University of Venda

MOLECULAR CHARACTERIZATION OF *ENTAMOEBA HISTOLYTICA* tRNA GENES

A dissertation submitted in fulfillment of the requirements for the award of Master`s Science degree in Microbiology

BY

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DECLARATION

I, Davhana Caroline Ndivhudzannyi (Student number: 11560723), hereby declare that the dissertation for Master's Degree in Microbiology at the University of Venda, hereby submitted by me, has not been submitted previously for a degree at this or any other university, that this is my own work in design and execution, and that all references contained therein have been duly acknowledged.

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DEDICATION

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degrees Celsius
µl	Micro liter
µm	micrometer
AIDS	Acquired Immunodeficiency Syndrome
ALA	Amebic liver abscess
bp	Base pairs
BSA	Bovine serum albumin
CD4	Cluster of differentiation 4
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphates
EhCP1	*Entamoeba histolytica* cysteine proteases 1
EhCP2	*Entamoeba histolytica* cysteine proteases 2

EhCP5	*Entamoeba histolytica* cysteine proteases 5
ELISA	Enzyme linked immunosorbent assay
et al	Et alia (and others)
Gal/GalNAc lectin	Galactose/N-acetylGalactosamine inhibitable lectin
HIV	Human Immunodeficiency Virus
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-10	Interleukin-10
Kb	Kilobase
kDA	Kilodalton
LPPG	Lipophosphopeptidoglycan
Mb	Megabytes
Ml	Milliliter
MUC2	Mucus layer

mRNA	Messenger ribonucleic acid
Nm	Nanometer
NO	Nitric oxide
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PGM	Phosphoglucomutase
RNA	Ribonucleic acid
ROS	Reactive oxygen specie
RPM	revolution per minute
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
SNP	Single nucleotide polymorphism
SPSS	Statistical Package for the Social Sciences
SREHP	Serine-rich *Entamoeba histolytica* protein

ssRNA	Single stranded ribonucleic acid
SSu- rDNA	Small subunit ribosaomal deoxyribonucleic acid
STD	Sexual transmitted diseases
STR	Short tandem repeat
Taq	an enzyme obtained from – Thermus aquaticus
TB	Tuberculosis
TNF	Tumor necrosis factor
tRNA	Transfer ribonucleic acid
Tyr	Tyrosine
UNESCO	United Nations Educational Scientific and Cultural Organization
USA	United States of America
UV	Ultraviolet
V	Volts
WHO	World Health Organization.

ABSRACT

BACKGROUND: *Entamoeba histolytica* is a eukaryotic protozoan parasite responsible for the disease called amoebiasis. Amoebiasis is a major cause of morbidity and mortality in the developing world. It is well established that about 500 million people are infected worldwide, resulting in up to 100,000 deaths annually. It is not completely understood why some individuals once infected with *E. histolytica,* develop clinical amoebiasis while others remain asymptomatic. Very few studies have been conducted in order to determine the potential role of the parasite genomic features on the outcome of the infection. No studies have been done in South Africa to show how parasite genotypes play a role in determining the outcome of infection. Therefore, the present study determined the molecular characteristics of tRNA genes of *E. histolytica* in relation to the occurrence of diarrhea.

METHOD: In this study, patients were recruited from rural primary health care clinics in Giyani, Limpopo Province and private clinics in Pretoria, Gauteng Province. The participants were supplied with a consent form and their information was kept confidential. Diarrheal and non-diarrheal stool samples were collected. All the stool samples were observed under a light microscope for the presence of *E. histolytica* cysts and trophozoites. The Techlab *E. histolytica* II kit was used to detect the antigen against *E. histolytica.* Genomic DNA was extracted from 78 stool samples that were positive by ELISA using ZYMO RESEARCH fecal DNA mini Prep kit from inqaba biotech. A multiplex PCR protocol was used for the identification of *E. histolytica.* Specific primers for the different loci (NK, RR, AL, DA and S^{TGA}-D) of the tRNA genes of *E. histolytica* were used for genotyping. In this study, 15 stool samples from the 42 positive

samples identified by ELISA and confirmed by PCR were amplified for the NK locus using the tRNA specific primers NK-3 and NK-5. The genotyping as well as the data were analyzed using the Statistical Package for Social Sciences (SPSS for WINDOWS version 18.0) program in order to determine the potential implications of *E. histolytica* infection.

RESULTS: A total of 774 stool samples were collected and it was found that, out of 774 stool samples examined by wet mount microscopically 16.7% were infected with *E. histolytica* cysts and trophozoites. The TechLab ELISA based antigen detection kit specific only for *E. histolytica* in stool samples revealed that 10.1% were positive for *E. histolytica*. The highest prevalence of *E. histolytica* was found in Pretoria with 10.5%, as compared to Giyani which was 5.4% and the difference was not statistically significant (X^2= 1.491; P= 0.222). *Entamoeba histolytica* was more common in males (12%) than in females (8.4%), but the difference was not statistically significant (X^2= 2.653; P= 0.103). Most of the participants who were infected were aged between 26-45 years with 21.2% followed by those who were in the age group 1-25 years with 16.8%. The least infected were of the age group 49-90 years with 8.2%. However, the difference was not statistically significant (X^2= 3.341; P= 0.188). According to samples consistency, the highest prevalence was found in watery stool samples with 13.6%, followed by soft with 11.1% and the least was formed with 5.9%, but the difference was not statistically significant (X^2= 5.781; P= 0.056).

Forty-two samples showed positive for *E. histolytica* small-subunit rRNA gene. Nine (9) different banding patterns were obtained for NK locus. The ratio of the profile and the number of samples tested was therefore three profiles for every 5 samples. Two hundred base pairs was the most common band that occurred five times in different samples as compared to 150bp and

250bp that occurred twice. Other bands observed included 120bp, 300bp, 350bp, 500bp, 600bp and 750bp which occurred only once each. Out of the 42 positive samples identified by ELISA and confirmed by PCR the RR locus was amplified in 30 samples using the tRNA specific primers RR-3 and RR-5. This gave a success rate of 71%. The product sizes of 150, 200, 250, 300, 400,450, 500, 550, 600 and 750bp were obtained. It was observed that 150bp and 250bp occurred only once. A total of 16 profiles were obtained giving a ratio of 0.53.

Out of the 42 positive samples identified by ELISA and confirmed by PCR, the AL locus was amplified in 25 samples using the tRNA specific primers AL-3 and AL-5. This gave an amplification success of 59.5%. The product size of 150, 180, 200, 220, 300, 350, 400,450, 500, 550, 600 and 1000bp were obtained. A total of 15 profiles were obtained for a ratio of 0.6. The band size of 200bp was seen in most of the samples, followed by 150, 180, 220, 300, 350, 400,450, 500, 550, 600 and 1000bp. It was observed that 150bp and 550bp occurred only once.

Thirteen (31%) stool samples from the 42 positive samples identified by ELISA and confirmed by PCR were amplified for the DA locus using the tRNA specific primers DA-3 and DA-5. The product sizes of 150, 200, 280, 300, 500 and 1200bp were obtained. One hundred and fifty base pair was seen in most of the samples, followed by 300 and 500bp bands. It was observed that bands of 200bp, 280bp, and 1200bp occurred only once.

Nine (21%) stool samples from the 42 positive samples identified by ELISA and confirmed by PCR were amplified for the S^{TGA}-D locus using the tRNA specific primers S^{TGA}-D -3 and S^{TGA}-D -5. The product size of 150, 180, 200, 220, 300, 350, 400,450, 500, 550, 600 and 1000bp were obtained. From the 9 samples that amplified for the S^{TGA}-D locus, bands of 150, 200, 280 and

400bp was seen in most of the samples. It was observed that 100, 240, and 300bp occurred only once. All samples were from Pretoria.

CONCLUSION: The present study indicates that infection caused by *E. histolytica* was prevalent in diarrheal samples obtained from Pretoria. *Entamoeba histolytica* infection was more prevalent in males than in females, and in the age group of 26- 45 years. The possible cause of infection in the stools of patients in this study could possibly be due to various factors such as drinking water from unprotected source or by direct contact with infected animals. According to our results, icroscopy is a simple method and it should be combined with other methods such as ELISA and PCR for identification of the species to avoid false and/or insufficient diagnosis and treatment applications.

Loci NK, RR, AL, DA and S^{TGA}-D of the tRNA genes identified in this study show promise as surrogate markers for prediction of infection outcome of *Entamoeba histolytica*. The findings of this study therefore suggest that further studies are needed to evaluate the prevalence, heterogeneity and combination of virulence-related genes in *E. histolytica* infection as well as their association with diarrhea and non-diarrhea.

Key words: *Entamoeba histolytica*, Genotyping, South Africa

CHAPTER 1

INTRODUCTION AND OBJECTIVES

1.1. General Introduction

Entamoeba histolytica is a eukaryotic protozoan parasite that infects the digestive tract of predominantly humans and other primate's, and is part of the genus *Entamoeba (*Ryan *et al.*, 2004). *E. histolytica* is responsible for a disease called amoebiasis, which remains an important health problem in developing countries, especially in areas where sanitation infrastructure and water supply are inadequate (Ximenez *et al.*, 2009). Infection by *E. histolytica* is normally initiated by the ingestion of fecal contaminated water and food which contain *E. histolytica* cysts. Approximately 10% of the world's population is stated to be infected with *E. histolytica*, resulting in up to 100 000 deaths worldwide each year (Haque *et al.*, 2003 and Petri *et al.*, 2000). The three species *E. histolytica*, *E. dispar* and *E moshkovskii* are morphologically identical but pathologically distinct. *Entemoeba histolytica* is the only pathogen that causes invasive diseases in humans, while *E. dispar* is a non- pathogenic commensal species that has been documented to occasionally cause disease in humans (Helmy *et al.*, 2007).

Some studies suggest that a few strains in *E. dispar* may be able to produce liver abscesses in hamsters (Shibayama *et al.*, 2007). However, *E. histolytica* and *E. dispar* species have a similar host range but they have different properties with regard to pathogenicity in vivo (Jetter *et al.*, 1997). Furthermore, both *E. histolytica* and *E. dispar* are able to colonize humans but only *E.*

histolytica is a potential pathogenic ameba that can cause serious damage to intestinal and other extra-intestinal organs, mainly the liver. Recent studies suggest that infection with *E. moshkovskii* which is a non- pathogenic intestinal parasite species, has been detected in individuals inhabiting endemic areas of amoebiasis and is also common in some areas of *E. histolytica* endemicity (Ali *et al.*, 2003; Fotedar *et al.*, 2008; Khairnar *et al.*, 2007 and Parija *et al.,* 2005). Light microscopy is still the only tool used for the detection of *E. histolytica* in most countries but it cannot distinguish between the morphologically identical yet genetically distinct three species of *Entamoeba*. Consequently, it is now believed that epidemiological figures on the disease and its spread are overemphasized since previous studies relied on microscopic identification only (Ali *et al.*, 2008). It has been reported that *E. dispar* acts as a potential agent capable of inducing intestinal and liver damage similar to that observed with *E. histolytica* (Ximenez *et al.*, 2010).

Entamoeba histolytica is a parasite of global distribution, but most of the morbidity and mortality from amoebiasis occurs in central and South America, Africa and Indian subcontinent. *E. histolytica* occurs primarily in developing countries due to poor sanitation, men who have sex with men and is more common in older patients (Haque *et al.*, 2003). It is well established that *E. histolytica* infections result in variable clinical outcomes. Most infections remain asymptomatic, only a few develop extra-intestinal complications such as liver abscesses, and some develop diarrhea and dysentery.

In tropical regions, *E histolytica* is more common among patients attending health care with diarrhea. *E. histolytica* is estimated to affect an estimated 50 million people worldwide (Ximenez

et al., 2009), but most of whom are children residing in developing countries (WHO, 2000). Diarrheal disease affects people of all ages and is one of the greatest causes of morbidity and mortality throughout the world. Diarrhea is a major contributor to childhood mortality and morbidity in the developing world, causing an estimated 2.5 million deaths each year and long-term effects on growth and cognitive function (Bern *et al.*, 2003 and Murray *et al.*, 1997). Similarly, in Dhaka Bangladesh, 80% of children studied prospectively were infected with *E. histolytica* at least once during four years of follow up. Furthermore, *E. histolytica* associated with diarrhea in these children was associated with low weight and height for age.

In order to investigate whether the parasite genotypes contribute to the outcome of infection with *E. histolytica*, a reliable genotyping method was required. A number of methods for detecting *E. histolytica* have been described over the years (Clark, 2006), but Ali *et al.*, (2005) have recently described a PCR-based approach that is highly sensitive and discriminatory. In these study six loci of the tRNA genes of *E. histolytica* was used for genotyping.

1.2. Rationale of the study

It is known that out of 100 people who get infected with *E. histolytica,* only 10 people get the disease. Very few studies have been conducted to determine the potential role of the parasite genomic features on the outcome of infection. Recent studies have investigated the relationship between parasite genotypes and the clinical outcome of infection using clinical samples from Bangladesh and a six-locus genotyping system based on tRNA-linked STRs (Haghighi *et al.*, 2002; Ali *et al.*, 2005 and Tawari *et al.*, 2008). Results of studies in Bangladesh and China have provided evidence that the parasite genome does influence the outcome of infection. The tRNA-linked STR genotyping was also behind the recent observation of differences between parasite genotypes in the intestine and the liver abscesses of the same patient (Ali *et al.*, 2007 and Feng *et al.*, 2012). Moreover, of the six pairs of tRNA-linked STRs, evidence emerged linking the tRNA-linked locus R-R sequence type with the outcome of infection (Ali *et al.*, 2012). In spite of these limited observations, no studies have been done in South Africa to show how parasite genotypes play a role in determining the outcome of infection.

1.3. Objectives of the Study

1.3.1. General objective

The primary objective of this study was to determine the molecular characteristics of *E. histolytica* in relation to the occurrence of diarrhea among patients attending a rural primary health care clinic and private clinics in the Giyani and Gauteng regions, of South Africa.

1.3.2. Specific objectives

(1) To determine the prevalence of *E. histolytica* infection from stool samples using microscopy and Enzyme-Linked Immunosorbent Assay (ELISA).

(2) To identify different genotypes of *E. histolytica* based on the tRNA genes that circulate in the population in a rural area and in an urban population.

(3) To identify any potential association that may exist between parasite genotype and the presence of diarrhea in the patients.

CHAPTER 2
LITERATURE REVIEW

2.1. History of *E. histolytica*

Entamoeba histolytica is the causative agent of amoebiasis that was first recognized as a deadly disease by Hippocrates who diagnosed a patient with fever and dysentery (460 to 377 B.C.). In 1875, Dr Friedrch Alexandravich Losch described the presence of motile trophozoites in the dysenteric stool of a Pietersburg labourer which he named *Entamoeba coli* (Kean, 1988). He successfully reproduced the clinical picture of dysentery in four out of five dogs by inoculating the amoebae obtained from his patient into the rectum of these experimental animals. Furthermore, he demonstrated ulcerative lesions teeming with *Entamoeba coli* in the colons of these dogs at autopsy. These lesions were identical to those found in his patient on post mortem examination. In spite of all this evidence Losch remained unconvinced that the *Entamoeba* was of any etiological importance in the causation of dysentery. He believed that the bacteria were the primary etiological agents in the ulcerative process resulting in dysentery and that the presence of *Entamoeba coli* served merely to perpetuate the inflammatory reaction.

The study done by Walker *et al*., (1913), demonstrated the cyst form of *E. histolytica* as an infective stage. In 1925, Brumpt proposed that *E. histolytica* and *E. dispar* were identical morphologically and suggested that they should be named as pathogenic and nonpathogenic species respectively.

2.2. Taxonomy and Classification

Entamoeba histolytica is an anaerobic protozoan parasite that has been classified as eukaryotes in the kingdom Protista, Subkingdom Protozoa, phylum Sarcomastigophora, subphylum Sarcodina (which contain both free-living and parasitic members characterized by the presence of pseudopodia that are used for the movement and uptake of food). Class Lobosea, Order Amoebida. *Entamoeba* contains many species, three of which are *E. histolytica, E. dispar* and *E. moshkovskii.* Out of the three species, *E. histolytica* is the only pathogen that causes invasive diseases in human, while *E. dispar* and *E moshkovskii* are considered as non- pathogenic commensal species which have never been documented to cause diseases in humans (Helmy *et al.*, 2007). The genus *Entamoeba* belongs to the Family *Entamoebidae*. It is described as distantly related to the genera *Mastigamoeba* and *Dictyostelium*, which together form the monophylum Conosa (Clark, 2000 and Bapteste *et al.*, 2002), and the species is *histolytica*.

