DETECTION OF β-TUBULIN SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS) ASSOCIATED WITH DRUG RESISTANCE AMONG SCHOOL-AGED CHILDREN INFECTED WITH SOIL-TRANSMITTED HELMINTHS IN BUNGOMA, KENYA

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Detection of β-Tubulin Single Nucleotide Polymorphisms (SNPs) Associated with Drug Resistance among School-Aged Children Infected with Soil-Transmitted Helminths in Bungoma, Kenya

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2024

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

Signature……..…… …Date ……..…….

Peterson Macharia Maingi

This research project has been submitted for examination with our approval as the University supervisors.

Signature……..…… …Date ……..…….

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Signature……..…… …Date ……..…….

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DEDICATION

This work is dedicated to my wife Grace and to my parents, Mr. and Mrs. Maingi, for their support, encouragement and prayers during my studies.

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I would like to acknowledge the almighty God for the gift of life and good health throughout my studies and research work.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ACRONYMS AND ABBREVIATIONS

ABZ	Albendazole.
BZAs	Benzimidazole anthelmintics.
CDC	Centers for Disease Control
CHV	Community Health Volunteers
CR	Cure Rate
DALYS	Disease Adjusted Life years
DNA	Deoxyribonucleic acid.
EPG	Egg per gram.
ERR	Egg Reduction Rate.
GIS	Geographical Information Systems
GMP	Good Manufacturing Practices.
NaCl	Sodium chloride.
MBZ	Mebendazole.
MDA	Mass Drug Administration.
MoE	Ministry of Education.
MoH	Ministry of Health
NTDs	Neglected Tropical Diseases.
PBS	Phosphate Buffered Saline

PCR	Polymerase chain reaction
SAC	School-aged Children
SNPs	Single nucleotide polymorphisms.
STH	Soil-Transmitted Helminths.
WHO	World Health Organization.
WRA	Women of Reproductive Age.

ABSTRACT

Soil-transmitted helminths (STHs) are parasitic nematodes associated with poor sanitation. These parasites are transmitted to humans by ingesting infective eggs or skin penetration by larvae found in feces-contaminated soil. Approximately 1.5 billion people across the globe are infected with STHs with another 4 billion people being at risk of infection. Although STH infections may occur in different demographic groups, the adverse effects of these infections are most significant among children, resulting in malnutrition, anemia, stunted growth and impaired cognitive development. STHs are part of the 20 neglected tropical diseases (NTDS) identified by the WHO as priority diseases for enhanced control and elimination. In Kenya, STH infections remain a significant public health concern, mainly among vulnerable populations, such as children. Despite efforts to control STH infections through mass drug administration (MDA) programs which have been running in Kenya since 2012, the prevalence still persists, posing a risk to the health and well-being of millions of individuals across the country This highlights the need for continuous monitoring of the prevalence and surveillance of the genetic diversity of the helminthes targeting the mechanisms known to cause drug resistance. Assessing the prevalence of STH infections pre and post MDA is crucial for evaluating the effectiveness of the MDA program. This can further inform the policy makers on the future policies concerning the STH control including the resource allocation and control approaches. Incorporating genetic surveillance into STH control programs allows for early detection of drug resistance early enough and facilitates timely modifications to treatment strategies. This study aimed to compare the prevalence of STH infections pre- and post-deworming in school-aged children in Bungoma County, Western Kenya and to detect SNPs associated with drug resistance in these infections. A longitudinal study was conducted in 414 school-aged children. Stool samples were collected one month pre and two weeks post-MDA with MBZ. STH prevalence and infection intensities were determined using the Kato-Katz technique. Genomic DNA was extracted from eggs obtained from the stool samples pre- and post-deworming that were positive for *A. lumbricoides* infection. Standard PCR generated 564bp amplicons surrounding the target codons 167, 198, and 200 of the *β*-tubulin gene of *A. lumbricoides,* which have been associated with drug resistance phenotype in various helminth species. The amplicons were sequenced using Sanger sequencing to detect the presence of SNPs at these loci. Two hundred and two and 212 pre- and post-MDA stool samples were collected, respectively. The overall pre-MDA and post-MDA STH prevalence was 33% and 6%, respectively. *A. lumbricoides* was the dominant infecting helminth species with a prevalence of *31*% and 4%, pre and post-MDA respectively. However, a few samples (<5) had hookworms, *T. trichiura* or mixed infections. Sequences were generated covering codons 167, 198, and 200 of the *β*-tubulin gene of *A. lumbricoides*. Sequencing of the DNA samples revealed that no β-tubulin SNPs were present in the *A. lumbricoides* infections. The significant reduction in prevalence post-MDA underscores the importance of continued investment in MDA interventions as part of integrated control strategies. The absence of detectable SNPs highlights the need for further research to understand the genetic diversity and population dynamics of STH in Bungoma County.

CHAPTER ONE

INTRODUCTION

1.1 Background

Soil-transmitted helminths (STH) is a collective term referring to nematode infections transmitted to man through contact with soil contaminated with the eggs or larval stages of these nematode species (Chong *et al*., 2022). They include the small intestinal roundworm (*Ascaris lumbricoides*), the whipworm (*Trichuris trichiura*), and the hookworms (*Necator americanus* and *Ancylostoma duodenale*) (Ásbjörnsdóttir *et al*., 2017; WHO, 2020b). STH infections are most prevalent in tropical and subtropical regions, with the highest prevalence in sub-Saharan Africa and Southeast Asia (Bronzan *et al*., 2018; WHO, 2020a).

World Health Organization (WHO) estimates that about four billion people are at risk of getting infected with STHs globally (WHO, 2017). Approximately one and a half billion people already have an infection with either hookworms, roundworms, or whipworms (WHO, 2020b). *A. lumbricoides* accounts for the highest morbidity among STH, followed by *T. trichiura* and hookworm infections. Although STH infections may occur in different demographic groups, the adverse effects of these infections are most significant among children, resulting in malnutrition, anemia, stunted growth and impaired cognitive development (Degarege *et al*., 2022; WHO, 2020b). In addition, STHs have resulted cumulatively in over 3.5 million disability-adjusted life years (DALYs)(WHO, 2020b) More than 613 million children of school- age are at risk of STH infections worldwide (Phuphisut *et al*., 2022).

STHs are part of the 20 neglected tropical diseases (NTDS) identified by the WHO as priority diseases for enhanced control and elimination in the recently published road map for NTD 2021-2030 (Malecela & Ducker, 2021). NTDs are a compendium of diseases found predominantly in poorer regions of the world, which for many years received minimal attention and support in their research and control. However, with substantial support from WHO and various stakeholders, increased resources have been directed towards community-based programs and research activities to understand better, control, and eliminate STHs. Management of NTDs is feasible using

many existing interventions, including drug therapies improved case management, health education, and WASH programs to enhance sanitation and hygiene practices in high-risk communities (Aruldas *et al*., 2023; Knopp *et al*., 2012).

The core strategic intervention for controlling STHs is preventive chemotherapy targeting pre-school-age children, school-age children (SAC), and women of reproductive age in endemic areas (WHO, 2020b). This deworming intervention aims at ensuring 75% coverage of targeted at-risk populations. High coverage is achieved following administration of the medications twice per year in areas where STH prevalence is equal to or greater than 50% and provision of a single administration per year in regions where STH prevalence is between 20% and 50% (Aruldas *et al*., 2023; WHO, 2020b).

In Kenya, STH control efforts have been primarily driven through the school-based deworming program targeting SAC and coordinated by the Ministry of Education (MoE) and the Ministry of Health (MoH) with support from partners (Okoyo *et al*., 2020a). Albendazole (ABZ) (400mg) and Mebendazole (MBZ) (500mg) are the recommended medications used in MDA programs due to their efficacy (when used as a single dose), affordability and ease of administration (Diawara, *et al*., 2013; Olliaro *et al*., 2022). The two drugs belong to the benzimidazole (BZ) group of compounds, which work by targeting *β*-tubulin protein monomers, which ordinarily occur as dimers in association with α-tubulin monomers that polymerize to form microtubules. The binding of these drugs to *β*-tubulin inhibits microtubule polymerization (Lacey, 1988, 1990). This binding interferes with the structural and cellular functions of the microtubules, leading to disruption of cellular transport, intracellular support, and DNA segregation, eventually resulting in the parasite's death (Lacey, 1988, 1990).

Despite the many benefits achieved by the MDA program over the past ten years since its inception in 2012, there is a growing concern about the possible emergence of drug-resistant parasite strains, which might erode these gains. These concerns are valid given that drug resistance species have been identified among veterinary nematodes such as *Haemonchus contortus,* where resistance to benzimidazoles occurs due to mutations of the beta β-tubulin gene (Schulz *et al*., 2018). The widespread occurrence

of drug-resistant STH species would be a devastating setback in the control and elimination efforts against these helminth infections.

1.2 Statement of the Problem

The continued and widespread use of medications creates selection pressure for drug-resistant pathogens. Drug resistance has been observed among bacteria, viruses, protozoans, and veterinary nematodes. There is a growing concern about the possible emergence of BZ-resistant STH parasite strains, which might erode the gains achieved by MDA programs such as reduced school absents and shifting of infection intensities from heavy to moderate and light infection. These concerns are valid given that drug resistance species have been identified among veterinary nematodes such as H. contortus, where resistance to BZ occurs due to SNPs within the β-tubulin gene (Ali *et al.*, 2018; Morrison *et al.*, 2022).

A study conducted in Kenya, Haiti and Panama in 2013 on human STHs demonstrated the existence of SNPs in the A. lumbricoides β-tubulin gene at codon 167 (Diawara, *et al.*, 2013). The study further demonstrated the existence of SNPs in T. trichura at codon 200, consistent with an earlier study conducted in Kenya in 2009 (Diawara *et al*., 2009). These studies indicated that the emergence of drug resistance is a valid concern. The widespread occurrence of such drug-resistant STH species would be a devastating setback in the control and elimination efforts against these helminth infections.

In Kenya, STH infections remain a significant public health concern, mainly among vulnerable populations, such as children. Despite efforts to control STH infections through mass drug administration (MDA) programs which have been running in Kenya since 2012, the prevalence still persists, posing a risk to the health and well-being of millions of individuals across the country (Mwandawiro *et al*., 2019*).* This is particularly the case in Bungoma County where despite several rounds of MDA among school aged children the prevalence never goes down to zero and bounces back after some time (Okoyo *et al.*, 2020a). This highlights the need for continuous monitoring of the prevalence and surveillance of the genetic diversity of the helminthes targeting the mechanisms known to cause drug resistance.

1.3 Justification

The emergence of drug-resistant STH strains poses a major challenge to control efforts. Monitoring the prevalence of resistance and identifying genetic markers associated with resistance can guide the selection and deployment of anthelmintic drugs, ensuring continued effectiveness of MDA programs. There is a need for broad and continued monitoring of STHs bearing drug-resistant mutations. Determining and tracking the extent of the spread of such parasites would be critical as part of efforts carried out to control and eliminate STHs from endemic areas. Molecular techniques enable the rapid and accurate detection of drug-resistance mutations early enough to mitigate the harmful impact of the widespread distribution of drug-resistant parasites, including STHs.

Assessing the prevalence of STH infections pre and post Mass Drug Administration is crucial for evaluating the effectiveness of the MDA program. This can further inform the policy makers on the future policies concerning the STH control including the resource allocation and control approaches. The β-tubulin gene is a target for many anthelmintic drugs, including Benzimidazoles, which are commonly used in MDA programs. Single nucleotide polymorphisms (SNPs) in this gene have been linked to resistance to Benzimidazoles in various parasitic species including the veterinary nematodes. Detecting SNPs associated with drug resistance can provide insights into the prevalence and spread of resistance SNPs within STH populations.

Incorporating genetic surveillance into STH control programs allows for early detection of drug resistance early enough and facilitates timely modifications to treatment strategies. By monitoring changes in SNP frequencies over time, we can identify hotspots of resistance and implement targeted interventions to prevent further spread. This may also inform potential future interventions as understanding the genetic basis of drug resistance in STHs opens avenues for the development of new control measures, such as new drug targets or molecular diagnostics. By elucidating the mechanisms underlying resistance, we can design more effective and sustainable strategies for combating STH infections in the long term.

1.4 Research Questions

1. What is the prevalence of soil-transmitted helminths (*A. lumbricoides*, *T. trichiura,* and the hookworms (*Necator americanus* and *Ancylostoma duodenale*)) among school-age children in Bungoma County, Kenya pre-MDA?
2. What is the prevalence of soil-transmitted helminths (*A. lumbricoides*, *T. trichiura,* and the hookworms (*Necator americanus* and *Ancylostoma duodenale*)) among school-age children in Bungoma County, Kenya post-MDA?
3. Which β-Tubulin SNPs are present in soil-transmitted helminth infection among school-age children in Bungoma County, Kenya?

