Genetic diversity of *Entamoeba* species, and the impact of the Madi Drop on the occurrence of *Entamoeba* among children in Vhembe, South Africa

BY

MAPONYA MARC MASILO

(11626662)

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To The

Department of Microbiology

The School of Mathematical and Natural Sciences

University of Venda

Private Bag X5050

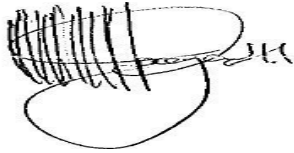Thohoyandou

0950

South Africa

Supervisor: Prof A. Samie

Co-Supervisor: Prof P.O Bessong

Declaration

I Maponya Marc Masilo declares that the present dissertation for MSc in Microbiology entitled: "Genetic diversity of *Entamoeba* species, and impact of the Madi Drop on the occurrence of *Entamoeba* among children in Vhembe, South Africa" hereby submitted by me to the University of Venda, has never been submitted to any other institution or existed before for this or any other degree. I am declaring that this is my work in purpose and execution with no other exudation. In the case where some of the information is from an external source, all the references that I have quoted, have been acknowledged using a reference list.

Signature: Date: 29 June 2020

Dedication

I dedicate this MSc research to my son Phokele Johannes Maponya.

Acknowledgments

I give all the glory to God for His grace and strength that sustained me throughout my study period. I will forever be grateful to my wonderful supervisor Professor Samie A. and my co-supervisor Professor Bessong P.O for having faith in my abilities and for taking me on board as one of their students. You have been a source of encouragement and strength throughout the program, thank you very much for your support and understanding. To my mentor, Dr. R Ngobeni thanks for being a good mentor and for your encouraging words during my experiments. To the parasitology laboratory team, I am sincerely grateful for your technical support and help during my time of the study, you all made the laboratory a great place for me to do my Master`s Degree. I would like to thank Madi-Trial field workers for collecting samples used in this study. Finally, to my wonderful friends, Zanele Esnart Mbewe and Tendamudzimu Harmless Dongola thank you for encouraging me all the time when I had lost hope. I will forever be grateful.

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DNA	Deoxyribose Nucleic Acid
PCR	Polymerase Chain Reaction
RNA	Ribose Nucleic Acid
EDTA	Ethylenediamine Tetra Acetic Acid
Kb	Kilo base pairs
Bp	Base pairs
µg	Microgram
µl	Microliter
µm	Micrometre
%	Percentage
°C	Degree Celsius
dNTP	Deoxyribonucleoside Triphosphate
RBC	Red blood Cells
IgA	Immunoglobulin A
IgG	Immunoglobulin G
AIDS	Acquired Immunodeficiency Syndrome
ALA	Amoebic liver abscess
CD4	Cluster of differentiation 4
CDC	Centres for Diseases Control and Prevention
Chit	Chitinase
ELISA	Enzyme-linked immunosorbent assay
et al	*Et al*ia (and others)
G	Gram

M	Molar
R	Reverse
F	Forward
WHO	World Health Organization
NHPs	Nonhuman primates
NGS	Next Generation sequencing

Abstract

Background: Amebiasis is a common parasitic disease that contributes to the burden of diarrhea in areas with poor sanitation and inadequate water supply worldwide. Water quality, therefore, plays an essential role in the transmission of amebiasis. The Madi Drop is a recent point of use water treatment tool that has not been tested for its efficiency against parasitic infections. Therefore, the present study sought to investigate the genetic diversity of *Entamoeba* species, and the impact of the Madi Drop utilization on the occurrence of *Entamoeba* among children in Vhembe, South Africa.

Materials and methods: The present study is part of the Madi Trial project entitled: The effectiveness of low-cost use water treatment technology to prevent stunting in children in Limpopo province. The trial included 4 groups of children recruited in different households to whom a filter with either the Madi Drop or not was given as well as a control group. Stool samples were collected every 3 months from (month 0 to 24 months follow up). For the present study, a total of 534 stool samples (months 18 and 24) from 313 participants both males and females, aged 3 years and below were used. All the stool samples were examined microscopically for the presence of *Entamoeba* cysts and trophozoites. DNA was extracted from all the samples by QIAamp Fast DNA stool mini kit and was subjected to conventional PCR for the presence of *Entamoeba* genus. Positive amplicons were sequenced by the Sanger method and 15 were selected for Next-generation sequencing in the MiSeq platform based on the *Entamoeba* 18S rRNA gene, using a MiSeq v3 (600 cycles) kit. Data analysis was performed at Inqaba in-house developed data analysis. The phylogenetic trees were constructed to determine the species relatedness.

Results: Of the 313 children recruited in the study, 163 were females and 150 were males, the age ranged between 1 to 3 years. Of 534 samples, 130/534 (24.3%) were microscopically positive for *Entamoeba* cysts. The month 18 sample had the highest presence of *Entamoeba* cysts 78/299

(26%) compared to month 24; 52/235 (22%). *Entamoeba* infections were high before the interventions of Madi Drop technology as compared to after intervention. Forty-three (8%) of the total samples were positive for *Entamoeba* genus by PCR. Twenty positive amplicons were sequenced by Sanger sequencing technologies. Of these, 11/20 (55%) were *E. polecki* 8(40%) followed by *E. coli* 2 (10%) and *E. muris* 1(5%). Next-generation sequencing showed a wide variety of organisms. Of note was the fact that about 3 different species of *Entamoeba* were found in most of the samples. The NGS results revealed that 14/15 (93%) of amplicon were positive for *Entamoeba* species, dominated by *E. coli* 9/15 (60%), followed by *E. polecki* 3/15 (20%) and *E. moshkovskii* 2/15 (13%). The phylogenetic tree showed the close relationship between isolated species and the ones in the GenBank.

Conclusion: The present study showed that the use of Madi Drop helped reduce the number of *Entamoeba* infections among children in a rural community north of South Africa. Even though the identified species are regarded or known to be non-pathogenic, their presence indicates intense fecal-oral transmission. Therefore, water could play a very important role in the transmission of *Entamoeba* and possibly other parasites in the region. Furthermore, in some cases, *E. polecki* and *E. moshkoviskii* have been isolated from patients that encountered an episode of bloody diarrhea associated with many *Entamoeba polecki* and *E. moshkovskii* cysts in the stool. The results suggested that the usage of Madi Drop and filters may decrease the level of parasitic infections and improved drinking water quality in these communities.

Keywords: Amebiasis; Madi Drop; Diversity; *Entamoeba* species; Microscopy; Next-generation sequencing; Parasites; phylogeny; PCR; Sanger sequencing; Water quality.

Chapter 1

General introduction

1.1 Background

The genus of *Entamoeba* contains the group of unicellular, anaerobic and parasitic microorganisms that infect the gastrointestinal tract of both humans and animals worldwide (Ngobeni *et al.,* 2017). *Entamoeba* genus contains several types of species which include, *Entamoeba histolytica, E. dispar, E. moshkovskii, E. coli, E. hartmanni, E. muris and E. polecki* (Hirashima *et al.,* 2017). To date, the identification, detection and differentiating of these species are depending mainly on their morphological characteristics (Verweij *et al.,* 2014).

The genetic diversity of *Entamoeba* species is one of the major processes followed to find a better understanding of the species (Kawano *et al.,* 2017). The genetic diversity of *Entamoeba* species leads to an understanding and assist in the re-description of the previous species (Partida-Rodríguez *et al.,* 2017). In the *Entamoeba*, some of the protein-coding genes with none coding show some variability. The identification of pathogenic species of *Entamoeba* may help in insight about the treatment, control, diagnosis, and the epidemiology of the species (Partida-Rodríguez *et al.,* 2017).

Due to humans' and animals' medicinal purposes, the diversity of these parasites has been investigated (Kawano *et al.,* 2017). Even though many *Entamoeba* species have been isolated by molecular techniques from humans and animals stool example, *E. histolytica*, *E moskovskii*, *E. dispar E. nuttalli*, and *E. gingivalis* and *E. invadens*, *E. insolita*, and *E. terrapinae*) respectively, but still the total genetic diversity of the *Entamoeba* species it is poorly known (Kawano *et al.,* 2017).

Of all known *Entamoeba* species, *E. histolytica* is documented to be associated with pathological sequelae in humans (Shirley *et al.,* 2018; Martinez, 2019). However, some studies have reported the discovery of *E. dispar* and *E. moshkovskii* in a human that were showing symptoms related to that of *Entamoeba histolytica.* Another case is that *Entamoeba polecki* has been isolated from the patient experienced several events of diarrhoea and bloody diarrhoea which associated with the high number of *Entamoeba polecki* cysts. Study done by Lozano, (2017) reported that the presence of *E. moshkovskii* can cause the weight loss in susceptible diarrhea in Bangladeshi children, however, his studies emphasizes the pathogenicity of this species. It can be suggested that 2 *Entamoeba* species might be considered as pathogenic *E. histolytica* and *E. moshkovskii*. The evidence concerning that is still under investigation that *E. histolytica* is not the only pathogenic member of the *Entamoeba* genus, but other species such as *E. moshkovskii* can also cause enteric diseases. However, there is still no definitive pathological characteristics of these three species and the symptoms on the host (McHardy *et al.,* 2014).

Entamoeba histolytica is responsible for amebiasis, a disease that may manifest in different forms including amoebic colitis, liver abscesses, bloody diarrhea and other extra and intra intestinal infections (Kantor *et al.,* 2018; Avila *et al.,* 2016). Amebiasis is responsible for the heavy burden of diarrhea in developing countries of less clean water and poor sanitation (Costa, 2018). Approximately 40 to 50 million humans are infected with invasive *Entamoeba histolytica* and about 100 000 die annually (WHO).

Dhaka and Bangladesh reported about 50% death cases of children under 6 years, and of those children 50% reported death had serological evidence of *Entamoeba histolytica* (Haque *et al.,* 1999; Haque *et al.,* 2002). Furthermore, the reported children had suffered from other infections with malnutrition and cognitive development.

Entamoeba histolytica associated diarrheal diseases have been reported recently that they harm the growth of children (Gilchrist *et al.,* 2015). Amebiasis infection ranges from asymptomatic to symptomatic infections such as amoebic dysentery and invasive amebiasis (Alum *et al.,* 2016). Previous studies have indicated that about 10% of people who become infected with this parasite are likely to progress to symptomatic diseases and others remain asymptomatic (Smith, 2016; Gathiram *et al.,* 1985).

A high prevalence of amebiasis infections is mostly seen in developing countries especially where there is inadequate water supply in certain regions (Burgess *et al.,* 2014). These infections are spreads mostly through the consumption of water contaminated by *Entamoeba* cyst. These parasites can be transmitted by oral and normal sexual intercourse, especially among gays (alum *et al.,* 2016). The geographically variable disease has been observed, and it has been reported that the invasive disease predominantly affects men (Abd-Alla and Ravdin, 2002; Acuna-Soto *et al.,* 2000; Bhattacharya *et al.,* 2002; Stauffer and Ravdin, 2003). There is no vaccine for amebiasis but nitroimidazole is the current medication used for the treatment of invasive amebiasis (Shirley *et al.,* 2018).

Microscopic examination is the most preferable when it comes to the diagnosis of amebiasis infections, it is known as a gold technique that is used for parasitic diagnosis (Verweij *et al.,* 2014). The limitation of this method is that it cannot distinguish between the different *Entamoeba* species. To date, several molecular diagnostic tests, which include PCR and ELISA are available for the identification, detection and differentiating the *Entamoeba* species in stool samples (Laude *et al.,* 2016). The molecular techniques provide clear information about the evolution, epidemiology of the amebiasis, mostly in the high endemic rate areas (Zeile *et al.,* 2015). Regardless of the availability of effective medication, death and disease cases associated with amoebic infections have persisted, and it is suggested that interventions designed to limit these infections are

ineffective (Williamson *et al.,* 2017). Currently, the most effective way to prevent and control these infections is to practice proper hygiene.

The higher number of *Entamoeba* infections is transmitted through contaminated water which contributes to the problem in children's cognitive development. Therefore, Lacking access to water quality in rural settings is a major problem and has led to an increase of parasitic infections in humans. Therefore, it is very important to treat water in an appropriate way to remove or reduce the pathogenic microbes especially in the rural communities. Affordable, safe and fast water treatment strategies in rural communities are very important.

In many areas, Various strategies for water treatment is utilized to provide safe drinking water quality. There are different common types of household water treatment systems which include, water softeners, distillation systems, disinfection, boiling and filtration systems. Water softeners are the devices which reduce the water hardness. It uses chemicals such as NA and K ions to remove the presence of Ca and Mg ion that are responsible for the hardness in the water (Applebaum, 2013).

Water treatment strategy involves the use of the chemical and physical processes of removing or killing pathogenic microbes (Ghernaout, 2018). The example of the chemical process may be the use of chlorine dioxide as well as ozone and the physical examples involve UV light, electronic radiation as well as the heat. Distillation is the process of purifying water through boiling, and the steam is collected by the condensation process in a separate container and leaves out the solid contaminants (Zhou, 2015). Boiling is a direct traditional method that is used by many communities for water treatment but may be very costly as it is energy intensive.

Filtration systems are a form of a device that removes impurities from drinking water through physical barriers and biological processes (Westerhoff, 2016). To date, many communities are using filtration techniques for the treatment of water. Filtration system involves devices such as Madi Drop which are currently used because they are effective, affordable, safe, and easy to use as compared to other methods.

The Madi drop was originated in the United States. It is an amazing and effective process in controlling and reducing the biological contaminations in the water at the household level (Carstea *et al.,* 2016). This method is very effective in preventing waterborne diseases without changing the water smell, taste, and colour. In terms of capacity, Madi drop can treat about 20 litres of water per day. It is guaranteed that it works for more than a year and gives out more than 6000 litres of quality water throughout. It is very easy to use; it is just a matter of placing it in the drinking water bucket and start drinking. The production of Madi drop does not involve chemicals or fillers, it was made from natural materials including clay soil and silver nanoparticles. It contains micro-pores and water permeable ceramic tablets which infused with silver nanoparticles. Madi drop contains advantageous features, highly effective, low cost, user-preferred, unlimited shelf life, small and portable as well as no chemicals involved (Smith *et al.,* 2015).

1.2 Study rationale

Diarrheal infection is the second leading cause of death worldwide after Malaria. It is responsible for the diseases and deaths particularly mostly to immunocompromised individuals and children under the age of 5 years (Farthing *et al.,* 2013). Diarrheal diseases are mostly seen in areas where there is not enough clean water supply and poor sanitation, and South Africa is one of the countries that have limited resources (Hunter *et al.,* 2010).

The occurrence of diarrheal diseases also reflects the economic status of the country and it has a tortuous effect on the countries' economy by reducing the health of its workforce (Birn *et al.,* 2017). Other than severe or unique clinical complications, in many cases, diarrheal diseases usually occur because of the consumption of ingestion and drinking contaminated water with microorganisms such as parasites (Ashbolt *et al.,* 2004). Few studies done by Samie *et al.,* 2008, 2009 and 2010 reported a high occurrence of diarrheal diseases in South Africa of which many are due to parasitic infection with the special reference to *Entamoeba* species.

Entamoeba species especially *Entamoeba histolytica* are responsible for amoebiasis, a disease that leads to bloody diarrhea, liver abscess, colitis, and other intestinal infections to humans. It can negatively affect the development of children, for example, growth, cognitive performances, and physical fitness (Mortimer *et al.,* 2010). These parasites are mostly transmitted through contaminated water because their cysts can survive a longer period in the environment.

Poor water quality remains a serious problem worldwide especially in the rural areas of developing countries. The MAL-ED study reported the prevalence of cognitive development problems in children as increased from 12% in the first month of life to 37% at 2 years of age overall, with several villages having close to 50% cognitive development problem at 2 years and all these cases were associated with a lack of access to water quality 9 (De Onis *et al*., 2013).

Since children are at high risk of becoming stunted and have poor access to clean water and drinking water quality, studying water treatment strategies and parasites will benefit the population as well as society at large. Since these parasites are mostly transmitted through contaminated water, lacking access to clean water may lead to an increase of parasitic infections and exposure to diarrheal diseases.

The list of microorganisms that can be found in contaminated water and which are responsible for causing diarrhea and other intestinal infection is dominated by common bacteria, viruses, and parasites. Among these causative agents, the parasitic aetiologies are often neglected because of their lesser severity (Tan *et al.,* 2012). *Entamoeba* species are one of the neglected parasites worldwide including Africa, with special reference to Dzimauli communities in the Vhembe district of Limpopo province. Investigations done in the study population were mostly focused on pathogenic *E. coli*, *Cryptosporidium*, *Shigella*, *Giardia*, *Campylobacter* and *Adenovirus*.

Studies on the genetic diversity of *Entamoeba* species have been reported worldwide. Feng *et al.,* (2013) reported the genetic diversity of *Entamoeba* species in China, in the report he highlighted the detection of 50% of *Entamoeba polecki* from pigs and humans. In South Africa, Samie *et al.,* (2008) reported the genetic diversity of *Entamoeba* African strains according to polymorphism of the serine-rich protein found in *E. histolytica*. Such studies have never been done in Dzimauli communities.

Affordable, fast and easy to use water treatment strategies have been developed. The combination of Ceramic filters and the Madi Drop may improve the capacity of the filters to efficiently remove parasitic organisms from water. However, such has not been evaluated in a community setting. Such may constitute a good solution for the parasitic infections in the communities. Several water treatment strategies can be used in many communities which involved, filtration, distillation, boiling, and disinfection. To date, technologies such as ceramic filters and Madi Drop are

preferable methods for water treatment because of their safety and it does not involve chemicals, unlike other techniques. It is very effective, cheap and easy to use as a method of cleaning water and removing pathogens mechanically.

Therefore, the present study is sought to determine the genetic diversity of *Entamoeba* species, and the impact of the Madi Drop on the occurrence of *Entamoeba* among children in Vhembe, South Africa.

1.3 Objectives of the study

1.3.1 Primary objective

- To determine the genetic diversity of *Entamoeba* species, and the impact of the Madi Drop on the occurrence of *Entamoeba* among children in Vhembe, South Africa

1.3.2 Secondary objectives

- To examine the presence of *Entamoeba* cyst using a light microscope.