2.3. Epidemiology of *Entamoeba histolytica*

2.3.1. Prevalence of *Entamoeba histolytica* in children

Entamoeba histolytica related diarrheal illnesses have been reported to have a negative impact on the growth of children (Mondal *et al.*, 2006). A study by Haque *et al.*, (1999) indicated that diarrheal diseases are the leading cause of childhood death, approximately 50% of children have serological evidence of exposure to *E. histolytica* by 5 years of age in Bangladesh. A prospective study of preschool children in a slum of Dhaka, Bangladesh demonstrated new *E. histolytica*

infection in 39% of children over a one year period of observation, with 10% of the children having an *E. histolytica* infection associated with diarrhea and 3% with dysentery (Haque *et al.*, 2006). Higher (41%) prevalence of *Entamoeba histolytica* between the age group of 6-14 years in South Kanara district, Karnataka was recorded by Subbannayya *et al.*, (1989). Similarly, Shetty *et al.*, (1990) recorded high prevalence of *Entamoeba histolytica* in children under the age group of 0-6 month (12.5%) and 7-12 month (20.3%) in a southern Indian population. Furthermore, 27% prevalence of *E. histolytica* in children < 15years has been reported by Waqar *et al.*, (2003) in Northern Pakistan. *Entamoeba histolytica* infection is more prevalent in younger age groups. These phenomena could be explained by the fact that younger children are more exposed to overcrowded conditions like playgrounds, schools, etc. Furthermore, the parasitic infections may be due to the poor sanitary conditions in the schools (Oguntibeju, 2006). Most people do not care about personal hygiene, for example, lack of fecal hygiene (Abu Mourad, 2004), playing in contaminated outdoor environments, in and around disposal sites, and lack of washing hands before meals (Nematian *et al.*, 2004).

2.3.2. Prevalence of *Entamoeba histolytica* in HIV positive patients

Multiple studies have reported a higher prevalence of amebic seroconversion, invasive amoebiasis and amebic liver abscesses (ALAs) among HIV-positive individuals compared with comparable groups of HIV-negative individuals (Park *et al.*, 2007 and Chen *et al.*, 2007).There have been controversies around the impact of HIV on the occurrence of invasive amoebiasis. Earlier reports suggested that invasive amoebiasis was not increased among patients with HIV infection (Jessurum *et al.*, 1992).

A study reported 18 (6.1%) cases of invasive amoebiasis (IA) in 296 HIV-infected Taiwanese patients, and suggested that invasive amoebiasis is an emerging parasitic infection in HIV-infected patients in non-disease-endemic areas as well as disease-endemic areas (Hung *et al.*, 1999). Furthermore, 31 patients suffering from amebic liver abscess at Seoul National University Hospital from 1990 to 2005, 10 (32%) were HIV-positive (Park *et al.*, 2007). In countries such as Japan, Mexico, Taiwan and South Africa, studies have shown an increase in the occurrence of *E. histolytica* among HIV patients (Moran *et al.*, 2005; Hung *et al.*, 2008; Samie *et al.*, 2009 and Watanabe *et al.*, 2011).

In a South African study in the Vhembe district, Limpopo Province, a positive association between *E. histolytica* infection and HIV positive individuals were been indicated. Among the HIV-positive individuals, those with CD4+ count less than 200 cells/µl, were found to be more likely seropositive for *E. histolytica* (Samie *et al.*, 2010). However, in a Chinese study, a higher seroprevalence of *E. histolytica* infections was also found in HIV-infected patients (Chen *et al.*, 2007). Furthermore, two studies conducted in Taiwan revealed a positive association as well (Hung *et al.*, 2005 and Tsai *et al.*, 2006).

In Japan, *E. histolytica* often occurs in institutions of mentally retarded individuals where outbreak of amoebiasis have been described with the prevalence and positive serology rate as high as 38.2% and 67.1%, respectively and occurring more often in HIV positive patients (Watanabe *et al.*, 2011). Men who have sex with men have higher rates of seropositive for *E. histolytica* based on a study from Taiwan within the HIV positive population, which examined HIV patients over an 11-year period (Hung *et al.*, 2008). Furthermore, a higher incidence of

amebic liver abscesses among men who have sex with men than in other HIV-positive populations have been reported (Park *et al.*, 2007; Hung *et al.*, 2008 and Stark *et al.*, 2007).

2.4. Transmission of *E. histolytica*

The active trophozoite stage of *E. histolytica* is known to exist only in the host and in fresh loose stool, while the cysts survive outside the host in water, soil and in foods (Gatti *et al.*, 1995). When the cysts are swallowed, they cause infection by existing in the digestive tract. The infection can be asymptomatic or can lead to symptomatic infections, that lead to amoebic dysentery or amebic liver abscesses (Moran *et al.*, 2005).

Transmission of *E. histolytica* generally occurs through ingestion of cysts from food or water contaminated by feces. The hands have been proven to be the common denominator in transferring pathogens from surface, food, animals to humans (Alum *et al.*, 2010). The consumption of unwashed fruits or contact with vegetables in the market could also lead to infection. Sometimes these fruits are brought from the remotest areas of the state where hygienic precautions are not taken during the packaging and transportation of the fruits. The vegetables consumed are mostly farmed along river banks during the dry season and are directly watered from the river without any form of water treatment thus could definitely lead to the contamination of the crops by these parasites.

Entamoeba histolytica infection can also be transmitted through unprotected oral-anal sexual contact. The infection is also confirmed to occur in homosexual men through oral-anal and oral-

genital (Stark *et al.*, 2008). This infection could also come from contaminated well water which most families depend on.

2.5. Life cycle of *E. histolytica*

Entamoeba histolytica exists in two forms which are trophozoites or the dividing form and the cyst which is the dormant form. Humans are infected with *Entamoeba histolytica* through the consumption of contaminated food and water containing the cysts (Marshall *et al.*, 1997). *Entamoeba histolytica* can also be transmitted from person to person contact (Petri *et al.*, 1999).

The cysts measure approximately 10-20μm in diameter. The trophozoites are responsible for tissue invasion and measure approximately 10-60μm in diameter and contain single nucleus with central karyosome (Gareia, 1999). Mature cysts in the large intestine leave the host in large numbers and remain viable and infective in a moist, cool environment for at least 12 days. Cysts can live in water for up to 30 days. Nonetheless, they are rapidly killed by temperatures below 5°C and above 40°C. Mature cysts are also resistant to chlorine levels normally used to disinfect water (Ximenez *et al.*, 2011).

When swallowed, cysts pass through the digestive system to the small intestine where excystation occurs and as a result of nuclear division, eight motile trophozoites are produced (Neva *et al.*, 1994; Katz *et al.*, 1989; Gareia, 1999 and Petri *et al.*, 1999). These motile trophozoites travel to the large intestine, where they multiply via binary fission and colonize the intestinal lumen. Encystation occurs. Both cysts and trophozoites are then passed in the feces to the external environment (Fig 1). Cysts can persist for weeks in the external environment, while

the trophozoites survive only for a few hours. Trophozoites play no role in transmission of the disease but are responsible for producing tissue pathology. Trophozoites may invade the intestinal mucosa causing dysentery and/or progress through the blood vessels to extra-intestinal locations like liver, brain and lungs, where they may form life-threatening abscesses. Amoebic abscesses (invasive infection) most commonly occur in the liver. Figure 1 show the life cycle of *E. histolytica.*

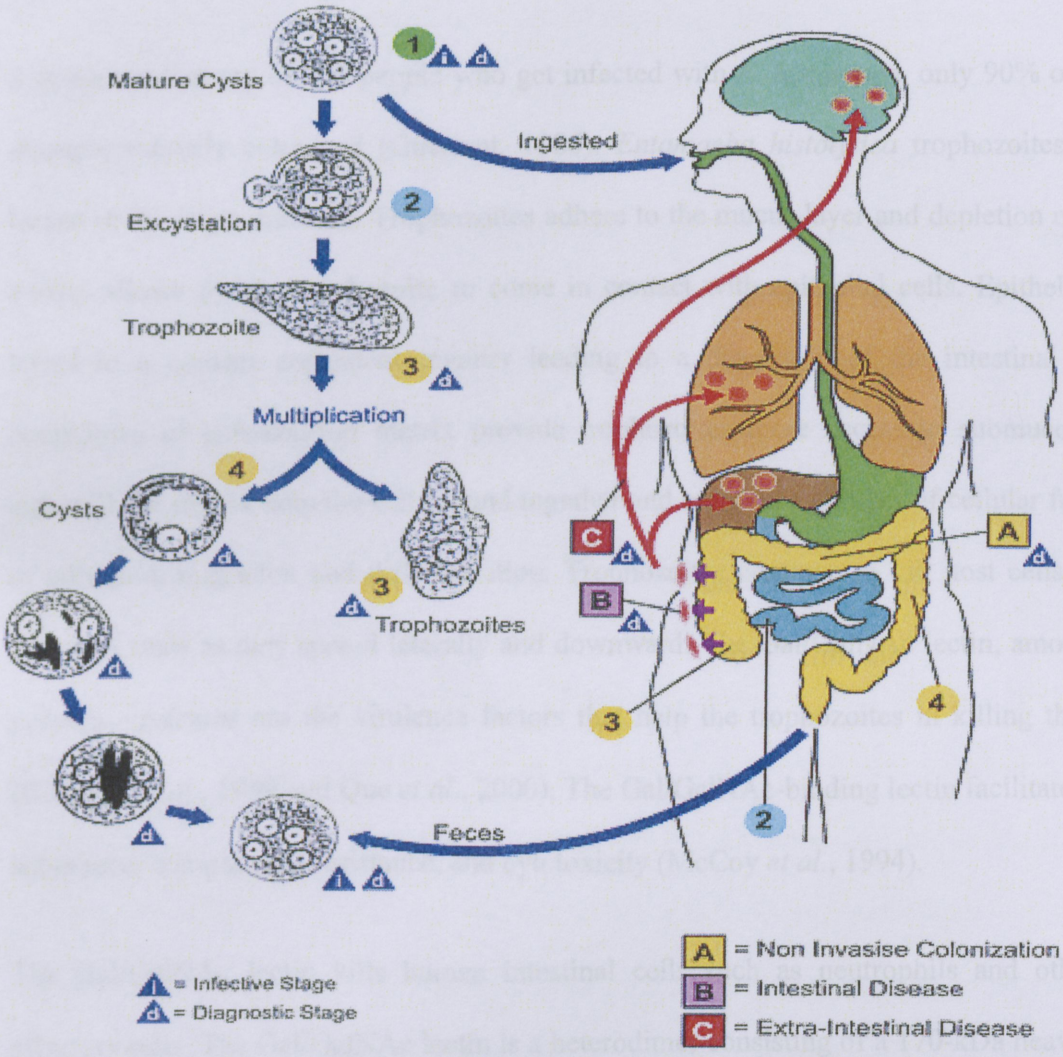


Figure 1: Life cycle of *Entamoeba histolytica* (Ximenez *et al.*, 2011)

2.6. Pathogenesis of *E. histolytica*

It is known that out of 100 people who get infected with *E. histolytica*, only 90% of people are asymptomatically colonized (Guerrant, 1986). *Entamoeba histolytica* trophozoites live in the lumen of the large intestine. Trophozoites adhere to the mucus layer and depletion of the mucus barrier allows for the trophozoite to come in contact with epithelial cells. Epithelial cells are killed in a contact dependent manner leading to a disruption of the intestinal mucosa. A breakdown of extracellular matrix provide trophozoites more access to submucosa, and the extracellular matrix help the cell to bind together and regulate a number of cellular function such as adhesion, migration and differentiation. Trophozoites continue to kill host cells and further disrupt tissues as they spread laterally and downward. The Gal/GalNAc lectin, amoebapore and cysteine proteases are the virulence factors that help the trophozoites in killing the host cells (Gilchrist *et al.*, 1999 and Que *et al.*, 2000). The Gal/GalNAc-binding lectin facilitates target cell adherence, complement resistance, and cytotoxicity (McCoy *et al.*, 1994).

The Gal/GalNAc lectin kills human intestinal cells such as neutrophils and other immune effectors cells. The Gal/GalNAc lectin is a heterodimer consisting of a 170-kDa heavy chain and 31 to 35 kDa light chain joined by disulfide bonds (Petri *et al.*, 1989) and non-covalently associated with a 150-kDa intermediate subunit (Clark, 1998). Once the trophozoites develop contact with the host cell, it causes cytolysis which then results in swelling and lysis of the 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 capable of killing metabolically

active eukaryotic cells and display antibacterial activity. Another virulence factor of *E. histolytica* are cysteine proteases. There are 84 proteinase genes from *Entamoeba* genome study, fifty of which encode for cysteine proteinases (Tillack *et al.*, 2007). Cysteine proteinases degrade the extracellular matrix of the colon, allowing for trophozoites invasion into the bloodstream (Hou *et al.*, 2010). Cysteine proteinases have also been shown to degrade IgA and IgG, antibodies needed to activate the host immune system (Que *et al.*, 2003). This attachment and penetration of host tissue and evasion of host immune system allows for potential dissemination of metastatic infections (Que *et al.*, 2000).

Although there are fifty cysteine proteinases genes in *E. histolytica*, 90% of the total proteinase activity in vitro is derived from three cysteine proteinases: EHCP1, EhCP2 and EhCP5 (Melendez-Lopez *et al.*, 2007). Of these three highly expressed and secreted proteinases, only EHCP1 and EhCP5 are present in the invasive strain *E. histolytica* versus the noninvasive strain *E, dispar*, implicating their potential importance in colonic invasion (Hou *et al.*, 2010). EhCP1 mRNA expression is increased two fold during invasion in the mouse cecal model, while EhCP5 is not (Que *et al.*, 2000). EhCP5 also plays an important role in the invasive process. EhCP5 is likely involved in the processing of other proteinases as over expression of EhCP5 by episomal transfection leads to the increased activity of EhCP1, EhCP2 and EhCP5 (Tillack *et al.*, 2006). 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). The trophozoites can also alter the integrity 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 parasite expresses surface perioxiredoxin, which neutralize both reactive oxygen species (ROS) and nitric oxide (NO) produced by the host to prevent the parasite from invading the sub-epithelium, thus facilitating parasite invasion (Davis *et al.*, 2006). Cytokines such as IL-1β, IL-1α, IL-8 and TNF-α are suspected of aggravating the disease process and driving the immunopathogenesis mechanism (Kammanadiminti *et al.*, 2003). Although neutrophils are known to cause intestinal tissue damage they are nevertheless critical for controlling the infection. Nonetheless, host and/or parasite factors normally play a role in determining whether the parasite is cleared or the disease becomes established (Asgharpour *et al.*, 2005). Figure 2 shows the pathogenesis of *E. histolytica.*