1.5 Objectives

1.5.1 General Objective

To determine the pre and post-MDA prevalence of STHs and to detect the β-tubulin SNPs associated with drug resistance among soil-transmitted helminth infected school-age children in Bungoma County, Kenya.

1.5.2 Specific Objectives

1. To determine the prevalence of soil-transmitted helminths (*A. lumbricoides*, *T. trichiura,* and the hookworms (*Necator americanus* and *Ancylostoma duodenale*)) among school-age children in Bungoma County in Kenya pre-MDA.
2. To determine the prevalence of soil-transmitted helminths (*A. lumbricoides*, *T. trichiura,* and the hookworms (*Necator americanus* and *Ancylostoma duodenale*)) among school-age children in Bungoma County in Kenya post-MDA.
3. To identify β-tubulin SNPs present in soil-transmitted helminth-infected children using Sanger sequencing1.7 Limitations of the Study

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction to Helminth Infections

Helminths, also called parasitic worms, are studied under a branch of science called helminthology. These worms are invertebrates; their bodies can be elongated, flat, or round. They are multicellular organisms and can cause diseases that can lead to human suffering (Grimes *et al*., 2016). Worldwide, helminths are known to cause high morbidity and mortality. In humans, helminths cause diseases ranging from asymptomatic infections to severe and life-threatening conditions. In extreme cases, helminths are associated with anemia and malnutrition. Helminths are also known to affect the intellectual development of children, which negatively impacts their academic performance. Children are more prone to helminth infection than adults; this is especially the case in soil-transmitted helminths and schistosomes and this parity is attributed to the predisposing behavioral and immunological status. Helminths have three developmental stages: egg, larval (juvenile), and adult.

2.2 Sources of Helminths Infections

Human infection with helminths occurs after exposure, which takes place in many different ways. For instance, some STH infections occur when consumed food and drinks are contaminated with soil containing eggs. Others, like most trematodes, infections occur through contact with contaminated water, where the infective larval stages penetrate the skin into the bloodstream, initiating the infection cycle. Food contaminated with larval stages of some helminths is another source of infection, for example, pork containing encysted *Taenia* larvae and fish with encysted *D. latum* larvae. Arthropod vectors may also serve as a source of human infection with some of the helminths, a good example being the filarial worms such as *Wuchereria bancrofti*, which is transmitted to humans through mosquito bites (Oswald *et al*., 2016). Other helminth infections, as in the case of *Enterobius vermicularis,* may be initiated through self-infection (auto-infection), while others, like *Hymenolepis nana*, may occur through transmission from person to person. Domestic or wild animals harboring the

parasites may also act as an infection source, for example, dogs being the source of *Echinococcus granulosus.* There being many different sources of helminth infections, there are, therefore, different routes through which they can enter the human body; these routes include the parenteral route (mouth), inhalation, or through the skin (hookworms)(Isaac *et al*., 2019).

2.3 Classification of Helminths

Helminths can be classified into Trematodes (Flukes), Nematodes (Roundworms), and Cestodes (Tapeworms).

2.3.1 Trematodes

Trematodes, commonly called flukes, have a dorsoventrally flat body, are non-segmented, and are bilaterally symmetrical. These helminths are leaf-shaped and range from a few millimeters to 8 centimeters in length. They lack a body cavity and have an incomplete digestive system (Ramlal *et al*., 2019). Most flukes are hermaphroditic, except for schistosomes, which have separate sexes. Apart from the schistosomes, which are blood-dwelling, most of the other trematodes are organ-dwelling, inhabiting different organs in the body (Clarke, *et al*., 2019b). Excluding schistosomes, all trematode eggs are operculated. In addition, most flukes require the snail as an intermediate host and usually undergo several larval stages before adulthood. Trematodes cause infections that may be asymptomatic but may also cause severe disease conditions and even lead to death. Examples of trematodes include *Fasciolopsis buski* and *Heterophyes heterophyes*, which are intestinal flukes, *Clonorchis sinensis*, and *Fasciola hepatica* (Liver Flukes), *Paragonimus spp* (Lung Flukes), and *Schistosoma spp* (Blood trematodes).

2.3.2 Cestodes

Cestodes are commonly referred to as tapeworms, and the adults have a flat and elongated bodies segmented. The body is divided into three sections: head (scolex), neck, and strobila, consisting of proglottids (Oswald *et al*., 2016). Tapeworms' sizes vary from a few millimeters to 10 meters, and the segments may range from three to

several thousand segments (Nasution *et al*., 2019). The proglottids can be classified as immature, mature, and gravid, with those near the neck being immature and those near the terminal end being gravid. The cestodes lack an alimentary canal and are hermaphroditic. They have worldwide distribution and cause a wide range of diseases ranging from asymptomatic infections to more severe conditions such as neurocysticercosis caused by *Taenia solium.* Examples of cestodes include *Diphyllobothrium latum* (Fish/Broad tapeworm), *Taenia solium* (pork tapeworm), *Taenia saginata* (beef tapeworm), *Hymenolepis diminuta* (rat tapeworm), and *Hymenolepis nana.*

2.3.3 Nematodes

Nematodes are also called roundworms; they are elongated, cylindrical, and have a non-segmented body. They are dioecious and have a complete digestive tract with oral and anal openings (Oswald *et al.*, 2017). Nematodes vary in size from a few millimetres to over a meter long; they are the largest human parasitic Helminths and have five stages in their life cycle, including four larval stages and an adult (Al-Tameemi & Kabakli, 2020). Unlike most cestodes and trematodes, nematodes are bisexual and undergo copulation to fertilize eggs. An exception is *Strongyloides stercoralis*, which is parthenogenetic (embryo growth and development occurs naturally without any fertilization) (Jourdan *et al*., 2018). Nematodes cause different human diseases, ranging from asymptomatic infections to life-threatening conditions. STH are found in this group of helminths. Other nematodes include *Enterobius vermicularis* and *Strongyloides spp, w*hich are intestinal dwelling*; Trichinella spp* and *Dracunculus medinensis,* which are tissue dwelling.

2.4 Neglected Tropical Diseases (NTDs)

Neglected tropical diseases (NTDs) afflict developing countries globally and have, for a long time, received little attention regarding financial support, control, and research compared to other diseases. The NTDs can be viral, parasitic, or bacterial and globally cause illness to more than one billion persons (WHO, 2020b). These diseases are closely associated with poverty. They may lead to the impairment of physical and cognitive ability and are also mainly contributing to child and mother deaths. NTDs

thrive more in developing countries with high poverty levels, poor sanitary conditions, and inadequate clean drinking water. In these developing countries, the level of healthcare is substandard and accessibility to it is also a challenge. NTDs rarely lead to death. However, they can lead to disabilities such as disfigurement and blindness. The effects of conditions caused by NTDs range from mild asymptomatic infections to extreme pain, permanent disability, and, in some cases, death. WHO has prioritized 20 NTDs for control and elimination, including STH, schistosomiasis, river blindness, and lymphatic filariasis. Over the past few years, great attention has been directed towards better understanding, research, and eliminating NTDs. The WHO, for instance, has established a ten-year roadmap on NTDs from 2021 to 2030. This road map targets STH infections for elimination as a public health problem by 2030 (WHO, 2020b).

2.5 Soil-Transmitted Helminths (STH)

Infections by soil-transmitted helminths rank among the most common infections globally, particularly in developing countries characterized by poverty and deprived communities (Karshima, 2018). These infections are transmitted to humans via eggs, typically found in soil previously contaminated with human feces. The situation is most common in areas with poor sanitary practices (WHO, 2017).

According to the WHO, estimates indicate that about four billion people worldwide risk getting infections caused by STHs. Approximately two billion people already have an infection with at least roundworms, hookworms or whipworms. The infections have resulted in over 5 million disability-adjusted life years (DALYs) (WHO, 2017). *A. lumbricoides* accounts for the highest morbidity among STHs, followed by *T. trichiura* and hookworm infections (Isaac *et al*., 2019). STH infections are more prevalent among children, adversely affecting their growth, school performance, and intellectual development, resistance to other infections and diseases, and many other effects. More than 613 million children of school- age are at risk of STH infections worldwide (Clarke, *et al*., 2019b).

2.6 Risk Factors for Soil-Transmitted Helminth Infections

Many factors determine STH transmission, including educational levels, poor environmental and personal hygiene, sanitary conditions, nutritional and health status, and poverty.

2.6.1 Poverty, Sanitation, and Urbanization as a Risk Factor for STH Infections

STH transmission is influenced by environments that have been contaminated with feces containing the eggs of the helminths. Subsequently, helminths are closely associated with poor sanitary conditions, poverty, and lack of clean drinking water (Clarke, *et al*., 2019b). Improved sanitary conditions and the provision of clean water are critical in controlling helminth infections. Even though STH infections are among the neglected diseases, occurring mainly in rural areas, social situations and environmental conditions in many urban areas where there are unplanned slums and areas of squatter settlements are ideal for the transmission of STHs in developing countries (Oswald *et al*., 2016).

2.6.2 Household Clustering, Behavior, and Occupation as a Risk Factor for STH Infections

STH prevalence is greatly influenced by behavior, specific occupations, and household clustering (Pickering, *et al*., 2019). This strategy principally affects the transmission of Hookworm, which has high frequencies occurring among adults (Amoah *et al*., 2018). Occupations such as engagement in agricultural activities are common factors witnessed in hookworm infections (Isaac *et al*., 2019).

2.6.3 Water, Season, and Climate as a Risk Factor for STH Infections

Suitable warmth and moisture are associated with the transmission of the STHs; these conditions are vital for hatching the eggs of hookworms and playing leading roles in the other STHs for their eggs embryonation (Pickering, *et al*., 2019). An increase in transmission rates is exhibited in wetter areas, and in some areas where STH infections are endemic, they exhibit noticeable seasonality (Ruberanziza *et al*., 2019). Distributional limits based on rainfall and temperature patterns have been identified

using remote sensing and geographical information systems (GIS) (Ruberanziza *et al*., 2019).

2.6.4 Genetics as a Risk Factor for STH Infections

Genes that can control human infection with STHs have not been identified. Nonetheless, recent genome scans have identified a locus that controls roundworm intensity on chromosomes 1 and 13 (Oswald *et al*., 2016).

2.7 The Life Cycle of STHs

2.7.1 Life Cycle of *Ascaris lumbricoides:*

Infection is initiated after the eggs are ingested through food or soil-contaminated hands. Hatching of larvae happens in the small intestine; the larvae penetrate the intestinal walls and wander through the liver-lung route, in the lungs, the larvae undergo further development before being coughed up and going back to the intestines, where maturation into adult worms occurs. Mating of the adult worms occur and the female stat producing eggs. *Ascaris lumbricoides* may produce up to Two Hundred thousand eggs daily (Isaac *et al*., 2019). The unfertilized eggs, which are ineffective are then passed with the feces. Depending on the environmental conditions, the fertilized eggs the Larvae develop to infectivity after 18 days to several weeks (WHO, 2017). Another infection cycle is initiated once these infective eggs are ingested. It takes between two to three months from the time of ingestion of the infective eggs to laying of eggs by the adult female. Adult roundworms can live 1 to 2 years.

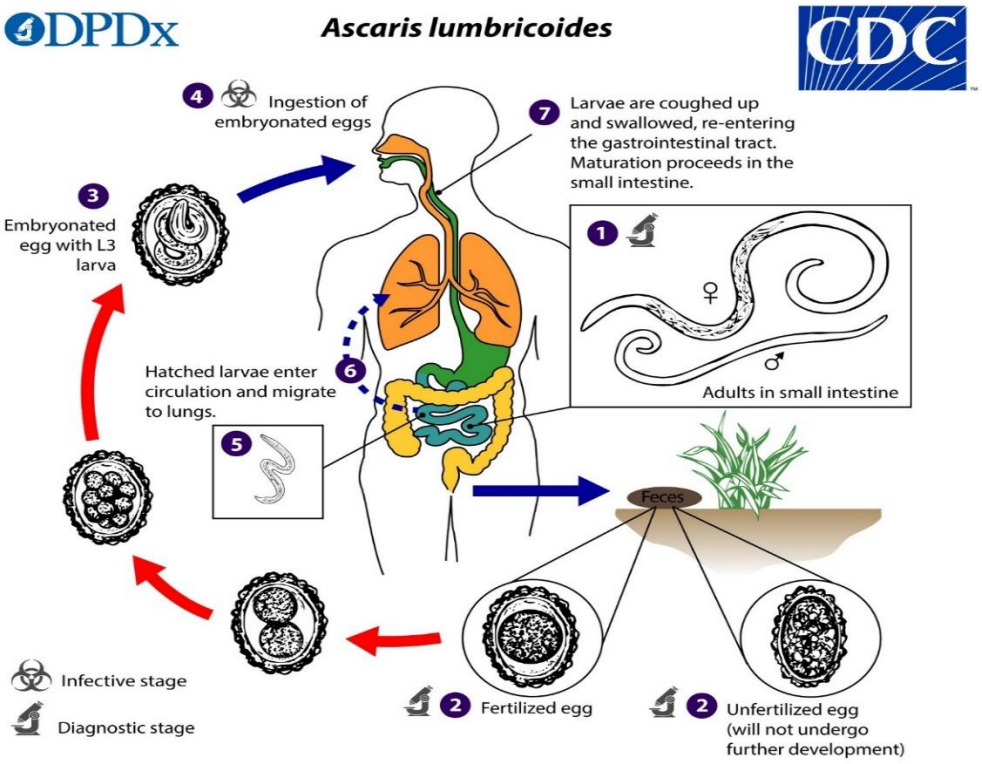


Figure 2.1: Life Cycle of Ascaris lumbricoides.