- To evaluates the efficacy of the Madi Drop to decrease the number of parasitic infections.

- To determine the molecular characterization of *Entamoeba* species by conventional PCR.

- To determine the genetic diversity of *Entamoeba* species by Sanger and Next-generation sequencing technologies.

Research questions

- How common and diverse are *Entamoeba* species in the study population?

- Does the usage of the Madi Drop reduce parasitic infections in rural settings?

Hypothesis

- The presence of *Entamoeba* infection is prevalent in the study population,

- The usage of the Madi Drop reduces the level of parasitic infections among children less than 5 years of age.

Chapter 2

Literature review

2.1 Background

Diarrheal diseases caused by parasitic infections especially *Entamoeba* species are closely related and associated with inadequate water supply, poor sanitation and environmental contamination with fecal matters in the developing countries (Calegar *et al.,* 2016; Ojha *et al.,* 2014, Turkeltaub *et al.,* 2015). Amebiasis is one of the parasitic infections that causes health problems and life-threatening diseases worldwide. These parasitic infections are mostly seen in children (male and female) under 5 years worldwide and are caused by *Entamoeba histolytica*, most commonly, which is distributed worldwide (WHO 1997; Jackson 1998).

Amebiasis infections are mostly transmitted by contaminated water and food with *Entamoeba* cysts (Al-Areeqi *et al.,* 2017). In the case of transmission, cysts are responsible because they can survive the external environment conditions (Al-Areeqi *et al.,* 2017). Therefore, water quality plays an essential role in the transmission of amebiasis. Poor drinking water quality can increase the level of parasitic infections in the communities. The most important route to control these parasites is to clean, treat and purify water before use. Therefore, it is important to consider water treatment strategies in the household to control parasitic infections in rural communities.

2.2 Common water treatment strategies used in households

2.2.1 Water softeners process

Water softeners are devices that are used to reduce the hardness of the water. They typically use sodium (NA) and potassium (K) ions to remove or reduce calcium (Ca) and magnesium (Mg) ions that are responsible for the hardness (Wachinski, 2016). It uses the ion exchange for chemical and ion removal to reduce the amount of hardness in water. Water softeners are designed in a way that they can remove also iron, magnesium, heavy metals, nitrates, and sulfate shown in figure 1. The

problem with this strategy is that it cannot remove some of the microbes such as protozoan, bacteria, and viruses (Wachinski, 2016).
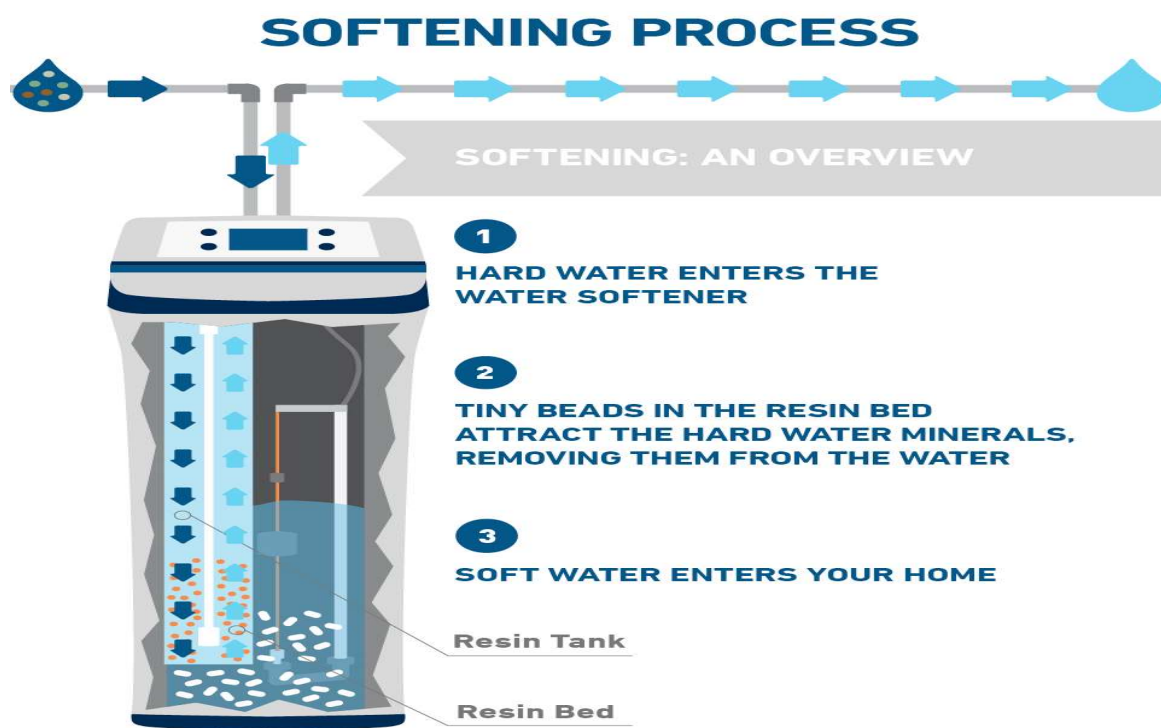
Figure 1: The representation of the softening water treatment strategy flow (https://homewater101.com/articles/water-softeners-work).

2.2.2 Distillation systems

The distillation system is a process of cleaning water by boiling and collect the steam into a separated separate flask and throw away the solid contaminants (Armarego, 2017). This system, water is been boiled until they reach boiling point, then water vapor is collected as it condenses shown in figure 2. This method is very effective in the removal of different types of microbes including, protozoan parasites such as *Cryptosporidium* and *Giardia,* bacterial microbes including salmonella and *E. coli* and *viruses* (*Norovirus and Rotavirus*). It can also reduce the contaminates including, sodium and many organic chemicals. It is expensive, slow and not easy to use because it involves heat in the process.
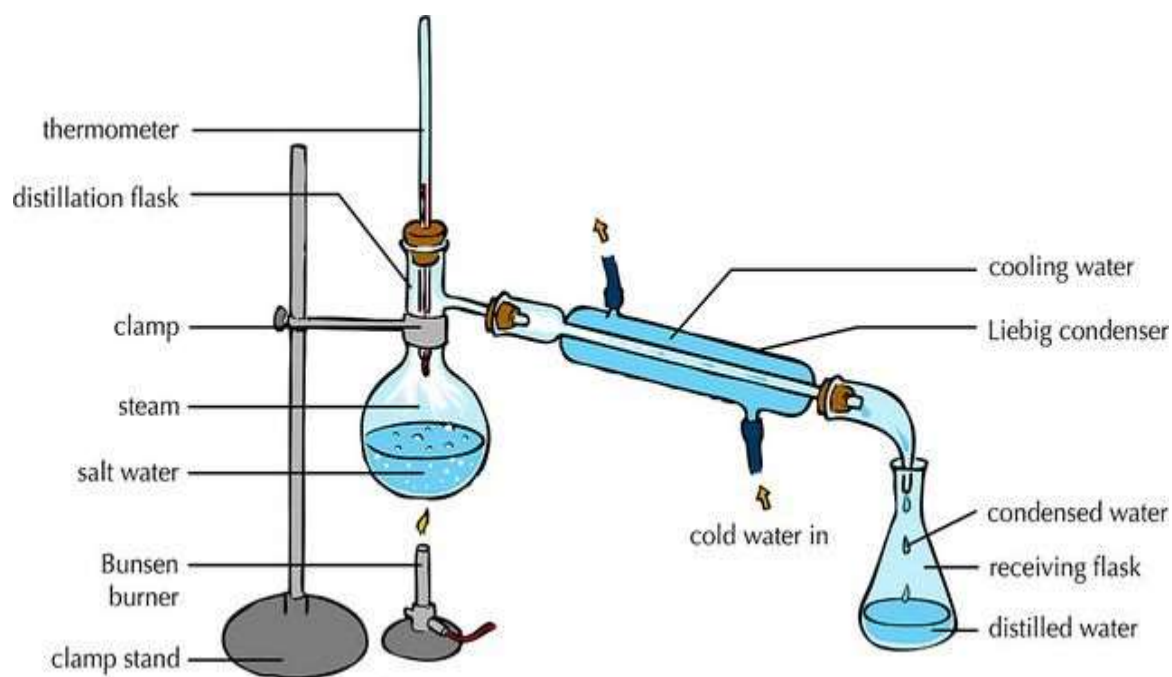
Figure 2: The representation of distillation system (https://za.pinterest.com/pin/835910380817166835/).

2.2.3 Disinfection method

Disinfection is the process of removing and killing pathogenic microbes using physical (UV light, radiation, and heat) and chemical (chlorine and ozone) materials (Meireles, 2916). In this process, the chemical is been added in the water before use shown in figure 3. It not user friendly because some individuals cannot uptake a certain amount of chlorine and some of the microbes such as cryptosporidium cannot be removed by chlorine.

Figure 3: Representation of disinfection by chlorine (http://techalive.mtu.edu/meec/module03/Sources-SurfaceWater.htm).

2.2.4 Reverse Osmosis Systems

The reverse osmosis system is a process that involves the reverse flow of water in a natural way of osmosis (Habimana *et al.,* 2014). Water can pass through the higher concentrated solution to a low concentrated solution through a permeable membrane. and it involves the post and pre-filters that are mostly combined along with the reverse osmosis membrane figure 4. It contains a pore size of about 0.0001 microns. This method can remove and reduce the level of *protozoans*, *bacterial* and *viral* infections, and sometimes can also remove chemical contaminants such as metal ions and aqueous salts. The limitation with this system is that it is very expensive, most of the rural villagers cannot afford it and requires time to time maintenance.

Figure 4: Reverse osmosis water system (https://www.aquazania.co.za/2019/08/the-best-reverse-osmosis-water-filters-to-keep-your-drinking-water-safe/).

2.2.5 Ultraviolet Treatment Systems (with pre-filtration)

The ultraviolet treatment system is a process that involves the pre-filtration, it uses UV light to clean the water or reduce the level of pathogenic microorganisms (Siegrist, 2017). The ultraviolet treatment system is very highly effective in the removal of the protozoa parasites such as *Giardia* bacteria including pathogenic *E. coli* and *viruses*, but it is limited to remove the chemical contaminants and it is expensive shown in figure 5.
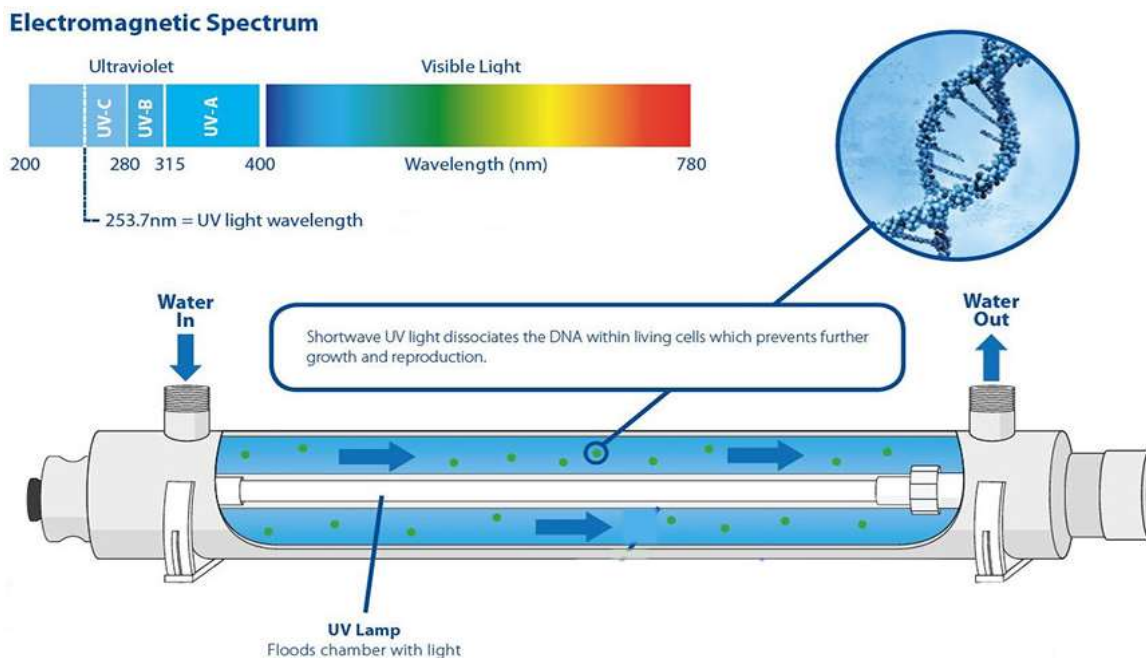
Figure 5: Ultraviolet treatment system flow (https://www.alfaauv.com/blog/all-about-uv-disinfection-systems-for-water-treatment/).

2.2.6 Filtration systems

The filtration process is a process of removing contaminations or impurities from the drinking water by a device through a physical barrier and biological process (Sharma *et al*., 2017). Filtration process takes place when the liquids and the suspended matter attached on the surface of the pores shown in figure 6 (Tien, 2013).

Filtration of contaminants is depending on the size and amount of the particles and their charges in that contaminant. In many cases, pre-treatment of the water before filtration is advisable, which includes, the addition of coagulants and powdered activated carbon. The Filtration system is divided into, microfiltration, Ultrafiltration, and nanofiltration.
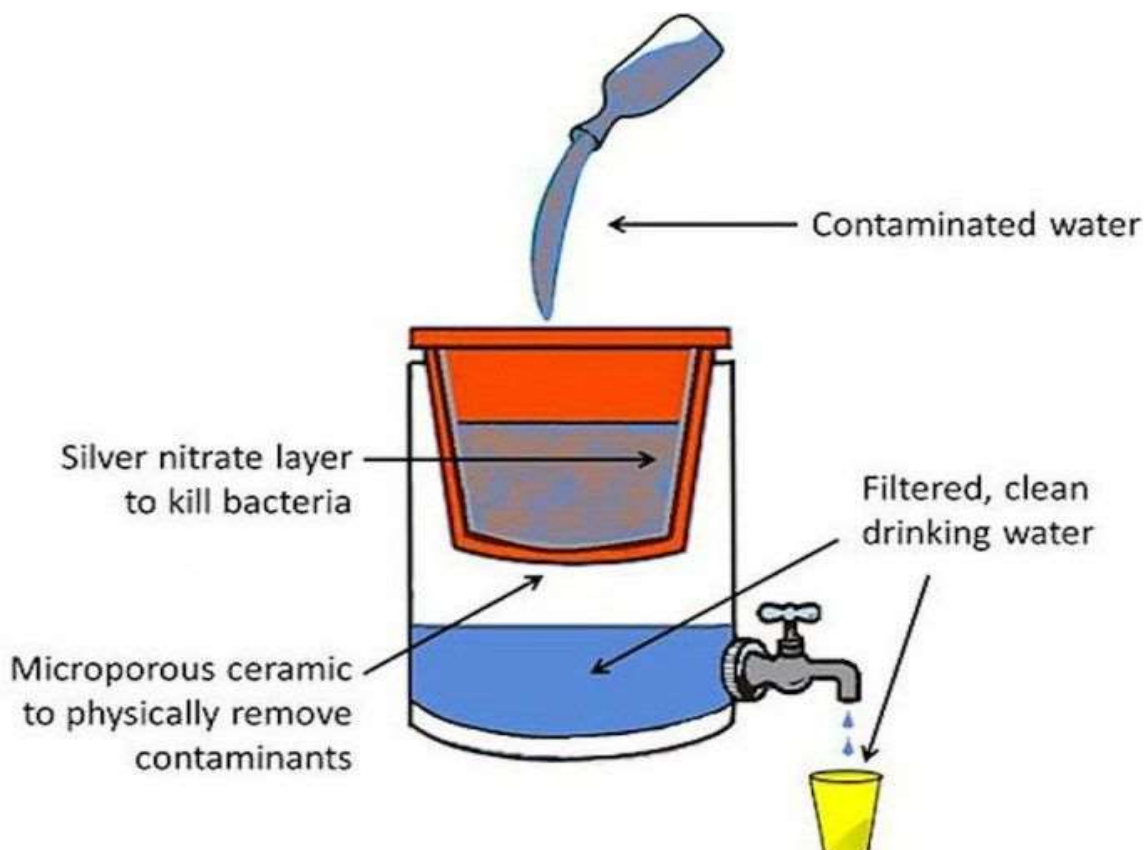
Figure 6: Representation of ceramic water filter flow (https://www.pinterest.ph/pin/11329436541069822/)

Microfiltration

They contain 0.1 microns pore size, but these filters differ with sizes (Adams *et al.,* 2014). It is highly effective to remove protozoa parasites, but partially effective to bacterial infections. It does not work on viruses (*Enteric, Hepatitis A, Norovirus, Rotavirus*) and chemicals.

Ultrafiltration

They contain 0.01 micron of pore size with a molecular weight of 13,000 Daltons). It removes contamination based on their different sizes, weights, and charges. It is highly effective in the removal of protozoa and bacteria, but moderate to viruses and chemicals

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Nanofiltration

The pore size of nanofiltration filters about 0.001 microns with the molecular weight of 2000 Daltons). it removes particles according to their size, weight, and charge. It is effective to remove protozoa, bacteria, and viruses, but limited chemicals.

2.2.7 Madi Drop strategy

Madi Drop is a method that continuously cleaning and disinfects water in household water storage buckets through diffusing silver into the water for daily treatment of 20 liters for at least 12 months Figure 7. Madi Drop is another commercially available technology that additionally removes different types of pathogens in water mechanically (Gupta *et al.,* 2018). As compared to other water treatment methods, Madi Drop is inexpensive, safe, healthy and easy-to-use. The container was designed with a spigot to limited contamination from hands and other external factors.

Figure 7: Representation of Madi Drop (right) and set up (left) (https://www.mdpi.com/1660-4601/9/9/3014).

Figure 8: The overview of the Madi Drop process (https://www.bing.com/images/search).

Water is a source of life and it is easily contaminated with pathogenic microorganisms that may lead to several intestinal diseases more especially in the rural communities. The above-mentioned water treatment strategies are used mostly in rural households to treat water. All the strategies have their pros and cons, but the Madi Drop is the recent water treatment strategy that is ben used. As compared to all methods, Madi Drop is essential, safe and it does not involve chemicals. It is cheap and very easy to maintain throughout the treatment. Other water treatment methods have limited in terms of microbe's removal but the Madi Drop strategy can remove almost all pathogenic microbes in water.