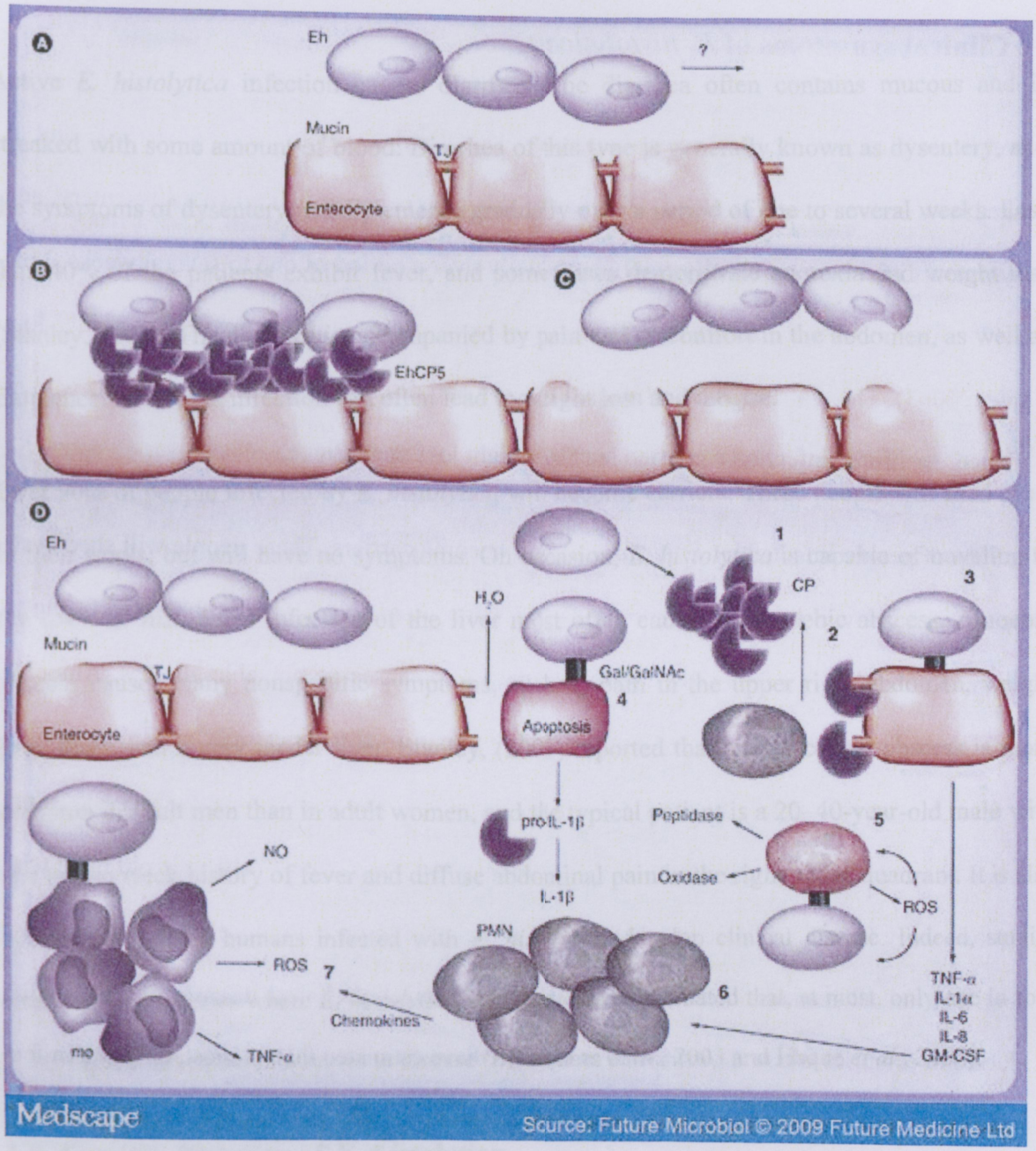


Figure 2: Systematic diagram of *E. histolytica* pathogenesis (Lejeune *et al.*, 2009).

2.7. Clinical symptoms of *E. histolytica*

Active *E. histolytica* infection causes diarrhea. The diarrhea often contains mucous and is streaked with some amount of blood. Diarrhea of this type is generally known as dysentery, and the symptoms of dysentery usually emerge gradually over a period of one to several weeks. Less than 40% of the patients exhibit fever, and some cases demonstrate anorexia and weight loss (Stanley, 2003). The diarrhea is accompanied by pain and discomfort in the abdomen, as well as flatulence. A chronic infection can often lead to weight loss and fatigue.

Over 90% of people infected by *E. histolytica* will become carriers. These people will shed cysts in their stools, but will have no symptoms. On occasion, *E. histolytica* is capable of traveling to the liver. *E. histolytica* infection of the liver most often causes an amoebic abscess. Amoebic abscess causes many nonspecific symptoms, such as pain in the upper right abdomen, weight loss, fever, and a very tender liver. Stanley, (2003) reported that amoebic liver abscess is more common in adult men than in adult women, and the typical patient is a 20–40-year-old male with one to two week history of fever and diffuse abdominal pain in the right upper quadrant. It is also evident that not all humans infected with *E. histolytica* develop clinical disease. Indeed, studies conducted in countries where *E. histolytica* is endemic have estimated that, at most, only one in four *E. histolytica* infections progresses to disease (Blessmann *et al.*, 2003 and Haque *et al.*, 2006).

2.8. Genetic diversity of *E. histolytica*

It is not completely understood why some individuals once infected with *E. histolytica* develop clinical amoebiasis while others remain asymptomatic. Host factors and strain variation of the *E.*

histolytica are probably involved. Polymerase chain reaction (PCR) and SNP typing are molecular tools to study genetic diversity observed in the various strains of *E. histolytica* and their pathological significance (Paul *et al.*, 2007). The studies using different genetic markers such as SREHP and tRNA gene linked STR have demonstrated that the parasite genotype does play a role in the outcome of infection in humans thus linking the parasite diversity and gene which will help in understanding the parasite virulence and pathogenesis (Clark, 1993; Ayeh-kumi *et al.*, 2001 and Ali *et al.*, 2007).

In general, studies using repetitive DNA as markers of diversity, have reported high diversity among genomes. A study by Samie *et al.*, (2008) indicated that certain SREHP profile might be responsible for the presentation of intestinal amoebic symptoms. PCR, restriction fragment length polymorphism and sequence analyses of SREHP and chitinase genes have also revealed the genetic diversity among the strains. A recent study by Ali *et al.*, (2007) using six tRNA linked polymorphic markers indicated a clear association between the genetic profiles of *E. histolytica* and disease presentation. A population differentiation in the genotype distribution was found in four of the six individual markers as well as in the combined genotypes, suggesting that the parasite genome does contribute in some way to the outcome of *E. histolytica* infection.

2.9. Diagnosis of *E. histolytica*

2.9.1. Microscopy

E. histolytica has been diagnosed by microscopy of the stool through identification of either cysts or trophozoites. Microscopy is the only method for diagnosing *E. histolytica* infection for over 100 years, but even though it is not possible to differentiate between two distinct species *E.*

histolytica and *E. dispar* by microscope due to their morphological similarity. Direct smear examples, either as wet mount or fixed and stained has been used for many years by microscopic examination of stool. Microscopic examination of a wet mount is a very insensitive method (<10%) which is performed on a fresh specimen (Huston *et al.*, 1999). The disadvantage of microscopy is that it is insensitive, nonspecific and less reliable in identifying *Entamoeba* species. Furthermore, microscopy is still the technique of choice used in many parasitology laboratories worldwide.

2.9.2. Stool culture

Stool culture technique followed by isoenzyme analysis has been considered as the "gold standard" for many years. This method has been used to distinguish between *E. histolytica* and *E. dispar*. A culture of *E. histolytica* can be performed from fecal specimens. *E. histolytica* or *E. dispar* cysts samples from the microscopy are washed and inoculated into sterile culture media. This can then be incubated at 37°c and examined for growth of cysts, and it can be seen on the wall of the test tube or in the debris if present. However, the process usually takes between one to four weeks to perform.

Monophasic media that have been developed include the egg yolk infusion medium of Balamuth (Balamuth, 1946), Jones's medium (Jones, 1946), and TYSGM-9 of Diamond (Diamond, 1982). Currently the most widely used media for xenic cultivation include the diphasic Locke-egg, Robinson, and the monophasic trypticase yeast extract serum gastric mucin (TYSGM-9) media (Haque *et al.*, 1995), while TYI-S-3332 is the most widely used medium for axenic cultivation. The major disadvantage is that none of the existing culture methods are selective for *E*

histolytica, and therefore they are not suitable or reliable for routine diagnosis. Culture of *E. histolytica* in a clinical diagnostic laboratory is not feasible as a routine procedure and is less sensitive than microscopy as a detection method (Clark *et al.*, 2002). Parasite cultures are difficult, expensive, and labor-intensive to maintain in the diagnostic laboratory (Clark *et al.*, 2002).

2.9.3. Polymerase chain reaction

Different type of PCR assays for the specific detection and differentiation of *E. histolytica*, *E. dispar* and *E. moshkovskii* DNA´s in fecal specimens are available (Tanyuksel *et al.*, 2003; Paul *et al.*, 2007; Gutierrez-Cisneros *et al.*, 2010 and Haque *et al.*, 2010). Several groups have developed a variety of excellent conventional PCR assays, targeting different genes, for the direct detection and differentiation of *E. histolytica*, *E. dispar*, and *E. moshkovskii* DNA in stool and liver abscess samples (Tanyuksel *et al.,* 2003 and Paul *et al.*, 2007). The SREHP gene is the target gene used in PCR to study the epidemiology and genotypes of *E. histolytica* in human (Stanley *et al.*, 1990). It was replaced by the use of PCR amplification of tRNA gene-linked STRs which in addition to providing details of the epidemiology of *E. histolytica*, also provides a tool to predict the outcome of the infection (Ali *et al.*, 2005).

PCR assays are powerful, highly sensitive, and useful for the differentiation of *E histolytica* and *E dispar* and for genetic typing of isolates. PCR methods can be used to detect *E. histolytica* in stool, tissues and liver lesion aspirates. Multiplex PCR, restriction fragment length polymorphism and real-time are also designed for differential detection of *E. histolytica* and *E. dispar* and have been developed (Clark *et al.*, 1992). PCR and real-time PCR are very sensitive

methods that can be used to detect several different pathogens and also to diagnose amoebic liver abscess (Singh *et al.*, 2009 and Roy *et al.*, 2005). However, Real Time PCR is faster and more sensitive than the conventional PCR, and is characterized by the elimination of gel analysis and other post-PCR analysis, thus reducing the risk of contamination and cost (Klein, 2002). Furthermore, real-time PCR is a quantitative method and allows the determination of the number of parasites in various samples (Fotedar *et al.*, 2007).

2.9.4. Enzyme-linked immunosorbent assay (ELISA)

ELISA is a test that uses antibodies and color change to identify a substance. ELISA is the only test on the market that specifically detects *E. histolytica*. ELISA indicates the presence of the organism in feces as a diagnostic aid. ELISA is reliable, easy to use and provides a rapid method for the diagnosis of *E. histolytica* infections especially in developing countries. It is widely used for the study of epidemiology and diagnosis of symptomatic amoebiasis (intestinal and/or extra intestinal). ELISA is the only method that is widely used in endemic areas because it is less costly and easy to perform (Buss *et al.*, 2008). The *E. histolytica* ELISA kit detection contains specific antibodies for *E. histolytica* that recognize an antigen on the surface of the trophozoites only, which are generally identified in diarrhea and not in the cystic stage of the parasite. This test is only used on fresh or frozen samples but not on preserved sample.

2.10. Treatment of *E. histolytica*

Asymptomatic carriers of *E. histolytica* should be treated in order to minimize the spread of the disease and the risk of developing invasive disease (Stanley, 2003). Paromomycin and

diloxanide furoate, the drugs recommended for the treatment of asymptomatic *Entamoeba histolytica* infection have been used for more than 30 years in clinical practice (WHO *et al.*, 1997). Due to the lower cure rate reported with the use of diloxanide furoate in symptomatic patients, some have suggested that paromomycin is more preferable (McAuley *et al.*, 1992). Nitroimidazole is highly effective against the trophozoite and has little effect on the cysts form of amoeba. Invasive disease responds to nitroimidazoles such as metronidazole or tinidazole, which have no effect on intraluminal carriage (Haque *et al.*, 2003).

2.11. How can *E. histolytica* be prevented?

When traveling in tropical countries where poor sanitation exists, only drink bottled or boiled water. Water can be made safe for drinking by filtering it through an absolute 1 micron or less filter and dissolving an iodine tablet in the filtered water. Clean uncooked fruits and vegetables with boiled or filtered water. Avoid eating the fruits and vegetables that are peeled by someone. Avoid milk, cheese or dairy products that may not be pasteurized, and also anything sold by street vendors. Safer sex measures, such as the use of condoms and dental dams for oral or anal contact, may help prevent infection. To prevent spreading the infection to others, one should take care of one´s personal hygiene. Always wash your hands thoroughly with soap and warm running water after using the toilet, changing nappies and before preparing, touching or eating food, and dry your hands properly after washing (Schuster *et al.*, 2007).

2.12. General structure of tRNA

A Transfer RNA is a small RNA molecule that is approximately 73 to 94 nucleotides long and folds back on itself to create three dimensional structures (Dirheimer *et al.*, 1995). However, tRNAs are present in all living organisms. Its structure is in the form of a cloverleaf, with the loop at the bottom containing the anticodon. The anticodon is a sequence which forms three bases which is meant to bind to the codon of a messenger RNA during the synthesis of a protein. However, mRNA encodes a protein as a series of contiguous codons, each of which is recognized by a particular tRNA (http://www.wiley.com/college/boyer/0470003790/structure/tRNA/trna_intro.htm).

Figure 3 shows the structure of tRNA.

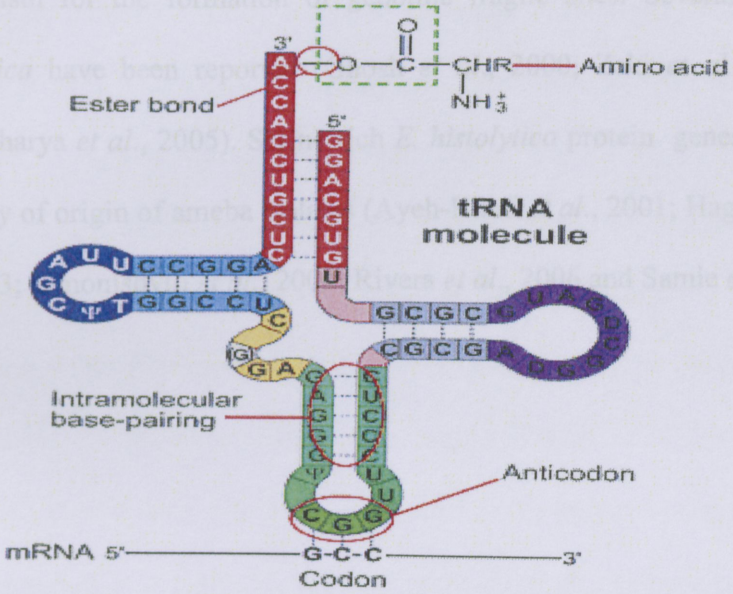


Figure 3: Transfer RNA structure

(http://www.wiley.com/college/boyer/0470003790/structure/tRNA/trna_intro.htm)

2.13. TRANSFER RNA GENES

From prokaryotic to eukaryotic organisms the tRNA genes ,which exist in multiple copies, are spread throughout the gene, but the number of gene copies for each tRNA species (tRNAs with the same anticodon) varies widely from species to species (Marck *et al.*, 2002). Transfer RNAs are among the most ancient genes. The principles that govern the evolution of tRNA gene populations remain unknown, but the factors that influence tRNA gene copy number within the genome have been studied mostly in individual species (Gonos *et al.,* 1990; Kanaya *et al.*, 1999

and Dong *et al.*, 1996). Recently studies show that all tRNA genes are homologs, deriving from an ancestral "proto-tRNA" (Eigen *et al.*, 1989). A recent study showed that tRNA genes may act as barriers to DNA replication fork progression (Mcfarlane *et al.*, 2009), providing a possible mechanism for the formation of genomic fragile sites. Several polymorphic markers of *E. histolytica* have been reported (Ghosh *et al.*, 2000; Zaki *et al.,* 2001; Ali *et al.*, 2005 and Bhattacharya *et al.*, 2005). Serine-rich *E. histolytica* protein genes are useful for analysis of the diversity of origin of ameba isolates (Ayeh-Kumi *et al.*, 2001; Haghighi *et al.*, 2002; Haghighi *et al.*, 2003; Simonishvili *et al.*, 2005; Rivera *et al.*, 2006 and Samie *et al.*, 2008).

2.14. *Entamoeba histolytica* with tRNA-linked short tandem repeat

Short tandem repeat (STRs) are short sequences of DNA, normally of length 2-8 base pairs, that are repeated numerous times in a head-tail manner. However, STRs are introns and they do not code for protein. The STRs are usually considered junk DNA. Furthermore, because the number of copies of repeats varies among individuals, STR is widely used in paternity testing and forensic cases. *Entamoeba histolytica* tRNA genes are unusually organized in 25 arrays containing up to 5 tRNA genes in each array, with intergenic regions between tRNA genes containing STRs (Blessmann *et al.*, 2003). A number of polymorphic loci containing diverse, noncoding short tandem repeats were investigated (Zaki *et al.*, 2001). All repeats were A+T rich and varied in size from 8 to 16 bp, and most of the polymorphism observed was due to variable repeat numbers. Subsequently, the developments of species-specific primers for these STRs were reported, and these primers allowed the simultaneous differentiation and strain typing of *E. histolytica* and *E. dispar* (Zaki *et al.*, 2002). This is important, because in some areas of *E. histolytica* and *E. dispar* endemicity, a significant number of individuals could be co-infected with both parasites (Mirelman *et al.*, 1997 and Haque *et al.*, 1998). These STRs were all found to be flanked by genes encoding tRNA.

