

Diagnostics and impacts of soil-transmitted helminth infections among populations from South-East Asia

by

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Table of Contents

Preface.....	6
I. Introduction – Literature review	6
II. Research aim 1 – Development and validation of a multiplexed-tandem qPCR tool for diagnostics of human soil-transmitted helminth infections	6
III. Research aim 2 – Effective low-cost preservation of human stools in field-based studies for helminth and microbiome analysis	6
IV. Research aim 3 – Soil-transmitted helminth beta-tubulin 1 amplicon sequencing for the detection of drug resistance associated SNPs in human populations	6
V. Summary – Importance of study and future directions	6
Acknowledgements	8
List of Manuscripts	10
Conference Presentations	11
Abstract	12
Abbreviations	14
List of Figures	15
List of Tables	17
List of Supplementary Figures	18
List of Supplementary Tables	18
Chapter 1 – Literature review	19
1. Overview of soil-transmitted helminth infections	19
2. Life cycles of the soil-transmitted helminths.....	20
2.1. Roundworms – <i>Ascaris spp.</i>	24
2.2. Whipworm – <i>Trichuris trichiura</i>	24
2.3. Hookworm – <i>Necator americanus</i> and <i>Ancylostoma spp.</i>	25
3. Global burden of STHs on human health	27
3.1. Acute symptomology	28
3.2. Chronic symptomology	31

4.	Global prevalence and disease burden of soil-transmitted helminths	37
4.1.	<i>STH prevalence and disease burden in South-East Asia</i>	37
4.1.1.	<i>Thailand</i>	39
4.1.2.	<i>Cambodia</i>	40
4.1.3.	<i>Vietnam</i>	41
4.1.4.	<i>Timor-Leste</i>	41
5.	Control of soil-transmitted helminth infections	43
5.1.	<i>Mass drug administration</i>	43
5.2.	<i>Emergence of benzimidazole drug resistance</i>	45
5.3.	<i>Water, sanitation and hygiene interventions</i>	47
6.	Diagnostics of soil-transmitted helminth infections	50
6.1.	<i>Microscopy-based methods</i>	50
6.2.	<i>Molecular-based methods</i>	51
7.	Thesis aims	54

Chapter 2 – Development and validation of a multiplexed-tandem qPCR tool for diagnostics of human soil-transmitted helminth infections		55
8.	Context	55
9.	Publication I	56
10.	Conclusion and future directions	82

Chapter 3 – Effective low-cost preservation of human stools in field-based studies for helminth and microbiome analysis		83
11.	Context	83
12.	Manuscript II	84
12.1.	<i>Abstract</i>	85
12.2.	<i>Importance</i>	86
12.3.	<i>Introduction</i>	87
12.4.	<i>Methods</i>	90
12.5.	<i>Results</i>	100
12.6.	<i>Discussion</i>	116

12.7. <i>Acknowledgements</i>	121
12.8. <i>Supplementary files</i>	122
13. Conclusion and future directions	123

Chapter 4 – Soil-transmitted helminth beta-tubulin 1 amplicon sequencing for the detection of drug resistance associated SNPs in human populations..... 124

14. Context.....	124
15. Manuscript III	125
15.1. <i>Abstract</i>	126
15.2. <i>Introduction</i>	127
15.3. <i>Materials and Methods</i>	130
15.4. <i>Results</i>	139
15.5. <i>Discussion</i>	146
15.6. <i>Acknowledgements</i>	149
15.7. <i>Supplementary files</i>	149
16. Conclusion and future directions	150

Chapter 5 – General discussion 151

17. Thesis outcomes.....	151
18. Reflections and future research.....	157

Appendix 163

Bibliography 174

Thesis Declaration

This declaration is to certify that:

- the thesis comprises only my original work towards the PhD except where indicated in the preface,
- due acknowledgement has been made in the text to all other material used,
- and the thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Katharina Stracke

Preface

- I. Introduction** – Literature review
- II. Research aim 1** – Development and validation of a multiplexed-tandem qPCR tool for diagnostics of human soil-transmitted helminth infections
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- IV. Research aim 3** – Soil-transmitted helminth beta-tubulin 1 amplicon sequencing for the detection of drug resistance associated SNPs in human populations
- V. Summary** – Importance of study and future directions

The work for this PhD thesis was undertaken at the Walter and Eliza Hall Institute for Medical Research in the laboratory of Associate Professor Aaron Jex (Melbourne, Australia). A field visit was undertaken to the Tha Song Yang district, Tak province in collaboration with Associate Professor Poom Adisakwattana from the Faculty for Helminthology at Mahidol University (Bangkok, Thailand). Funding, including that for travel, was provided from the Melbourne Research Scholarship (The University of Melbourne), Riady Scholarship (private funding entity), the Researcher Exchange, Travel and Training Award, and Student Conference Travel Award (Australian Society for Parasitology Inc.).

Publications appear in part and in complete original format within the body of this thesis. My individual contributions to these publications are as follows below. I acknowledge the contributions of the following individuals to the work in this thesis, as declared in the collaborator's authorisation forms.

Chapter 2. Published by Stracke *et al.*, in PLoS Neglected Tropical Diseases on 17th June 2019 (75%)

Dr Naomi Clarke Provided Timor-Leste samples and critiqued manuscript

Ms Camille Awburn Assisted with experimental design and initial assay testing

A/Prof Susana Vaz Nery Critiqued manuscript

Dr Virak Khieu Provided Cambodian samples and critiqued manuscript

Prof Rebecca Traub Critiqued manuscript

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Dr Stephen Wilcox Assisted with experimental design

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List of Manuscripts

The following scientific papers were published or are in preparation as part of the here presented work:

- Peer-reviewed original research article “Development and validation of a multiplexed-tandem qPCR tool for diagnostics of human soil-transmitted helminth infections” (published by Stracke *et al.*, in *PLoS Neglected Tropical Diseases* on 17th June 2019)
- Original research article “Effective low-cost preservation of human stools in field-based studies for microbiome and STH infection analysis” (submitted for publication by Stracke *et al.*, in *Gut Microbes* on 1st March 2020)
- Original research article “Soil-transmitted helminth beta-tubulin 1 amplicon sequencing for detection of drug resistance associated SNPs in human populations” (unpublished material not submitted for publication)
- Peer-reviewed mini-review article “Zoonotic ancylostomiasis – an update of a continually neglected zoonosis” (accepted for publication by Stracke *et al.*, in *American Journal for Tropical Medicine and Hygiene* on 16th March 2020)

Conference Presentations

The following scientific conference contributions were published as part of the work presented here:

- Australian Society for Parasitology Inc. 15-minute conference talk 2017 with the title “Development and validation of a multiplexed-tandem qPCR tool for detection of human soil-transmitted helminth infections”
- Australian Society for Parasitology Inc. 3-minute speed poster presentation and poster presentation 2018 with the title “Effective, low-cost preservation of human stools for nucleic acid extraction, helminth detection and microbiome investigation”
- Joint International Tropical Medicine Meeting 15-minute invited speaker conference talk 2018 with the title “Effective, low-cost preservation of human stools for nucleic acid extraction, helminth detection and microbiome investigation”
- Australian Society for Parasitology Inc. 5-minute conference talk 2019 with the title “Soil-transmitted helminth beta-tubulin 1 amplicon sequencing for detection of drug resistance associated SNPs in human populations”
- Victorian Infection and Immunity Network (VIIN) Young Investigator Symposium 2019 10-minute oral presentation with the title “Effective, low-cost preservation of human stools for gut microbiome investigation in a helminth endemic region”

Abstract

Gastrointestinal parasitic infections, in particular soil-transmitted helminths (STHs), are a major cause of morbidity in tropical developing countries worldwide. Globally, infections are estimated to cause disease-related morbidity of approximately 3.4 million disability adjusted life years and an infection prevalence of around 1.5 billion people. More than 3.5 million people manifest acute clinical symptoms, although the majority of infections represent asymptomatic cases. Severe infections cause acute clinical symptoms including malnutrition, abdominal pain, diarrhoea, fever, skin conditions and iron deficiency anemia. Most importantly, long-term sequelae in pre-school and school-aged children and pregnant women can lead to stunting, wasting and an impaired cognitive development. Inadequate sanitation, impoverished conditions, poor public health systems and population overcrowding all contribute to high infection prevalence levels. STH infections occur predominantly in sub-tropical to tropical regions worldwide, such as South America, Sub-Saharan Africa and South-East Asia. Diagnosis is confirmed by microscopy-based tools (Kato Katz Thick Smear method), which is both, cost-effective and applicable in remote settings, but has significant drawbacks in terms of labour intensity and diagnostic accuracy. The World Health Organization recommends annual or biannual mass drug administration of the front-line benzimidazole drugs, albendazole and mebendazole, to reduce overall infection prevalence and disease-related morbidity. However, there is an increasing concern that ongoing mass drug administration may lead to the emergence of drug resistance. There is an urgent need for accurate and streamlined diagnosis of STH infections, in particular among subpopulations harbouring low-intensity infections in order to effectively treat populations at risk. Additionally, an improved evaluation of drug treatment efficacy including a subsequent monitoring

of potentially emerging drug resistance is crucial for future studies. A global multi-factorial intervention approach is required to effectively move towards transmission interruption and ultimately eradication of STH infections.

The here presented thesis has resulted in the successful development of a molecular diagnostic tool for qualitative and quantitative estimation of STH infections. A novel, semi-automated qPCR-based tool (multiplexed-tandem qPCR (MT-PCR)) was developed and validated using two cross-sectional cohorts from South-East Asia (Timor-Leste, Cambodia). The MT-PCR subsequently was used to investigate the infection prevalence and intensity in a cross-sectional cohort study of Karen ethnic pre-school and school-aged children from the Tha Song Yang district, Tak province, in Thailand. Furthermore, the effectiveness of three different preservation methods for faecal samples was compared in terms of soil-transmitted helminth infection recovery and gut microbiome characterisation. The impacts of infection on overall and gut microbial health were investigated. Thirdly, faecal DNA samples from three cohorts (Cambodia, Vietnam, Thailand) were screened for single nucleotide polymorphisms at codons Phe167Tyr, Glu198Ala and Phe200Tyr, that may be associated with the emergence of benzimidazole drug resistance. Using this amplicon sequencing approach, the variant allele frequency of the beta tubulin isotype 1 gene was analysed.

Abbreviations

ALB	Albendazole
<i>A. ceylanicum</i>	<i>Ancylostoma ceylanicum</i>
<i>A. duodenale</i>	<i>Ancylostoma duodenale</i>
<i>A. lumbricoides</i>	<i>Ascaris lumbricoides</i>
BZ	Benzimidazole
Ct-value	Cycle threshold value
DALY	Disability-adjusted life years
DESS	DMSO, EDTA and saturated NaCl
Epg	Eggs per gram (faeces)
FF	Fresh frozen
KKTS	Kato Katz Thick Smear
MEB	Mebendazole
<i>N. americanus</i>	<i>Necator americanus</i>
NTD	Neglected tropical disease
MDA	Mass drug administration
MT-PCR	Multiplexed-tandem polymerase chain reaction
PC	Preventive chemotherapy
PD	2.5% potassium dichromate
qPCR	Quantitative polymerase chain reaction
SEA	South-East Asia
SNP	Single nucleotide polymorphism
STH	Soil-transmitted helminths
<i>T. trichiura</i>	<i>Trichuris trichiura</i>
WHO	World Health Organization

List of Figures

Figure 1.1. Global proportion of 1 – 14-year-old children requiring preventive chemotherapy for soil-transmitted helminth infections in 2014.....	20
Figure 1.2. General soil-transmitted helminth life cycle	21
Figure 1.3. Map of soil-transmitted helminth studies conducted in South-East Asia ...	38
Figure 2.1 Overall infection prevalence of the major STH species comparing multiplexed qPCR and MT-PCR diagnostic approaches in cohorts from Timor-Leste and Cambodia	72
Figure 2.2. Cycle threshold value scatterplot for all investigated STH species showing agreement of multiplexed qPCR and MT-PCR.....	76
Figure 3.1. Hierarchical clustering of 48 trial samples preserved in DESS, PD or FF with corresponding bacterial gut community structure as per phyla and genera	104
Figure 3.2. Alpha-diversity measure as per observed richness and inverted Simpson measure. Beta-diversity measure as calculated by principal coordinate analysis (PCoA) with Bray-Curtis distance clustering and non-metric multidimensional scaling (NMDS) using weighted UniFrac distance measure	105
Figure 3.3. Bacterial gut community abundance to phyla and genera level, hierarchical clustering, STH infection status by MT-PCR and microscopy, gender, age and faecal score.....	112
Figure 3.4. Significant increased and decreased abundance of operational taxonomic units as per log fold change influenced by STH infection status as per MT-PCR and sex	113
Figure 3.5. Alpha-diversity with analysis of variance statistics of the complete cohort of faecal samples preserved in DESS	114
Figure 3.6. Beta-diversity measure as calculated by principal coordinate analysis (PCoA) with Bray-Curtis distance clustering and non-metric multidimensional scaling (NMDS)	

using weighted UniFrac distance measure of the complete cohort of faecal samples preserved in DESS..... 115

Figure 4.1. Mean Shannon’s entropy measures per amplicon, STH species and cohort 143

Figure 4.2. Baseline and follow-up variant allele frequency of *Necator americanus* positive samples from Cambodia and Vietnam..... 144

Figure 4.3. Variant allele frequencies of *Trichuris trichiura* and *Ascaris lumbricoides* positive samples from Thailand and species-specific variant allele frequencies and minor allele frequencies for all cohorts..... 145

List of Tables

Table 1.1 Summary of life cycle characteristics, disease burden and symptoms caused by STH infections.....	22
Table 2.1. 2x2 contingency table for any STH infections	73
Table 2.2. Interrater reliability (Cohen’s kappa, κ) including standard error, 95% confidence interval (CI) and significance; and coefficient of determination for qPCR vs MT-PCR	75
Table 3.1. STH infection prevalence and intensity among SAC children from Tha Song Yang, Tak district, Thailand including all DESS preserved samples.....	109
Table 4.1. Species-specific primers with and without adapter overhangs targeting the beta-tubulin isotype 1 gene of human soil-transmitted helminth species (<i>Ascaris lumbricoides</i> , <i>Trichuris trichiura</i> , <i>Necator americanus</i>)	134
Table 4.2. Variant allele frequencies among all cohorts (Cambodia, Vietnam, Thailand) and STH species (<i>A. lumbricoides</i> , <i>T. trichiura</i> , <i>N. americanus</i>) at codons 167, 198 and 200	143

List of Supplementary Figures

S1 Figure. STARD flow-diagram.....	81
S2 Figure. Stool collection workflow after STARD guidelines	122
S3 Figure. Statistical analysis of MT-PCR STH infection prevalence and intensity data	122
S4 Figure. Quantity and quality control of protein extracts	122
S5 Figure. Quantity and quality control of RNA extracts	122

List of Supplementary Tables

S1 Table. Raw data file of MT-PCR and qPCR diagnostics	81
S2 Table. STARD checklist.....	81
S3 Table. Statistical power analysis.....	81
S4 Table. Microbiome metadata	122
S5 Table. Height-for-weight, height-for-age and weight-for-age z-score analysis	122
S6 Table. Relative abundance of bacterial phyla and genera including statistical analysis	122
S7 Table. OTU data tables for alpha and beta-diversity measures	122
S8 Table. Differential OTU abundance analysis for STH infection status and sex.....	122
S9 Table. MT-PCR data of faecal gDNA samples	149
S10 Table. Variant allele frequency data for human soil-transmitted helminths	149
S11 Table. <i>Tub-1</i> sequencing read analysis.....	149
S12 Table. Shannon's entropy data per sample, amplicon, STH species and cohort..	149

Chapter 1 – Literature review

1. Overview of soil-transmitted helminth infections

Soil-transmitted helminths (STHs) are gastrointestinal parasitic nematodes that include roundworms (*Ascaris lumbricoides*), whipworms (*Trichuris trichiura*) and hookworms (*Necator americanus* and *Ancylostoma* spp.) [1]. These parasites are transmitted through faecal contamination of the environment, leading to ingestion of a mature egg (*A. lumbricoides* and *T. trichiura*) and penetration of the skin by a soil-dwelling larval stage (hookworms) [2]. STH infections are among the most prevalent chronic infectious diseases worldwide and represent the three most significant neglected tropical diseases [3]. Around 1.5 billion people are infected with an STH worldwide, of which 1.2 billion, 800 million and 700 million are attributed to *A. lumbricoides*, *T. trichiura* and hookworms, respectively [1]. Global disease burden due to STHs is estimated at around 3.4 million disability adjusted life years (DALYs) in 2016 [4] and disproportionately borne by young children aged 2 – 10 years and women of childbearing age or pregnant women [5] in low-socioeconomic tropical to sub-tropical regions of the world (Figure 1.1) [6, 7]. STH control is dependent on global programs delivering oral benzimidazoles to 75% of at-risk school-aged children (873 million) in endemic regions until 2020 [8]. The threat of emerging benzimidazole (BZ) resistant STHs is a significant global health concern that impedes these programs. Halting STH transmission and sustainably reducing their burden requires new drug classes, improved sanitation and hygiene infrastructure, and better tools to monitor their prevalence, distribution and the emergence of drug resistance.

Proportion of children (1-14 years of age) in the country requiring preventive chemotherapy (PC) for soil-transmitted helminthiases, worldwide, 2014

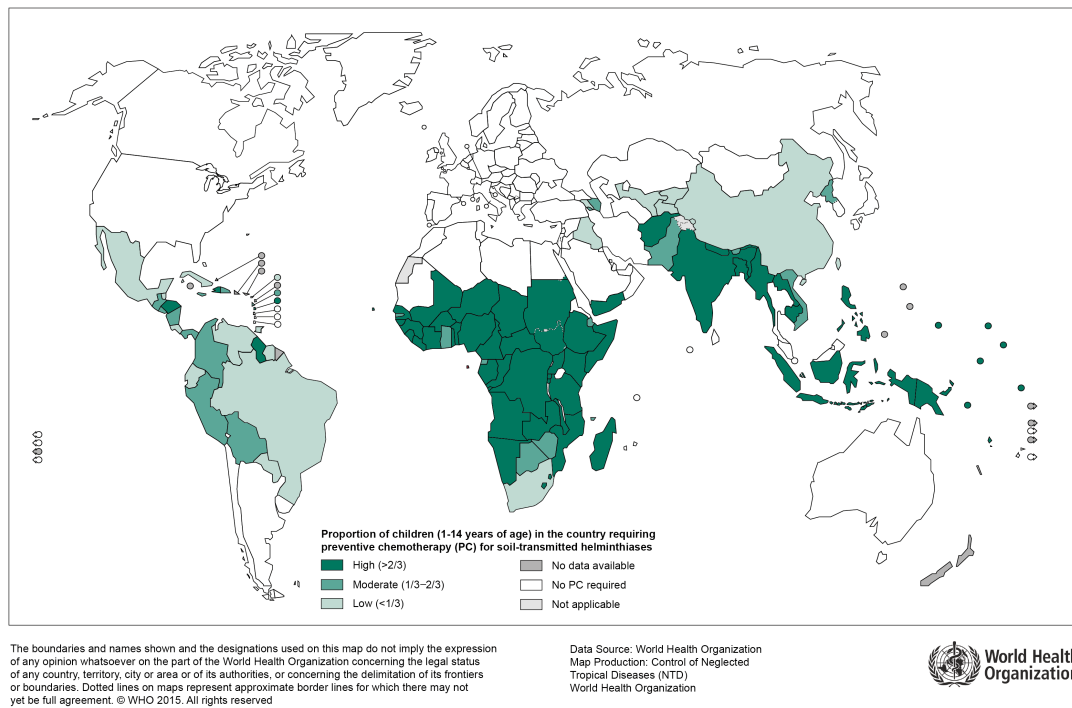


Figure 1.1. Global proportion of 1 – 14-year-old children requiring preventive chemotherapy for soil-transmitted helminth infections in 2014. Figure adapted from WHO 2015 (http://gamapserver.who.int/mapLibrary/Files/Maps/STH_2014.png?ua=1, accessed 30th October 2019).

2. Life cycles of the soil-transmitted helminths

The life cycles of human infectious STH species are similar in that they all require an environmentally resistant stage, which infects the human host either via direct skin contact (third-stage hookworm larvae) or ingestion through contaminated food or water (embryonated roundworm and whipworm eggs) [9]. Adult helminths reside in the gastrointestinal tract, where they cause clinical symptoms, undergo sexual reproduction and the adult female nematode excretes parasitic eggs that are passed back into the

environment via the faeces [10]. Parasitic eggs embryonate in the environment under species-specific optimal conditions (8 – 37 days at 35 – 39°C for *A. lumbricoides*, 20 – 100 days at 37 – 39°C for *T. trichiura* and 2 – 14 days at 40°C for hookworms [3]) and complete the transmission cycle once infection has been established in a suitable host (Figure 1.2). However, each species exhibits specific life cycle characteristics and can lead to varying symptomology as described below.

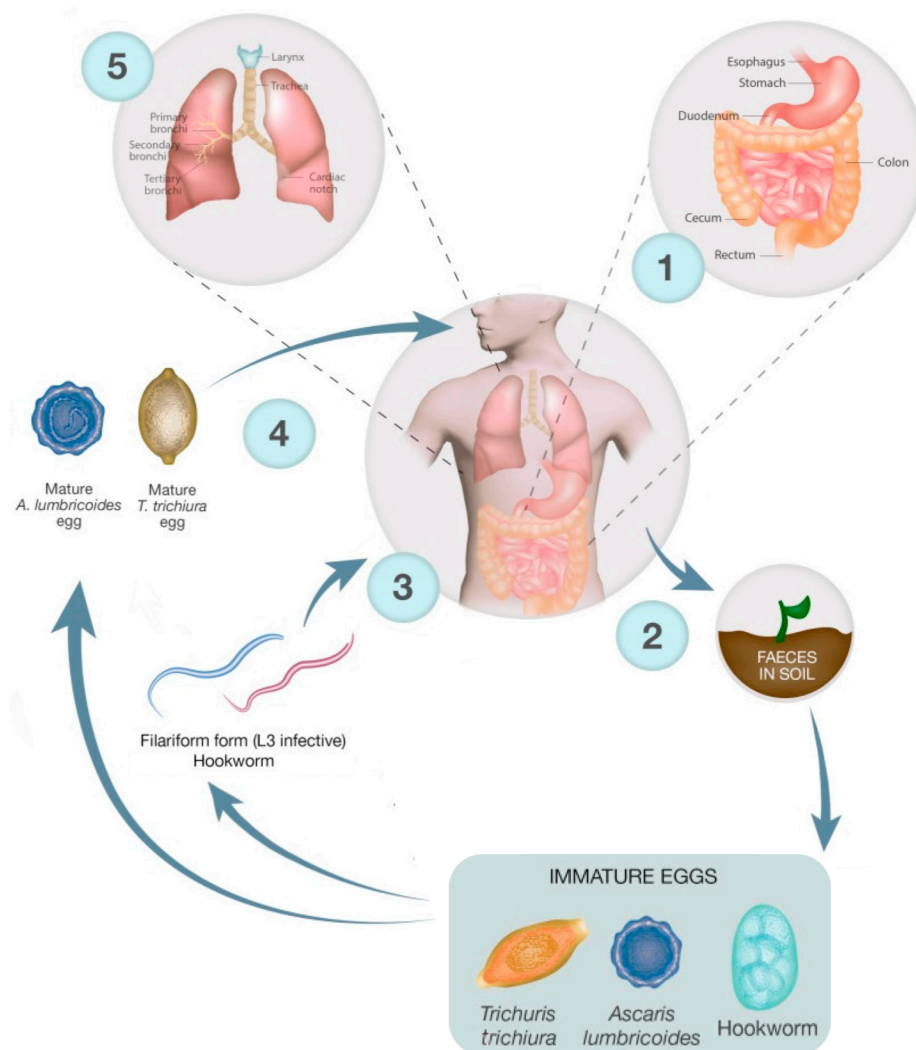


Figure 1.2. General soil-transmitted helminth life cycle. Species-specific life cycles are depicted for *Ascaris lumbricoides*, *Trichuris trichiura* and the hookworms *Necator americanus* and *Ancylostoma* spp. Figure adapted from [10].

Table 1.1 Summary of life cycle characteristics, disease burden and symptoms caused by STH infections worldwide (summarised from [1, 3, 11-15]). Epg = eggs per gram faeces.

	Roundworm <i>Ascaris lumbricoides</i>	Whipworm <i>Trichuris trichiura</i>	Hookworm <i>Necator americanus</i> <i>Ancylostoma</i> spp.
Life cycle			
Egg size (μm)	40 – 80 x 25 – 50	50 – 54 x 22 – 23	62 – 67 x 40 – 42
Development in soil (<i>days</i>)	28 – 84	10 – 30	3 – 10
Larval development (<i>weeks</i>)	9 – 11	12	5 – 9
Adult length (<i>cm</i>)	35	3 – 5	5 – 10
Adult life span (<i>years</i>)	1 – 2	1	1 – 5
Location of adult life stage	Small intestine	Caecum, colon	Upper small intestine
Average egg count per day	> 50,000	> 10,000	> 4,000
Global disease burden			
Infection prevalence (<i>million</i>)	1221 – 1472	795 – 1049	740 – 1298
Infection intensity (<i>epg</i>)			
Low intensity	1 – 4,999	1 – 999	1 – 1,999
Medium intensity	5,000 – 4,9999	1,000 – 9,999	2,000 – 3,999
High intensity	> 50,000	> 10,000	> 4,000
Disease cases (<i>million</i>)	335	220	159
DALYs (<i>million</i>)	10.5	6.4	22.1

Mortality rate (<i>annually</i>)	60,000	10,000	65,000
Symptoms			
Acute	Abdominal pain, coughing, pneumonia	Diarrhea, rectal prolapse	Abdominal pain, iron deficiency anaemia, skin irritation
Long-term	Stunting, wasting, cognitive impairment, anaemia		

2.1. Roundworms – *Ascaris* spp.

Roundworms (*Ascaris* spp.) include two human-infective species, *Ascaris lumbricoides* and *Ascaris suum*, that are highly similar, morphologically and at the DNA sequence level, and are both likely to be important in humans worldwide [16]. *Ascaris* infection occurs by ingestion of embryonated eggs, typically through contaminated food or water [2]. Upon passage through the stomach, the L3 larval stage hatches and penetrates the wall of the small or large intestine to enter the host bloodstream [17]. Once in the bloodstream, the L3 larvae travel to the liver and move into the parenchyma [18], where they reside for two days. The L3s then return to the blood, move towards the lungs, and burrow through the lung tissue where they are coughed up and re-swallowed. At some point during this larval migration the L3 moults to the L4 stage. Upon reaching the small intestine, the L4 matures to adulthood and undergo sexual reproduction [19]. Female *Ascaris* spp. live for up to two years (Table 1.1) [20] and are the most fecund of the STHs, producing more than 50,000 eggs per day [21]. Embryonated eggs are secreted into the environment and require 8 – 37 days to reach maturation and infectivity, during which the embryo develops to the L3 larvae [3]. *Ascaris* eggs are highly resilient and under suitable conditions (~4°C [22]) may be viable for several months to years in the environment [23, 24].

2.2. Whipworm – *Trichuris trichiura*

The whipworm (*Trichuris trichiura*) has a direct life cycle transmitted through third stage larvated eggs that are ingested, typically in contaminated food or water. The L3 larvae hatch in the small intestine and burrow into the intestinal wall, but do not migrate into the bloodstream [2]. *Trichuris* L3s undergo two cuticular moults to adulthood within the

intestinal mucosa. At adulthood, the worms move to the large bowel, and embed their highly modified and absorptive oesophagus (the ‘stichosome’) into the mucosal tissues [25]. *Trichuris* secretes a variety of digestive proteases, pore-forming proteins and other enzymes through the stichosome into the bowel epithelium [26], forming a syncytial mass of host tissue upon which they feed. Some studies suggest adult *Trichuris* also blood feeds [27], which may explain their contribution to bloody diarrhoea, iron deficiency anaemia (IDA) and abdominal pain [19, 28]. Adult *Trichuris* may live for up to 1 year and produce more than 10,000 eggs per day. These eggs are secreted in the faeces and, like *Ascaris*, these embryos must develop to the L3 stage within the egg, before it is infective. Also, like *Ascaris*, *Trichuris* eggs are highly resilient and have significant environment longevity (10 – 30 days [3] with an increased viability at low temperatures [22]) [25].

2.3. Hookworm – *Necator americanus* and *Ancylostoma* spp.

Human infective hookworms include *Necator americanus*, *Ancylostoma duodenale* [29] and *Ancylostoma ceylanicum* [30]. *Ancylostoma ceylanicum* is listed as a neglected hookworm species (e.g. not included in declarations by the Centre for Disease Control (CDC) or WHO) with a high zoonotic potential [30]. Hookworm infections occur when L3 larvae penetrate the skin of the host, subsequently travel through the blood stream to the lungs, where they penetrate lung tissue, get coughed up and swallowed into the intestines (hepatopulmonary migration) [31]. Adult worms live in the small intestine, attach to the villi and feed on blood [32]. Hookworms are the least fecund of the STHs and produce >4,000 embryonated eggs per day, which are secreted into the environment. Once in the soil, under suitable conditions (dependent upon temperature and soil moisture e.g. tropical and subtropical climates are ideal [33]), the embryo matures to a L1 larvae, which

hatches and lives within the soil. They develop to an L2 and then an infective L3 stage over the period of two weeks [3]. L3 larvae can live in the environment for a long time (3 – 10 days [3]) [34].