Adopted from: https://www.cdc.gov/dpdx/ascariasis/index.html

2.7.2 Life Cycle of *Tichuris trichura*

Infection is initiated after the eggs are ingested through food or soil-contaminated hands. Hatching of larvae happens in the small intestine where the larvae are released. Unlike the roundworms, the larvae of *Trichuris trichura* does not migrate through the liver-lung route but instead migrate to the large intestines where maturation into adults occur (WHO, 2017). The adult whipworms attach to the mucosa of the large intestine. Reproduction occurs in the large intestines and the female whipworms start laying thousands of eggs which are deposited into the environment through feces. Embryonation of the eggs occurs in the environment in the soil, the eggs embryonate, becoming infective to humans after within 15 and 30 days (Isaac *et al*., 2019). It takes sixty to seventy days for the adult whipworms to start depositing eggs after an infection

and the adult worms can live for about one year.

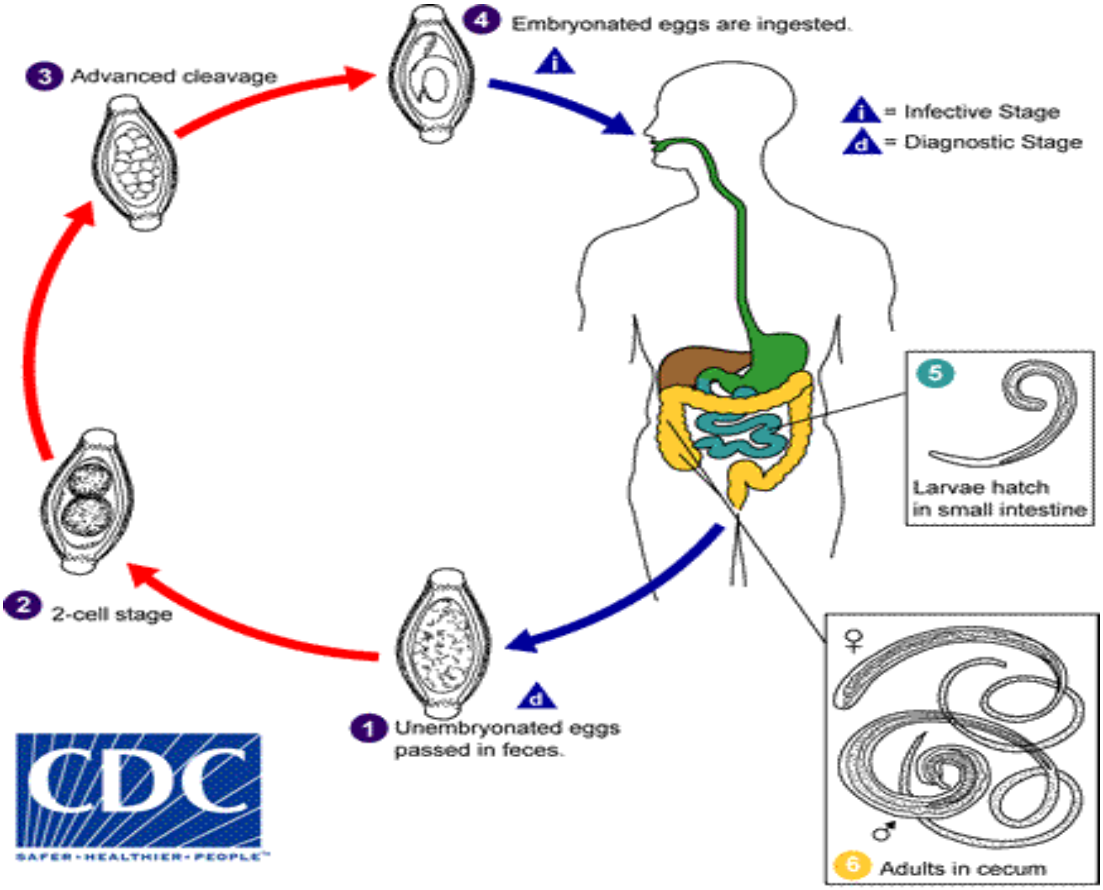


Figure 2.2: Life Cycle of Trichuris trichura.

Adopted from: https://www.cdc.gov/dpdx/trichuriasis/index.html

2.7.3 Life Cycle of Hookworms.

Infection by hookworms occurs when the filariform penetrates the human skin, typically after coming into contact with bare feet. They reach the lungs via the blood vessels and are coughed up, after which they are swallowed, maturing into adults upon reaching the small intestines (WHO, 2017). Adult hookworms inhabit the jejunum, mate, and start daily egg production. The eggs are passed in stool after which they hatch in one to two days into rhabditiform larvae; the hatching depends on the availability of favorable conditions (Isaac *et al*., 2019). Rhabditiform larva then molts twice into a third-stage larval form (filariform) (Clarke, *et al*., 2019b). The third-stage

larvae, the infective form, can survive for up to weeks in favorable environmental conditions. Adult hookworms have a life span of 1 to 2 years.

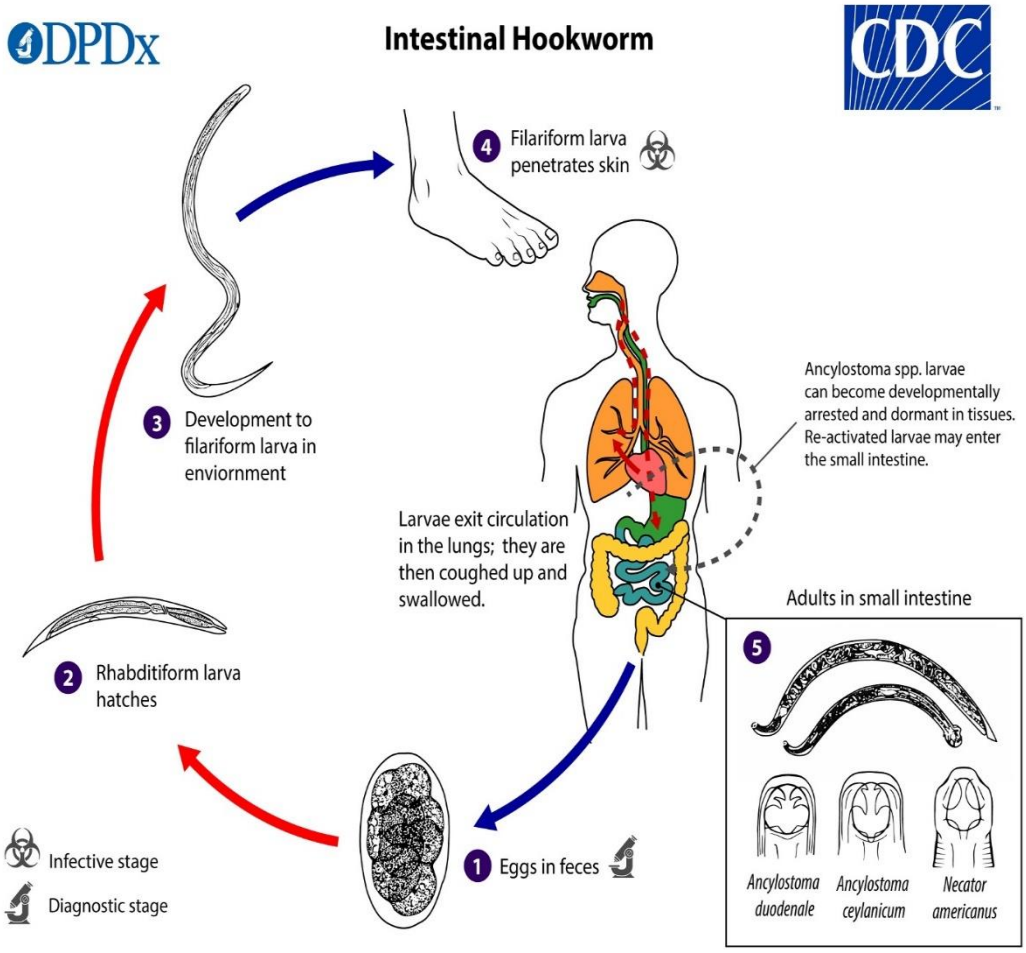


Figure 2.3: Life Cycle of Hookworms.

Adopted from: https://www.cdc.gov/dpdx/hookworm/index.html

2.8 Diagnosis of STHs

Detection of characteristic helminth eggs in human stool specimens using microscopic techniques is the primary method of diagnosing STH infection (Ngwese *et al*., 2020). This technique is sensitive, provided trained personnel perform the diagnosis and follow the appropriate method (Isaac *et al*., 2019). Wet saline preparations, as well as iodine preparations, are usually prepared for egg detection. Concentration techniques like floatation or sedimentation can also ensure accurate diagnosis (Papaiakovou *et al*.,

2019). Other methods used for stool examination for STHs include the *Kato-Katz* and *McMaster* techniques, widely used in research. Although they are not readily used, other methods that can be useful for diagnosis are molecular diagnosis, antibody tests, and antigen detection (Papaiakovou *et al*., 2019).

2.9 Clinical Disease Due to STHs

The number of worms directly relates to the morbidity due to STHs (Farrell *et al*., 2018). Few worm burdens (light intensity) cause little or no infection amongst the people infected. High worm burden is associated with a range of symptoms: Intestinal manifestations such as diarrhea, bloating, flatulence, and abdominal pain. In extreme cases, malnutrition, impairment of physical development and growth, general weakness, and malaise may be witnessed (Tinkler, 2020). STH infections hardly cause mortality; instead, they are associated with long-lasting and insidious nutritional and health effects on the host (Tinkler, 2020). For long, hookworms have been known to be a significant cause of intestinal blood loss, leading to protein malnutrition and iron deficiency. After the depletion of the host's iron stores, the magnitude of iron deficiency anemia is directly associated with the burden of hookworm infection (Molla & Mamo, 2018). The groups at the most significant risk of developing hookworm anemia are women of reproductive age and expectant women and children due to their underlying poor iron status. During pregnancy, iron deficiency anemia has been associated with severe consequences for the child and the mother, including low birth weight, prematurity, and impaired lactation (Mutombo *et al*., 2019). In children, chronic STH infections may dramatically affect physical, mental, and educational development. Although worm burden is directly related to children's symptoms, light infections can cause significant symptoms in poor community nutritional status (Molla & Mamo, 2018). High-intensity infections by STHs can cause intestinal obstruction, and the parasites are also associated with chronic malnutrition in children. In cases of intestinal obstruction, surgery is advised to avert significant consequences that may be involved (Morrison *et al*., 2022).

2.10 Treatment of STH Infections

According to various studies conducted in the past, chronic STH infection deficits in growth and physical fitness in children can be reversed after treatment with anthelmintic drugs (Jourdan *et al*., 2018). Albendazole (ABZ) and Mebendazole (MBZ) are the most common drugs currently in use against the STHs. These two drugs generally have high efficacy and few side effects (Ojja *et al*., 2018). Current WHO guidelines for STH treatment target morbidity control through the preventive chemotherapy strategy, mainly through repeated deworming programs among school-age children and treating women of reproductive age (WRA) (WHO, 2017). The two WHO-recommended medicines are not expensive, and teachers and other non-medical personnel such as Community health volunteers (CHV) can conduct drug administration since it can be quickly done and with ease. Donation of ABZ and MBZ is usually made through WHO to National Ministries of health in all countries where STHs are endemic for the treatment of school-age children (Morrison *et al*., 2022),(Truscott *et al*., 2014).

2.11 Prevention and Control

2.11.1Treatment with Anthelmintic Drugs

Deworming reduces the worm burden, helping reduce the morbidity of STHs. Periodic deworming at regular intervals in areas of endemicity and to the groups at higher risk can ensure that infections are kept at low levels, hence low morbidity and immediately resulting in improved health, growth, and development in children (Farrell *et al*., 2018). Deworming has also been known to help avert the development of any irreversible consequences of STH infections, especially in children (Schulz *et al*., 2018).