CHAPTER 3

MATERIALS AND METHODS

3.1. Ethical considerations

This study was approved by the research and ethics Committee of the University of Venda and MEDUNSA campus of the Limpopo University, South Africa. Permission to use public health facilities to source participants was obtained from the Department of Health and Welfare. The objectives of the study were clearly explained to the participants and they were requested to sign consent forms before the collection of stool samples. The information obtained from the participants was kept confidential.

3.2 Study sites and sample collection

A total of 774 stool samples were collected from different clinics in Pretoria, Gauteng province, and Nkomo, Giyani, Limpopo province, South Africa. Nkomo is a village situated 40 KM from Giyani located in the Northern part of the Limpopo Province under the Greater Giyani Municipality (Figure 3). This area is rural with people of different religious, educational and socio-economics backgrounds, living in neighbourhood's with distinctly different level of sanitation. The road distance from Thohoyandou to Giyani is 80km. Pretoria is a city in the northern part of Gauteng Province under City of Tshwane Metropolitan Municipality, South Africa. The road distance from Thohoyandou to Pretoria is 448 km. The population of Pretoria

is estimated to be composed of about 74.40% Black African, 20.08% White, 2.01% courlered and 1.8% Indian. Both diarrheal and non- diarrheal stool samples were collected in Nkomo while in Pretoria mostly diarrheal samples were collected. The stool samples were transported without delay to the University of Venda, Microbiology parasitology laboratory in cooler boxes with ice.

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

Iodine wet mounts of fresh unpreserved stool samples were examined microscopically for demonstrating *E. histolytica* cysts and trophozoites as described by Parija *et al.*, (1995). Iodine wet mounts were briefly prepared by adding a portion of each unpreserved stool specimen to a drop of Lugol's iodine on a glass microscope slide and placing a cover slip on the stool suspension. The wet mounts were microscopically examined initially by using a low-power (10×) objective and then using a high-power (40×) objective of a compound light microscope. Unpreserved stool samples were stored at -20°C until further use for ELISA and DNA extraction.

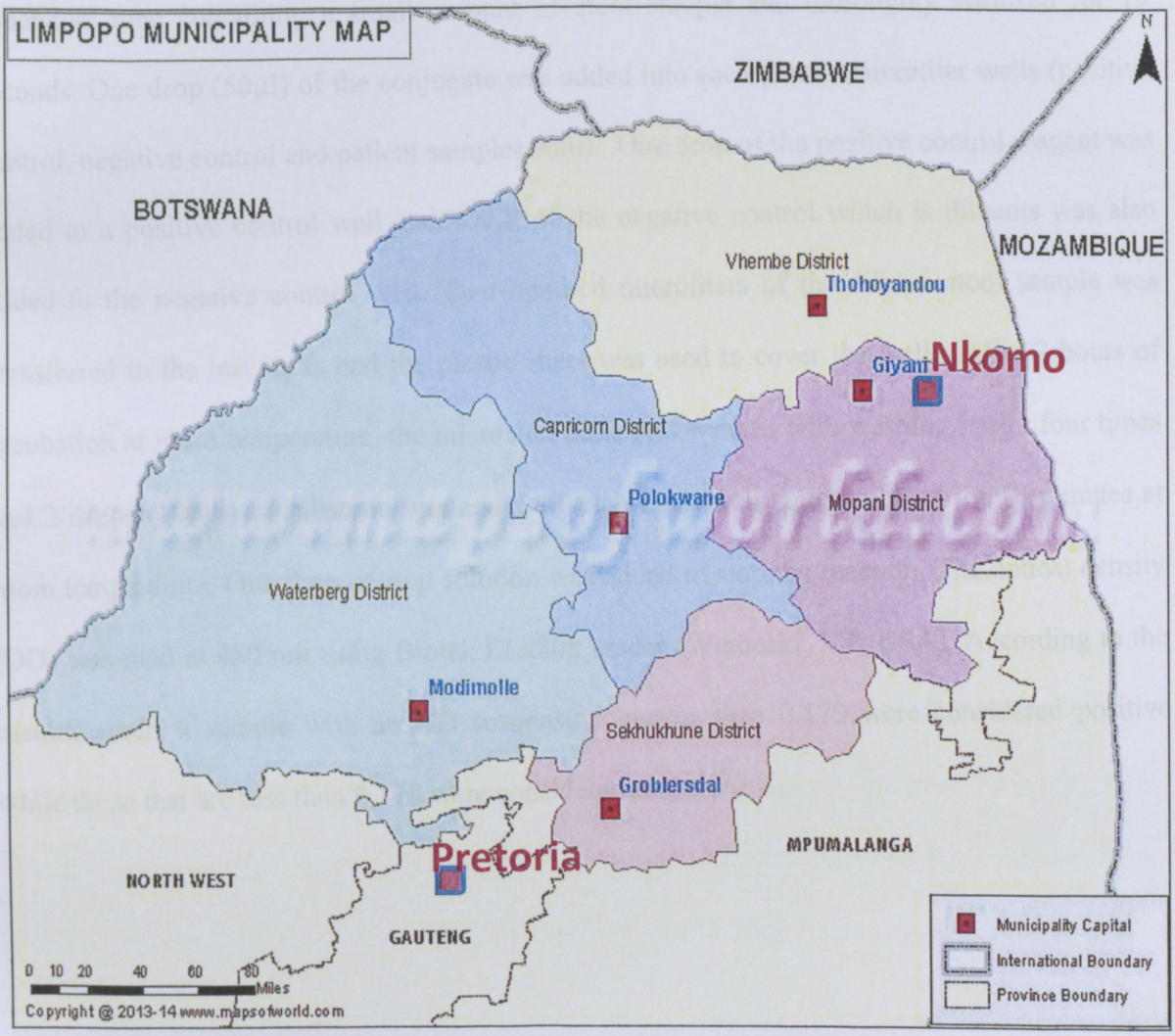


Figure 4: Limpopo and Gauteng Province map showing in the study site (www.mapsofworld.com)

3.4. Antigen detection of *E. histolytica* in stool samples by ELISA

Entamoeba histolytica was detected using *E. histolytica* II enzyme-linked Immunosorbent assay (ELISA) antigen detection kit (TechLab, Inc. Blacksburg, VA, USA) as per the manufacturer's instructions. In brief, 400µl of *E. histolytica* sample diluents was transferred into the 2ml eppendorf tube containing a small amount of stool sample and thoroughly vortexed for 15 seconds. One drop (50µl) of the conjugate was added into each plastic microtiter wells (positive control, negative control and patient samples well). One drop of the positive control reagent was added to a positive control well and 100µl of the negative control which is diluents was also added to the negative control well. Two hundred microliters of the diluted stool sample was transferred to the test wells and the plastic sheet was used to cover the wells. After 2 hours of incubation at room temperature, the microwell plate was washed with washing buffer four times and 2 drops (100µl) of substrate was added to each microwell and incubated for 10 minutes at room temperature. One drop of stop solution was added to stop the reaction. The optical density (OD) was read at 450 nm using Biotek ELx808 reader (Winooski, VT, USA). According to the manufacturer, a sample with an OD suspension greater than 0.170 were considered positive while those that are less than 0,170 were considered negative.

3.5. Extraction of Genomic DNA from stool samples.

The genomic DNA of *E. histolytica* was extracted from stool samples using ZYMO RESEARCH fecal DNA mini Prep kit from inqaba biotech. Briefly, 150mg of stool sample was added to ZR bashing bead lysis tube and 750µl of the lysis solution was also added and then disrupted at a maximum speed for 5 minutes. The ZR bashing bead lysis tube was centrifuged at 10000rmp for 1 minute, the supernatant of the samples were transfered to a Zymo- spin ^{TM} IV spin filter in a collection tube. The samples were centrifuged at 7000rmp for 1 minute, thereafter 1200µl of fecal DNA binding buffer was added to the filtrate in the collection tube. The mixtures were transfered to a Zymo- spin TM IIC column in a collection tube and centrifuged at 10000rmp for 1 minute. The flow from the collection tube was discarded and 200µl of DNA pre-wash buffer was added to the Zymo-spin ^{TM}IIC column in a new collection tube. The samples were then centrifuged at 10000rpm for 1 minute and 500µl of fecal DNA wash buffer was then added to the Zymo- spin ^{TM}IIC column and then centrifuged at 10000rpm for 1 minute. Zymo- spin ^{TM}IIC column was transferred to a clean 2ml microcentrifuge tube and 100µl of DNA elution buffer was added directly to the column matrix and then centrifuged at 10000rpm for 30 seconds to elute the DNA. The eluted DNA was then transferred to a prepared Zymo-spin^{TM} IV HRC spin filter in a clean 2ml microcentrifuge tube and then centrifuged at 8000rpm for 1 minute. After centrifugation the filtered DNA was stored at -20°C for further analysis.

3.6. Molecular detection of *E. histolytica*, *E. dispar*, and *E. moshkovskii* by PCR

The multiplex PCR assay was performed according to a protocol previously described by Hamzah *et al.*, (2006). The multiplex PCR reaction was performed in a total volume of 25µl. The reaction mixture briefly contained 2.5µl of 10x PCR buffer with 1.5µl of $MgCl_2$, 0.5µl of deoxynucleoside triphosphate mix, 0.2µl of Taq polymerase, 0.25µl of BSA, 0.6µl of each reverse primer (EhR, EdR and EmR), 12.65µl of distilled water 0.6µl of forward primer (EntaF) (Table 1) and 5µl of the extracted DNA samples. Samples were gently vortexed and then placed in a thermocycler (ThermcHybaid, United Kingdom). The conditions for PCR were set at: heat denaturation 1 cycle at 94°C for 3 minutes, followed by 30 cycles of 94°C for 1 minute, annealing temperature at 58°C for 1 minute, extension at 72°C for 1 minute, with a final extension at 72°C for 7 minutes. The PCR products were separated by electrophoresis in 1.5% agarose gel at 100 volts for 45 minutes in Tris-acetate buffer, visualized by UV-transilluminator. A 100bp DNA ladder was used as a size reference for PCR assay. The forward primer in combination with the appropriate reverse primer generated a 166-bp PCR product with *E. histolytica* DNA, a 752-bp PCR product with *E. dispar* DNA, and a 580-bp product with *E. moshkovskii* DNA.

Table 1: Primers used for the detection of *E. histolytica*, *E. dispar*, and *E. moshkovskii*

PRIMER NAME	PRIMER SEQUENCE
EntaF	5′-ATG CAC GAG AGC GAA AGC AT-3′
EhR	5′-GAT CTA GAA ACA ATG CTT CTC T-3′
EdR	5′-CAC CAC TTA CTA TCC CTA CC-3′
EmR	5′-TGA CCG GAG CCA GAG ACA T-3′

3.7. PCR Genotyping of *E. histolytica*

The PCR reaction was performed in a total volume of 25µl. The reaction mixture briefly contain 2.5µl of 10x PCR buffer with 1.5µl of $MgCl_2$, 0.5µl of deoxynucleoside triphosphate mix, 0.2µl of Taq polymerase, 0.25µl of BSA, 13.85µl of distilled water, 5µl of the extracted DNA samples and 0.6µl of each six pairs of primers (Table 2) were used: locus RR (R-R5 and R-R3), locus NK (NK5 and NK3), locus AL (A-L5 and A-L3), locus D-A (D-A5 and D-A3), locus SD (S^{TGA}-D5 and S^{TGA}-D3), and locus SQ (S-Q5 and S-Q3), under the conditions previously described (Ali *et al.*, 2005). The PCR products were separated electrophoretically in 1.5% agarose gels after staining with ethidium bromide and visualized by UV light and photographed.

Table 2: Primers sequences used for genotyping PCR

PRIMER NAME	SEQUENCE (5` TO 3´)	ANNEALING (°C)
A-L5	GGATCGATACCCCTCATCTCCA	64
A-L3	CGCATCTTGCGATAGCCGAG	
D-A5	CTGGTTAGTATCCTTCGCCTGT	56
D-A3	GCTACACCCCCATTAACAAT	
N-K5	CGAACGGCTGTTAACCGTTA	55
N-K3	TTCCTAGCTCAGTCGGTAGA	
R-R5	AGCATCAGCCTTCTAAGCTG	55
R-R3	CTTCCGACTGAGCTAACAAG	
STGA-D5	CTCTGGATGCGTAGGTTCAA	58
STGA-D3	GTATCTTCGCCTGTCACGTG	
S-Q5	GTGGTCTAAGGCGTGTGACT	56
S-Q3	GAGATTCTGGTTCTTAGGACCC	

3.8. Statistical Analysis

The results were compiled using an excel spread sheet and edited appropriately (Microsoft office package) and analyzed using the Statistical Package for Social Sciences (SPSS) program, version 17.1 with the fisher chi square test and the difference between two variables was considered significant if the p value was less than 0.05.

Chapter 4: RESULTS

4.1. Demographic characteristics of the study population.

Table 3 shows the demographic characteristics of the study population based on the origin, age group, gender and sample consistency. A total of 774 stool samples were collected from 774 participants from different health centers from the beginning of April, 2013 to the end of November, 2014. From the study population, most were males with 50.5% followed by female with 48.6%. The type of the stool samples was indicated by the physical presentation of the sample at the time of the collection. From the 774 samples, 467(60.3%) were loose, 202 (26.1%) were formed and 104 (13.4%) were watery. Only a small proportion of the participants provided the ages between1-25 years contributed the highest percentage of samples 107(13.8), followed by those who were 26- 45 years old and 46-90 years old.

Table 3: Demographic characteristics of the study population

Characteristics		Frequency	Percent (%)
Origin	Giyani	56	7.2
	Pretoria	718	92.8
Gender	Female	376	48.6
	Male	391	50.5
Age group	1-25 years	107	13.8
	26-45 years	56	7.2
	46-90 years	49	6.3
Samples Consistency	Formed	202	26.1
	Loose	467	60.3
	Watery	104	13.4
	Total	774	100.0

4.2. Detection of *Entamoeba histolytica* by microscopy

In the present study, 774 samples were examined microscopically for the presence of *E. histolytica* cyst in stool samples and 16.5% samples were found to be positive.

4.3. Enzyme- Linked Immunosorbent Assay (ELISA) for the detection of *E. histolytica* antigen

Out of the 774 samples analyzed, 10.1% samples were found to be positive for *E. histolytica* antigen using the ELISA method (**Figure 5**). Table 4 indicates the prevalence on *E. histolytica* based on age, origin, and gender and sample consistency. The highest prevalence of *E. histolytica* was found in Pretoria with 10.5%, as compared to Giyani which was 5.4% and the difference was not statistically significant ((X^2= 1.491; P= 0.222). *Entamoeba histolytica* was more common in males (12%) than in females (8.4%), but the difference was not statistically significant (X^2= 2.653; P= 0.103). Most of the participants who were infected were the ages of between 26-45years by 21.2% followed by those who were in the age group 1-25 years by 16.8%. The least infected were of the age group 49-90 years by 8.2%. However, the difference was not statistically significant (X^2= 3.341; P= 0.188). According to samples consistency, the highest prevalence was found in watery stool samples by 13.6%, followed by soft with 11.1% and the least was found with 5.9%, but the difference was not statistically significant (X^2= 5.781; P= 0.056).