3. Global burden of STHs on human health

Global disease burden, or morbidity, is measured in DALYs, which is defined as the life years an individual has lived with the disease and those lost due to early death [35]. Globally, the estimated number of DALYs lost due to soil-transmitted helminths in 2016 was around 3.4 million years [4]. Although their primary impact is through chronic morbidity, STH infections cause around 135,000 deaths per year [2, 36]. Morbidity caused by STH infections typically correlates positively with infection intensity [37], which is classified by the WHO as light, medium and heavy infections based on the estimated numbers of eggs per gram of faeces (Table 1.1) [12]. The morbidity threshold for children is lower than that for adults [37] and under the age of 5 years ranges from 10 – 20 *A. lumbricoides*, 90 – 250 *T. trichiura* and 50 – 105 hookworm eggs per gram of faeces [37].

Approximately 450 million people suffer from STH infection related illness resulting in clinical symptoms requiring medical treatment [38]. Symptoms may vary according to infectious species, infection intensity, host age, immunological and nutritional status and correlates with gender [1, 39-42] Less severe STH infections, in particular *Ascaris* spp., are often asymptomatic, but nonetheless impact negatively on the host [43]. The majority of an infected population exhibits low intensity infections, while typically only a small proportion harbours high intensity infections (over dispersed distribution) [44, 45]. As most asymptomatic infections are low intensity, these cases may go undiagnosed [24], likely contributing to a significant underestimation of the global infection prevalence and can act as important sources of transmission [46]. Clinical symptoms are diverse, can be divided into those occurring during acute and chronic disease stages, and range from coughing, pneumonia, diarrhea, fever, abdominal pain, malnutrition and allergy response

to stunting, wasting, iron deficiency anaemia, protein loss and decreased cognitive development; all of which are particularly significant in pre-school and school-age children [24, 47-50].

3.1. Acute symptomology

Acute symptoms from STHs include malnutrition, abdominal pain, diarrhea and fever [36, 45], which may lead into long-term conditions such as stunting, wasting and an impaired cognitive development [51]. Acute symptomology is predominantly caused by a host-parasite competition leading to a decreased nutritional uptake by the host [51], and the infection-specific immune response of the host caused by parasite life stages in different tissues [52].

Malnutrition

Malnutrition, specifically undernutrition, is one of the most acute effects of soil-transmitted helminth infections. Malnutrition is most commonly defined as a nutritional deficiency due to a lack of protein-energy or calories [5]. Broadly, there are four types of undernutrition: stunting and wasting, underweight, and vitamin and mineral deficiencies [53]. Underweight is defined as a child with low weight-for-age compared to their age group [54]. A deficiency in vitamins and minerals is defined as micro-nutrient related malnutrition, causing problems with enzymes, hormone and other substance production, such as Vitamin A and iodine [55]. Malnutrition is a result of a reduced nutrient absorption and loss of appetite [56]. Globally, there are 50 million malnourished children under five years old [57]. Of the 10 million children that die annually worldwide, more than 50% die due to malnutrition [58]. A decrease in growth rate, consequently described as stunting, has been widely used

as a marker to characterize malnutrition [59]. Further, on-going malnutrition can also lead to wasting, a lowered weight-for-height ratio. Notably, malnutrition correlates positively with an increased susceptibility to other infections and further exacerbates undernutrition [60, 61]. STH infections correlated with iron and Vitamin A deficiency in pre- and school-aged children from Sub-Saharan Africa [62]. These effects however, appear to be STH species specific [63]. Multi-nutrient supplementation in addition to mass drug administration (MDA) showed mixed effects in terms of child health, with lower rates of *A. lumbricoides* and *T. trichiura* re-infections [60]. However, these results were not statistically significant and effects of supplementation on patient health status are debatable [60].

Abdominal pain

Abdominal pain and complications are one of the most common symptoms associated with STH infections [64], which in severe high-intensity cases can result in internal obstructions [65]. In particular heavy *A. lumbricoides* infections can further lead to intestinal [66] and biliary complications [67]. Although less common, some studies have reported abdominal pain caused by high intensity hookworm [68, 69] and *T. trichiura* [25] infections.

Coughing and pneumonia

Ascaris lumbricoides infections often cause coughing and subsequent pneumonia due to the hepatopulmonary migration of L2 larvae within the host [48]. These conditions may also cause Loeffler's syndrome, which involves an accumulation of eosinophils in the lungs due to migration of infective larvae within the human host [48, 70]. *Ascaris lumbricoides* and hookworms are most commonly associated with this symptom [71]. This symptom is

associated with prior levels and duration of STH exposure and can last several months [48]. Loeffler's syndrome often increases existing respiratory symptoms by causing additional coughing and difficulty breathing [72]. As with many other symptoms related to parasitic infections, diagnosis can be difficult due to the generalised nature of the symptomology [73].

Skin conditions

Hookworm infections typically cause skin itchiness, inflammation and rashes resulting from the dermal penetration of infective L3 larvae [33]. Infection with other zoonotic *Ancylostoma* spp. species can lead to cutaneous larvae migrans around the area of parasite entry, which grow several centimetres per day, but will gradually decline and are self-limiting as the parasite develops and migrates [71].

Other species-specific symptoms

Additional acute symptoms are generally diverse and can include fatigue [74] and diarrhea [75]. The non-specific nature of many STH-related symptoms makes it challenging to accurately diagnose the causal agent. The remote location, low socioeconomic status and poor access to health care for many patients compound this problem [76]. High intensity *T. trichiura* infections may also cause rectal prolapse in addition to watery diarrhea due to the *Trichuris* dysentery syndrome (TSD), which contributes to dehydration and malnutrition [25].

3.2. Chronic symptomology

Chronic symptoms caused by STH infections are predominantly stunting, wasting, iron deficiency anaemia and impaired cognitive development [1]. Infected patients often show signs of decreased growth and poor physical and cognitive function when compared to uninfected individuals [59, 77-79]. Some of these symptoms (stunting and wasting) are caused by undernutrition due to a reduced food intake and loss of appetite [80]. MDA, as the predominant intervention method, shows little to no effect on health measures such as height, weight, stunting and cognition [81]. However, this meta-analysis included non-normalised data as well as limited data in terms of attendance [81]. In contrast, several other studies have shown some benefits regarding cognitive and motor development, iron status and morbidity as reviewed in [82].

Stunting

Stunting, defined as an inappropriate height for the recorded age (height-for-age z-score < -2 for reference population), is a decreased growth rate compared to uninfected children [59, 83, 84]. Stunting in young children can be caused by a multitude of factors such as undernutrition, nutrient deficiencies and socioeconomic factors [85]. Approximately 161 million children globally suffered from stunting in 2013 [86]. The estimated amount of stunted children below the age of 5 globally was 26% (165 million) in 2011 worldwide [57]. Among children infected with *A. lumbricoides*, *T. trichiura* or hookworms, stunting as an effect of STH infections was 5, 6 or 8 times more likely to occur, respectively [85, 87]. STH infections, in particular multi-parasitism, are reported to be significantly correlated with stunting among children in endemic regions [88, 89]. Further, children who have been infected with STHs in addition to other diarrhea-related disease during their first

two years of life exhibit an overall childhood growth shortfall, compared to uninfected children, of up to 5 cm [58], which may affect stunting later in life [90]. Under favourable conditions, e.g., STH intervention and nutritional supplementation, children may return to a non-stunted height-for-age during their teenage years [87]. It is difficult to pin-point a single cause of stunting as many factors in developing countries contribute to a negative growth rate, such as lack of sufficient food in food-insecure households, unbalanced diet, lack of education, large family size leading to uneven distribution of available resources and low socioeconomic income; in addition to STH infections [59, 87]. Thus, the extent to which the relationship between STH infections and stunting is causal is not clear.

Wasting

Wasting as a consequence of malnutrition is defined as an inappropriate weight for the recording age (weight-for-age z-score < -2 for the reference population) [54, 91]. Globally, approximately 17 million children under 5 are severely wasted [92]. Similar to stunting, wasting is caused by on-going malnutrition of the host and has a large potential impact on lifelong health outcomes among young developing children [93]. The correlation between STH infections and wasting is controversial, with some studies indicating a causal relationship [94] and others finding no significant impact [95]. Interestingly, intensity of infection does not correlate with wasting [96].

Iron deficiency anaemia

Anaemia as a symptom of iron deficiency is defined as an insufficient amount of oxygen-carrying red blood cells due to low haemoglobin concentrations within a host [97]. Iron deficiency anaemia is caused by hookworm infections due to the mechanical attachment of the worm to the intestinal mucosa and associated blood loss [33]. Although adult

hookworms on average take up around 0.001 mL of blood per day, the majority of blood is lost due to tissue disruption around the attachment site [33]. IDA alone causes an annual global disease burden of 12 million DALYs [7]. Hookworm infected populations exhibit overall lower iron levels, irrespective of the intensity of infection, when compared to uninfected populations [32]. Successful BZ treatment results in increased iron levels and gives a comparable outcome to a combination therapy of iron supplementation with deworming treatment [32]. Although the prevalence of anaemia in children under 5 years has reduced from the 1980s to 1990s (41% to 25%), infants are still at high risk and additional strategies such as education, evaluation of policies and food fortification are likely to be beneficial [98]. Despite these efforts, it is questionable if universal iron supplementation is sufficient to restore iron levels in the long-term without combined deworming treatment.

Impaired cognitive development

Impaired cognitive development is defined as mild to severe difficulties in remembering, learning, concentrating and decision making [99]. Multiple factors affect cognitive development, highlighting the importance of integrated multi-factorial studies [100]. The socioeconomic status and anaemia levels on a national population scale have a significant impact on children's development and learning curve [101]. Impaired cognitive development may lead to increased health problems in older age [102] and correlates with decreased height, weight, cognitive ability [103] and economic productivity [80].

Impaired cognitive development due to STH infections is most severe in pre-school and school-aged children [103, 104] and is caused by infection through effects on patient health and subsequent school/work absenteeism [105]. Considering the high levels of

polyparasitism among endemic populations, this impairment might be additive and hence underestimated [106]. All major STH pathophysiology mechanisms may contribute to impaired cognitive development, with anaemia being particularly important [59]. Boivin and colleagues showed that uninfected children had a greater cognitive performance compared to STH infected children indicating a negative correlation between gastrointestinal parasitic infections and brain development [101]. Further studies conducted between 1992 to 2016 showed that infection is associated with deficits in the majority of evaluated domains [102]. Interestingly, successful STH treatment may not improve intellectual or cognitive performance [101, 107]. Similar results have been reported for memory and reasoning among Jamaican children infected with *T. trichiura* [108]. Only two studies, conducted in Guatemala and Jamaica respectively [109], have found positive changes associated with BZ treatment.

Alterations of the host gut microbiome

The human body contains more microbial cells than human cells, with most of these being bacterial [110]. These microbes partition into communities or microbiomes by host organ, tissue and between and among individuals [111]. The human gut microbiome is the richest community compared to all other organs of the body [112]. Correct function and compositional balance (homeostasis) of the gut microbiome contributes to digestion, mental health, the immune response and protection from pathogens [113]. Disturbance of the microbial balance can lead to dysbiosis [114], which contributes to negative health outcomes for the host such as inflammatory bowel disease, arthritis, diabetes [52] and an impaired cognitive development [114]. The gut microbiome is particularly relevant to STH infections due to their co-habitation of the gut as an environmental niche [115]. The gut

microbiome is shaped by a range of environmental factors including diet [116], antibiotic usage [117], mode of birth [118], age [114, 119] and geographical location [120].

Although research correlating STH infections (prevalence and intensity) with alterations of the gut microbiome is limited, some evidence for an interaction has been found [121]. STH infections may influence the gut microbiome directly (mechanical attachment) or indirectly (STH induced immune response and subsequent symptomology). Helminth infections may indirectly influence the gut microbiome through nutritional strategy and immunopathology [114] and increase the diversity of the bacterial gut community [122]. Consistent with this STH infections were associated with an increase in bacterial alpha-diversity (diversity within a sample) and beta-diversity (diversity among samples) among patients from Liberia and Indonesia [123]. A study in 2006 by Mai and colleagues found a different microbiota profile in patients suffering from long-term diarrheal episodes compared to healthy individuals [124]. However, it was unclear if these changes were the cause or result of diarrhea, which is one of the major symptoms of STH infections [124].

STH infections may associate also with specific changes in the taxonomic composition or relative abundance of bacteria in the gut microbiome [122, 125]. STH infections correlate negatively with the presence of *Lactobacillus* and *Lachnospiraceae* species in infected humans [126]. However, Cooper *et al.*, [127] found no correlation between *Trichuris trichiura* infections and changes in the gut microbiome of school-aged children. STH induced gut microbiome alterations can subsequently impact on brain function [114], immune-inflammatory disease [70] and other diseases [128]. Differences in study conditions including a lack of standard operating protocols, sample collection and processing procedures, diagnostic tools and sample preservation make it difficult to

accurately compare results from different cohort studies. Overall, there is no clear evidence for the impact of STH infections on the gut microbiome and this area requires further study.

4. Global prevalence and disease burden of soil-transmitted helminths

STH prevalence is highest in endemic, tropical to sub-tropical countries such as Sub-Saharan Africa, South-America and South-East Asia (SEA); in particular within low-socioeconomic regions (Figure 1.1) [44]. A species-specific geographical distribution has been found with *A. lumbricoides* predominantly occurring in Africa, whereas *T. trichiura* and hookworms are found in Asia [29], and concurrent, multi-parasitic STH infections are common [45]. Rural populations with overcrowded living conditions are most vulnerable due to contributing factors such as inadequate sanitation and personal hygiene, impoverished conditions and little to no infrastructure [2]. Further, environmental and geographical factors such as high humidity correlate with infection prevalence [129]. Global infection prevalence has decreased over the past 12 years from 807 – 1,221 to 804 million for *A. lumbricoides*, 604 – 795 to 477 million for *T. trichiura* and 576 – 740 to 472 million for hookworms [1, 43]. Although a high reduction in infection intensity has also been observed from 2011 [2] to 2018 [130], the largest proportion of children requiring chemotherapy are from Sub-Saharan Africa and South-East Asia [21].

4.1. STH prevalence and disease burden in South-East Asia

Previous research has focussed predominantly on countries such as Brazil, Sub-Saharan Africa and Indonesia; although one to two-thirds of the global helminth disease burden occurs in South-East Asia [10, 58, 101, 131], which is highlighted by the large amount of research studies conducted in this region (Figure 1.3) [132]. Jex *et al.* [2] reviewed STH burden and distribution in all South-East Asian countries in 2010. My study focusses on four SEA countries; Thailand, Cambodia, Vietnam and Timor-Leste; and a current synopsis of the STH burden in these countries can be found here.

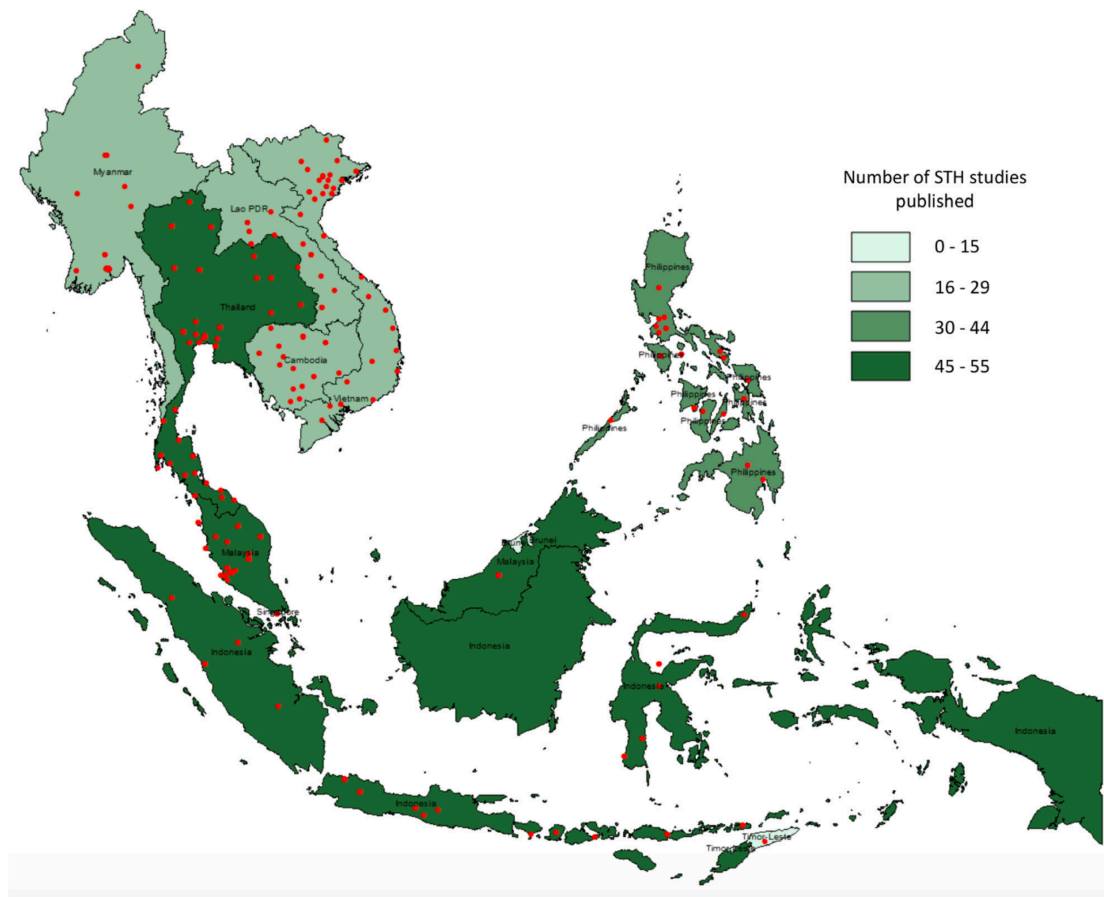


Figure 1.3. Map of soil-transmitted helminth studies conducted in South-East Asia. Number of published STH infection prevalence and/or intensity studies per country correlates approximately with national STH infection prevalence measures. Red dots highlight the study locations [132].

4.1.1. Thailand

In 2010, the national infection prevalence of STHs in Thailand was estimated to be below 10% for *T. trichiura* and *A. lumbricoides*, and between 10 and 20% for hookworms [29]. Since then, the STH infection prevalence has decreased in certain regions to <0.3% [133]. Although Thailand has been classified as an upper middle-income country in 2012 [134], with an increased socio-economic status correlating with a reduced prevalence of parasitic infections [135], there are still sub-populations (rural regions, refugee camps, immunocompromised people) that have a high STH infection prevalence [2]. Geographical factors such as remote forested areas and an extended annual wet season also contribute to high infection rates [136]. However, there are many gaps in this data. Levels of IDA among young children (4-6 months) has been found to range from 32% up to 62% [98]. Although policies have been implemented in an attempt to decrease IDA among Thai populations, universal iron supplementation is difficult to efficiently supply in children and pregnant women in particular among remote communities [98]. The number of repeated diarrheal incidences ranges from 20,000 to 50,000 in children (<5 years) with STH infections identified as one of the causative factors [79]. Overall, the importance of hygiene education and improved sanitation in combination with available chemotherapy treatment is highlighted [136, 137].

Ethnic and regional sub-populations, such as the Karen ethnic people residing at the Thai-Myanmar border, are at higher risk of infection due to their lower socioeconomic status compared to the rest of the country. The Thai government supports control strategies in this remote community, whereby they receive regular deworming,

education and medical care [2]. Nonetheless gastrointestinal infection prevalence has been reported of above 47% in children, with many children experiencing multi-parasitic infections [138]. The predominant soil-transmitted helminth species are *A. lumbricoides* and *T. trichiura* [138].

A cross-sectional study among 2,171 school children in northern Thailand in 2001 showed a species-specific helminth prevalence of 21.7% for *A. lumbricoides*, 16.3% for *T. trichiura* and 18.5% for hookworms [136]. Anantaphruit and colleagues confirmed a high STH prevalence among school-children six years later with hookworms as the most prevalent parasites (11.4%) [137]. Although infection prevalence among the Tungsor Hongsa population (North-Thailand) is relatively low with 7.5%, *N. americanus* could be identified as the main infective hookworm species due to the inadequate use of footwear and close housing proximity to cattle farming [139]. Another study reported an overall hookworm prevalence of 48.7% with a hookworm specific infection prevalence of 26.3% [140]. More recent prevalence studies revealed a large decrease compared to early 2000s of <1.25% for any STH species [141]. In comparison, the most abundant parasitic infection in Southern Thailand among school-children (Nakhon Si Thammarat province) was hookworms followed by *T. trichiura* [40]. Infrastructure improvement, including piped water supply, reduced the incidence rate of diarrhoea within this region [142].

4.1.2. Cambodia

Data for STH burden in Cambodia is significantly less readily available than for Thailand. Hookworms are reportedly the predominant STH species in Cambodia [143],

with high proportions of zoonotic *A. ceylanicum* infections [144]. The national geostatistically modelled infection prevalence of *A. lumbricoides* and *T. trichiura* lies below 25% [143]. Another study has found similar species-specific results, but reported a lower overall infection prevalence, in particular for *A. lumbricoides* and *T. trichiura*, highlighting the positive effect of on-going national control programs [145]. Although overall the disease episodes that required drug treatment decreased, total infection prevalence among children increased from 2006 to 2011 [146].

4.1.3. Vietnam

Similarly to Cambodia, hookworms are the predominant STH species (58.1%) in Vietnam, followed by *T. trichiura* (45.2%) and *A. lumbricoides* (13.5%) [147]. A review in 2003, estimated the national STH infection prevalence to be 33.9 million *A. lumbricoides* (44.4 %), 17.6 million *T. trichiura* (23.1 %) and 21.8 million hookworm (28.6 %) infections [148]. Interestingly, roundworm and whipworm co-infections appear to be positively correlated [149]. STH prevalence is highest in the northern regions of Vietnam, averaging above 45% [150] and 47% [151] for any species. Sub-populations living in rural agricultural settings are at higher risk of infections in Vietnam, with high *T. trichiura* infection prevalence (40%) across the country [151].

4.1.4. Timor-Leste

STH infection prevalence data are sparse within Timor-Leste due to the resource constraints of the country. However, a study in 2012 of school-aged children found a

STH infection prevalence as high as 29% [152]. A study in 2016 found an overall STH prevalence of 69%, with 24% *A. lumbricoides*, 0.33% *T. trichiura* and 60% *N. americanus* infections, respectively; this increase in prevalence is likely due to the use of a molecular diagnostic tool [153]. Effects of MDA are limited due to an inadequate implementation of preventive chemotherapy intervention caused by a lack of available resources [152]. Studies investigating risk factors [153] and the effects of water, sanitation and hygiene (WASH) interventions on STH prevalence have been conducted in this region [154], with reportedly limited success of WASH implementation [155].

5. Control of soil-transmitted helminth infections

Control strategies for reduction of STH infection prevalence and intensity are outlined in the second WHO report on neglected tropical disease [156], 2012 London Declaration on Neglected Tropical Disease [157] and the 2013 World Health Assembly recommendations [158]. These documents outline strategic MDA approaches based on preventive chemotherapy and target resource poor, at-risk populations worldwide [159]. In addition, interventions providing safe access to drinking water, sanitation facilities and hygiene education are becoming increasingly important [160].

5.1. Mass drug administration

Benzimidazole drugs (BZ) are a class of broad-spectrum anthelmintics that are used for treatment of parasitic infections in livestock [161] and humans [162]. MDA of the BZs, albendazole and mebendazole, aims to reduce STH morbidity and prevalence and is focussed on the most at-risk population, children [82, 163]. The current WHO-recommended treatment regime of endemic at-risk populations is annual (infection prevalence 20% – 50%) or biannual (infection prevalence >50%) administration of BZs to up to 75% of school-aged children [21, 46]. Due to the cost effectiveness and easy application especially in remote areas of the world, MDA as a form of preventive chemotherapy is consistently utilized as the main treatment approach in remote areas [164, 165].

Long-term MDA has significantly reduced infection-related morbidity in several countries [166]. Nonetheless, the current MDA approach has several limitations, such as coverage,

compliance, drug efficacy and resistance [167]. Although the 2020 WHO roadmap aims to target 75% of children in at-risk populations in order to eliminate moderate to heavy STH infections (<1%), the current coverage is only 50% among endemic countries [168]. The WHO estimated in 2012 that more than 370 million people are still in need of STH treatment in SEA alone [8]. Extending community-wide routine treatment is economically [169] and logistically feasible [170], in order to remove parasite refugia and interrupt disease transmission [171]. However, community acceptance presents an essential challenge for successful MDA, because religion [172], hygiene practise [173], sanitation facilities [174], employment [174], income [174] and remote housing [175] of a given population all influence STH infection status. Thus, appropriate and effective interventions require a thorough understanding of each populations cultural background.

Nevertheless, it is debatable if MDA alone can interrupt disease transmission, with some mathematical modelling studies estimating that high-pressure chemotherapy can achieve this [176] and others showing conflicting data [177]. Interruption of transmission relies on several factors such as drug efficacy, community coverage and age groups [178]. Although mebendazole treatment proves to be highly effective against *Ascaris*, it has little to no effect against *Trichuris* and hookworm infections [179]. Single-dose oral mebendazole treatments show limited success, with cure rates of below 45% for *T. trichiura* [179]. Generally, albendazole treatment has shown to be effective against . Treatment efficacies vary greatly depending on the study design including drug quality, dosage, timeframe and application method [180]. Further, MDA does not prevent STH reinfections, provides only a short-term reduction in the absence of continuous treatment [177] and the longevity of environmental reservoirs of STH stages may contribute to ongoing-disease transmission [178].

5.2. Emergence of benzimidazole drug resistance

Reduced BZ efficacy is emerging as a significant global health concern that threatens the sustainability of MDA programs and may be a forewarning of emerging BZ resistance, as has been seen in animal populations [181]. Although the exact molecular mechanism of the mode of action of BZ has not been identified yet, it has been shown that BZs bind to the guanosine-5'-triphosphate (GTP) binding domain of beta-tubulin 1 [182, 183]. This process leads to a conformational change that inhibits polymerisation of beta-tubulin 1 and beta-tubulin 2 to microtubule strands within the parasite cells [184]. This non-polymerization and conformational change of microtubules results in the parasites being unable to take up glucose due to disruption of cellular activities, glucose transport and secretion, ultimately resulting in starvation and death of the adult parasite and prevention of larval hatching [185]. Interestingly, although many veterinary studies suggest single nucleotide polymorphisms (SNPs) at codons 167 and 200 to be responsible for drug resistance, an *in silico* modelling approach did not find any changes in the binding affinity of BZs when mutations are present [182]. To date crystallography of nematode beta-tubulin has not been performed, with only a handful of studies performing structural modelling and autodocking experiments [182].

Drug resistance in livestock populations

Drug efficacy is evaluated by measuring the cure rate (CR, rate of individuals that clear the infection) or faecal egg count reduction test (FECRT, reduction in number of excreted eggs pre- and post-treatment) [162]. Drug efficacy evaluation relies on application of appropriate diagnostic timeframes and techniques. Ideally, post-treatment follow-up sampling should

be conducted between 2 – 3 weeks after initial drug administration [186]. BZ resistance has been documented in livestock parasitic nematodes, including *Haemonchus contortus*, for many years [187] and spread rapidly [188, 189]. The mechanism of BZ drug resistance has been linked to the beta-tubulin isotype 1 gene, mainly through three SNPs at codons 167, 198 and 200 (Phe167Tyr, Glu198Ala and Phe200Tyr) [190-192]. These mutations are selected for during high-pressure mass drug treatment [181]. Using the same drugs for routine treatment in human populations, this raises the concern of the potential of emergence of BZ drug resistance [193].

Evidence for drug resistance in human populations

Efficacy of benzimidazole drugs naturally varies according to parasite and host factors [194]. Generally, single-dose albendazole cure rates are higher for hookworm and roundworm than whipworm [194]. In comparison mebendazole is effective for clearance of roundworm infections, but less so for whipworm and hookworms (mean CR <40%) [194]. Further, a drop in BZ cure rate has been observed from 2008 [179] to 2017 [195] comparing summarised data of two systematic reviews and meta-analyses on albendazole and mebendazole efficacy. A human population focussed study in Mali has found drug resistance to mebendazole in 1997 indicating a potential rapid increase in drug resistances [196]. In contrast, Prichard *et al.* showed that drug resistance in humans may be emerging, but requires further investigation [197]. Steinmann and colleagues critically reviewed current drug treatments, and concluded that non-targeted treatments against a variety of gastrointestinal parasites in the absence of subsequent screening of patients might increase the risk of drug resistances and waste valuable resources [45]. Conclusive identification of BZ drug resistance in humans remains controversial. In summary, it is evident that the development of novel anthelmintics needs to be a main focus of research due to ongoing

high-pressure BZ application in endemic countries and the resulting high selection pressure for beta-tubulin 1 resistance alleles [198]. In addition, the development of new, highly sensitive techniques for drug resistance screening would aid in on-going monitoring and early detection of potentially emerging drug resistance [199].