2.2 Genetic diversity

Genome is the total genetic makeup of a specific species (Nielsen *et al.,* 2014). The genetic diversity of *Entamoeba* species has been explored only in few places around the world. This variation helps the organisms to survive in their respective environments (Bernatchez, 2016). The population will continue to reproduce because they adapt to the environment (Bernatchez, 2016). The neutral theory of evolution stated that genetic diversity resulted from an accumulation of neutral substitutions (Wright *et al.,* 2014). This process occurs between pathogen-host interaction where a high frequency of a defensive allele among the host means that it is more likely to overcome that allele (Thrall *et al.,* 2016; Wright *et al.,* 2014).

The genetic diversity of *Entamoeba* species has been studied for medical purposes (Poulsen *et al.,* 2016; Kawano *et al.,* 2017). Scientists named Clark and colleagues have suggested that the making use of the lineage in the ribosomal for discovery of DNA sequences is close enough to understand the *Entamoeba* species, but not independent *Entamoeba* species (Kawano *et al.,* 2017; Poulsen *et al.,* 2016). In contrast, although quite a few *Entamoeba* species were identified at the molecular level from humans (*E. histolytica*, *E moskovski*, *E. dispar E. nuttalli*, and *E. gingivalis*), animals (*E. invadens*, *E. insolita*, and *E. terrapinae*), and environments (*E. moshkovskii*, *E. ecuadoriensis*, and *E. marina* (Kawano *et al.,* 2017).

2.3 History of *Entamoeba histolytica*

Entamoeba histolytica parasites are causative agents of amebiasis infections which were recognized as a dangerous disease by the scientist known as Hippocrates during the diagnosis of a patient with fever and dysentery (Garmie *et al.,* 2016). In the year 1875, another scientist by the name of Dr. Fredrich Losch reported the presence of motile trophozoites in the dysenteric stool of a Pietersburg laborer and he named it *Entamoeba coli* (Gathiram, 1989). He experimented on dogs and he successfully produced the picture of dysentery which was the same with those obtained from a human. He demonstrated the ulcerative lesions teeming with *Entamoeba coli* in the colon of dogs at autopsy and the same lesions were obtained from his patient on post-mortem examination (Gathiram, 1989).

After all this evidence, he remained unconvinced that the *Entamoeba* was of any aetiological importance in the causation of dysentery (Gathiram, 1989). Furthermore, another scientist known as Walker in 1913, reported the cysts of *Entamoeba histolytica* as an infective agent from the human stool, followed by Brumpt in 1925 where he proposed that *E. histolytica* and *E. dispar* they have same morphology and he suggested that they should be called pathogenic and non-pathogenic species (Dumevi, 2017).

2.4 Taxonomy and classification

Entamoeba family are divided into several domains, which include, *Eukarya*. They belong to a kingdom of the *Protista* and sarcomastigophora phylum and the subphylum of *Sarcodina*, which contains both free-living and parasitic members (Hendrix, 2016). *Entamoeba* belongs to the class of *Sarcodines* and subclass *Lobosea* because they undergo locomotion process and feeding using pseudopods. They belong to the *amoebida* order and family *Endamoebidae,* and the genus is *Entamoeba*. Among the eight species of human intestinal amoeba (*E. histolytica*, *E. dispar*, *E. moshkovskii*, *E. hartmanii*, *E. Bangladeshi*, *E. polecki* also called *E. chattoni*, *E. hartmanni,* and

E. gingivalis), *Entamoeba histolytica* is classified as a pathogen and the others are considered non-pathogenic and rarely cause disease in humans (Siddiqua, 2016).

2.5 Morphology

The morphology of *Entamoeba* consists of three forms, trophozoites, pre-cyst and cysts (Bogitsh *et al.,* 2018). Trophozoites (tropho=food), zoites=zoon) are an invasive form of the parasite which passes only in faces of patients with active dysentery (Hodges *et al.,* 2010). They measure around 08-30µm. Their cytoplasm has a clear ectoplasm and a granular endoplasm (Hodges *et al.,* 2010). Endoplasm contains red blood cell and food vacuoles; the ingested RBCs are characteristic features of *E. histolytica* but not *E. dispar.* The nucleus is single, spherical, 4-6µm in size, contains central dot-like compact karyosome surrounded by fine peripheral chromatin (Acquah *et al.,* 2010). Pseudopodia are long finger-like projections of endoplasm through ectoplasm, it is the organ of locomotion. Trophozoites are motile with active unidirectional progression and purposeful movement (they go with the intention of feeding) (Acquah *et al.,* 2010).

Pre-cysts are the intermediate stage between trophozoites and cysts (Acquah *et al.,* 2010). They are oval with blunt pseudopodia. Cysts are the infective form/stage of the parasite which are mostly found in informed stools (Lun *et al.,* 2015). They are spherical in shape and measure around 10-20µm, they are covered with a smooth chitinous wall that makes them resistant to gastric acid and other adverse conditions (Lun *et al.,* 2015). Their nuclear structure is the same as those of trophozoites Figure 9.

The mature cysts have 4 nuclei that characteristically have centrally located karyosomes and fine, uniformly distributed peripheral chromatin and cyst usually measure 12 to 15 µm shown in figure 10 (Bogitsh *et al.,* 2018). The most active, motile and feeding form is called trophozoite and has an average of about 25 µm in diameter (Bogitsh *et al.,* 2018). The cytoplasm consists of a clear

ectoplasm and a finely granular endoplasm that contains food vacuoles filled with RBC in different stages of digestion shown in figure 9 (Mahmud *et al.,* 2018).

The nucleus is single and vesicular, measures about 3 to 5 μm in diameter and within it lies the endosome (nucleolus), which may be a single granule or a closely packed cluster of minute granules (Pannese *et al.,* 2015). A ring appears to surround the endosome. Spoke-like lines radiate from the endosome to the nuclear membrane.
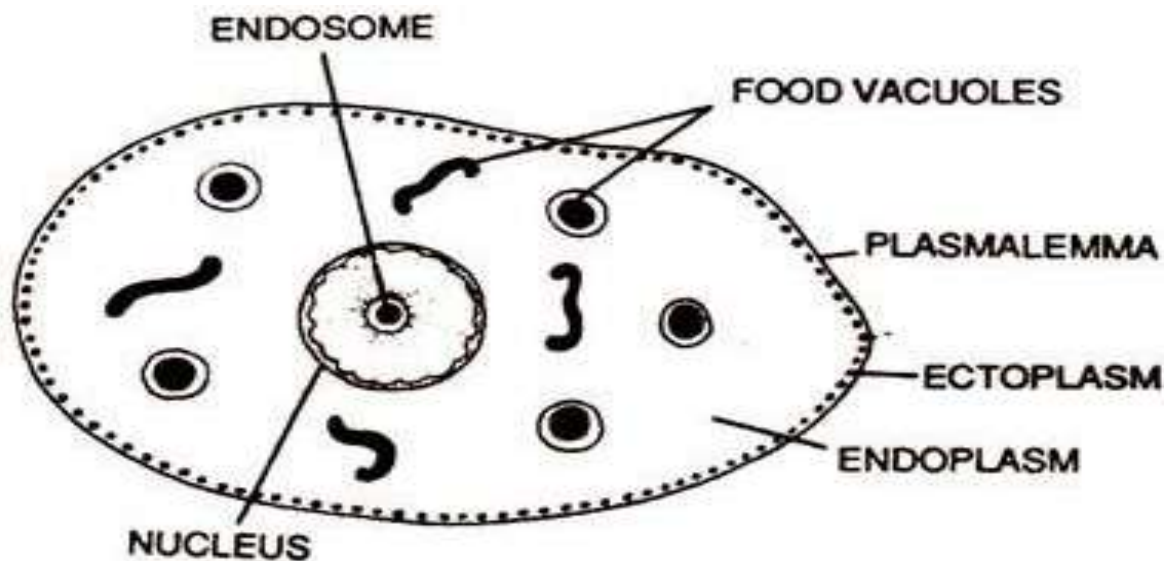
Figure 9: Representation of the *Entamoeba histolytica* cell (www.biologydiscussion.com/micro-biology/species-of-*Entamoeba*-with-diagram-microbiology/34319).

The trophozoite produces finger-like pseudopodium and movement is irregular. As there used to be only one pseudopodium, it is typically monopodial. The type and rapidity of movement vary depending upon the consistency of surrounding medium, age of the parasite, temperature, etc. The nutrition in trophozoite is holozoic. It feeds by phagocytosis. Food usually consists of bacteria or other organic material found in the intestine. RBCs are found only in the food vacuole of pathogenic forms.
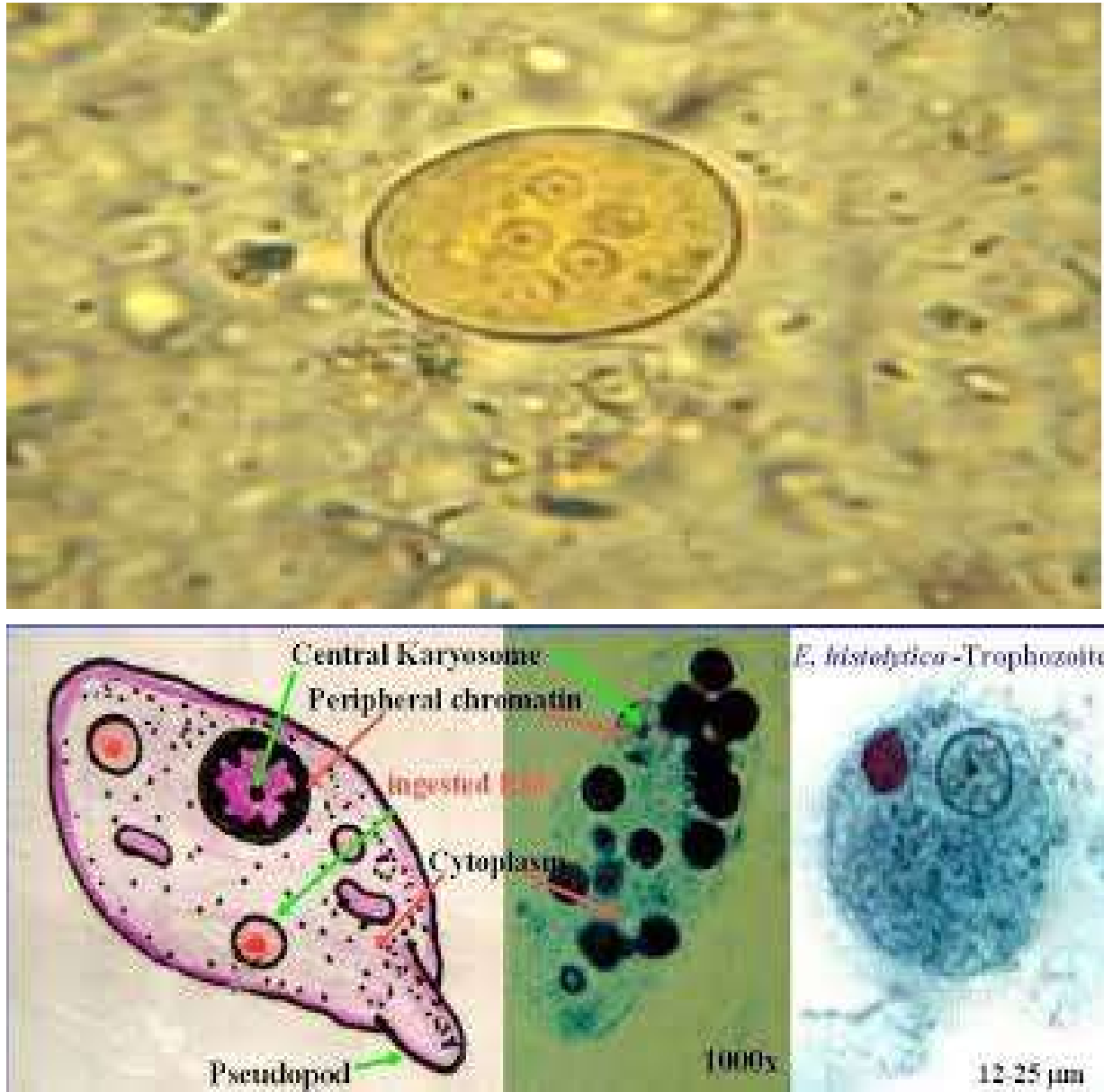
Figure 10: Representations of *Entamoeba* cyst and trophozoites
(https://www.waterpathogens.org/book/*Entamoeba*-histolytica).

Table 1: Representation of the differences between trophozoites and cysts.

	Trophozoite	Cyst
Size	5-12 μm	5-10 μm
Nucleus	Single nucleus	Mature cyst contains 4 nuclei, only 2 nuclei frequently seen
Cytoplasm	Finely granular cytoplasm	Finely granular cytoplasm
Peripheral chromatin	Evenly distributed peripheral chromatin	Evenly distributed peripheral chromatin
Karyosome	Small compact karyosome, may be centrally located or may be eccentric	Small compact central karyosome
Inclusion	Bacteria, no RBC	-
Motility	Non-progressive motility	-
Chromatoidal body	-	Elongated chromatoidal bars with blunt and smooth ends, known as Cigar-shaped.

Table 1: represents the difference between *Entamoeba* cysts and trophozoites based on their characteristics*(https://www.google.co.za/search?q=cysts+and+trophozoites&tbm=isch&ved=2ahukewiyy rdhu6_qahxyarqkhzjjcj8q2-ccegqiabaa&oq=cysts+and+&gs_lcp=cgnpb).*

Trophozoites are large as compared to the cyst, and they cannot survive on the environment for a long time. In terms of internal structure, in most cases, cysts contain 4 nuclei and trophozoites with only one nucleus.

2.6 Epidemiology

Entamoeba spp are distributed throughout the worldwide shown in figure 11, *Entamoeba* infections have been described in the North part of America (21.5%) followed by Africa with a prevalence of 10.73%. With specific to the countries, Southern Africa is the second country in Africa with a higher prevalence of *Entamoeba* infections.
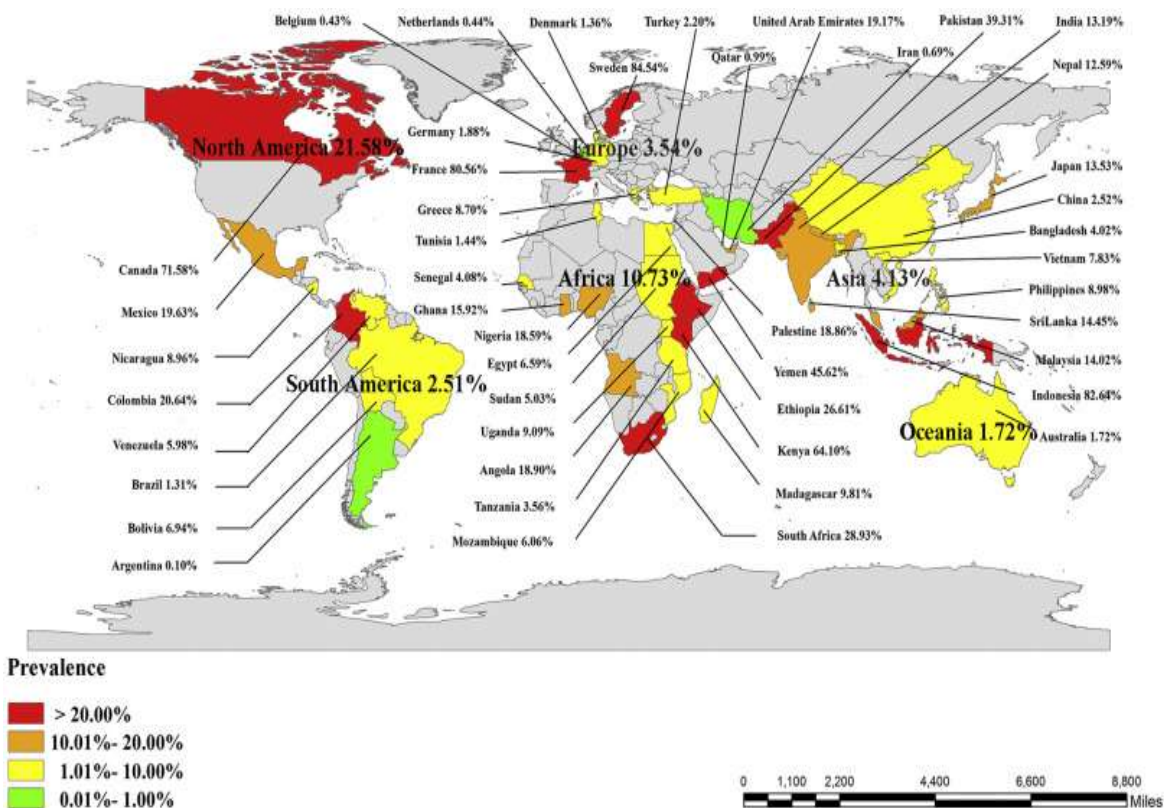
Figure 11: Representation of the epidemiology of *Entamoeba* infection worldwide (https://ars.els-cdn.com/content/image/1-s2.0-S156713481930245X-ga1_lrg.jpg).

2.6.1 Global distribution

The host for these parasites is humans and few animals for both symptomatic and asymptomatic (Hill *et al.,*2018). Water is a source of transmission for these parasites, the matured cysts are the source of infection (Smith *et al.,* 2010). They can also be transmitted sexually and vectors (files and cockroaches) mechanisms as well as sexual contact (homosexual partners) (Smith *et al.,* 2010). *Entamoeba* infection is mostly seen in tropical and subtropical areas (Najafi *et al.,* 2019). For about 4 to 10% of human carriers, they turn to show some symptoms with a period of 12 months (Mortimer and Chadee, 2010; Ghasemi *et al.,* 2015). These diseases contribute to a heavy burden of diarrheal illness in developing and some of the developed countries (Prüss-Üstün *et al.,* 2016).

although many cases of death are due to *E. histolytica* infections, in other parts of the world, some information on the prevalence of *E. histolytica* might be an overestimation or predicted (Diamond and Clark, 1993; Tengku Shahrul *et al.,* 2012). Since 1986, the published information concerning the frequency of this infection caused by *Entamoeba histolytica*, Walsh reported that about 10-20% of the world's population were infected *Entamoeba* parasites, among them 1% developed the symptoms.