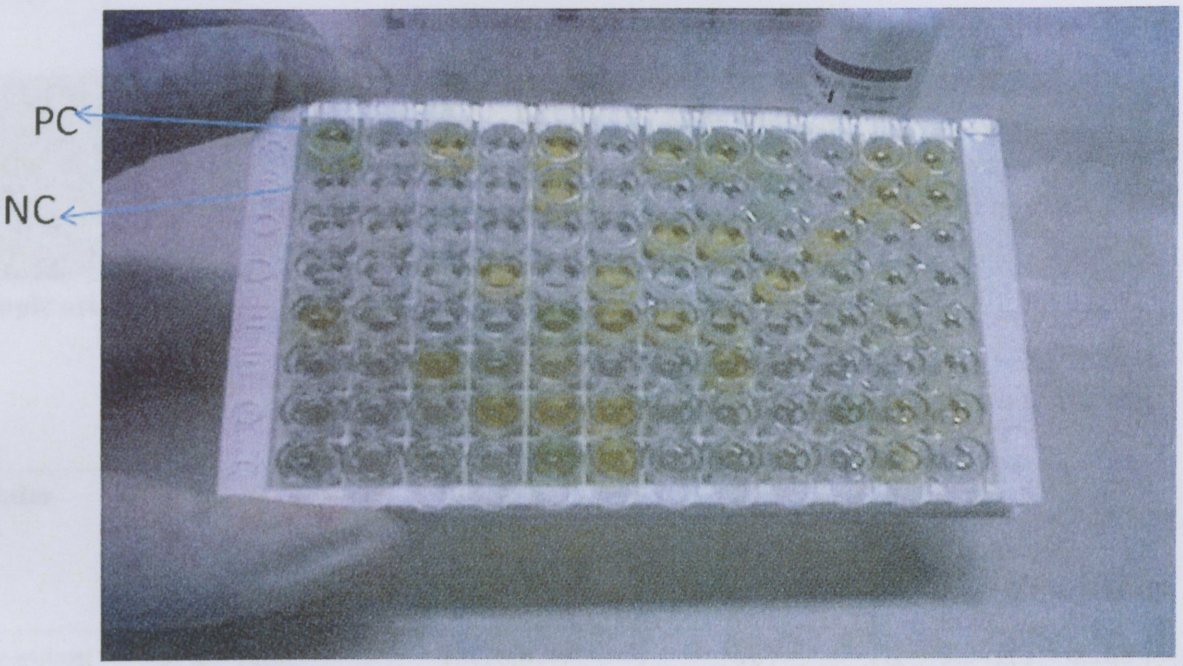


Figure 5: Micro-titer plate showing some of the positive samples from ELISA assay

PC- Positive Control; NC- Negative Control

Table 4: Prevalence of *E. histolytica* according to the origin, gender, age and, sample consistency of the participants using ELISA method.

Samples characteristics		*E. histolytica* Positive	Total	Statistics
Sample origin	Giyani	3(5.4%)	56	X^2= 1.491, P= 0.222
	Pretoria	75(10.5%)	717	
Gender	Male	45(12%)	375	X^2= 2.653, P= 0.103
	Female	33(8.4%)	391	
Age group	1-25 years	18(16.8%)	107	X^2= 3.341; P= 0.188
	26-45 years	11(21.2%)	52	
	46-90 years	4(8.2%)	49	
Consistency	Formed	12(5.9%)	202	X^2= 5.781; P= 0.056
	Soft	52(11.1%)	467	
	Watery	14(13.6%)	103	

4.4. Molecular detection of *E. histolytica* by PCR method

The 78 samples that were positive by ELISA were used in the PCR protocol for the detection of *E. histolytica* DNA. From these, 42 samples showed positive for *E. histolytica* small-subunit rRNA gene. **Figure 6** shows the results obtained from the PCR detection of *E. histolytica* from stool samples.

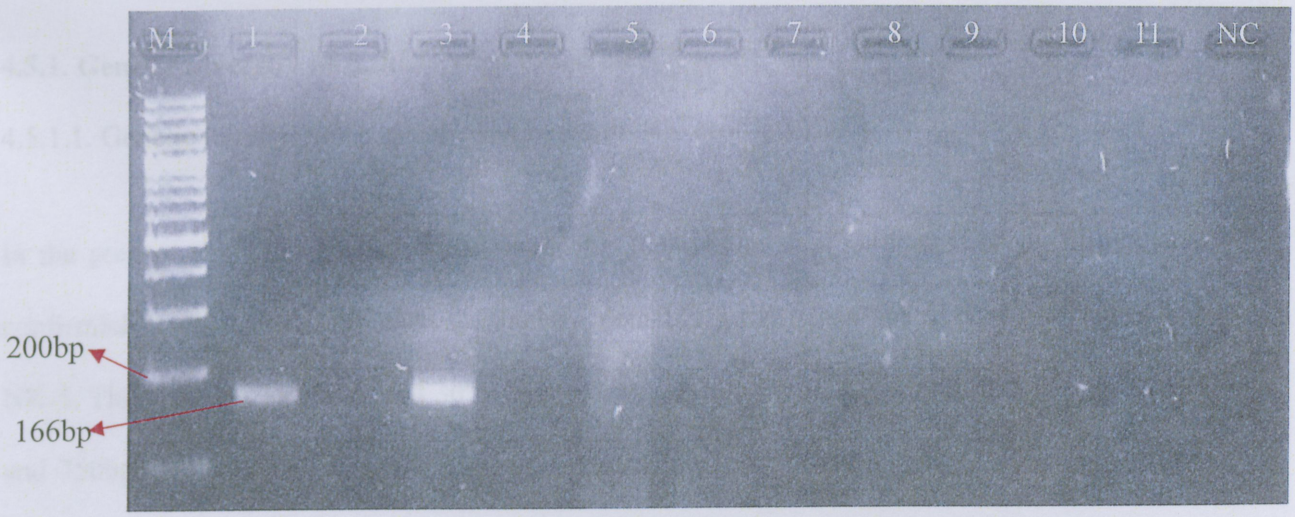


Figure 6: An agarose gel picture showing the PCR results for the detection of *E. histolytica* in the stool samples.

M- Molecular marker 100bp, NC- negative control, 1-11 DNA samples

4.5. Genotyping of *E. histolytica* based on PCR profile

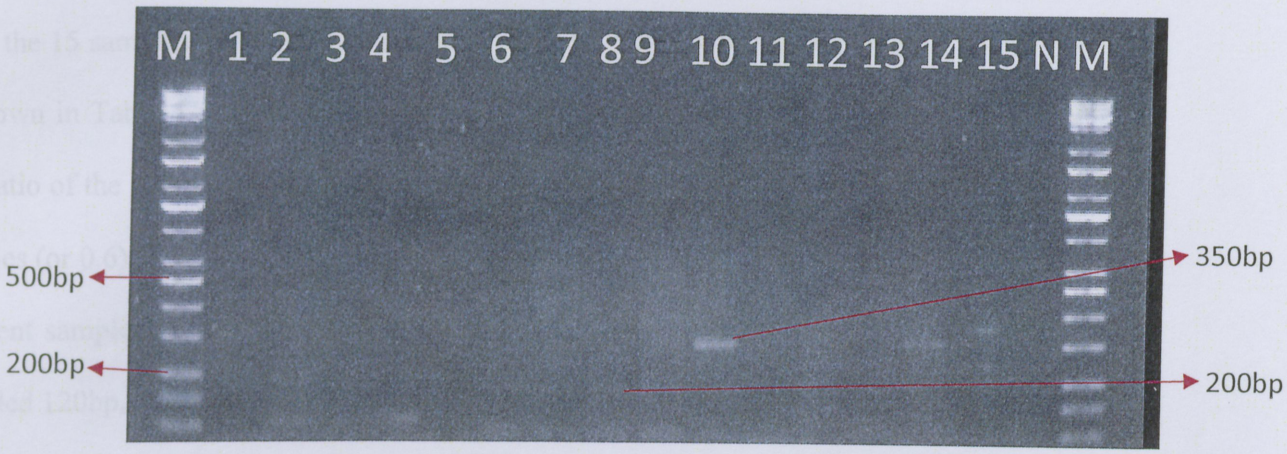
In order to investigate genetic diversity of the *E. histolytica* based on the six tRNA-linked STR loci in genotyping, 78 DNA samples were used in different PCR reactions targeting the six STR loci. The PCR was unsuccessful in some of the samples for certain loci of the six tRNA-linked STR. Loci S-Q did not show any bands in all tested samples and was excluded from the study. In the present study different profiles were observed in different samples of different origins (Giyani and Pretoria).

4.5.1. Genetic diversity of *E. histolytica* based on NK locus

4.5.1.1. Genetic diversity of *E. histolytica* based on NK profile

In the present study, 15 stool samples from the 78 positive samples identified by ELISA and confirmed by PCR were amplified for the NK locus using the tRNA specific primers NK-3 and NK-5. This gave a success rate of 36%. The product sizes of 120, 150, 200, 250, 300, 500, 600 and 750bp were obtained. Figure 7 shows some of the gels obtained after amplification of the NK locus.

A



B

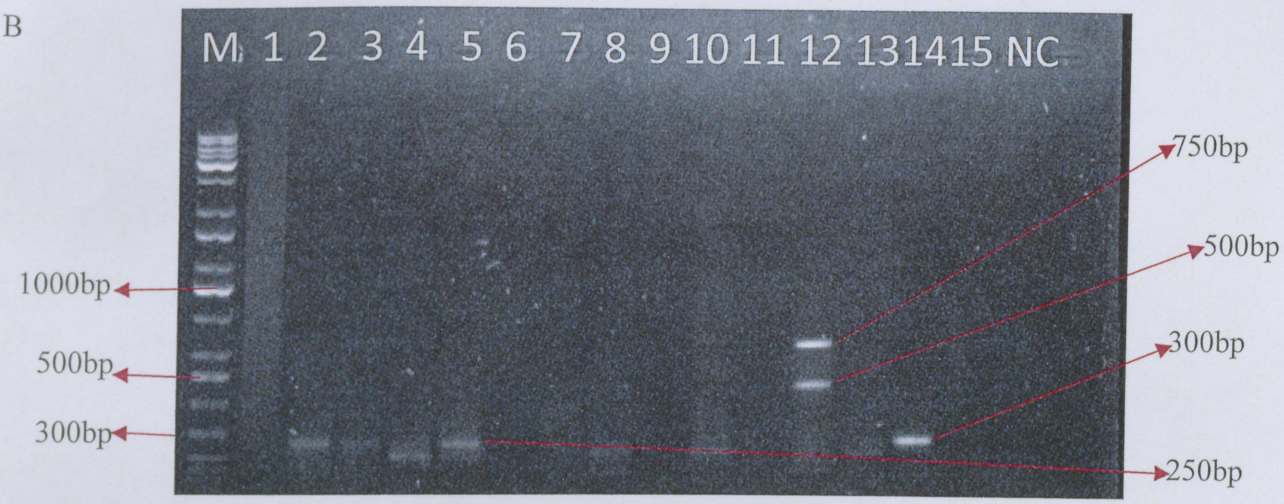


Figure 7: Pictures of the Agarose gel electrophoresis showing the profiles of the NK locus (A and B).

M- Molecular marker 100bp, NC- negative control, 1-15 DNA samples

4.5.1.2. Genetic profiles obtained for the NK locus of *E. histoltytica.*

From the 15 samples that amplified for the NK locus, 9 different banding patterns were obtained as shown in Table 5 which indicated extensive genetic diversity among *E. histolytica* samples. The ratio of the profile and the number of samples tested was therefore three profiles for every 5 samples (or 0.6). Two hundred base pairs was the most common band that occurred five times in different samples as compared to 150bp and 250bp that occurred twice. Other bands observed included 120bp, 300bp, 350bp, 500bp, 600bp and 750bp which occurred only once each.

Table 5: Summary of the samples that produced different profiles for the NK locus of *E. histolytica*.

CODE	TYPE	GENDER	ORIGIN	NK bands	NK Profile No	Frequency
NS06861	watery	male	Pretoria	120 and 200bp	1	1
NS05704	loose	female	Pretoria	120bp	2	1
MS4	loose	male	Giyani	150bp	3	2
MS32	loose	male	Giyani	150bp	3	2
GY106	formed	female	Giyani	200 and 350bp	4	1
GY90	loose	male	Giyani	200bp	5	5
MS30	loose	Female	Giyani	200bp	5	5
MS39	loose	Female	Giyani	200bp	5	5
MS41	loose	Female	Giyani	200bp	5	5
MS47	formed	Female	Giyani	200bp	5	5
NS02226	loose	male	Pretoria	250bp	6	2
MS35	loose	male	Giyani	250bp	6	2
NSS03183	loose	Male	Pretoria	300bp	7	1
MS34	formed	Female	Giyani	500bp, 750bp	8	1
NNS08850	loose	male	Pretoria	600bp	9	1

4.5.1.3. Distribution of the different profiles for the NK locus of *E. histolytica*

Of all nine different profiles obtained for the NK locus, profile number 5 was found to be the most common profile compared to other profiles. This profile occurred in five different samples in Giyani and 4 were females and 1 was male. Of these five samples, four samples had loose consistency and one had formed consistency. Therefore this profile was not associated with diarrhea. Another profile that occurred in two samples was profile number 3 and both samples were from Giyani and were both from male participants, and had loose consistency. Out of all profile obtained in NK locus, only profile number 1 had watery consistency and was from Pretoria. It is possible that this profile could be associated with diarrhea.

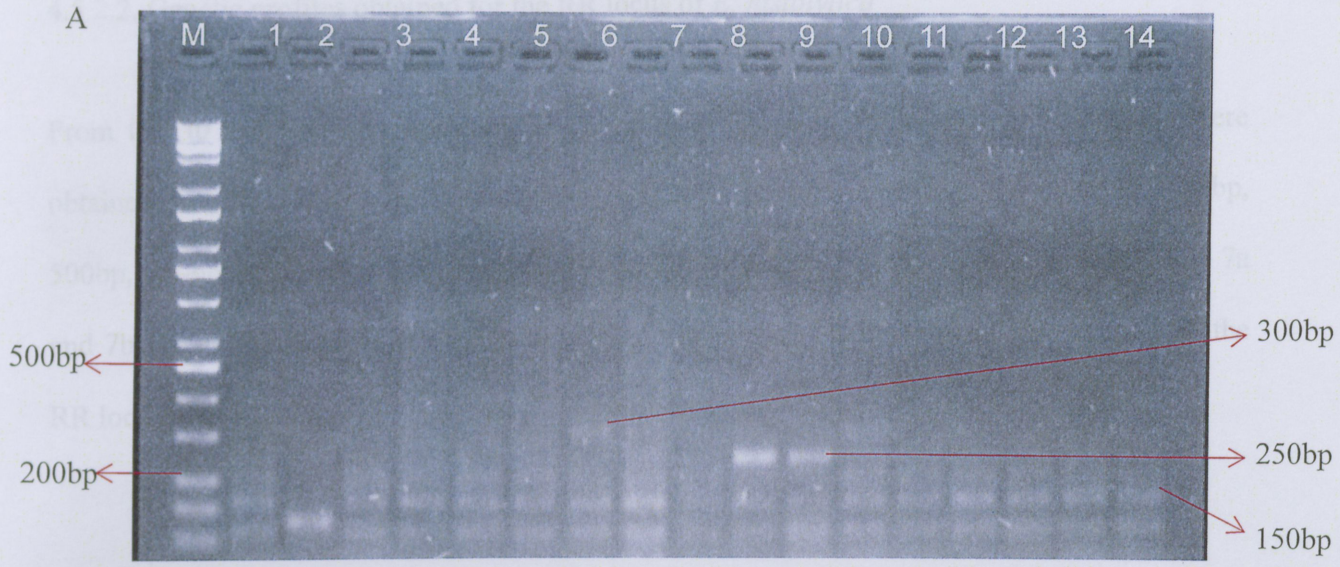
Table 6: Distribution of the different profiles for the NK locus of *E. histolytica.*

	Sample origin			Consistency				Gender		
NK Profile	Giyani	Pretoria		Formed	Loose	Watery		Male	Female	Total
1	0	1		0	0	1		1	0	1
2	0	1		0	1	0		0	1	1
3	2	0		0	2	0		2	0	2
4	1	0		1	0	0		0	1	1
5	5	0		1	4	0		1	4	5
6	1	1		0	2	0		2	0	2
7	0	1		0	1	0		1	0	1
8	1	0		1	0	0		0	1	1
9	0	1		0	1	0		1	0	1
Total	10 (66.7%)	5 (33.3%)		3 (20%)	11 (73.3%)	1 (6.7%)		8 (53.3%)	7 (46.7%)	15 (100%)

4.5.2. Genetic diversity of *E. histolytica* based on the RR locus

4.5.2.1. Genetic diversity of *E. histolytica* based on the RR profile

Out of the 78 positive samples identified by ELISA and confirmed by PCR the RR locus was amplified in 30 samples using the tRNA specific primers RR-3 and RR-5. This gave a success rate of 71%. The product sizes of 150, 200, 250, 300, 400,450, 500, 550, 600 and 750bp were obtained. Figure 8 shows some examples of the gels obtained after amplification of the RR locus.