5.3. Water, sanitation and hygiene interventions

It is unlikely that preventive chemotherapy alone can interrupt STH transmission; a multi-factorial approach of water quality improvement, improved sanitation and hygiene education is essential for a holistic intervention approach [200]. Ideally this approach would follow an early high-sensitive molecular detection of infected populations. Access to safe water and sanitation are crucial in the economic development of a country; however in 2015, 663 million people still lacked access to safe drinking water worldwide [201]. Another 2.4 billion people lacked appropriate sanitation, requiring as many as 1 billion people to defecate openly due to lack of the aforementioned [201]. Hygiene and health education significantly improve child health (knowledge and infection status), in particular among those who are at high risk of infection [202]. Socio-economic development is a crucial factor for improved health outcomes as this correlates positively with improved sanitation and living standards [91].

The WHO has suggested that improvements in WASH will contribute significantly to a decrease in STH infection prevalence [203]. These interventions should disrupt oral ingestion and faecal excretion of fertilized STH eggs into the environment by providing access to safe drinking water, implementing latrine availability and acceptability, and hygiene education [204]. Surprisingly, two studies have shown that WASH interventions

in combination with MDA have little to no additional benefit on infection prevalence of STH species in endemic countries compared to MDA alone [155, 205]. However, these studies were limited by sample size, with other studies showing a correlation between WASH interventions and decreased STH prevalence [206]. A larger number of in-depth, integrated longitudinal studies identifying specific beneficial WASH components and coordinated universal, standardised studies are required [204].

It is crucial for the community to accept these WASH interventions in order for long-term benefits to be maintained. Often local communities do not implement continued behavioural changes, although improved sanitation is available [207]. Some trials including local community health care workers to aid in intervention acceptability, reported one third of participants practising on-going open defecation as well as problematic sustainability of latrine usage [155]. Outcomes may be further improved, if local authorities are more actively involved in decision making in collaboration with international field teams/funding agencies to implement changes.

It has been shown that knowledge among early school-aged children is limited in terms of STH infections and their mode of transmission [208]. Health education packages such as the “Magic Glasses” show promising results in terms of an improved hygiene behaviour and increased knowledge [209]. Certain regions of the world still practise open defecation as reported in field studies performed in East-Africa where 100% of participants practise open defecation [208]. Inclusion of local communities in the decision-making and on-going implementation of WASH interventions is important for program uptake [210]. Although novel resources had been installed, other studies found that large proportions of the community did not accept latrines and continued their original open defecation practice

[155]. Therefore, financial support and acceptance of cheap gender-separated latrines is critical for reducing the prevalence of STH infections in communities with a low socioeconomic standard [211].

6. Diagnosis of soil-transmitted helminth infections

Overall, diagnostic methods can be divided into two subclasses: microscopy-based methods and molecular-based methods [212].

6.1. Microscopy-based methods

The Kato-Katz Thick Smear (KKTS) is the current WHO-recommended diagnostic for STHs [213]. This method involves fixation of up to 1 gram of faeces in glycerin-soaked cellophane, which is microscopically evaluated for STH eggs [214] and has major limitations. KKTS is heavily reliant on parasitology expertise to efficiently and accurately perform helminth egg counts and species differentiation, which is particularly difficult for hookworm eggs [215]. For large cohort studies this process may be too time-consuming and costly [216]. Fixed hookworm eggs can collapse after 60 minutes, potentially leading to an underdiagnosis [214, 217]. The fertile female adult excretes eggs at irregular time intervals, which contributes to an underestimation of the true infection prevalence and intensity using KKTS, hence reducing its sensitivity [218, 219]. Other microscopy-based diagnostic methods include direct smear/microscopy [220], McMaster technique [219], mini-FLOTAC [221] and FLOTAC [222-224]. Many of these techniques have been developed for veterinary parasitology with a translation into clinical settings. Although, the KKTS is relatively cost-effective in terms of immediate resources, novel tools are required to overcome sensitivity, time and large-scale study constraints [225].

6.2. Molecular-based methods

Molecular diagnostic tools provide improved diagnostic sensitivity and specificity for identification of soil-transmitted helminths in faecal DNA [226, 227]. Over the past 10 years, molecular assays using DNA amplification [228] such as conventional PCR [229], qPCR [230], multiplexed qPCR [231] and loop-mediated isothermal amplification (LAMP) [232, 233] have been developed. These have been further expanded to high throughput sequencing assays using pyrosequencing [190, 191, 234] and other next-generation sequencing assays [235]. Molecular assays present an increased diagnostic sensitivity, specificity and reproducibility compared to microscopy assays. Due to on-going high-pressure MDA as proposed by the WHO in 2012 (873 million children, 75% of school-aged children) [21], there has been a significant decrease in disease-related prevalence, but more importantly infection intensity [132]. This reduction in infection intensity on a national scale, leads to undiagnosed, low-intensity sub-populations that have the potential to contribute to on-going disease transmission. An increase in diagnostic sensitivity is especially important among these sub-populations harbouring low-intensity infections, which are likely to be missed using traditional microscopy-based methods (KKTS) [236].

The most widely used molecular technique for STH diagnosis is the multiplexed qPCR, which targets gene markers such as the internal transcribed spacer region 1 and 2 (ITS1, ITS2) [230, 231]. Advantages of this technique are the ability to run samples in parallel (reduction of cost), increased sensitivity (detection of one egg per gram faeces, gene copy number), and faster sample processing (faecal DNA extraction to diagnosis ~2 hours) [226]. Compared to microscopy (Kato Katz Thick Smear, FLOTAC, McMaster), molecular tools show an increase in sensitivity to 85.7 – 100.00% for *A. lumbricoides*, 75.7 – 100.00%

for hookworms and 100.00% for *T. trichiura* as reviewed elsewhere [236]. In veterinary settings the multiplexed qPCR has been further developed by the so called multiplexed-tandem qPCR assay (MT-PCR) [237]. The MT-PCR utilizes a two-step PCR protocol to firstly perform multiplexed amplification of a gene of interest followed by a dilution step and secondly uses a nested PCR amplification followed by high-resolution melt curve analysis [237-239]. The increased sensitivity of these molecular tools implies a higher detection rate of positive infections as well as ability to differentiate to a species level e.g. hookworms [226]. Ideally these tools are cost-effective for application in endemic low-income countries, while being user-friendly for non-scientific application [240]. Therefore, molecular tools can improve diagnostic resolution of STH prevalence by identifying asymptomatic and low-intensity infections more accurately [231] and provide an early detection of drug resistance [188].

Although molecular diagnostics have many advantages, there are limitations such as a potentially higher cost of resources relative to KKTS, a lack of standardised approaches and infrastructure, and the lack of a molecular gold standard [226]. These limitations constrain their application in endemic areas with a low to moderate socioeconomic status where the majority of infections occur [228]. Usage of multiple diagnostic techniques (microscopic versus molecular) may further exacerbate diagnostic disagreements and underrepresentation of true infection data [241]. This implies, that past field studies are unlikely to be comparable to current strategies due to the difference in diagnostic application. Further, conversion from cycle threshold values (Ct-values) or gene copy number to eggs per gram is currently not available [242]. Such a translation formula is required for successful correlation of quantitative molecular data output into infection intensity measures. This is in particular important in terms of administered drug treatment

regime, which relies on an epg intensity measure (annual for low-intensity infection, bi-annually for high-intensity infections) [21]. Some mathematical modelling or correlation attempts for such a conversion formula have been attempted previously [243, 244]. Unavailability of diagnostic tools in endemic regions implies a sample transportation nationally or internationally to a research facilities [228]. Prolonged timeframes and temperatures increase potential of sample degradation in terms of loss of helminth eggs [245] or bacterial species [246]. Lastly, community acceptance is another important factor to ensure ongoing compliance and integration of practises and tools. In many endemic countries insensitive microscopic tests are still used due to the familiarity with the tool and the resource limitation. Importantly, there is no universal molecular diagnostic gold standard that has the ability to be applied in remote field settings, while also being cost-effective, time-efficient and highly sensitive [212].

Molecular diagnosis of benzimidazole drug resistance

As discussed above, diagnosis of BZ resistance can be determined using microscopy-based approaches such as CR and FECRT. However, due to cohort study limitations and host-parasite interactions such as aggregation of faecal egg counts across hosts [247], molecular-based approaches are becoming increasingly important [248]. Molecular tools can accurately screen for drug resistance, discover novel drug resistance associated genes and have the ability to be performed in economical, high-throughput screening of samples [249]. However, as previously highlighted these tools are currently limited to research settings only. Further, molecular tools available for screening of drug resistance in humans include pyrosequencing [250] and qPCR [250]. However, these are limited in terms of genotyping of known target SNPs only [251] and assay error rates for low allele frequencies [190].

7. Thesis aims

Based on this critical summary of the relevant literature, there is a clear lack of cost-effective and user-friendly molecular diagnostic tools for STH infections and potentially emerging BZ drug resistance. Samples further require adequate preservation for accurate infection testing and evaluation of STH induced long-term health impacts, in particular in terms of large-scale international cohort studies.

Therefore, the aims of my PhD thesis are:

- 1) to develop and test a novel, high-sensitivity, semi-automated multiplexed-tandem qPCR tool for diagnosis of STH infections,
- 2) to compare three faecal sample preservation methods for STH infection recovery and host gut microbiome characterisation, and
- 3) to develop a high-throughput amplicon sequencing assay targeting codons 167, 198 and 200 of the beta-tubulin 1 gene.

Chapter 2 – Development and validation of a multiplexed-tandem qPCR tool for diagnostics of human soil-transmitted helminth infections

8. Context

Global soil-transmitted helminth infection prevalence and intensity data are an important informative source for deployment of targeted disease-related treatment plans through health agencies. In order to accurately estimate infection prevalence, in particular within endemic countries where MDA treatment has reduced morbidity significantly, a high-sensitivity diagnostic tool is necessary for diagnosis of sub-populations. As covered in the literature review, microscopy-based approaches such as the Kato-Katz Thick Smear are too insensitive, time-consuming and require technical expertise, making it impractical to apply for large cohort studies. Novel diagnostic tools which are cost-effective, applicable in the context of field studies and have the ability to screen patients for multiple infectious nematode species in multiplex are necessary. Such a diagnostic tool is required as a complementation to a multifactorial intervention approach for STH prevention, which includes socio-economic development, improved sanitation and hygiene education.

9. Publication I

Development and validation of a multiplexed-tandem qPCR tool for diagnostics of human soil-transmitted helminth infections

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Abstract

Soil-transmitted helminths (STH) are a major cause of morbidity in tropical developing countries with a global infection prevalence of more than one billion people and disease burden of around 3.4 million disability adjusted life years. Infection prevalence directly correlates to inadequate sanitation, impoverished conditions and limited access to public health systems. Underestimation of infection prevalence using traditional microscopy-based diagnostic techniques is common, specifically in populations with access to BZ mass treatment programs and a predominance of low intensity infections. In this study, we developed a multiplexed-tandem qPCR (MT-PCR) tool to identify and quantify STH eggs in stool samples. We have assessed this assay by measuring infection prevalence and intensity in field samples of two cohorts of participants from Timor-Leste and Cambodia, which were collected as part of earlier epidemiological studies. MT-PCR diagnostic parameters were compared to a previously published multiplexed qPCR for STH detection. The MT-PCR assay agreed strongly with qPCR data and showed a diagnostic specificity of 99.60-100.00% (sensitivity of 83.33-100.00%) compared to qPCR and kappa agreement exceeding 0.85 in all tests. In addition, the MT-PCR has the added advantage of distinguishing *Ancylostoma* species, namely *A. duodenale* and *A. ceylanicum*. This semi-automated platform uses a standardized, manufactured reagent kit, shows excellent run-to-run consistency/repeatability and supports high-throughput detection and quantitation at a moderate cost.

Author Summary

Soil-transmitted helminthiases are among the most prevalent and damaging neglected tropical diseases and have a significant global health impact. Accurate identification and quantitation of STH infection is a cornerstone of effective control. Direct observation and counting of eggs in faeces is the current gold-standard method for diagnosis of infection. This approach is time consuming and has poor sensitivity. As ongoing oral BZ therapy across many endemic regions leads to a reduction in STH prevalence and intensity, these sensitivity limitations become an increasingly relevant issue, particularly with respect to monitoring treatment efficacy, identifying reductions in parasite transmission, and accurately quantifying infection burden in discrete populations in middle-income countries. PCR-based detection has long been proposed as an alternative approach to STH diagnosis and many protocols, including quantitative PCR-based methods, have been developed. However, these methods are largely bespoke and use non-standardized reagents that can greatly impact on the transferability and relative consistency of their performance. In the current study, we evaluate an automated, commercially-produced molecular diagnostic tool for validation of the major soil-transmitted helminths, including *A. lumbricoides*, *T. trichiura*, *N. americanus*, *A. duodenale* and *A. ceylanicum*, and evaluate its performance in comparison to an established multiplexed qPCR using faecal samples from endemic settings.

Introduction

Soil-transmitted helminths (STH), including roundworms (*A. lumbricoides*), whipworms (*T. trichiura*) and hookworms (*N. americanus*, *A. duodenale*, *A. ceylanicum*) represent a major cause of morbidity in tropical to sub-tropical developing and low-income countries [21]. In 2016, the Global Burden of Disease Study estimated that as many as 3.4 million DALYs are lost globally due to STH infections each year, of which 1.1, 0.5 and 1.8 million DALYs accounted for roundworm, whipworm and hookworm infections within all age groups in 2015 [4]. Total global infection prevalence is estimated to lie slightly above 1.9 billion infections depending on the diagnostic tool used, and as many as 5.3 billion people are at risk of infection worldwide [1, 29]. Infection risk directly correlates with inadequate sanitation, impoverished conditions, limited access to public health systems and population overcrowding [3]. Symptomatology and severity of infection correlates with species of STH and intestinal burden, as well as host age, nutritional and health status [21]. Acute clinical symptoms are less prevalent, but include, for hookworm (*N. americanus* and *Ancylostoma* spp.) and whipworm (*T. trichiura*), anaemia and diarrhoea, and for roundworm (*A. lumbricoides*), intestinal blockage and/or rupture, leading to ~135,000 deaths per year [2, 252]. Ultimately, the burden of disease caused by morbidity is far more significant than the impact caused by mortality, with long-term sequelae including malnutrition, stunting, wasting and decreased cognitive development [7].

Control of STH infection is dependent on oral anthelmintic therapy using BZ [46]. Currently, the WHO recommends, regional MDA programs in endemic populations to deliver 400 mg single dose albendazole or 500 mg single dose mebendazole annually or biannually to reduce both infection prevalence and intensity [46]. These MDA programs follow the London Declaration on Neglected Tropical Disease (NTD) 2012 endorsement of the WHO goal to scale

up global deworming in order to treat 75% of pre- and school-aged children at least once a year until 2020 in order to decrease STH burden in endemic areas [21]. Quantifying STH burden and the efficacy of BZ MDA programs is dependent on accurate and sensitive diagnosis and quantification of infection, particularly in regions where infection intensity is low but prevalence remains relatively high. Methods used include direct microscopy, formalin-ether concentration method, the McMaster egg counting technique, simple sodium nitrate flotation (SNF) including FLOTAC, the KKTS and, more recently, PCR-based approaches [132]. The observation and enumeration of STH eggs in faecal samples is the current gold standard for diagnosis of STH infection, with the WHO-recommended, KKTS the most widely used approach [203]. Advantages of the KKTS method are its cost-effectiveness and its application in remote settings [203]. However, there are several drawbacks of this method, such as the requirement for immediate microscopic examination of multiple freshly collected faecal samples and technicians exhibiting parasitological expertise, a lack of standard protocols (e.g., for the amount of faecal matter examined, fixation method, calculation of eggs per gram faeces) and hence reproducibility, time consumption, labour intensity, the need for rapid assessment of faecal samples to avoid hookworm egg clearance and most importantly the underestimation of infection prevalence due to limited sensitivity [253]. The technique also requires large teams of technicians and equipment to be dispatched to remote communities with an additional requirement for electricity and running water, which is logistically challenging in remote areas. More recently, Inpankaew and colleagues (2014) have established the sodium nitrate flotation method, a direct faecal microscopy-based method used widely in veterinary settings in the past, of which a single application has a 6% higher sensitivity than KKTS (performed in quadruplicate over a two day period to reduce sensitivity limitations) for the detection of hookworm eggs in human stool at any given time point [254].

Copro-microscopic diagnostic methods in combination with regularly administered drug treatment are ideal in highly endemic regions; however, they are not representative in areas containing sub-populations with low prevalence and intensity infections [255]. Further, STH infections are overdispersed, with a majority of the infected harbouring moderate to light intensity infections (roundworm 1 – 49,999 epg, whipworm 1 – 9,999 epg, hookworm 1 – 3,999 epg) and only a minority suffering from high intensity infections (roundworm > 50,000 epg, whipworm > 10,000 epg, hookworm > 4,000 epg) [21, 23]. Consequently, even within highly endemic regions worldwide, the majority of infections may not be readily detectable by microscopy [6, 37]. Although the disease burden in regions of the world has decreased significantly over years of MDA control and increased socioeconomic development, STHs remain a major global human health issue [44]. It is assumed that with successful implementation of MDA treatment a larger decrease in infection intensity than infection prevalence can be observed [12], making the sensitivity limitations of the diagnostic tool a more significant issue than the cost associated with molecular diagnostic approaches [225], as a greater proportion of the population harbours low intensity infections that can potentially be missed by copro-microscopic diagnostic approaches [255]. While these low-grade STH infections may be of a lesser consequence in terms of disease burden, they are highly relevant in terms of any effort to interrupt infection transmission [220], seeing that adults in endemic countries represent infection reservoirs that inhibit the chance of an interruption of transmission cycle by school-based MDA [256]. One major limitation of MDA treatment is the inability to prevent re-infection once treatment has ceased, resulting in rebounding infection levels among targeted communities [177, 257]. Transmission of infection, with a particular focus on populations in low intensity settings, needs to be interrupted to stabilize control of STH infections [258], but is influenced by infection prevalence and intensity, human migration, regional demography, diagnostic tool application and drug efficacy [259]. Diagnostic tools

contribute to reducing transmission by providing a knowledge basis for the specific, targeted and sustainable treatment of sub-populations at risk which serve as a transmission reservoir [260]. Notably, MDA treatment should be administered in combination with other intervention methods to reach satisfactory health outcomes [178]. There is a global need for an appropriate, rapid, cost-effective and sensitive tool for detection of STH infections in order to decrease burden of disease by identifying low-intensity infections and a subsequent targeted sustainable reduction in worm burden [43].

Real-time qPCR methods have recently been tested for STH diagnosis, targeting species-specific gene markers such as ITS-1 or ITS-2 [229, 230, 261]. However, these methods currently have limitations that make them not applicable in field settings such as the need for trained scientific personal [261]. Particularly challenging is the transfer of customized qPCR methods among laboratories with a requirement for optimization via significant molecular biological expertise. A reliable, automated diagnostic tool could have the potential to overcome issues related to reproducibility and create a standardized method of STH infection detection.

In the current study, we evaluate a multiplexed-tandem PCR (MT-PCR) based assay to differentiate, identify and quantify each major STH species in genomic DNA isolated directly from stool samples. The method is user-friendly, has high sensitivity and specificity and is produced as a standardized kit that is commercially available and readily transferrable to other laboratories. The method is semi-automated and requires little *a priori* expertise in molecular diagnostics or parasitology. Although unlikely to be cost-effective for routine diagnostics at the present time, the method provides a useful research tool for epidemiological studies of STHs in endemic regions, particularly in populations where prevalence and intensity of

infection is highly variable and the limitations of direct egg counting by microscopic examination is impractical or insufficient.

Methods

Ethics statement

Written informed consent for this prospective study was received from all study participants, or from parents or guardians for participants under the age of 18 years in Timor-Leste and Cambodia respectively. Ethics approval for the Cambodian based part of this study was provided by the National Ethics Committee for Health Research of the Ministry of Health in Cambodia (269NECHR, 27th of June 2016) as well as by the Human Research Ethics Committee of the University of Melbourne (1647208). Ethic approval for the Timor-Leste based part of this study has been received from the Human Research Ethics Committees at the Australian National University (2015/111) and the Timor-Leste Ministry of Health (2015/196).

Study areas, faecal sample collection, DNA isolation and multiplexed qPCR validation

Timor-Leste

Field sampling structure, sample processing and multiplexed quantitative PCR (qPCR) validation for Timor-Leste samples have been described elsewhere [231, 262]. Briefly, stool samples were obtained from 462 school children attending six primary school in Aileu and Manufahi municipalities, Timor-Leste, at the baseline of the (S)WASH-D for Worms pilot study. A 2-3 g aliquot of each sample was preserved in 5 mL of 5% potassium-dichromate (weight/volume) and transported at room temperature to QIMR Berghofer Medical Research Institute (Brisbane, Australia), where DNA extraction was performed using the PowerSoil DNA isolation kit after the manufacturer's instructions (Qiagen, Germany). All samples were stored at -20°C following DNA extraction. Initial STH multiplex qPCR targeted *A. lumbricoides*, *T. trichiura*, *N. americanus* and *Ancylostoma* spp. A total of 462 baseline

samples of isolated genomic DNA were used for validation of STH infection prevalence and intensity using the MT-PCR method.

Cambodia

Field sampling structure and sample processing of Cambodian samples are as follows. A total of 166 faecal samples from participants originating from ten remote villages in Preah Vihear province, Cambodia were obtained from collaborators in 2016 for testing the MT-PCR method at baseline and 3-month follow-up (n=332) and had been previously tested by multiplex qPCR targeting hookworm species only using a published protocol (quantification and identification of *N. americanus*, *A. ceylanicum* and *A. duodenale*) [243]. Faecal samples were collected in the morning by each participant and transported to the laboratory at room temperature within 60 minutes of sample collection. As much as 3 mL faeces was preserved in 6 mL 5% potassium dichromate for subsequent shipment to the University of Melbourne, Australia. In preparation for DNA isolation, preserved faeces were centrifuged at 2,000 g for 3 minutes, supernatant decanted, and the faecal pellet washed in 15 mL sterile H₂O (twice) in order to eliminate preservative and avoid interference with downstream molecular assays. 250 mg of the washed faecal pellet was used for DNA isolation using the ISOLATE Faecal DNA Kit (Bioline, UK) according to the manufacturer's instructions. All samples were stored at -20°C before validation using an established multiplexed qPCR protocol [243]. A total of 302 samples of the cohort described above has been used to validate the developed MT-PCR assay.

Molecular multiplexed-tandem PCR assay assessment

In collaboration with an industry partner (AusDiagnostics Ptd. Ltd., Australia) we have used an established commercially available multiplexed molecular diagnostic platform, the Easy-Plex system, to develop an assay targeting human STH infections in faecal DNA samples [237].

Multiplexed-tandem polymerase chain reaction (MT-PCR) tests were developed targeting the β -tubulin 1 locus of *A. lumbricoides* (GenBank accession number FJ501301.1), *T. trichiura* (GenBank accession number AF034219.1), *N. americanus* (GenBank accession number EF392851.1), *A. duodenale* (GenBank accession number EF392850.1), and targeting the second internal transcribed spacer region of the nuclear ribosomal RNA gene (ITS-2) for *A. ceylanicum* (GenBank accession number JN164660.1). The PCR primers for these assays are held in commercial confidence by AusDiagnostics Pty. Ltd.

The MT-PCR is a nested PCR method consisting of a primary PCR performed in multiplex on a Gene-Plex CAS1212 liquid handling robot (AusDiagnostics, Pty Ltd., Australia), followed by a secondary, tandem real-time qPCR (in which testing is conducted in single-plex in a tandem battery of reactions) run on a LightCycler 480 system (Roche, Switzerland). At the initiation of testing, DNA samples are placed on the Gene-Plex robot deck in a designated loading area and provided a unique sample ID (entered manually into a computer interphase linked to the Gene-Plex robot) which is carried over throughout the MT-PCR testing. Upon initiation of the amplification protocol, the robot distributes 5 μ l of each sample DNA to a separate 20 μ l reaction well in the “multiplex reaction strip tubes” provided by the STH MT-PCR kit manufacturer. No template controls (dH₂O) are included with each MT-PCR run and carried over through both amplification phases. Each well of this strip contains lyophilized standardized quantities of each external primer pair for each STH species and an internal spike (consisting of 10,000 copies of a 120 bp, heterologous, synthetic oligonucleotide) to control for PCR inhibition and provide a quantification standard for each sample. The initial multiplex step is performed in a conventional thermocycler unit installed on the Gene-Plex robot deck. This amplification phase consists of 15 cycles with the following parameters: denaturation at

95°C for 10 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 20 seconds with no initial denaturation or final extension phase.

Following the initial multiplex PCR, all first round product amplicons are diluted by the GenePlex robot (this is done to eliminate primary carry-over and PCR inhibition) and used as a template for the secondary tandem real-time PCR step. Tandem PCR is conducted on a 384-well plate in 20 µl reactions in a reaction master mix containing SYBR Green I/HRM dye. Each well of the 384-well plate contains lyophilized internal PCR primers for one target species or the internal spike control per reaction arranged in tandem array (i.e., multiplex amplicon from one sample is loaded onto a tandem array of six consecutive reaction wells, with each well containing one amplicon specific primer pair). Following loading of the tandem PCR plate, the plate is sealed using a self-adhesive heat-adherent film (MSB1001, BioRad, USA) and then loaded onto a LightCycler 480 system (Roche, Switzerland). The secondary amplification consisted of 30 cycles with the following profile: denaturation at 95°C for 10 seconds, annealing at 60°C for 15 seconds and extension at 72°C for 15 seconds with initial denaturation at 95°C for 10 minutes and no final extension phase.

Following the tandem real-time PCR phase, each amplicon is subjected to high-resolution melt-curve (HRM) analysis (from 72°C to 95°C). Following HRM, the melt profile for each amplicon for each sample is assessed for quality, purity (based on melt peak number, height and width) and specific identity (based on estimated melting temperature relative to control parameters determined using purified positive control DNAs for each targeted species during initial assay development by AusDiagnostics Pty. Ltd.). A positive or negative test call was determined based on these HRM results. Quantity of each test-positive amplicon was then calculated based on its amplification cycle threshold (Ct-value) relative to the cycle threshold

of the sample-specific internal spike control. From the initial loading of the sample DNAs onto the Gene-Plex robot, all steps of the MT-PCR method are automated, including the determination of positive test results and their quantitation, which is provided at the end of the reaction in a tabular and graphical format by the software (MT Analysis Software, AusDiagnostics Pty. Ltd., Sydney) used to run the reaction protocol (MT Assay Setup Software for the multiplex-PCR phase, AusDiagnostics Pty. Ltd., Sydney; and LightCycler 480 Software Version 1.5 for the tandem-PCR phase, Roche, Switzerland). Upon initiation of the initial multiplex reaction protocol, the only additional operator input required prior to final test results is the application of a heat-sealing film between the multiplex and tandem-PCR phases and the transfer of the sealed tandem-PCR reaction plate from the Gene-Plex robot to the Lightcycler.

Evaluation of MT-PCR diagnostic performance

All samples tested in the current study were assessed previously for STH infections by multiplex qPCR [231, 262]. We evaluated both the quantitative and qualitative diagnostic performance of the MT-PCR method against the previous molecular test results. Run-to-run variation was assessed for the assay by testing all 462 (5 µl) faecal DNA samples from Timor-Leste in duplicate, with disagreements (n=31; 6.7%) tested in triplicate. As this testing showed high run-to-run consistency between replicates, subsequent testing of samples from Cambodia (n=302) has been performed in single replicate (5 µl). Diagnostic sensitivity of the MT-PCR was assessed using the previously published multiplex qPCR as the diagnostic gold standard; i.e., we defined a “true positive” as samples that are positive by both molecular diagnostic methods (multiplex qPCR and MT-PCR) and a “true negative” as samples that are negative by both methods. MT-PCRs that disagreed with the multiplex qPCR were defined as “false positive” or “false negative” respectively. These potentially “false positive” or “false negative” samples were retested via the previously published conventional qPCR with slight

modifications. Briefly, we tested all disagreements using a single-plex qPCR approach for the respective gene targets (*A. lumbricoides* n=31, *A. ceylanicum* n=1, *T. trichiura* n=1 and *N. americanus* n=25). Tests were evaluated using 1x SensiFAST SYBR No-ROX mastermix (Bioline, UK), 2 µl template DNA and optimized primers as described elsewhere in a total reaction volume of 20 µl [230, 231]. The DNA quantification and melt-curve analysis for all tests was performed on the LightCycler 480 instrument (Roche, Switzerland) using the following conditions: 3 minutes at 95°C followed by 40 cycles of 9 seconds at 95°C and 30 seconds at 60°C with a final step-wise denaturation from 60°C to 97°C in 1.1°C/second increments. On retesting, we consistently found primer dimer formation at Ct values equal to 35 (n=18) within the negative control of the *N. americanus* qPCR test after 35 cycles. For the purpose of verifying that sample degradation had not influenced MT-PCR testing, we considered any single-plex qPCR retests as negative if amplification was detected above Ct = 35.

Data analysis

Data and statistical analysis was performed using the Stata 12.1 (StataCorp LP, USA), Prism 7.0b (Graphpad Software Inc., USA), RStudio 1.1.463 (RStudio, Inc., USA) and Excel 15.4 software (Microsoft, USA). Power calculations for chi-squared tests were performed in RStudio 1.1.463 (RStudio, Inc., USA) using the *pwr* package 1.2-2 [263]. All tests used a significance level of $p = 0.05$ with a degree of freedom (df) of 2-1 (i.e., 1) (S3 Table). STH infection prevalence and intensity data were analysed in Excel 15.4 with visualization in Prism 7.0b. Interrater agreement values - described as Cohen's κ - between both molecular diagnostic tests, 95% confidence intervals, significance of κ (using a p-value threshold for statistical significance of 0.05), sensitivity and specificity of MT-PCR have been determined using the Stata 12.1 software package. Cycle threshold values of MT-PCR vs qPCR were plotted against

each other in a scatterplot and a simple linear regression analysis was performed using Prism 7.0b with R^2 values confirmed through regression analysis in Stata 12.1.