2.11.2 Sanitation

Improved sanitary conditions are intended to reduce soil and water contamination, hence controlling transmission of the parasites. For STH infections to be eliminated, sanitation must be an ultimate intervention and cover a high population percentage for

it to be effective (Pickering, *et al*., 2019). Unfortunately, sanitation strategy implementation could be problematic with limited resources due to the high costs involved. Moreover, when sanitation is used as the chief control strategy, it can take a long time to be an effective option (Mwandawiro *et al*., 2019).

2.11.3 Health Education

Health education intends to reduce transmission rates and the risk of re-infection of STHs by advocating healthy behaviors. To manage STH infections, health education aims to reduce soil and water contamination by encouraging personal and community hygiene and using latrines (Clarke, *et al*., 2019a). Periodic deworming programs cannot achieve a stable decline of transmission or re-infection without changing behavior, for example, defecation habits (Grimes *et al*., 2016). Health education presents no contraindications or risks and is economical and straightforward to provide. Furthermore, its benefits are not just confined to the control of STH infections. Based on its benefits, it is rational to include health education in all the programs aimed at helminth control.

2.12 MDA Program

Mass drug administration (MDA), which is a preventive chemotherapy strategy, aims to target school-age children (SAC) and is the recommended method by WHO for controlling morbidity due to STH infections in all countries with high endemicity (WHO, 2017). However, this program does not prevent parasite re-infection between treatment intervals. Also, only targeting SAC will not be enough to lower the prevalence or possibly interrupt the transmission of these infections (Vercruysse *et al*., 2011). In countries where the endemicity of the infections is high, the coverage, targeted groups, and re-infection rates will determine the effectiveness of MDA in bringing down the transmission in the long term (Anderson *et al*., 2014).

2.13 Global Target on STHs

WHO has developed a roadmap of global targets for STH by 2030 (WHO, 2020b). These targets include achieving and maintaining low soil levels of STHs reported cases

in pre-school and SAC. Increasing individual countries financial support for preventive chemotherapy, lowering the number of tablets necessary for preventive treatment for these infections (WHO, 2020b), establishing an efficient program for controlling STHs in adolescent, lactating mothers, and pregnant women, and lastly, guaranteeing worldwide access to basic hygiene and sanitation in areas of high endemicity of STHs by the year 2030 (WHO, 2020b).

2.14 Anthelmintic Vaccines

Alternative control methods for STHs have been prompted by the emerging drug resistance cases and the high degrees of re-infection witnessed after treatment with anthelmintic drugs (Jourdan *et al*., 2018). For vaccine development, reduction of the burden of adult worms is considered to be the gold standard. However, developing an effective anti-STH vaccine has proved to be a big challenge compared to developing other vaccines, such as anti-viral or antibacterial vaccines. This challenge is primarily due to several factors: the complexity of the helminth's life cycle, incomplete immunological knowledge of the host-parasite interactions, and the protection conferred to the parasites by immune mechanisms. Furthermore, STH genomes and proteomes are complex. This complexity makes it hard to pinpoint antigenic targets for developing an effective vaccine. Notwithstanding these obstacles, however, the development of vaccines against STHs has advanced over the decades. There are ongoing efforts by the Human Hookworm Vaccine Initiative to develop and test a hookworm vaccine (Mascarini-Serra, 2011). Na-ASP-2 hookworm vaccine is the first to be developed using the current Good Manufacturing Practices (GMP) and tested for toxicity and quality control. This vaccine was developed from research demonstrating human correlates of immunity and partial protection data in vaccinated laboratory animals (Zhan *et al*., 2014). Additional research is required to determine how vaccines against STH can be integrated into existing control programs and how they would be used for populations at high risk of infections not presently targeted for regular deworming programs (Zawawi & Else, 2020).

2.15 Anthelmintic Drug Resistance in STHs

Anthelmintics are drugs used to treat and control parasitic worms' infections (nematodes, trematodes, and cestodes) in animals and humans. The absence of effective vaccines and poor sanitary conditions in some highly endemic areas has made it difficult to break the life cycles of these parasites (Ojja *et al*., 2018). As an alternative, treatment and prophylaxis have been the recommended control approach for at-risk populations. With increased treatment frequency, the likelihood of drug selection pressure occurring is high, and this, in turn, leads to drug resistance. Other factors that may lead to drug resistance include using the same drugs over a long period and under-dosing in other cases. Drug resistance may occur in four ways: reduced drug uptake, increased drug efflux, detoxification and altered drug target. ABZ and MBZ, both of which belong to a class of compounds known as Benzimidazoles (BZ), are the most commonly used anthelmintics for the treatment of STHs; this is due to their low cost and the fact that they are administered in single doses. BZ's mechanism of action involves binding to the β-tubulin molecule (Ruberanziza *et al*., 2019). This binding interferes with cellular structures and functions as they inhibit microtubule polymerization, leading to the parasite's death. Mutations to the β-tubulin molecule alter the target of the BZ leading to resistance.

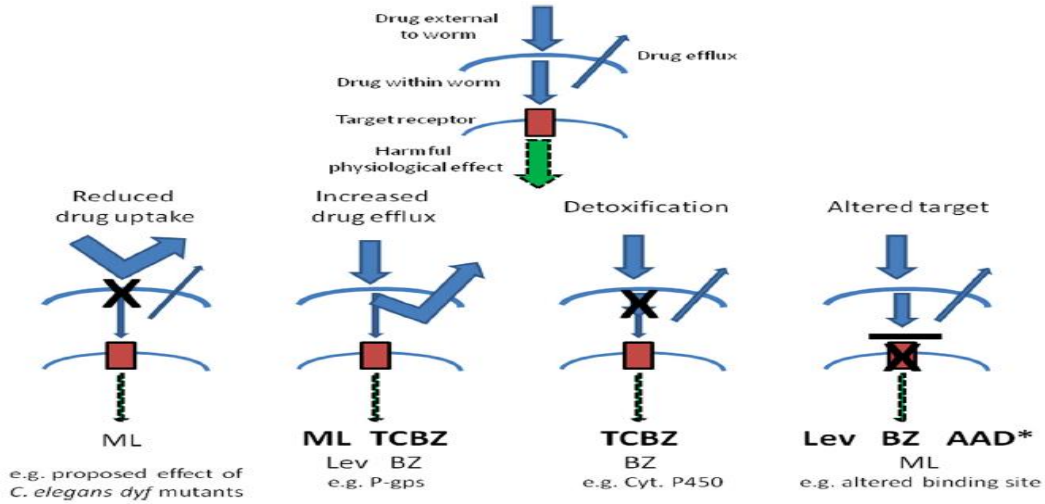
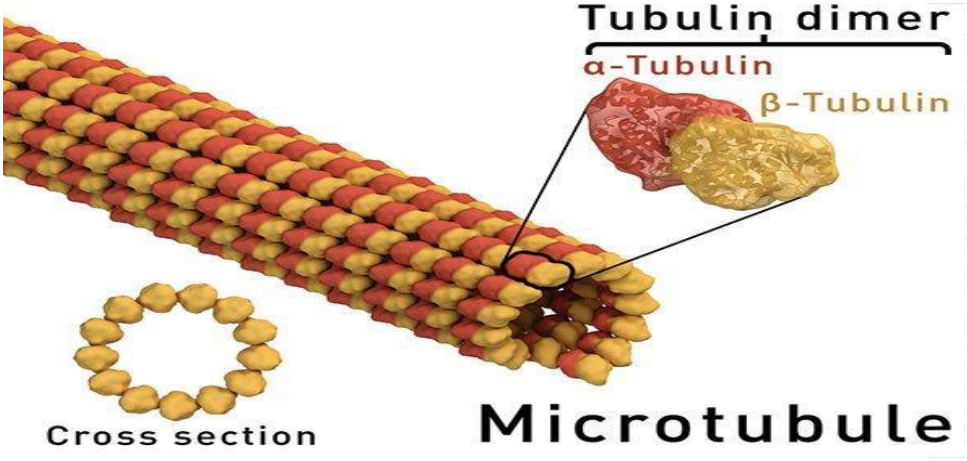


Figure 2.4: Mechanisms of Drug Resistance

Adopted from (Kotze et al., 2014)

2.16 Role of the β -Tubulin Protein Molecule

β-tubulin is one of the two protein molecules that join to make the microtubules; these microtubules are hollow microscopic tubes of a tubulin dimer (beta-tubulin and alpha-tubulin) and form part of the cytoskeleton. The microtubules play critical roles in the cell, one of which is cell division. During cell division in eukaryotic cells, the microtubules are involved in the formation of the mitotic spindles during meiosis. These mitotic spindles organize and separate chromosomes, leading to the formation of daughter cells. Also, the microtubules are involved in cell movement. They are essential in the flagella and cilia's structure, enabling various cell types to move ((Palma *et al*., 2020). Further, microtubules are also involved in cell transport. As part of the cytoskeleton, they facilitate the movement of organelles into and out of the cytoplasm. They are also involved in the way cells communicate with each other. Lastly, microtubules also provide the cell with a distinct shape and structure. Beta-tubulin protein molecules act as the BZs' target, a group of compounds used to treat STHs. These drugs inhibit microtubule polymerization by interfering with cellular structures and functions. This inhibition leads to the death of the parasites. Mutations to the beta-tubulin molecule may confer the parasite's resistance against the benzimidazoles (Furtado *et al*., 2020).



Adopted from (Aryal & Nandish, 2021)

Figure 2.5: Structure Of the Microtubule Comprising α- and β-tubulin Dimers

2.17 Evidence of Anthelmintic Drug Resistance in STHs

Regular and widespread usage of BZs for helminth treatment in veterinary nematodes has led to exceedingly high resistance rates in several nematodes of veterinary importance over a wide range of hosts (Zuccherato *et al*., 2018). The possibility of drug resistance emerging among STH in humans is a major cause of alarm concerning the feasibility of sustainable control of STH infections with BZs (Vercruysse *et al*., 2011). BZ resistance arises from the spreading of the nematode-tubulin allele point mutations. Although direct proof of human STH resistance against BZ is still not available, such resistance could be why MBZ's failure to treat human hookworms and reduced efficacy has been observed in southern Mali and Zanzibar, respectively, after repeated and periodic use of the drug (Savioli *et al*., 2017).

2.18 Molecular Studies on the Emergence of Resistance in STHs

Different studies on BZ resistance in parasitic nematodes have indicated that this resistance occurs due to SNPs in the Beta-tubulin gene. In these SNPs, amino acid phenylalanine is substituted by tyrosine, which occurs at codons 167 and 200 (Figure 2.3). Likewise, the amino acid alanine replaces glutamate at codon 198. These mutations have often been associated with drug resistance in STHs (Mutombo *et al*., 2019).

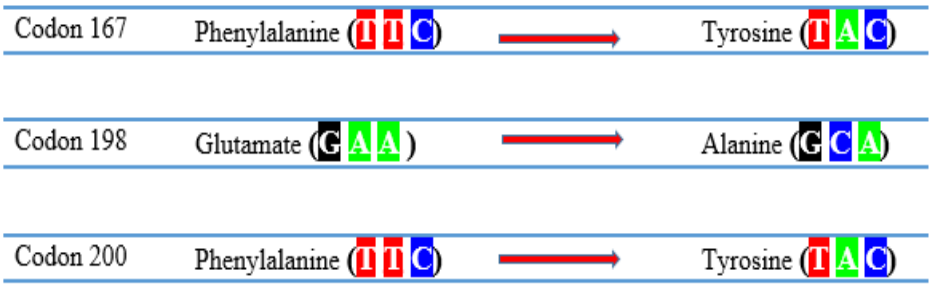


Figure 2.6: B-tubulin Isotype 1 Gene Showing SNPs that Lead to BZA Resistance.

