JIMMA UNIVERSITY COLLEGE OF HEALTH SCIENCES DEPARTMENT OF MEDICAL LABORATORY SCIENCES AND PATHOLOGY

COMPARISON OF FOUR DNA EXTRACTION METHODS FROM STOOL SAMPLES FOR THE DETECTION OF INTESTINAL PARASITES WHILE OPTIMIZING MOLECULAR TOOLS AT JIMMA UNIVERSITY MOLECULAR BIOLOGY LABORATORY

By:

Mio Ayana (BSc. in MLs)

A THESIS SUBMITTED TO JIMMA UNIVERSITY COLLEGE OF PUBLIC HEALTH AND MEDICAL SCIENCES, DEPARTMENT OF MEDICAL LABORATORY SCIENCES AND PATHOLOGY AS PARTIAL FULFILMENT OF MASTERS IN MEDICAL PARASITOLOGY

October, 2014

Jimma, Ethiopia

COMPARISON OF FOUR DNA EXTRACTION METHODS FROM STOOL SAMPLE FOR THE DETECTION OF INTESTINAL PARASITES WHILE OPTIMIZING MOLECULAR TOOLS AT JIMMA UNIVERSITYMOLECULAR BIOLOGY LABORATORY

By:

Mio Ayana (BSc. in MLs)

Advisors:

Zeleke Mekonnen: MSc, PhD Scholar

Abdissa Bruksew: BSc, MSc

Bruno Levecke: PhD

October, 2014
Jimma, Ethiopia

Summary

Intestinal helminths and protozoan parasites are among the most prevalent parasites causing a significant morbidity and mortality to humans in developing countries. Commonly used diagnostic methods for these parasites rely mainly on microscopical examination of stool samples for the identification of helminth eggs and protozoan trophozoites and cysts. However, this method is not efficient enough to differentiate morphologically indistinguishable interspecies and strains of parasites. Thus, the present study was aimed to evaluate different DNA extraction methods for the identification and further advanced molecular based studies of intestinal parasites in terms of their effectiveness, cost and time. Moreover, the present study was also aimed at optimizing basic molecular biology techniques and tools to be used in newly established molecular biology laboratory at Jimma University.

We compared and evaluated two commercially purchased DNA extraction kits (QIAamp stool mini kit and DNeasy blood and tissue kit) with and without their modifications of beat beating. The comparison of these four methods were done by collecting and examining 195 stool samples from schoolchildren attending primary schools in Jimma Town where high prevalence of soil-transmitted helminth (STH) infections has been reported recently. These stool samples were categorized in to three groups of 10 based on the intensity of infection as (high, low and no) eggs per gram of stool (EPG) for each of the three STH (Ascaris lumbricoides, Trichuris trichiura, and hookworms) and subjected to four different DNA extraction methods. Finally, the agreements of the results were compared using nested PCR followed by electrophoresis and visualization of DNA bands using UV trans-illuminator.

The comparison of these different DNA extraction methods was also performed for Giardia duodenalis. Since we don't have a recommended gold standard diagnostic or DNA extraction method from stool samples for diagnosis of intestinal parasites, we have used measure of agreement "kappa" using SPSS version 20 for the evaluation of effectiveness of different DNA extraction methods. Finally, DNeasy blood and tissue kit with bead beating method is appeared to be the most effective DNA extraction method in terms of DNA yield, cost and time. Therefore, we would like recommending this kit as relatively effective DNA extraction methods for soil transmitted helminthes and G. duodenalis in resource poor regions like our country Ethiopia. Moreover, we recommend further evaluation of those kits for other parasites.

Table of contents

Acronyms

AR – Anthelminthic Resistance

BZ – Benzimidazole

DNA – Deoxy Ribonucleic Acid

dNTPs – Deoxy Nucleoside Triphosphates

EDTA – Ethylene Diaminetetra Acetic acid

EPG – Eggs per Gram

GI – Gastrointestinal

HIV/AIDS – Human Iimmune Deficiency Virus/ Acquired Immune Deficiency Syndrome

ITS – Internal Transcribed Spacers

MDA – Mass Drug Administration

PCR – Polymerase Chain Reaction

PCR-RFLP – Polymerase Chain Reaction –Restricted Fragment Length Polymorphism

rDNA – ribosomal Deoxy Ribonucleic Acid

RNA – Ribonucleic Acid

SOP – Standard Operating Procedures

STH – Soil Transmitted Helminths

TAE – TrisAacetic acid and EDTA

UV – Ultra Violet

WHO – World Health Organization

qPCR – quantitative Polymerase Chain Reaction

rPCR – real time Polymerase Chain Reaction

FW – Forward

RV - Reverse

List of tables

List of figures

Chapter One

1. Introduction

1.1 Back ground

Intestinal parasitic infections, caused by intestinal helminths and protozoan parasites, are among the most prevalent infections of humans in developing countries. Intestinal parasites cause a significant morbidity and mortality in endemic countries. Among intestinal parasites, there are four species of soil transmitted helminthes (STH), also known as geohelminths: *Ascaris lumbricoides* (roundworm), *Trichuris trichiura* (whipworm), *Ancylostoma duodenale*, and *Necator americanus* (hookworms). These parasites are most prevalent in tropical and subtropical regions of the developing world where adequate water and sanitation facilities are lacking (Bethony *et al*., 2006). In addition to their health effects, intestinal helminthic infections also impair physical and mental growth of children and hinder economic development (Utzinger *et al*., 2009). Pre-school as well as school-aged children and pregnant women are the groups at highest risk of morbidity due to these infections (Glinz *et al*., 2010)

Significant progress has been made in the control of soil-transmitted helminthiasis by means of large-scale administration of anthelminthic drugs targeting high-risk groups or entire populations. A number of initiatives to reduce helminths-related morbidity are currently under way in different countries (Savioli *et al*., 2004). Single-dose anthelminthic treatment, usually without prior diagnosis administered to high risk groups is the strategy of choice. This approach has been termed mass drug administration (MDA) (WHO, 2006). Furthermore, a detailed understanding of the epidemiology of these parasitic worm infections is important for the design, implementation, monitoring, and evaluation of helminth control programs (Utzinger *et al*., 2003).

The other most common causes of intestinal parasitic infections are: intestinal protozoan parasites; *G. duodenalis* , *E. histolytica*, and *Cryptosporidium spp.* The diseases caused by these intestinal protozoan parasites are known as *giardiasis*, *amoebiasis*, and *cryptosporidiosis* respectively, and they are associated with diarrhea (Hotez *et al*., 2008). Gastrointestinal (GI) protozoa cause significant morbidity in children and as opportunistic infections in human immunodeficiency virus (HIV)/AIDS and immune suppressed patients in developing countries who are already malnourished or have limited access to medical services. Consequently, these

patients will suffer from repeated severe diarrheal episodes that can be fatal (Gupta *et al*., 2008). *G. duodenalis* is the most prevalent parasite and cause of diarrhea in developing countries as well as in the developed world due to the fact that cysts of giardia are highly resistant to environmental conditions, being able to survive in cold mountain streams, stomach acid, and chlorine and even in UV-treated wastewater (Li *et al*., 2009).

Amoebiasis is the third leading cause of death from parasitic diseases worldwide, with its greatest impact on the people of developing countries. The World Health Organization (WHO) estimates that approximately 50 million people worldwide suffer from invasive amoebic infection and resulting in 40-100 thousand deaths annually. However, these numbers are partly estimated based on data using diagnostic methods that did not effectively differentiate *E. histolytica* from the nonpathogenic *E. dispar* (Hotez *et al*., 2008).

Cryptosporidiosis is becoming most prevalent in both developed and developing countries among patients with HIV/AIDS and among children aged less than five years. Spread of these protozoan parasites in developing countries mostly occurs through faecal contamination as a result of poor sewage disposal and poor quality of drinking water supply. Cryptosporidium and Giardia are also potential zoonotic threats from commercial livestock and domestic animals (Langkjær *et al*., 2007). This is an important health risk factor to consider in children living in more underprivileged settings where animals and livestock are closely integrated in the community (Harhay *et al*., 2010).

To control and prevent these parasitic infections, application of a more sensitive and accurate diagnostic methods appears critical especially as one moves towards elimination programs. To this end, a range of DNA based methods for the detection of intestinal parasites has been described. Mainly, the remarkable impact of the implementation of automated DNA isolation and combination of multiplex real-time PCR assays for the detection of parasites are widely appreciated (Espy *et al*., 2006).

1.2 statement of the problem

In developing countries, diagnosis of intestinal parasitic infections still depends mainly on microscopical examination of stool samples for the identification of helminth eggs and protozoan trophozoites and cysts. Nevertheless, the use of microscopy in diagnostic laboratories has several shortcomings. Some parasite species cannot be differentiated based on microscopy alone, while detection of other species may need well trained and experienced laboratory personnel. Moreover, the overall diagnostic sensitivity of microscopy is low especially for light intensity infections. Developed countries have changed their diagnostic methods of parasitic infections to relatively sensitive and specific test methods from time to time. Such as, concentration methods, triple faeces test (TFT) protocol and the development of immunoassays. However, these test methods may have also their own limitations (Espy *et al*., 2006).

For example, the Kato-Katz technique is the most widely used copromicroscopic method in epidemiological surveys of human intestinal helminthic infections because of its simplicity and low cost (McCoy *et al.*, 2009). However, a Kato–Katz test method will probably only pick up 50% of all low-intensity infections (Harhay *et al*., 2010). Hence, the development and adoption of sensitive methods like PCR based diagnostic method is key for adequate patient management and for guiding the design, implementation, and monitoring of intestinal parasitic disease control programs (Glinz *et al*., 2010).

In addition, protozoan /helminthes zoonosis is an important feature of public health and has significant socio economic concerns. For example, most of the species of hookworms which infect dogs and cats are zoonotic. Using traditional microscopy, identification of *Ancylostoma* species infecting dogs and cats based on egg morphology is impossible. However, recently developed species-specific and sensitive PCR-RFLP technique detects and differentiates canine *Ancylostoma* species directly from eggs (Palmer *et al*., 2007). Until these diagnostic discrepancies resolved, it is likely that the prevalence of *A. ceylanicum* in human population especially in developing world, will continue to be under reported and the significance of this important canine and feline parasitic zoonosis will remain unknown in humans (Traub *et al.*, 2008).

Thus, molecular based diagnostic tools should be in practice aiding differential diagnosis between species or intraspecific variants that is often not achieved by microscopy alone. It can also provide important information about the parasite's zoonotic potential and transmission dynamics with in a community (Traub *et al*., 2005). Moreover, the scale up of chemotherapy programmes with anthelminthic currently underway in various parts of Africa, Asia and South America, will likely exert a drug pressure on the parasites and this has the potential to select for parasite genotypes that can resist anthelminthics. This emergence of anthelminthic resistance can only be investigated and confirmed through application of molecular techniques as part of monitoring drug efficacies.

Therefore, effective parasitic disease control could be achieved through developments and application of DNA technology based diagnostic techniques which includes but not limited to, next generation anti-parasitic drugs and anti- parasitic vaccine developments. Finally, understanding the molecular biology of host–parasite interactions using the tools of genomics and proteomics offer the vision of improving our success in preventing and controlling parasitic diseases (Olliaro *et al*., 2011).

1.3 Significance of the study

Recently, molecular biology laboratory has been established at Jimma University. However, the molecular tools for use in the laboratory have not been yet optimized within our context and became functional to its full capacity. Thus, the present study has optimized certain basic molecular tools like DNA amplification, electrophoresis and detection steps of DNA bands for intestinal parasites and then at the end compared four different DNA extraction methods. At present, we are confident enough that this newly established molecular laboratory infrastructure and all basic techniques we have optimized coupled with our recommendation of efficient DNA extraction method can be used as a base for any molecular based researches targeting intestinal parasites. Moreover, as part of this thesis, we have modified and prepared different standard operating procedures (SOPs) and put on table that can be used in research or teaching purpose towards parasitic infection prevention and control.

Chapter Two

2. Literature review

2.1 General overview of intestinal parasites

Intestinal parasitic infections are widely distributed throughout the world causing significant pressure to the public health, economy, physical and cognitive development particularly among children in developing countries. The poor personal hygiene, poor environmental hygiene, and poor health system commonly observed in developing countries make the prevalence to be highest among these populations (Okyay *et al*., 2004). Among these intestinal parasites, STH (*A. duodenale, N. americanus, A. lumbricoides and T. trichiura*) are the most prevalent organisms, estimated to infect almost one-sixth of the global population (Hall *et al.,* 2008) and GI protozoa parasites (e.g. *E.histolytica, G.duodenalis and cryptosporidium* species) which can cause significant morbidity in children and act as opportunistic infections in HIV/AIDS and immune suppressed patients are highly prevalent in developing countries (Gupta *et al.*, 2008).

2.2 Diagnostic methods for intestinal parasites

For diagnosis and control of parasitic diseases most of the attention has been focused on development of diagnostic methods, anti-parasitic drugs development, understanding resistance to anti-parasitic drugs and vaccines. So far, impact of these measures for diagnosis and control of parasitic diseases is not so much encouraging. For instance, for identification of helminthes species, microscopical examination of eggs and third stage larva (L3) cultured in vitro are considered to be gold standard test but, most of parasite eggs having closely similar morphological characteristics, and identification up to species level is not always easy by microscopy (Mochizuki *et al*., 2006).

Therefore, the diagnosis and treatment of intestinal helminth infections especially in developing countries have not been changed much; due to identification of major intestinal helminthes infections are still solely dependent on microscopy. As for protozoan parasite infections, many are confirmed by the use of microscopy in conjunction to other methods of diagnosis including serology-based assays and more recently molecular-based assays (Ndao, 2009).

However, in the last three decades, we have seen new approaches to diagnose intestinal protozoan parasite like antigen-detection tests which are currently commercially available for the diagnosis for intestinal protozoan parasites. (Roy *et al*., 2005). For instance, diagnosis of *E. histolytica* cannot be done any longer by microscopy, since this parasite is morphologically similar to the non-pathogenic parasite *E. dispar*. In addition to the antigen-detection test, several PCR-based tests specific for *E. histolytica* have been developed and used for specific detection of *E. histolytica (*Roy *et al., 2005).*

For giardiasis, diagnosis is best accomplished by detection of Giardia antigen in stool, since the classic microscopic examination is less sensitive and specific. In addition to antigen detection tests, PCR-based test for the detection of *G. duodenalis* has also been used for strain characterization since population genetics of Giardia are complex. For example, a recent genetic linkage study has confirmed the distinct grouping of Giardia into five major types/assemblages. The two main human parasite genotypes/assemblages of *G. duodenalis* are commonly known as: assemblage A and assemblage B; while feline and canine parasites are categorized as assemblage C, D and E of *G. duodenalis*. Differentiation of these assemblages of *G. duodenalis* can only be done by PCR-based tests (Ng *et al*., 2005).

In the case of cryptosporidiosis, there are two main species that infect humans; *C. hominis* (genotype I) and *C. parvum* (genotype II). The PCR-based diagnostic method is required for differentiation of these two types of *Cryptosporidium* species. Thus, it is recommended that these modern antigen-detection and PCR-based diagnostic tests need to be used for understanding the actual prevalence and epidemiology of these protozoan parasites (Chalmers *et al*., 2005)

Nevertheless, in developing countries, the most commonly used diagnostic methods for these intestinal parasites still rely on microscopic detection of helminths eggs or larvae, cyst and trophozoites in human stool. These copromicroscopic approaches have drawbacks, such as low sensitivity for the detection of light-intensity infections (Glinz *et al*., 2010). To minimize these inherent limitations of traditional diagnostic test; advanced, sensitive and specific molecular based diagnostic method has to be developed and/or adopted. Thus, a comprehensive understanding of the epidemiology of these intestinal parasitic infections at their molecular level using molecular biology tools is important for the design, implementation, monitoring, and evaluation of parasitic disease control programs.

These all molecular based diagnostic methods for parasitic infection that rely on DNA analysis are based on the assumption that individuals from a same species carry specific DNA sequences that are different from those found in other species. Moreover, it should be realized that a continuous genetic variability does always exist among individuals of a species (Ahmed *et al*., 2011)

Currently a wide range of DNA based technology applications in medical and veterinary parasitology are growing rapidly whose specificity and sensitivity have gradually increased to detect parasites that were previously difficult to be diagnosed using conventional techniques. The accumulation of more information on the DNA sequences of parasites will reveal many more unique sequences which can be used for identification, diagnosis, molecular epidemiology, vaccine development and for studying of anti-parasitic drug receptors of parasites. The study of the molecular biology of anti-parasitic drug receptors, potential targets for chemotherapy, and the molecular genetics of drug resistance will allow molecular screens to be used in the search for new anti-parasitic drugs, improvements to existing chemotherapeutic families and better diagnosis and monitoring of drug resistance (Prichard and Tait, 2001).