Results

This study evaluated a total of 764 faecal DNA samples (462 Timor-Leste and 302 Cambodia) that had been previously tested by qPCR examination [231, 262]. Each sample was tested by MT-PCR as described above, of which 20.3% were positive for *A. lumbricoides*, 36.4% were positive for hookworm (32.3% were positive for *N. americanus*, 4.1% *A. ceylanicum*) and 1.4% were positive for *T. trichiura*. No infections were detected for *A. duodenale*. Infection prevalence in Timor-Leste with any STH was 42.9% with species-specific infections of 33.5% for *A. lumbricoides*, 2.4% for *T. trichiura*, 10.4% *N. americanus* and 1.1% *A. ceylanicum*. Hookworm infections dominated in the Cambodian cohort with prevalence of 65.9% for *N. americanus* and 8.6% for *A. ceylanicum* infections. Overall infection prevalence with any STH species was generally higher in Cambodia (70.5%) than Timor-Leste (42.9%) (Figure 2.1).

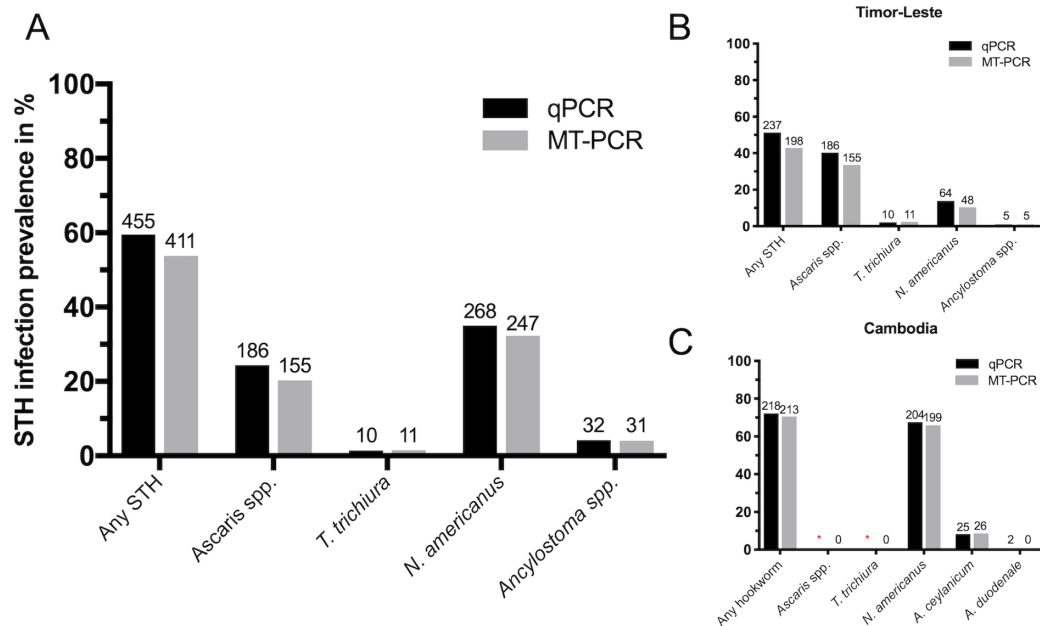


Figure 2.1. Overall infection prevalence of the major soil-transmitted helminth species comparing multiplexed qPCR and MT-PCR diagnostic approaches in cohorts from Timor-Leste (B) Cambodia (C) and combined (A). (A) Representation of combined infection prevalence for Timor-Leste and Cambodian cohort in percent with total numbers of positive infections as seen on individual bars. (B) Estimation of infection prevalence in Timor-Leste cohort of 462 stool samples with percentage infection prevalence on y-axis and STH species by diagnostic method on x-axis. (C) Estimation of infection prevalence in Cambodian cohort of 302 stool samples with percentage infection prevalence on y-axis and STH species by diagnostic method on x-axis. Estimation of infection by qPCR has been conducted for hookworm infections only. Field study setting is known to show very little to no *A. lumbricoides* or *T. trichiura* infections as to why these infections have not been included in estimation of infection prevalence by qPCR with confirmation of zero positive infections by MT-PCR within the scope of this study (*).

Table 2.1. 2x2 contingency table for any soil-transmitted helminth infections. Any STH infections is defined as a positive infection by molecular diagnostic analysis with one or more STH species.

	MT-PCR positive	MT-PCR negative	Total
qPCR positive	409	46	455 (59.55%)
qPCR negative	2	307	309 (40.45%)
Total	411 (53.80%)	353 (46.20%)	764

Chi-square table values for all investigated species are as follows: *A. lumbricoides* 276 true negative, 31 false negative, 0 false positive, 155 true positive (n=462); *N. americanus* 494 true negative, 23 false negative, 2 false positive, 245 true positive (n=764); *T. trichuris* 451 true negative, 0 false negative, 1 false positive, 10 true positive (n=462); *Ancylostoma* spp. 732 true negative, 1 false negative, 0 false positive and 31 true positive (n=764). Total diagnostic infection values for any STH species are 307 true negative, 46 false negative, 2 false positive and 409 true positive (n=764) as shown in Table 2.1.

Interrater reliability values, kappa (κ), showed a very good agreement between MT-PCR and conventional qPCR (Table 2.2), as defined by Cohen [264], as follows: <0.2 poor agreement, 0.20-0.40 fair agreement, 0.40-0.60 moderate agreement, 0.60-0.80 good agreement and 0.80-1.00 very good agreement. Total amount of agreement between both molecular diagnostic methods for true positive and true negative validation shows a percentage of agreement for all tests > 93.29%. Diagnostic specificity (true negative rate) of the MT-PCR method relative to multiplex qPCR was above 99.60% for all tests (Table 2.2). Diagnostic sensitivity (true positive rate) relative to multiplex qPCR ranged from 83.33% (*Ascaris*) to 100.00% (*Trichuris*) with three tests above 91.42%. The quantitative capacity of the MT-PCR method was compared by linear correlation of MT-PCR vs qPCR considering samples that were infection positive by both diagnostic methods (Table 2.2, Figure 2.2). Quantitative capacity was visualized considering all samples independent of infection prevalence by the various diagnostic methods (Figure 2.2). MT-PCR gene copy number estimates ranged from 28 – 70,059,042 with a median of 590,956.3 for *A. lumbricoides*, 41 – 1145.5 with a median of 84.5 for *T. trichura* and 15 – 781,465 for hookworms, with species-specific medians of 4228 for *N. americanus* and 4940 for *A. ceylanicum*, providing a possible initial starting point of inferring infection intensity measure/intestinal burden from gene copy number counts.

Table 2.2. Interrater reliability as defined by kappa agreement values (Cohen's kappa, κ) including standard error of κ , 95% confidence interval (CI) of κ and significance of κ ; and coefficient of determination (R^2) for qPCR vs MT-PCR to determine closeness of data to fitted linear regression line. Standard errors (SE) range from 0.0361 to 0.0465 with a 95% confidence interval ranging from 0.808 to 1.00 among all tests. All p-values for κ are <0.00001 assuming interrater reliability measurements to be significant. R^2 values are given in percentages of the total number of samples validated for each STH species (N). Only samples deemed infection positive by both methods were included in this analysis (1 *A. lumbricoides* and 3 *N. americanus* outliers were removed (highlighted in Figure 2)). All *A. ceylanicum* positive infections represented co-infections with *N. americanus* and were subsequently discarded from this analysis.

	Total Agreement (%) Agreement)	Sensitivity (%)	Specificity (%)	κ	SE of κ	95% CI of κ	p-value of κ	N	qPCR vs MT-PCR R^2 (%)
<i>A. lumbricoides</i>	431 (93.29)	83.33	100.00	0.8566	0.0460	0.808-0.905	$p<0.00001$	155	97.36
<i>T. trichiura</i>	461 (99.78)	100.00	99.78	0.9513	0.0465	0.856-1.00	$p<0.00001$	10	75.48
<i>N. americanus</i>	739 (96.73)	91.42	99.60	0.9268	0.0361	0.899-0.955	$p<0.00001$	245	80.63
<i>Ancylostoma</i> spp.	763 (99.87)	96.88	100.00	0.9834	0.0362	0.951-1.000	$p<0.00001$	-	-

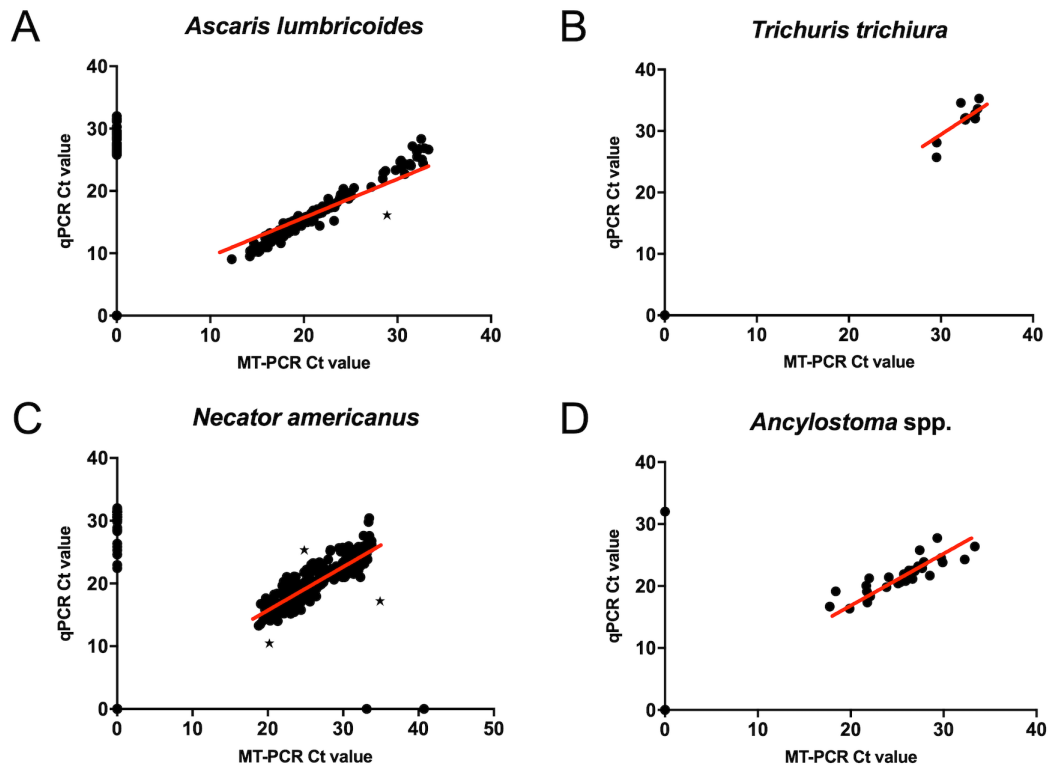


Figure 2.2. Cycle threshold (Ct) value scatterplot for all investigated STH species showing agreement of multiplexed qPCR and MT-PCR for all samples tested infection positive by either none, one or both molecular diagnostic methods. *Ascaris lumbricoides* (A) *Trichuris trichiura* (B) *Necator americanus* (C) *Ancylostoma* spp. combined values for *A. duodenale* and *A. ceylanicum* (D). Highlighted samples were removed for analysis of coefficient of determination which determines the closeness of data to a fitted linear regression line using only samples deemed infection positive by both molecular diagnostic methods (*).

Discussion

We have evaluated the multiplex-tandem PCR as a semi-automated diagnostic tool suitable for human STHs, comparing its performance to a multiplexed qPCR assay. Although copro-microscopic analysis is overwhelmingly the predominant method for diagnosis of STH infections, there is no clear gold standard for determining “true positives” in STH diagnostics [220, 226]. Based on this, we adopted the approach of considering a “false positive” or “false negative” by MT-PCR based on previous multiplex qPCR results for this dataset [231, 262], followed by re-testing of the disagreeing samples using a single-plex qPCR with an established ITS-1 or ITS-2 gene marker (GenBank accession numbers AB571301.1, FM991956.1, AJ001599.1, EU344797.1) [231]. Using this approach, two “false positive” tests (n=2) in the MT-PCR validation (n=764) determined relative to qPCR were confirmed as true positives, yielding a total diagnostic specificity of above 99.78% for each species assay.

We note that all faecal samples tested in the current study are samples collected from field sites in Timor-Leste and Cambodia in 2016 and have been stored at -20°C since their original testing by qPCR. After several freeze-thaw cycles, it is possible that the samples were influenced by DNA degradation, which may influence their subsequent detection. To test this, we retested all samples that were “false negative” by MT-PCR (n=55), using the conventional qPCR protocol they originally tested positive by. Per the above, 38 of these samples retested as positive with an average Ct value of 30.80 (S1 Table). The remaining 17 samples yielded faint positives with the majority of Ct values between 30 to 35 (S1 Table). This indicates that *A. lumbricoides* MT-PCR has a slightly lower sensitivity than the conventional qPCR, but that sample degradation may have impacted on some of our test results, and requires further evaluation with fresh field samples. Using this approach, total diagnostic sensitivity of the MT-PCR ranged from 90.64 (*Ascaris*) to 100.00% (*Trichuris*).

Overall, our study supports recent efforts to develop qPCR as a diagnostic alternative to or complement of faecal microscopy [228]. Each MT-PCR assay had very good agreement ($\kappa > 0.85$) and strong quantitative correlation ($R^2 > 0.7548$) to a recently published multiplexed qPCR for these species [231, 243]. Limitations of the quantitative correlation include limited numbers of *T. trichuris* positive samples ($n=10$) as well as no *Ancylostoma* spp. single infection positive samples, which are required to present a complete assay evaluation (Table 2.2). Both standard qPCR and the MT-PCR methods have clear advantages over faecal microscopy in terms of sensitivity. The limited sensitivity of faecal microscopy for STH detection, particularly hookworm, is well documented in the literature [265, 266]. This has increasingly been noted as a challenge to sustainable STH control in regions where prolonged oral MDA programs have resulted in a substantial reduction in worm burden such that the majority of infections now fall below the WHO definition for “light intensity” infections [21]. This reduction will clearly impact on the global STH disease burden and with the increased economic development in many STH endemic countries as well as the potential for the emergence of drug resistant helminths, the focus needs to begin to shift toward sustainable control and STH transmission interruption [267]. Programs to monitor transmission and to allow efficient control of STHs in populations where regional MDA programs are no longer sensible will require more sensitive diagnostic approaches than faecal microscopy can provide [255]. As shown in previous studies of parasitic nematode infections, nested qPCR diagnostics show a higher sensitivity measure compared to that of a standard qPCR while including a quantitative estimation missing in traditional PCR approaches [268].

The primary limitations associated with a shift to a molecular or PCR-based diagnostic for STH infections include (i) the complexity of PCR application in endemic settings, (ii) a limitation

in the transferability or reproducibility of bespoke PCR techniques and a need for standardization for clinical applications, (iii) the lack of clear relatability (conversion of molecular diagnostic Ct values to epg counts) of PCR-based test results to WHO treatment/burden guidelines, which is based on faecal egg densities and found to be on average 4x lower than egg intensity counts by molecular diagnostic for *N. americanus* [243], and (iv) the relative cost differences between microscopic and PCR-based detection. The MT-PCR method developed here is built around a user-friendly, largely automated robotic platform that requires minimal molecular biological expertise. All kit reagents are produced and standardized by a commercial entity accredited for clinical diagnostic assay production, supporting rapid and reliable transfer among laboratories. The MT-PCR method is quantitative and further evaluation of the correlation between copy numbers and eggs per gram will provide meaningful information on helminth intensity as well as assist in its translation to assess intensity-related morbidity. However, as found in other studies investigating the use of multiplexed qPCR for STH diagnosis [226], correlations between qPCR results and egg intensities are moderate. These results can be inferred via logistic regression, but lack of knowledge about target gene copy number per genome limits our ability to make conclusions about helminth burden (particularly for loci, such as the nuclear ribosomal RNA gene, that are of an unstable copy number) [269]. Establishing these threshold levels will require additional screening of well characterized control samples of known egg densities as well as further field evaluation including patient specific health records to assess burden.

Finally, regarding cost, recent estimates for multiplex qPCR-based testing of STH infected stools put the cost per sample at US\$2.61 [230] as a single-well and qPCR mastermix are used per sample (run in duplicate). The same study estimated true costs per sample for microscopy at US\$2.60, indicating that the cost-effectiveness of microscopy is likely over-estimated. At

present, the MT-PCR method is not competitive with these costs, with testing costing approximately US\$7.31 (AUS\$10.17) per sample for the current assay configuration following DNA extraction (this calculation does not include cost of liquid handling robot or qPCR thermocycler; approximate cost of DNA extraction US\$ 6.85 (AUSD\$ 9.64)). However, our focus here was on assay evaluation not on maximizing the efficiency or economics of the system. Overall, we acknowledge the current limitation of the platform, considering all resources associated, to laboratory-based most likely non-endemic settings. We have addressed the issue of to date limited data availability of PCR based helminth diagnostics approaches which perform with an increased sensitivity compared to currently performed copro-microscopic approaches [220].

In summary, we find the MT-PCR method to be a rapid, semi-automated and user-friendly molecular diagnostic tool for STH infection that provides comparable performance to conventional multiplex qPCR and superior sensitivity to faecal microscopy.

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Supplementary files

S1 Table. Raw data file of MT-PCR and qPCR diagnostics

S2 Table. STARD checklist

S3 Table. Statistical power analysis

S1 Figure. STARD flow-diagram

10. Conclusion and future directions

Within the scope of this chapter, I have developed and validated a novel multiplexed-tandem qPCR (MT-PCR) assay for detection of STH infections. Advantages of the here presented assay are its high specificity (direct nested amplification approach), high sensitivity (one gene copy number), multiplexing (screening of up to 24 biological samples targeting up to 8 infectious species by their gene marker), semi-automation using a robotic liquid handling platform (accessible customer usage), speed (excluding DNA extraction 2.5 hours to diagnosis) and easy implementation with little prior knowledge (automation). I used this molecular diagnostic tool to screen faecal samples from two cross-sectional cohort studies in order to determine STH infection status (prevalence and intensity) compared to a standard qPCR. I found high infection rates among participants with a good agreement between diagnostic tools. This MT-PCR tool provides a platform that can aid in a more sensitive diagnosis of STH infections, updated prevalence mapping and ultimately lead to screen-and-treat strategies in the future.

Chapter 3 – Effective low-cost preservation of human stools in field-based studies for helminth and microbiome analysis

11. Context

STHs co-inhabit the same environmental niche as the bacterial gut community within the body. The gut microbiome serves multiple important functions from digestion to regulation of the immune system. The homeostatic balance between commensal and pathogenic bacteria is what regulates a healthy or disease state. Some studies have found impacts of infection on the bacterial diversity and abundance. To investigate microbiome alterations, stool sample collection is important and heavily reliant on effective preservation until downstream processing.

Here, I have compared a non-toxic chemical preservative to preservation in 2.5% potassium dichromate and fresh freezing at low temperatures. I have characterised the gut microbiome of a cross-sectional cohort of 273 pre-school and school-aged children to evaluate the impact of faecal sample preservation methods on the bacterial gut diversity and abundance. These data were correlated to environmental factors such as sex, age, school and STH infection status. I established the overall STH infection prevalence and intensity status using the above developed MT-PCR tool.

12. Manuscript II

Full length title: Effective low-cost preservation of human stools in field-based studies for helminth and microbiome analysis

Short length title: Stool preservation for helminth and microbiome analysis

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Key words: Gut microbiome, 16S rRNA sequencing, soil-transmitted helminths, preservative

12.1. Abstract

Molecular studies of gastrointestinal infections require either rapid sample processing or effective sample preservation. This is difficult in remote settings in low-income countries, where the majority of the global infectious disease burden exists. Refrigeration or freezing of samples upon collection is often not feasible and the cost of commercial preservatives is prohibitive. We investigated three low-cost preservation methods: a salt-based buffer consisting of DMSO, EDTA and NaCl (DESS), 2.5% potassium dichromate (PD) and rapid fresh freezing at -80°C (FF), for qPCR-based diagnosis of STH infection and microbiome characterisation in school-aged children from North-West Thailand. DESS performed best for STH infection (*A. lumbricoides*, *A. ceylanicum* and *T. trichiura*). All methods performed similarly for microbiome preservation as measured by alpha-diversity. DESS and FF methods were also suitable for RNA and protein preservation. We found DESS is an effective and low-cost (<AUD\$0.20 per mL) preservative that eliminates the need for frozen storage, hence making it particularly applicable for studies in remote and resource poor settings. We then applied DESS to STH and microbiome characterisation in a larger cross-sectional study (n=273) of children in the same schools in Thailand. *Ascaris lumbricoides* (107/273) and *T. trichiura* (100/273) were the predominant helminths detected, with 36.17% of *A. lumbricoides* infections exceeding egg per gram intensities of >1,000. STH infection status did not influence alpha-diversity or overall abundance, but was correlated with changes in beta-diversity.

12.2. Importance

Molecular tools are now a cornerstone of diagnostic and epidemiological studies. However, they are no better than the quality of samples they are used on. Studies of gastrointestinal infections and the impacts of infection on gut microbial health require rapid sample processing and/or effective preservation of a range of molecular media, including DNA, RNA and proteins. This preservation must introduce minimal disruption or bias into the data. These challenges are particularly difficult in the field of neglected parasitic infections, which overwhelmingly affect impoverished populations in remote, tropical to subtropical, regions worldwide. We compared two chemical preservation methods to the current low-temperature freezing gold standard to provide a more practical and cost-effective alternative for use in at-risk populations.

12.3. Introduction

Soil-transmitted helminths (STHs), namely roundworms (*Ascaris* spp.), whipworms (*T. trichiura*) and hookworms (*Ancylostoma* spp. and *N. americanus*), are among the most common parasitic causes of neglected tropical disease worldwide [43, 270]. These helminthiases disproportionately affect children, resulting in a global estimated loss of 1.2 million DALYs in children alone [271]. Depending on the infective species, infection intensity and host nutritional and immunological status, symptoms include malnutrition, diarrhea, abdominal pain and anaemia, which may lead to stunting, wasting and impaired physical or cognitive development [272].

Since 2012, in response to the UN Millennium Development Goals, there has been a concerted global effort to reduce STH burden in children through annual or biannual treatment with BZ targeting >75% of at-risk, school-aged children in endemic regions [21]. These programs have reduced the intensity of infection and disease-related morbidity [166]. However, quantifying the magnitude of this change and its sustainability is limited by the availability of methods with adequate sensitivity to diagnose infection under conditions of low worm burden and low population prevalence [273]. As endemicity rates fall, there is a need to move from low-sensitivity and -throughout diagnostic tools such as KKTS method [220] toward molecular-based methods such as quantitative PCR [230]; meaning that effective sample preservation gains importance [274]. Furthermore, in addition to currently quantified measures of disease, there is increasing evidence that STHs impact on the host gut microbiome [121], as has been shown for other parasitic infections [275]. This may lead to changes in bacterial diversity and abundance [122, 123], which can have a range of important impacts on child development such as brain function [114, 115, 276], immune-inflammatory disease [70], obesity, bowel disease, allergies and diabetes [128, 277]. It is

also possible that a converse relationship exists, where gut microbial composition influences susceptibility to STH infection [278], although this remains unclear. Not all STH studies support these findings of infection induced gut microbiome alterations [127], and their potential pathology has not been determined [115].

Application of molecular diagnostic tools for faecal samples relies on appropriate sample collection procedures that preserve samples integrity, and support extraction of nucleic acids and other macromolecules [279]. This requires snap freezing of the sample upon collection and then either cold-transport [280], or chemical preservation [281]. Due to the remote and resource-poor locations of many STH endemic populations, cold-storage is not always available or is impractical [282]. Inadequate sample preservation and prolonged processing times can lead to a rapid loss of detectable infections, particularly for hookworms [214], and impact on STH infection prevalence and intensity estimates [283]. Commercial preservatives for faecal samples are cost prohibitive in resource poor settings [281]. Many chemical preservatives (e.g. Tris-EDTA, 5% formalin or 70% ethanol) have significant limitations, including leading to a loss of bacterial diversity [246] or poor parasite recovery [226, 245], being unsuitable for DNA/RNA preservation [282] or being toxic [284]. Whereas others (e.g. FTA cards, OMNIgene Gut, RNAlater, 95% ethanol,) have shown adequate sample preservation over short time timeframes [282, 285], the majority of which are again cost-prohibitive. A safe, effective and low-cost preservative is required for human stools for molecular diagnostic and epidemiological studies. Ideally samples should be able to be stored for several weeks at warm temperatures [245] without significant degradation of DNA, RNA or proteins. Such preservative has the potential to contribute moving toward an universal and transferable sample processing protocol from

collection, preservation and storage to DNA extraction, library preparation and finally sequencing [286].

In this study, we evaluated two low-cost chemical stool preservation methods, a DMSO, EDTA and NaCl based buffer (DESS) [287] and 2.5 % potassium dichromate (PD), compared to conventional rapid fresh freezing at -20°C/-80°C (FF) for the preservation of DNA, RNA and proteins from stool for STH diagnosis and microbiome characterisation. We then used the lowest-cost, best overall preservative method to characterise STH infection and faecal microbiome composition in a population of pre-school and school-aged children in a remote community in Tha Song Yang, Tak province, Thailand.

12.4. Methods

Ethics statement

Oral informed consent was received from all parents or legal guardians for participants under the age of 18 years. Ethics approval was provided by Mahidol University (FTM ECF-019-05) as well as by the Human Research Ethics Committee of the Walter and Eliza Hall Institute of Medical Research (HREC Project #16/10).

Study area, faecal sample collection and processing

A total of 273 faecal samples were collected from 11 children's day care centers located in the Mae Song subdistrict, Tha Song Yang district, Tak province, Thailand in 2017 from pre-school and school-aged children (2 to 6 years old). Eligibility criteria, sample collection, processing, storage and transport can be found within the supplementary files (S1 Figure, S2 Figure). Epidemiological data (age, sex, school, date of birth, height, weight) for each participant were collected during sample collection from 28th to 30th of March 2017.

Each parent or legal guardian was provided with an infant stool collection kit containing one faecal sample collection container (TechnoPlas Cat.No. P5744F), a pair of single-use disposable gloves, a zip-locked bag and information sheet describing the guidelines for infant stool collection procedure at first appointment. One double-contained sample (maximum of 30 mL or as much stool as possible whilst minimising the environmental contamination) was requested to be returned within 24 hours of the collection kit drop off. Upon collection, a source data sheet including the participants ID, date and time of sample collection, sample processing date and preservation method was compiled. At collection (<12h), one gram of stool was used for STH diagnosis by direct duplicate Kato-Katz Thick

Smear and light microscopy performed by two independent examiners. Concurrently, faecal samples were screened for parasitic protist infections via iodine stained direct faecal smear fixed in sodium acetate-acetic acid-formalin [288].

Each stool sample was transported on ice within 12 hours of sample drop-off to the laboratory field site in Mae Sot, Thailand. Each sample was classified in accordance with the Bristol Stool Chart for stool consistency [289]. If the participant's stool was classified as type 5-7, the stool was transferred into 50 mL falcon tubes for centrifugation at 14,000 x g for 5 minutes. The supernatant was discarded while avoiding contact with the remaining faecal pellet. Each pellet was split into three equal aliquots of 10 mL stool in fresh 50 mL falcon tubes of which one was stored fresh frozen at -20°C, one in 10 mL DESS at room temperature (1:1 sample to preservative ratio; 20% dimethyl sulfoxide, 0.25 M ethylenediaminetetraacetic acid, saturated sodium chloride) [287] and one in 30 mL 2.5% potassium dichromate at room temperature (1:3 sample to preservative ratio). Each sample was shaken vigorously to ensure an appropriate sample to preservative ratio.

Upon preservation, all samples were transferred on ice within 72 hours to the main laboratory located at Mahidol University (Bangkok, Thailand). On arrival, each sample for each preservation method was split into two 1 to 1.5 g aliquots (one for analysis and one for back-up) into 2 mL universal Nunc tubes (i.e., six tubes in total for each sample, each containing 1 to 1.5 g of stool). Upon splitting, fresh frozen (FF) samples were stored at -20°C and DESS and 2.5% potassium dichromate preserved samples were stored at 4°C. All samples were shipped to the Walter and Eliza Hall Institute of Medical Research with fresh frozen samples transported on dry-ice and DESS or PD preserved samples on cold packs.

Immediately after arrival all faecal samples were transferred into long-term storage with fresh frozen samples at -80°C and chemically preserved samples at -20°C.

DNA extraction

Prior to DNA extraction, each sample was thawed and thoroughly homogenized using a sterile wooden spatula. To remove chemical preservatives each sample stored in DESS or PD was washed twice with 50 mL sterile water at room temperature (RT). After centrifugation at 2,000 x g for 3 minutes the supernatant was decanted and a maximum of 150 mg of the remaining pellet used for DNA extraction. Left-over preservative-free faecal pellets were stored at -80°C. Extraction of DNA was performed using the Bioline ISOLATE Fecal DNA Extraction kit according to the manufacturer's instructions (Bioline, Cat. No. BIO-52082). To test each preservation method, an initial representative subset of 48 samples were selected (based on Kato-Katz Thick Smear STH infection data and participant age) and compared for each of the three preservatives (n=144). DNA was extracted from these samples, quantified using the Qubit 2.0 fluorometer (Thermo Fischer Scientific, USA) and used for helminth detection as well as microbiome characterisation. Following testing (see below), DNA was extracted from all remaining DESS (n=225) preserved samples, quantified using the Qubit 2.0 fluorometer (Thermo Fischer Scientific, USA) and used for the complete cohort analysis.