In human STHs, concerns are emerging, fearing that regular and widespread MDA with the same BZA drugs may utilize selection pressure similar to the one witnessed in veterinary nematodes, favoring the emergence of drug resistance. Some of the non-

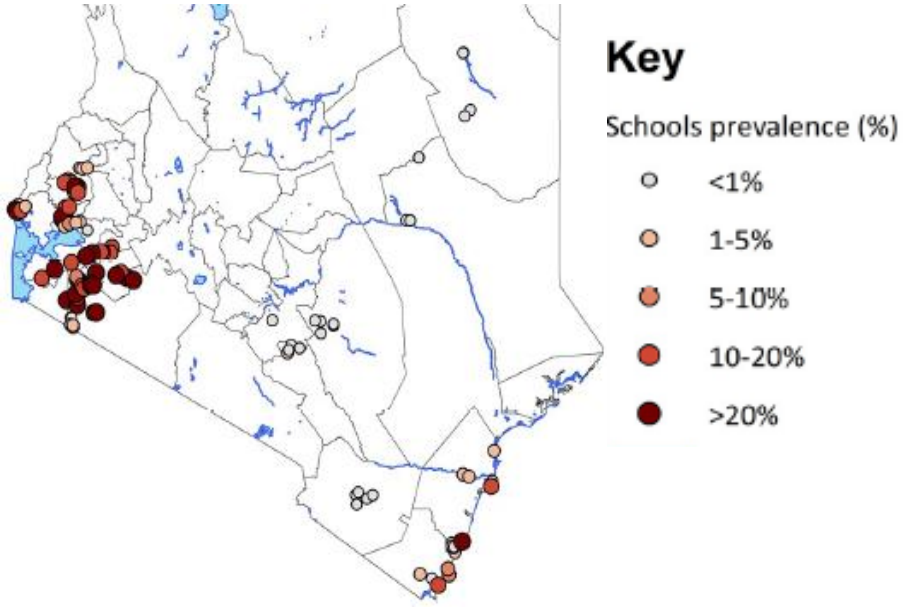
molecular parameters, such as reduced cure rate (CR) and reduction of fecal egg count (egg reduction rates, ERR) after BZ treatment, have been used in some studies to suggest the emergence of drug resistance in STH. Nonetheless, these key indicators, used for evaluating the efficacy of the drugs, lack satisfactory precision and are less accurate in determining resistance development (Diawara, *et al*., 2013). Consequently, molecular techniques have been employed to determine the SNPs associated with drug BZ resistance. These molecular methods involve DNA extraction from individual STH parasite eggs and genotyping of the beta-tubulin gene to check for polymorphisms at positions 200, 198, and 167 (Diawara,a *et al*., 2013). The mutant sequence of the parasite is then compared with the wild-type parasite.

CHAPTER THREE

METHODOLOGY

3.1 Study Area.

Samples for the study were obtained from two schools in Bungoma County (Bukirimo RC Primary School and Chongweyo Primary School). The two schools were purposively selected in consultation with the Division of Vector-Borne and Neglected Tropical Diseases (DVBNTD) at the Kenya Ministry of Health based on the prevalence data collected by the DVBNTD in preparation for the MDA exercise. According to previous research, Bungoma County in Western Kenya has a 7.3% prevalence of STH infections (Okoyo *et al*., 2020b). Over the last ten years, the county has been through multiple rounds of MDA targeting school-age children.



Adopted from (*Okoyo et al., 2020)*

Figure 3.1: Prevalence of the Combined Soil-Transmitted Helminths in 2017 after Five Years of MDA in Kenya

3.2 Study Design

This study was a longitudinal study undertaken in Bungoma County, western Kenya. Stool samples were collected one month before (October 2021) and two weeks after the deworming exercise (January 2022).

3.3 Study Population

School-age children from grade 1 to Class 6 were recruited into the study after obtaining parental consent through a sample opt-out form and children's verbal assent. This exercise was consistent with previous practice in studies conducted in the region (Okoyo *et al*., 2020a)

3.4 Inclusion Criteria

i. Primary SAC between grade one and class six;
ii. Resident in Bungoma County
iii. Children at risk of STH infections who assented to the study and whose parents had given consent were incorporated into the study.
iv. Children who had not taken ABZ or MBZ for the last six months.

3.5 Exclusion Criteria

i. Selected children who could not provide a stool sample during the collection time were excluded from the study.
ii. Selected children absent on the day of sample collection were also exempted from the study.
iii. Children whose parents didn't sign the sample opt out form were excluded.

3.6 Sample Size Determination

The sample size for the number of school children who were enrolled from each school was calculated using Fisher's formula below:

$$N = Z^2pq/d^2$$

Where;

n = desired sample size (where the population is greater than 10,000),

Z = the standard normal deviation set at 1.96, corresponding to the 95% confidence level,

p = the prevalence of STH parasites in the Bungoma county. An estimated 7% prevalence was utilized (Okoyo *et al.*, 2020)

q = 1 – p

d = degree of accuracy desired, usually set at 0.05.

Z=1.96, P=0.07, q=0.94, d=0.05.

$N= (1.96^2 \times 0.07\ (0.94)) \div 0.05^2$.

N= 100

A total of 414 samples were collected for the study. Two hundred and two samples were collected before the MDA exercise, 102 from Bukirimo RC Primary School and 100 from Chongeywo Primary School. Two hundred and twelve samples were collected after the MDA exercise, 106 from Bukirimo RC Primary School and 106 from Chongeywo Primary School.

3.7 Sampling Method

The two selected schools in Bungoma County were visited three days before the actual sample collection date; during this visit, the purpose of the study was explained to the head teacher. School-level permission was sought. Sample opt-out forms were given to the class teachers who gave them to the children. On the day of sample collection convenience sampling was used, every eligible child who met the inclusion criteria was provided with a sample container (polypot) labelled with a specific code assigned to each participant for anonymity and instructed on collecting approximately 25 grams of their stool sample. When receiving the collected samples, the age and gender of the children were recorded. The samples collected were treated as infectious and placed in a cooler box with ice packs and transported to the nearest Level IV hospital laboratory (Bumula Sub-County Hospital Laboratory for samples from Bukirimo RC Primary School and Chwele Sub-County Hospital Laboratory for samples from Chongweyo Primary School).

3.8 Laboratory Procedures

3.8.1 Stool Examination Procedures

Collected stool samples were examined for STH eggs using the Kato-Katz. Recovered eggs (using the sodium chloride floatation technique (NaCl).) were then preserved in 70% ethanol for subsequent DNA extraction.

3.8.2 Kato-Katz Technique

A standard *Kato-Katz* template (41.7mg) was placed on a microscope previously labelled with a corresponding sample identification number. Two slides were prepared per sample. Roughly 3 grams of a fresh fecal sample was scooped onto a piece of aluminum foil. The sample was then sieved using a wire mesh to remove the sizeable fecal debris. The sieved material was scooped using a spatula and used to fill the *Kato-Katz* template hole. The material was then levelled using a spatula to remove the excess sample. The *Kato-Katz* template was carefully lifted vertically to avoid disrupting the sample that adhered to the microscope slide. A cellophane tape previously soaked in Glycerol-malachite green solution for 24 hours was then used to cover the stool sample on the glass slide. The microscope slide was inverted and pressed over another clean slide to ensure an even distribution of the sample into a round, thick smear. To allow clearing of the fecal material, the smear was allowed a clearing time of thirty to sixty minutes, after which they were examined by two laboratory technologists independently. Each helminth species' number of helminth eggs was counted using a tally counter and recorded separately. The average number of eggs by the two independent technologists was used to calculate the fecal egg count. The final EPG was calculated by multiplying the mean egg count by a factor of 24 (WHO, 1994).

3.8.3 DNA Extraction from STH Eggs

Recovered helminth eggs were preserved in 70% ethanol in 1.5ml tubes at room temperature. A 1.5ml Eppendorf tube containing the preserved eggs was centrifuged at 5,000xg for 2 minutes, and the supernatant was removed by pipetting to remove the ethanol. The pellet was washed in two cycles of adding 1ml Phosphate

buffered saline (PBS), vortexed at 3500xg for 1 minute, and centrifuging at 5,000xg for 2 minutes. The supernatant was discarded by pipetting, and the pellet was re-suspended in 500µl of distilled water for DNA extraction from the eggs using a commercial kit by Meridian Bioscience® (Bioline ISOLATE II Genomic DNA Kit) according to the manufacturer's protocol with some modifications which included: initial step of freezing the eggs in liquid nitrogen for 10 minutes followed by thawing at 100°C for 10 minutes for five cycles. Next, 0.1mm Zirconia silica beating beads were added, and the sample vortexed at maximum speed (3500xg) for 5 minutes to mechanically break the egg shells.

3.8.4 PCR Amplification of *β*-Tubulin Gene

Extracted DNA was amplified to generate an amplicon covering the region where the SNPs at codon positions 167, 198 and 200 in the *β*-tubulin gene of *A. lumbricoides* are located. Positive control from adult *A. lumbricoides* and a negative control without any DNA were included in the amplification. Standard PCR for *A. lumbricoides* was done with Forward (5`-CCAGCTGACGCACTCGCTTGG-3`) and reverse (5`-ATGGTTGAGGTCTCCGTATGTG-3`) primers that were specific to the flanking regions as designed by Diawara *et al.*, (2013). The PCR reaction mix was prepared by adding 10x standard reaction buffer, 2.5µl 10nM dNTPs, 0.5µl Forward Primers, 0.5µl Reverse primers, 0.125µl Taq DNA polymerase, 5µl DNA template, and 15.875µl Nuclease free water to make a total volume of 25µl. PCR reactions were performed with the following cycling parameters: 95°C for 5 minutes followed by 30 cycles of 95°C for 45 seconds, 59°C for 45 seconds, and 72°C for 1 minute with a final extension step at 72°C for 5 minutes.

3.8.5 Detection of *β*-Tubulin 167, 198 and 200 SNPs

Following PCR, the generated amplicons were purified before sequencing using a commercial kit by Meridiam Bioscience® (Bioline ISOLATE II PCR and Gel Kit) according to the manufacturer's protocol. The total volume of the amplified DNA sample was adjusted to 50ul by adding PCR water. 100ul of binding buffer was then added and loaded to ISOLATE II PCR and GEL column. The column was then centrifuged at 11,000xg for 30 seconds after which the flow through was discarded.

700ul of the wash buffer was added to the column and centrifuged at 11,000xg for 30 seconds to wash the sample. The flow-through was discarded and the column centrifuge at 11,000xg for 30s to dry the membrane. DNA was then eluted by adding 30ul PCR water and incubating the sample for 1 minute followed by centrifuging at 11,00xg for 30seconds. A 20µl aliquot of each amplicon was submitted to the Sequencing, Genotyping and Oligosynthesis platform (Segolip) unit at The International Livestock Research Institute (ILRI) Kenya. Multiple sequence alignment was conducted online using Clustal Omega, and SNP frequencies were reported as a percentage.

3.9 Data Analysis

The prevalence (calculated with a 95% confidence interval using R statistical programming) of helminth infection according to the STH species was calculated as the proportion of those infected out of those examined. The intensity of STH infection was also enumerated and expressed as eggs per gram (EPG) and categorized according to WHO-proposed thresholds for classifying individuals with helminth infections (WHO, 2002). Using Chi-Square, the pre-MDA and post-MDA prevalence were compared. Prevalence by gender was also calculated and compared. Multiple sequence alignment was conducted online using Clustal Omega, and SNP frequencies were reported as a percentage.

3.10 Ethical Approval

Ethical approval for the study protocol was obtained from the Jomo Kenyatta University of Agriculture and Technology Ethical Review Committee under approval number **JKU/IERC/02316/0411**. A research license for the study was also obtained from NACOSTI under license number **NACOSTI/P/21/14436**. At the county level, approval was sought from the County Health and Education authorities. At school approval was obtained from the head teachers, parental consent was also obtained based on passive opt-out consent rather than written opt-in consent due to the routine and low-risk nature of the study procedure, consistent with the previous research on STH in SAC in Kenya. In addition, individual assent was obtained from each child before participation in the study.

CHAPTER FOUR

RESULTS

4.1 Prevalence of STHs Pre and Post-MDA.

Two hundred and two pre-MDA samples were collected from grade 1 to grade 6 children. The SAC age range was 8 to 15 years, with a mean age of 10.6 years (SD 1.65). The baseline STH prevalence pre-MDA was 33% (95% CI: 26.7-39.7), whereas that for *A. lumbricoides* was 31% (95% CI: 24.8-37.6). Two weeks post-MDA exercise, where the children were treated with MBZ, 212 stool samples were collected. The age range for the children was 7 to 15 years, and the mean age was 11.0 years (SD 1.60). The post-MDA STH prevalence dropped to 6% (95% CI: 2.6-8.8), while that of *A. lumbricoides* dropped to 4% (95% CI: 1.5-7) (Table 4.1). This study showed a significant difference in prevalence Pre and post-MDA $X^2 = 48.5$ P = <0.00001.

Table 4.1: Prevalence of Soil-Transmitted Helminths Infections Pre- and Post-MDA (N=414).

STH species	Pre-MDA, n (%)	Post-MDA, n (%)
Ascaris lumbricoides	63 (31)	9 (4)
Hookworms	2 (1)	2 (1)
Trichuris trichiura	0 (0)	0 (0)
Ascaris lumbricoides and *Trichuris trichiura*	1 (0.5)	0 (0)
Ascaris lumbricoides and Hookworms	1 (0.5)	1 (0.5)
No STH observed	135 (67)	200 (94)
TOTAL	202 (100)	212 (100)

Pre-MDA, Chongeywo primary school had a total prevalence of 51% with *A. lumbricoides* having a prevalence of 50%. The prevalence drastically dropped post-MDA to a total prevalence of 3% with *A. lumbricoides* being the only STH identified post-MDA (Table 4.2).