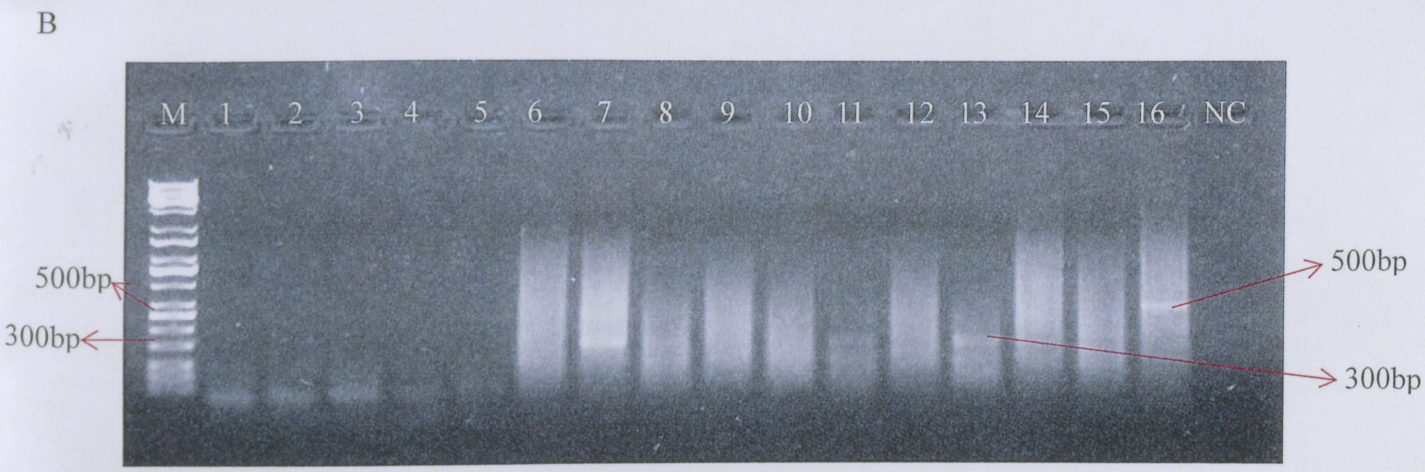


Figure 8: Pictures of the Agarose gel electrophoresis showing the profiles of the RR locus (A and B).

M- Molecular marker 100bp, NC- negative control, 1-17 DNA samples.

4.5.2.2. Genetic profiles obtained for the RR locus of *E. histolytica*

From the 30 samples that were amplified for the RR locus, different banding patterns were obtained as represented in table 7a and 7b. The band sizes varied between 200, 400bp, 450bp, 500bp, 600bp and 750bp. It was observed that 150bp and 250bp occurred only once. Table 7a and 7b shows the summary of the samples that produced the different profiles obtained for the RR locus of the *E. histolytica.* A total of 16 profiles were obtained giving a ratio of 0.53.

Table 7(a): Summary of the samples that produced the different profiles for the RR locus of *E. histolytica.*

CODE	TYPE	GENDER	ORIGIN	RR	RR profile	Frequency
GY93	loose	x	Giyani	150bp and 200bp	1	1
MS34	formed	Female	Giyani	200bp and 600bp	2	2
MS42	loose	Female	Giyani	200bp, 600bp	2	2
NS09951	loose	female	Pretoria	200bp	3	2
NNS11884	watery	male	Pretoria	250bp	3	2
NS08609	loose	male	Pretoria	300,400 and500bp	4	1
MS29	loose	Female	Giyani	300bp	5	1
NS03019	loose	male	Pretoria	400 and 500bp	6	1
NS09644	loose	female	Pretoria	400bp	7	3
MS4	loose	male	Giyani	400bp	7	3
NS02159	loose	male	Pretoria	400bp	7	3
NS08523	loose	female	Pretoria	450 and 500bp	8	1
MS8	formed	Female	Giyani	450bp	9	4

Table 7 (b): Summary of the samples produced different profiles for the RR locus of *E. histolytica*

CODE	TYPE	GENDER	ORIGIN	RR	RR profile	Frequency
NS06861	watery	male	Pretoria	450bp	9	4
NNS08583	watery	male	Pretoria	450bp	9	4
NNS03624	loose	female	Pretoria	450bp	9	4
NS03305	loose	female	Pretoria	500 and 600bp	10	3
NS07121	formed	male	Pretoria	500 and 600bp	10	3
NS00335	loose	male	Pretoria	500 and 600bp	10	3
MS45	formed	male	Giyani	500bp	11	4
MS30	loose	Female	Giyani	500bp	11	4
NNS08850	loose	male	Pretoria	500bp	11	4
NS02492	loose	female	Pretoria	500bp	11	4
NS00333	loose	male	Pretoria	500bp and 550bp	12	1
MS46	loose	male	Giyani	500bp, 750bp	13	1
MS44	formed	male	Giyani	550bp	14	1
MS47	formed	Female	Giyani	600bp	15	3
NSS03183	loose	Male	Pretoria	600bp	15	3
NS01022	loose	male	Pretoria	600bp	15	3
NS05704	loose	female	Pretoria	750bp	16	1

4.5.2.3. Distribution of the different profiles for the RR locus of *E. histolytica*

Of all sixteen different profiles obtained in RR locus (**Table 8**), profile number 9 occurred four times in different samples. Out of four samples observed in profile number 9, three samples were from Pretoria (two males and I female) and one was from Giyani (female). Of these four samples, only two males from Pretoria had watery consistency. Another profile that occurred in four samples was profile number 11 where two samples were from Giyani (male and female) and another two were from Pretoria (male and female). Of these four samples, three samples had loose consistency and one had formed consistency. Profile number 7 occurred three times in different samples. Out of three samples observed in profile number 7, two samples were from Pretoria (male and female) and one was from Giyani (male). Both samples had loose consistency. Another profile that occurred in three samples was profile number 15 where two samples were from Pretoria (two males) and another one was from Giyani (female). Of these three samples, two samples had loose consistency and one had formed consistency. Another profile observed in three samples from Pretoria was profile number 10 and two samples had loose consistency (male and female) and another one had formed consistency (male). Profile number 3 occurred in two samples (male and female) and both samples were from Pretoria. Out of those two samples, only one sample had watery consistency. Other profiles occurred only once were profile number 1, 4, 5, 6, 8, 12, 13, 14 and 16 and all these profiles were not associated with water.

Table 8: Distribution of the different profiles for the RR locus of *E. histolytica.*

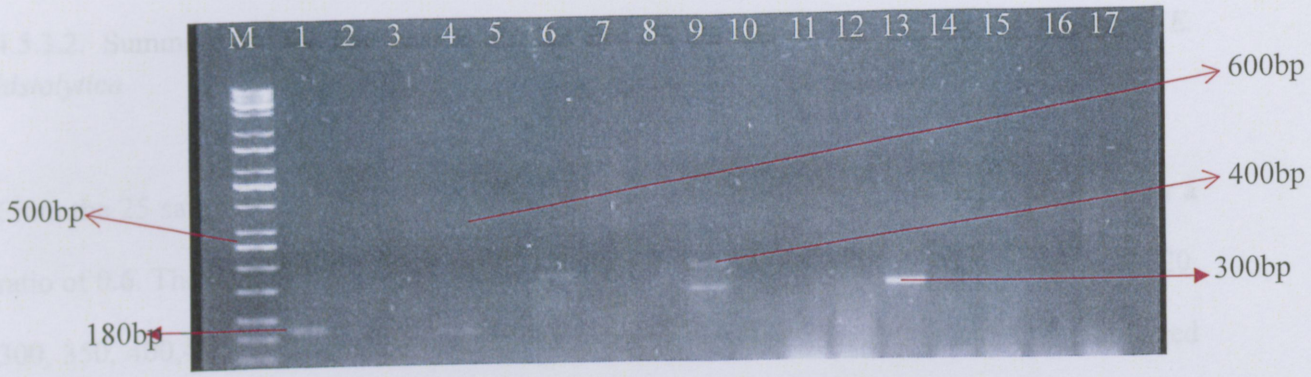
	Origin			Consistency				Gender		
RR profile	Giyani	Pretoria		Formed	Loose	Watery		Male	Female	Total
1	1	0		0	1	0				1
2	2	0		1	1	0		0	2	2
3	0	2		0	1	1		1	1	2
4	0	1		0	1	0		1	0	1
5	1	0		0	1	0		0	1	1
6	0	1		0	1	0		1	0	1
7	1	2		0	3	0		2	1	3
8	0	1		0	1	0		0	1	1
9	1	3		1	1	2		2	2	4
10	0	3		1	2	0		2	1	3
11	2	2		1	3	0		2	2	4
12	0	1		0	1	0		1	0	1
13	1	0		0	1	0		1	0	1
14	1	0		1	0	0		1	0	1
15	1	2		1	2	0		2	1	3
16	0	1		0	1	0		0	1	1
Total	**11 (36.7%)**	**19 (63.3%)**		**6 (20%)**	**21 (70%)**	**3 (10%)**		**16 (55.2%)**	**13 (44.8%)**	**30 (100%)**

4.5.3. Genetic diversity of *E. histolytica* based on AL locus

4.5.3.1. Genetic diversity of *E. histolytica* based on AL profile

Out of the 78 positive samples identified by ELISA and confirmed by PCR, the AL locus was amplified in 25 samples using the tRNA specific primers AL-3 and AL-5. This gave an amplification success of 59.5%. The product size of 150, 180, 200, 220, 300, 350, 400,450, 500, 550, 600 and 1000bp were obtained. Figure 9 shows the gels obtained after amplification of the AL locus.

A



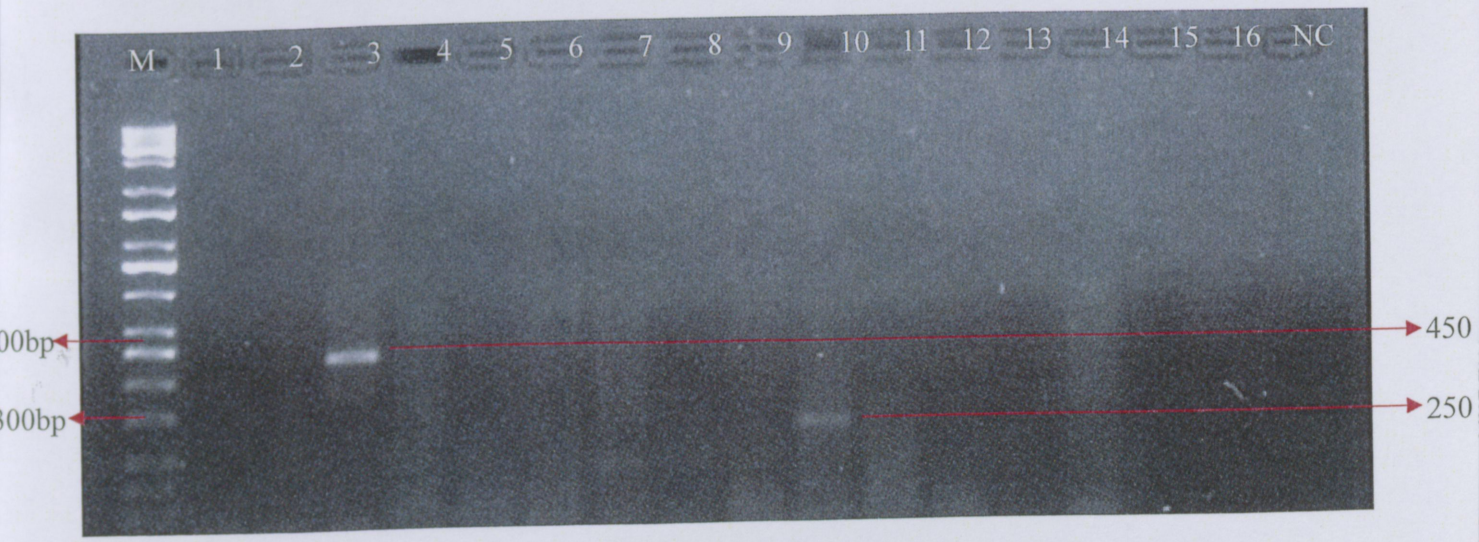


Figure 9: Picture of the Agarose gel electrophoresis showing the profiles of the AL locus (A and B).

M- Molecular marker 100bp, NC- negative control, 1-17 DNA samples.

4.5.3.2. Summary of the samples that produced different profiles for the AL locus of *E. histolytica*

From the 25 samples that amplified for the AL locus (**Table 9**), 15 profiles were obtained for a ratio of 0.6. The band size of 200bp was seen in most of the samples, followed by 150, 180, 220, 300, 350, 400,450, 500, 550, 600 and 1000bp. It was observed that 150bp and 550bp occurred only once. Table 9 shows the summary of the samples that produced different profiles for the RR locus of the *E. histolytica*

Table 9: Summary of the sample produced different profiles for the AL locus of *E. histolytica.*

CODE	TYPE	GENDER	ORIGIN	AL	Al profile	Frequency
MS29	loose	Female	Giyani	1000bp	15	1
NNS09797	loose	Male	Pretoria	150 and 200bp	1	1
NNS10459	watery	Male	Pretoria	180 and 300bp	2	2
NS05704	loose	female	Pretoria	180and 600bp	2	2
NSS03183	loose	Male	Pretoria	180bp	3	2
HI088	loose	female	Pretoria	180bp	3	2
NNS09777	watery	Female	Pretoria	200 and 400bp	4	1
MS28	formed	Female	Giyani	200bp	5	6
MS4	loose	male	Giyani	200bp	5	6
MS32	loose	male	Giyani	200bp	5	6
HI051	loose	female	Pretoria	200bp	5	6
NNS06388A	loose	female	Pretoria	200bp	5	6
NNS11274	loose	male	Pretoria	200bp	5	6
MS36	loose	Female	Giyani	200bp, 550bp	6	1

4.5.3.3. Distribution of the different profiles for the AL locus of *E. histolytica*

Of all fifteen different profiles obtained in AL locus (**Table 10)**, profile number 5 occurred six times in different samples. Out of those six samples observed in profile number 5, three samples were from Pretoria (one male and two females) while three were from Giyani (two males and one female). Of these six samples, only one sample from Giyani had formed consistency and the rest had loose consistency. Another profile that occurred in three samples was profile number 14 and two samples were from Pretoria (two males) and one was from Giyani (female). Of these three samples, two samples from Pretoria had loose consistency and one from Giyani had formed consistency. Profiles 2, 3 and 8 were observed in two samples each and all of them were from Pretoria (female and male), and only one sample observed in profile number 2 had watery consistency and the rest had loose consistency. Other profiles occurred once in the sample and included profile number 1, 4, 6, 7, 9, 10, 11, 12 and 13. Out of all these profiles, only profiles number 4 and 7 had watery consistency.

Table 10: Distribution of the different profiles for the AL locus of *E. histolytica.*

	Origin			Consistency				Gender		
Al profile	Giyani	Pretoria		Formed	Loose	Watery		Male	Female	Total
1	0	1		0	1	0		1	0	1
2	0	2		0	1	1		1	1	2
3	0	2		0	2	0		1	1	2
4	0	1		0	0	1		0	1	1
5	3	3		1	5	0		3	3	6
6	1	0		0	1	0		0	1	1
7	0	1		0	0	1		1	0	1
8	0	2		0	2	0		1	1	2
9	0	1		0	1	0		1	0	1
10	1	0		1	0	0		0	1	1
11	0	1		0	1	0		0	1	1
12	0	1		1	0	0		1	0	1
13	0	1		0	1	0		0	1	1
14	1	2		1	2	0		2	1	3
15	1	0		0	1	0		0	1	1
Total	**7 (28%)**	**18 (72%)**		**4 (16%)**	**18 (72%)**	**3 (12%)**		**12 (48%)**	**13 (52%)**	**25 (100%)**

4.5.4. Genetic diversity of *E. histolytica* based on the DA locus

4.5.4.1. Genetic diversity of *E. histolytica* based on the DA profile

In this study, 13 (31%) stool samples from the 42 positive samples identified by ELISA and confirmed by PCR were amplified for the DA locus using the tRNA specific primers DA-3 and DA-5. The product sizes of 150, 200, 280, 300, 500 and 1200bp were obtained. Figure 10 shows the gels obtained after amplification of the DA locus.