According to the world health organization (WHO) report, *Entamoeba histolytica* infects about 500 million humans worldwide and 50 million experience some with symptoms. Later stated, about 100,000 people died (WHO, PAHO, UNESCO, 1997). About 80-90% of the infections remain asymptomatic because of the none pathogenic species such as *E. dispar* and *E. moshkovskii,* the worldwide incidence of *E. histolytica* estimated 5 million cases per year, with the global death of 100 000 humans per year (Jackson, 1998).

Entamoeba histolytica causes amebiasis infections which lead to an amoebic liver abscess, bloody diarrhea, and abdominal pains in South Africa, the part of Egypt and Asia, the manifestation of the same symptoms is the predominant form (Ravdin *et al.,* 2003). The studies were done by Samie *et al.,* 2008 have reported 26.1% presence of *Entamoeba* infections and the species distribution in the Vhembe district.

Epidemiology of the *Entamoeba* species can sometimes be complicated by the existence of three different forms of *Entamoeba* species (*E. histolytica*, *E. dispar,* and *E. moskovskii*) that possess the same morphology but differ with genetics. Since *E. histolytica* is known to be a pathogen, some species such as *E. dispar* and *E. moshkovskii* which are non-pathogens but possess the same morphology as *Entamoeba histolytica* (Ali *et al.,* 2008). These cases are mostly seen in Africa as well as many other developed places in the world includes Latin American and Asian countries, where there is a lack of specific diagnostic tools (Ali *et al.,* 2008).

Places such as central and Latin America these parasites display endemic behavior (Bacon *et al.*, 2013). In Mexico for example, the case rates of the amebiasis from the year 1995 to 2000 were between 1000 and 5000 per annually. In the year 2002 to 2006 approximately 1128.8 to 615.85/100,000 were infected per year. In another part of the countries, individuals with less than 15 years of age were frequently infected, with a notable increase in children aged between 5 and 9 (Ximenez, 2009).

In the part of India, the rate prevalence of amebiasis among the population was found to be around 11.7% by microscopic examination. However, PCR results showed that *E. histolytica* reported to be 3.5% of those infected (Khairnar *et al.,* 2007). In Bangladesh, the ELISA antigen detection kits for *E. histolytica* reported a prevalence of 4.2% among children under 5 years of age living in Dhaka (Haque *et al*. 2006). Thus, the epidemiology of amoebiasis remains very uncertain particularly in this part of the world.

2.6.2 Prevalence of *Entamoeba* species in children

Entamoeba species, especially *Entamoeba histolytica* which is related to diarrheal illness have recently been reported to harm the growth of children (Gilchrist *et al.,* 2015). Approximately 50% of children have serological evidence of exposure to *Entamoeba* species by 5 years of age in Bangladesh (Lin *et al.,* 2018). studies than in preschool children in the part of the slum of Dhaka, Bangladesh demonstrated a new *Entamoeba histolytica* infection in 39% of children over one year of observation (Lin *et al.,* 2018).

The higher 41% prevalence of *Entamoeba* species between the age group of 6 to14 years in the South Canara district in British India has been recorded (Zeb *et al.,* 2018). Furthermore, a 27% prevalence of *E. histolytica* in children less than 5 years has been reported by in Northern Pakistan (Anuar *et al.,*2018). *Entamoeba* infections are mostly seen in younger age groups, However, this could be explained on the basis that children are more exposed to overcrowded conditions such as

the playground, school, and their immune systems are weak and they can easily come in contact with animals. Furthermore, the parasitic infection may be due to poor sanitary conditions in the schools (Al-Hindi *et al.,* 2015).

2.6.3 Prevalence of *Entamoeba* species in HIV positive patients

Several studies have reported the prevalence of amebiasis infections in HIV positive humans compared to HIV negative (Schuster *et al.,* 2019). However, a recent study reported 6.1% of cases of amebiasis as an emerging parasitic infection in HIV positive infected humans in non-endemic areas as well as disease-endemic areas (Juma, 2018).

In a recent study, 31 patients with amebiasis at Seoul National University Hospital from 1990 to 2005 were HIV positive (Malla *et al.,* 2016). In countries like Japan, Mexico, Taiwan, and South Africa, recent data has shown an increase in the occurrence of *Entamoeba* infections among HIV positive patients (Abdollahi *et al.,* 2015). In South Africa, with special reference to the Vhembe district, the association between *Entamoeba* infections and HIV positive individuals has been reported. Among HIV positive patients, those with CD4+ counts less than 200 cells/µl, were relatively more likely to be seropositive for *Entamoeba histolytica* (Samie *et al.,* 2010).

2.7 Modes of transmission

Trophozoites' stage of *E. histolytica* is active and found in humans and animals' gastrointestinal tract as well as fresh stools (Hamad *et al.,* 2016). Cysts can survive in the external environment for a few days and infections can take place if the cyst has been swallowed and reach the digestive tract (Hamad *et al.,* 2016).

The primary hosts for *E. histolytica* are humans and the transmission occur through consumption of water contaminated with feces containing cysts (Hamad *et al.,* 2016). Hands have been proven to be the common denominator in transferring pathogens from surface water, food, and animals to humans (Bunia *et al.,* 2018). *Entamoeba histolytica* can also be transmitted by unprotected oral-anal sexual intercourse and infection was also confirmed in homosexual men through oral-anal and oral-genital (Van Wagoner *et al.,* 2017).

Transmission of these parasites occurs through the consumption of cysts from water contaminated by feces (Bhunia, 2018; Carmena *et al.,* 2010). The contamination could also come from contaminated wells water in the household on which most of the families depend as shown in figure 12. However, most the people get water from contaminated water sources using water pots and jerry-cans in developing countries (Taylor *et al.,* 2015).

Figure 12: The representation of the transmission of *Entamoeba* infections through water (https://saraprecipolio.weebly.com/transmission.html).

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2.8 Life cycle of *Entamoeba histolytica*

The life cycle of *Entamoeba histolytica* begins when the trophozoites and the cysts pass in feces to the environment (soil and water). In most cases, Cysts are found or passes in the formed feces and the trophozoites are mostly found in diarrheal feces. The infection by this parasite occurs when one is ingesting the matured cysts in fecal contaminated water and food. When the cysts reach the small intestine, the excystation occurs and release the trophozoites that will then migrate to a large intestine.

Trophozoites will undergo binary fission to multiply and produce more cysts. Since cysts have a protective cell wall, they can survive the external environment for days to weeks and are responsible for transmission. Trophozoites are passed in the diarrheal feces but they are easily destroyed once they reach an external environment and if they are ingested with, they might not survive the presence of gastric acid. Trophozoites remain confined in the intestinal lumen where they remain noninvasive but, in some cases, they invade the intestinal mucosa and cause intestinal diseases. Some of the trophozoites will pass through the bloodstream and cause extra-intestinal infection such as live absences shown in figure 13 (Kucik *et al.,* 2004).
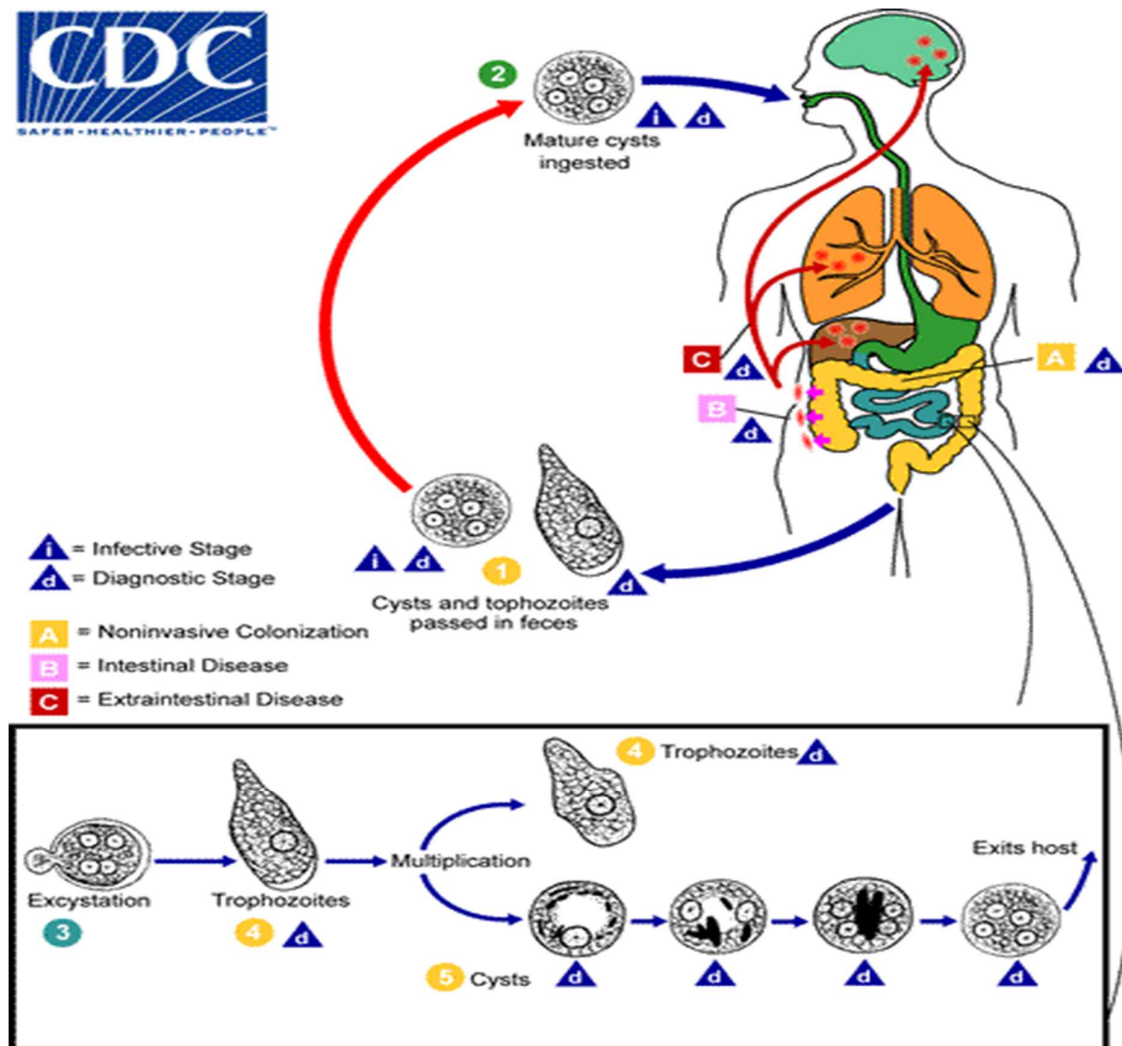
Figure 13: Representation of *Entamoeba histolytica* Life cycle

(https://i0.wp.com/microbeonline.com/wp-content/uploads/2016/06/Life-Cycle-of-Entamoeba-histolytica.png)

2.9 Virulence factors

Trophozoites play a major role in the pathogenesis of intestinal as well as the extra-intestinal amebiasis (Nakada-Tsukui *et al.,* 2016). Amoebic Lectin antigens are proteins that are present on the surface of trophozoites of pathogenic *E. histolytica* and can also be produced by *E. dispar* (Aguirre Garcia *et al.,* 2015). Lectin (carbohydrate-binding protein) is a 260kDa surface protein that consists of 170 and 35-kDa sub-units (Aguirre Garcia *et al.,* 2015). The 170-kDa subunit protein participates in mediating the adhesion in the host while 35-kDa mediates the production

of cytotoxicity and complement resistance (Christy *et al.,* 2011). The other proteins such as amoebic pore-forming proteins and cysteine proteinase are responsible for IgA and IgG degradation shown in figure 14. Hydrolytic enzymes are responsible for RNAse, neutral proteases and phosphate degradation (Singh *et al.,* 2016).
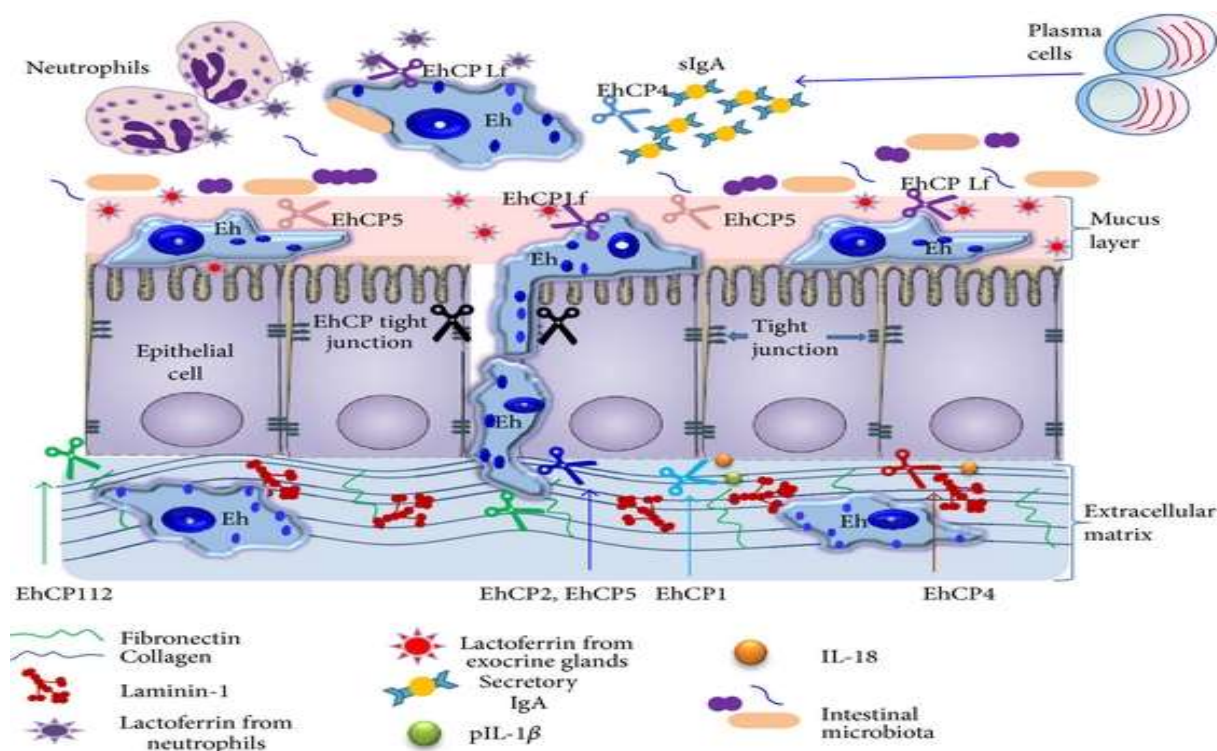
Figure 14: Proteases of *Entamoeba histolytica* as virulence factors during intestinal amoebiasis. (https://www.researchgate.net/figure/Transferrin-endocytosis-and-signaling-pathways-in-protozoan-parasites-A-Trafficking_fig1_277915038)

2.10 Pathogenesis

The trophozoites are the infective stages of *Entamoeba histolytica* life cycle, they colonize the intestinal lumen of humans and animals and remain in the mucus layer and feeding on available nutrients and bacteria (Leon Coria, 2018). With time trophozoites move through that layer and meet up with the epithelial cell layer where they start the pathological process (Leon Coria, 2018). Lectin of *Entamoeba histolytica* binds to the galactose and N-acetyl galactosamine, which is a sugar found on the surface of the epithelial cells (Leon-coria *et al.,* 2019; Jantscher-Krenn *et al.,*

2012). These parasites contain several enzymes including lipases, and the cysteine proteases, they are used to lysis the epithelial cell through inducing cellular necrosis and apoptosis when they bind to it (Leon-coria *et al.,* 2019; Jantscher-Krenn *et al.,* 2012). After that, the released enzymes will allow the penetration of trophozoites into the lumen blood vessels liver and other organs in the body. (Leon-coria *et al.,* 2019; Jantscher-Krenn *et al.,* 2012). The damage of the epithelial cell layer destroys the human immune cells and releases the immune cell's enzymes on the surroundings tissues create destructions (Leon-coria *et al.,* 2019; Jantscher-Krenn *et al.,* 2012). The destruction causes some ulcers in the tissues and it is known as the flask shaped. (Solerio *et al.,* 2017). This tissue destruction is involved in the blood vessels which later lead to bloody diarrhea. *Entamoeba* trophozoites enter the bloodstream and transported to the liver and cause amoebic liver abscesses (Mim, 2018). In all locations that trophozoites reach, similar pathology can occur (Mim, 2018). They invade the submucosa by dissolving the mucosa of the intestinal wall. They multiply by binary fission and produce flask-shaped ulcers containing cellular debris, RBCs, lymphocytes and bacteria. These ulcers rupture and discharge blood and mucus into the intestine that passes to outside with the stool. This results in amoebic dysentery or amebiasis (Mim, 2018; Khubchandani *et al.,* 2019).

2.11 Pathology

The pathology of intestinal amebiasis occurs when *E. histolytica* invades the colonic mucosa and start producing; ulcerative lesion and profuse bloody diarrhea (amoebic dysentery) (Shirley *et al.,* 2016). An amoebic ulcer is a flask in shapes and varies in sizes from a pinhead to an inch and they may be localized to the ileocecal region or generalized (Shirley *et al.,* 2016). They may be superficial or deep and are scattered with intervening normal mucosa.

Pathology of extraintestinal amebiasis involves the amoebic liver abscess, which is a single or multiple abscess found on the surface of the right lobe of a liver (Khannathasan *et al.,* 2017). They

contain three zones namely, inner central zone-necrotic and consists of lysed hepatocytes, intermediate zone-degenerated liver cells, tissues, RBCs and occasional trophozoites and lastly outer zone which is a layer of normal inner tissue invaded by amoebic trophozoites. Liver abscess pus is thick and chocolate brown is known as anchovy sauce pus and pleuropulmonary amebiasis are a direct extension from liver or metastatic foci (Muthusamy, 2013; Guzmán-Silva *et al.,* 2013).