Table 4.2: Prevalence of Soil-Transmitted Helminths Infections Pre- and Post-MDA in Chongeywo Primary School (N=206).

STH species	Pre-MDA, n (%)	Post-MDA, n (%)
Ascaris lumbricoides	50 (50)	3 (3)
Hookworms	0 (0)	0 (0)
Trichuris trichiura	0 (0)	0 (0)
Ascaris lumbricoides and *T. trichiura*	1 (1)	0 (0)
Ascaris lumbricoides and Hookworms	0 (0)	0 (0)
No STH observed	49 (67)	103 (97)
TOTAL	100 (100)	106 (100)

Pre-MDA, Bukirimo RC primary school had a total prevalence of 16% with *A. lumbricoides* having a prevalence of 13%, followed by hookworms at 2%. The prevalence dropped post-MDA to a total prevalence of 5% post-MDA (Table 4.3).

Table 4.3: Prevalence of Soil-Transmitted Helminths Infections Pre- and Post-MDA in Bukirimo Primary School (N=208).

STH species	Pre-MDA, n (%)	Post-MDA, n (%)
Ascaris lumbricoides	13 (13)	6 (5)
Hookworms	2 (2)	2 (2)
Trichuris trichiura	0 (0)	0 (0)
Ascaris lumbricoides and *T. trichiura*	0 (0)	0 (0)
Ascaris lumbricoides and Hookworms	1 (1)	1 (1)
No STH observed	86 (84)	97 (92)
TOTAL	102 (100)	106 (100)

There was no significant difference in prevalence based on gender pre- and post-MDA $X^2 = 0.051$ P = 0.82 and $X^2 = 1.41$ P = 0.23, respectively.

Table 4.4: Prevalence of Soil-Transmitted Helminthes by Gender Pre- and Post-MDA (N=414).

STH species	Pre-MDA, n (%)		Post-MDA, n (%)	
	Male	**Female**	**Male**	**Female**
Ascaris lumbricoides	32 (31)	31 (31)	4 (4)	5 (5)
Hookworms	1 (1)	1 (1)	1 (1)	1 (1)
Trichuris trichiura	0 (0)	0 (0)	0 (0)	0 (0)
A. lumbricoides and *T. trichiura*	1 (1)	0 (0)	0 (0)	0 (0)
A. lumbricoides and Hookworms	1 (1)	0 (0)	1 (1)	0 (0)
No STH observed	67 (66)	68 (68)	98 (94)	102 (94)
TOTAL	102 (100)	100 (100)	104 (100)	108 (100)

Following stool sample analysis using the Kato-Katz technique, the intensity of infection (eggs per gram) was also calculated based on the WHO guidelines (World Health Organization, 2012). Most infections pre and post-MDA were light-intensity, with no heavy infections (Table 4.5).

Table 4.5: Infection Intensity of the Positive Samples Pre and Post-MDA (N 414).

STH species	Infection Intensity Pre-MDA, n, (%)			Post-MDA, n, (%)		
	Light[a]	Moderate[b]	Heavy	Light[a]	Moderate[b]	Heavy
A. lumbricoides	43(68)	20 (32)	0 (0)	6 (67)	3 (33)	0 (0)
T. trichiura	1(100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Hookworms	3(100)	0 (0)	0 (0)	3(100)	0 (0)	0 (0)

[a]Light infections: *A. lumbricoides* 1 – 4999 eggs per gram (epg), *T. trichura*: 1 – 999 epg, Hookworms 1 – 1999 epg

[b]Moderate infections: *A. lumbricoides* 5000 – 49,999 epg, *T. trichura* 1000 – 9,999 epg, Hookworms 2,000 – 3,999 epg

[c]Heavy infection: *A. lumbricoides* > 50,000 epg, *T. trichura*: > 10,000 epg, Hookworms > 4,000 epg

4.2 Identification of *B*-Tubulin SNPs Associated with Drug Resistance in *A. lumbricoides*

For the nine samples where *A. lumbricoides* infection persisted post-MDA, eggs were isolated from 8 samples using the sodium chloride floatation technique (NaCl). One sample was inadequate and was all used up in the Kato-Katz technique. For comparison, eight samples were selected from the positive samples pre-MDA and eggs isolated. Before DNA extraction using the commercial kit (Bioline ISOLATE II

Genomic DNA Kit), mechanical breakage of the egg shells was confirmed microscopically (Figure 4.1).

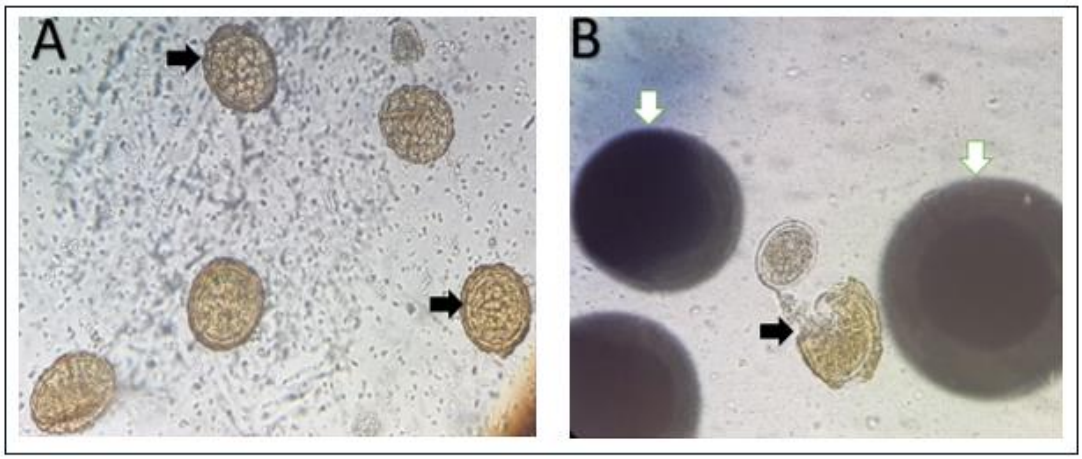


Figure 4.1: Microscopic Analysis of Stool Samples

(A) Showing intact *A. lumbricoides* eggs (black arrows) before the freeze-thawing and bead beating step. (B) Showing 0.1mm Zirconia silica beating beads (White arrows) and *A. lumbricoides* egg with a broken egg shell (black arrow) after the freeze-thawing and bead beating step.

After confirmation of mechanical breakage of the egg shells, genomic DNA was extracted and amplified by Standard PCR where 564bp amplicons surrounding the target codons 167, 198, and 200 of the *β*-tubulin gene of *A. lumbricoides*, associated with the drug-resistant phenotype were generated (Figure 4.2).

Figure 4.2: Agarose Gel Showing Amplified A. lumbricoides DNA.

L is the 1000bp ladder, P is the positive control, and N is the negative control, BA- Bukirimo Primary school post-MDA, CA- Chongeywo Primary school post-MDA CB- Chongeywo Primary school pre-MDA, BB- Bukirimo Primary school pre-MDA

Following Sanger sequencing to detect the presence of SNPs at these loci, sixteen sequences were generated covering codons 167, 198, and 200 of the *β*-tubulin gene of *A. lumbricoides*. Aligned results from the Sanger sequencing demonstrated the wild-type *A. lumbricoides* with 0% SNP frequency. All the three codons targeted (167, 198, and 200) were monomorphic for all the samples (Figure 4.3)

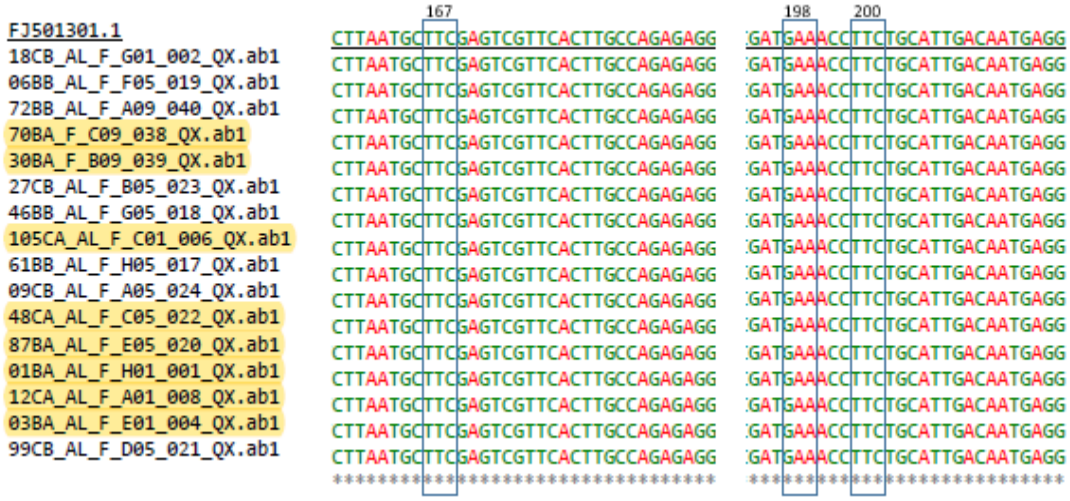


Figure 4.3: Multiple Sequence Alignments of β-tubulin Amplicon.

The first underlined sequence shows the wild-type A. lumricoides (GenBank accession number FJ501301.1) sequences surrounding the target codons 167, 198, and 200 of the β-tubulin gene. Highlighted sequences show the post-MDA sequences surrounding the target codons 167, 198, and 200 of the β-tubulin gene (BA- Bukirimo Primary school post-MDA, CA- Chongeywo Primary school post-MDA). Un-highlighted shows the pre-MDA sequences surrounding the target codons 167, 198, and 200 of the β-tubulin (CB- Chongeywo Primary school pre-MDA, BB- Bukirimo Primary school pre-MDA).

CHAPTER FIVE

DISCUSSION

The pre-MDA prevalence rate indicates a significant burden of STH infections in Bungoma County. This finding is in contrast with a previous studiy conducted in Kenya by (Okoyo *et al*., 2020a) which had observed a prevalence of 7.3%. Pre-MDA the prevalence at Chongeywo Primary School was way higher than the prevalence reported by the previous study. These rates underscore the urgent need for intervention strategies to control and reduce the transmission of STH.

Post-MDA, there was a significant decrease in prevalence, this suggests the effectiveness of the MDA program in reducing the burden of STH infections. This finding is consistent with studies conducted in other regions, such as those by (Ojja *et al*., 2018; Tefera *et al*., 2017), which demonstrated the impact of MDA on reducing STH prevalence rates. A study conducted in Kenya had also demonstrated similar findings (Mwandawiro *et al*., 2019). However, it's crucial to note that despite the significant reduction, a 6% prevalence rate is still high and highlights the importance of sustained intervention efforts. It is also of concern that despite there being a decline in the prevalence of *A. lumbricoides* and *T. trichiura*, there was no decline in the prevalence of Hookworms. This lack of decline may suggest MBZ being ineffective towards Hookworms.

The study further observed that *A. lumbricoides* was the most prevalent STH, accounting for 94% of the observed prevalence in the study area, followed by hookworms and *T. trichura*. This observation is consistent with a previous study in the region, which showed that *A. lumbricoides* was the most prevalent STH, followed by hookworms and *T. trichiura*, respectively (Mwandawiro *et al*., 2019). The findings are also consistent with the global statistics of STH, where *A. lumbricoides* account for the highest morbidity. There was no difference in prevalence based on gender both pre and post-MDA, similar finding have been reported in previous studies (Tinkler, 2020; Papaiakovou *et al*., 2019).

On a positive note, however, the study found that of all the positive infections in the area under study, none was a case of heavy infection intensity, and a majority of the

infections (77%) were light infections, suggesting a positive impact of the MDA control efforts in this setting. Studies conducted previously where MDA programs have been in place have shown a similar trend where there is a considerable shift of infection from heavy infection intensity towards moderate and light infection intensities (Ojja *et al*., 2018; Tefera *et al*., 2017). Infection intensity directly relates to the morbidity due to STHs(Farrell *et al*., 2018). Few worm burdens (light intensity) cause little or no infection amongst the people infected. One possible explanation for the lack of heavy infection intensities post-MDA is the effectiveness of mass drug administration programs in reducing the overall worm burden within the population. MDA aims to target both moderate and heavy infection intensities, thereby reducing the overall transmission of STH. The absence of heavy infections may indicate that MDA has successfully targeted individuals with higher parasite burdens, leading to a reduction in the prevalence of heavy infections within the study population.