Three-way comparison of DESS, PD and FF for STH infection diagnosis and microbiome characterisation

Samples for three-way comparison of preservatives were chosen based on reported STH infection prevalence by KKTS covering a range of low to high infection intensities. Molecular diagnosis and quantitation of STH infections were conducted for each of these

48 representative samples for each preservation method, by multiplexed-tandem qPCR (MT-PCR) [290] for roundworm (*A. lumbricoides*), whipworm (*T. trichiura*) and hookworm (*Ancylostoma* spp. and *N. americanus*) species following the manufacturer's instructions (AusDiagnostics, Ltd. Py., Australia). Briefly, the multiplexed-tandem qPCR approach combined a total of 8 gene markers targeting known STH species (*A. lumbricoides* GenBank accession number FJ501301.1, *T. trichiura* GenBank accession number AF034219.1, *N. americanus* GenBank accession number EF392851.1, *A. duodenale* GenBank accession number EF392850.1, *A. ceylanicum* GenBank accession number JN164660.1, *S. stercoralis* GenBank accession number AH013160.2, Pan Hookworm GenBank accession number KJ543495.1, spike control). A nested qPCR approach was applied to determine infection prevalence (first round multiplex amplification on a liquid handling robot with a second round species-specific singleplex amplification) as previously described elsewhere [237]. 5 µl of each genomic DNA was tested using this assay including a no template control (dH₂O) in each run.

Recovery of the faecal microbial community was characterised for the same sample subset (n=144) by preservation method through 16S rRNA gene sequencing. Two technical replicates per sample were sequenced, using the universal bacterial 16S V4 primers (515F 5'-CTGAGACTTGACATCGCAGCGTGYCAGCMGCCGCGGTAA-3' and 806R 5'-GTGACCTATGAACTCAGGAGTCGGACTACNVGGGTWTCTAAT-3') on the Illumina MiSeq platform (Illumina, USA) [291, 292]. Demolecularized water (dH₂O) was used as a negative control and ZymoBIOMICS microbial community DNA standard (Cat. No. D6300, Zymo Research, USA) as a positive control on each plate. Each PCR was conducted with the following conditions: 10 µl OneTaq master mix with standard buffer (2x) (New England Biolabs, USA), 0.4 µl of each primer (10 µM) and 2 µl DNA template

made up to a total reaction volume of 20 µl with water were amplified for 3 minutes at 95°C, 45 seconds at 95°C, 60 seconds at 50°C, 90 seconds at 72°C in 20 cycles with a final extension time of 10 minutes at 72°C. PCR products were diluted 1:5 in molecular grade H₂O and diluted products used as a template for a second PCR amplification using unique adapter barcodes (8-mer) for each sample. 10 µl of diluted PCR product was amplified using 15 µl OneTaq master mix with standard buffer (2x) (New England Biolabs, USA), 1.5 µl of each primer (10 µM) and made up to a total reaction volume of 30 µl using H₂O. Cycling conditions were as follows: 3 minutes at 95°C, 45 seconds at 95°C, 60 seconds at 55°C, 90 seconds at 72°C in 25 cycles with a final extension time of 10 minutes at 72°C. A representative selection of PCR products was quality controlled throughout the library preparation using gel electrophoresis by size separation on a 1.5% agarose gel. Five µl of each sample was pooled and the pooled library cleaned up using the NucleoMag NGS clean-up and size-select kit (0.8X) as per the manufacturer's instructions (Machery-Nagel, Germany). Pooled libraries were eluted in 55 µl of sterile H₂O and quantified on the Agilent 4200 TapeStation (Agilent, USA) and amplicon sequenced on the Illumina MiSeq platform using the MiSeq Reagent Kit V3 2 x 300bp as per the manufacturer's instructions (Illumina, USA).

Data analysis

STH infection prevalence and intensity

MT-PCR data were processed in Excel 16.13.1 (Microsoft, USA), Stata 12.1 (STATA Corp, USA) and visualized using GraphPad Prism 7.0d (GraphPad Software Inc., USA). Samples were deemed infection positive or negative as per MT Analysis Software output (AusDiagnostics Pty. Ltd., Sydney). Kappa statistics (interrater reliability), sensitivity, specificity and correlation with epidemiological variables were calculated to evaluate

performance of the MT-PCR method compared to the gold standard KKTS using Stata 12.1 (STATA Corp, USA). Statistical analysis (one-way ANOVA) of matched samples for each preservative was performed in GraphPad Prism 7.0d (GraphPad Software Inc., USA). P-values of <0.05 were assumed to represent statistical significance following a p-value correction for multiple testing by the Benjamini-Hochberg false discovery rate (FDR) method [293]. Impacts of STH infections defined as stunting and wasting were calculated as height-for-weight, height-for-age and weight-for-age z-scores compared to the 2006 WHO child growth standard [294]. Anthropometric z-score calculations were performed in Stata 12.1 (STATA Corp, USA) using the “zscores06” command [295].

Gut microbiome characterisation of the V4 16S rRNA gene

Microbial community analysis has been performed using an established protocol [296]. Briefly, metadata for each replicate were compiled in a mapping file based on their unique index barcodes using QIIME1 [297]. Paired-end reads were merged with a minimum overlap of 100 bp, a maximum assembly length of 600 bp and a minimum assembly length of 80 bp using the paired-end read merger PEAR [298]. Fastq sequences were demultiplexed and sequence orientation adjusted using QIIME with p=0.90 and a Phred quality score cut-off at 29 (base call accuracy >99.9%). 16S V4 rRNA gene universal primers were trimmed using a trim_fasta_amplicons script (Jocelyn Penington, Papenfuss Laboratory, https://github.com/PapenfussLab/Jocelyn_tools/blob/master/amplicon_tools/trim_fasta_amplicons.py). The trimmed reads were aligned to the Silva bacteria database, the alignment cut and sequences filtered to remove potential overhangs at both ends using the Mothur 1.39.5 software (Mothur, USA) [299].

After quality filtering, sequence deduplication, operational taxonomy unit (OTU) clustering, reference generation and chimera filtering was performed using VSEARCH with a minimum sequence length of 64 bp. Taxonomy was assigned to unique sequences using the Greengenes database [300]. In order to generate a phylogenetic tree, sequences were aligned to the reference in Mothur and a phylogenetic tree was built with the QIIME script `make_phylogeny.py` which employs the FastTree tool. Finally, a biom file was generated containing all trimmed and quality filtered sequences including metadata, taxonomy and phylogeny. The full pipeline is available online (https://github.com/PapenfussLab/RothSchulze_microbiome).

In RStudio 1.1.453 (RStudio, USA) technical replicates were merged and data rarefied to even depth for alpha-diversity and differential abundance analysis using phyloseq 1.26.1 [301]. Merged replicates were normalised using the cumulative sum squaring (CSS) normalization in QIIME1 for beta-diversity. Alpha-diversity was calculated by plotting observed richness, inverted Simpson and Shannon estimated diversity indexes using the `plot_richness` function in phyloseq 1.26.1 and visualized with microbiomeSeq 0.1 (<https://github.com/umerijaz/microbiomeSeq>). Statistical analysis for alpha-diversity measures, were performed by using pairwise analysis of variance (ANOVA) tests on a fitted, linear and releveled regression model on observed richness data as calculated with the `estimate_richness` function in phyloseq 1.26.1. Beta-diversity was calculated using the `ordinate` and `distance` function with Bray Curtis measure in phyloseq 1.26.1. Statistical analysis for beta-diversity measures were performed with Euclidian distance and 999 permutations using the `adonis` function in the `vegan` package 2.5-4 and `pairwise.perm.manova` function in RVAideMemoire package 0.9-73. Relative taxonomic abundance analysis for phyla and genera was conducted by estimating the mean proportion,

fitting a linear model and applying a pairwise ANOVA test in RStudio 1.1.453 (RStudio, Inc., USA). Differential abundance analysis of OTUs was performed using QIIME1 with the 'DESeq2_nbinom' algorithm. A p-value threshold of <0.05 was used for statistical significance following p-value correction for multiple testing by the Benjamini-Hochberg FDR method [293] for all tests. Data visualization such as alpha-diversity measure (observed richness and inverted Simpson diversity), beta-diversity measure (Bray-Curtis distance for PCoA and weighted UniFrac for NMDS), relative abundance and differential abundance were visualised using the phyloseq 1.26.1 and ggplot 3.1.0 packages in RStudio 1.1.453 (RStudio, Inc., USA).

Cohort analysis for STH infection diagnosis and microbiome characterisation

Following comparative analysis of DESS, PD and FF preservation methods, we examined STH infection prevalence and intensity, as well as microbial community structure in all study participants (n=273), based on the DESS preserved stool aliquots only. For microbiome characterisation, we tested for sequence batch effects in the initial 48 and subsequent 225 sequenced DESS preserved samples. We did this by merging both datasets before the OTU clustering step in vsearch and plotted alpha- (observed richness p-value 0.9654; inverted simpson p-value 0.9088) and beta-diversity (p-value 0.001). Because the intra-sample diversity showed no significant change among sequencing runs, we combined the data for all subsequent analyses. All molecular procedures and data analysis steps were performed as described above.

Data availability

Raw fastq data files for all 16S rRNA gene amplicon sequenced samples for the complete study, were deposited in the NCBI Sequence Read Archive (SRA) under BioProject ID PRJNA606654.

RNA and protein extraction

Although not the focus of the current study, we also undertook comparative testing of the suitability of each preservation method for the recovery of RNA and proteins. RNA (n=5) was extracted using the Quick-DNA/RNA Microprep Plus kit after the manufacturer's instructions (Zymo Research, USA). A maximum of 200 mg faecal material per sample, was lysed using 1 mL DNA/RNA shield in ZR BashingBead Lysis Tubes for 50 seconds at 4,000 g using a MOBIO Powerlyzer (MOBIO, USA). Lysed samples were centrifuged at 11,000 x g for 30 seconds and the supernatant transferred into a clean microcentrifuge tube. An equal volume of DNA/RNA Lysis Buffer (1:1) was added to non-preserved samples and 4 volumes (4:1) to samples in preservative. Subsequently, all samples were processed according to the RNA purification protocol (Quick-DNA/RNA Microprep Plus kit, Zymo Research, USA) with half the ethanol volume added during RNA purification. Extracted RNA was quantified on the 4200 TapeStation System (Agilent, USA) (S9 Figure).

Proteins were extracted using the RNA purification flow-through for acetone precipitation (Quick-DNA/RNA Microprep Plus kit, Zymo Research, USA). Protein pellets were resuspended in 100 µl 2.5% SDS + 100 mM Tris + 5 mM DTT. Total protein extracts were quantified using a bicinchoninic acid (BCA) assay (Thermo Scientific, Pierce™ BCA Protein Assay Kit, Cat. No. 23225) following the manufacturer's instructions. Following from quantification, quality of total protein extracts (5 µg) were separated via

polyacrylamide gel (SDS-Page) gel electrophoresis (S8 Figure). RNA and total protein extracts were stored at -80°C and -20°C respectively for future analysis.

12.5. Results

Cohort epidemiology

All 273 participants (133 male and 132 female) included in this study were pre-school and school-aged children from 3 to 6 years, distributed as follows: 3 years (18 female, 27 male), 4 years (45 female, 38 male), 5 years (35 female, 35 male), 6 years (24 female, 24 male) and 27 individuals with no age information (10 female, 9 male). We note that 8 individuals had no gender information provided. Participants were from 11 schools from Tha Song Yang province, Tak district in Thailand, namely: Mae Song, Thi Mo Ko Tha, Mae Salid Khi, Mae Salid Luang, Mae Salid Noi, Krae Khi, Mae Kho, Mae Nil, Mae Nil Khi, Huay Manhok and Huay Manhok Khi. Stool samples were collected from the 28th to 30th of March 2017 as part of an ongoing local field study.

Total infection prevalence of any helminth species by microscopy was 48.7% by KKTS (Table 1). Infection intensity as determined by microscopic egg count ranged from 1 – >1,000 *As. lumbricoides* fertilized/unfertilized eggs (mean egg count 595.02) and 1 – 190 *T. trichiura* eggs (mean egg count 21.23). We note that 51 (46.36%) *As. lumbricoides* infected samples had egg densities reaching a threshold of 1,000 eggs per gram at which counting ceased. Hookworm prevalence was not assessed as it was not feasible to process samples within one hour after drop-off (per [214]). Each sample was assessed for additional parasitic infections via field-based microscopy, with the results as follows: 2 *Enterobius vermicularis*, 41 *Giardia duodenalis*, 5 *Entamoeba histolytica*, 69 *Entamoeba coli*, 29 *Blastocystis* spp., 5 *Iodamoeba bushlii*, 53 *Endolimax nana*, 10 *Chilomastix mesnili* and 1 *Cytoisospora bellii* infections.

Child growth measures were available for 246 (90.11%, aged 0 – 5 years) of the 273 children with average measures as follows: mean age 59.19 completed months (36 – 82), mean weight 15.79 kg (8.2 – 21.7) and height 103.47 cm (78 – 122). Of this subset 27 (10.98%) children were stunted (height-for-age < -2 standard deviation from median) (n=15 any STH infection), 6 (2.44%) were wasted (weight-for-height < -2 standard deviation from median) (n=2 any STH infection) and 17 (6.91%) were underweight (weight-for-age < -2 less than standard deviation from median) (n=9 any STH infection). Further, 4 (1.63%) children were deemed too tall for their age (height-for-age > 2 standard deviation from median) and 1 (0.41%) had a weight-for-height measure above 2 standard deviation from median (n=2 any STH infection).

Comparative analysis of DESS, PD and FF for stool preservation

The impact of sample preservation on molecular studies was assessed in 48 representative samples, comparing DMSO, EDTA and saturated sodium chloride (DESS), 2.5% potassium dichromate (PD) or fresh frozen on-site in dry ice (FF). These samples included 26 females and 22 males, aged from 3 to 6 years (8 3-year old, 11 4-year old, 19 5-year old, 10 6-year old), and were selected based on STH infection status by KKTS (44 *As. lumbricoides* and 33 *T. trichiura*, with 29 co-infections) and participant age.

STH infection prevalence and intensity

By MT-PCR, STH infection rates for *As. lumbricoides* and *T. trichiura*, respectively, was 41 (85.42%) and 29 (60.42%) for DESS, 40 (83.33%) and 31 (64.58%) for PD, and 27 (56.25%) and 7 (14.58%) for FF. Infection intensity, based on MT-PCR calculated gene copy number, ranged from 57 to 1,343,337 for *As. lumbricoides* (mean Ct values 24.33 DESS, 25.23 PD, 27.04 FF) and 18 to 3,206 for *T. trichiura* (mean Ct value 30.83 DESS,

31.55 PD, 31.52 FF). Among this sample subset, *As. lumbricoides* positive DESS preserved samples showed a significant increase in MT-PCR gene copy number (p-value 0.004) and decrease in Ct value (p-value 0.0091) compared to the matched fresh frozen gold standard (S4 Figure). DESS and PD preserved *T. trichiura* positive samples both showed an increased gene copy number (p-values 0.0001, 0.0022) and decrease in Ct values (p-values <0.0001) compared to FF. We found no hookworm (*N. americanus*, *A. duodenale* or *A. ceylanicum*) positive individual within this sample subset regardless of the preservation method (S4 Table).

Gut microbiome characterisation

In addition to examining the effect of preservation on STH recovery, we assessed its impact on the inferred richness and diversity of the faecal gut microbiome of each sample based on sequencing of the V4 region of the 16S rRNA gene. Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria and Tenericutes were the predominant bacterial phyla in all samples, with no significant difference in relative abundance among preservatives (Figure 3.1, S6 Table). We note that relative abundance measure of bacterial genera of the same samples cannot reach 100% due to the presence of unassigned taxonomy during data processing. No significant difference was observed among preservation methods in intra-sample (alpha) diversity based on observed richness for DESS (p-value 0.758) and PD (p-value 0.176) or inverted Simpson measure (a measure considering the evenness among samples) for DESS (p-value 0.562) or PD (p-value 0.851) compared to FF as the gold standard (Figure 3.2, S7 Table). Inter-sample (beta) diversity was calculated via Bray-Curtis distance (quantification of compositional dissimilarity) and principal coordination analysis (PCoA). Statistical analysis found a significant clustering by preservative (p-value

0.012) for DESS (p-value 0.021) and PD (p-value 0.048) preservatives compared to FF (Figure 3.2, S7 Table).

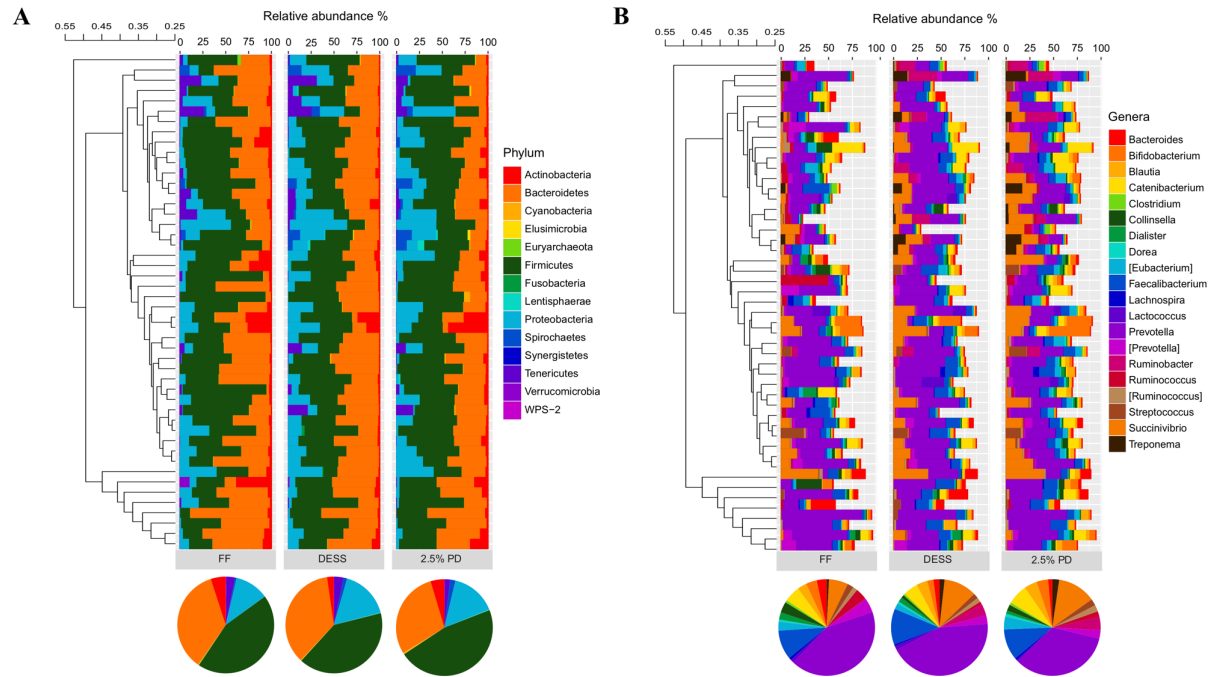


Figure 3.1. Hierarchical clustering of 48 trial samples preserved in DESS, PD or FF (n=144) with corresponding bacterial gut community structure as per phyla (A) and genera (B). Pie charts represent the mean relative abundance of each bacterial phyla per sample preservation method. Scale bar represents evolutionary distance.

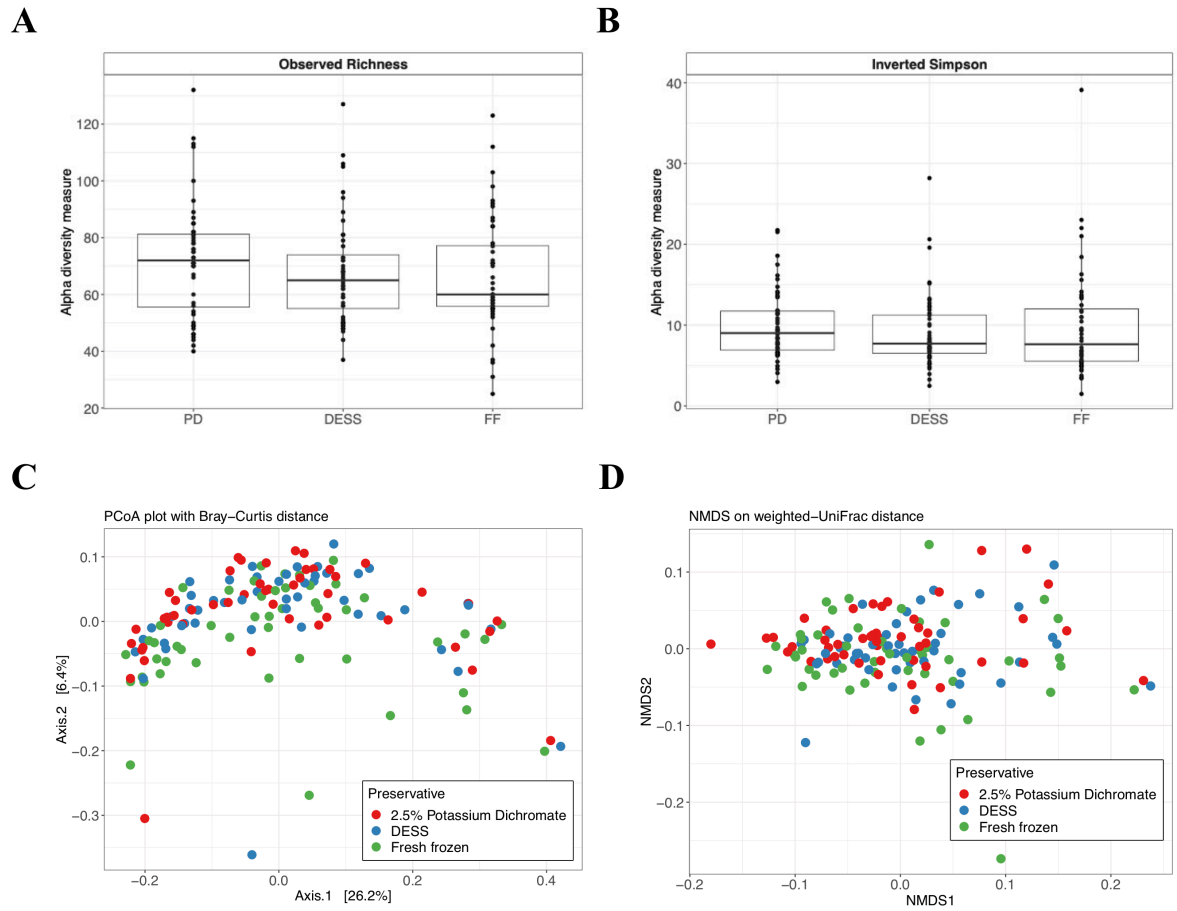


Figure 3.2. Alpha-diversity measure as per observed richness and inverted Simpson measure (A, B). Beta-diversity measure as calculated by principal coordinate analysis (PCoA) with Bray-Curtis distance clustering and non-metric multidimensional scaling (NMDS) using weighted UniFrac distance measure (C, D). Pairwise ANOVA analysis for alpha-diversity measures found no statistically significant difference among either preservative (S5 Table).

RNA and protein preservation

Although not the focus of the current study, we tested five faecal samples for the preservation and recovery of high-quality RNA and proteins for subsequent molecular study. For DESS and PD preserved samples, we tested extraction with and without prior removal of the preservative by centrifugation and washing (see methods). Extracted RNA ranged from 15.4 to 41.6 ng/μl, with fresh frozen supporting the highest overall yield. RNA quality, as inferred by gel electrophoresis, was highest for DESS preserved samples (with and without prior preservative removal) as inferred by the presence of clear and intact large and small ribosomal RNA bands (S5 Figure). Fresh frozen preservation provided the highest yield (mean 215.8 μg per sample) and overall quality protein extracts (per SDS-Page and Silver Staining; S4 Figure). In contrast, chemical preservation resulted in a loss of ~50-75% of protein yield and increased peptide fragmentation. PD gave slightly higher yields and DESS slightly higher protein quality. Nonetheless, either chemical method appears sufficient for protein preservation for conventional proteomics analysis [302]. We cannot infer whether protein extracts from chemically preserved faecal samples will be of sufficient quality for immunodiagnostics; this needs to be tested using antibodies specific to this purpose and these are, at present, not commercially available.

Cohort study based on DESS preserved stools only

As DESS performed as well as or better than PD and FF methods for STH diagnosis and microbiome preservation, we undertook baseline analyses of all study participants (n=273) using DESS preserved samples only.

STH infection prevalence and intensity

A total of 273 samples including 133 male, 132 female and 8 participants with no gender data were analysed (S2 Table). Based on KKTS, 133 samples were positive for either *As. lumbricoides* (n=110) or *T. trichiura* (n=86) with 63 instances of co-infection. Egg densities for these infections ranged from 1 to >1,000 for *As. lumbricoides* and 1 to 190 for *T. trichiura*, noting that egg counting was capped at 1,000 eggs per gram (there were 51 such samples, all for *As. lumbricoides*) and no testing was undertaken for hookworm due to the time between sample collection and processing exceeding 1 hour (per [214, 303]).

Based on molecular testing, STH prevalence in the study participants was 39.19% (n=107), 36.63% (n=100) and 1.10% (n=3) for roundworm (*As. lumbricoides*), whipworm (*T. trichiura*) and hookworm (*An. ceylanicum*) respectively (Table 3.1). Diagnostic target gene copy number for STH positive samples ranged from 39 – 4,711,749 for *As. lumbricoides* (mean copy number 273,375.38 and Ct value 23.82), 85 – 5,251 *An. ceylanicum* (mean copy number 3,058.33 and Ct value 28.85) and 37 – 6,343 *T. trichiura* (mean copy number 710.00 and Ct value 30.89) as determined by MT-PCR concentration (S4 Table). For the 51 *As. lumbricoides* samples containing >1,000 epg, target copy number ranged from 0 – 4,711,749.00 (mean copy number 505,851.45).

Diagnostic agreement between the microscopy-based method (KKTS) and MT-PCR was 91.58% and 88.28% for roundworm and whipworm. Diagnostic sensitivity (microscopy gold standard) was 88.18% for roundworm and 89.53% for whipworm with a diagnostic specificity of 93.87% and 87.70%, respectively. Interrater reliability (kappa) values ranged from 0.740 to 0.824 with confidence intervals from 0.656 to 0.893 (Table 3.1). Sixty-six infected individuals (46.81%) had co-infections with *As. lumbricoides* and *T. trichiura* by MT-PCR. One individual was infected with *As. lumbricoides*, *T. trichiura* and *An.*

ceylanicum. We detected no *N. americanus* or *An. duodenale* infections in any sample by KKTS or MT-PCR. We found no correlation between schools and STH infection status, except for one school in Mae Kho having the significantly highest number of infected children (p-value 0.000). Prevalence of infection by sex was 55 *As. lumbricoides* and 55 *T. trichiura* infections in male; and 54 *As. lumbricoides* and 44 *T. trichiura* infections in female participants. All *An. ceylanicum* infections (n=3) were found in males. We found no correlation between sex and STH infection status (p-values 0.2809, 0.2043, 0.1431), MT-PCR Ct value (p-values 0.3924, 0.2080, 0.1138) and MT-PCR gene copy number (p-values 0.7997, 0.3510, 0.6873) for *As. lumbricoides*, *An. ceylanicum* or *T. trichiura*, respectively. Overall, we found a relatively even distribution of species-specific infections among all age groups: 16 *As. lumbricoides*, 1 *An. ceylanicum* and 15 *T. trichiura* among 3-year olds; 30 *As. lumbricoides*, 0 *An. ceylanicum* and 28 *T. trichiura* among 4-year olds; 29 *As. lumbricoides*, 0 *An. ceylanicum* and 31 *T. trichiura* among 5-year olds; and 27 *As. lumbricoides*, 2 *An. ceylanicum* and 21 *T. trichiura* among 6-year olds.

Table 3.1. STH infection prevalence and intensity among SAC children from Tha Song Yang, Tak district, Thailand including all DESS preserved samples (n=273). Interrater reliability measure or kappa values are classified as follows: κ 0.0-0.2 non to slight, 0.2-0.4 fair, 0.4-0.6 moderate, 0.6-0.8 substantial, 0.8-1.0 almost perfect [304]. Sensitivity is classified as the rate of true positive samples and specificity as the rate of true negative samples compared to a known gold standard (Kato-Katz Thick Smear).