For this molecular based diagnosis purposes, DNA is extremely useful molecule of the parasite; for one thing, DNA is an extremely stable and long-lived biological molecule that can be recovered from biological material, even after it exposed to extreme stress conditions (processed food products, coprolites, mummified plant tissues, blood stain, etc.). Second, DNA is found in all biological tissues or fluids having nucleated cells (or non-nucleated cells), enabling its analysis from almost all kinds of biological substrates (saliva, feces, plant seeds, milk, etc.). Third, DNA can provide more information than other molecular materials due to non-degeneracy of genetic code (Nsubuga *et al*., 2004)

These molecular applications have been fully applied in the fields of bacteriology, virology and mycology in developed developing countries. And even in our country, Ethiopia, molecular diagnosis of polio virus, measles virus, HIV, few veterinary important viruses and on some bacterial infections are on progress in different medical and veterinary laboratories. However, in our country the attempt of molecular diagnosis of intestinal parasitic infections are still far away from practical application (Hove, 2009).

To perform these molecular based diagnosis for intestinal parasites; isolation of representative genomic DNA in sufficient amount, free from inhibitory substances and high quality intact DNA from stool sample is the critical step. For this isolation of DNA there are several procedures and commercially available extraction kits have been used for stool sample. Among these extraction kits, the recently developed QIAamp DNA stool mini Kit and DNeasy blood and tissue kits are the most commonly used methods to extract DNA from organisms of stool and other biological samples. These extraction kits have their own strength and weakness to extract purified DNA of different parasites form fecal samples (Smith *et al*., 2011).

It is, therefore, essential to check for each target parasite whether the isolation of DNA procedure is capable of extracting the purified DNA from the stool sample or not. For example, PCR was successful for the detection of *T. trichiura* when DNA has been extracted from an adult worm. On the other hand, though, several rough treatments (e.g. sonification and microwaving) have been used; isolation of DNA from the *T. trichiura* eggs in stool was the most challenging procedure, as some of the extraction method used was ineffective to release the purified DNA from the eggs for PCR procedure. Thus, this study was aimed at comparing the capability of four DNA extraction methods to isolate purified DNA some of the intestinal parasite including the most difficult parasite *T. trichiura* from stool samples.

2.3 Basic aspects of PCR

Polymerase chain reaction (PCR) - based techniques have revolutionized many areas of study because the enzymatic amplification of DNA can be performed in vitro from small amounts of bilogical material. This is particularly relevant to parasitology because it is frequently impossible to obtain or isolate a sufficient amount of material from parasites at their different life-cycle stages by conventional analysis. These techniques provide alternative methods for detecting specific pathogens in different parasitological specimens. PCR-based methods have also been combined with other techniques such as restriction fragment length polymorphism (RFLP) or nested PCR to genotype and characterize organisms. The detection sensitivity of PCR is higher than that of light microscopy; therefore, this technique is useful for detecting a low number of parasites in parasitological samples. (Antinori *et al*., 2007).

This PCR -based method of diagnosis consist a design of a primer (small nucleotide sequence of 18-30 base length) that will originate an amplification product in the presence of specific DNA sequence from the target species. The process of designing species-specific primer is done from available genomic sequences of target parasite from gene bank central repository with the help of software that assist in primer designing. The amplified segments of DNA are separated in a conventional electrophoretic gel and visualized under UV trans-illuminator (Fantaccione *et al*., 2008)

By the help of these primers, the PCR makes it possible to perform selective amplification from complex genomes. This technique is based on the process of denaturing a double-stranded genomic DNA template using heat. Next, the temperature is lowered to ensure that primers can anneal to their complementary sequences into the template. Thus, the elongation of DNA template follows in both directions from the primer site by means of enzymatic catalysis with a thermo-stable DNA polymerase, generating double-stranded products (Gasser, 2006).

The specificity and sensitivity of this conventional PCR can be enhanced by performing a nested PCR, in which the target region is first amplified with an outer primer pair followed by a second amplification using an internal primer pair. Applying two rounds of PCR markedly enhances the specificity of PCR analysis because the inner primers only anneal if the proper template has been amplified with the outer primers. The chance of amplifying unspecific genomic regions is reduced with nested PCR as compared to conventional PCR since undesired sequences amplified in the first round of PCR are unlikely to contain a sequence to which the primers for the second amplification reaction will bind.

Chapter Three

3. Objectives

3.1 General objective

To compare and evaluate four different types of DNA extraction methods from stool samples for detection of intestinal parasites while optimizing basic molecular tools at the newly established Molecular Biology Laboratory in Jimma University.

3.2 Specific objectives

- ✓ To compare and evaluate four different DNA extraction methods from stool samples.
- ✓ To amplify and detect the DNA of the parasites namely (*A. lumbricoides, T. trichiura*, Hookworms and *G. duodenalis*
- ✓ To optimize a molecular biology tools that includes DNA amplification, electrophoresis, and detection of specific target DNA from intestinal parasites.
- ✓ To modify and prepare standard operating procedures (SOPs) of basic molecular techniques.

Chapter Four

4. Materials and Methods

4.1. Study area

The study was conducted at Jimma University Molecular Biology Laboratory, Jimma University, Jimma, Ethiopia.

4.2. Study design and period

From March to June 2014, four different DNA extraction kits for stool samples were comparatively evaluated with the 'manufacturers' protocol for duration of extraction, purity, yield and cost for detection of intestinal parasites DNA. In the meantime, optimizations of molecular tools were carried out for efficient use of those tools in our context.

4.3. Sample size

We have collected stool samples from schoolchildren of three elementary schools in Jimma Town (Hamle 19, Jirem N^{o}1 and Hermata) and examined microscopically until we get 10 positive samples with high EPG and 10 with low EPG for each of the three soil-transmitted helminthes (*A. lumbricoides*, *T. trichiura* and hookworms). Finally we have considered 10 stool samples negative for each of the above three parasites, altogether making the total sample sizes of 195 stool samples.

4.4. DNA extraction methods

1. QIAamp stool mini kit
    a) With bead beating
    b) without bead beating
2. DNeasy blood and tissue kit
    c) With bead beating
    d) without bead beating

4.5 Ethical Consideration

Ethical clearance was obtained from the Research Ethics Review Board of College of Public Health and Medical Sciences, Jimma University. An official letter was written to Jimma town educational office and selected elementary schools. The purpose of the study, procedure of the research and confidentiality letter was attached to each participant information sheet. The participants and/or their guardians were informed that they have full right to participate or not. The directors and teachers of the schools were informed about the purpose and procedures of the study. Additionally, oral assent was obtained from children. At the end of sample collection, all children positive for any helminths were treated for free with albendazole (400 mg) single oral dose according to WHO recommendations (WHO, 2006).

4.6. Data collection procedure

4.6.1 Microscopic examination of stool samples

A total of 195 stool samples were collected from schoolchildren attending three elementary schools (Jirem N^{o}1, Hamle 19 and Hermata) in Jimma Town where intestinal parasites are highly prevalent. Stool specimens were processed and examined by McMaster stool examination technique as described by (Levecke *et al*., 2011). Briefly, saturated sodium chloride (33.3%) was prepared 24 hr ahead of stool sample collection and stored at room temperature until it was used. The day stool samples were collected, 2gm of fresh stool specimen from each sample was weighed and suspended in 30ml of saturated sodium chloride floatation solution and emulsified well. The emulsified suspension was sieved using tea plastic strainer to another clean cup. The sieved stool suspension was homogenized by pouring from one cup to other repeatedly for 10 times and immediately filled into both side of McMaster slide using pasture pipette. Then, after waiting for 2 min to allow eggs to float, we examined and counted for STH eggs in both sides of the slide under x10 microscope objectives. Total counted eggs was multiplied by the dilution and volume factor of the slide (50) which finally gives us eggs per gram (EPG).

4.6.2 Stool samples selection and preservation for DNA extraction

Finally, we selected 60 positive samples based on the intensity (EPG) of parasites [30 with high EPG, which means 10 for each of the three species of STH (*A. lumbricoides, T. trichiura*, and

hookworms), and similarly 30 with low EPG] and as well 10 negative samples for any STH. Lastly, those selected stool samples based on microscopic results were processed and preserved in absolute (95%) ethanol for DNA extraction later. Shortly, 3gm of stool samples were weighed and transferred into 15ml tube and then 95% ethanol was added to each tube till it reaches 10ml mark. After being thoroughly emulsified, the samples were kept at room temperature until it was processed for DNA extraction.

4.6.3 DNA extraction steps and procedures for each kits

Two commercially available DNA extraction kits (QIAamp® DNA stool mini kit and DNeasy® blood and Tissue kit) with different in prices and protocols have been used (**Table 1**) for comparison purpose. In addition we have modified each DNA protocol with and without bead beating steps, making the comparison among 4 different extraction methods.

Table 1 Cost and protocol steps of commercially purchased DNA extraction kits

DNA extraction kits	№ of protocol steps	Cost for 50 samples/Birr	Origin
QIAamp® DNA stool mini kit	19	4284.00	Germany
DNeasy® Blood and Tissue kit	11	2790.00	Germany

4.6.3.1 Washing step (ethanol from stool sample)

This washing step was the same throughout DNA extraction for all methods. But, the volume of stool-ethanol suspension to be processed depends up on the protocol of each kit. For example, 666µl (0.2gm stool) for QIAamp® DNA stool mini kit and 333 µl (0.1gm stool) for DNeasy® blood and Tissue kit of an ethanol stool suspension was pipetted to a clean Eppendorf tube and centrifuged for 1min at 10,000 rpm to remove the supernatant ethanol and the pellet was washed by adding 1000µl phosphate buffer saline (PBS) and centrifuged for 1mn at 10,000 rpm. After whole supernatant was removed, 200µl PBS was added to the pellet and kept in -80^{o}c for 30 min to freeze. Immediately the sample was put in 100^{0}c heat block with shaking for 10 min.

4.6.3.2 QIAamp® DNA stool mini kit (QIAGEN Germany)

2ml of ASL buffer (cell lysis buffer) was added to each stool sample tube and thoroughly homogenized from which 1.6ml of stool lysate was added to a clean 2ml Eppendorf tube and

heated at 70^{o}c for 5 min. The lysate was mixed using high speed vortex and centrifuged at 10,000 rpm for 2 min. Then, 1.2ml of supernatant was pipetted to a new 2ml Eppendorf tube to which 1inhibitEX tablet was added to each sample tube and mixed by vortex continuously until the tablet was completely suspended. The added tablet supposed to be adsorbing the PCR inhibitors in its matrix. Stool and tablet suspension was centrifuged at 10,000 rpm for 3 min to pellet stool particles and inhibitors bound to inhibitEX tablet matrix. 15µl of proteinase k was pipetted to 1.5ml Eppendorf tube containing 200µl stool lysate supernatant and 200µl AL buffer (cell lysis buffer) and incubated for 10 min at 70^{o}c and 200µl of ethanol (96 – 100%) was added to the lysate. A complete lysate was added to QIAamp spine column and centrifuged until the lysate completely passed through spine column membrane enabling nucleic acid attached to the membrane.

Two different washing steps were carried out simultaneously; 500µl of AW1 and AW2 buffer was added to spine column and centrifuged at 10,000 rpm for 2 min. Finally, the repeatedly washed QIAamp spine column was transferred to new 1.5 Eppendorf tube and 200µl AE buffer was added to spine column to elute attached DNA to collecting tube and stored in -20^{o}c until they were used for PCR amplifications.

4.6.3.3 QIAamp® DNA stool mini kit (QIAGEN, Germany) with bead beating

This DNA extraction modification method was employed by following the same step with QIAamp® DNA stool mini kit. But, after the first ethanol-stool suspension washing steps, 200µl PBS was added to stool pellet and kept in -80^{o}c for 30 min. Then after, the suspension was defrosted at room temperature, the tubes were subjected to vigorously manual MagNa lyser green bead (Roche, Germany) beating using high speed vortex (3150 rpm) for 5 min. The rest steps, from the addition of 2ml of ASL buffer up to the storage of collected DNA in -20^{o}c were the same with the above QIAamp® DNA stool mini kit method.

4.6.3.4 DNeasy® Blood and tissue kit (QIAGEN, Germany)

The stool-ethanol suspension washing steps were the same with the above extraction methods. 200µl PBS was added to the stool pellet and kept in -80^{o}c for 30 min. immediately the sample was put in 100^{o}c heat block with shaking for 10 min.

In case of this kit, 200µl of tissue lysis buffer (ATL) containing 20µl of proteinase k was added to stool lysate and incubated at 55^{o}c for 2 hrs. in heat block. 400µl AL buffer (cell lysis buffer) was added to the lysate and incubated in heat block at 70^{o}c for 10 min. Then, 600µl of the mixture was pipetted to spine column supported with 2ml collecting tube and centrifuged until the lysate completely passed through spine column membrane enabling nucleic acid attached to the membrane of the column.

Two different washing steps were carried out simultaneously; 500µl of AW1 and AW2 buffers were added to spine column and centrifuged at 10,000 rpm for (1 min and 3 min) respectively. Finally, the repeatedly washed spine column was transferred to new 1.5 Eppendorf tube and 200µl AE buffer was added to spine column to elute attached DNA to collecting tube and stored in -20^{o}c until they were used for PCR amplifications.

4.6.3.5. DNeasy® Blood and Tissue extraction kit (QIAGEN, Germany) with bead beating

This DNA extraction method was done by the same step with DNeasy® Blood and Tissue kit except this procedure additionally involved the bead beating step. After the first ethanol - stool suspension washing steps, 200µl PBS was added to stool pellet and kept in -80^{o}c for 30 min and defrosted at room temperature, the tubes were subjected to vigorously manual MagNa lyser green bead (Roche, Germany) beating using high speed vortex (vortex mixer VM-300, Taiwan) with 3150 rpm for 5 min. The rest steps, from the addition of 400 µl of AL buffer up to the collection and storage of DNA were the same with the above DNeasy® Blood and Tissue extraction kit method.

4.6.4 PCR of parasites DNA

Master mixes were done for every PCR steps to amplify DNA of each parasite from each DNA extraction methods.

Table 2 Amounts of required constituents to make master mix for the amplification of target DNA of each parasite per sample

PCR products	**Amount/ µl**	
	T. trichiura, *A. lumbricoides*, hookworm	*G. duodenalis*
Primer 1(forward primer)	0.5	1.0
Primer 2 (reverse primer)	0.5	1.0
dNTPs	0.5	1.0
$MgCl_2$	1.0	2.0
Buffer	5.0	10.0
Distilled water	14.875	29.75
Taq polymerase enzyme	0.125	0.25
DNA sample	2.5	5.0
Total PCR volume	25	50.0

This mater mix steps requires very sensitive and critical attention to avoid risk of contamination and miss pipetting of constituents. The required amount of the constituents were added to one sterile Eppendorf tube (1.5ml) and well mixed to ensure even distribution of the constituents for each PCR sample tube. The required constituents and their amounts are described on **Table 2**. The well mixed mixture was proportionally distributed to each PCR sample tubes to which samples of DNA were added for amplification step.