The absence of heavy infection intensities may also be influenced by environmental and socioeconomic factors within the study area. Access to clean water and sanitation facilities, as well as hygiene practices, can play a crucial role in reducing the risk of heavy STH infections (Pickering, *et al*., 2019). Understanding the distribution of infection intensities within a population is essential for designing targeted control strategies. The absence of heavy infection intensities suggests that interventions focusing on reducing moderate infections may be sufficient for controlling STH transmission in the study area. However, it's important to continue monitoring infection intensities over time to detect any changes in transmission dynamics and adapt control strategies accordingly.

The study also checked for mutations in the *β*-tubulin gene for the positive samples post-MDA. *β*-tubulin is one of the two protein molecules that join together to make the microtubules; these microtubules are hollow microscopic tubes made up of a tubulin dimer (*β*-tubulin and alpha-tubulin) and form part of the cytoskeleton (Eyamo *et al*., 2019). The microtubules play critical roles in the cell: cell division, cell movement, and cell transport (Binarová & Tuszynski, 2019). *B*-tubulin protein molecules act as the target of the BZ, a group of compounds used to treat STHs, and MBZ, the drug used for MDA in this study, belongs (Makaula *et al*., 2022). These

drugs inhibit microtubule polymerization by interfering with cellular structures and functions. This interference leads to the death of the parasites (Lacey, 1988). Mutations to the *β*-tubulin molecule may confer the parasite's resistance against the BZ (Furtado *et al*., 2019).

The study analyzed all the sequences obtained from the DNA isolated from positive samples post-MDA and eight sequences obtained from randomly selected samples pre-MDA. The samples sequenced demonstrated the wild-type *A. lumbricoides* and all the targeted codons were monomorphic for all the samples with 0% SNP frequency. No *β*-tubulin SNPS associated with drug resistance in STH were identified. A recent study conducted in Honduras demonstrated similar results (Matamoros *et al*., 2019). It is noteworthy that even after over ten years of use of BZ drugs in the school-based deworming program, there is no evidence of drug resistance SNPs. This result supports the continued use of the current anti-helminthic drugs in this setting. However, in contrast, a previous study has identified high-frequency mutations and the *β*-tubulin gene of *A. lumbricoides*, especially at codon 167 (Diawara, *et al*., 2013). Different studies on BZ resistance in parasitic nematodes have indicated that this resistance occurs due to SNP in the *B*-tubulin gene. In these SNPs, amino acid phenylalanine is substituted by tyrosine, which occurs at codons 167 and 200. Likewise, the amino acid alanine replaces glutamate at codon 198. These mutations have often been associated with drug resistance in STHs (Makaula *et al*., 2022; Mutombo *et al*., 2019).

The lack of detectable SNPs could imply a relatively homogeneous STH population in the study area, possibly due to factors such as limited gene flow or selective pressures exerted by anthelmintic treatment. The absence of detectable SNPs highlights the need for further research to understand the genetic diversity and population dynamics of STH in different geographical contexts. Additionally, future studies could explore the impact of other factors, such as environmental conditions and host immunity, on STH transmission dynamics.

With widespread resistance to BZ in veterinary nematodes having been reported since their first use in the early 1960s and their resistance having been reported in 1990 (Cheng *et al*., 2016; Potârniche *et al*., 2021), it is encouraging to find no such mutations

in human nematodes after their usage for several decades having first been used in humans in 1974(MBZ) and 1982 (ABZ) (Chai *et al*., 2021; Horton, 2000).

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

1. The Pre-MDA prevalence rate was high and highlights the importance of sustained intervention efforts.
2. The prevalence significantly dropped Post-MDA, this suggests the effectiveness of the MDA program in reducing the burden of STH infections in the study area.
3. There were no identified b-tubulin single nucleotide polymorphisms linked to drug resistance in the study area. This highlights the need for further research to understand the genetic diversity and population dynamics of STH in the study area.

6.2 Recommendations

1. Continued monitoring of prevalence of soil transmitted helminthes among school age children in Bungoma county Kenya, this is essential to ensure sustained STH control efforts.
2. Sustain the Mass Drug Administration program and possibly expand it to the community level to further reduce the prevalence of the STHs in Bungoma.
1. Continued genetic surveillance of STHs to monitor for the emergence of any drug resistance SNPs.

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APPENDICES

Appendix I : Sample Opt-Out Form (English).

Dear [Parent/Guardian]

I am Peterson Maingi, a master's student at Jomo Kenyatta University of Agriculture and Technology. I am collaborating with the Ministry of Health and The Ministry of Education to conduct research titled "DETECTION **OF *B*-TUBULIN SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS) ASSOCIATED WITH DRUG RESISTANCE AMONG CHILDREN INFECTED WITH SOIL-TRANSMITTED HELMINTHS IN BUNGOMA, KENYA"**

The research involves evaluating eggs of parasitic worms isolated from stool for mutations leading to drug resistance to the drugs commonly used for their treatment. We will be collecting stool samples from your son/daughter's school on _____/202__ and we plan to involve your son/daughter.

If you do not wish your son/daughter to take part in the study, kindly sign this letter and let your son return it to their class teacher by _____/202__.

Yours faithfully

Peterson Maingi

Peterson Maingi will conduct the above study at my son/daughter's school. I **do not** wish my son/daughter to be included in this study.

Signed

(Parent/Guardian)

_____

Please return this form as soon as possible if you do not wish your son or daughter to participate in the study.

Appendix II: Ufafanuzi wa Idhini (Kiswahili).

Mpendwa [Mzazi / Mlezi]

Mimi ni Peterson Maingi mwanafunzi wa Chuo Kikuu cha Kilimo na Teknolojia cha Jomo Kenyatta. Ninafanya kazi kwa kushirikiana na Wizara ya Afya na Wizara ya Elimu kufanya utafiti uliopewa Jina la "**DETECTION OF *B*-TUBULIN SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS) ASSOCIATED WITH DRUG RESISTANCE AMONG CHILDREN INFECTED WITH SOIL-TRANSMITTED HELMINTHS IN BUNGOMA, KENYA "** Utafiti huu unajumuisha kutathmini mayai ya minyoo ya vimelea iliyotengwa kutoka kinyesi kwa mabadiliko ya uwepo yanayosababisha upinzani wa dawa kwa dawa zinazotumiwa kwa matibabu yao. Tutakusanya sampuli za kinyesi kutoka shule ya mwanao / binti yako mnamo _____ / 202__ na tunapanga kumshirikisha mwanao / binti yako.

Ikiwa hutaki mwanao / binti yako kushiriki katika utafiti, saini barua hii na umruhusu mwanao / binti yako airudishe kwa mwalimu wao wa darasa ifikapo _____ / 202__.

Wako mwaminifu

Peterson Maingi

Ninaelewa kuwa Peterson Maingi atakuwa akifanya masomo hayo hapo juu katika shule ya mtoto wangu. Sitamani mwana / binti yangu kujumuishwa katika utafiti huu.

Imesainiwa_____ (Mzazi / Mlezi)

<u>Tafadhali rudisha fomu hii haraka iwezekanavyo ikiwa hutaki mwanao au binti yako kushiriki katika utafiti huu.</u>

Appendix III: Kato-Katz Technique Bench-Top Protocol.

World Health Organization

Bench aids for the diagnosis of intestinal parasites, second edition

Plate 6
Kato-Katz

Cellophane faecal thick smear

Kato–Katz technique for soil-transmitted helminths

The Kato–Katz technique is the diagnostic method recommended for monitoring large-scale treatment programmes implemented for the control of soil-transmitted helminth infections because of its simple format and ease of use in the field. Alternative techniques are McMaster and FLOTAC.

Commercial diagnostic kits are available for immediate use in the field.

Materials and reagents

1. Wooden applicator sticks.
2. Screen (stainless steel, nylon or plastic: 60-105 µm mesh) (Fig. 1).
3. Template (stainless steel, plastic, or cardboard) (Fig. 1). A hole of 9 mm on a 1 mm thick template will deliver about 50 mg of faeces; a hole of 6 mm on a 1.5 mm thick template, 41.7 mg; and a hole of 6.5 mm on a 0.5 mm thick template, 20 mg. The same size of templates should always be used to ensure repeatability and comparability of prevalence and intensity data.
4. Spatula (plastic) (Fig. 1).
5. Microscope slides (75 x 25 mm).
6. Hydrophilic cellophane (40–50 µm thick, strips 25 x 30 or 25 x 35 mm in size).
7. Flat-bottom jar with lid, forceps and toilet paper or absorbent tissue.
8. Newspaper.
9. Glycerol-malachite green (1 mL of 3% aqueous malachite green is added to 100 mL of glycerol and 100 ml of distilled water and mixed well). This solution is poured onto the cellophane strips in a jar and left for at least 24 hours prior to use.

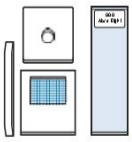


Fig. 1


Fig. 2


Fig. 3


Fig. 4


Fig. 5


Fig. 6

Procedure

1. Place a small amount of the faecal sample on a newspaper and press a piece of nylon screen on top. Using a spatula, scrape the sieved faecal material from the screen (Fig. 2).
2. Label a glass slide with the sample number and place a template with hole on the centre of a microscope slide. Fill the hole in the template with the sired faecal material, avoiding air bubbles and levelling the faeces off to remove any excess material (Fig. 3).
3. Carefully lift off the template and place it in a bucket of water mixed with concentrated detergent and disinfectant so that it can be reused.
4. Place one piece of cellophane, which has been soaked overnight in glycerol solution, over the faecal sample (Fig. 4).
5. Invert the microscope slide (Fig. 5) and firmly press the sample against the cellophane strip on another microscope slide or on a smooth hard surface to spread the faeces in a circle (Fig. 6).
6. Carefully pick up the slide again by gently sliding it sideways to avoid separating the cellophane strip or lifting it off. Place the slide on the bench with the cellophane upwards. Water evaporates while glycerol clears the faeces. When clarified it should be possible to read newspaper print through the stool smear (Fig. 7).
7. For all except hookworm eggs, keep the slide for one or more hours at room temperature to clear the faecal material prior to examination under the microscope. To speed up clearing and examination, the slide can be placed in a 40 °C incubator or kept in direct sunlight for several minutes.
8. *A. lumbricoides* and *T. trichiura* eggs will remain visible and recognizable for many months. Hookworm eggs clear rapidly and will no longer be visible after 30–60 minutes. Schistosome eggs may be recognizable for up to several months but it is preferable to examine the slide preparations within 24 hours.
9. The smear should be examined systematically. Then, multiply by the appropriate number to give the number of eggs per gram of faeces (by 20 if using a 50 mg template; by 50 for a 20 mg template; and by 24 for a 41.7 mg template).

Appendix IV: Jomo Kenyatta University of Agriculture and Technology Ethical Approval.

JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY
P.O BOX 62000(00200) NAIROBI, Tel:(067) 58700001-4
(Office of the Deputy Vice Chancellor, Research Production and Extension Division)

JKUAT INSTITUTIONAL ETHICS REVIEW COMMITTEE

REF: JKU/2/4/896B **Date: 3rd March 2022**

MAINGI PETERSON MACHARIA
DEPARTMENT OF MEDICAL LABORATORY SCIENCES, JKUAT

Dear Mr. Maingi,

RE: DETECTION OF β-TUBULIN SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs) ASSOCIATED WITH DRUG RESISTANCE AMONG CHILDREN INFECTED WITH SOIL TRANSMITTED HELMINTHS IN BUNGOMA, KENYA

This is to inform you that JKUAT Institutional Ethics Review Committee has reviewed and approved your request for amendment to read as above and change of study sites to include Bungoma County only in line with your earlier ethics application approval number **JKU/IERC/02316/0411** dated 28th October 2021.

This approval is subject to compliance with the following requirements;

i. Only approved documents including (informed consents, study instruments, MTA) will be used
ii. All changes including (amendments, deviations, and violations) are submitted for review and approval by JKUAT IERC.
iii. Death and life threatening problems and serious adverse events or unexpected adverse events whether related or unrelated to the study must be reported to JKUAT IERC within 72 hours of notification
iv. Any changes, anticipated or otherwise that may increase the risks or affected safety or welfare of study participants and others or affect the integrity of the research must be reported to JKUAT IERC within 72 hours
v. Clearance for export of biological specimens must be obtained from relevant institutions.
vi. Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. Attach a comprehensive progress report to support the renewal.
vii. Submission of an executive summary report within 90 days upon completion of the study to JKUAT IERC.

Prior to commencing your study, you will be expected to obtain a research license from National Commission for Science, Technology and Innovation (NACOSTI) https://oris.nacosti.go.ke and also obtain other clearances needed.

Yours sincerely

Dr Patrick Mburugu
Chair, JKUAT IERC

INSTITUTIONAL ETHICS REVIEW COMMITTEE
03 MAR 2022
APPROVED
P.O. Box 62000-00200 NAIROBI
www.jkuat.ac.ke
J K U A T

Appendix VIII: NACOSTI Research License.