<i>Total cohort</i> (<i>n</i> =273)	Prevalence by KKTS	Mean total egg count	Prevalence by MT-PCR	Mean gene copy number	Mean cycle threshold value	Agreement		Sensitivity	Specificity	Kappa statistic (95% CI)
						(expected agreement) with standard error				
Any STH infections	133 (48.72%)	-	141 (51.65%)	-	-	89.74% (49.96%) 0.0604		92.48%	87.14%	0.795 (0.723 – 0.867)
<i>A. lumbricoides</i>	110 (40.29%)	595.02	107 (39.19%)	273,375	23.82	91.58% (52.10%) 0.0605		88.18 %	93.87 %	0.824 (0.755 – 0.893)
<i>T. trichiura</i>	86 (31.50%)	21.23	100 (36.63%)	710	30.89	88.28% (54.95%) 0.0601		89.53%	87.70%	0.740 (0.656 – 0.824)
<i>A. ceylanicum</i>	-	-	3 (1.10%)	3,058	28.85	-		-	-	-

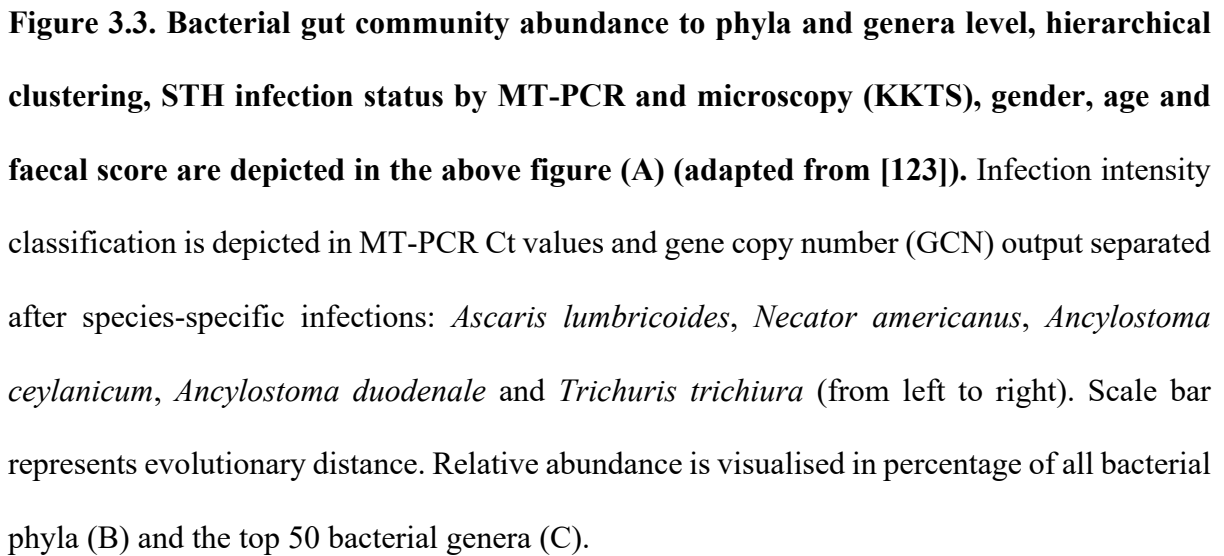
Gut microbiome characterisation

In addition to exploring STH infections, we assessed the cohort (n=273) for development and composition of the gut microbiome (inferred from faecal samples). Overall, Firmicutes, Bacterioidetes, Proteobacteria and Actinobacteria were the most abundant bacterial phyla across the complete cohort (Figure 3.3). The majority (56.53%) of operational taxonomic units (OTUs) assigned to bacterial genera were classified as *Prevotella*, *Faecalibacterium*, *Succinivibrio* and *Catenibacterium*. Before characterising differences in the microbiome associated with STH infection, we examined the data at a cohort level, considering participant sex, age and geographic location (Figure 3.4 – 3.6). Our study had an even representation of males and females. We observed no significant differences in alpha-diversity or beta-diversity based on participant sex and age. Further, we found no significant differences at a taxonomic level between male and female participants. However, two OTUs (OTU 65 *Bacteroides coprophilus*, OTU 9 Mollicutes) were more and six OTUs (OTU 214 *Prevotella*, OTU 84 *Parabacteroides*, OTU 89 *Bacteroides uniformis*, OTU 23 *Bacteroides fragilis*, OTU 200 *Ruminococcus* and OTU 20 *Lactococcus garvieae*) less abundant comparing male versus female participants (Figure 4, S7 Table).

Geographical location of the schools appeared to influence inverted Simpson alpha-diversity, with children from Mae Nil Khi and This Mo Ko Tha having significantly lower alpha-diversity compared to children from Huay Manhok (p-value < 0.05); and children from Mae Nil Khi showing a lower alpha-diversity than those from Mae Kho and Mae Song (Figure 3.5). Beta-diversity also significantly differed (p-value 0.032) among schools (Figure 3.6; S7 Table). These differences were also highlighted on a taxonomic level, with children from Huay Manhok Khi having a significant increase in Actinobacteria (p-value 0.0217) and those from

Mae Salid Noi, Mae Nil Khi and Mae Kho having significantly less Eubacterium (p-values 0.0311, 0.0385, 0.0433).

Among STH infected and uninfected study participants, at a community level, we found no significant difference in bacterial alpha-diversity (Figure 3.5). However, their faecal microbiomes did differ significantly in beta-diversity (p-value 0.021). Although, we found no significant changes at higher taxonomic levels, there were changes in differential abundance at the OTU level. Five OTUs, one Verrumicrobia (OTU 91, *Akkermansia muciniphila*), two Bacteroidetes (OTU 124, Prevotella; OTU 65, *Bacteroides coprophilus*), one Firmicutes (OTU 118, Ruminococcaceae) and one Tenericutes (OTU 9) were significantly more abundant and one OTU (OTU 527, *Bifidobacterium adolescentis*) was less abundant when comparing infected versus uninfected participants (p-value 8.00e-08) (Figure 3.4).



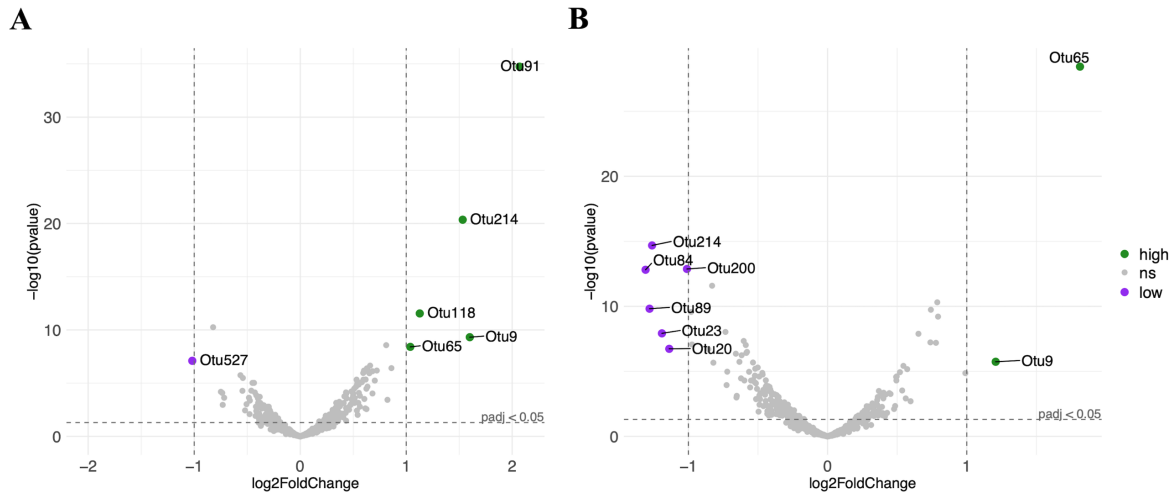


Figure 3.4. Significant increased and decreased abundance of operational taxonomic units as per log fold change influenced by STH infection status as per MT-PCR (A) and sex (B). Green OTUs are found to be significantly more abundant and purple OTUs significantly less abundant when comparing female versus male and infected versus uninfected participants (ns=non-significant). For STH infection status (A) OTU 91 *Akkermansia muciniphila*, OTU 124 *Prevotella*, OTU 65 *Bacteroides coprophilus*, OTU 118 Ruminococcaceae, OTU 9 Tenericutes are more and OTU 527 *Bifidobacterium adolescentis* is less abundant. For sex (B) OTU 65 *Bacteroides coprophilus*, OTU 9 Mollicutes, OTU 214 *Prevotella*, OTU 84 Parabacteroides, OTU 89 *Bacteroides uniformis*, OTU 23 *Bacteroides fragilis* are more and OTU 200 Ruminococcus, OTU 20 *Lactococcus garvieae* are less abundant.

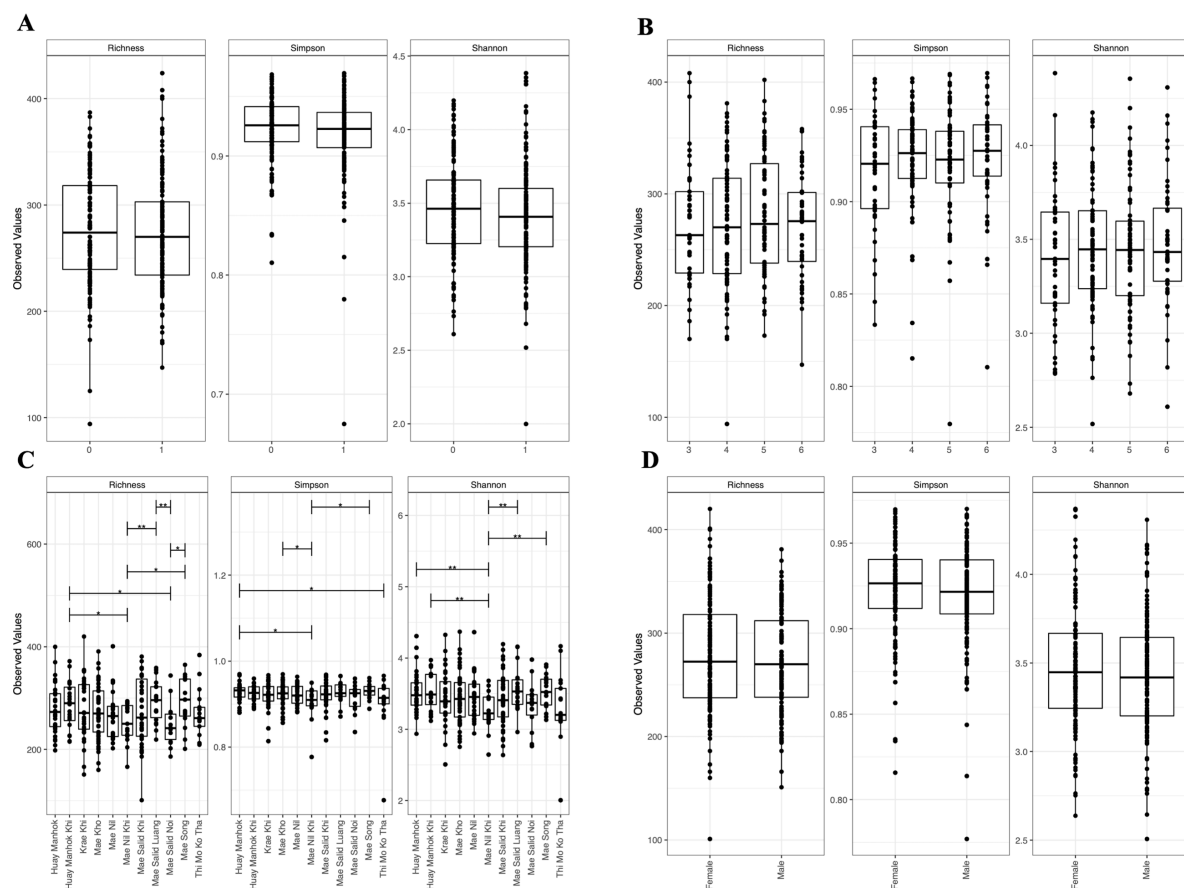


Figure 3.5. Alpha-diversity with analysis of variance (ANOVA) statistics of the complete cohort of faecal samples preserved in DESS (n=273). Statistical analysis was performed using a p-value cut-off of 0.05 (*0.05 **0.005 ***0.0005) for STH infection status as estimated by MT-PCR (0 uninfected, 1 infected) (A), age (3 to 6 years) (B), school (C) and sex (female, male) (D).

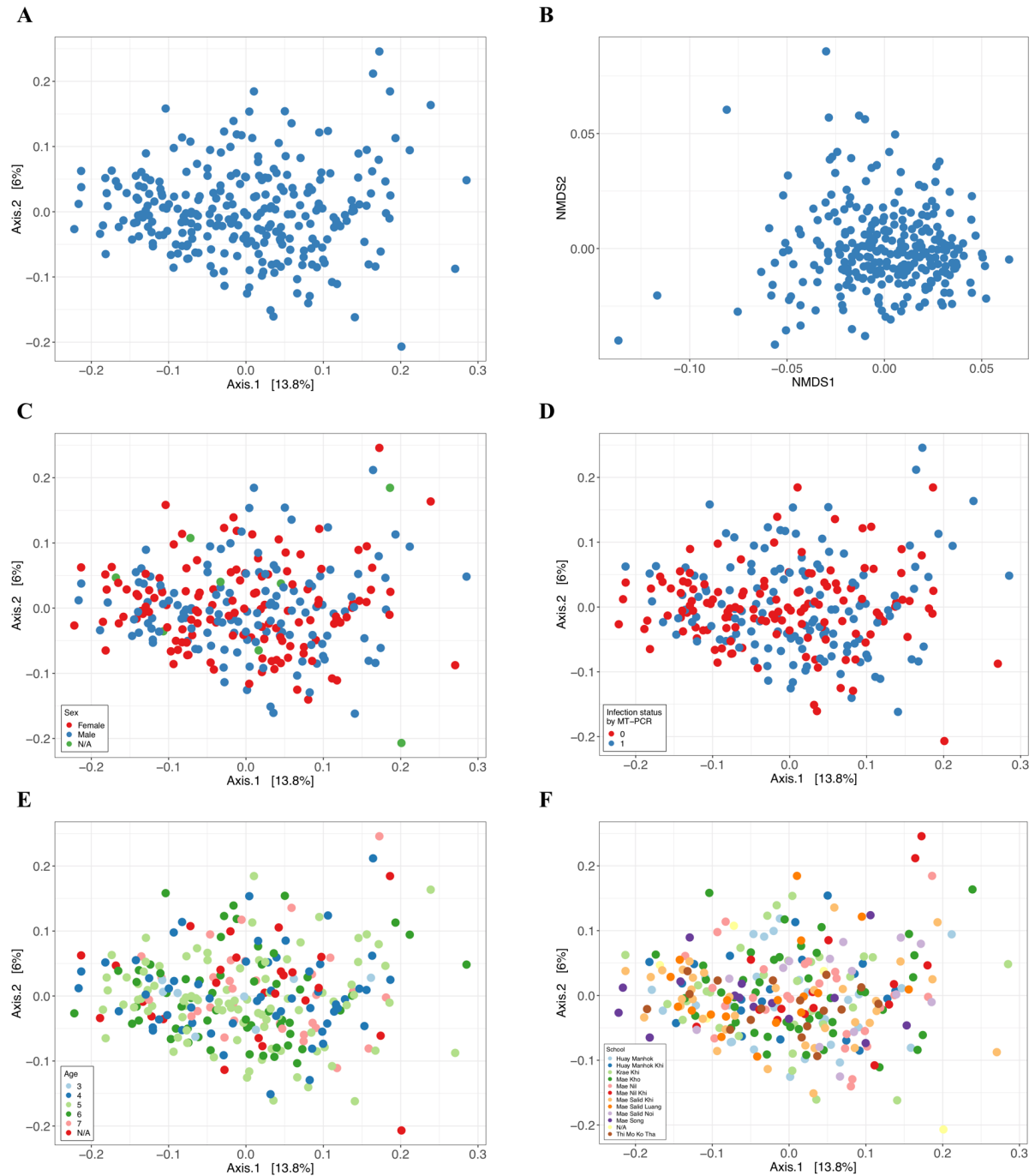


Figure 3.6. Beta-diversity measure as calculated by principal coordinate analysis (PCoA) with Bray-Curtis distance clustering (A) and non-metric multidimensional scaling (NMDS) using weighted UniFrac distance measure (B) of the complete cohort of faecal samples preserved in DESS (n=273). PCoA with Bray-Curtis distance clustering for sex (C), STH infection status (D), age (E) and school (F) are shown (p-values 0.571; 0.021; 0.778; 0.032).

12.6. Discussion

In this study, we compared three methods (DESS and PD versus FF sample storage) for preservation of human stools in resource poor-settings, with a focus on molecular diagnosis of STH infection and analysis of the faecal microbiome. Particularly in remote and resource-poor field settings, where the majority of intestinal parasitic nematode infections occur, sample freezing is difficult to implement and often unavailable. Chemical preservation provides an important alternative approach, but commercial preservatives are often prohibitively expensive. Previous studies have found DESS performs well for microbial community preservation [305, 306] and helminth recovery from canine faeces [307], but to our knowledge, this method has not been assessed for humans, nor has it been tested for preservation of STH eggs or recovery of other macromolecules, such as RNA or protein. In contrast, PD is been shown to be suitable for preservation of STH eggs for molecular diagnostics [231], but, to our knowledge, has not been assessed for microbiome or non-DNA macromolecular preservation.

We found that DESS provided the best overall performance for STH detection and sensitivity (as inferred by quantitative MT-PCR). All three preservatives performed similarly for 16S rRNA-based microbiome profiling, showing no statistically significant differences in intra-community composition (alpha-diversity). Differences in inter-community composition (beta-diversity) were significant comparing DESS and PD preserved samples to a fresh frozen standard. Fresh frozen samples provided the best RNA yield, but DESS provided the best RNA quality (based on evidence of RNA degradation on gel electrophoresis). DESS also provided acceptable protein recovery, but with some evidence of protein fragmentation. The highest quantity and quality of extracted proteins was found in fresh frozen samples. Overall, we find that DESS-based preservation provided

a high-quality and low-cost method to store faecal samples. DESS is low-cost (<\$0.20 per 1mL samples 1:1 ratio) and, unlike PD [308], non-toxic [287]. Future evaluation of DESS could interrogate larger sample sizes, a broader range of infection intensities and microbial pathogens, and long-term storage at a range of ambient (sub-tropical to tropical) and cold temperatures (4°C, -20 °C and -80 °C) (per [305]). Although not a focus of our study, further evaluation of the quality and any preservative induced biases in RNA or protein composition and yield for either target specific (e.g. quantitative reverse transcription PCR or western blot) or systems-based (e.g., metatranscriptomics or metaproteomics) is needed.

Our subsequent use of DESS in a larger cross-sectional study of STH prevalence and faecal microbiome composition showed that molecular diagnosis of STH infection had a strong agreement of 89.74% (overall kappa value 0.795; 0.824 for *As. lumbricoides* and 0.740 for *T. trichiura*) with Kato-Katz Thick Smear based microscopy. Quantitative-PCR-based inference of infection intensity yield estimated target gene copy numbers ranging from 39 – 4,711,749 for *As. lumbricoides* (39.19%) and 37 – 6343 for *T. trichiura* (36.63%). Additional studies of populations with moderate to high level infections are required to determine the gene copy equivalents to egg-density based infection thresholds; noting this we found a significant difference in Ct value (p-value 0.0003838) and gene copy number (p-value 5.343e-09) comparing KKTS *As. lumbricoides* positive samples with an egg count of <1,000 or >1,000. A large proportion of infected participants (46.81%) had co-infections with *As. lumbricoides* and *T. trichiura*. Co-infections with these parasites are common in STH-endemic populations globally [3], in particular in South-East Asia [130]. No hookworms were detected by the Kato-Katz Thick Smear method, but three *An. ceylanicum* infections were identified by MT-PCR. This is consistent with known STH epidemiological

data for Thailand, which suggests that hookworms are rare in most regions, but that *An. ceylanicum* is significantly more common than *An. duodenale* [309, 310].

In addition to assessing the prevalence and relative intensity of STH infections in Tha Song Yang school-aged children, we assessed their faecal microbiome composition and explored these data for an association with STH infection in a larger cohort study compared to previous studies [278]. Overall, the predominant bacterial phyla, with Bacterioidetes, Firmicutes, Proteobacteria and Actinobacteria being most abundant, were consistent with similar studies of enteric bacteria elsewhere [113, 125, 311]. A multitude of factors are able to shape the gut microbiome composition. Besides infection [121, 312], this can include diet [116], age [313, 314], caesarean versus vaginal birth [118], medication, particularly antibiotic usage [117] and numerous other factors [315]. In our study, we noted statistically significant differences in alpha-diversity with school location, possibly reflecting dietary differences. Noting these host factors, we found no evidence for an association between sex or age and STH infection and thus, are able to consider the effect of infection despite the confounding influence they had on microbial composition.

The acute and chronic influence of STH infections on child microbial health is not yet clear. This is due to cross-sectional studies comparing impacts between infected versus uninfected participants, which may have a history of past infections; therefore, these studies measure acute effects only. We found no difference in alpha-diversity or relative abundance with STH infection. However, we found an association in bacterial beta-diversity depending on STH infection status (infected versus uninfected); this is consistent with a previous study of STH infections in Sri Lanka [125]. Recently, field studies conducted in Malaysia, Liberia and Indonesia found STH infections correlated with an increase in the

host gut microbial diversity and richness [122, 123]. Another study found *T. trichiura* infections did not correlate with increased microbial diversity among school-aged children from Ecuador [127]. Previous studies investigating alpha- and beta-diversity alterations are conflicted with one reporting a significant increase in bacterial diversity and species richness associated with helminth, *T. trichiura*, colonization [122] and another none [127]. The majority of these studies compares infected versus uninfected children at a single timepoint; which implies currently uninfected children may have been infected several times in the past. Therefore, these studies measure acute effects only and highlight the need for longitudinal comparison of children from endemic versus non-endemic regions.

We found that other variables such as age (except for observed differences in beta-diversity) and sex did not influence the gut microbiome community among 3-6 year olds; this microbiome stabilisation has also been observed in studies investigating other parasitic infections [275]. Overall, OTUs identified as significantly more abundant in relation to infection status or sex have been linked to multiple confounding factors such as early childhood development (*Bacteroides fragilis*) [119], geographical location including diet (rural populations consuming plant-rich foods) (*Prevotella*) [122, 127, 316] and impacts caused by parasitic infections (*Parabacteroides*, *Tenericutes*, *Ruminococcaceae*, *A. muciniphila*) [125, 278, 317].

Lastly, we acknowledge the current limitations of commonly used bacterial taxonomic databases (Greengenes [300] and Silva [318-320]) in terms of species identification, public data availability and reference genomes [321, 322]. Although an improved curation of currently available databases has been undertaken for human intestinal 16S rRNA gene sequences, other large reference databases may not be able to provide a low-resolution to

a species or genus level [323]. Advantages is Greengenes taxonomic classification to a species level, which is not feasible using Silva [324]. Future directions may include metagenomic and metatranscriptomic sequencing rather than targeted 16S metabarcoding [325] and, where possible, culturing of unassigned microbes in order to determined taxonomic classification [326]. This is important as post-infectious disorders such as a change in susceptibility to microbial infections has been linked to helminth infections [327]. A predisposition (altered gut microbiome) in children can have a significant impact on their future development and has been linked to chronic disease such as asthma and inflammatory bowel diseases [328]. Importantly, large-scale longitudinal cohort studies are required to investigate chronic STH impacts [123] and long-term DESS preservation, as well as studies in endemic high intensity settings [275].

In conclusion, we have compared three faecal preservatives using a cross-sectional cohort of school-aged children aged 3-6 years from Tha Song Yang district, Tak region, Thailand. We evaluated these in terms of STH infection recovery, macromolecule preservation and gut microbiome characterisation. Overall, DESS performed as well as, or better than the other methods, and we find DESS adequate, low-cost and safe preservative for STH infection recovery, gut microbiome characterisation and RNA/protein extraction. Once DESS was deemed suitable we performed total cohort analysis of STH infection prevalence and intensity as well as microbial community characterisation. STH infection status did not alter the gut microbiome diversity within samples, but the diversity and composition among samples. Relative abundance of bacterial phyla and genera was similar to other communities worldwide and OTU abundance influenced by diet, geographical location and parasitic infection.

12.7. Acknowledgements

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12.8. Supplementary files

S4 Table. Microbiome metadata

S5 Table. Height-for-weight, height-for-age and weight-for-age z-score analysis

S6 Table. Relative abundance of bacterial phyla and genera including statistical analysis

S7 Table. OTU data tables for alpha and beta-diversity measures

S8 Table. Differential OTU abundance analysis for STH infection status and sex

S2 Figure. Stool collection workflow after STARD guidelines

S3 Figure. Statistical analysis of MT-PCR STH infection prevalence and intensity data

S4 Figure. Quantity and quality control of protein extracts

S5 Figure. Quantity and quality control of RNA extracts

13. Conclusion and future directions

In conclusion, the here provided information aid in further elucidating the impact of soil-transmitted helminth infections on the gut microbiome. I detected no changes in alpha-diversity between infected and uninfected children, possibly due to past infections. These impacts highlight an incomplete understanding of STH-related morbidity. Future studies require comparison to children from the same population that have never been infected in the past. Validation of DESS as a cost-effective, non-toxic preservative may advance future studies in endemic regions, in particular those with limited resources. This is likely going to help with a more accurate description of microbiome changes and STH infection prevalence and intensity estimates.

Chapter 4 – Soil-transmitted helminth beta-tubulin 1 amplicon sequencing for the detection of drug resistance associated SNPs in human populations

14. Context

BZ drug resistance against the two most commonly used drugs, albendazole and mebendazole, is a significant problem in livestock worldwide. Drug resistance is caused by high-pressure, long-term drug treatment. The SNPs Phe167Tyr, Glu198Ala and Phe200Tyr of the beta-tubulin 1 gene are causal for drug resistance in livestock. Similarly, human populations in STH endemic countries experience an on-going MDA as proposed by the WHO. There is rising concern that this treatment might lead to the emergence of drug resistance. Early detection is key to combating resistance as it (i) provides timely and immediate information about the current efficacy status and (ii) informs health organizations and stakeholders.

Here, I developed a high-throughput deep amplicon sequencing approach sequencing the beta-tubulin isotype 1 gene for SNP variants at codons 167, 198 and 200. I have tested this assay using three different cohorts from South-East Asia; Cambodia, Vietnam and Thailand, screening 376 faecal gDNA samples infected with *Necator americanus*, *Ascaris lumbricoides* and *Trichuris trichiura*.

15. Manuscript III

Full length title: Beta-tubulin 1 amplicon sequencing for detection of benzimidazole resistance associated SNPs in human soil-transmitted helminth populations

Short length title: STH beta-tubulin amplicon sequencing

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Key words: Amplicon sequencing, beta-tubulin isotype 1, benzimidazole drug resistance, soil-transmitted helminths, *Ascaris lumbricoides*, *Trichuris trichiura*, *Necator americanus*

15.1. Abstract

Soil-transmitted helminths (STHs) infect more than 1 billion people and have a global burden of 3.4 million DALYs. STH control depends predominantly on MDA with single oral-dose BZs, namely albendazole or mebendazole. BZ resistance is widespread among parasitic nematodes of livestock and caused by SNPs at codons 167 (Phe to Tyr), 198 (Glu to Ala) or 200 (Phe to Tyr) of the beta-tubulin isotype 1 (*tub-1*) gene. Amid increasing concerns about the global health implications of BZ resistance STHs, we have developed an amplicon sequencing approach targeting *tub-1* of three major STH species (*A. lumbricoides*, *T. trichiura*, *N. americanus*) allowing low-cost genotyping of codons 167, 198 and 200. We have used this tool to genotype STH eggs in faecal samples collected from 376 patients from South-East Asia (Cambodia, Vietnam, Thailand). Some participants were from populations that have never received drug treatment (Vietnam) and others have received annual BZ treatment for more than 5 years (Cambodia, Thailand), including a cohort with poor treatment efficacy at follow-up (Cambodia). We explored these data for selection under drug treatment. We saw no significant selection for known BZ resistance SNPs, but found a statistically significant reduction in variant allele frequency in a Cambodian population on follow-up, indicating some evidence for a selective pressure from BZ MDA (p-value 0.03955), which may preface emergence of resistant parasites.

15.2. Introduction

Soil-transmitted helminths (STHs) infect more than 1 billion people [29], and have a global burden of 3.4 million DALYs [4]. These infections disproportionately affect children in resource-poor populations [329], with around 270 million pre- and 550 million school-aged children considered at risk of infection living in high transmission settings [46, 330]. In 2012, the World Health Organization proposed an MDA approach to annually or biannually deworm 75% of school-aged children in STH endemic regions [21, 331]. BZs, namely albendazole or mebendazole, are the front-line drugs used in these programs [332].

Reduced BZ efficacy for STHs has been observed worldwide, although there is no clear consensus if this represents emerging drug resistance [188]. BZ resistance is common and verified in livestock and companion animals [183], which may suggest a risk in human populations [197]. For nematodes, drug efficacy is evaluated by egg reduction rate (ERR) and CR, i.e., the complete clearance of infection [179]. In veterinary fields, threshold ERR and CR levels are defined and failure to achieve a minimum reduction in a standardized treatment trial, the FECRT [333], is considered evidence of drug resistance [334]. However, these threshold levels are not established for human STHs, and FECRT is not standard practise in endemic settings [247].