A total of 30 extracted DNA samples for each STH (10 from each three infection level) and 10 qPCR positive DNA samples for *G. duodenalis* assemlages'A and B have been used to amplify their respective target DNA their respective species specific primers

Table 3 Conserved genes used to design species specific primers and their sequences at different stages of PCR (1st PCR and 2nd PCR/nested PCR) to amplify target DNA of each parasite species

Parasites	**PCR**	**Primer sequence**	**Target gene**	**Reference**
A. Lumbricodes	1st PCR	FW: 5' CCG GGC AAA AGT CGT AAC AA3' RV: 5'CAT ATA CAT CAT TAT TGT CAC G C3'	rDNA –ITS1	(George S.*et al*.), unpublished data
	2nd PCR	FW: 5' TCC GAA CGT GCA CAT AAG TAC 3' RV: 5' CAT ATA CAT CAT TAT TGT CAC G C3'		
T. trichiura	1st PCR	FW: 5' TGA CAA CGG TTA ACG GAG AAT 3' RV: 5' TCA AGT CGC CAA GGA CAC TC 3'	rDNA –ITS1	(George S.*et al*.), unpublished data
	2nd PCR	FW: 5' TGA CAA CGG TTA ACG GAG AAT 3' RV: 5' CGA CTC CTG CTT AGG ACG AC 3'		
Hook worm	1st PCR	FW: 5' GTT GGG AGT ATC RCC MMC CK 3' RV: 5' AAC AAC CCT GAA CCA GAC GT 3'	rDNA –ITS1	(George S.*et al*.), unpublished data
	2nd PCR	FW: 5' GTT GGG AGT ATC RCC MMC CK 3' RV: 5' ATG CGT TCA AAA TTT CAC CA 3'		
***G. duodenalis* Assemblage A**	1st PCR	FW: 5' CCC TTC ATC GGI GGT AAC TT 3' RV: 5' GTG GCC ACC ACI CCC GTG CC 3'	TPI	(Geurden *et al*., 2008)
	2nd PCR	FW: 5' CGC CGT ACA CCT GTC A 3' RV: 5' AGC AAT GAC AAC CTC CTT CC5 3'		

For these amplification steps, two pairs of primers have been used (**Table 3**). These primers were designed by using the sequence of their respective conserved region of rDNA- ITS1 for STH and TPI for *G. duodenalis*. The amplifications of target DNA of each parasite were initiated by the external target site amplification steps using the 1st pair primers (forward and revers primers) and followed by internal target site amplification steps using the 2nd pair primers (forward and reverse primer) for 35 cycles. This two round DNA amplification technique using two pairs of primers is called nested PCR which can increase the specificity of PCR.

Table 4 PCR protocols to amplify target DNA of each parasite

Parasites	**PCR steps**						**Reference**
	Denaturation		Annealing		*Elongation*		
	Temp (^{0}c)	Time	Temp (^{0}c)	Time	Temp (^{0}c)	Time	
T. trichiura	95	30sec	55.0	30sec	72	4min	(George S.et al.),
A. lumbricoides	95	30sec	54.3	30sec	72	5min	unpublished data
Hookworm	95	30sec	53.0	30sec	72	5min	
G. duodenalis	95	30sec	56.0	30sec	72	6min	(Levecke et al., 2009)

Next, according to the parasites respective optimized protocols, thermal cycler was programed for each parasites amplification steps. Then, amplification of target DNA of each parasite from each extraction methods was carried out turn by turn. The optimized protocols of DNA amplification for each parasite were summarized on **Table 4**

4.6.5 Electrophoresis and detection of amplified DNA bands for each parasites

After the end of each 2nd PCR amplification cycle (internal region target DNA amplification), 15µl of amplified DNA from each samples were loaded on sample wells formed by comb on 1.5% agarose gel (1.5 gm of agarose powder suspended in 100ml of x1TAE (Tris Acetic acid and EDTA)) and subjected to electrophoresis for 30 min. at 100 V. Then electrophoresed gels were stained with ethidium bromide-1 x TAE suspension for 15min, and the DNA bands were visualized on UV trans-illuminator from which the images of bands on the gel were captured using digital camera.

4.7 Data quality assurance

All the reagents used in each steps (like, NaCl saturated solution, ethidium bromide, 1xTAE buffer, agarose gel, and its running buffer, etc.) were prepared and stored according to SOPs. To avoid risk of contamination: sample processing and master mix preparation were done in their respective rooms; tables were cleaned daily using 70% ethanol; clean gowns were worn for each rooms; gloves were changed at the intervals of each steps and rooms; micropipettes were allocated for each rooms; sterilized micropipettes tips, calibrated Eppendorf micropipettes, and sterilized Eppendorf tubes (2ml and 1.5ml) were used for each steps. Moreover, collected data were handled both in hard copy and soft copy, checked for completeness with each date and authorized signature by of principal investigator and cross checked by supervisor (s). Finally, the captured images of the gels (DNA bands) were transferred to PC and labeled with all necessary information (ladder, size of DNA band, species of the parasites, etc.) and cross-checked with the label for the gel before the gel get discarded.

4.8 Data analysis

Descriptive data analyses were carried out to calculate measure of agreement "Kappa" using SPSS version 20. Finally the DNA extraction methods were evaluated based on their respective PCR results of kappa agreement with microscopically positive samples. Whereas, for microscopically negative samples, recovery rates of PCR from each DNA extraction methods were compared.

4.9 Interpretation of measure of agreement kappa

Kappa coefficient degree of agreement

- < 0 Less than chance agreement
- 0.01–0.20 Slight agreement
- 0.21– 0.40 Fair agreement
- 0.41–0.60 Moderate agreement
- 0.61–0.80 Substantial agreement
- 0.81–0.99 Almost perfect agreement (Viera and Garrett, 2005)

4.10 Strategy for dissemination and utilization of the study findings

The research report will be submitted to Jimma University SRP office and department of medical laboratory science and pathology, Jimma University. The result will be disclosed to a wider audience during public defense of this master thesis and the copy of the thesis including supplementary documents (like the SOPs, optimized protocols will be kept in the newly established molecular laboratory of Jimma University). Finally, we will make an effort to prepare a manuscript that will be published on a local or international journal.

Chapter Five

5. Results

5.1 Comparison of DNA extraction methods

Over all 70 DNA samples, 10 from each three infection level (high, low and Negative) EPG for each STH and 10 qPCR positive for *G. duodenalis* from different extraction methods were amplified by nested PCR

Table 5 nested PCR results versus different DNA extraction methods for each parasite with different infection level

DNA extraction kit		**PCR detection**							
		A. lumbricoides (10 for each infection level)			*T. trichiura* (10 for each infection level)			qPCR positive *of G. duodenalis*	**Total**
		High	Low	Neg	High	Low	Neg	10 samples	
QIAamp Stool mini kit	Bead	10	5	-	10	5	4	5	39
	No bead	9	1	-	6	2	2	2	22
DNeasy blood and tissue kit	Bead	10	9	1	10	10	6	8	54
	No bead	9	4	1	10	8	4	3	39

The PCR results of each parasite infection level by different extraction method were compared with parasite detection capacity of microscopy and evaluated using descriptive measure of agreement "kappa" **Table 5** shows the overall detection capacity of PCR of parasites by different extraction methods from three infection levels for STHs and qPCR positive samples for *G. duodenalis*.

5.1.1 Nested PCR of *A. lumbricoides*

The detection variability of DNA of A. lumbricoides by PCR among four types of DNA extraction methods was demonstrated

Table 6 Nested PCR results of *A. lumbricoides* from the samples of three infection levels (high, low and negative EPG) extracted by different DNA extraction methods

DNA extraction methods		**PCR for *A. lumbricoides*: 10 from each infection level**		
		High EPG	Low EPG	Negative
QIAamp stool mini kit	Bead	10	5	-
	No bead	9	1	-
DNeasy blood and tissue kit	Bead	10	9	1
	No bead	9	4	1

Based up on the results of PCR described in **Table 6**, the parasite DNA detection capability of PCR were compared among each extraction methods with microscopy. Based up on these data, the detection capability of PCR using all DNA extraction methods for high EPG samples shows almost perfect agreement (kappa coefficient >0.86). However, for microscopically low EPG samples, DNeasy® Blood and tissue kit with bead beating PCR result shows substantial agreement (kappa coefficient = 0.887) followed by QIAamp stool mini kit with bead beating shows moderate agreement (kappa coefficient = 0.60). For microscopically negative samples, though, the detection capability of PCR among different extraction methods is minimal; the extraction method which shows almost perfect agreement for low EPG samples detected 10% from microscopically negative samples.

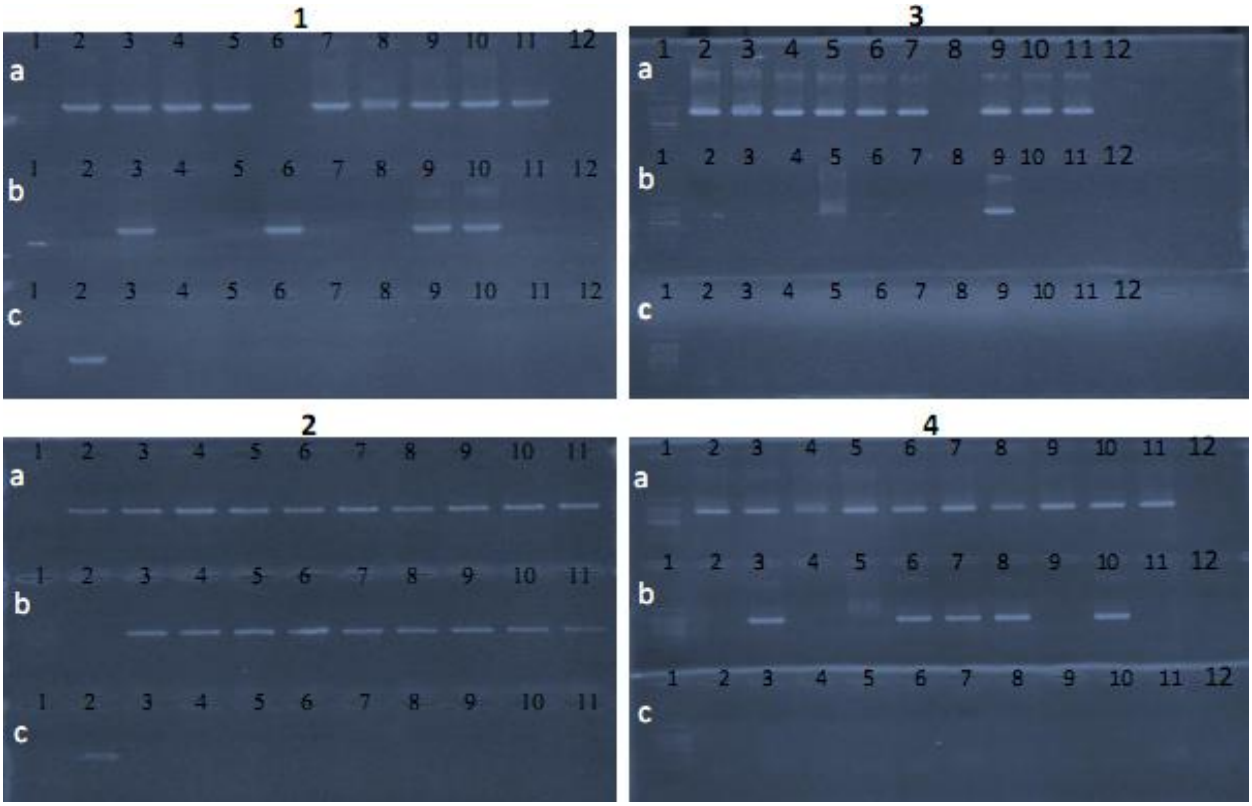

Figure 1 PCR - DNA band of *A. lumbricoides* on agarose gel electrophoresis using four different extraction methods.

1) DNA extracted by DNeasy® Blood and tissue kit, 2) DNA extracted by DNeasy® Blood and tissue kit with bead beating, 3) DNA extracted by QIAamp® DNA stool mini kit, 4) DNA extracted by QIAamp® DNA stool mini kit with beat beating. a), High EPG; b), low EPG and c) negative. For gel No 1, 3, 4: Lane 1, DNA ladder; lane 2-11, DNA samples; lane 12, Negative control. 2a): lane 1, DNA ladder; lane 2-11, DNA samples from high EPG 2b): lane 1, DNA ladder; lane 2-11, DNA samples from low EPG 2c): lane 1-10, DNA samples from low EPG; lane 11, Negative control.

Figure 1 illustrates, the similarity in detection of all DNA extraction methods for high EPG (label 1a, 2a, 3a and 4a) and Negative stool samples (label 1c, 2c, 3c, and 4c) for *A. lumbricoides* but difference in detection capacity for low EPG samples (label 1b, 2b, 3b, and 4b)

5.1.2 Nested PCR of *T. trichiura*

The detection variability of DNA of *T. trichiura* by PCR among four types of DNA extraction methods was demonstrated.

Table 7 Nested PCR results of *T. trichiura* from the samples of three infection levels (high, low and negative EPG) extracted by different DNA extraction methods

DNA extraction methods		PCR for *T. trichuira*: 10 samples for each infection level		
		High EPG	Low EPG	Negative
QIAamp stool mini kit	Bead	10	5	4
	No bead	6	2	2
DNeasy blood and tissue kit	Bead	10	10	6
	No bead	10	8	4

Based up on the data described in **Table 7**, QIAamp stool mini kit without bead beating shows moderate agreement for high EPG samples (kappa coefficient =0.486). The PCR result of the rest three extraction methods, show almost perfect agreement with microscopically high EPG samples (kappa coefficient 1.00). For microscopically low EPG samples, PCR results of DNeasy blood and tissue kit with bead beating shows almost perfect agreement (kappa coefficient =1.00) followed by DNeasy blood and tissue kit without bead beating (kappa coefficient = 0.857). Moreover, PCR of DNeasy blood and tissue kit with bead beating shows perfect agreement for high and low EPG samples detected 60% from microscopically negative samples.

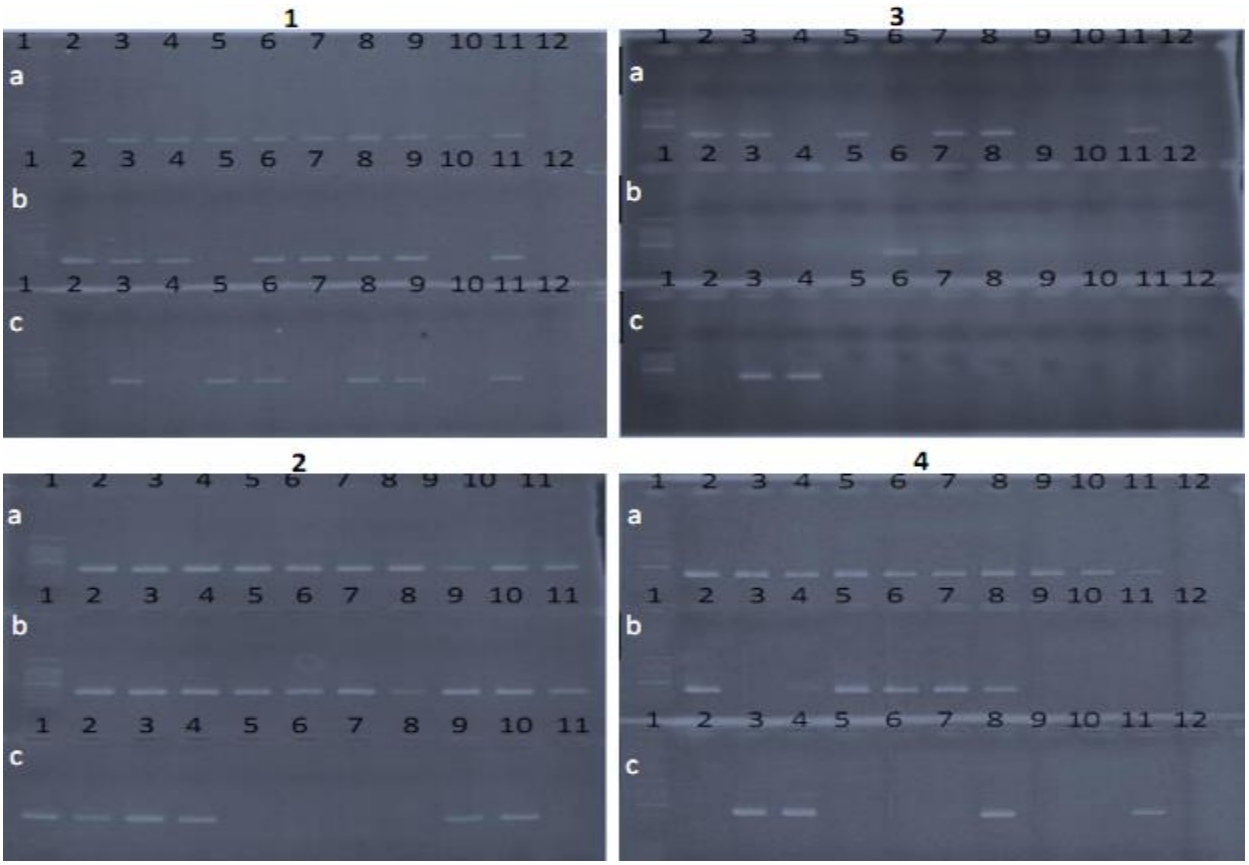

Figure 2 PCR - DNA band of *T. trichiura* on agarose gel electrophoresis using four different DNA extraction methods.

