal hygiene. In order to avoid an epidemic situation the following recommendations become important:

- ✓ Awareness campaign on *E. histolytica* for transmission is needed.
- ✓ People should be educated about safe storage of food and handling.
- ✓ Water from untreated source should be boiled.
- ✓ Toilets should be cleaned to avoid the spread of the disease

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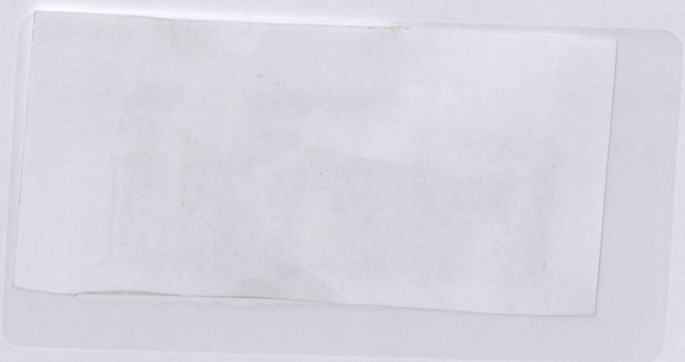

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