The ambiguity of defining treatment failure as drug resistance is a common and challenging problem for infectious disease. *Trichuris trichiura* infections show a decrease in average cure rate from 2008 to 2017 of 12.9% (43.6% – 30.7%) when treated with single-dose albendazole [179, 195]. Hookworms exhibit low cure rates when treated with mebendazole (22.9% in 2008, 32.5% in 2017) [179, 195]. However, there are a variety of possible causes of reduced ERR and CR in STH infection besides drug resistance. These include poor

compliance [335], the use of counterfeit tablets [336], and the logistical and cost difficulties of accessing patients for repeat testing in remote and resource poor settings. Larval motility and egg hatching assays (EHA) are widely used in veterinary medicine, and allow direct assessment of drug sensitivity in a controlled test [337]. These are not practical in resource poor settings and cannot detect resistance below 25% prevalence in the population [338], which is too late to prevent its emerging. Recent studies have suggested that by the time drug resistance appears in clinical practice (i.e., is phenotypically evident), the genetics underpinning resistance have already reached ~50% prevalence in the parasite population [338].

The emergence of widespread BZ resistance would have major implications for global MDA programs and STH control and requires ongoing standardised diagnostic surveillance as a control strategy [338]. Recently, there has been a move towards the development of molecular diagnostic methods for the detection of BZ resistance in STHs [190], building on extensive research on this topic in veterinary fields [183]. There is a recognized need for a high sensitivity PCR-based genotyping tool to test for BZ-resistant STHs [188, 339]. BZs are suggested to bind the GTP binding pocket of the beta-tubulin isotype 1 gene (*tub-1*), which subsequently prevents assembly of microtubules by blocking beta-tubulin 1 and 2 dimerization [182, 183] and ultimately causing starvation of adult worms and preventing metabolism in larvae and eggs [340]. Among veterinary nematodes BZ resistance is associated with three causative single nucleotide polymorphisms (SNPs) at codon positions 167 (Phe/Tyr), 198 (Glu/Ala) and 200 (Phe/Tyr) [250]. A variety of molecular tools have successfully been developed for BZ resistance in veterinary parasites, for example real-time PCR and pyrosequencing [250] and amplicon sequencing [341] in *Haemonchus contortus* and other sheep nematodes. For human STHs, pyrosequencing has been used for

identification of BZ drug resistance in the beta tubulin 1 gene (*A. lumbricoides*, *T. trichiura*, *N. americanus*) [190, 191, 234]. Although, this method successfully sequenced the desired gene targets, it is limited to identification of known, targeted SNPs [251]. Further limitations are associated cost and unreliable results for low allele frequencies due to the assay's error rate [190].

Here, we developed an amplicon sequencing approach to genotype the beta-tubulin 1 gene for three major human STH species, *A. lumbricoides*, *T. trichiura* and *N. americanus*, in faecal samples. We then tested this approach in stools collected from three South-East Asian cohorts (Cambodia, Vietnam, Thailand).

15.3. Materials and Methods

Three cohorts originating from Thailand, Vietnam and Cambodia, respectively were used for this study. Work at the Walter and Eliza Hall Institute of Medical Research was conducted under human ethics permit by the WEHI Human Research Ethics Committee (HREC Project #16/10). Thailand samples under the ethics permit FTM ECF-019-05 provided by Mahidol University, Bangkok, Thailand. Vietnamese samples under human ethics permit #1443512 provided by the Behavioural and Social Sciences Human Ethics Sub-Committee (The University of Melbourne, conducted under the supervision of the Center for Public Health, Dak Lak, Vietnam). Cambodian sampling under human ethics permit 269NECHR provided by the National Ethics Committee for Health Research of the Ministry of Health in Cambodia.

Sample collection, processing and DNA extraction

Thailand samples

Thai samples were collected in 2017 as part of a cross-sectional study investigating the infection prevalence and intensity of STH infections in the area. In collaboration with researchers from Mahidol University (Bangkok, Thailand) we have collected 273 faecal samples, which were preserved three ways in DMSO, EDTA and saturated sodium chloride (DESS), 5% potassium dichromate and fresh frozen and shipped to the Walter and Eliza Hall Institute (Melbourne, Australia). Sample processing and preservative evaluation has been described elsewhere (Chapter 3 – Effective low-cost preservation of human stools in field-based studies for helminth and microbiome analysis). Briefly, 273 DESS (DMSO, EDTA, saturated sodium chloride) preserved samples were centrifuged twice for 3 minutes at 2,000 x g, supernatant decanted and faecal pellet resuspended in sterile H₂O. Total

genomic DNA was extracted from faecal samples using the ISOLATE II Faecal DNA extraction kit after the manufacturer's instructions (Bioline, UK). Isolated DNA was quantified using the Qubit 3.0 assay following the manufacturer's instructions and stored at -20°C. Unpreserved faecal pellets were subsequently stored at -80°C.

Vietnam samples

Samples of this study were collected as part of a cross-sectional study in Vietnam as published elsewhere [342]. Briefly, stool samples were collected from eligible participants and screened by KKTS prior to treatment of infection positive samples and had not received any drug intervention in the past. Stool samples were stored in 5% potassium dichromate in the field. To remove the preservative, samples were washed thrice in distilled water. DNA extraction was performed using the PowerSoil DNA Isolation Kit after the manufacturer's instructions (Mo Bio, USA) using 1 g Silica/Zirconia 0.5 mm beads (Daintree Scientific, Australia). Subsequently, extracted faecal DNA was used for molecular biological analysis such as multiplex qPCR and enzyme-linked immunosorbent assay (ELISA).

Cambodia samples

Cambodian samples were collected as part of a longitudinal study in 2016 (Khieu *et al.*, in prep). Briefly, stool samples were collected at baseline and 500 mg single-dose albendazole administered followed by a 3-day follow-up collection. Faecal samples were preserved in 5% potassium dichromate until shipped to the main laboratory (Faculty for Veterinary and Agricultural Sciences, The University of Melbourne). Preservative was removed via twice washing in sterile water followed by centrifugation at 2,000 x g for 3 minutes. DNA extraction was performed using the ISOLATE II Faecal DNA extraction kit after the

manufacturer's instructions (Bioline, UK). Hookworm infection prevalence was determined via Kato-Katz Thick smear (KKTS) and multiplex qPCR.

Sample size and cohort sub-setting

Sample size and precision

To determine the minimum level of precision for determining *tub-1* allele frequency for each species, we used a modified Cochran's formula $e = \sqrt{Z^2 pq/n_0}$, where Z is a value from the normal distribution table (95% confidence interval), p the population proportion to have the variant, q is $1-p$, e the desired level of precision and n_0 the sample size. Using this formula, with 171 *N. americanus* positive samples, 106 *A. lumbricoides* positive samples and 99 *T. trichiura* positive samples, we were powered to detect an allele with a 1% frequency for each species to be between $1 \pm 1.49\%$, $1 \pm 1.89\%$ and $1 \pm 1.96\%$ respectively.

Soil-transmitted helminth infection prevalence and intensity testing

All samples ($n=376$), including 132 from Cambodia (66 *Necator americanus* at baseline, 66 *Necator americanus* at follow-up), 39 from Vietnam, 205 from Thailand (106 *Ascaris lumbricoides*, 99 *Trichuris trichiura*), were retested for STH infections using an established multiplexed-tandem qPCR (MT-PCR) assay [290]. MT-PCR positive samples for any STH species were used for SNP genotyping. Cambodian samples included in this study, were matched individuals that had retained their infection positive status at follow-up (S9 Table).

Primer design and evaluation

Species-specific primers targeting the beta-tubulin isotype 1 (*tub-1*) gene were designed for *A. lumbricoides* (GenBank accession number FJ501301.1), *T. trichiura* (GenBank accession number AF118385.1) and *N. americanus* (GenBank accession number

EF392851.1) in Geneious 6.1.8 (<https://www.geneious.com>). To be compatible with the Illumina MiSeq sequencing platform, we required an amplicon size of <300 bp, but considering prior research on BZ resistance in veterinary nematodes, we aimed to be able to genotype key BZ-resistance SNP loci at codons 167, 198 and 200, as well as, as much of *tub-1* gene as practical. For *T. trichiura*, this could be achieved with a single primer pair (Table 4.1, amplicon size 289 bp). However, due to the presence of introns between codons 167 and 198, we required two amplicons for *A. lumbricoides* and *N. americanus*. The primers target an amplicon range of 133 – 247 bp to allow for sufficient overlap during deep amplicon sequencing (Table 4.1).

All primers were tested by conventional PCR using total gDNA extracted from adult helminth tissue for *A. lumbricoides* and *T. trichiura*, and faecal gDNA from an infection positive faecal sample for *N. americanus*. PCR products were confirmed via Sanger sequencing (AGRF, Melbourne). Conventional PCR was undertaken per the following conditions: *Taq* 2x Master Mix New England BioLabs (Catalogue #M0270L), 10 µM forward primer, 10 µM reverse primer, 2 µl DNA template in a total reaction volume of 20 µl made up with sterile H₂O. Both a standard and adapter-overhang ligated primer pair was tested for each amplicon. The adapter-overhang allowed the addition of sample specific barcoded oligonucleotides (index primers) for each amplicon during library preparation to support sample multiplexing and reduce cost (see below).

Table 4.1. Species-specific primers with and without adapter overhangs targeting the beta-tubulin isotype 1 gene of human soil-transmitted helminth species (*Ascaris lumbricoides*, *Trichuris trichiura*, *Necator americanus*). Underlined base pairs highlight the adapter overhangs for Illumina MiSeq amplicon sequencing.

	Forward 5'-3'	Reverse 5'-3'
<i>Ascaris lumbricoides</i> codon 167	GCACAGGATCTGGCATGGG	AGCAACCACACTTGAACCTGC
<i>Ascaris lumbricoides</i> codon 167 adapter primer	<u>CTGAGACTTGCACATCGCAGC</u> GCACAGGATCTGGCATGGG	<u>GTGACCTATGAACTCAGGAGTC</u> AGCAACCACACTTGAACCTGC
<i>Ascaris lumbricoides</i> codon 198/200	CACTTGCCAGAGAGGGATGG	TGAGGTCTCCGTATGTGGGA
<i>Ascaris lumbricoides</i> codon 198/200 adapter primer	<u>CTGAGACTTGCACATCGCAGC</u> CACTTGCCAGAGAGGGATGG	<u>GTGACCTATGAACTCAGGAGTC</u> TGAGGTCTCCGTATGTGGGA
<i>Trichuris trichiura</i> codon 167, 198 and 200	GGAGTGGAATGGGTACGCTT	AGTCTCCGTAAGTTGGTGTGT
<i>Trichuris trichiura</i> codon 167/198/200 adapter primer	<u>CTGAGACTTGCACATCGCAGC</u> GGAGTGGAATGGGTACGCTT	<u>GTGACCTATGAACTCAGGAGTC</u> AGTCTCCGTAAGTTGGTGTGT
<i>Necator americanus</i> codon 167	TGACCCACTCTCTTGGAGGA	CCAGGCTGATGCCCTCAATG
<i>Necator americanus</i> codon 167 adapter primer	<u>CTGAGACTTGCACATCGCAGC</u> TGACCCACTCTCTTGGAGGA	<u>GTGACCTATGAACTCAGGAGTC</u> CCAGGCTGATGCCCTCAATG
<i>Necator americanus</i> codon 198/200	GTTGGATACTGGAGTTTCGC	RAGTTTCAADGTWCGGAAGC
<i>Necator americanus</i> codon 198/200 adapter primer	<u>CTGAGACTTGCACATCGCAGC</u> GTTGGATACTGGAGTTTCGC	<u>GTGACCTATGAACTCAGGAGTC</u> RAGTTTCAADGTWCGGAAGC

Beta-tubulin isotype 1 amplicon sequencing

Sequencing libraries were prepared using a two-step PCR approach. In the first step, five µl of genomic faecal DNA was amplified using 10 µl *Taq* 2x Master Mix New England BioLabs (Catalogue #M0270L), 0.5 µl species-specific forward adapter primer and 0.5 µl species-specific reverse adapter primer and made up to a total reaction volume of 20 µl with sterile H₂O including a non-template control (sterile H₂O) on each plate. Reactions were denatured at 95°C for 2 minutes followed by 20 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 60 seconds and elongation at 72°C for 60 seconds with a final extension of 5 minutes at 72°C. All first step PCR products were cleaned-up and size selected using a concentration of 1x NucleoMags NGS magnetic beads after the manufacturer's instructions (Machery-Nagel, Germany). Samples were washed twice using 180 µl of 80% ethanol and eluted in a final volume of 40 µl RNA/DNAse-free water.

To support high-throughput multiplexing during sequencing, two µl of cleaned-up PCR products was amplified in a second PCR using 15 µl *Taq* 2x Master Mix New England BioLabs (Catalogue #M0270L), 1.5 µl forward index primer (10 mM), 1.5 µl reverse index primer (10 mM) made up to a total reaction volume of 30 µl with sterile H₂O. Index primers each contained a sample unique 8-mer oligonucleotide barcode and a complimentary sequence corresponding to each species-specific adapter primer. Reactions were denatured at 95°C for 3 minutes followed by 25 cycles of denaturation at 95°C for 45 seconds, annealing at 55°C for 60 seconds and elongation at 72°C for 90 seconds with a final extension of 10 minutes at 72°C. A representative selection of second PCR products covering all columns and rows were size separated on a 1.5% agarose gel to confirm correct-sized product amplification. Five µl of each product was separated for 60 minutes at 110 V and 400 mA, gel pre-stained using 2 µl ethidium-bromide and imaged on the GelDoc (BioRad, USA).

Finally, samples were pooled into three sequencing libraries according to species, by combining 5 µl of each product in a new 1.5 mL Eppendorf tube. Pooled sequencing libraries were cleaned-up and size select using a 0.7x concentration of NucleoMag NGS magnetic beads (Machery-Nagel, Germany) after the manufacturer's instructions. Samples were washed twice using 180 µl of 80% ethanol and eluted in a final volume of 55 µl RNA/DNase-free water. Library quantity and quality was confirmed using the TapeStation device (Agilent, USA). Final libraries were sequenced on the Illumina MiSeq using the V3 2 x 300 bp sequencing kit according to the manufacturer's instructions (Illumina, USA). Two sequencing runs were performed, one using the *A. lumbricoides*/*N. americanus* codon 167 library and the other for the *A. lumbricoides*/*N. americanus*/*T. trichiura* codon 198/200 library.

Data processing and analysis

Raw fastq sequencing data were demultiplexed by species and sample. Forward and reverse reads for each sample were aligned to the respective reference sequences (*A. lumbricoides* GenBank accession number FJ501301.1, *T. trichiura* GenBank accession number AF118385.1, *N. americanus* GenBank accession number EF392851.1) using the parallel BWA-MEM algorithm. The output sequence alignment map (SAM) files were converted into binary sequence alignment map (BAM) files using the SamFormatConverter in the picard tools package 2.20.2. The bam files were sorted and indexed using sambamba 0.6.6. Here, we did not mark duplicates as amplicon sequencing data by nature of the technique consist of large amounts of sequencing reiteration over the target region, resulting in a high number of similar amplicons or duplicates. Duplicate removal would lead to a loss of sequencing depth and has been shown to have little effect on dataset accuracy [343]. Read coverage was analysed using the bedtools package 2.26.0 and samtools 1.7 package.

The mpileup algorithm in samtools 1.7 package was used to predict variant likelihood occurrence and create a multiple sequence alignment for each sample. Finally, variants (SNPs/indels) with a minimum variant allele frequency of 1% were called using VarScan 2.3.9 with default filtering parameters and binary variant call format (BCF) files converted into variant call format (VCF) using the mpileup2cns command. Individual VCF files were imported into RStudio 1.1.453, adapter primers filtered and converted into a data frame using varikondo 0.6.5. Statistical analysis between groups was performed using a two-sided Wilcoxon ranked test in RStudio 1.1.453 (RStudio Inc., USA). Shannon's entropy for variant allele frequency measures was estimated for each variant and sample using the diverse package 0.1.5. with euclidean method in RStudio 1.1.453 (RStudio Inc., USA) [344]. Processed data were visualised using the Integrated Genomics Viewer 2.3.81 and ggplot 3.2.0 in RStudio 1.1.453 (RStudio Inc., USA).

To determine assay specificity, *tub-1* only sequencing reads were filtered and written to a new bam file. Bam files were sorted to name and converted into two paired-read fastq files while removing singletons using the samtools 1.7 package. These *tub-1* only sequencing reads were aligned to the species-specific whole genomes (GenBank assembly accession GCF_000507365.1 *N. americanus*, GCA_000613005.1 *T. trichiura*, WormBase ParaSite assembly accession GCA_000951055.1 *A. lumbricoides*), including the amplicon reference (GenBank accession numbers EF392851.1, AF034219.1, FJ501301.1), using the parallel BWA-MEM algorithm; and analysed using the samtools package 1.7.

Data availability

Raw fastq data files from amplicon sequenced samples for the complete study, were deposited in the NCBI Sequence Read Archive (SRA) under BioProject ID PRJNA623462.

15.4. Results

A total of 376 STH infection positive samples were analysed, including 106 *A. lumbricoides* and 99 *T. trichiura* positive infections from Thailand, 39 *N. americanus* positive infections from Vietnam, and 66 matched samples positive for *N. americanus* prior to and 3 days after oral benzimidazole treatment. Gene copy numbers based on MT-PCR ranged from 39 – 4,711,749 (mean Ct-value 23.82), 37 – 6,343 (mean Ct-value 30.89), 344 – 441,747 (mean Ct-value 23.38), 15 – 182,255 (mean Ct-value 28.19) and 18 – 24,919 (mean Ct-value 28.22) for *A. lumbricoides*, *T. trichiura*, *N. americanus* Cambodia baseline, *N. americanus* Cambodia follow-up and *N. americanus* Vietnam, respectively.

Single nucleotide polymorphism analysis

Out of 376 STH infection positive samples, all but 7 *T. trichiura* and 3 *N. americanus* positive samples with reportedly low infection intensity (n=9) as shown by gene copy number (mean GCN 86) and MT-PCR Ct-value (mean Ct value 33) were successfully sequenced at *tub-I* using this approach. We generated a total average of 98,972 sequence reads per amplicon over two sequencing runs (mean 149,783 reads for 106 *A. lumbricoides* samples and 55,989 reads for 171 *N. americanus* samples over two sequencing runs; and 140,063 reads for 98 *T. trichiura* samples,). Mapping the data to known *tub-I* references (GenBank accession numbers EF392851.1, AF034219.1, FJ501301.1), an average proportion of 37.74% *A. lumbricoides* (mean coverage 56,521 reads), 32.45% *T. trichiura* (mean 45,125 coverage reads) and 25.20% *N. americanus* (mean coverage 15,722 reads) of reads mapped to the *tub-I* gene. To test amplification specificity, we re-aligned all *tub-I* only reads for each sample to the whole genome assemblies of each species including the *tub-I* amplicon reference, respectively. Of the *tub-I* only reads, an average of 92.93 %, 78.32% and 71.62 % of reads per sample re-aligned to *tub-I*, for *N. americanus*, *T. trichiura*

and *A. lumbricoides*, respectively (S3 Table). SNPs were called using variant allele frequency measures for all reads successfully mapped to the *tub-1* reference. A total of 305 (Cambodia *N. americanus*; 4 indels), 126 (Vietnam *N. americanus*; 2 indels), 336 (Thailand *A. lumbricoides*; 0 indels) and 279 (Thailand *T. trichiura*; 3 indels) variants were detected, respectively.

Thailand

We detected a total of 336 *Ascaris lumbricoides* variants (61 segregation sites, 224 SNPs in coding regions) in 106 and 279 *Trichuris trichiura* variants (80 segregation sites, 200 SNPs in coding regions) in 99 confirmed infection positive faecal samples from pre-school and school-aged children from Tha Song Yang, Thailand. The average variant allele frequency per SNPs was 3.87% and 11.30%, respectively. Two coding variants were detected at codon positions known to be associated with BZ resistance, these are one at codon 167 (Phe to Ser; VAF 0.0424) and one at codon 200 (Phe to Ser; VAF 0.0215) (each detected on a different 1 of 99 *T. trichiura* positive Thai samples). The mean Shannon's entropy measure for *T. trichiura* was 0.60 (0.00 – 3.48) per variants over all segregation sites and 0.37 (0.00 – 2.05) per sample over all segregation sites. For *A. lumbricoides* codon 167 amplicon, the mean Shannon's entropy was 0.00 per variant and sample, whereas the mean Shannon's entropy for codon 198/200 variants was 0.85 (0.00 – 3.69) per amplicon over all segregation sites and 0.39 (0.00 – 2.11) per sample over all segregation sites.

Vietnam

We detected a total of 126 variants (79 segregation sites, 57 variants within coding region) within 39 *N. americanus* infection positive samples from Vietnam with an average variant allele frequency of 18.22% (Table 2). Except for one sample (Glu/Gly codon 198), no

variants were detected at codon 167, 198 or 200 within this subset. Shannon's entropy measures for variants at both, codon 167 and codons 198/200 amplicons were as follows: *N. americanus* codon 167 amplicon had a mean Shannon entropy of 0.18 (0.00 – 1.09) per variants over all segregation sites and 0.34 (0.00 – 1.58) per sample over all segregation sites and *N. americanus* codons 198/200 amplicon had a mean Shannon entropy of 0.16 (0.00 – 3.41) per variants over all segregation sites and 0.13 (0.00 – 1.37) per sample over all segregation sites.

Cambodia baseline versus follow-up

A total of 132 *Necator americanus* infection positive Cambodian samples were sequenced. 66 were collected at baseline and matched to a participant specific sample that retained their infection positive status at follow-up (3 days post treatment). Mean variant allele frequency (VAF) for this combined dataset was 25.43% (33.51% baseline, 20.39% follow-up). Mean VAF decreased to 13.12% at follow-up (p-value 0.03955). 118 samples had a variant allele at amplicon position 99 with an average variant allele frequency of 58.32% at baseline and 60.20% at follow-up (Figure 4.2). We note that this variant lies within the non-coding region of the *tub-1* gene. No variants were detected at codons 167, 198 or 200, respectively. Only 103 variants were detected within the beta-tubulin coding region as shown in Figure 4.2. Overall, we found an increased number of variants at follow-up (n=188 at 87 segregation sites) compared to baseline (n=117 at 42 segregation sites). Mean Shannon's entropy measure was calculated for each cohort subset (baseline versus follow-up) separately. At baseline, *N. americanus* codon 167 amplicon had a mean Shannon entropy of 0.11 (0.00 – 1.05) per variant over all segregation sites and 0.19 (0.00 – 0.69) per sample over all segregation sites and *N. americanus* codons 198/200 amplicon had a mean Shannon entropy of 0.40 (0.00 – 4.13) per variants over all segregation sites and 0.03

(0.00 – 0.69) per sample over all segregation sites. At follow-up, *N. americanus* codon 167 amplicon had a mean Shannon entropy of 0.23 (0.00 – 1.33) per variant over all segregation sites and 0.19 (0.00 – 1.59) per sample over all segregation sites and *N. americanus* codons 198/200 amplicon had a mean Shannon entropy of 0.25 (0.00 – 3.94) per variants over all segregation sites and 0.07 (0.00 – 1.09) per sample over all segregation sites.

Table 4.2. Summary of detected variant allele frequencies among all cohorts (Cambodia, Vietnam, Thailand) and STH species (*A. lumbricoides*, *T. trichiura*, *N. americanus*) at codons 167, 198 and 200. Variant allele frequencies (VAF) were calculated using the mean frequency among all called variants.

Cohort	Sample number	STH species	Total VAF	Variant number	Segregation sites
Cambodia	66 (baseline)	<i>N. americanus</i>	33.51%	117	42
	66 (follow-up)	<i>N. americanus</i>	20.39%	188	87
Vietnam	39	<i>N. americanus</i>	18.22%	126	79
Thailand	106	<i>A. lumbricoides</i>	3.87%	336	61
	99	<i>T. trichiura</i>	11.30%	279	80

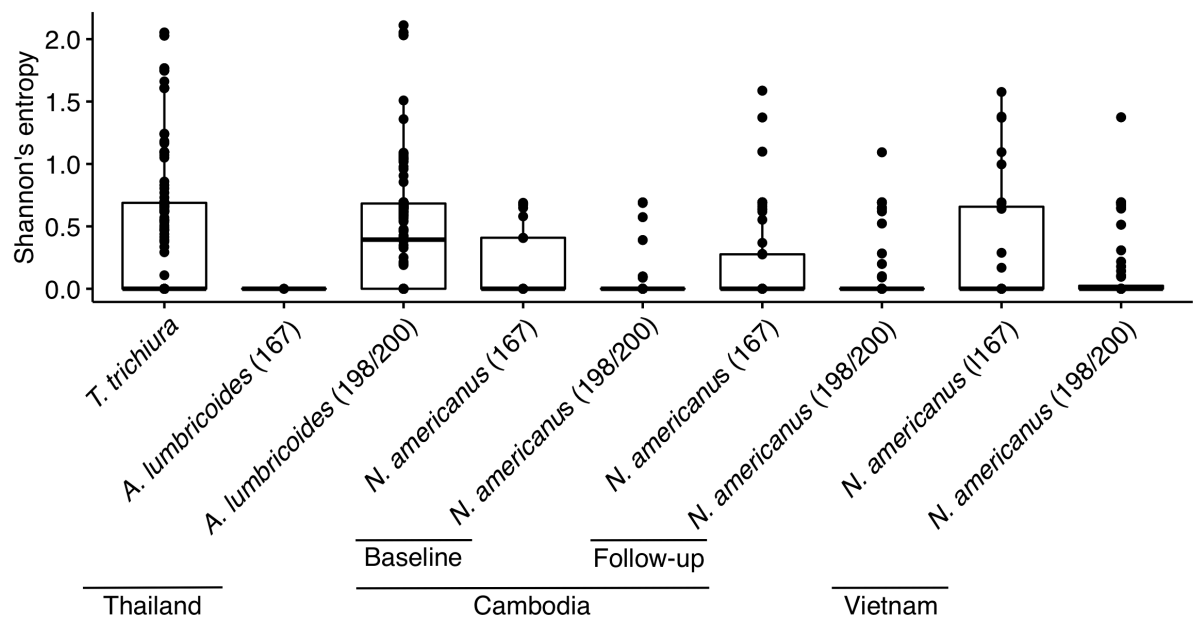


Figure 4.1. Mean Shannon's entropy measures per amplicon, STH species and cohort.

The mean Shannon's entropy measure shown was estimated by calculating the average diversity per variant site across each sample for each sub-cohort

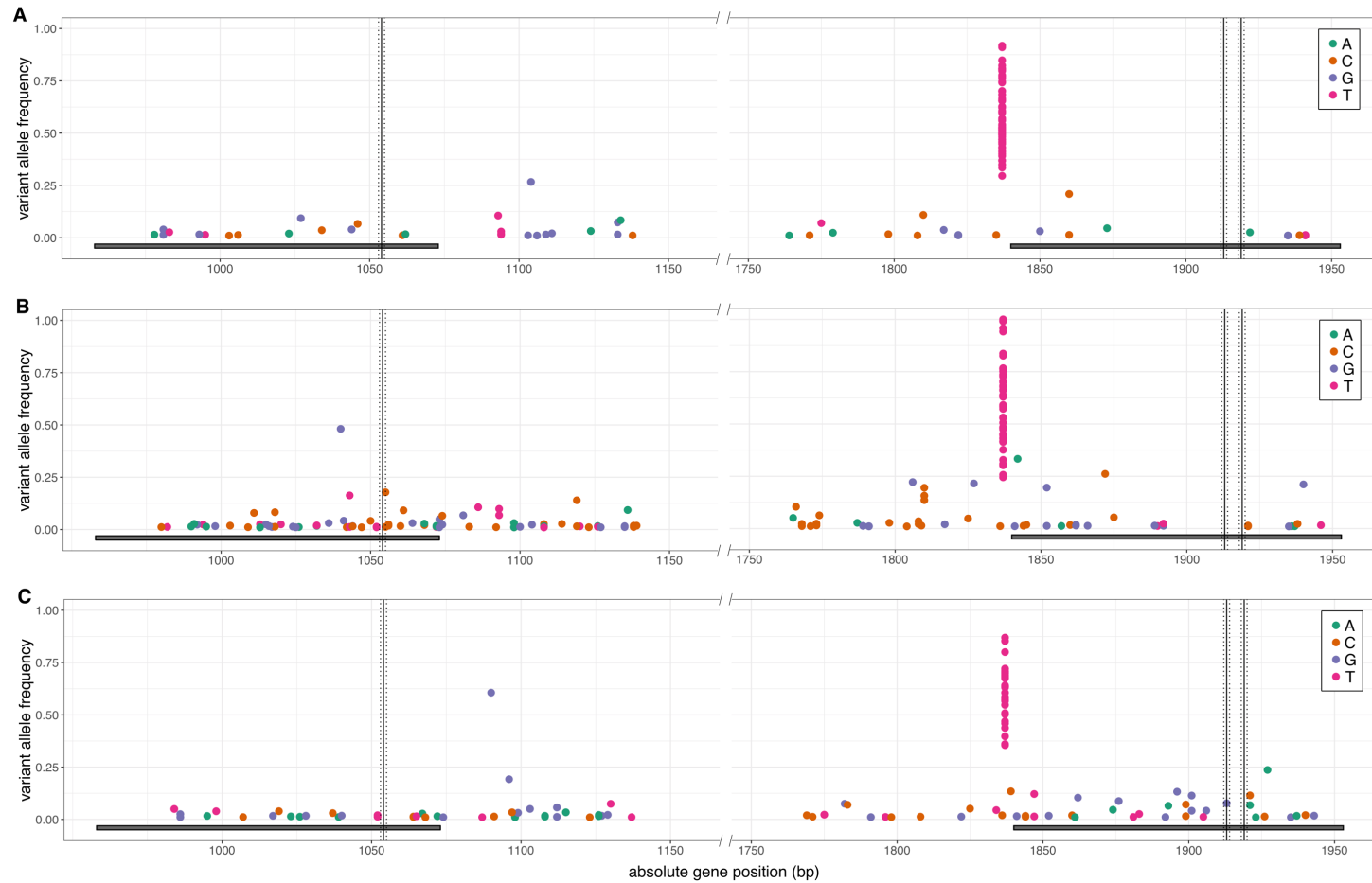


Figure 4.2. Baseline (A) and follow-up (B) variant allele frequency (y-axis) of *Necator americanus* positive samples from Cambodia and Vietnam (C). Absolute *tub-1* gene position is depicted on the x-axis. Coding regions are highlighted with dark grey boxes. Codon 167, 198 and 200 boundaries are highlighted with dotted lines and the SNP with a straight line, respectively. Raw variant allele frequency measures are summarised elsewhere (S2 Table).