1) DNA extracted by DNeasy® Blood and tissue kit, 2) DNA extracted by DNeasy® Blood and tissue kit with bead beating, 3) DNA extracted by QIAamp® DNA stool mini kit, 4) DNA extracted by QIAamp® DNA stool mini kit with beat beating. a), High EPG; b), low EPG and c) negative. For gel No 1, 3, 4: Lane 1, DNA ladder; lane 2-11, DNA samples; lane 12, Negative control. 2a): lane 1, DNA ladder; lane 2-11, DNA samples from high EPG. 2b): lane 1, DNA ladder; lane 2-11, DNA samples from low EPG. 2c): lane 1-10, DNA samples from negative; lane 11, Negative control.

Figure 2 illustrates, the similarity in detection of PCR in three DNA extraction methods (DNeasy® Blood and tissue kit without and with bead beating, and QIAamp® DNA stool mini kit with bead beating) for high EPG (label 1a, 2a and 4a) and difference in detection for low EPG and negative stool samples on label 1(b, c), 2 (b, c), 3 (b, c), and 4 (b, C),

5.1.3 Nested PCR of hookworm

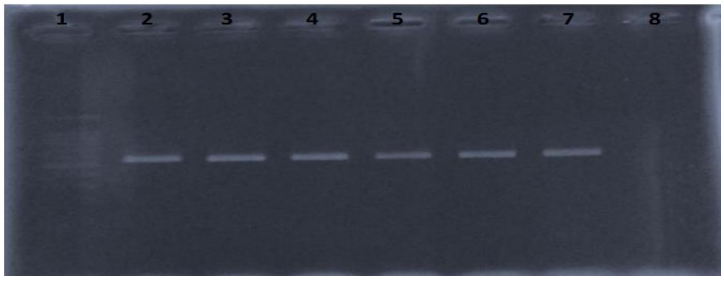

Fig. 3 PCR - DNA band of hookworm on agarose gel electrophoresis using DNeasy® blood and tissue kit DNA extraction method

Lane 1, DNA ladder; lane 2-6, DNA sample from high EPG of hookworm; lane 7, DNA of *N. americanus* (from Gent University, Belgium); lane 8, Negative control.

Figure 3 illustrates, PCR-DNA bands on lane № 2-6 extracted from single sample with high EPG (2000 eggs per gram of stool sample) and band on lane № 7 is DNA of *Necator americanus* as a positive control (obtained from the Department of Parasitology, Virology and Immunology, Gent University, Belgium).

5.1.4 Nested PCR of *G. duodenalis*: assemblage A

The detection variability of DNA of *G. duodenalis*: assemblage A by PCR among four types of DNA extraction methods was demonstrated

Table 8 PCR result of *G. duodenalis* (assemblages A) extracted by different extraction method versus qPCR

DNA extraction methods		PCR of G duodenalis: 10 qPCR positive samples
QIAamp stool mini kit	Bead	5
	No bead	2
DNeasy blood and tissue kit	Bead	8
	No bead	3

Based up on the data described on **Table 8**, PCR of different extraction methods gives us variable results for 10 qPCR positive samples of *G. duodenalis* assemblage A. according to these data, DNeasy® Blood and tissue kit with bead beating shows substantial agreement (kappa coefficient = 0.800) followed by QIAamp® DNA stool mini kit with bead beating (kappa

coefficient =0.500) which shows moderate agreement with qPCR. Whereas, QIAamp® DNA stool mini kit without bead beating shows slight agreement (kappa coefficient = 0.200).

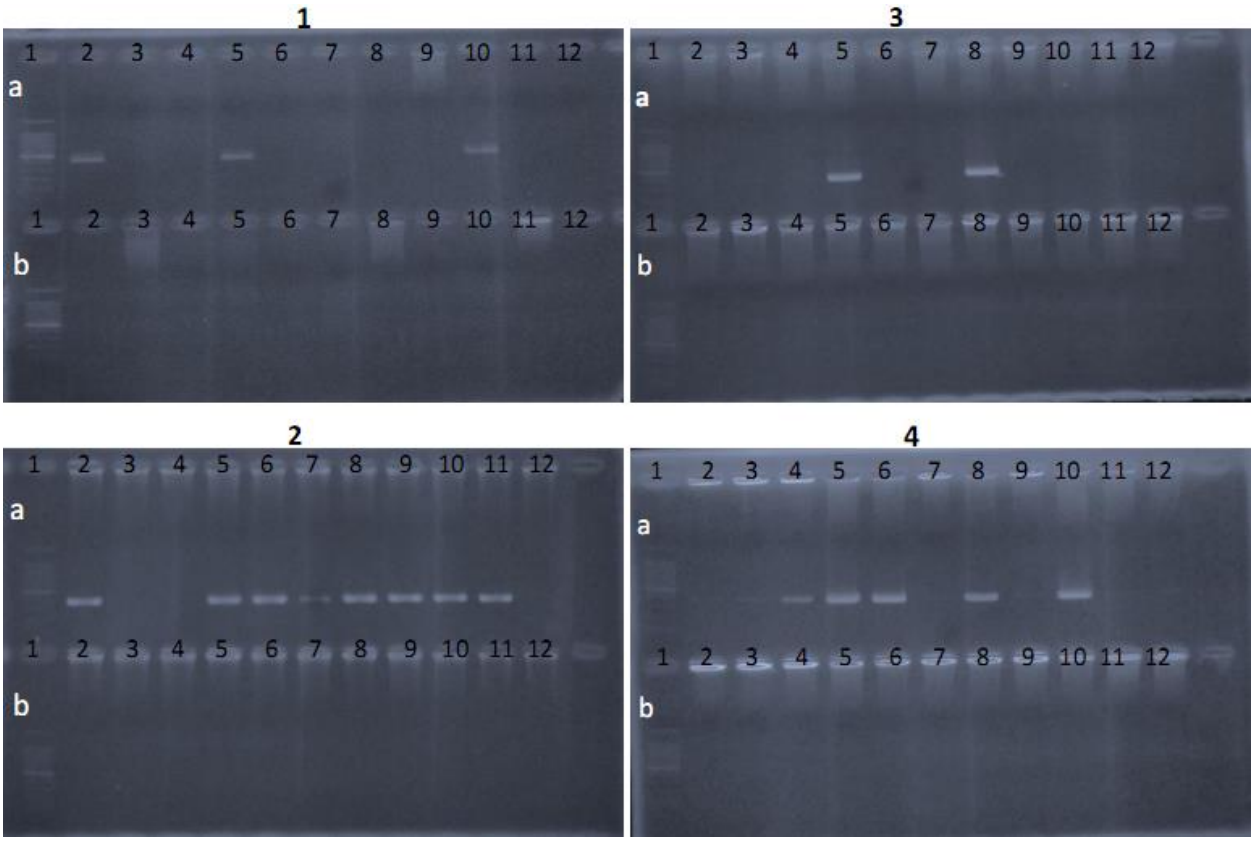

Figure4 PCR - DNA band of *G. duodenalis* assemblage A & B on agarose gel electrophoresis using four different DNA extraction methods

1) DNA extracted by DNeasy® Blood and tissue kit, 2) DNA extracted by DNeasy® Blood and tissue kit with bead beating, 3) DNA extracted by QIAamp® DNA stool mini kit, 4) DNA extracted by QIAamp® DNA stool mini kit with beat beating. a): Lane 1, DNA ladder; lane 2-11, DNA samples for Giardia assemblage A; lane 12, Negative control. b): lane 1, DNA ladder; lane 2-11, DNA samples for Giardia assemblage B; lane 12, Negative control. .

Figure 4 illustrates PCR-DNA bands of Giardia duodenalis assemblages A. (label 1a.) and no band for assemblage B (label b.) using four different DNA extraction methods.

Chapter Six

6. Discussion

Studies of intestinal parasitic infections have been performed conventionally by microscopic identification of eggs, larvae and adult parasites. However, the introduction of advanced molecular techniques, particularly the use of PCR- has revealed even the existence of more complex intestinal parasites strains and variants (Favier et al., 2002). To date, several studies have adopted different DNA extraction methods for some protozoan and very few helminthic intestinal parasites, such as QIAamp DNA stool Mini Kit and DNeasy blood and tissue kit with some other modifications (Smith *et al*., 2011).

The detection of intestinal parasites in stool specimens by PCR usually requires a highly sensitive DNA extraction method because of the presence of inhibitors in stool (Smith *et al*., 2011). So, it is crucial in parasitology to find a reproducible procedure for obtaining sufficient amounts of DNA, with no degradation and inhibitory products, from even a very small stool sample. There were some comparative studies of different DNA extraction methods for organisms, such as fungi, bacteria, viruses and protozoa (Yamada et al., 2002, Van Burik et al., 1998, Klerks et al., 2006). Although there are different DNA extraction methods for intestinal parasites, there is scarcity of studies that systematically compared the different DNA extraction methods from intestinal parasites. Hence, the present study was the first for comparison of DNA extraction methods for intestinal parasites in which four methods (QIAamp stool mini kit with and without bead beating and DNeasy blood and tissue with and without bead beating) with some additional modifications (like incubation in -80^0c) were compared and evaluated. Our aim was to find out a quick, easy to perform and cost effective intestinal parasites DNA extraction method for application in any molecular biology laboratory. In all methods, DNA were isolated and purified by spine column and AE buffer (QIAGEN®, Germany).

The present study showed that the detection limit varied between microscopically high EPG of STHs to no EPG, depending on the DNA extraction methods. For high EPG of two parasite species (*A. lumbricoides* and *T. trichiura*) all extraction methods have recovered sufficient yield of DNA for PCR amplification; and for low EPG stool samples of both parasite species, QIAamp stool mini kit with bead beating and DNeasy blood and tissues kit with bead beating yielded

sufficient DNA quantity for PCR amplification. Whereas, for no EPG stool samples of intestinal parasite species (*T. trichiura*), DNeasy blood and tissues kit with bead beating was the most DNA extraction method yielding sufficient DNA for PCR- DNA amplification. Although the QIAamp stool mini kit has been widely used as a standard method for microorganisms DNA extraction from stool samples, our data showed that, it was less effective method and could detect only when stool samples contain more than 5000 EPG for *A. lumbricoides*. This less effectivity might be due to incomplete removal of inhibitors during DNA extraction.

The quality and yield of DNA recovered using different extraction methods was evaluated using PCR as shown in **Figure 6, 7** and **8** with the expected band size for each parasite species. Although, PCR is not a quantitative method, the PCR products and their respective intensities were qualitatively evaluated in a descriptive manner. The specificity of the PCR for each parasite species were enhanced by running Nested PCR in which the target region is first amplified with an outer primer pair followed by a second amplification using an internal primer pair. Applying two rounds of PCR markedly enhances the specificity of PCR analysis because the inner primers only anneal if the proper template has been amplified with the outer primers. The chance of amplifying unspecific genomic regions is reduced with nested PCR as compared to conventional PCR since undesired sequences amplified in the first round of PCR are not likely to contain a sequence to which the primers for the second amplification reaction will bind (McNAMARA *et al*., 2006).

Although, QIAamp stool mini kit without bead beating DNA extraction method has been found as one of the best method in some comparative studies of fungi and some intestinal protozoa, (Van Burik *et al.*, 1998), in this study, it does not seem a proper method for extracting DNA from intestinal parasites. DNeasy blood and tissue with bead beating is relatively a new method used for intestinal parasites (Rahimi *et al*., 2007) which gave us high DNA yield and PCR bands in our study. However, since an automatic bead beater is not available in our molecular biology laboratory, the manual hand held vortex bead beating method may has some limitations in which each sample should be bead beaten using vortex mixer separately, and consequently, it is difficult to beat samples uniformly, and takes more time and laborious. Overall, considering different aspects of suitability for a DNA extraction method such as PCR band quality, cost effectiveness and simplicity, our result shows DNeasy blood and tissue with bead beating was

the most suitable methods for the DNA extraction of four intestinal parasites species we have examined. So, this DNA extraction method can be recommended for future studies based on PCR amplification of ITS1-rDNA or probably other suitable target genes of intestinal parasites from fecal sample.

For all DNA extraction methods we have compared, especially for QIAamp stool mini kit, the extraction protocol involved several steps; thus, limited numbers of specimens could be tested each time. Though, single-step PCR method is convenient and less time consuming, We evaluated the extraction methods using nested PCR methods for the detection of intestinal parasites in stool specimens to enhance the specificity of the PCR to avoid the non-specific binding of the primers to many different types of DNA fragments found in stool specimens which are from (human, nutrition, pathogenic and non-pathogenic organisms) origin DNA.

The PCR of the DNA extraction method, DNeasy blood and tissue kit with bead beating which showed a perfect agreement (kappa =1.0) with high and low infection level, detected 60% of *T. trichiura* microscopically negative samples. This can be happened due to less sensitivity of microscopy.

Thus, to extract DNA of intestinal parasites from stool sample, DNeasy® Blood and tissue kit with bead beating is the most recommended extraction methods to be used for low infection intensity and microscopically negative samples. Moreover, this extraction method is preferred than the other kit in less cost and time to extract DNA.

In other side, though, we have tried many times by modifying every aspect of DNA amplification steps; our PCR couldn't detect DNA of hookworms from all categorized ethanol preserved stool samples (high EPG, low EPG and no EPG). At first, this has presented a challenge to figure out the reasons for the failure. We have carefully checked for all possible underlying factors contributing for the failures of PCR to detect hook worm DNA like specificity of the primers, effect of chemical preservation etc. Finally, we have noticed that preserving sample with ethanol has effect on the viability of hookworm unlike other parasites as confirmed by re-examining the preserved stool sample microscopically. Moreover, we have further confirmed this effect by extracting hookworm DNA from fresh unpreserved hookworm positive stool samples.

Limitations of the study

There was no Magna bead beater (automatic bead beater) so, we have used hand held vortex for bead beating, and also there was no gel scanner so, we have captured the gel image directly from UV trans-illuminator using digital camera which is difficult to focus and to detect faint DNA bands that may lead to false negative results.

Conclusion

The molecular tools and some basic techniques in newly established molecular biology laboratory, Jimma University, is now optimized and became functional for some intestinal parasites (STH and *Giardia duodenals*). In this comparison, the DNeasy blood and tissue kit with bead beating appears to be the most sensitive DNA extraction method in terms of cost, easy to perform and DNA yield for PCR from very few eggs of parasites even unable to be detected microscopically to high EPG and also for *G. duodenalis*. So, the present study provides useful information on the extraction methods for intestinal parasites DNA from fecal sample which was very challenging in many molecular biology laboratories failing to purify DNA from inhibitory substances and yielding high quality DNA for PCR.

Recommendations

We recommend for scientific communities interested to conduct their research work on intestinal parasites at molecular level, DNeasy blood and tissue kit (QIAGEN®, Germany) modified with bead beating and -80^{O}c is the efficient, sensitive and cost effective DNA extraction method for intestinal parasites especially for STH and *G. duodenalis*.

Acknowledgments

- I would like to extend my deepest appreciation to my advisors: Mr. Zeleke Mekonnen, Mr. Abdissa Bruksew and Dr. Bruno Levecke for their unreserved support and encouragement during development of proposal, data collection and finalizing the thesis.
- I would like to pass my appreciations to Medical Laboratory Sciences and pathology department for giving me a chance to carry out this MSc study.
- I would like to pass my thanks to Prof. Joszef Vercruysse and his laboratory staffs for giving me a hand on molecular biology techniques at Ghent University, Belgium
- Again, I would like to show my gratitude to Jimma University for giving me the chance to undertake this MSc study and providing me financial support to carry out this research.
- Finally, I would like pass my gratitude to VLIR–IUC-JU research project for the effort to establish Molecular Biology laboratory and supporting me by providing me necessary reagents, primers and kits.
- Last but not least, my deepest heartfelt thank goes to my beloved wife Jalene Benti for her unreserved support in all aspects of my life including my educational carrier.

References

AHMED, M., SINGH, M., BERA, A., BANDYOPADHYAY, S. & BHATTACHARYA, D. 2011. Molecular basis for identification of species/isolates of gastrointestinal nematode parasites. *Asian Pacific journal of tropical medicine,* 4**,** 589-593.

ANTINORI, S., CALATTINI, S., LONGHI, E., BESTETTI, G., PIOLINI, R., MAGNI, C., ORLANDO, G., GRAMICCIA, M., ACQUAVIVA, V. & FOSCHI, A. 2007. Clinical use of polymerase chain reaction performed on peripheral blood and bone marrow samples for the diagnosis and monitoring of visceral leishmaniasis in HIV-infected and HIV-uninfected patients: a single-center, 8-year experience in Italy and review of the literature. *Clinical infectious diseases,* 44**,** 1602-1610.