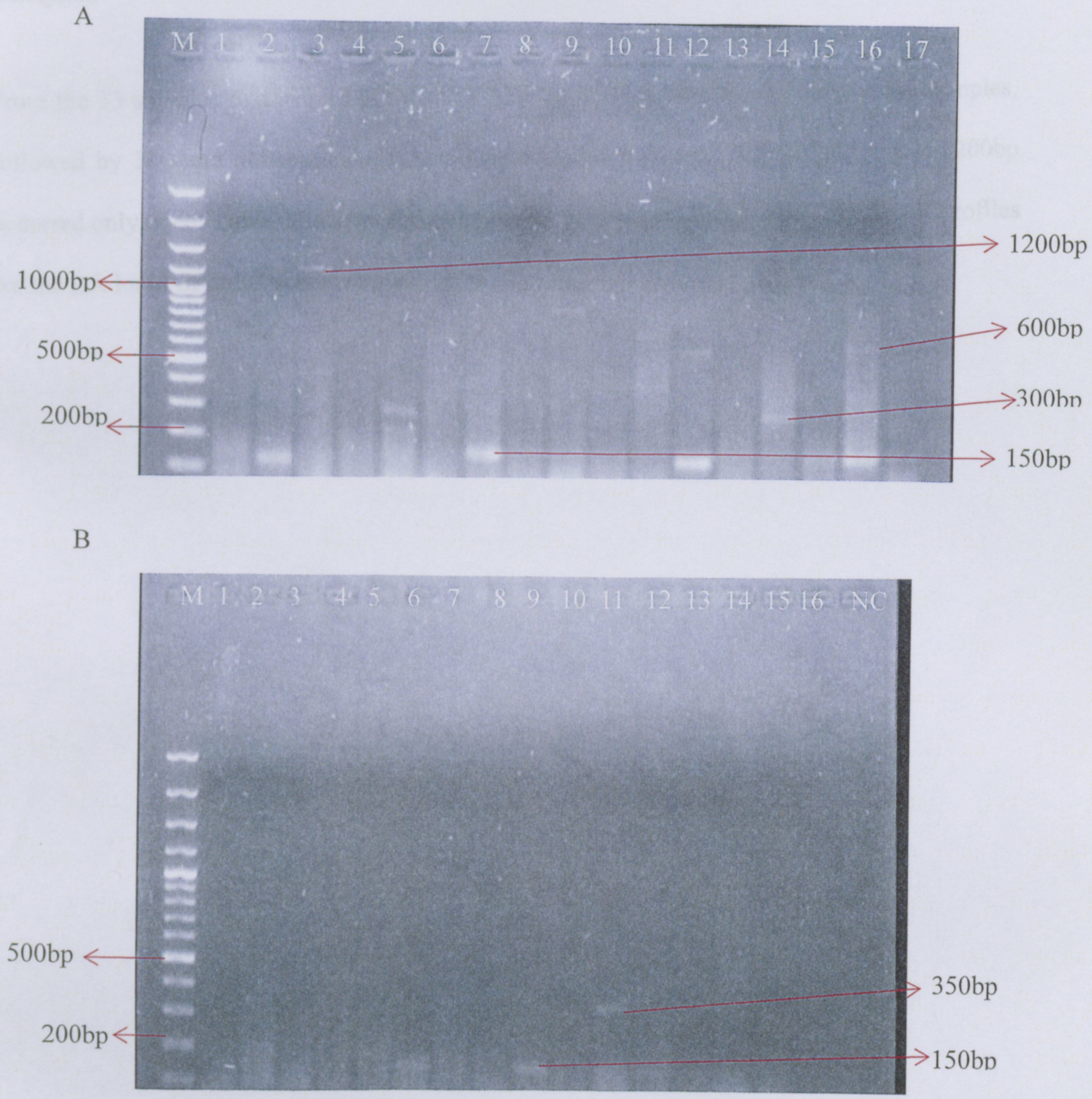


Figure 10: Picture of the Agarose gel electrophoresis showing the profiles of the DA locus (A and B).

M- Molecular marker 100bp, NC- negative control, 1-17 DNA samples.

4.5.4.2. Summary of the samples that produced different profiles for the DA locus of *E. histolytica*

From the 13 samples that amplified for the DA locus, 150bp was seen in most of the samples, followed by 300 and 500bp bands. It was observed that bands of 200bp, 280bp, and 1200bp occurred only once. **Table 11** shows the summary of the samples that produced different profiles for the DA locus of the *E. histolytica.*

Table 11: Summary of the samples produced different profiles for the DA locus of *E. histolytica.*

CODE	TYPE	GENDER	ORIGIN	DA	DA	Frequency
NNS10459	watery	male	Pretoria	1200bp	6	1
NNS09797	loose	male	Pretoria	150 and 500bp	1	2
NS08098	formed	male	Pretoria	150 and 500bp	1	2
NNS08605	loose	female	Pretoria	150bp	2	5
NNS09872	loose	male	Pretoria	150bp	2	5
HI043	formed	male	Pretoria	150bp	2	5
NS02968	loose	female	Pretoria	150bp	2	5
GY85	loose	female	Giyani	150bp	2	5
GY93	loose	-	Giyani	200bp	3	1
NNS09777	watery	female	Pretoria	280bp	4	1
NNS04644	watery	male	Pretoria	300bp	5	3
NS06859	loose	male	Pretoria	300bp	5	3
NNS06055	loose	male	Pretoria	500bp	5	3

4.5.4.3. Distribution of the different profiles for the DA locus of *E. histolytica*

Of all six different profiles obtained in DA locus (**Table 12)** from the 13 samples that amplified giving a ratio of 0.46. Profile number 2 occurred five times in different samples. Out of those five samples observed in profile number 2, four samples were from Pretoria (two males and two females) and one was from Giyani (female). Of these five samples, only one sample from Pretoria had formed consistency and the rest had loose consistency. Profile 5 occurred in all three samples, and both samples were from Pretoria and, were males. Of these three samples, two samples had loose consistency and one had watery consistency. Again profiles number 4 and 6 were observed in one sample each and were from Pretoria (male and female). Both of them had watery consistency. Another profile that occurred in two samples was profile number 1 and, both samples were from Pretoria (males). Of these two samples, one had loose consistency and another one had formed consistency.

Table 12: Distribution of the different profiles for the DA locus of *E. histolytica.*

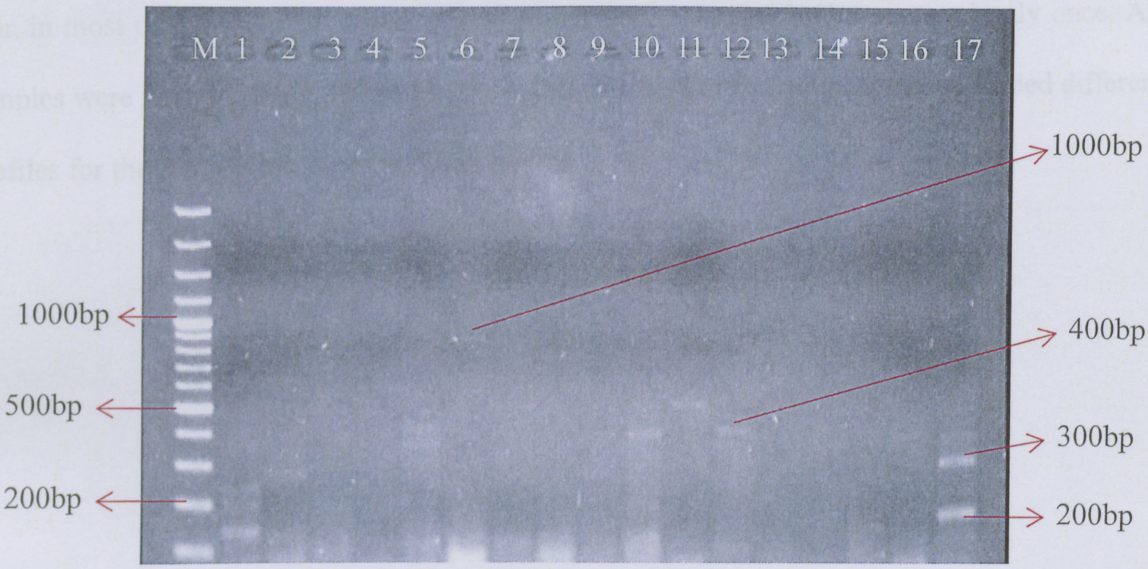
	Origin			Consistency				Gender		
DA profile	Giyani	Pretoria		Formed	Loose	Watery		Male	Female	Total
1	0	2		1	1	0		2	0	2
2	1	4		1	4	0		2	3	5
3	1	0		0	1	0				1
4	0	1		0	0	1		0	1	1
5	0	3		0	2	1		3	0	3
6	0	1		0	0	1		1	0	1
Total	**2 (15.4%)**	**11 (84.6%)**		**2 (15.4%)**	**8 (61.5%)**	**3 (23.1%)**		**8 (66.7%)**	**4 (33.3%)**	**13 (100%)**

4.5.5. Genetic diversity of *E. histolytica* based on the S^{TGA}-D locus

4.5.5.1. Genetic diversity of *E. histolytica* based on the S^{TGA}-D profile

In this study, 9 (21%) stool samples from the 78 positive samples identified by ELISA and confirmed by PCR were amplified for the S^{TGA}-D locus using the tRNA specific primers S^{TGA}-D -3 and S^{TGA}-D -5. The product size of 150, 180, 200, 220, 300, 350, 400,450, 500, 550, 600 and 1000bp were obtained. Figure 11 shows the gels obtained after amplification of the DA locus.

A



B

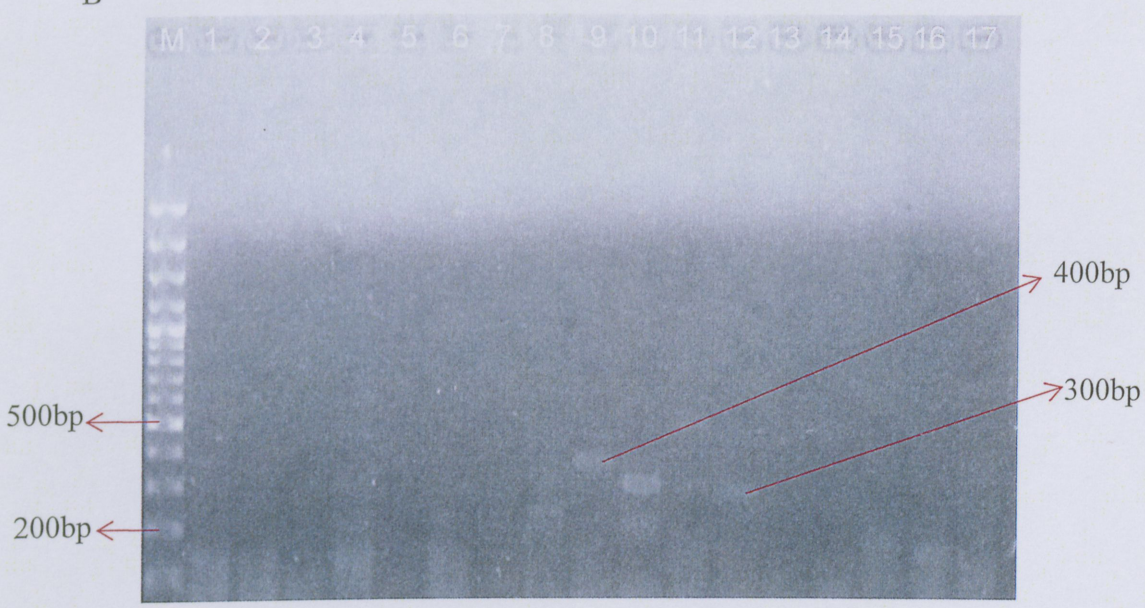


Figure 11: Pictures of the Agarose gel electrophoresis showing the profiles of the S^{TGA}-D locus (A and B). M- Molecular marker 100bp, NC- negative control, 1-17 DNA samples.

4.5.5.2. Summary of the samples produced the S^{TGA}-D locus of *E. histolytica*

From the 9 samples that amplified for the S^{TGA}-D locus, bands of 150, 200, 280 and 400bp were seen in most of the samples. It was observed that 100, 240, and 300bp occurred only once. All samples were from Pretoria. **Table 13** shows the summary of the samples that produced different profiles for the S^{TGA}-D locus of the *E. histolytica.*

Table 13: Summary of the samples produced the S^{TGA}-D profiles.

CODE	TYPE	GENDER	ORIGIN	S^{TGA}-D	S^{TGA}-D profile	Frequency
NNS05264	formed	female	Pretoria	100bp	1	1
HI051	loose	female	Pretoria	150, 200 and 240	2	1
NNS09872	loose	male	Pretoria	150bp	3	2
NNS05966	loose	male	Pretoria	150bp	3	2
NNS05256	watery	female	Pretoria	200 and 300bp	4	1
NNS08605	loose	female	Pretoria	280bp	5	2
NNS10683	loose	female	Pretoria	280bp	5	2
NNS10721	loose	female	Pretoria	400bp	6	2
NNS06388 A	loose	female	Pretoria	400bp	6	2

4.5.5.3. Distribution of the different profiles for the S^{TGA}-D of *E. histolytica.*

Of the 9 samples that amplified, 6 profiles were generated. Of all six different profile obtained in S^{TGA}-D locus as represented in **Table 14**, profile number 3, 5 and 6 occurred in two different samples each and all samples had loose consistency. Two samples observed in profile number 3 were males while the rest were females. Profiles number 1, 2 and 4 were observed in one sample each and were female but only one sample observed in profile number 4 had watery consistency.

Table 14: Distribution of the different profile for S^{TGA}-D locus of *E. histolytica.*

	Sample origin		consistency				Gender		
STGA-D profile	Pretoria		Formed	Loose	Watery		Male	Female	Total
1	1		1	0	0		0	1	1
2	1		0	1	0		0	1	1
3	2		0	2	0		2	0	2
4	1		0	0	1		0	1	1
5	2		0	2	0		0	2	2
6	2		0	2	0		0	2	2
Total	**9 (100%)**		**1 (11.1%)**	**7 (77.8%)**	**1 (11.1%)**		**2 (22.2%)**	**7 (77.8%)**	**9 (100%)**

CHAPTER 5

DISCUSSION, CONCLUSSION & RECOMMENDATIONS

5.1. Discussion

The objectives of this study were to determine the prevalence of *E. histolytica* and to determine the molecular characteristics of *E. histolytica* in relation to the occurrence of diarrhea among patients attending a rural primary health care clinic in Giyani and private clinics in the Gauteng region. *Entamoeba histolytica* is the causative agent of one of the most important infectious diseases called amoebiasis that affects mainly tropical and subtropical countries. *Entamoeba histolytica* is the pathogenic species that has been documented to cause diseases in humans and can also cause serious damage to the intestinal and other extra-intestinal organs, such as the brain and liver (Ximenez *et al.*, 2010). Amoebiasis remains a major cause of morbidity and mortality worldwide (Petri *et al.*, 2000). It is estimated that 50 million people have invasive diseases due to *E. histolytica* (Choudhuri *et al.*, 2012).

Microscopy is still the only tool used for the detection of *E. histolytica* in most countries, but cannot distinguish between the morphologically identical yet genetically distinct *E. histolytica* and *E. dispar*. Consequently, it is now believed that epidemiological figures on the disease and its spread are overemphasized since they relied on microscopic identification (Ali *et al.*, 2008). In the present study, 774 samples were examined microscopically for the presence of *E. histolytica* cyst in stool samples, only 129 (16.7%) were positive. Microscopic examination of a wet mount is very insensitive and nonspecific method which is performed on a fresh specimen

(Huston *et al.*, 1999). Repeated stool sample examinations may be needed in order to avoid the confusion with other forms of amoeba, polymorphonuclear leucocytes, *E. nana*, *E. coli, Giardia* and *cryptosporidium*.