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY & INNOVATION

Ref No: **903984**

Date of Issue: **20/November/2021**

RESEARCH LICENSE

This is to Certify that Mr.. Peterson Macharia Maingi of Jomo Kenyatta University of Agriculture and Technology, has been licensed to conduct research in Bungoma, Kiambu, Kwale on the topic: Evaluation of ?-Tubulin Single nucleotide polymorphisms (SNPs) associated with drug resistance in soil-transmitted helminths for the period ending : 20/November/2022.

License No: **NACOSTI/P/21/14436**

903984

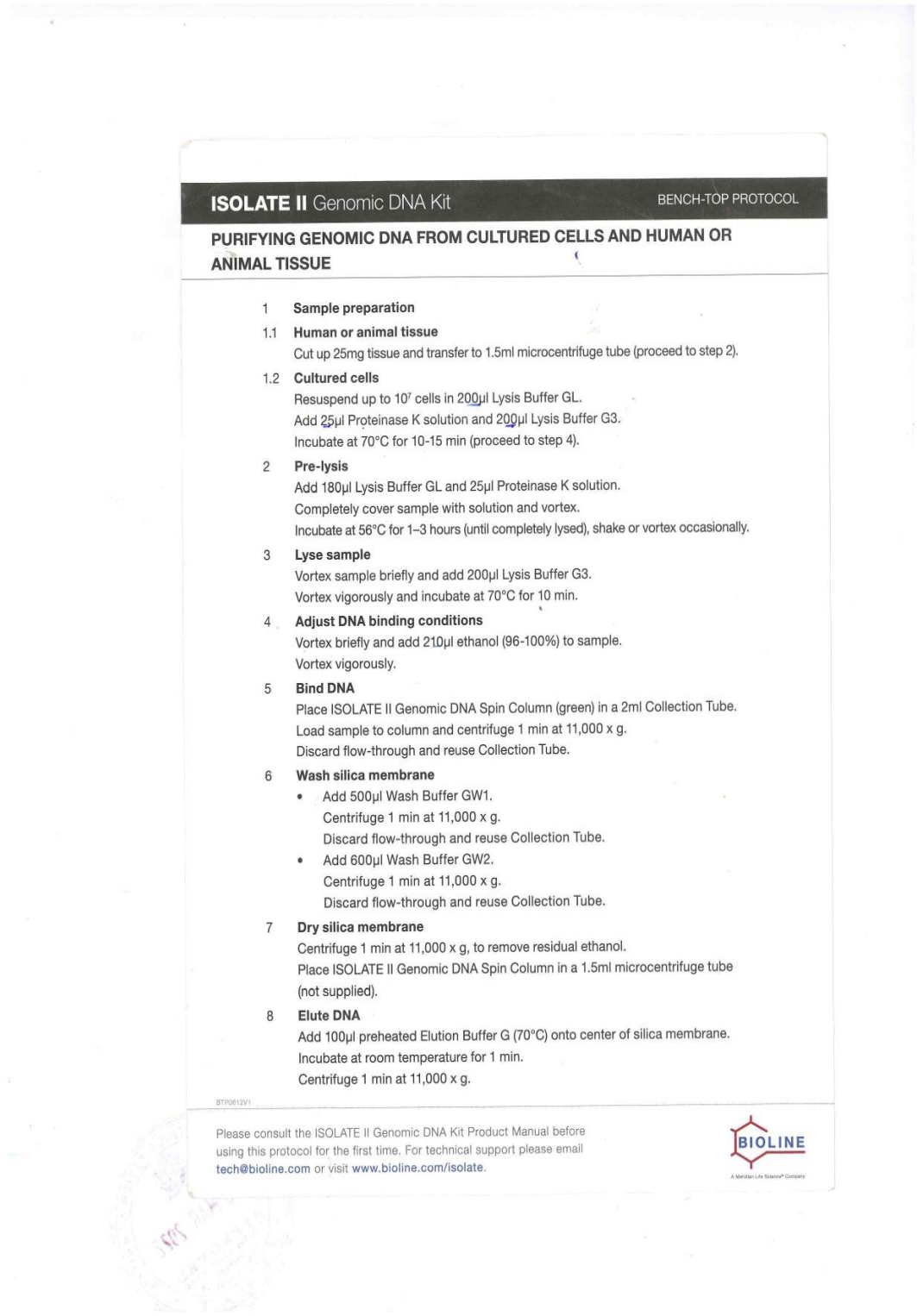
Applicant Identification Number

Director General
NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY & INNOVATION

Verification QR Code

NOTE: This is a computer generated License. To verify the authenticity of this document, Scan the QR Code using QR scanner application.

Appendix VI: Bioline DNA Extraction Bench-Top Protocol.



ISOLATE II Genomic DNA Kit — BENCH-TOP PROTOCOL

PURIFYING GENOMIC DNA FROM CULTURED CELLS AND HUMAN OR ANIMAL TISSUE

1 **Sample preparation**

1.1 **Human or animal tissue**
Cut up 25mg tissue and transfer to 1.5ml microcentrifuge tube (proceed to step 2).

1.2 **Cultured cells**
Resuspend up to 10^7 cells in 200µl Lysis Buffer GL.
Add 25µl Proteinase K solution and 200µl Lysis Buffer G3.
Incubate at 70°C for 10-15 min (proceed to step 4).

2 **Pre-lysis**
Add 180µl Lysis Buffer GL and 25µl Proteinase K solution.
Completely cover sample with solution and vortex.
Incubate at 56°C for 1–3 hours (until completely lysed), shake or vortex occasionally.

3 **Lyse sample**
Vortex sample briefly and add 200µl Lysis Buffer G3.
Vortex vigorously and incubate at 70°C for 10 min.

4 **Adjust DNA binding conditions**
Vortex briefly and add 210µl ethanol (96-100%) to sample.
Vortex vigorously.

5 **Bind DNA**
Place ISOLATE II Genomic DNA Spin Column (green) in a 2ml Collection Tube.
Load sample to column and centrifuge 1 min at 11,000 x g.
Discard flow-through and reuse Collection Tube.

6 **Wash silica membrane**
- Add 500µl Wash Buffer GW1.
  Centrifuge 1 min at 11,000 x g.
  Discard flow-through and reuse Collection Tube.
- Add 600µl Wash Buffer GW2.
  Centrifuge 1 min at 11,000 x g.
  Discard flow-through and reuse Collection Tube.

7 **Dry silica membrane**
Centrifuge 1 min at 11,000 x g, to remove residual ethanol.
Place ISOLATE II Genomic DNA Spin Column in a 1.5ml microcentrifuge tube (not supplied).

8 **Elute DNA**
Add 100µl preheated Elution Buffer G (70°C) onto center of silica membrane.
Incubate at room temperature for 1 min.
Centrifuge 1 min at 11,000 x g.

BTP0812V1

Please consult the ISOLATE II Genomic DNA Kit Product Manual before using this protocol for the first time. For technical support please email tech@bioline.com or visit www.bioline.com/isolate.

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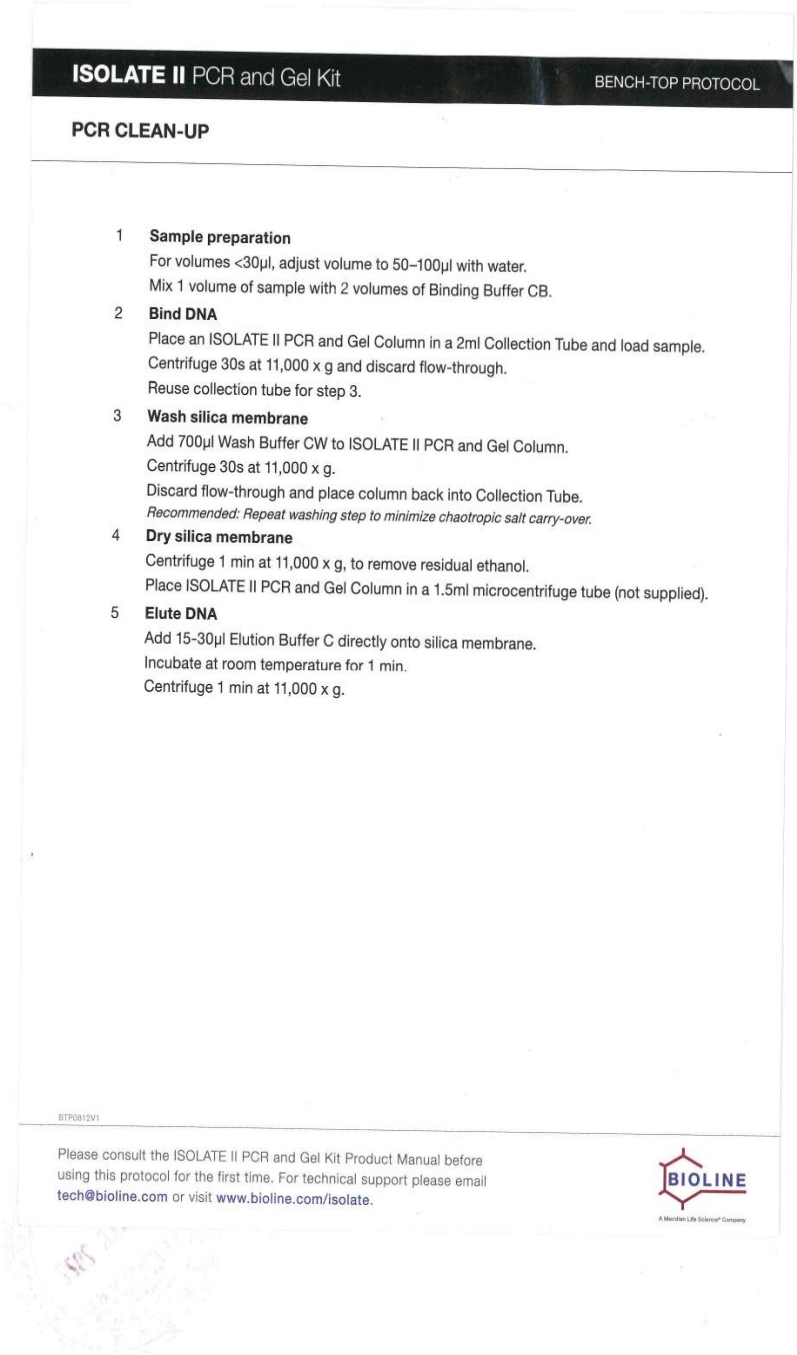
Appendix VII: Bioline DNA Purification Bench-Top Protocol.



ISOLATE II PCR and Gel Kit — BENCH-TOP PROTOCOL

PCR CLEAN-UP

1 **Sample preparation**
For volumes <30µl, adjust volume to 50–100µl with water.
Mix 1 volume of sample with 2 volumes of Binding Buffer CB.

2 **Bind DNA**
Place an ISOLATE II PCR and Gel Column in a 2ml Collection Tube and load sample.
Centrifuge 30s at 11,000 x g and discard flow-through.
Reuse collection tube for step 3.

3 **Wash silica membrane**
Add 700µl Wash Buffer CW to ISOLATE II PCR and Gel Column.
Centrifuge 30s at 11,000 x g.
Discard flow-through and place column back into Collection Tube.
Recommended: Repeat washing step to minimize chaotropic salt carry-over.

4 **Dry silica membrane**
Centrifuge 1 min at 11,000 x g, to remove residual ethanol.
Place ISOLATE II PCR and Gel Column in a 1.5ml microcentrifuge tube (not supplied).

5 **Elute DNA**
Add 15-30µl Elution Buffer C directly onto silica membrane.
Incubate at room temperature for 1 min.
Centrifuge 1 min at 11,000 x g.

BTP0812V1

Please consult the ISOLATE II PCR and Gel Kit Product Manual before using this protocol for the first time. For technical support please email tech@bioline.com or visit www.bioline.com/isolate.

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Appendix VIII: Published Article

Journal of Advances in Medicine and Medical Research

Volume 35, Issue 20, Page 292-301, 2023; Article no.JAMMR.106051
ISSN: 2456-8899
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Absence of β-tubulin SNPs Associated with Drug Resistance in *Ascaris lumbricoides* Infections of School-Age Children in Bungoma County, Kenya

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[b] Neglected Tropical Diseases Unit, Centre for Global Health Research, Kenya Medical Research Institute, Kisumu, Kenya.
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Authors' contributions

This work was carried out in collaboration among all authors. Author AM conceptualized the study. Authors PMM, AM and WWA contributed to development of methodology, sample collection and preliminary analysis. Authors AM and MRO supervised the study. Authors AM and PMM conducted data analysis wrote the original draft preparation. Author AM was involved in project administration. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: This study compared the prevalence of *Ascaris lumbricoides* infections pre- and post-deworming in school-age children in Bungoma County, western Kenya, to detect β-tubulin gene single nucleotide polymorphisms (SNPs) associated with drug resistance in these infections.

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