2.12 Clinical features

Entamoeba histolytica causes intestinal infections which may lead to diarrheal infections that contain mucous and bloody (Kelly, 2015). This kind of infection is generally known as dysentery, which can emerge gradually throughout one to several weeks. Worldwide, about 40-45% of the patients develop a fever and some may lose weight.

Acute amoebic dysentery is the most common symptom of amebiasis which includes pleuropulmonary abscess, acute onset of abdominal pains, liver abscess, tender cutaneous and genital amoebic lesions, hepatomegaly, rectal tenesmus, fever, and stool containing blood and mucus (Berger, 2018).

About 95% of the population infected by *E. histolytica* show no symptoms but will shed cysts in their stool (Verweij *et al.,* 2014).

Table 2: The three main kinds of the disease manifestations of amebiasis

Non-invasive disease	Invasive disease	Extra-intestinal disease	References
Amoeba colony on mucosa surface	Necrosis of mucosa leading to ulcer	Metastasis via the bloodstream	(Aristizabal *et al.*, 1991)
- Asymptomatic cyst excreted	- Amoebic colitis - dysentery - blood diarrheal	- Primarily liver abscess, - Several weeks of fever	Seeto and Rockey, 1999
- No dysenteric diarrhea	- An ameboma - Colonic lesions	All but less frequently in lungs and brain,	Petri and Singh, 1999)
Presence of *E. histolytica* in stool	Diagnosis is only `reliable when there is the detection of *E. histolytica* antigens in stool	The detection of *E. histolytica* in the patient's serum	Seeto and Rockey, 1999
Equally distributed between genders	All groups	Mostly common in men	Petri and Singh, 1999)

2.13 Diagnosis

Diagnosis is a process of identifying the nature of the disease other health complications by examining the symptoms (Liberman *et al.,* 2018). Recently, the diagnosis of amebiasis relies commonly on the microscope (Paulos *et al.,* 2016). Since it has been discovered that microscopic examination is not sensitive and reliable in terms of distinguishing the *Entamoeba* species, to date several methods such as PCR and ELISA are now used for the diagnosis of *Entamoeba* infections (Hamad *et al.,* 2016).

2.13.1 Microscopic examination

Microscopic examination is a process of using a microscope to examine a specific symptom or object that cannot be seen by naked eyes (Boas *et al.,* 2016; Bradbury, 2014). Three common known microscopic which include, the optical, electron, and scanning probe microscopy as well as the X-ray microscopy (Raghuwanshi *et al.,* 2019).

Entamoeba infections are diagnosed using the microscopic techniques for the presence of either cysts or trophozoites (Van den Bossche *et al.,* 2015). Microscopic examination is neither sensitive nor specific. However, microscopic techniques remained the only method for diagnosing *Entamoeba* infections for over 100 years, though it is difficult to differentiate between the distinct species of *Entamoeba*, more especially *dispar* and *histolytica* because of their similarities morphologically shown in figure 16. The direct smear is examined under a microscope shown in figure 16 (Madison-Antenucei *et al.,* 2016). Microscopic examination is not sensitive, and it ranges between 20% to 40% which performed on a fresh specimen and microscopic examination is still the technique of choice in many parasitology laboratories (Beyhan *et al.,* 2017).

2.13.2 Stool culture

The stool culture technique is known as a gold standard for many years (Llewellyn *et al.,* 2016). This technique was used to differentiate *Entamoeba histolytica* and *dispar* because of their similar characteristics (Rock, 2016). *Entamoeba histolytica* or *dispar* cysts sample from the microscopy are washed and inoculated into culture media and then incubated at 37 °C and examined for growth of cysts, and the results are viewed on the wall of the test tube or in the debris if present (Rock, 2016; Garcia *et al.,* 2018).

Currently, the most widely used media for xenic cultivation include the diphasic lock-egg, Robinson, and the monophasic trypticase yeast extract serum gastric mucin (TYSGM-9) media (Garcia *et al.,* 2018). The limitation with this method is that it takes weeks to perform and none of

the existing culture methods are selective for *Entamoeba histolytica*, and therefore are not suitable or reliable for routine diagnosis (Shane *et al.,* 2017). *Entamoeba* culture is difficult, expensive, and labor-intensive to maintain in the diagnostic laboratory (Ryan *et al.,* 2017).

2.13.3 Polymerase chain reaction

A PCR is a powerful, sensitive, and very useful to differentiate the microorganisms based on their molecular characteristics and genetic typing (Rothman *et al.,* 2010). These assays can be used to detect the microorganisms in stool, tissue, and blood. There are several PCR assays which include Multiplex, Conventional, Nested, Reverse transcriptase, Asymmetric and Quantitative PCR (Glushakova *et al.,* 2015; Van Eeden *et al.,* 2014).

The polymerase chain reaction is the current method used to differentiate *E. histolytica* from other *Entamoeba* species (Efunshile *et al.,* 2015). Real-time PCR is a sensitive method that can be used to detect several pathogens and used to diagnose amoebic liver abscess (Weitzel *et al.,* 2017). The real-time PCR is also faster and more sensitive compared to other PCRs and is characterized by the elimination of gel analysis level. Real-time PCR is a quantitative method that allows the determination of the number of parasites in various samples (Weitzel *et al.,* 2017).

2.13.4 Sanger sequencing method

The Sanger sequencing method is also called the chain termination method. This method is used for the determination of the nucleotide sequence of the DNA (Heather and Chain, 2016). Historically, it was developed by Laureate Frederick Sanger together with the colleagues in the year 1977. This method can take place either manual or automatic, but in most cases, the automatic route is commonly used with a sequencing machine that followed three steps described in figure 18.
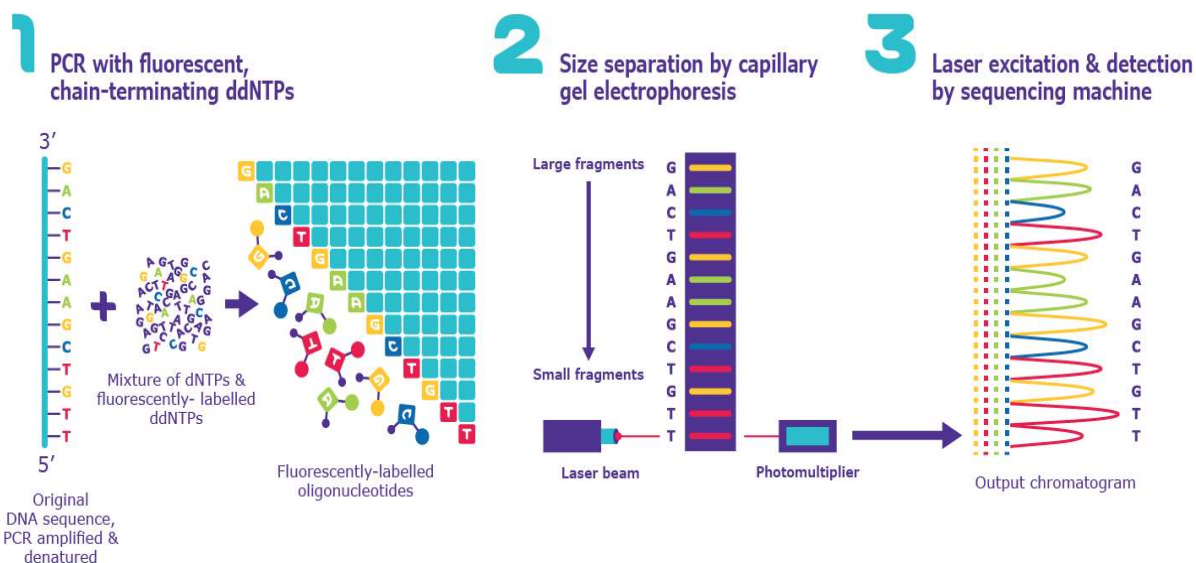
Figure 15: Representation of the summary of steps involved Sanger sequencing method
(https://www.sigmaaldrich.com/technical-documents/articles/biology/sanger-sequencing.html)

2.13.5 Next-generation sequencing technology

Next-generation sequencing is the term that describes the DNA sequencing technology that has revolutionized genomic research. It involves 3 steps, which include library preparation, sequencing, and finally data analysis. It is faster, guarantee and effective as compared to Sanger sequencing technology.

Next-generation sequencing is also known as the massively parallel sequencing technology that can sequence millions of small DNA fragments in unison (Ref). it generates a massive pool of data. It was discovered at the beginning of the 20th century, whereby the first draft of the human full genome was completed. Since then, more than ten this technology showed the ability to produce 20 000 times data in a single run. This technology can help cancer patients to benefit from the discovery of medication (Ref).
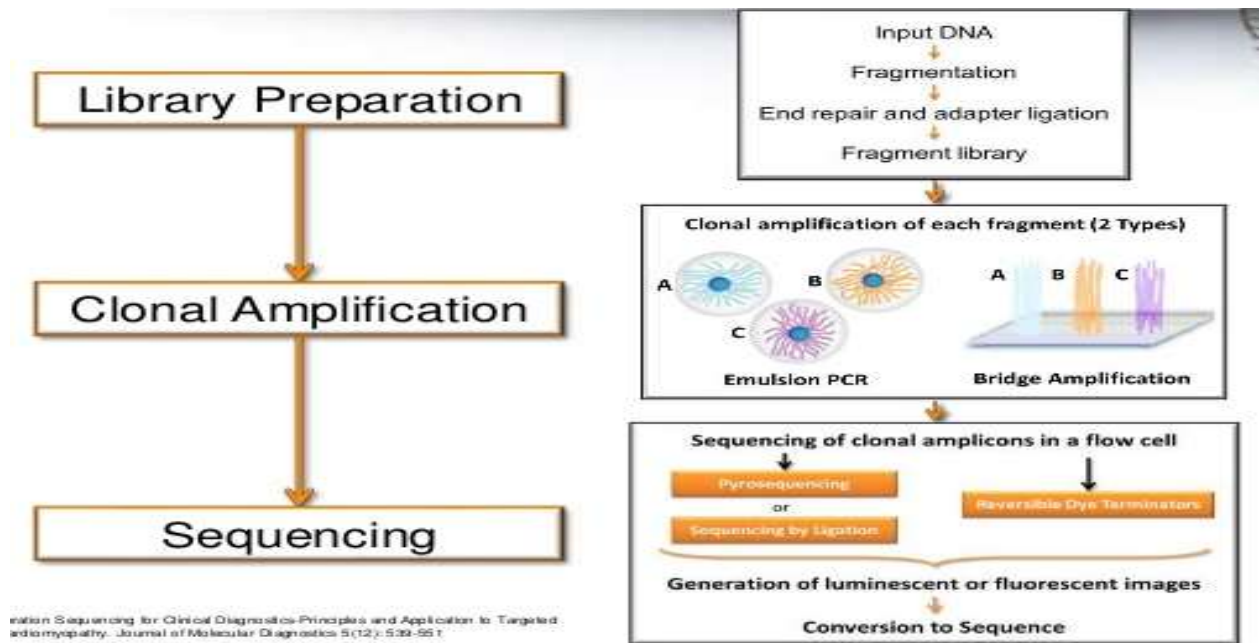
Figure 16: Representation of the overview of NGS technologies
(https://www.slideshare.net/USDBioinformatics/basic-steps-of-ngs-method)

2.13.6 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay is a test that uses antibodies and antigen to detect the presence of parasites and it uses color change to identify the presence of microbes (Chen *et al.,* 2017; Kaittanis *et al.,* 2010). This test indicates the presence of the organism in feces as a diagnostic kit. ELISA is a reliable, easy to use and rapid method for the diagnosis of *Entamoeba* infections especially in developing countries (Abdollahi *et al.,* 2015). It has been used widely for the study of the epidemiology and diagnosis of symptomatic amoebiasis (intestinal and extraintestinal). ELISA is the only method that is widely used in endemic areas because they are not expensive (Parkash *et al.,* 2015).

Tech-Lab *E. histolytica* II test is one of the examples that were reported to be sensitive and specific (El-Dib, 2017). The *Entamoeba histolytica* ELISA kit detection contain 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 (El-Dib, 2017). This kit is only used on a fresh or frozen sample, not preserved samples.

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2.14 Treatment

Luminal amebicides (Diloxanide furoate, Iodoquinol, and Paromomycin) are the anti-parasitic drug that kills intestinal parasites (Martínez-Castillo *et al.,* 2018). Tissue amebicides (Metronidazole, Tinidazole, and Emetine hydrochloride) are the anti-parasitic drug that kills *Entamoeba* from the tissue. For liver abscess, only chloroquine can be used, and intestinal wall erythromycin is recommended (Islam, 2011).

Table 3: Antibiotic drugs recommended for the infections of amebiasis

Disease	Drug	Dosage (mg/day)		Side effects	References
		Adult	**Paediatric**		(Haque *et al*., 2003)
Liver abscesses	Luminal agent	750	35–50 mg/kg of body weight	Primarily gastrointestinal: anorexia, nausea, vomiting, diarrhea, abdominal discomfort, unpleasant metallic taste; disulfiram-like intolerance reaction with alcohol, etc.	(Haque *et al*., 2003)
		3 times	in 3 divided doses		(Haque *et al*., 2003)
		For 7–10 days			
	Tinidazole followed by a luminal agent	800	60 mg/kg (maximum 2 g)	Primarily gastrointestinal and disulfiram-like intolerance reaction as for metronidazole	(Haque *et al*., 2003)
		3 times			(Haque *et al*., 2003)
		For 5 days			
	Paromomycin	25–35 mg/kg		Primarily gastrointestinal: diarrhea, gastrointestinal Upset	(Haque *et al*., 2003)
		3 divided doses			
		For 7 days			
	Diloxanide furoate	500	20 mg/kg	Primarily gastrointestinal: flatulence, nausea, vomiting, pruritus, urticaria	(American Academy of Paediatrics, 2012
		3 times a day	3 divided doses		
		For 10 days			

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Amoebic colitis	Metronidazole followed by a luminal agent (as for amebic liver abscess)	750	35–50 mg/kg	As for amoebic liver abscess	(American Academy of Paediatrics, 2012
		3 times a day	in 3 divided		
		For 7–10 days			
Asymptomatic intestinal Colonization	Paromomycin	25–35 mg/kg		Primarily gastrointestinal: diarrhea, gastrointestinal upset	(American Academy of Paediatrics, 2012
		3 divided doses			
		For 7 days			

2.15 Prevention and control

Individual prophylaxis involves the improvement of personal hygiene and the safe consumption of water and food (WHO, 2019). For example, uncooked vegetables like salad should be eaten only after thoroughly washing, Avoiding the irrigation of vegetables by contaminated water (Alum *et al.,* 2016). Community prophylaxis involves proper sanitation of surroundings, proper disposal of sewage, prevention of fecal contamination of water and better management of cases and carriers (Lewnard *et al.,* 2016).

Entamoeba species is known as a neglected parasite due to its lesser severity as compared to other microbes such as bacteria, viruses, and fungi. However, worldwide *Entamoeba* infection causes a serious health problem. Since it can be transmitted through consumption of contaminated water and food, water treatment, and good sanitation must be prioritized in the rural communities. it can also be transmitted sexually, especially from men who have sex with me, therefore oral sex should be avoided as well. Understanding the life cycle and genetic diversity of these parasites is a measuring key for controlling, diagnosing, and preventing it.

Chapter 3

Materials and methods

3.1 Ethical clearance

Ethical clearance was obtained from the University of Venda Ethics Committee (SMNS/15/MBY/27/0502) and University Virginia Institutional Review Board to perform this study. Authorizations to collect stool samples were also obtained from village leaders (Chiefs). Before sample collection, confidentiality was maintained by assuring that all patient identification (IDs) such as names and dates of birth are deleted and replaced with anonymous IDs and age, respectively. Recommendations made by the Ethics committee were strictly adhered to.

3.2 Study site

Dzimauli area situated in the Vhembe district, Mutale municipality showed in figure 21. It is situated in the northern part of Limpopo Province, South Africa. The Vhembe district is bordered by Botswana and Zimbabwe to the north and Mozambique to the East.

Figure 17: A map showing the Vhembe district where the study was undertaken.

The present study took place in 19 villages of the Dzimauli area in Vhembe District, Limpopo from February 2017 until November 2018. Generally, these villages are occupied by members of

(Vha-Venda people). Every village has a very small population of people with approximately 90 to 110 residents. Most of the villages are living in a bad condition such as overcrowding, poor sanitation, lack of access to clean water, low level of education and inadequate water supply. Even though some basic facilities are provided by the municipality, still most of the villagers cannot the borehole in their households. Therefore, the nearby rivers are used as a source of water needs.

The environmental condition of the villages is very poor with limited resources such as latrine. Therefore, this will cause villagers especially children to defecate around and in the nearby rivers

Pigs, cattle, dogs, and poultry are the most common domestic animals that are found in the villages and some of the animals are just rooming around and defecate in the surrounding property without supervision. Villagers are close in contact with the animals, and they feed them with their bare hands.

3.3 Samples collection

The present study is part of the Madi Trial project entitled: Effectiveness of low-cost point-of-use water treatment technologies to prevent stunting among children in Limpopo, South Africa. It is also investigating the occurrence of pathogenic microorganisms in children's stool. The study population was divided into 4 groups including Madi Drop and filters (these group of participants was given Madi Drop and filter for water treatment), Madi Drop only (these participants were given Madi Drop only), filter only (The only filter were used in this group) and No intervention (control, the group did not receive anything), and samples were collected quarterly or every 3 months from month 0 to 24 for 2 years. Since Madi-Trial was a bigger project, a sub-section of the samples was used with a total of 534 stool samples (month 18 and 24) of 313 participants both males and females, aged 5 years and below were used in the present study

To ensure that all the interventions were working properly, all households or groups that received interventions were visited every 3 months for 2 years following the initial receipt of the

interventions. A short questionnaire was given to caregivers to ascertain adherence to the appropriate use of the interventions. Fieldworkers were also encouraging the participants to use the interventions for all drinking water in the household.

Height and weight measurements were taken on all children (male and female) under 5 years in the household and a stool sample was collected from the children. Treated water samples from the safe-storage water container were collected from a random subset of 50 households receiving the filter or ceramic disk. A questionnaire was given to caregivers to ascertain adherence to the appropriate use of the interventions, satisfaction with the interventions, diarrhea prevalence in the past 7 days, and breastfeeding practices.