BETHONY, J., BROOKER, S., ALBONICO, M., GEIGER, S. M., LOUKAS, A., DIEMERT, D. & HOTEZ, P. J. 2006. Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. *The Lancet,* 367**,** 1521-1532.

CHALMERS, R. M., FERGUSON, C., CACCIÒ, S., GASSER, R. B., ABS EL-OSTA, Y. G., HEIJNEN, L., XIAO, L., ELWIN, K., HADFIELD, S. & SINCLAIR, M. 2005. Direct comparison of selected methods for genetic categorisation of< i> Cryptosporidium parvum</i> and< i> Cryptosporidium hominis</i> species. *International journal for parasitology,* 35**,** 397-410.

ESPY, M., UHL, J., SLOAN, L., BUCKWALTER, S., JONES, M., VETTER, E., YAO, J., WENGENACK, N., ROSENBLATT, J. & COCKERILL, F. 2006. Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clinical microbiology reviews,* 19**,** 165-256.

FANTACCIONE, S., WOODROW, P. & PONTECORVO, G. 2008. Molecular authentication of three Italian melon accessions by ARMS-PCR and ITS1 (internal transcribed spacer 1) secondary structure prediction. *Bioinformation,* 2**,** 311.

FAVIER, C. F., VAUGHAN, E. E., DE VOS, W. M. & AKKERMANS, A. D. 2002. Molecular monitoring of succession of bacterial communities in human neonates. *Applied and Environmental Microbiology,* 68**,** 219-226.

GASSER, R. B. 2006. Molecular tools—advances, opportunities and prospects. *Veterinary parasitology,* 136**,** 69-89.

GEURDEN, T., GELDHOF, P., LEVECKE, B., MARTENS, C., BERKVENS, D., CASAERT, S., VERCRUYSSE, J. & CLAEREBOUT, E. 2008. Mixed< i> Giardia duodenalis</i> assemblage A and E infections in calves. *International journal for parasitology,* 38**,** 259-264.

GLINZ, D., SILUÉ, K. D., KNOPP, S., LOHOURIGNON, L. K., YAO, K. P., STEINMANN, P., RINALDI, L., CRINGOLI, G., N'GORAN, E. K. & UTZINGER, J. 2010. Comparing diagnostic accuracy of Kato-Katz, Koga agar plate, ether-concentration, and FLOTAC for Schistosoma mansoni and soil-transmitted helminths. *PLoS neglected tropical diseases,* 4**,** e754.

GUPTA, S., NARANG, S., NUNAVATH, V. & SINGH, S. 2008. Chronic diarrhoea in HIV patients: prevalence of coccidian parasites. *Indian journal of medical microbiology,* 26**,** 172.

HALL, A., HEWITT, G., TUFFREY, V. & DE SILVA, N. 2008. A review and meta-analysis of the impact of intestinal worms on child growth and nutrition. *Maternal & child nutrition,* 4**,** 118-236.

HARHAY, M. O., HORTON, J. & OLLIARO, P. L. 2010. Epidemiology and control of human gastrointestinal parasites in children. *Expert review of anti-infective therapy,* 8**,** 219-234.

HOTEZ, P. J., BRINDLEY, P. J., BETHONY, J. M., KING, C. H., PEARCE, E. J. & JACOBSON, J. 2008. Helminth infections: the great neglected tropical diseases. *The Journal of clinical investigation,* 118**,** 1311.

HOVE, R. J. T. 2009. *Molecular detection of intestinal parasites for clinical diagnosis and epidemiology.* Department of Parasitology/Epi-Diag, Faculty of Medicine, Leiden University Medical Center (LUMC), Leiden University.

KLERKS, M., VAN BRUGGEN, A., ZIJLSTRA, C. & DONNIKOV, M. 2006. Comparison of methods of extracting Salmonella enterica serovar enteritidis DNA from environmental substrates and quantification of organisms by using a general internal procedural control. *Applied and Environmental Microbiology,* 72**,** 3879-3886.

LANGKJÆR, R. B., VIGRE, H., ENEMARK, H. L. & MADDOX-HYTTEL, C. 2007. Molecular and phylogenetic characterization of Cryptosporidium and Giardia from pigs and cattle in Denmark. *Parasitology,* 134**,** 339.

LEVECKE, B., GELDHOF, P., CLAEREBOUT, E., DORNY, P., VERCAMMEN, F., CACCIÒ, S. M., VERCRUYSSE, J. & GEURDEN, T. 2009. Molecular characterisation of< i> Giardia</i>< i> duodenalis</i> in captive non-human primates reveals mixed assemblage A and B infections and novel polymorphisms. *International journal for parasitology,* 39**,** 1595-1601.

LEVECKE, B., RINALDI, L., CHARLIER, J., MAURELLI, M. P., MORGOGLIONE, M. E., VERCRUYSSE, J. & CRINGOLI, G. 2011. Monitoring drug efficacy against gastrointestinal nematodes when faecal egg counts are low: do the analytic sensitivity and the formula matter? *Parasitology research,* 109**,** 953-957.

LI, D., CRAIK, S. A., SMITH, D. W. & BELOSEVIC, M. 2009. Infectivity of< i> Giardia lamblia</i> cysts obtained from wastewater treated with ultraviolet light. *Water research,* 43**,** 3037-3046.

MCCOY, D., KEMBHAVI, G., PATEL, J. & LUINTEL, A. 2009. The Bill & Melinda Gates Foundation's grant-making programme for global health. *The Lancet,* 373**,** 1645-1653.

MCNAMARA, D. T., KASEHAGEN, L. J., GRIMBERG, B. T., COLE-TOBIAN, J., COLLINS, W. E. & ZIMMERMAN, P. A. 2006. Diagnosing infection levels of four human malaria parasite species by a polymerase chain reaction/ligase detection reaction fluorescent microsphere-based assay. *The American journal of tropical medicine and hygiene,* 74**,** 413-421.

MOCHIZUKI, R., ENDOH, D., ONUMA, M. & FUKUMOTO, S. I. 2006. PCR-based species-specific amplification of ITS of Mecistocirrus digitatus and its application in identification of GI nematode eggs in bovine faeces. *The Journal of veterinary medical science/the Japanese Society of Veterinary Science,* 68**,** 345-351.

NDAO, M. 2009. Diagnosis of parasitic diseases: old and new approaches. *Interdisciplinary perspectives on infectious diseases,* 2009.

NG, C. T., GILCHRIST, C. A., LANE, A., ROY, S., HAQUE, R. & HOUPT, E. R. 2005. Multiplex real-time PCR assay using Scorpion probes and DNA capture for genotype-specific detection of Giardia lamblia on fecal samples. *Journal of clinical microbiology,* 43**,** 1256-1260.

NSUBUGA, A., ROBBINS, M., ROEDER, A., MORIN, P., BOESCH, C. & VIGILANT, L. 2004. Factors affecting the amount of genomic DNA extracted from ape faeces and the identification of an improved sample storage method. *Molecular Ecology,* 13**,** 2089-2094.

OKYAY, P., ERTUG, S., GULTEKIN, B., ONEN, O. & BESER, E. 2004. Intestinal parasites prevalence and related factors in school children, a western city sample-Turkey. *BMC Public Health,* 4**,** 64.

OLLIARO, P., SEILER, J., KUESEL, A., HORTON, J., CLARK, J. N., DON, R. & KEISER, J. 2011. Potential drug development candidates for human soil-transmitted helminthiases. *PLoS neglected tropical diseases,* 5**,** e1138.

PALMER, C. S., TRAUB, R. J., ROBERTSON, I. D., HOBBS, R. P., ELLIOT, A., WHILE, L., REES, R. & THOMPSON, R. 2007. The veterinary and public health significance of hookworm in dogs and cats in Australia and the status of< i> A. ceylanicum</i>. *Veterinary parasitology,* 145**,** 304-313.

PRICHARD, R. & TAIT, A. 2001. The role of molecular biology in veterinary parasitology. *Veterinary parasitology,* 98**,** 169-194.

RAHIMI, H., KIA, E., MIRHENDI, S., TALEBI, A., HARANDI, M. F., JALALI-ZAND, N. & ROKNI, M. 2007. A new primer pair in ITS1 region for molecular studies on Echinococcus granulosus. *Iranian Journal of Public Health,* 36**,** 45-49.

ROY, S., KABIR, M., MONDAL, D., ALI, I. K. M., PETRI, W. A. & HAQUE, R. 2005. Real-time-PCR assay for diagnosis of Entamoeba histolytica infection. *Journal of clinical microbiology,* 43**,** 2168-2172.

SAVIOLI, L., ALBONICO, M., ENGELS, D. & MONTRESOR, A. 2004. Progress in the prevention and control of schistosomiasis and soil-transmitted helminthiasis. *Parasitology international,* 53**,** 103-113.

SMITH, B., LI, N., ANDERSEN, A. S., SLOTVED, H. C. & KROGFELT, K. A. 2011. Optimising bacterial DNA extraction from faecal samples: comparison of three methods. *The open microbiology journal,* 5**,** 14.

TRAUB, R. J., INPANKAEW, T., SUTTHIKORNCHAI, C., SUKTHANA, Y. & THOMPSON, R. 2008. PCR-based coprodiagnostic tools reveal dogs as reservoirs of zoonotic ancylostomiasis caused by< i> Ancylostoma ceylanicum</i> in temple communities in Bangkok. *Veterinary parasitology,* 155**,** 67-73.

TRAUB, R. J., ROBERTSON, I. D., IRWIN, P. J., MENCKE, N. & THOMPSON, R. 2005. Canine gastrointestinal parasitic zoonoses in India. *Trends in parasitology,* 21**,** 42-48.

UTZINGER, J., MÜLLER, I., VOUNATSOU, P., SINGER, B. H., N'GORAN, E. K. & TANNER, M. 2003. Random spatial distribution of Schistosoma mansoni and hookworm infections among school children within a single village. *Journal of Parasitology,* 89**,** 686-692.

UTZINGER, J., RASO, G., BROOKER, S., DE SAVIGNY, D., TANNER, M., ORNBJERG, N., SINGER, B. & N'GORAN, E. 2009. Schistosomiasis and neglected tropical diseases: towards integrated and sustainable control and a word of caution. *Parasitology,* 136**,** 1859.

VAN BURIK, J.-A., SCHRECKHISE, R., WHITE, T., BOWDEN, R. & MYERSON, D. 1998. Comparison of six extraction techniques for isolation of DNA from filamentous fungi. *Medical mycology,* 36**,** 299-303.

VIERA, A. J. & GARRETT, J. M. 2005. Understanding interobserver agreement: the kappa statistic. *Fam Med,* 37**,** 360-363.

YAMADA, Y., MAKIMURA, K., MERHENDI, H., UEDA, K., NISHIYAMA, Y., YAMAGUCHI, H. & OSUMI, M. 2002. Comparison of different methods for extraction of mitochondrial DNA from human pathogenic yeasts. *Japanese journal of infectious diseases,* 55**,** 122-125.

Annexes

Annex 1 Optimization of molecular tools

All basic molecular tools for DNA amplification, electrophoresis and detection of DNA bands have already optimized by extracting intestinal parasites target DNA using those commercially purchased DNA extraction kits (QIAamp® DNA stool mini kit and DNeasy® Blood and tissue kit (QIAGEN, Germany) modified with the combination of MagNA lyzer green beads (Roche, Germany), -80^{o}c freezer, heat block thermo mixer and vortex for bead beating techniques. To optimize molecular tools, first we have used directly the protocols of DNA amplification which other studies previously have used successfully. The protocols to be optimized were; timing and temperature of pre- denaturation, cycles denaturation, primers annealing and sequence extension for each intestinal parasites species target DNA.

For *A. lumbricoides* target DNA - ITS1 amplification, the protocol we have used directly from other study have already gave us exactly the expected DNA band size (850bps) from PCR product using a minimum PCR volume (25µl). For the rest intestinal parasite species target DNA amplification, the protocols what we have used directly from other studies were not gave us the exact and expected band size with our new molecular tools and laboratory set up even there was no any visible DNA band for some parasite species. For those parasite species there was no visible and unexpected DNA band size were appeared, we had been modifying the timing and temperature of the amplification steps for our thermo cycler program until they gave us the visible and expected DNA band size for each parasite species.

So, what we have described on methodology part; the protocols for amplification of different parasite species target DNA, are the optimized and modified for our molecular biology tools and laboratory settings in which we have got visible and expected band size for the respective parasite species

Annex 2 stool samples categorized in to three infection level from which DNA was extracted

code No	*A. lumbricoides* EPG	code No	*T. trichuira* EPG	code No	Hook worm EPG
38	56500	6	4450	86	900
6	40350	38	4300	18	900
13	35900	1	2150	87	750
1	29900	84	1900	20	650
54	20550	13	1700	38	500
23	19350	65	1400	61	500
68	18750	16	1250	88	450
16	18600	15	1250	12	450
55	15900	36	1100	1	400
48	13500	60	1050	81	350
15	4850	14	250	48	300
36	4050	88	250	84	200
22	3500	40	250	69	200
60	3400	30	200	58	200
14	3150	48	150	82	200
43	2800	64	150	83	200
44	2650	68	100	22	100
65	2200	50	100	7	50
7	2200	55	50	50	50
30	250	42	50	55	50
84	0	54	0	6	0
20	0	12	0	13	0
86	0	69	0	65	0
88	0	22	0	16	0
40	0	43	0	15	0
64	0	18	0	36	0
50	0	87	0	60	0
42	0	61	0	44	0
18	0	81	0	23	0
87	0	58	0	14	0

key: grey = high EPG, blue = low EPG, green = No EPG

Annex 3. Materials

1.1 Basic equipment and materials in molecular biology laboratory:

- Equipment for DNA extraction from fecal sample: thermo mixer, liquid nitrogen, micro centrifuge(2ml capacity), different size capacity micropipettes, eppendorf tubes(1.5ml and 2ml)
- Equipment for DNA amplification: thermal cycler, ice cube machine, PCR tubes,
- Equipment for electrophoresis: electrophoresis unit, agarose gel cast, TAE ingredients(tris, acetic acid and EDTA)
- Equipment for visualization of DNA on gel: gel scanner/UV ray, ethidium bromide

1.2 Materials required for this particular study:

- Extraction kits (stool mini kit and tissue kit)
- Primers for A. lumbricoides, A. duodenale, N. americanus, Trichuristrichiura, G. duodenalis (including for its strains), E. histolytica/dispar, cryptosporidium spp.
- dNTPs
- PCR buffer
- Ladder (100bp)
- Loading dye
- MgCl
- Positive controls(known DNA for an organisms)
- Deionized water
- Gloves

Annex 4. Laboratory procedure

4.1 DNeasy blood and tissue kit DNA extraction procedure

1. 0.1 gram faeces is suspended in 200 µl of PBS with 2% pvpp (polyvinylpolypyrolidone)**
2. Freeze sample overnight at -20 °C.
3. Heat faeces-suspension for 10 minutes in heatblock at 100 °C.
4. Add an equal volume of Tissue Lysis Buffer (ATL) containing 20 µl proteinase K (so that is 180 µl of ATL and 20 µl of prot K from the kit) solution to the sample (vortex). Incubate 2 hours or overnight at 55 °C in heatblock (vortex after 1hr briefly).
5. Add 400 µl AL Buffer (with spike) to 400 µl sample, mix thoroughly by vortexing, and incubate at 70 °C for 10 minutes.
6. Centrifuge 30 seconds full speed and transfer supernatant to an eppendorf containing 400 µl of ethanol (96-100 %), and mix thoroughly by vortexing (short spin)
7. Number a spin column in a 2 ml collection tube. Carefully apply 600 µl of the mixture from step 6 to the spin column without moistening the rim, close the cap and centrifuge at 10000 rpm. for 1 minute.
8. Place spin column in a clean 2 ml collection tube and repeat with the rest of the mixture from step 6
9. Place the spin column in a clean 2 ml collection tube.
10. Carefully open the spin column and add 500 µl of AW1 buffer. Centrifuge at 10000 rpm for 1 minute.
11. Empty the collection tube and add 500 µl of AW2 buffer. Centrifuge 3 minutes, 1minute at 10.000 rpm and 2 minutes at full speed.
12. Place the spin column in a numbered, clean 1.5 ml microfuge tube (with lit).
13. Carefully open the spin column, elute the DNA with 200 µl AE buffer, leave the buffer on the filter for 1 minute and centrifuge at 8000 rpm for 1 minute.