The prevalence of *E. histolytica* infection varies in different parts of the world. *Entamoeba histolytica* was found to be more prevalent in Pretoria by 10.5% than in Giyani by 5.4%, but the difference was not statistically significant (X^2= 1.491; P= 0.222). In the present study, there was a high prevalence of *Entamoeba histolytica* infection in males (12%) than in females (8.4) and the difference was not statistically significant (X^2= 2.653; P= 0.103). These results agree with the results of the study done by Zurainee *et al.*, (2003), in which they demonstrated the high prevalence of *E. histolytica* infection in male with the rate of 24.6% as compared to the female with a low rate of 8.8%. The possibility of males having high prevalence of *E. histolytica* may be due to the fact that male's body fails to produce enough hormone that helps fighting the infection. Therefore, women suffer less from infection or disease due to the protection of estrogen which help in preventing the infection. (http://www.sharecare.com/health/endocrine-system/what-difference-male-female-hormones) Reports from other geographical locations revealed the same thing. The findings in this study were similar to those reported by Ozyurt *et al.*, (2007), who reported 67% prevalence of *E. histolytica* in male and 33% in female among patients attending training hospital. However, it disagreed with the results of the study done by Ozgumus *et al.*, (2007), in which he reported a high prevalence of *E. histolytica* infection for female with the rate of 64% and 36% for male among patients attending health care in Turkey, also a hospital-based study in Pakistan, observed a high prevalence of *E. histolytica* infection in female which was 31.5% compared to 19.6% of male (Ejaz *et al.*, 2011).

With regards to the results of *E. histolytica* infection among different age groups, the results showed that the participants of aged 0 – 25 years had a lower rate of infection (16.8%) and it is almost the same with the study conducted in Saudi Arabia (Al-Shammari *et al.*, 2001). This finding could be as a result of the parents being responsible for their children's hygiene (Al-Saeed *et al.*, 2006). The present study showed that the participants of age groups between 26- 45 years had higher rate of *E. histolytica* infection as compare with other ages 0-25 and 46-90 although this difference was not statistically significant (X^2= 3.341; P= 0.188). This is possibly due to poor housing conditions, poor sanitary practices, such as not washing hands after playing with soil or gardening, indiscriminate defecation in the river or bush, poor access to supplies of safe water. *Entamoeba histolytica* infection is a fecal oral disease, thus improper hygiene practice play a major role in the transmission of *E. histolytica* (Rivera *et al.*, 1998). A study conducted in Vietman also shown that the transmission of *E. histolytica* was more than three - fold risk increase if hands were not properly washed (Pham Duc *et al.*, 2011). While the results of this study disagree with the results of the study done by Dawood *et al.*, (2002), in which he demonstrated the high prevalence of *E. histolytica* infection at the rate of 66% among ages of 1-5 years.

Several PCR assays designed to differentiate *E. histolytica* from *E. dispar* have been described (Evangelopoulos *et al.*, 2000; Nunez *et al.*, 2001 and Gonin *et al.*, 2003). Most of them targeted either the small subunit ribosomal RNA gene or specific episomal repeats species. The PCR amplification for detection of small subunit ribosomal RNA genes is almost 100 times more sensitive than currently available ELISA kit for detection of *E. histolytica* antigens, when parasite forms isolated from cultured stool were used (Freitas *et al.*, 2004 and Troll *et al.*, 1997).

The NK locus successfully amplified 15 samples out of 42 positive samples identified by ELISA and confirmed by PCR. Nine different profiles were obtained in the present study. In the present study, the profiles were slightly different from those obtained from previous studies. However, we obtained higher diversity than those described in the Philippines by Rivera *et al.*, (2003) who found only six different profiles from 74 positive samples. Another finding in the present study also showed the occurrence of five identical samples having the same profile number (profile number 5). All five samples were from Giyani and 4 were females and 1 was male, and this profile was not associated with diarrhea. The fact that profile number 5 was only found in Giyani, may be due to unprotected water source and presence of domestic animals in almost all rural house hold. This finding corroborated previous findings in a study carried out by Obi *et al.*, (2002), in which it was reported that microbial quality of water from river used by the Venda rural communities of South Africa showed that the water from the source was unsafe for human consumption.

Another finding in the study was the occurrence of two different samples in NK locus profile number 6 (from Giyani, sample MS35 and from Pretoria, sample NS02226) and both samples were males and were not associated with diarrhea, and this indicates that this profile is found both in Giyani and Pretoria and it is non-pathogenic. This shows similarities between the strains found in Pretoria and those found in Giyani. The possibility of finding the same profile affecting individuals in the different areas may be due to prepared food sold in street. Previous study have demonstrated that *E. histolytica* is an indicator organism of fecal contamination and are frequently present in street food, and street food may cause outbreaks of amoebiasis and cholera

(Ajero *et al.*, 2008). Another finding in the study was the occurrence of two different samples having the same NK locus profile number 3 (MS4 and MS32) and was found in Giyani only. All two samples were males and both of them had loose consistency. As diarrhea is mainly transmitted via fecal oral route through the consumption of water and food contaminated with human excreta, the present study found that out of all those six different profiles, only NK locus profile number 1 had watery consistency indicating that it is associated with diarrhea and the rest were not associated with diarrhea. Transmission of agents that cause diarrhea are usually by the fecal oral route, which include the ingestion of fecal contaminated water or food, person to person contact and direct contact with infected feces (Andu *et al.*, 2002).

It was also found that the profile number was more prevalent in Giyani with 66.7% than in Pretoria with 33.3%, with high distribution in males than females. The results obtained in the present study indicate that there is an existence of the same strain infecting individuals from the Giyani and Pretoria, and it also shows that there are strains clustering from Giyani. The results of this study suggest that the NK locus might have a role in the presentation of amoebiasis (symptomatic and asymptomatic infections) depending on the infecting profile. The RR locus successfully amplified 30 samples out of 42 positive samples identified by ELISA and confirmed by PCR. In the present study, the profiles were slightly different from those obtained from previous studies. However, we obtained lower diversity than those obtained by Haghighi *et al.*, (2003) who obtained 22 profiles from 79 isolates, while we obtained 16 profiles from 30 positive stool samples.

Another finding in the present study showed the occurrence of three different samples having the same RR locus profile numbers 7 (sample MS4 was from Giyani and samples NS09644 and NS02159 were from Pretoria). Both samples had loose consistency indicating that they are not associated with diarrhea. The possibility of this profile affecting individuals from rural and urban areas may be due to the consumption of unwashed fruits or contact with vegetables sold in the street of two distinct geographical areas. Previous study have demonstrated that *E. histolytica* is an indicator organism of fecal contamination and are frequently present in street food, and street food may cause outbreaks of amoebiasis and cholera (Ajero *et al.*, 2008).

Another finding was the occurrence of four different samples having the same RR locus profile 9 (sample MS8 from Giyani and samples NS06861, NNS08583 and NNS03624 were from Pretoria). Out of those four samples, only two samples from Pretoria were associated with diarrhea indicating that this profile is pathogenic to Pretoria and non-pathogenic to Giyani. Diarrheal disease affects people of all ages and is one of the greatest causes of morbidity and mortality throughout the world (Bern *et al.*, 2003).

Another finding was the occurrence of three different samples having the same RR locus profile number 10 (samples NS03305, NS07121 and NS00335 from Pretoria). Out of those three samples, two samples were males and one was female. Of these three samples, two samples had loose consistency and another one had formed consistency indicating that it is not associated with diarrhea. The possibility of this profile affecting individuals from rural and urban areas may be due to poor personal hygiene. Previous studies reported that fecal oral route is significant in the transmission of *E. histolytica* to humans via poor personal hygiene (Okyay *et al.*, 2004).

Another finding in the present study was the occurrence of four different samples having the same RR locus profile number 11. Out of those four samples, two were from Giyani (male and female) and another two were from Pretoria (male and female). Of these four samples, three samples had loose consistency and one sample had formed consistency, indicating that all four samples are not associated with diarrhea. Another finding in the study was the occurrence of three different `samples having the same RR locus profile number (profile 15). Out of those three samples, two were from Pretoria (males) and another one was from Giyani (female). Of these three samples, two samples had loose consistency and one sample had formed consistency, indicating that both samples are not associated with diarrhea. The fact that profile number 11 and 15 affect individuals from rural and urban areas may be due to prepared food sold in market. Foods sold in markets may be contaminated from hands that have not been washed after defecation or from flies that land on both food and feces hence increasing risks of transmission of intestinal parasites to consumers (Nichols, 1999)

Interestingly, another finding in the study was the occurrence of two different samples having the same RR locus profile number 2. Both samples were from Giyani (females) and are not associated with diarrhea because they had loose and formed consistency. The possibility of this profile affecting individuals from Giyani only may be due to consumption of drinking contaminated water from the river. A previous report on the microbial quality of river water used by the Venda rural communities of South Africa showed that water from the source was unsafe for human consumption (Obi *et al.*, 2002).

A study done by Gabon *et al.*, (2004) indicated that the transmission of agents that cause diarrhea are usually by the fecal oral route which include the ingestion of fecal contaminated water or food, person to person contact and direct contact with infected feces. It was found that the profile number was more prevalent in Pretoria with 63.3% than in Giyani with 36.7% and with high distribution in males with the rate of 55.2% compared to females with the rate of 44.8%. The results obtained in the present study indicate that there is an existence of the same strain infecting individuals from the Giyani and Pretoria, and it also shows that there are strains clustering from Pretoria. The results obtained in the present study suggest that the RR loci might have a role in the presentation of amoebiasis (symptomatic and asymptomatic infections) depending on the infecting profile.

The AL locus successfully amplified 25 samples from 42 positive samples by ELISA and confirmed by PCR. Due to the small sample size used in this study, the profile number were low compared to the profile numbers obtained by Haghighi *et al.,* (2003), where 22 profiles were obtained from 74 positive isolates. Another study done by Samie *et al.*, (2008), also showed a high profile number of 26 profiles from 38 isolates. A total of 15 different profiles of AL locus were obtained in the samples from Giyani and Pretoria. The profiles were associated with gender, two distinct geographical areas and sample consistency of the study participants. High diversity of the AL profiles was obtained mostly in Pretoria with the rate of 72% than in Giyani with the rate of 28% and this indicates that this profile is circulating in the peoples of Pretoria and most of the samples were associated with diarrhea. The presentation of infection depending on the infecting profile.

Another finding was the occurrence of two different samples having the same AL locus profile number 2 and both of them were from Pretoria. Out of those two samples, one was males and another one was female. Of these samples, only a male had watery consistency indicating that it is associated with diarrhea, therefore it is the cause of amoebiasis. Previous studies reported that fecal oral route is significant in the transmission of *E. histolytica* to humans via poor personal hygiene (Okyay *et al.*, 2004). Another finding was the occurrence of two different samples having the same profile number 3. Both samples were from Pretoria (males and female) and had loose consistency. Another finding was the occurrence of profile number 5 that was seen in most of the samples and was found in Giyani and Pretoria. Both samples were not associated with diarrhea. Another finding was the occurrence of two different samples having the same AL locus profile number from Pretoria. Out of those two samples one was a male and another one was a female and both of them had loose consistency indicating that they are not associated with diarrhea.

Another finding was the occurrence of two different AL loci profile numbers (profile 4 and 7) that were found only in Pretoria. Both profiles had one sample each (male or female) and had watery consistency. The finding of the same profile affecting individuals in the same area was also demonstrated in a study conducted by Ali *et al.*, (2014). In the present study, it was found that the profile number 2, 3 and 5 were more prevalent in Pretoria with 72% than in Giyani with 28% and with high distribution in females with the rate of 52% than males with the rate of 48%. The results obtained in study also indicate that there is an existence of the same strain infecting individuals in both Giyani and Pretoria, and some strains were specific to Pretoria.

The DA locus successfully amplified 13 isolates from 42 positive samples identified by ELISA and confirmed by PCR. Six different profiles were obtained in the present study. In this study, the profiles were slightly different from those obtained from by Haghighi *et al.*, (2003) who obtained 22 profiles from 79 isolates and those obtained by Samie *et al.*, (2008), who obtained 26 profiles from 38 isolates. However, our results are similar to those obtained by Rivera *et al.*, (2006) who found only six different profiles from 79 isolates.

Another finding in the present study was the occurrence of two different samples having the same DA locus profile number (NNS09797 and NS08098) and both samples were from Pretoria. Both sample were males from Pretoria and were not associated with diarrhea. Another finding was the occurrence of five different samples having the same DA locus profile numbers 2 (NNS08605, NNS09872, HI043 and NS02968 from Pretoria and GY85 from Giyani). Out of those five samples, four samples had loose consistency and one sample had formed consistency. Another finding was the occurrence of three different samples having the same DA locus profile number 5 (NMS04644, NS06859 and NNS 06055) and all the samples were from Pretoria. Out of those three samples, only one male had watery consistency indicating that it is associated with diarrhea and the other two are non-diarrhea.

Another finding was the occurrences of two different DA locus profiles having one sample each (profile number 4 and 6). Both profiles were found only in Pretoria and were associated with diarrhea, therefore it is the cause of amoebiasis in the study population. In the present study, it was found that the profile number was more prevalent in Pretoria with 84.6% than in Giyani with 15.4% and with high distribution in male with the rate of 66.7% than female with the rate of

33.3%. The results obtained in study also indicate that there is an existence of the same strain infecting individuals from Giyani and Pretoria, and it also shows that there are strains clustering from Pretoria.

The S^{TGA}-D locus successfully amplified 9 samples from 42 positive samples identified by ELISA and confirmed by PCR. Six different profiles were obtained in the samples from Giyani and Pretoria (Table 13). In the present study, the profiles were slightly different from those obtained by Haghighi *et al.,* (2003), who obtained 22 profiles from 74 positive isolates and another study by Samie *et al.*, (2008), who obtained 26 profiles from 38 isolates. However, our results are similar to those obtained by Rivera *et al.*, (2006) who found only six different profiles from 79 isolates.

Another finding in the present study was the occurrence of two different samples having the same S^{TGA}-D locus profile numbers 3 (NNS09872 and NNS05966) and both of them were from Pretoria. Both samples were two males and had loose consistency indicating that they are not associated with diarrhea. This indicates that the present of infection depend on the infective profile. Another finding was the occurrence of two different samples having the same S^{TGA}-D locus profile number 5 (NNS08605 and NNS10683 from Pretoria). Both samples were two females and had loose consistency indicating that they are not associated with diarrhea.

Another finding was the occurrence of two different samples having the same S^{TGA}-D locus profile number 2 (NNS10721 and NNS06388) and both samples were from Pretoria. Both samples were females and had loose consistency. The finding of the same profile affecting

individuals in the same area was also demonstrated in a study conducted by Ali *et al.*, (2014). Of all nine different profile obtained in the study, only profile number 4 that was a female had watery consistency, indicating that this profile was pathogenic. In the present study, it was found that there is an existence of the same strain infecting individuals from Pretoria.

5.2. Overall Conclusions and Recommendations

5.2.1. Conclusions

The present study indicates that infection caused by *E. histolytica* was more prevalent in Pretoria than Giyani. *Entamoeba histolytica* infection was more prevalence in males than in females, and in the age group of 26- 45 years. The possible risks of infection in this study might be drinking water from unprotected source or by direct contact with infected animals (especially cats, dogs, pigeons and hens). Therefore, further impact of drinking water quality and hygiene is still an important factor as *E. histolytica* can also be transmitted through drinking water which is contaminated by fecal materials. According to our results, microscopy is a simple method and it should be combined with other methods such as ELISA and PCR for identification of the species to avoid false and/or insufficient diagnosis and treatment applications.

We are confident that the data will improve the understanding of the genotype contribution to the outcome of *E. histolytica* infection in relation the occurrence of diarrhea and non- diarrhea. The tRNA genes identified in this study was associated with *E. histolytica* infection. Some of the tRNA genes identified in this study show promise as surrogate markers for prediction of infection outcome of *Entamoeba histolytica*. Further investigations are required to confirm these findings using larger samples collected from different locations.

5.2.2. Recommendations

It is necessary to examine more than one slide from each stool sample to get more accurate diagnosis. Because transmission is frequently associated with contaminated food and water, people should be educated about safe storage of food and handling. The importance of personal hygiene and its role in prevention of infections will help to reduce the transmission of infection. Improved water supplies should be used, including protection of community wells. The analysis of genotypes of *E. histolytica* should help in determining the geographic origins of isolates and the potential routes of transmission.

CHAPTER 6: REFERENCES

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