At the 6, 12, 18- and 24-month home visits, the ceramic disk was replaced with new disks in households in the 2 intervention arms. For a random subset of 80-100 participants, treated water samples were collected at the subject's residence along with either stored, untreated water from the home or source water. At the 24-month home visits, the filter was replaced in households randomized to the filter arm. A more comprehensive questionnaire concerning the caregiver's satisfaction with the technologies and an assessment of willingness-to-pay were also be completed at these visits. The 24-month visit was including a cognitive assessment using age-appropriate developmental tests that have already been adapted, translated, and piloted in this population. All participants were offered new ceramic disks and water filters in safe-storage water containers at the 24-month visit.

3.4 Sample processing and microscopic examination

The labeled plastic containers containing fresh fecal samples were transferred to the lab and stored at 4°C in a cooler box with ice. Upon arrival at the laboratory, samples were aliquoted into 2ml screw tubes and stored at -20°C until further analysis.

The small portion of the fecal sample was mixed with a drop of iodine dye on the new clean. The specimen was covered with the coverslip and observed and was observed microscope with the

magnification of (10×) to identify the presence of *Entamoeba* cyst and other available intestinal parasites. The presence of *Entamoeba* cyst was identified based on their shape and presence of 4 nucleic

3.5 Madi drop technologies

A bucket that was used strictly for water storage and treating drinking water was used. Madi Drop showed in figure 22 with different colors based on the material that was used to make them was placed in the bucket and 20 liters of water was filled in the bucket. It was advised to leave the Madi drop in the bucket unless the bucket needed to be cleaned. The bucket was left for about 8 hours (overnight), the longer the waiting period before water use, the better the decomposition. After a waiting period then the water was readily used. The Madi drop was checked for every 3 months and it worked continuously for over 12 months.

Figure 18: The picture of Madi Drop+ that was placed in the bucket for water treatment.

3.6 Genomic DNA extraction (QIAamp® Fast DNA Stool Mini Kit)

DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen). The about 180-220mg stool was weighed in a 2ml microcentrifuge tube and 200mg of glass beads were added. The amount of 800μl of InhibitEX Buffer was added into the tube that contained the beads and the stool. The solution was subjected to the bead's beater for about 3minutes. The solution was placed in the vortex mixer for 1 minute until the solution was thoroughly homogenized and then the tube was

placed on the heat suspension for 5 minutes at 95°C and centrifuged for 1 minute at 13500 rpm to pellet the stool particles. A 25µl volume of proteinase K was added into a new 1.5ml microcentrifuge tube, followed by the addition of 600µl supernatant solution and 600µl of Buffer AL, then subjected to the vortex for about 15 seconds. The mixture solution was then incubated for 10 minutes at 70°C after that 600µl of 100% ethanol were added into the lysate and mixed by the vortex. The amount of 600µl of lysate from the mixture above was transferred into the QIAamp spin column and the cap was carefully closed, and the tube centrifuged for 1 minute at a high speed.

The new QIAamp spin column was placed on a 2ml collection tube and the tube that contained the filtrate was discarded (this step was repeated until the lysate was all used). The amount of 500µl buffer AW1 was added into the QIAamp spin column and centrifuge for 1minute at a high speed. The same QIAamp spin column was used after the addition of 500ml of buffer AW2 and centrifuged for 3minutes and the high speed. The QIAamp filter was placed on a 1.5ml centrifuge tube and pipet 200µl buffer TAE/ATE directly into the QIAamp filter membrane and incubated for 3 minutes at room temperature. Finally, the tube was centrifuged for 1minute at a high speed to elute DNA. The extracted DNA was stored at −20°C until further use.

3.7 Genus-specific PCR assay

Polymerase chain reaction assays for the detection of *Entamoeba* species were performed using genus-specific PCR primers based on small-subunit rRNA gene sequences. Primer sequences previously described by Verweij *et al.,* (2001) were used including Entam1: 5´GTT GAT CCT GCC AGT ATT ATA TG 3´ and Entam2: 5´CAC TAT TGG AGC TGG AAT TAC 3´ which produce a fragment of 550bp. Genus-specific PCR amplifications were performed in a final volume of 25µl containing 12.5 µl of Dreamtaq master mix (SYBR Green Master Mix), 0.25µl of BSA, 0.6µl of each primer and 6.05µl deionized water. An amount of 5µl stool DNA was mixed

with 20µl of master mix to a final volume of 25 µl. The reaction took place in the thermal cycler (P100™ Thermal Cycler, BIO-RAD). Initial denaturation occurred at 94 ˚C for 5 minutes followed by 35 cycles of 94 ˚C for 1 minute, 55 ˚C for 1 minute and 72 ˚C for 1 minute, the final extension occurred at 72 ˚C for about 7 minutes. The PCR products were separated by electrophoresis in 2% agarose gel at 100 V for 45 min in Tris-acetate buffer and visualized by UV-transilluminator.

3.8 Sequencing and phylogenetic analysis

All positive PCR amplicons were sequenced by Sanger and Next Generation Sequencing methods (NGS) (Inqaba Biotech, Pretoria, South Africa). Amplicons were gel purified, end-repaired and Illumina specific adapter sequences were ligated to each amplicon.

The samples were subjected to the quantification process whereby, the samples were individually indexed, and another purification step was performed. Amplicons were then sequenced on Illumina's MiSeq platform, using a MiSeq v3 (600 cycles) kit and 20Mb of data (2x300bp long paired end reads) were produced for each sample. The BLAST-based data analysis was performed using an Inqaba in-house developed data analysis pipeline. Sequence data were analyzed by Bioedit and MEGA 7 software. The phylogenetic tree was computed for species relatedness.

3.9 Statistical analysis

Data entry and analysis were performed by SPSS v. 26. The student tTest and Multiple logistic regression methods were used with a level of significance of $P<0.05$.

Chapter 4

Results

4.1. Sociodemographic characteristics of the study population

A total of 313 participants from 19 villages of the Dzimauli area were recruited in the present study, and 534 stool samples were collected from them. The sample collection was divided into four groups, namely Madi drop and filters, Madi Drop only, filters only, no intervention) shown in table 6. The number of female participants was slightly higher as compared to that of males. The age of participants ranged between 1 to 3 years with a mean of 1.39 years. 73% of the participants were from the household that receives the total income of R1000 – R1500 per month. Most participants obtained their water from the public tap and store them in their households. Most of the households of participants had access to proper toilets and only 7% were still using open defecation shown in table 4.

Table 4: Total data of the sociodemographic characteristics of the study population according to the participants

Characteristics	Number of participants
Gender	
Male	150 (47.9%)
Female	163 (52.1%)
Total	**313 (100%)**
Age group (years)	
0-1	117 (36%)
1-2	115 (36%)
3>	81 (28%)
Total	**313 (100%)**
Income monthly	
1000 - 1500	230 (73%)
1500 - 2000	43 (14%)
2500>	40 (313%)
Total	**313(100%)**
Source of drinking water	

Piped into house	107 (34%)
Neighbor's pipe	77 (24%)
Public tap	121 (39%)
Truck	8 (3%)
Total	**313 (100%)**
Sanitation facilities	
Latrine	290 (93%)
Open defecation	23 (7%)
Total	**313 (100%)**

The characteristics of the samples collected are shown in Table 5. A total of 155/534 (29%) of the sample was diarrhea, while (13.5%) and (1%) of the stool samples had mucus and blood, respectively.

Table 5: Characteristics of the stool samples used in the study.

Characteristics	Number of samples
Stool type	
Bloody	7 (1.0%)
Mucus	72 (13.5%)
Diarrhea stool	155 (29%)
Non-diarrhea stool	300 (56%)
Total	**534 (100%)**
Groups	
Madi Drop and filters	133 (24.9%)
Madi Drop only	126 (23.8%)
Filter only	149 (27.7%)
No intervention	126 (23.8%)
Total	**534 (100%)**
Month of collection	
Month 18	299 (56%)
Month 24	234 (44%)
Total	**534 (100%)**

4.2. Prevalence of *Entamoeba* infection by microscope

Of all 534 stool samples, a total 130 (24.3%) were microscopically positive for *Entamoeba* cysts either singly and combined with other intestinal parasites such as *Endolimax nana,* and *Trichuris*

trichiura. Month 18 samples had the highest *Entamoeba* infections with 78/299 (26%) as compared to month 24 with 52/235 (22%).

4.3. Prevalence of *Entamoeba* infection by PCR

All microscopic positive and negative samples were subjected to conventional PCR assays using *Entamoeba* genus-specific PCR primers that generate a 550-bp amplicon. that the results showed that 43/534 (8%) samples were positive for *Entamoeba* genus shown in figure 23.
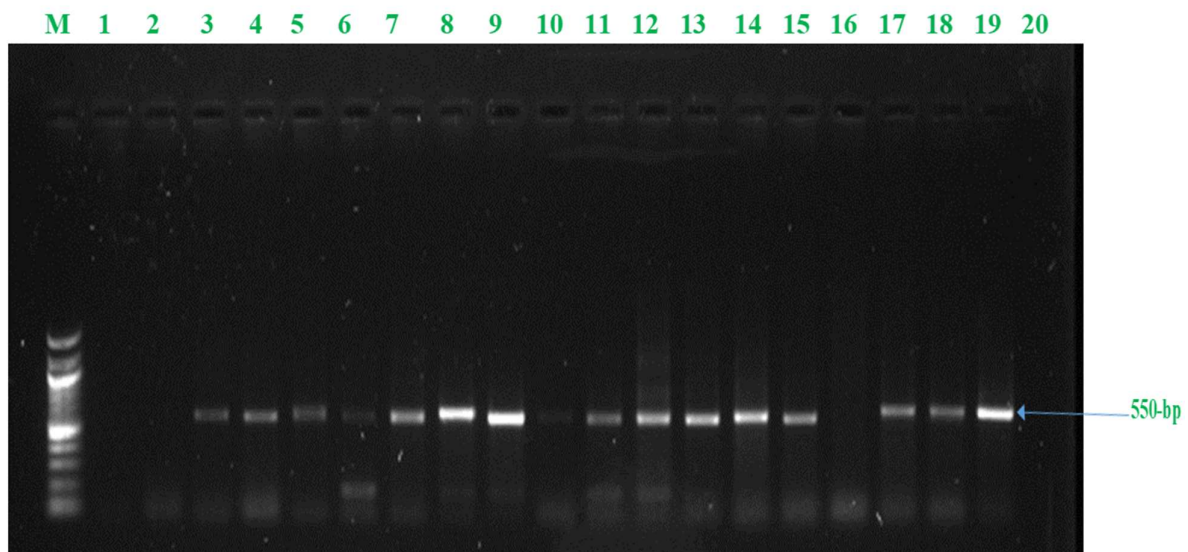
Figure 19- PCR amplification of *Entamoeba* DNA using genus-specific primers. Lane M= molecular marker (100-bp), Lane 1= negative control (PCR water), Lanes 2 to 20 = amplified products (550bp) indicating positive specimens

4.4. Prevalence of *Entamoeba* infections among the different groups of intervention.

The efficacy of the different groups including the Madi Drop and filters group, Madi Drop only group and filters only group was tested and the results are shown in Table 7. The results showed a reduction of infection from the 18 months to the 24-month collection indicating a reduction in the level of infection probably due to the intervention compared to the control group where there was no change. *Entamoeba* infections for months 18 months in the Madi drop and filters intervention groups were higher as compared to month 24 after intervention with both microscopic

and PCR results. The decrease level of *Entamoeba* infections was seen in Madi Drop only group but not as effective as compare to Madi drop and filters. The least decrease of *Entamoeba* infection was seen in the filter only intervention and no intervention/ -control showed no change in the PCR but slightly change in microscopic results, data showed in table 6.

Table 6: Frequency of infection by *Entamoeba* in the study population

Groups	Month of collection	No. of samples	*Entamoeba* infection	
			By Microscope	**By PCR**
Madi Drop and filters	18 24	67 66	35/67 (55%) 15/67 (22%)	10/67 (14%) 2/67 (2%)
Madi Drop only	18 24	63 63	23/63 (36%) 8/63 (12%)	7/63 (11%) 1/63 (1%)
Filter only	18 24	75 74	20/75 (26%) 16/75 (21%)	12/75 (16%) 7/75 (9%)
No intervention (-control)	18 24	63 63	7/33 (21%) 6/33 (18%)	3/33 (9%) 3/33 (9%)
Total		**534**		

4.5. The results of Sanger and Next-generation sequencing technologies.

A total of 20 samples were successfully sequenced by the Sanger method and 11 (55%) of amplicons were *Entamoeba* species as shown in table 7. *Entamoeba polecki* dominated by 40% and the other species identified were *Entamoeba muris* with 5%. The remaining 9 amplicons were not *Entamoeba* species but other microorganisms (results not shown).

Table 7: Representation of Sanger sequencing results

Species	Number of positive (N)	Percentage (%)
Entamoeba polecki (EP)	9	45
Entamoeba Coli (EC)	2	5
Entamoeba muris (Emur)	1	5
Other microorganisms	9	45
Total	**20**	**100**

For the NGS technologies, 15 positive samples were successfully sequenced, and different *Entamoeba* species were described as shown in table 8. *Entamoeba coli* was the most common followed by *E. polecki*. Thirteen percent of the amplicons were *Entamoeba moshkovskii*. One sample failed quality control (QC) and was not analyzed.

Table 8: Representation of NGS sequencing results according to the species

Species	Number of positive (N)	Percentage (%)
Entamoeba coli	9	60
Entamoeba polecki	3	20
Entamoeba moshkovskii	2	13
Failed at QC level	1	7
Total	**15**	**100**

The sequencing results revealed that *Entamoeba polecki* was the most dominant species shown in Table 9. *Entamoeba* infections were mostly found among the children in the homes in the filter only group and the least infection was found in Madi Drop only. This shows that the Madi Drop has much more effective.

Table 9: Occurrence of *Entamoeba* infections in different study groups.

Groups	*Entamoeba* infection	Infection frequency for specific *Entamoeba* (%)			
		E. coli	*E. polecki*	*E. muris*	*E. Moshvkovskii*
Madi drop and filter	8	2	5	1	0
Madi drop only	7	5	2	0	0
Filters only	11	3	7	1	3
No intervention	8	2	4	2	0
Total		**12**	**18**	**4**	**3**

4.6. Phylogenetic analysis of the organisms in the study population

Briefly, a phylogenetic tree is a form of a diagram that represents the evolutionary relationship between organisms, but they are not definitive facts. Every pattern branching in the tree reflects on how the species and others evolved from common ancestors. Mostly in the phylogenetic tree,

if two or more species are seeming to be more related in terms of recent common ancestors, then they are set to be more related. In the present study, the evolutionary analyses were conducted from obtained Sanger and Next-generation sequences using MEGAX software.

4.6.1. Phylogenetic analysis of Sanger sequencing

REF, SEQUENCES	COUNTRIES
KJ870232Em	CAMEROON
MN536494Em	USA
AB426549Eh	JAPAN
FN666249Eb	SWEDEN
MH133209Ech	CHINA
FN396613Emur	SPAIN
AB444953EC	JAPAN
AB845671Ep	CAMEROON
AB845670Ep	CAMEROON
AB845674EC	CAMEROON
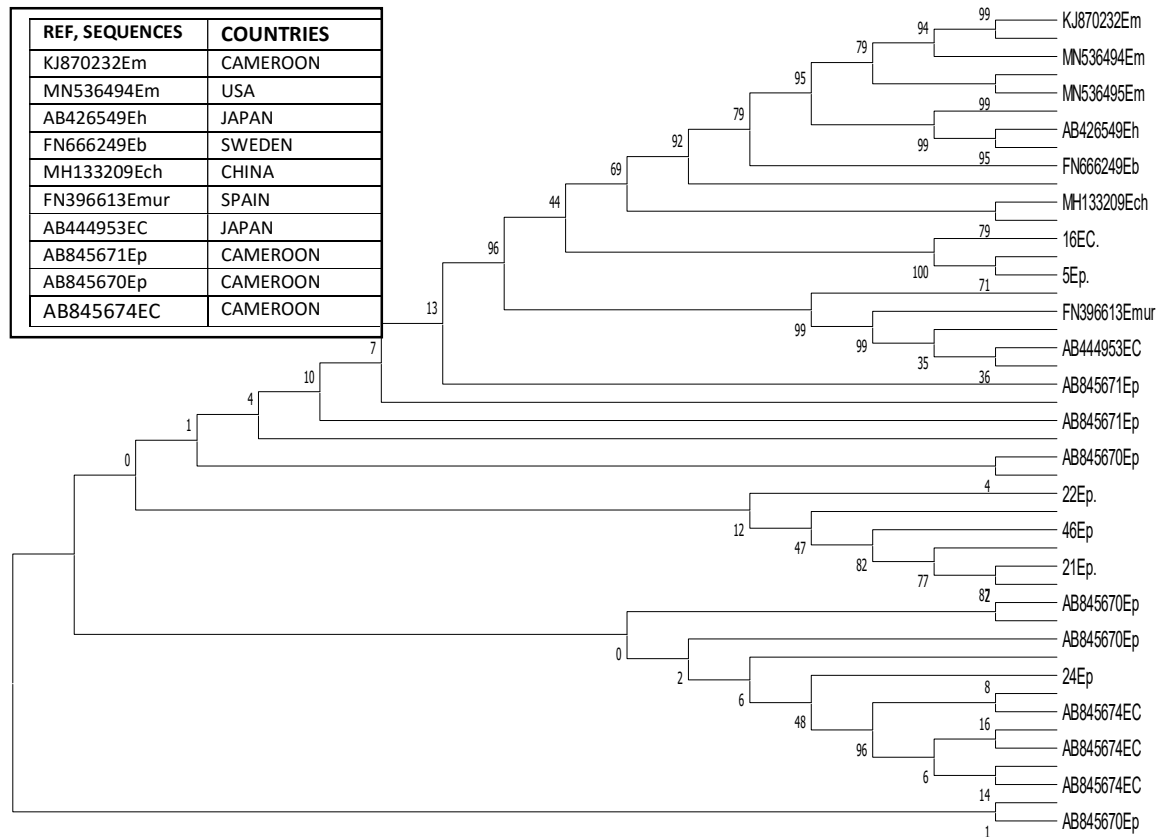
Figure 20: The representation of the evolutionary relationship by Maximum likelihood tree.