4.2 QIAamp DNA stool mini kit DNA extraction procedure

1. Weigh 180–220 mg stool in a 2 ml micro-centrifuge tube (not provided) and place the tube on ice. This protocol is optimized for use with 180–220 mg stool but can also be used with smaller amounts. There is no need to reduce the amounts of buffers or InhibitEX matrix when using smaller amounts of stool. If the sample is liquid, pipet 200 µl into the micro

centrifuge tube. Cut the end of the pipet tip to make pipetting easier. After addition of Buffer ASL, all following steps can be performed at room temperature (15–25°C).

2. Add 1.4 ml Buffer ASL to each stool sample. Vortex continuously for 1 min or until the stool sample is thoroughly homogenized.
3. Heat the suspension for 5 min at 70°C. This heating step increases total DNA yield 3- to 5-fold and helps to lyse bacteria and other parasites. The lysis temperature can be increased to 95°C for cells that are difficult to lyse (such as Gram-positive bacteria).
4. Vortex for 15 s and centrifuge sample at full speed for 1 min to pellet stool particles.
5. Pipet 1.2 ml of the supernatant into a new 2 ml micro centrifuge tube (not provided) and discard the pellet.
6. Add 1 InhibitEX Tablet to each sample and vortex immediately and continuously for 1 min or until the tablet is completely suspended. Incubate suspension for 1 min at room temperature to allow inhibitors to adsorb to the InhibitEX matrix.
7. Centrifuge sample at full speed for 3 min to pellet inhibitors bound to InhibitEX matrix.
8. Pipet all the supernatant into a new 1.5 ml micro centrifuge tube (not provided) and discard the pellet. Centrifuge the sample at full speed for 3 min. Transfer of small quantities of pelleted material from step 7 will not affect the procedure.
9. Pipet 15 µl proteinase K into a new 1.5 ml micro centrifuge tube (not provided).
10. Pipet 200 µl supernatant from step 8 into the 1.5 ml micro centrifuge tube containing proteinase K.
11. Add 200 µl Buffer AL and vortex for 15 s.
12. Incubate at 70°C for 10 min. Centrifuge briefly to remove drops from the inside of the tube lid (optional).
13. Add 200 µl of ethanol (96–100%) to the lysate, and mix by vortexing. Centrifuge briefly to remove drops from the inside of the tube lid (optional).
14. Label the lid of a new QIAamp spin column placed in a 2 ml collection tube. Carefully apply the complete lysate from step 13 to the QIAamp spin column without moistening the rim. Close the cap and centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2 ml collection tube, and discard the tube containing the filtrate.Close each spin column to avoid aerosol formation during centrifugation. If the lysate has not

completely passed through the column after centrifugation, centrifuge again until the QIAamp spin column is empty.

15. Carefully open the QIAamp spin column and add 500 µl Buffer AW1. Close the cap and centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2 ml collection tube, and discard the collection tube containing the filtrate.
16. Carefully open the QIAamp spin column and add 500 µl Buffer AW2. Close the cap and centrifuge at full speed for 3 min. discard the collection tube containing the filtrate.
17. Recommended: Place the QIAamp spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min. This step helps to eliminate the chance of possible Buffer AW2 carryover.
18. Transfer the QIAamp spin column into a new, labeled 1.5 ml micro centrifuge tube (not provided). Carefully open the QIAamp spin column and pipet 200 µl Buffer AE directly onto the QIAamp membrane. Close the cap and incubate for 1 min at room temperature, then centrifuge at full speed for 1 min to elute DNA.

Annex 5. SOP for DOPPIO Thermal Cycler:

Fig.5. Home page of thermal cycler

1. Programming thermal cycler for amplification

Programming is simply a matter of "adding" command elements required from a command list into the program window. This opens a window where you can define further command-specific settings such as time, temperature, lid temperature, etc. In this way, you can create your program according to the planned programming sequence.

1.1. To generate new program, click on the home page **"new program"** this will open compose a program screen to write your program. This screen consists of a blank area in which commands are entered to create the program **(Figure.3).**

Fig.6. compose and edit program or command list window

1.2. To begin writing the program, click on the list of available commands:

1.3. Select the command that you require and this will open a new window for the selected command

1.4. Enter your settings in the relevant fields of the selected command.

1.5. Confirm your settings by clicking OK. This will return you to the Compose a Program window

1.6. To add another command to your program, click on the next list available command and repeat steps 1.2 – 1.5 for each required command.

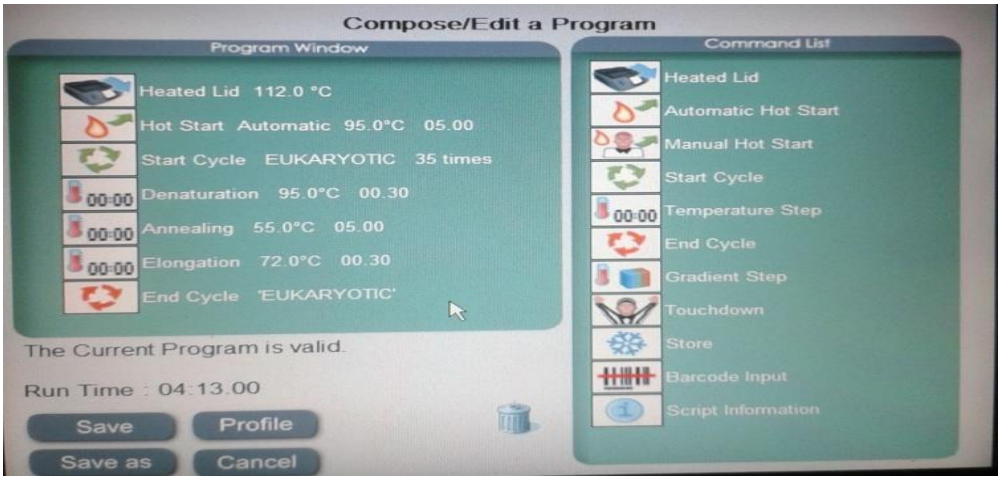

Fig.7. required program composed.

1.7. Commands may be edited or deleted by selecting the command and then clicking either the **EDIT STEP or DELETE** buttons.

1.8. Once you have finished creating your program, save it by clicking on **"SAVE AS"** enter the filename in the text box and click **"OK".**

1.9. Name your file with in windows naming conventions.

2. The following program steps are available in the Command List for creating customized programs in the Program Window:

2.1. **Heated lid:** use this command to set the required lid temperature. The temperature may range from 80°C to 115°C. The minimum increment is 1°C **(Figure.5).**

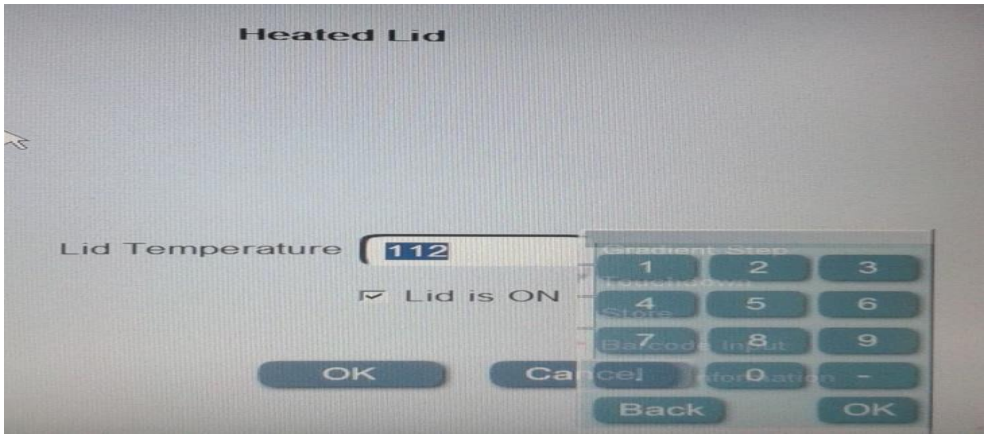

Fig.8. heated lid command window

2.2. Annealing Temperature: Minimum Annealing Temperature: sets the minimum annealing temperature. **HINT** The temperature difference is commonly between 5°C and 10°C starting with a temperature that is 2°C above the higher Primer melting temperature. Example: if Tm Primer 1 is 60°C and Tm Primer 2 is 54°C, the resulting Touchdown will be from 62°C to 52°C.

2.3. Elongation Temperature: Sets the elongation temperature. Duration of the temperature step in seconds.

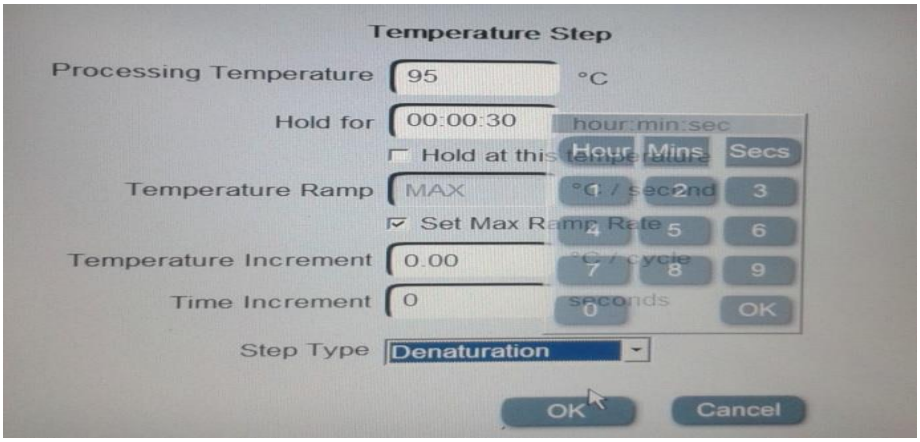

Fig.9. temperature step command window (denaturation, annealing and elongation temp. steps)

2.4. Cycle name: enter the name of the cycle and the required number of cycle

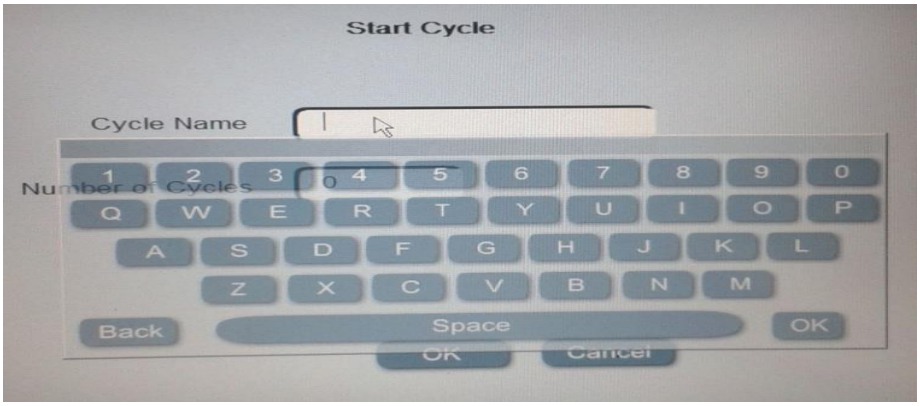

Fig.10. name of the cycle window

2.5. End cycle: insert this command to terminate the cycle. Note: all programs with a start cycle must have an end cycle.

2.6. Store: cools the thermal cycler to a temperature between 4 – 12^0c for a specified period of time.

2.7. Hold: duration of the store step. Note: "infinite" holds the specified temperature for an unlimited period of time.

2.8. Saving programs: to save a program script, click on save as in compose a program window. Enter a program name and click ok to save a file.

2.9. Editing program: you can edit the parameters of existing program and store the program with the same name or rename the edited program. Note: select the command you wish to edit and click "EDIT STEP". This will open the window of the respective

command and click "ok" to save your changes and to return to the compose program screen.

2.10. Deleting a program step: to remove a program step from a program, select the step then, press "DELETE"

2.11. Adding a program step: to add a program step, select below the position where the new step is to be inserted and select the appropriate command. Save the program as described above.

2.12. Deleting the programs: to delete an existing program, click on the program page. Select the program you wish to remove. Click "delete" this will remove the program from the script folder.

3. Running a program:

3.1. Opening and closing the lid: the lid can be opened gently twisting anti clock wise and closed by clock wise the lid top manually at any range.

3.2. Loading tubes: this thermal cycler can be loaded up to 48tues (0.2ml) per block.

3.3. Setting the lid pressure: close the lid and the spring mechanism will ensure contact is made between the heated lid and tubes.

3.4. Starting a run: to start a run, click on home page **"run program"**. The program list will open. Select the program you wish to run and click **"Run selected"(Figure.8)**

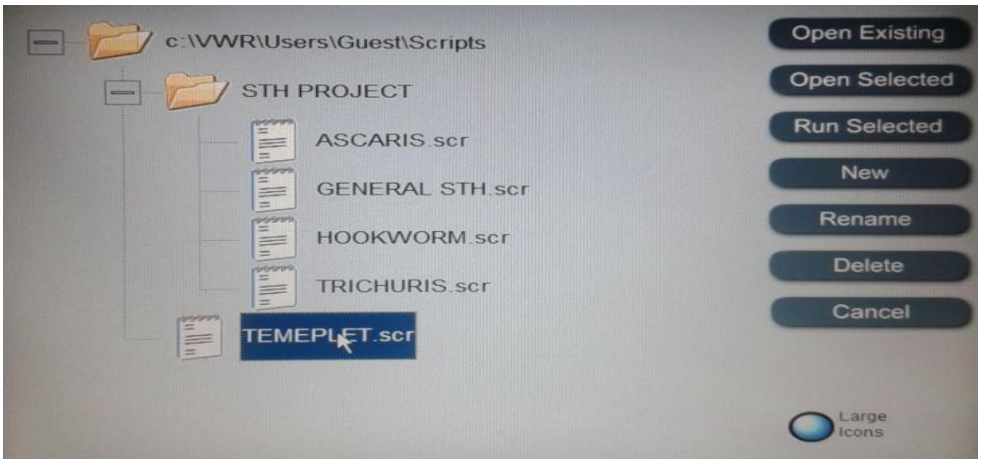

Fig.11. Program list windows

3.5. Enter your sample volume (2-150ul) and click "Ok" to start the selected program **(Figure.9).**

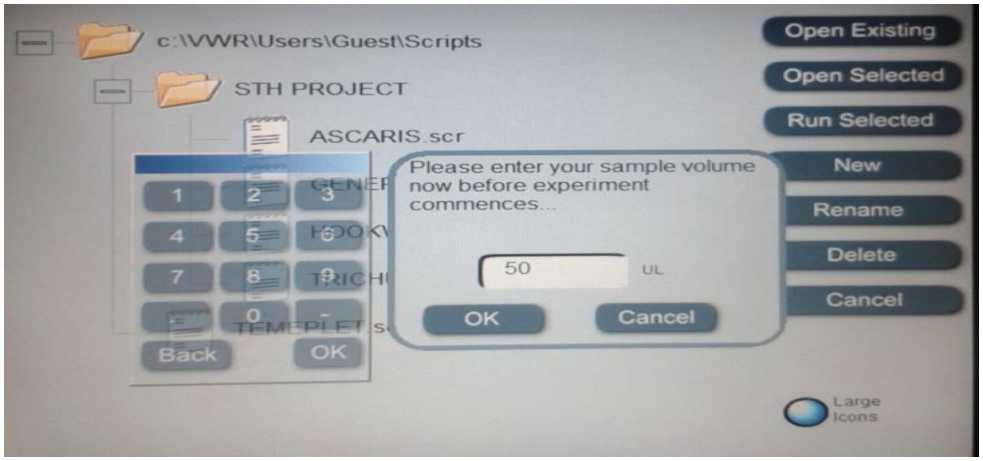

Figure.12. sample volume entering window

3.6. Viewing current program step information: After a run has started, information is displayed on the screen providing the following details.

a) Target temperature and duration of current steps

b) Program name

c) Elapsed time – also shows current cycle number e.g. cycle 1 of 35

d) Time remaining

3.7. Terminating a run: to stop a program manually before it completes, click the "stop" button on the run info screen followed by yes at the prompt.

3.8. Switching off the instrument: the unit can be switch off at any time during operation using the power button at the rear of the instrument. There is no need to exit the thermal cycler software prior to switching off the instrument. If the instrument is switched off, or there is power failure during a run, the run will continue automatically when power is resumed.

Annex 6. SOP for VWR® Mini Gel (electrophoresis unit)

Fig.1 electrophoresis unit

1. General information

1.1 specification

Overall electrophoresis unit	
Product description	VWR® Mini Gel
Catalogue numbers	700-003 EU, 700-004 UK, 700-005CH
Unit dimensions (WxLxH)	24.5cm x 17.0cm x 6.2cm
Maximum sample capacity	112 samples (4combs, 28 sample each)
Power supply	
Dimension (WxLxH)	7.5cm x 17.0cm x 3.8 cm
Weight	410gm
Input voltage	Ac100 - 240v, 50/60Hz
Output voltage	10 – 150; v constant peak voltage of 150v
Output amperage	10 – 400mA
Maximum voltage	45W
Timer	99 hours 59 min, and continuous model
Safety switch	Micro-sensor in the power supply. No output without safety lid
Memory function	Automatic memory (the last used V and T)

1.2 parts of the instrument

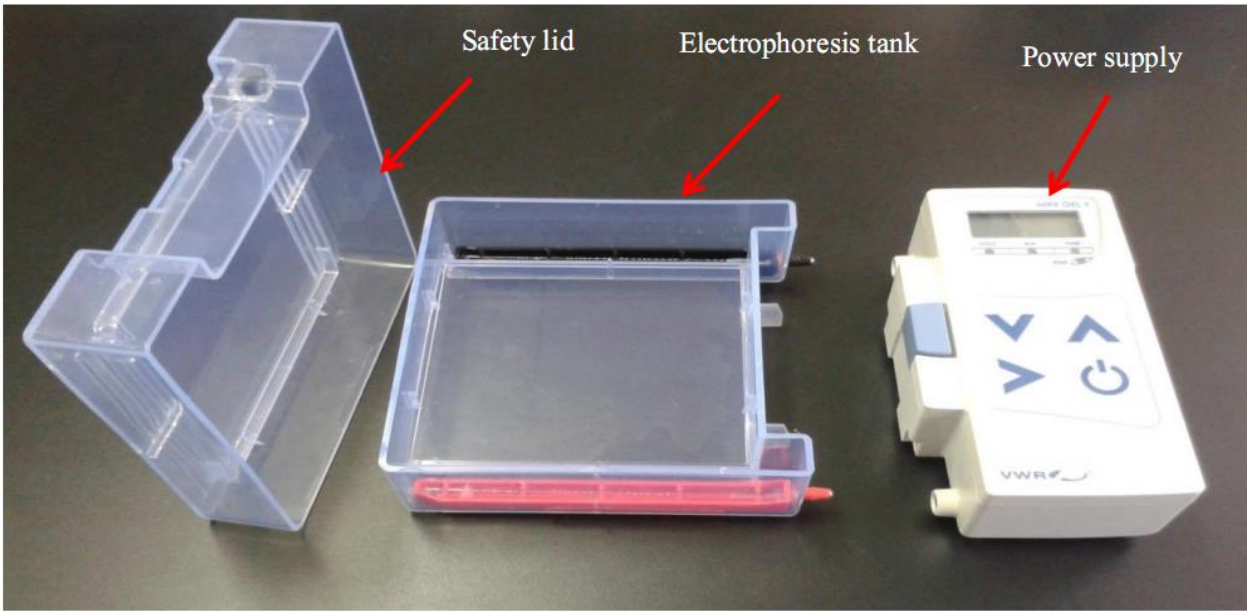

Fig.2 Lid, tank and power supply of electrophoresis unit

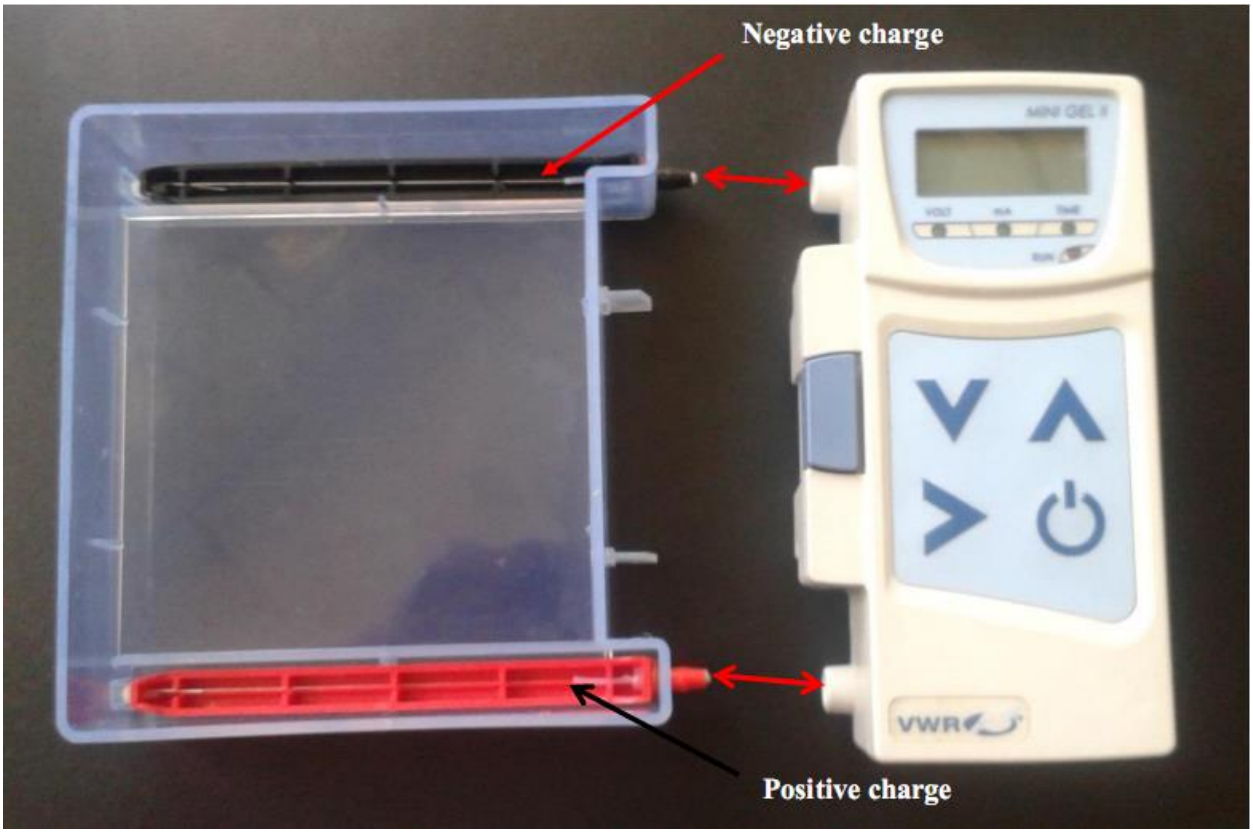

Fig.3 negative and positive charge of electrodes

Principle:

Agarose gel electrophoresis separates DNA molecules by size. DNA is negatively charged and will pass through the gel matrix towards the positive electrode when subjected to an electric field. Short molecules will move more quickly than larger ones and will migrate further through the gel.

This SOP describes the method used to separate and visualize amplified intestinal parasites DNA (*A. lumbricoides*, *T. trichuira*, hook worms and *G. duodenalis* strains DNA) extracted from absolute ethanol preserved fecal samples.

Required materials:

- Agarose powder
- Distilled water
- Electrophoresis unit
- Measuring Cylinder (250 – 1000ml)
- Tris, glacial acetic acid and EDTA
- 2 – 20 micropipettes
- Micro pipette tips for 2 – 20 micropipettes
- 10x TAE (tris, acetic acid and EDTA) buffer
- 1xTAE (tris, acetic acid and EDTA) buffer
- Loading dye if PCR buffer used is colourless (optional)
- Gel tray (gel casting mold)
- Gel combs
- 100bp DNA (ladder)
- Microwave heater or hot plate
- Laboratory Scale 0.1gm sensitive
- Reagent bottles (250 – 1000ml)
- UV Transilluminator
- Edithium bromide fluorescent dye

2. TAE (tris, acetic acid and EDTA) Stock/ working solution preparation

2.1 TAE 10 x stock solution preparation

To prepare 1L 10x TAE, we need

- 48.5 g Tris
- 11.4 mL glacial acetic acid
- 20 mL 0.5M EDTA (pH 8.0)
- Measuring cylinders
- Reagent bottles

Procedure

1. Dissolve Tris in about 800 mL of distilled water.
2. Add acetic acid and EDTA.
3. Add distilled water up to 1L.
4. Mix and Store at room temperature

2.2 TAE 1x working solution preparation

Dilute 100ml of stock solution (10x TAE) in 900 ml distilled water to make 1x TAE working solution.

3. Agarose gel preparation

To prepare agarose gel, you need

- Agarose powder
- 1xTAE working solution
- Laboratory scale sensitivity 0.01gm
- Glass flask (200 – 500ml)
- Microwave heater/ hot plate
- Gel tray (gel casting mold)
- Combs

Procedure

***NB**. DNA of intestinal parasites can migrate well and visualized in 1.5% agarose gel matrix*

1. Weigh agarose powder to make 1.5% of the volume you desire in 1x TAE

2. Add to glass flask and Dissolve by heating the mixture in a microwave or heating plate until all the agarose particles have dissolved
3. Cool to approximately 60^{o}c
4. Assemble gel tray with combs and pour in molten agarose according to your sample size
5. Allow agarose gel to cool to room temperature and solidify. The agarose will change from being transparent to being opaque
6. Pour 2 ml of TAE buffer on top of agarose gel to prevent dehydration.
7. Remove the gel well-forming comb by carefully lifting it vertically out of the agarose gel.

4. Loading DNA on agarose gel

To load DNA on gel we need

- Pre prepared gel according to our sample size
- 1x TAE buffer
- Electrophoresis unit
- Amplified DNA of target organism
- 100bp DNA (ladder)
- 2 – 20 micropipette
- 2 – 20 micropipette tips

Procedure:

1. Put your gel in electrophoresis unit.
2. Pour 1 x TAE over gel to cover it completely
3. Label your samples on work sheet which directly represents the gel lane numbers.
4. Pipette 5 µl of DNA ladder and load on 1st lane of the gel
5. Pipette 15µl of samples DNA and load on lane corresponds to the work sheet.

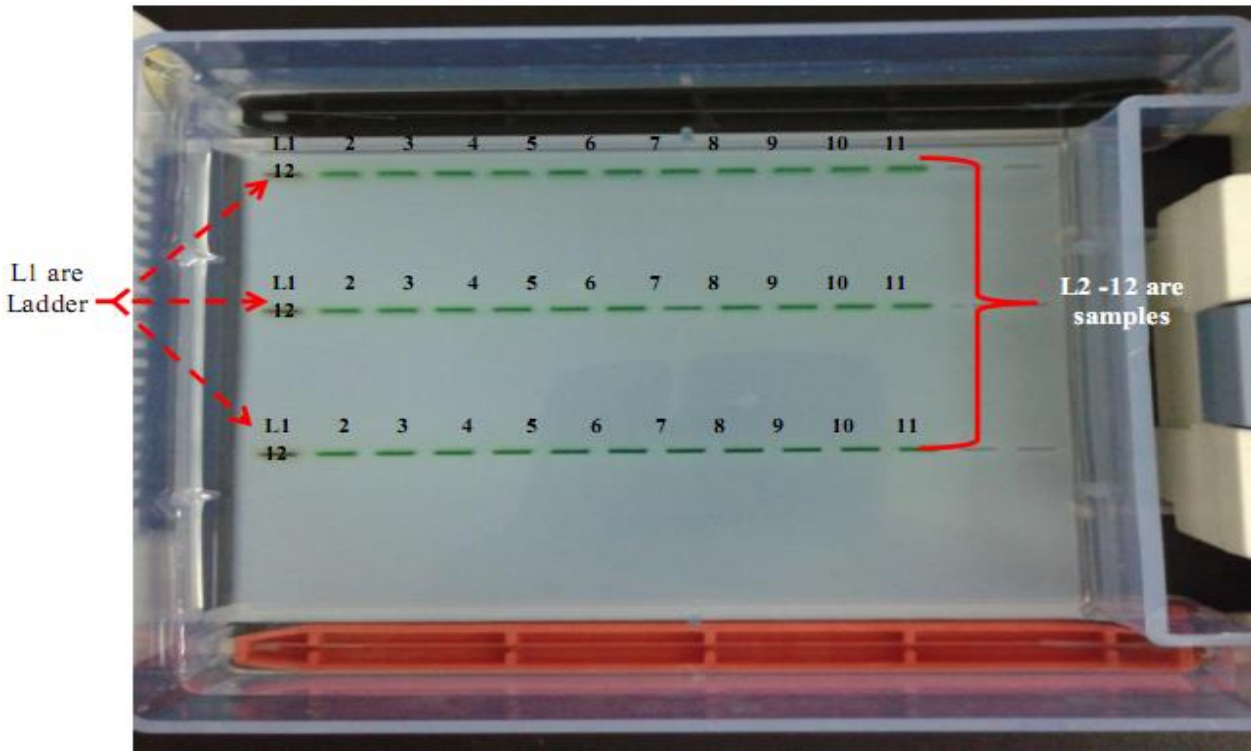

Fig. 4 sample loaded agarose gel in the electrophoresis unit

*NB. ***The ladder must be loaded for each gel row.*
*NB. ***Take care when pipetting to prevent sample mix up. Always follow the worksheet order when loading gels*

5. Running gel

1. Switch on the power supply of the unit
2. Run the gel according to recommended time and volt/ampere
3. Adjust your recommended time (30 minutes) and voltage (100V) by pressing the arrows shown on the unit down, up and across.
4. Start running by pressing start button.
5. When adjusted time completed the machine gives you piping sound
6. Switch off the main power of power supply of the unit.

Start

Fig.5 adjusted recommended time and voltage/ampere of the instrument

6. Staining DNA in gel

To stain DNA in gel, we need

- Ethidium bromide
- 1x TAE working solution
- Tank having enough space to hold the gel
- Flat spoon/plastic or metal spatula
- Nitrile gloves

Procedure

1. Prepare staining solution by diluting 10µl of ethidium bromide in 100ml of 1xTAE in staining tank
2. Carefully, take off the gel to avoid breakage of gel from electrophoresis unit and Immerse in ethidium bromide staining solution for 15 minutes.
3. Take off the gel from staining solution by using spoon or spatula and put on UV transilluminator and take a photograph using a digital camera.

NB. ****UV safety goggles must be worn when working at the UV transilluminator.*

4. Compare the bands appeared on the gel with the size of the ladder whether the band is the same size with your target DNA or not.

Important points to remember:

- **Ethidium bromide is a known carcinogen. Take special care not to contaminate yourself and laboratory work surfaces.
- **Ethidium bromide is extremely toxic by inhalation therefore; do not use the powdered form in the laboratory. Ethidium bromide solutions are available and should be purchased

Note: Several factors influence how fast the DNA migrates. These include:

- DNA molecule size
- Time and Amper used
- Concentration of agarose in the gel.

Annex 7. Consent form (English version)

FOR CHILDREN PARTICIPATING IN A STUDY IN HAMLE 19, HERMATA, AND JIREN #1 (JIMMA TOWN) ELEMENTARY SCHOOL IN JIMMA ZONE .

Investigators: Mio Ayana (PI)
Zeleke Mekonnen
Abdissa Bruksew
Bruno Levecke

Organization: Department of Medical Laboratory Science and Pathology, College of Public Health and Medical Sciences, Jimma University, Ethiopia.

Investigators : Mio Ayana (PI)
Zeleke Mekonnen
Abdissa Bruksew
Bruno Levecke

Part I- Information sheet (statement)

Introduction:

My name is Mio Ayana (Medical Laboratory Technologist & principal investigator) and I and my colleagues are from Jimma University. This information sheet is prepared by group of researchers whose main aim is to Optimize molecular tools by comparing two different DNA extraction methods for intestinal parasites at Jimma university, South West Ethiopia from March to June; 2014.

Purpose:

The purpose of this research is to make functional molecular tools and techniques and evaluate the efficient DNA extraction methods for intestinal parasites at Jimma University, South West Ethiopia from March – June; 2014.

Intestinal helminths and protozoan parasites are among the most prevalent Parasites causing a significant morbidity and mortality to humans in developing countries. Commonly used diagnostic methods for these parasites rely mainly on microscopical examination of stool samples for the identification of helminth eggs and protozoan trophozoites and cysts, which do not effectively differentiate morphologically indistinguishable interspecies and strains of parasites. So, a detailed epidemiological understanding of these parasitic infections using

advanced DNA based technology is important for the design, implementation, monitoring, and evaluation of intestinal parasitic disease control programs. Therefore in this study, by comparing two different DNA extraction methods molecular tools will be optimized for further DNA based researches for intestinal parasites.