The phylogenetic tree is showing the species relatedness among the *Entamoeba* species (**16EC, 5Ep, 22Ep, 46Ep, 21Ep, and 24Ep**) isolated in the present study shown in **figure 20** along with the species obtained from other countries. The reference sequences and the countries are shown on diagram (figure 23) in a small table.

REF, SEQUENCES	COUNTRIES
KJ870232Em	CAMEROON
MN536494Em	USA
AB426549Eh	TOKYO
FN666249Eb	SWEDEN
MH133209Ech	CHINA
FN396613Emur	SPAIN
AB444953EC	JAPAN
AB845671Ep	CAMEROON
MN536495Em	USA
AB845670Ep	CAMEROON
FR686378Ehur	DENMARK
FR686413EC	DENMARK
AB845674EC	CAMEROON
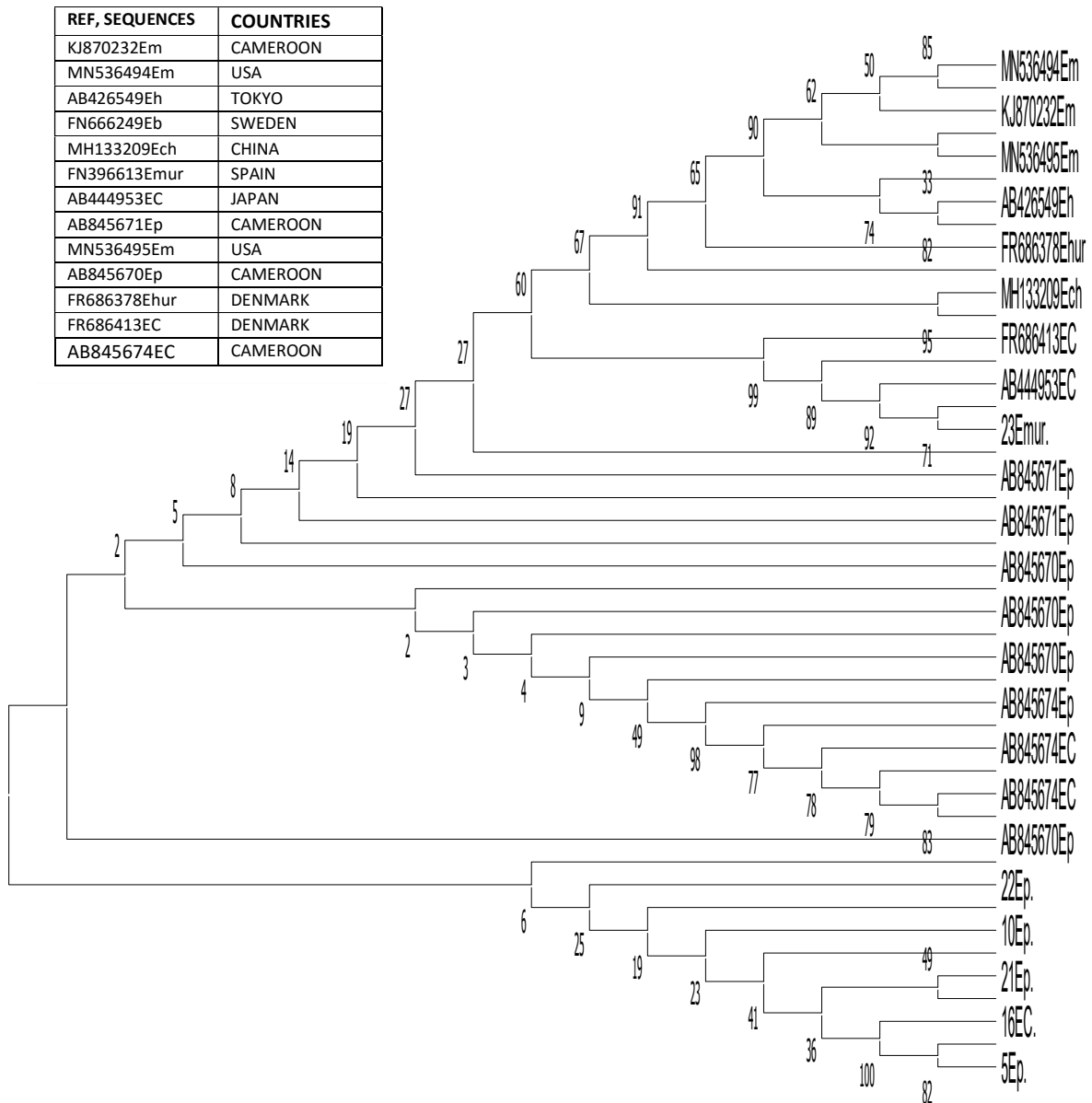
Figure 21: The representation of evolutionary relationships of taxa by Neighbour-Joining tree.

A phylogenetic tree is showing the species relatedness of the *Entamoeba* isolated in the present study (**23Emur, 22Ep, 10Ep, 21Ep, 16EC, and 5Ep**) and other species isolated from other countries or obtained from GenBank. The reference sequences and the countries are shown on diagram (figure 23) in a small table.

REF, SEQUENCES	COUNTRIES
KJ870232Em	CAMEROON
MN536494Em	USA
AB426549Eh	JAPAN
FN666249Eb	SWEDEN
MH133209Ech	CHINA
FN396613Emur	SPAIN
AB444953EC	JAPAN
AB845671Ep	CAMEROON
MN536495Em	USA
AB845670Ep	CAMEROON
FR686378Ehur	DENMARK
FR686413EC	DENMARK
AB845674EC	CAMEROON
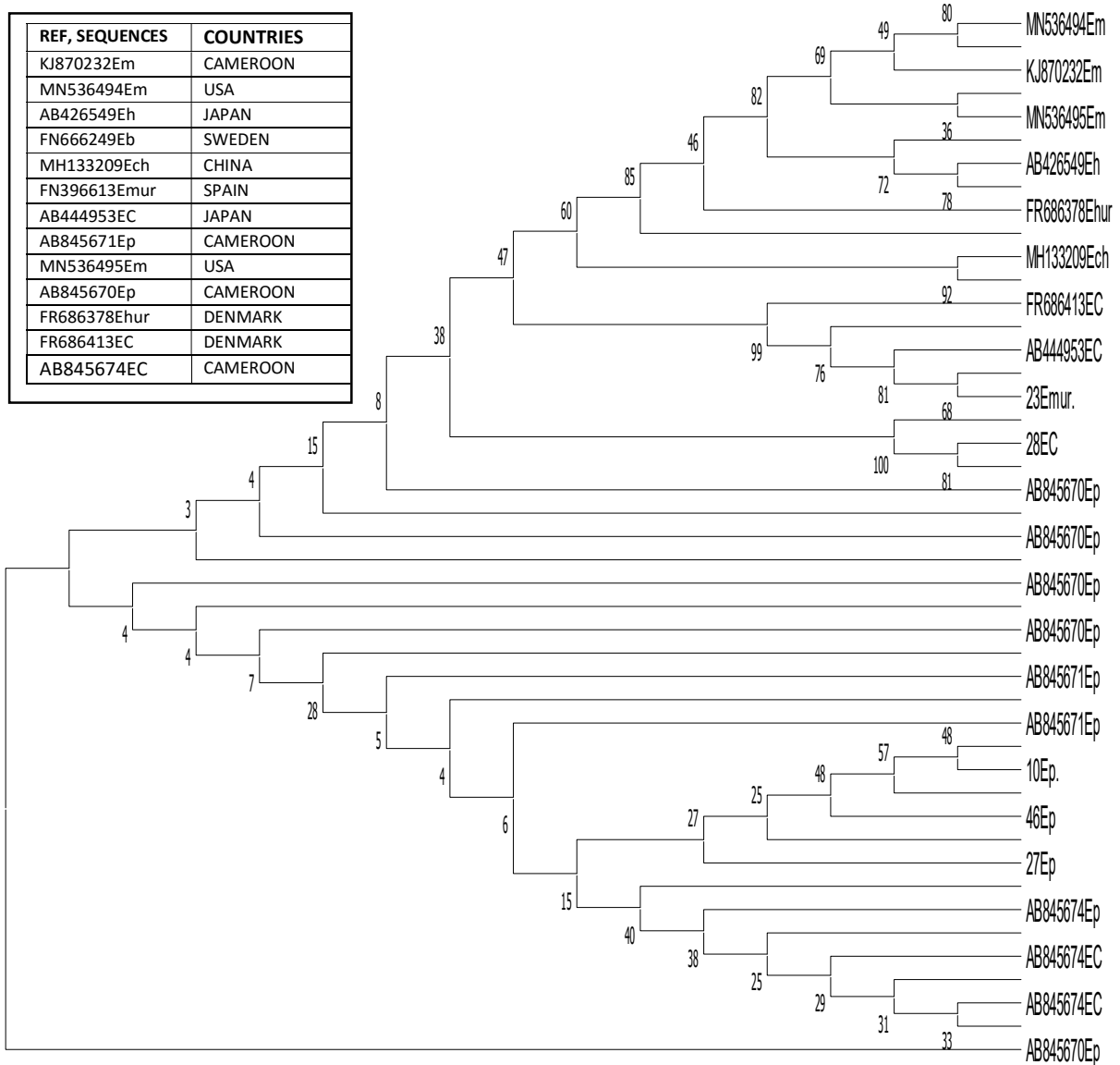
Figure 22: The representation of evolutionary relationships of taxa using a minimum likelihood tree.

A phylogenetic tree is showing the species relatedness amongst the once obtained from this present study (**23Emur, 28EC, 10Ep, 46Ep, 27Ep**) as well as the species isolated from other countries. The sequences obtained from the GenBank where used as the references in the study. The reference sequences and the countries are shown on diagram (figure 23) in a small table.

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4.6.2. Phylogenetic analysis of Next-Generation Sequencing

REF, SEQUENCES	COUNTRIES
AB845671Ep	CAMEROON
AB845670Ep	CAMEROON
LC230016Ep	JAPAN
MN536499Em	USA
AB845674EC	CAMEROON
MN536496Em	USA
FR686440Ec	DENMARK
FR686438Ec	DENMARK

Figure 23: The representation of evolutionary analysis by Maximum Likelihood tree.

A phylogenetic tree is showing the species relatedness among the species identified in the present

study (**3694Ec, 2719EC, 99974Ecp and 85407Ecp**) and the ones obtained from a GenBank. The

reference sequences and the countries are shown on diagram (figure 23) in a small table.

REF, SEQUENCES	COUNTRIES
AB845671Ep	CAMEROON
AB845670Ep	CAMEROON
LC230016Ep	JAPAN
MN536499Em	USA
AB845674EC	CAMEROON
MN536496Em	USA
FR686440Ec	DENMARK
FR686438Ec	DENMARK
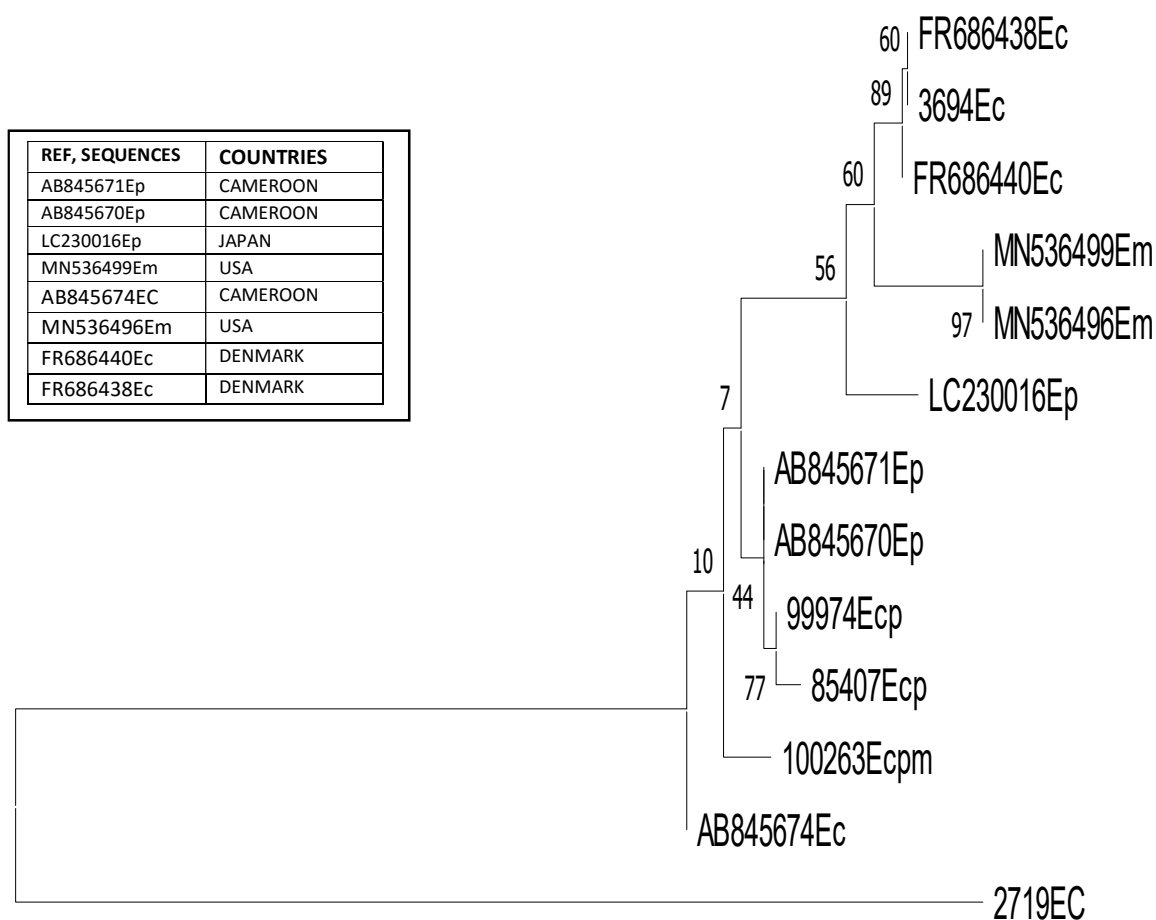
Figure 24: The representation of evolutionary relationships of taxa using the Neighbor-Joining tree.
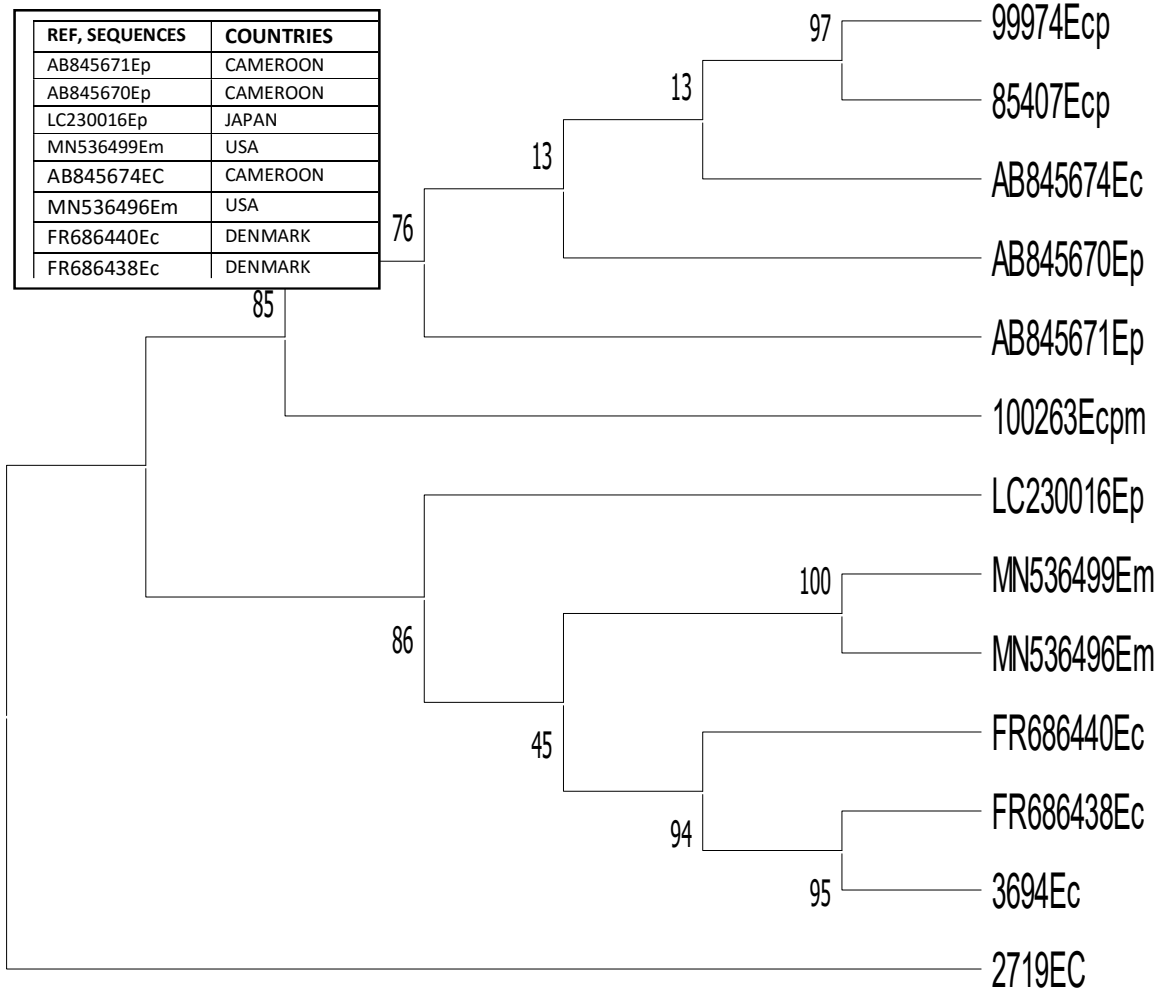A phylogenetic tree is showing the relationships between the isolated species in the present study
(**3694Ec, 99974Ecp, 85407Ecp, 2719EC**) and the ones from the GenBank/ obtained from other
countries. The reference sequences and the countries are shown on diagram (figure 23) in a small
table.