Procedures:

If you agree to participate, we will visit you at the school and give you a small plastic container and ask you to collect your fresh stool sample (about 3 gm). Then we will check the stool to see if you have infection of intestinal worms.

Confidentiality:

The information obtained during the conduct of this study will remain confidential. The results of the research study may be published, but subjects' names or identities will not be revealed. Records will remain confidential. Only the researchers doing the study and principal investigator will use these forms. All will have a duty of confidentiality to you as a research participant.

Safety:

For this survey, you are inquired to provide your feaces only, which is a noninvasive procedure. Therefore, we do not expect any harm to occur on you. If you are infected with any of the soil transmitted helminthes you will be treated with albendazole. The drug will only be administered by qualified health professional.

Benefits:

By participating in the survey you will directly benefit by being investigated for intestinal worm infections and receiving appropriate treatment free of charge.

Right to refuse or withdraw:

We assure you that our best care will be taken for you if you agree to take part in the study. You are free to withdraw from the study at any time and no one will force you to participate and you will not be discriminated in any form for education or health services. If you have questions, feel free to ask

Mr. Mio Ayana (Medical Laboratory Technologist & principal investigator, (Tel. 0917805479) or Mr. Zeleke Mekonnen (Tel: 09 17 76 54 27)

Part II: Certificate of consent

I have read the information above, or it has been read to me. I have been given the opportunity to ask questions and my questions have been answered to my satisfaction. I voluntarily consent that

I will participate in this study by giving my stool for intestinal worm diagnosis and management and I understand that I have the right to withdraw from the study at any time.

Print name of subject, date and signature
_____ ____/____/____ (dd/mm/yy)

Annex 8. Consent form (Amharic version)

ቅን 7:5 □እድሜያቸው ከ12 ዓመት በላይ የሆኑትና በጥናቱ ላይ በሚሳተፉት ተማሪዎችየስምምነት ቅፅ

የጥናቱ ርዕስ፡ **በጅማ ዩኒቨርሲቲ ሞለኩላር ባዮሎጂ ላቦራቶሪ ለሆድ ትላትል ሁለት የዲኤንኤ መለያ ዘዴዎችን በማወዳደር የሞለኩላር መሳሪያዎችን በትክክል ስራ ላይ እንድዉሉ ማድረግ**

የተመራማሪዎች ስም፡ **ሚኦ አያና (ዋና ተመራማሪ)**
ዘለቀ መኮንን
አብዲሳ ብሩክሰዉ
ቡሩኖ ሌቪኬ

□አል ሀ፡ ማብራሪ□

ጤና ይስጥልኝ፡፡ ሚኦ አያና እባላለሁ፡፡ እኔ በጅማ ዩኒቨርሲቲ የህክምና ፓራሳይቶሎጂ የማስተር ተማሪ ነኝ፡፡ እንደሚታወቀዉ በሀገራችን ኢትዮጵያም ሆነ በሌሎች በአድገት ላይ ላሉ ሀገሮች ጥገኛ የሆድ ትላትሎች በሰዎች ላይ ይልቁኑ በህጻናት ላይ ከፍተኛ የጤና ችግር በማስከተል ላይ ይገኛሉ፡፡ ከብዙ አመት ጀምሮ የመመርመሪያ ዘዴዎቹ የቆዩና የመመርመር አቅማቸዉ ዉሰን ነዉ፡፡ ስለዚህ ዲኤንኤ ላይ የተመረኮዘ የምርመራ ዘዴ ያስፈልጋል፡፡ ለዝህ ደግሞ የሰገራ ናሙና ተወስዶ በዘመናዊ የዲኤንኤ ምርመራ መመርመር አለበት፡ ስለ□ህ አንተ ወይም አንቺ በ□ህ ዓ ናት ውስጥ እንድትሳተፍ/ፊ አ ቃ□ኛ እንድትሆን/ኝ በትህትና እ□□ቃለሁ፡፡

አላማ

ከብዙ አመት ጀምሮ ጥገኛ የሆድ ትላትሎችን ለመለየት የመመርመሪያ ዘዴዎቹ የቆዩና የመመርመር አቅማቸዉ ዉሰን ስለሆኑ በዲኤንኤ ላይ የተመረኮዘ የምርመራ ዘዴ ያስፈልጋል፡፡ ለዝህ ደግሞ የሰገራ ናሙና ተወስዶ በአሁኑ ጊዜ ባሉት የመመርመሪያ ዘዴዎች ተመርምሮ

ለዘመናዊ የዲኤንኤ ምርመራ ምርምር ምቹ መደረግ አለበት፡ ስለ□ህ አንተ ወይም አንቺ በ□ህ ዓ ናት ውስጥ እንድትሳተፍ/ፊ አ ቃ□ኛ እንድትሆን/ኝ በትህትና እ□□ቃለሁ፡፡

የአካሄድ ቅድመ ተከተል

በጥናቱ ላይ ለመሳተፍ ከተስማማህ/ሽ/ በጥናቱ ቀን የሠገራ ናሙና እንድታመ□/ቸ □ስቱራ ማም□ እቃ እንሰጥሃለን፡፡ የሰገራው ናሙናዉ የሆድ ውስጥ ትላትሎች እንቁላ□ ለማረፉ ዓ ላቦራቶሪ ተወስዶ ምርመራ ይደረግበታል፡፡ በሽታ ከተገኘበት መድሐኒት እንሰጥሃለን/ሻለን ፡፡

ሚስጥራዊነት

ከምርምሩ የምንሰበስበው መረጃ በሚስጥር ይያዛል፡፡ ከጥናቱ በሚገኘው ውጤት የግለሰብ ማንነት በማይታወቅ መልኩ ሊታተም ይችላል መዝገቦች በሚስጥርነት ይያዛሉ እና የተለየ የጥናቱን ስነምግባር የሚከታተል ኮሚቴ ስለ አንተ መረጃ ሊሰጣቸው ይችላል፡፡ ይህ ደፅሞ ጥናቱ ደረጃወን የጠበቀ እንዲሆን ለማድረግ ነው፡፡ እነዚህ አካላትም ምስጢርነቱን የመጠበቅ ኃላፊነት አለባቸው ፡፡

ጥንቃቄ ስጋትና ጉዳት

ጉዳት፡ የሰገራ ናሙና መስጠት ምንም አይነት ጉዳት አይኖረውም

ጥቅሞች

በዚህ ጥናት የሚሳተፉ ልጆች ከሆድ ትላትል ነፃ እንዲሆኑ ይደረጋል፡፡ በተፊ ማሪ የበሽታው ስርጭት እና ጠቃሚ የመመርመሪያ ዘዴ ለማወቅ ይረዳል፡፡ የመመርመሪያ ዘዴዎችን በማመላከት በሃገራችን በሽታዉን ለመከላከል በሚደረገው ጥረት ከፍተኛ አስተዋጽኦ ያደርጋሉ፡፡

ያለመሳተፍና ከተሳታፊነት የማቋረጥ መብቶች

በዚህ ጥናት ላይ ብትሳተፍ /ብትሳተፊ/ ምንም አይነት ጉዳት እንደማይደርስ ለማረጋገጥ እንወዳለን፡፡በ□ሮ□□ቱ ላለመሳተአ □ቃ□ኛ ባትሆን/ኚ የሚደርስብህ/ሽ ምንም አይነት ተጽንኦ □ለም፡፡ በጥናቱ መሳተፍ ከጀመርክ/ሽ/ በኃላ በማናቸውም ሰዓት ከጥናቱ መዉጣት ወይም ማቋረጥ ይቻላል፡፡ ከጥናቱ በመውጣትህ/ሽ/ በማቋረጥህ/ሽ/ የሚገባህን/ሽ/ የህክምና አገልግሎት

አይነፈግህም/ሽም/ ። ጥያቄ ካለህ/ሽ ሚኦ ኢያና /ዋና ተመራማሪ 0917805479 ወይም ዘለቀ መኮነን፡ 09 17 76 54 27 **መጠየቅ ይቻላል።**

□አል ለ፡ □ተሳታፊዎች የስምምነት ሰነድ

ከላይ የተገለፁትን ነገሮች በሙሉ አንብቤያለው ወይም ተነቦልኛል። ግልፅ ያልሆነልኝ ነገር ካለ ጥያቄዎች እንድጠይቅ እድል ተሰቶኛል ለጥያቄም በቂ ምላሽ አግኝቻለሁ። በሙሉ ፍቃደኝነት በዚህ ጥናት ላይ የሰገራ ናሙና በመስጠት በመሳተፍ በፍቃደኝነት ተስማምቼአለሁ።

□ተሳታፊ ስም፡ ቀን፡ ፊርማ/የጣት አሻራ

ቀን----------ወር----------□ም

Annex 9. Consent form (Afan Oromo version)

Guca haayyama Hirmaannaa ijoollee waggaa 12 fi isaa oli

Qorattoonni: Mio Ayana (Qorataa duraa)

Zeleqee Mekonnen

Abdisa Bruksew

Bruno Leveke

Dhaabata: Yuniversiitii Jimmaatti Kollejjii Saayinisii fayyaa Hawaasafi Meedikaala Dipartmeenti Labooratoory fi Patoologii

Matadureen Qowaanichaa: Laaboraatoorii molekulaar bayooloojii Yuniversiitii jimmaatti DNA ramoolee garaa keessaa adda baasuuf tooftaalee lama dorgomsiisuudhan meshalee molekularii sirriitti hojii irra olshuuf .

Kutaa 1- Odeefannoo/ibsa

Seensa:

Maqaan Koo mi'oo Ayyaanaa (Medikal Laboratory Tekenologistii fi qorataa isa duraati). Ani fi qorattoonni kannen yuunivarsiitii Jimmaa. Duraan dursa nagaan isin gaafadha ; akkam jirtu. Akkuma beekamu biyya keenya keessattis ta'e biyyoota guddataa jirran irratti ramooleen garaa keessaa rakkina fayyaa namoota irra geesisaa jira kesumayyuu da'iman irratti. Garuu tooftaan ittiin sakatta'man tooftaa waan hintaneef qulquleesee sakata'a hinjiru kanaafuu, DNA irratti kan hunda'e tooftaan ittiin sakata'an bayyee barbaachisaadha.

Kaayyoo qorannichaa

Tooftalee DNA ramoolee garaa kessaa ittiin adda baasan lama dorgomisiisuudhaan meshalee molekulaar bayoolooji Yuniversiitii Jimmaa sirriitti akka hojii irra oolu goochudha.

Adeemsa

Yoo fedha qabaattan hojjetoota qorannoo keenya mana barumsa keessan dhaquun qabdu iddattoo pilaastikii irraa tolfamte sinii kennuun akka iddattoo bobbaa ho'aatilmaamaan gm 5 fiddan isin gaafatu. Firii qorannoo keessan irratti hundaa'uudhaan qorichi praziquaanteli fi albendaazoli isin tola kenname ni fayyitu.

Icitii

Odeeffannoon yommuu qorannoo kanaa argamu icitiidhaan eegama. Odeeffannoon argame kun qaama biraaf dabarfame hin kennamuu. Argannoo qo'annoo kanaa maxxansumuu ni danda'a haata'u malee maqaaf eenyummaan namootaa hin ibsamu. Galmeewwn icitiin eegamu. Iciitii

kana eeguudhaaf, qorataan galmeewwan kana sanduqa furtuu qabu keessa Biiroo Yunivarsitti Jimmaatti argamu keessa kaa'a. Friin qorannoo taasifamuu kun koodiin itti kennamee akka eenyummaan namoota fedhiitiin hirmatamanii akka hin beekamnne ta'a. Namoota firii laaboraatorii ilaalani allatti akka ragaan kampiitera keessa taa'u ni taassifama. Kompuuterichis koodiitiin cufaa ta'a. odeeffannoon namoota dhuunfaa yoo fedhii saanii ta'e qofa ogeessota fayyaaf kennamuu danda'a. Bobbaa funaanamu haala protokolii keessaatti ibsameen alatti qo'annoo biraatiif osoo hin ooliin haala miidhaa hin finneen qo'annoon kun erga dhumee booda ni gatama.

Of-eegannoo

Kan isin irra dheegamu bobba furdaa qofaa kennuu dha. Yaaliin kun Praziquantalii fi albendaazolii yeroo ammaa kana gabaa irratti argaman waan laatuuf bu'aa malee miidhaa hin qabu.Qorichis kan kennamu ogeessa fayyaati qofaani dha.

Faayidaawwan

Isin ijoleen kana keessatti hirmaattan raamolee marri'imaaniif faalamuu fi hin faalamne qorannoon adda baasa fi wala'ansa barbaachisaa argachuun ni fayyadamtu. Kan dhuma irrattis raamootiin qabamtanis haala tokko tokko irratti hunda'uun yaalii atattamaa ni argattu.

Mirga diduu fi addan kutuu

Yoo qorannoo kana keesatti hirmaattan of eegannoo guddoo akka goonu isinii mirkaneesina. Akkasumas yeroo barbaadanii hirmanaa qoranichaa addan kutuuf mirga guutuu qabda. Kanaaf immoo miidhaan/loogiin karaa barnootaa fi waalansaa isin irraan ga'u hin jiru. Yoo gaaffi qabaatte battaluma Oboo Mi'oo Ayyaanaa (Bil. 0917805479) fi Zeleqee Mekonnen (Bil: 09 17 76 54 27) gaafachuu dandeessa.

Kutaa II – Guca raga Heeyyamaa

Odeefannoo armaan olii dubbiseera ykn naa dubbisameera carraan gaafii gaafachuu naa kennameera gaaffii kootifisi deebii gahaan argadheera. Fedhii kootiin qorannoo keessan keesatti hirmaachuu akkan barbaade isin mirkaneessa:

Maqaa hirmaata _____Mallato _____

Guyyaa _____ g/j/w

Maqaa qorataa guyyaa fi mallattoo qorataa _____guu/j/w_____

Annex 10. Questionnaire (English Version)

JIMMA UNIVERSITY COLLEGE OF PUBLIC HEALTH AND MEDICAL SCIENCES
DEPARTMENT OF MEDICAL LABORATORY SCIENCE AND PATHOLOGY
A RESEARCH QUESTIONNAIRE, 2014

AIM: Dear participant, first I wonder your participation that you think your Participation will help us to provide a rapid and effective diagnostic method for the assessment of helminthiasis. As I told you the objective of the study is to optimize molecular tools by comparing two different DNA extraction methods at Jimma University molecular biology laboratory.

1. Subject code No _____
2. Name of School _____
3. Name of the student _____
4. Grade /section of student_____
5. Age (in years) _____

Annex 11. Questionnaire (Amharic Version

ጅማ ዩኒቨርሲቲ የህብረተሰብ ጤናና ሕክምና ሳይንስ ኮሌጅ የሜዲካል ላቦራቶሪ ሳይንስና ፓቶሎጂ ትምሕርት ክፍል መጠየቂያ፡ 2014

ዓላማ፡- ውድ ተሳታፊያችን በጥናቱ ላይ በመሳተፎ እጅግ እያመሰገንን የእርሶ መሳተፍ ጥናቱን በትክክል እንድናካሀድ ይረዳናል፡፡ በመሆኑም የጥናቱ አላማ እንደገለጽነው ሁለት የሆድ ጥገኛ ትላትል ዲኤንኤ መለያ ዜደን በማወዳደር የሞለኩላር መሳሪያዎችን በትክክል ስራ ላይ እንድዉለ ለማድረግ ነዉ፡፡

1. **የተሳታፊው መለያ**----------
2. **የትምህርት ቤት ስም** ----------
3. **የተማሪው ስም**----------
4. **ክፍል** ----------
5. **ዕድሜ** ----------
6. **ዖታ** ----------

Annex 12. Questionnaire (Afan Oromo Version)

Yuniversiitii Jimmaatti Kollejjii Saayinisii fayyaa Hawaasafi Meedikaala Dipartmeenti Labooratoory fi Patoologii, Guca gaaffii: 2014

Hirmaannaa keessaniif guddaa isin galateeffanna, hirmaannan keessan kunis qowannicha akka sirriitti qowannu nugargaara. Akkum duraan dhibisuu yaalle kayyoon isaa tooftaa DNA raamoolee garaa keessa adda baasan dorgomsiisuudhaan meshaalee molekulaarii sirritti akka hojjetan gochuudha.

1. Koodii Barataa: ----------
2. Maqaa M/ barumsaa:----------
3. Maqaa barataa:----------
4. Kutaa :----------
5. Umurii:----------
6. Saala :----------