REF, SEQUENCES	COUNTRIES
AB845671Ep	CAMEROON
AB845670Ep	CAMEROON
LC230016Ep	JAPAN
MN536499Em	USA
AB845674EC	CAMEROON
MN536496Em	USA
FR686440Ec	DENMARK
FR686438Ec	DENMARK
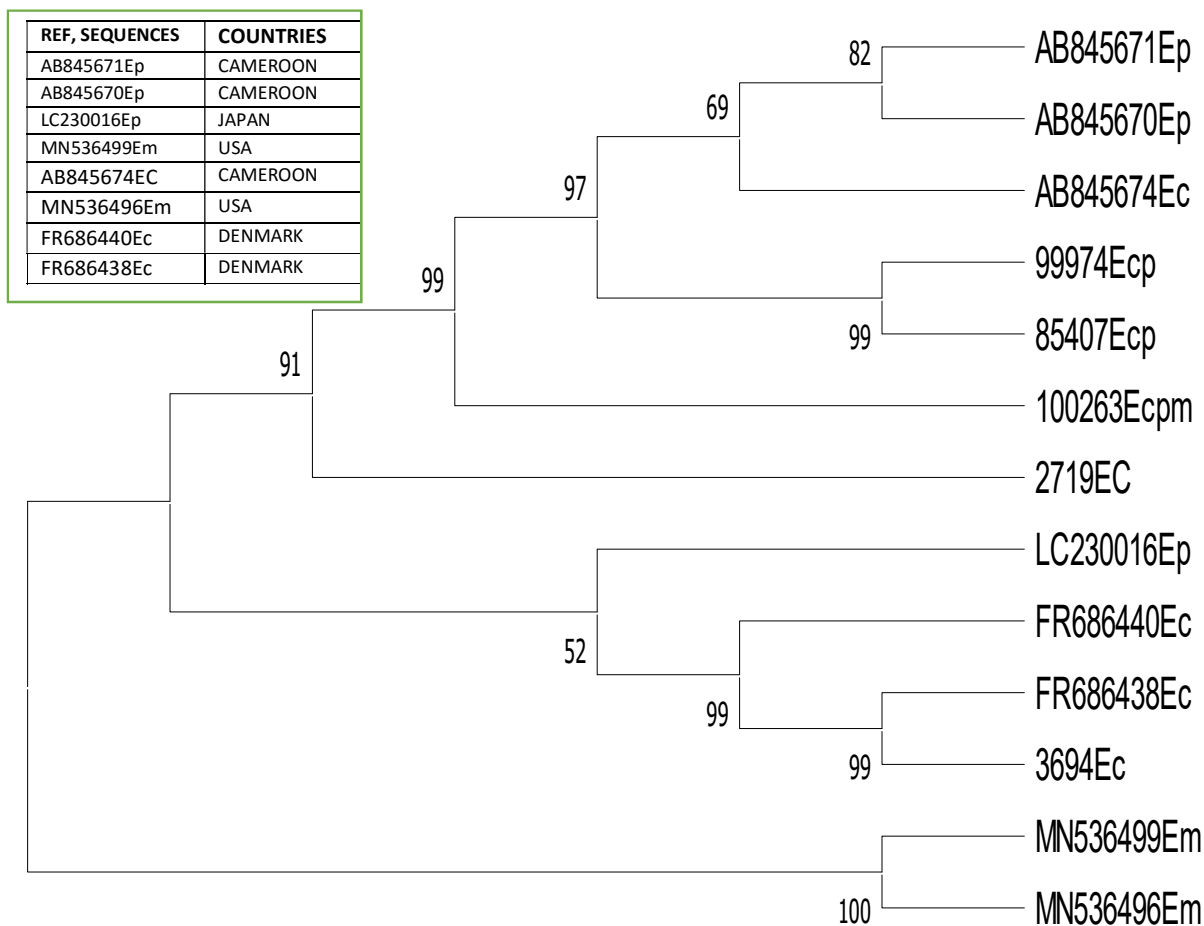
Figure 25: The representation of evolutionary history using the Minimum evolutionary method.

A phylogenetic tree is showing the relationships of the *Entamoeba* species isolated (**2719EC, 3694Ec, 85407Ecp, 99974Ecp, 100263Ecpm**) and the ones obtained from the GenBank or other countries. The reference sequences and the countries are shown on diagram (figure 23) in a small table.

Figure 25 it is also showing the relationship and species relatedness of the *Entamoeba polecki* (99974Ecp and 85407Ecp) isolated in the present study aligning with the one from the GenBank (AB845674Ec and 85407Ecp), and the evolved from closest ancestors. *Entamoeba coli* (2719Ec) branched out, which shows that it evolved from the same ancestor but not closely related to other species.

Neighbor-joining and minimum likelihood have shown that species in the GenBank shared some characteristics with the isolated ones. The phylogenetic tree showed the original cluster of the *Entamoeba polecki* and *coli* strains besides the *E. Moshkovskii* and *histolytica* branch. The sequences were highly conserved within each of the *Entamoeba species*, particularly for the strains *E. polecki* and *E. coli*. All the identified alignments in the present study were then compared ones reported in GenBank and the isolated species in the study showed the relationship with the one in the GenBank and the only homologous region corresponding to the sequences obtained in our study was considered. Although *E. moshkovskii* sequences were shorter than all other sequences, their overlapping sequences were similar and only two minor differences at about the end of the sequenced region were observed.

Of all 15 samples subjected to next-generation sequences (NGS), 14 came out positive for *Entamoeba* species with different reads. Only 3 different species were isolated (*Entamoeba coli, E. polecki* and *E. moshkovskii*) from all 14 samples. *Entamoeba coli* was isolated from 9 samples (60%) followed by *Entamoeba coli* with (20%) and *E. moshkovskii* was the lowest with (13%). Sample 14 had the highest reads of (99.50%) with *Entamoeba polecki* followed by sample 14B with the reads of (97.74%) with *Entamoeba coli* and sample 16 had read of (95.96%) with the *Entamoeba moshkovskii.*

Table 11: Identified *Entamoeba* species by next-generation sequencing in the different samples.

Sample code	Family	Reads (%)	Species
Sample 1	*Entamoeba*	10 (0.55)	*Entamoeba coli* *Entamoeba polecki*
Sample 2	*Entamoeba*	132 (0.33)	*Entamoeba coli*
Sample 4	*Entamoeba*	250 (1.17)	*Entamoeba coli*
Sample 5	*Entamoeba*	140 (0.52)	*Entamoeba polecki* *Entamoeba coli* *Entamoeba moshkovskii*
Sample 6	*Entamoeba*	25 (0.06)	*Entamoeba coli* *Entamoeba polecki*
Sample 7	*Entamoeba*	30 (0.25)	*Entamoeba coli*

			Entamoeba polecki
			Entamoba moshkovskii
Sample 9	Entamoeba	904 (4.09)	Entamoeba coli
			Entamoeba polecki
Sample 10	Entamoeba	17 (0.12)	Entamoeba coli
			Entamoeba moshkovskii
Sample 14	Entamoeba	24111 (99.50)	Entamoeba polecki
			Entamoeba coli
Sample 14B	Entamoeba	4059 (97.74)	Entamoeba coli
			Entamoeba polecki
Sample 16	Entamoeba	14638 (95.96)	Entamoeba moshkovskii
			Entamoeba polecki
Sample 18	Entamoeba	10748 (86.31)	Entamoeba coli
			Entamoeba polecki
Sample 18B	Entamoeba	2195 (90.89)	Entamoeba moshkovskii
			Entamoeba coli
Sample 23	Entamoeba	3784 (92.92)	Entamoeba coli

Chapter 5

Discussion

The present study aimed to determine the genetic diversity of *Entamoeba* species, and the impact of the Madi Drop on the occurrence of *Entamoeba* among children in Vhembe District, South Africa. The prevalence of *Entamoeba* infections was found to be 24% as determined by microscopic examination. One cannot exactly conclude based on the microscopic results because most of the parasites have the same morphological characteristics (Van Wyk *et al.,* 2013). To conclude on the detection and identification, one should use molecular techniques. Therefore, PCR was used as a molecular technique and the results reported 8% of *Entamoeba* genus which was characterized based on the amplicon size of 550 bp. Since PCR is reliable and sensitive, therefore *Entamoeba* infections in the study population were found to be 8%.

Four types of stool samples (stools with some blood in it, with some mucus, diarrheal and non-diarrheal stool) were used in the present study. High prevalence of *Entamoeba* infections was seen in bloody, mucus and diarrheal stool samples. Almost all stool samples that came out positive for *Entamoeba* infections in both microscopic and PCR were containing blood, mucus, and diarrheal samples. Non-diarrheal stool samples had a lower prevalence of *Entamoeba* infections. Even though some of the samples that were positive for *Entamoeba* infection under the microscope were not positive in PCR, some of the samples showed corresponding results in both methods. In the case of no- correspondence, it is known that the microscopic technique is not sensitive and reliable as compared to PCR (Kaya *et al*., 2013).

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Lack of access to water quality, poverty, and poor sanitation, as well as a lack of education are known factors that contribute to the prevalence of parasitic infections with special reference to *Entamoeba* species (Faria *et al.,* 2017). *Entamoeba* infections are easily and mostly transmitted through contaminated water and food by *Entamoeba* matured cysts (Tengku *et al*., 2011). Therefore, factors such as inadequate water supply increase the level of parasitic infections in a rural area (Omarova *et al.,* 2018). It was noted that some participants' households in this study did not have toilets facilities which then led the villagers to defecate nearby the rivers and sources may be contaminated with *Entamoeba* cysts from human faces which are responsible for transmission (Omarova *et al.,* 2018). This reported prevalence agrees with previous studies carried out among Vhembe district communities in Limpopo province, which reported the prevalence rate of 21. 6% of *Entamoeba* species (Samie *et al.,* 2006).

The present study is situated in a rural sociodemographic and environmental setting characterized by deficits in sanitation infrastructure and water stress. The study area has a problem with the access of water or drinking water quality which is subjecting them to access water from nearby rivers, trucks, public taps and other rainwater is stored in household tanks for later use. Three methods and control have been used for water treatment including, Madi Drop and filters, Madi Drop only, Filter only and no intervention (control). We found that the rate of *Entamoeba* infections was three times higher before the interventions. After the intervention, the microscopic and PCR results showed the reduction of *Entamoeba* infections. From our results, it can be suggested that the use of the Madi Drop and filters, and Madi Drop impregnated with silver can be used to improve drinking water quality and decrease the level of parasitic infections.

Our findings agree with the study that reported the effectiveness of the filters in Mashamba village, Vhembe district. The study indicated that in "rural settings, water contamination occurs mainly at the point-of-use or household level and water purification in Limpopo province is still a huge

challenge". The study has also shown that locally manufactured the utilization of water filters been proven to remove pathogenic microbes in water and improve drinking water quality (Brown *et al.,* 2008). A ceramic water filter can remove up to 99.9% of pathogenic microbes.

From water treatment strategies, Madi Drop is the preferable technology that can be practiced in rural households because of its characteristics. Madi Drop is affordable, easy to use and also safe for example, no additional chemicals that may lead to health complications in the process (Applebaum *et al.,* 2012). From the literature, all methods are limited to *Entamoeba* species in terms of protozoan removal, but Madi Drop has shown some positive impact on the removal of *Entamoeba* infection in the study population.

To investigate the present and genetic diversity of *Entamoeba* species in the study population, PCR amplicons were sequenced by Sanger and Next-generation sequencing technologies. For tested amplicons, Sanger sequencing reported the presence of three *Entamoeba* species dominated by *Entamoeba polecki, E. coli and E. muris*, and NGS results revealed three different *Entamoeba* species *E. coli*, *E. polecki* and *E. moshkovskii*. The sequencing results have shown that *Entamoeba Polecki*, *E. coli, E. muris,* and *E. moshkovskii* are the circulating species in the study population. The results further suggested that cysts producing *Entamoeba* infections in the study population are dominated by *Entamoeba polecki*.

Due to the similarities of morphological characteristics, it is difficult sometimes to differentiate between *Entamoeba polecki* cysts with other *Entamoeba* species commonly found in human fecal samples, including immature cysts of *Entamoeba histolytica* (Stensvold *et al.,* 2018). Genetic analysis identified the parasites as *E. polecki* (100% identity), *Entamoeba coli* (100%), *Entamoeba muris* (99%) and *Entamoeba moshkovskii* (90%). The molecular techniques showed that *E. polecki* (75.0%) was the most common species detected in the study areas, followed by *E. coli* (30.8%), *E. muris* and *E. moshkovskii* (5.8%). Although these identified species of *Entamoeba* might be

less pathogenic or non-pathogenic to humans and animals in the case of a single infection, the coinfections with other pathogens including bacterial, fungal and viral infection may increase the severity of the disease (Fletcher *et al.,* 2012).

In many cases, *Entamoeba polecki* has been detected from humans with severe symptoms, for example, bloody diarrhea and abdominal pains (Verweij *et al*. 2001). Studies are done Stensvold *et al.,* (2011) demonstrated human infections with *E. polecki*, in which a novel 18S rRNA gene sequence was identified in a species of Sulawesi macaque. Similar study was done in Malayisa and reported *E. polecki* (13.2%) was more prevalent compared to *E. coli* (5.6%) (Sahimin *et al.,* 2019). On the other hand, the high prevalence of *E. polecki* in the present study was in contrast to the worldwide distribution of *Entamoeba* species, which indicated that *E. coli* is ten times more common as compared to *E. polecki and Entamoeba muris* (Elsheikha *et al.,* 2018). However, in many cases, the local prevalence of these species may vary significantly based on the different geographical regions. Similarly, the other studies also reported that about 70% of patients were infected with *Entamoeba coli* as compared *Entamoeba polecki* and *Entamoeba moshkovskii* in Australia (Ngui *et al.,* 2012).

Study done in South African reported that *E. polecki* (90%) was more frequent compared to *E. coli* (10%) among infected individuals (Sylvain *et al.,* 2015). Furthermore, another study was reported in India which reported 49.5% of patients were infected by *E. polecki* and only 7.4% with *E. coli and E. moshkovskii* (Sylvain *et al.,* 2015). Studies were done by Meurs *et al.,* (2017), reported that cases of intestinal parasitic infection in the rural population of north of Ghana with a single microscopic stool examination, from all the cases of infections, *Entamoeba polecki,* and *Entamoeba coli* is detected in more than half of all individuals and their presence indicates the possibility of fecal-oral transmission (McHardy *et al.,* 2014). *Entamoeba polecki* is mostly isolated from domestic animals especially pigs (Matsubayashi *et al*., 2017). Therefore, looking at the study

population setting we can also suggest that the infection might be transmitted from pigs to water than humans.

The sequencing results from both Sanger and Next-generation sequencing revealed almost the same *Entamoeba* species. The identified species had slight differences in their sequences. Sanger method revealed the most common species with the variant and frequency of 20% and above. The Next-generation sequencing revealed the *Entamoeba* species with the minor species of 20% and below. Though the sequences were slightly different, due to the frequency percentage and variant on sequences, it can be suggested that NGS technologies can be useful in revealing the novel species as well as other parasites as compared to the Sanger method (Knief, 2014). Other microorganisms that were obtained in NGS were not available in Sanger.

In terms of the number of reads per sample, we found that *Entamoeba polecki* had the highest reads of 99.5% followed by *E. coli* with 97.74% and *E. moshkovskii* 95.56%. These species were picked up or identified by NGS technologies. Since NGS is likely to pick more microorganisms from the lower level as compared to Sanger, we can suggest that these 3 species are the once dominating and circulating in the study population. In general, dominated species in the study population is found to be *Entamoeba coli* and *E. polecki* because they were almost found in most of the samples.

To obtain better characterization and species relatedness, sequences obtained in the present study were compared with available sequences from the GenBank. Phylogenetic trees were constructed from Sanger and Next-generation sequencing technologies using different methods (Neighbour-joining, minimum likelihood, and maximum likelihood method). The maximum likelihood method has shown that the isolated species were sharing the same characteristics with the ones from the GenBank sequences and they shared the same ancestors. *Entamoeba polecki* **(24Ep)**

isolated from the study was shown to be aligned with other *Entamoeba polecki* **(AB845670Ep and AB845671EP**) in the GenBank.

Conclusion and recommendations

An important key issue in the understanding of morbidity and mortality associated with amebiasis infections aligns with the proportion of infections associated with the pathogenic *Entamoeba* species. To the best level of our knowledge, this study report for the first time the genetic diversity of *Entamoeba* species, and the impact of the Madi Drop on the occurrence of *Entamoeba* among children in Vhembe, South Africa.

We successfully characterized different types of *Entamoeba* species using microscopic and molecular techniques such as PCR followed by Sanger and Next-generation sequencing technologies. We chose to use NGS techniques to validate our finding because the previous experience in this area showed that it can be able to detect even minor species of these parasites and is much more efficient to use. In the present study, we managed to assess the efficiency of Madi Drop technology for the removal and decrease the level of parasitic infections

It would be noteworthy to consider the parasitic infections such as amebiasis during diagnosis, treatment, controlling and prevention of other infections in the study population. This will establish a better understanding of *Entamoeba* infections, epidemiological data and diagnosis methods for amebiasis. The findings of the study also highlighted a need for developing a better *Entamoeba* infection diagnostic tool, to improve the control and treatment procedures for the study population because there are higher possibilities of pathogenic strains circulating in the study population. Our findings further emphasize the need for the re-evaluation of the pathogenicity of species such as *E. polecki* and *E. moshkovskii* which are quite common in the study population and might be responsible for some of the morbidity.

It is known that in developing countries, lack of access to water quality is a major problem and generally lead to the transmission of several parasitic infections. Therefore, our results suggest that usage of the Madi Drop and filters can be a good approach since they showed a positive

response in terms of removing and reducing the level of parasitic infections. Unlike other methods or technologies, Madi Drop technology is affordable, easy to use, fast and less exposed to external contamination, people are advised to use it for cleaning water. Our findings brought out several questions concerning virulence of the identified amoebas in children, including whether these strains isolated in children may infect other animals and humans or may result in similar symptoms. Therefore, more studies need to be done to answer the questions concerning the virulence of the identified Entamoeba species in the current study.

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