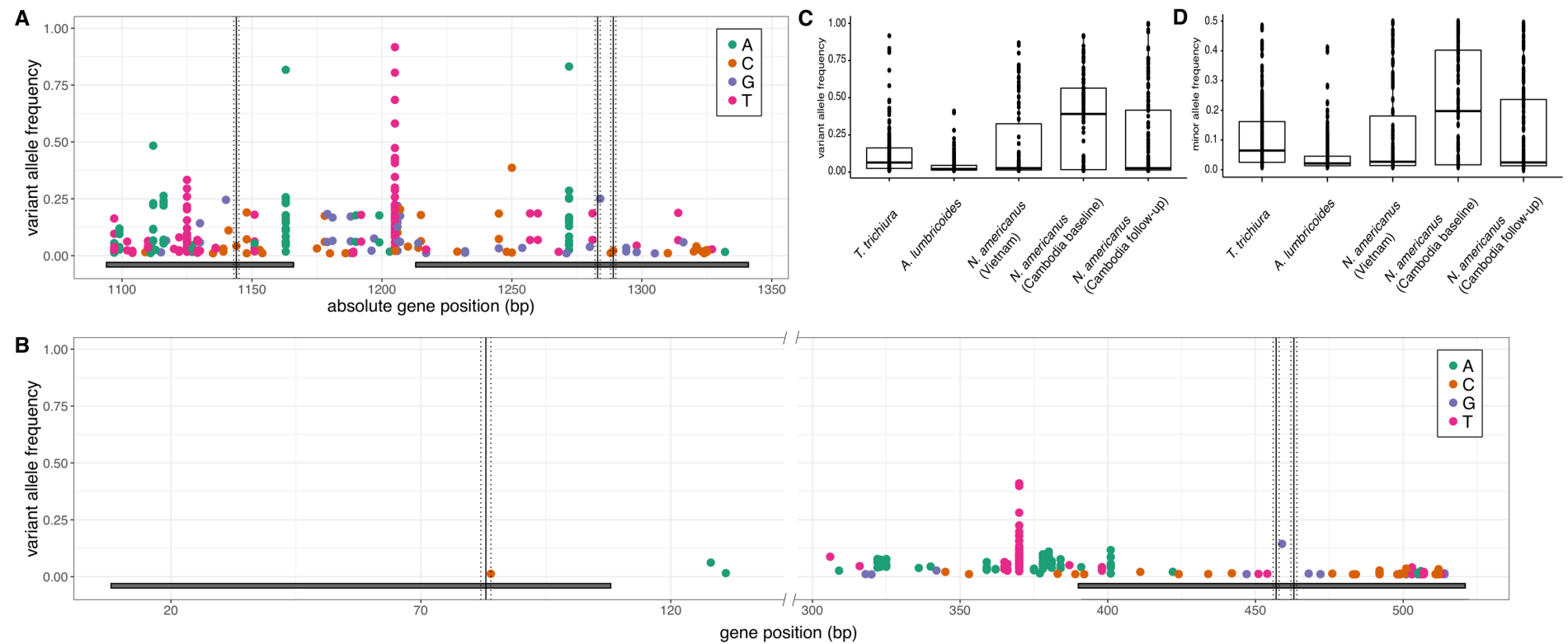


Figure 4.3. Variant allele frequencies (y-axis) of *Trichuris trichiura* (A) and *Ascaris lumbricoides* (B)* positive samples from Thailand and species-specific variant allele frequencies (C) and minor allele frequencies (D) for all cohorts. Absolute or partial *tub-1* gene position is depicted on the x-axis. Coding regions are highlighted with dark grey boxes. Codon 167, 198 and 200 boundaries are highlighted with dotted lines and the SNP with a straight line, respectively. Raw variant allele frequency measures are summarised elsewhere (S2 Table). *X-axis values for *A. lumbricoides* are amplicon base pair values due to incomplete reference sequence annotation.

15.5. Discussion

Expanding on the need for novel diagnostics to monitor for beta-tubulin associated BZ drug resistance [199], we present a novel amplicon sequencing approach for the detection of SNPs of the beta-tubulin 1 gene of human STHs. This assay provides a platform that allows for detection of all three BZ resistance associated codons of the major human STHs, as well as novel SNP discovery in a region with around 230 bp of these codons.

We used this assay to screen for BZ variant allele frequency in 376 samples representing known positives (re-confirmed here by a recently published MT-PCR assay [290]) for *A. lumbricoides*, *T. trichiura* or *N. americanus* from populations from STH endemic regions in Thailand, Vietnam and Cambodia. Seven *T. trichiura* and 3 *N. americanus* positive samples failed at *tub-1* amplification (despite amplification by MT-PCR); eight of which had low infection intensity based on MT-PCR. This likely reflects the difference in sensitivity between the single-phase *tub-1* PCR and the nested MT-PCR method, rather than variants that cannot be amplified using our primers. Although these samples failed to amplify using the *tub-1* amplicon sequencing approach compared to the MT-PCR screening, platform sensitivity is relatively good with 97.34% of samples (366 out of 376) amplifying. With an average total read depth of 99,123 reads (average *tub-1* read depth 33,889) per sample this high-throughput assay provides an increased sequencing coverage compared to other platforms [345].

Among all sample sets we found levels of variant allele frequencies below 34% and detected three variants associated with known SNP marker positions for BZ resistance; Phe167Ser (codon 167 *T. trichiura* from Thailand), Glu198Gly (codon 198 *N. americanus* from Vietnam) or Phe200Ser (codon 200 *T. trichiura* from Thailand). These variants may

represent novel resistance associated SNPs worth further investigation. Identification of novel markers within the *tub-1* gene and other potential BZ resistance associated genes using whole-genome approaches may become increasingly important [249]; in particular to define genomic diversity surrounding genes that may contribute to anthelmintic resistance [346].

Since the Cambodian cohort received 5+ years of annual BZ treatment and persistence of *N. americanus* positive infections was observed at 3-day follow-up sampling, we hypothesized the presence of variants that may be indicative of BZ resistance. Although, we did not detect variants at known codons 167, 198 or 200 in Cambodia, we did detect a decrease in variant allele frequency, which may highlight selection for resistance-associated mutations with ongoing MDA treatment (bottleneck selection of resistant type alleles) [347, 348]. High prevalence at follow-up may also be present, because as shown within other populations in South-East Asia, single-dose benzimidazole drug treatment exhibits low efficacy against hookworm infections [349]. In saying this, drug resistant parasitic nematodes exhibit a lower allele frequency compared to susceptible strains [350].

The here presented study was limited in terms of the Cambodian cohort with follow-up sampling timeframe of 3 days after baseline, which should ideally be 2-3 weeks for complete successful worm and egg expulsion from the host [186]. Therefore, future cohort studies using prolonged timeframes between drug administration at baseline to follow-up are required to draw accurate conclusions about drug efficacy and allele frequency. Future evaluation may include long read sequencing to estimate individual haplotypes [351], while sequencing all three codons concurrently and whole genome sequencing for identification of novel SNPs or genes as markers of BZ resistance [249]. Although, we were unable to

detect BZ resistance associated SNPs at codons 167, 198 and 200; we cannot conclusively confirm absence or presence of drug resistance using this assay. Therefore, novel drugs are required to ensure successful STH infection clearance in the future [352, 353]. Further, we were restricted to variable population data on overall frequencies, since our study is limited in terms of positive control samples. On-going monitoring of emergence of drug resistance must be a key consideration in future policies and field studies in order to perform rapid detection and intervention once drug resistance has emerged [354].

We have developed a beta-tubulin 1 amplicon sequencing assay using human faecal DNA infected with soil-transmitted helminth eggs from South-East Asia. This assay aids in screening for single nucleotide polymorphisms in the beta-tubulin 1 gene associated with benzimidazole drug resistance. Advantages of the here presented platform are multiplexing of samples and sequencing depth which consequently reduces cost associated. Additionally, this assay can concurrently be used for SNP genotyping as well as estimation of STH infection prevalence. This assay presents an ideal tool for periodical monitoring of drug resistance in sub-populations worldwide.

15.6. Acknowledgements

We are grateful for the contribution of the field teams and participants involved with sample collection and processing.

15.7. Supplementary files

S9 Table. MT-PCR data of faecal gDNA samples

S10 Table. Variant allele frequency data for human soil-transmitted helminths

S11 Table. *Tub-1* sequencing read analysis

S12 Table. Shannon's entropy data per sample, amplicon, STH species and cohort

16. Conclusion and future directions

I developed an amplicon sequencing tool for human STHs that can be used to test for SNPs known to cause BZ drug resistance. I found low levels of variant allele frequency and discovered three novel SNPs at codons 167, 198 and 200. These findings contribute to an updated knowledge of BZ markers among populations in areas with ongoing transmission and mas drug administration. Further validation using large cohort studies, follow-up sampling timepoints of 2 – 3 weeks, as well as on-going monitoring are essential for early detection of drug resistance.

Chapter 5 – General discussion

17. Thesis outcomes

Approximately a quarter of the global population is infected with at least one species of STH (*A. lumbricoides*, *T. trichiura*, *N. americanus* and *Ancylostoma* spp.), which cause a significant disease-related morbidity [43]. Global infection intensity has been reduced over the last decade through WHO-recommended mass drug administration with benzimidazoles [355]. This treatment regime, although partially successful, has raised concerns about long-term treatment efficacy and the potential for the emergence of drug resistance in human cohorts [188]. Traditional diagnostic tools applied to measure infection prevalence and intensity underestimate the true infection prevalence [220], are time-consuming and require parasitological expertise [253]. Further, as global prevalence and worm burden falls [43], the limited sensitivity and specificity of microscopy-based diagnostics becomes a major impediment to control [220]. Even the efficiency and relevancy of national MDA versus targeted screen-and-treat programs comes into question. Although the majority of infections globally are classified as low-intensity, they can cause a variety of moderate to severe symptoms that impact upon a patient's quality of life [1]. In addition, these infections may escape microscopic detection, but still provide a source for environmental transmission, considering the tremendous fecundity of STH species [178]. Therefore, they provide a major challenge to efforts to eradicate these parasites from endemic environments, leading to continues reliance on unsustainable national MDA programs [200].

The aims of my research were (1) to establish a semi-automated and commercially standardised molecular diagnostic tool for STHs, (2) to explore low-cost faecal preservatives for molecular diagnosis of STHs and gut microbiome characterisation and (3) to develop a low-cost and

accurate molecular tool to screen for the selection or emergence of benzimidazole resistance in national STH control programs. I successfully developed and implemented these aims in the context of this PhD thesis using four South-East Asian field cohorts.

Development and validation of a multiplexed-tandem qPCR tool for diagnostics of human soil-transmitted helminth infections

Several studies, as reviewed in Chapter 1 (Literature review of the current status of soil-transmitted helminth infections) and 2 (Development and validation of a multiplexed-tandem qPCR tool for diagnostics of human soil-transmitted helminth infections), suggest an underestimation of the true STH infection prevalence caused by the low sensitivity of gold standard diagnostics. This is becoming increasingly important in the context of long-term MDA, where a decrease in STH infection intensity has been observed [132]. Resources, time and expertise associated with testing of multiple KKTs are unlikely to increase sensitivity. Although molecular diagnostic tools identify low-intensity infections more sensitively; they lack in gold standard, transferability and cost-effectiveness [226]. This highlights the urgent need for a universal diagnostic tool which is rapid, sensitive and resource-effective [226].

Here, I developed a novel molecular diagnostic tool – the multiplexed-tandem qPCR – for the detection of human STHs [290]. This tool overcomes several limitations of microscopy-based diagnostics, such as time constraints, sensitivity and parasitological expertise [255]. In addition to identifying *A. lumbricoides* and *T. trichiura* infections, which is also achievable through microscopic examination, the method allows species-specific identification of hookworm eggs, which cannot be done through non-molecular techniques, including allowing differentiation of human-infective *Ancylostoma* spp. [356]. The high-sensitivity of the tool allows detection of sub-clinical infections, that contribute to on-going disease transmission [357], but are

undetectable by microscopy. Further, this approach provided species-specific STH infection prevalence data via MT-PCR for populations that had previously only been screened for hookworms (Cambodia). Although conversion of gene copy number into eggs per gram faeces is currently not feasible, I was able to show prove-of-concept for a positive correlation. This study primarily contributes to availability and updated knowledge of STH infection data on a sub-national level, which may contribute to an improved global disease mapping (as highlighted in other endemic regions [358, 359]) and aid in awareness, education, diagnosis and treatment of these neglected tropical disease. In addition, the method can be readily expanded to include other gastrointestinal pathogens.

Effective low-cost preservation of human stools in field-based studies for helminth and microbiome analysis

Molecular diagnostic tools generally require total gDNA extraction of faecal samples, which implies either (a) immediate sample processing in the field or (b) sample storage and transport to a research facility for downstream processing. The former is often not feasible in endemic, resource-poor settings; hence samples need to be shipped nationally or internationally. The main limitation in this context is sample degradation, which can be influenced by environmental factors such as temperature [279] and time since collection [360], leading to a loss of bacterial species [246] and STH eggs [214]. In addition, comparison between multiple studies or cohorts relies heavily on consistent sample collection, storage and processing. Thus, development of a universal standard preservative may contribute to improved study outcomes.

Here, I compared two chemical preservatives, DESS and PD, to the fresh frozen gold standard preservation for inference of the most effective stool sample preservation technique. I did this by establishing a cross-sectional cohort study among pre-school and school-aged Karen ethnic

children from a helminth endemic region in North-West Thailand in collaboration with researchers from Mahidol University (Bangkok). I successfully developed and implemented standard operating procedures for collection, preservation and processing of faecal samples for STH infection recovery and host gut microbiome characterisation in (i) a subset of samples preserved 3 ways and (ii) the total cohort using DESS preserved samples only (Chapter 3 Effective low-cost preservation of human stools in field-based studies for helminth and microbiome analysis).

This study provides the first comprehensive assessment of STH prevalence in this region using the MT-PCR assay. These results vastly expand our knowledge on performance of this novel molecular diagnostic tool and provide a basis for future establishment of a PCR-based gold standard. High-sensitivity, molecular data complement current KKTS-based infection data and contribute to accurate global STH infection prevalence mapping on subnational levels [273]. Although Thailand is now classified a developed country, I detected a relatively high infection prevalence and significant number of co-infections. Multiple factors may be causal for the high infection prevalence, such as non-compliance [335], lack of knowledge and hygiene practises [361], lack of sanitation [362], zoonotic infections [30] or a decrease in drug efficacy [363]. Further, the refugee status of many of the tested children is likely to contribute to a high STH prevalence; with other Karen populations in North-West Thailand harbouring gastrointestinal parasites [364]. Data further complement previously reported species-specific geographical distribution patterns in South-East Asia [130], as well as a correlation between hookworm infections in male participants [365]. With an expansion of molecular diagnostics to the whole community, infection prevalence estimates are likely to increase.

I evaluated DESS as a cost-effective alternative to fresh freezing that only requires short-term fridge storage and introduces no bias into curated microbiome data. DESS is non-toxic [287] and further expandable to other sample types as shown for other studies and preservatives [305, 307, 366, 367]. This is the first study using DESS as a preservative for faecal samples in a human cohort. Further, I performed the first study investigating STH induced alterations within the gut microbiome in this particular population. The outcomes of this research contribute to an expanding, but yet limited, body of research into the interaction between STHs and the gut microbiome [278]. Because past infections have the ability to alter the microbial diversity prior to cross-sectional sampling, there is a clear need for comparing samples from infected participants and those that have never been infected. Other factors such as diet [116], antibiotic usage [117] and environmental factors [315] can additionally induce microbiome modifications and require further investigation. Quantitative and qualitative characterisation of faecal proteins/RNA provide a starting point that may aid in translation of these into future studies.

Soil-transmitted helminth beta-tubulin 1 amplicon sequencing for the detection of drug resistance associated SNPs in human populations

The BZs, albendazole and mebendazole, are the frontline compounds for mass drug administration of STHs, targeting 873 million at-risk children until 2020 [21]. Although BZ treatment is not as high-pressure in humans, there is rising concern about emergence of drug resistance and the future implications for intervention strategies, similarly to that observed in livestock [368]. Although the emergence of drug resistance is not yet conclusively confirmed in clinical settings, the current treatment regime may select resistance-associated alleles [191]. This threatens the sustainability of BZ-based STH programs and requires robust monitoring. Direct evaluation of drug resistance through the FECRT is difficult [247] and few human

cohort studies used total gDNA extracted from stool samples. Recent authors have developed sequencing-based assays for genotyping of SNPs in the beta tubulin 1 gene, however the sequencing-by-synthesis technique limits this assay to typing of known loci [190].

Here, I developed the first beta-tubulin 1 amplicon sequencing assay that supports high-sensitivity sequencing of faecal gDNA samples of the major human STH species (*A. lumbricoides*, *T. trichiura*, *N. americanus*) (Chapter 4 Soil-transmitted helminth beta-tubulin 1 amplicon sequencing for the detection of drug resistance associated SNPs in human populations). Screening of three cohorts from South-East Asia (Cambodia, Vietnam, Thailand) provides insight into BZ resistance-associated SNPs in these regions for future field studies. This tool may contribute to on-going monitoring studies for early detection of drug resistance in human populations and concurrently be used for qualitative estimation of infection prevalence [188]. It further complements already existing tools in livestock populations [341] and provides a more cost-effective, high-throughput alternative to other sequencing tools [191, 192]. The high sequencing read coverage provides a basis for high-confident variants calling, concurrent genotyping of already known and discovery of novel SNPs surrounding these. Decreased next-generation sequencing cost, paired with an increase in data capacity, may make screening more affordable in the future [369]. Further, other unidentified genetic markers can contribute to emerging drug resistance [249], as well as environmental reservoirs which add to on-going infection transmission [354].

18. Reflections and future research

Diagnostic techniques for low-intensity infections

The microscopic KKTS is often not sensitive enough for accurate qualitative and quantitative STH infection testing. Although several molecular diagnostic tools have been developed previously [45], these were often limited in reproducibility, quantitative capacity and standardization. Additionally, required resources and cost make many molecular diagnostic tools prohibitive for application in endemic, resource-poor regions [225]. The semi-automated MT-PCR assay I have developed, which is now commercially available through AusDiagnostics Pty Ltd, addresses limitations around sensitivity, user-friendliness and time constraints. In the future, an implementation of molecular diagnostics in endemic settings is necessary to test its deployment in the field and the expanded application of these tools outside of research-based settings [226, 370]. Specifically, applications that require little prior scientific knowledge, such as the MT-PCR assay [290] or LAMP assays [232, 233] may aid in this translation. Further, this study was limited to long-term stored faecal gDNA samples, with evaluation using fresh faecal samples potentially increasing overall diagnostic performance of the MT-PCR. This limitation additionally highlights the need for rapid stool sample processing after collection in the field. Importantly, the molecular data output requires a conversion from GCN into EPG faeces to accurately translate into STH infection intensities. Some attempts have been made to establish such a formula [243]; however no standardized method is available at this point in time. Statistical estimation and modelling of infection intensity using prevalence data may only suffice for endemic populations [244]. To accurately make inferences about long-term health impacts caused by STH infections, large-scale longitudinal cohort studies are required [169, 371].

Standardised procedures for endemic cohort studies

Testing of DESS for stool preservation is promising for future standardisation of field studies and the adoption of molecular diagnostic tools. The availability of this low-cost, universal and safe preservative enables conservation of macromolecules for a range of downstream applications, with DESS potentially being utilised for other sample types [366, 367]. Additional work is required to establish the viability of DESS preservation under ambient temperatures and over prolonged storage timeframes. The future of STH research more broadly, relies on a better understanding, planning and implementation of field studies such as establishment of standard operating procedures (SOPs) for population studies. I have developed and implemented such a SOP for collection and processing of stool samples (Chapter 3 – Effective low-cost preservation of human stools in field-based studies for helminth and microbiome analysis). Validation of a universal preservative, such as DESS, may aid in establishment of a standardised study protocol in the future. Ideally, a global standardised cohort study procedure encompassing study design, sample collection, processing and validation would be available; which aids to provide comparability of studies, expansion of knowledge and collaboration among communities [273]. Further, the MT-PCR additionally provides a standardised infection diagnosis with reduced operator influence by being commercially produced and semi-automated.

Chronic impacts of STH infections on child health

There are various aspects of the exact physiological impacts of STH infections that remain unidentified and require an intensified research focus. The incomplete understanding of the exact impacts of infections on host microbial and overall health is just one example [115]. The presented microbiome data provide a basic understanding and starting point of in-depth investigation of these impacts. Large cohort studies comparing species-specific infections in

different endemic settings worldwide, including extensive patient health records, are essential. These undetected effects may only be discovered using more sophisticated techniques and are particularly concerning as they contribute unaccounted to disease-related morbidity. To determine effects of STHs on overall health over time, large-scale longitudinal cohort studies are necessary that include participant health records. Followingly, to investigate the impact of STH infection on the host gut microbiome, a comparison of infected participants to others that have never been infected is required. Since other environmental factors influence the gut microbiome, matched samples from the same cohort are required to reduce the variable effect.

On-going monitoring of potentially emerging benzimidazole drug resistance

The wide-spread emergence of BZ resistant STHs would present a major obstacle to global control programs. Although recommended by the WHO, phenotypic assays for drug resistance, including EHAs and FECRTs, are rarely used in practise to test for BZ-resistant STHs [247]. This is due to their cost, laboriousness, the practical challenges associated with follow-up access and testing of children in often remote regions, and their need for specific parasitological expertise. Early detection of emerging drug resistance is essential for preserving BZs in global DMA programs and requires molecular diagnostics built on robust genetic markers [188, 190]. This monitoring is essential, because drug resistance is typically irreversible, if drug pressure is on-going [372]. Genotyping tools often use PCR and sequencing based approaches, which provide a high read coverage and ability to test samples in parallel.

These molecular assays contribute to addressing the requirement for early detection, but are limited in terms of field application due to the resources associated [226]. Other solutions may include, (ii) investigation into novel drugs or vaccines [353, 373], and (iii) improved health education around hygiene behaviour for STH intervention [202]. The high-throughput

capability and cost-effectiveness of the amplicon sequencing assay, contribute to global efforts of developing a universal screening tool. Additional testing using larger sample sets as well as testing using multiplexed assays targeting all major STHs is required for establishment of a gold standard tool. Although currently costly, whole genome sequencing may help identify novel gene markers that are associated with benzimidazole drug resistance in addition to beta-tubulin isotype 1 [249]. Long-read screening of samples for detection of drug resistance associated haplotypes may provide further information [351].

Sustainable and integrated control strategies for transmission interruption and disease eradication

The ultimate goal of STH intervention more broadly is interruption of disease transmission and eradication. For this, accurate sample testing to avoid underestimation of infection prevalence is important. Diagnostic tool development and STH infection prevalence data may contribute to an increased knowledge to contribute to targeted intervention strategies. Although MDA treatment effectively reduces STH infection prevalence and intensity, it requires long-term application to prevent rebounding infection levels. Because of this, diagnostics allowing for early detection of drug resistance are increasingly important. It is debatable if MDA alone can achieve transmission interruption [178, 374]. Thus, a combination of sustainable (multi-year intervention commitment and resources) and integrated (multi area combination such as molecular diagnostics, drug treatment and WASH interventions) approaches is likely to be required [204]. In the long-term, increased education levels to combat non-compliance of drug usage [335] and open defecation [375], health and hygiene education to prevent a resurgence of infection prevalence [376], and improvement of infrastructure, sanitation and socioeconomic factors become increasingly important [91]. Currently, predominantly school-aged children receive MDA treatment due to ease of delivery and risk of infection [21].

Although this may be true for the majority of populations, adults can also harbour relatively high disease burdens in at-risk populations, which contribute to on-going disease transmission and environmental reservoirs [377]. Here, I provide infection data on a range of South-East Asian cohorts including whole communities, with a focus on a large cohort study of a majority of pre-school children. Thus, it is of relevance to consider expansion of drug delivery regimes to extend MDA to entire at-risk communities to interrupt transmission [355].

Extensive on-going interventions are important in order to eliminate environmental reservoirs [178]. This becomes increasingly important with an increase in environmental temperatures and overpopulation, which contribute to ongoing high levels of disease transmission [94]. Lastly, longitudinal studies investigating STH infection prevalence in correlation to MDA and WASH interventions are required to attain on-going information about effectiveness of these. To combat reduced drug efficacy, administration of drug combinations e.g. ivermectin and albendazole may be beneficial as a short-term solutions [352, 378], with a current need for new anthelmintics [179] or pan-anthelmintic vaccines [373]. The developed *tub-1* amplicon sequencing assay may aid in monitoring drug efficacy qualitatively as well as detect BZ resistance at an early stage.

Concluding remarks

In summary, my presented research aims and outputs complement multiple knowledge gaps. Specifically, the semi-automated and commercially molecular MT-PCR tool for STHs contributes to an increased detection of low-intensity infections, standardisation of study protocols and provide a user-friendly tool. The DESS preservative provides a safe, low-cost media in the context of large-scale cohort studies and effectively preserves a range of STHs and macromolecules. Lastly, the beta-tubulin sequencing assay aids in early detection of BZ

resistance by identifying known and potentially novel SNPs with high confidence. All of these tools and data shall contribute to moving towards integrated control and reduction of STH infection prevalence and disease burden.

Appendix

Mini-review article “Zoonotic ancylostomiasis – an update of a continually neglected zoonosis”

Full-length title: Zoonotic ancylostomiasis – an update of a continually neglected zoonosis

Short-length title: Zoonotic ancylostomiasis

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Key words: *Ancylostoma ceylanicum*, ancylostomiasis, hookworm, soil-transmitted helminths, zoonosis

Abstract

Hookworms are classified as the most impactful of the human soil-transmitted helminth infections, causing a disease burden of ~4 million DALYs, with a global prevalence of 472 – 740 million infections. Until a decade ago, epidemiological surveys largely assumed *Necator americanus* and *Ancylostoma duodenale* as the human relevant hookworm species implicated as contributing to iron-deficiency anemia. This assumption was based on the indistinguishable morphology of the *Ancylostoma* spp. eggs in stool and absence of awareness of a third, zoonotic hookworm species, *Ancylostoma ceylanicum*. The expanded use of molecular diagnostic assays for differentiating hookworm species infections during soil-transmitted helminth (STH) surveys, has now implicated *A. ceylanicum*, a predominant hookworm of dogs in Asia, as the second most common hookworm species infecting humans in the Asia Pacific. Despite this, with exception of sporadic case reports, there is a paucity of data available on the impact of this emerging zoonosis on human health at a population level. This situation also challenges the current paradigm, necessitating a One Health approach to hookworm control in populations in which this zoonosis is endemic.

Review

Hookworm infections in humans occur predominantly in low socio-economic countries located in tropical and subtropical areas of the world [43]. Symptomology is measured in disability-adjusted life years (DALYs) which include the years a human host has lived with the infection (YLD) as well as the years that are lost due to early death (YLL) [35]. The global infection prevalence for any hookworms in 2016 lay at 450.68 million (1297 million in 1994 [37]) with a disease burden of around 1.8 million disability adjusted life years (DALYs) in 2015 [4, 379]. Primary syndromes associated with infection include iron-deficiency anemia caused by intestinal blood loss within the small intestine [1, 71], which particularly impacts pregnant women [380] and children [381]. For hookworms, as well as other soil-transmitted helminths (STH), disease burden has fallen significantly over the last two decades [37]. This decrease can be attributed principally to targeted mass drug administration (preventive chemotherapy) in populations at risk of infection [21, 46, 82], as well as integrated intervention programs targeting improved access to safe water, sanitation and hygiene [206, 262] and development of molecular high-sensitivity diagnostic methods (improved infection prevalence estimation) [226] and socio-economic development [44]. The World Health Organization (WHO) recommended and approved diagnostic tool for STH detection is the Kato Katz Thick Smear [203]. However, this microscopy-based diagnostic method has important limitations; these include its innately low diagnostic sensitivity in regards to hookworm detection [382], the requirement for rapid sample screening to avoid the over-clearance of hookworm eggs following slide preparation [214] and the inability of microscopy-based diagnostics to identify the human hookworms to a species level [215]. These factors combined suggest an underestimation of the true species-specific prevalence and intensity of human hookworms.

Hookworm infections in humans have largely been attributed to *Necator americanus* and *Ancylostoma duodenale* [71]. However, contrary to this belief, recent molecular studies have demonstrated unequivocally that *Ancylostoma ceylanicum* is highly endemic, comprising the second most common species of hookworm, after *N. americanus* in many parts of the Asia Pacific and estimated to infect ~100 million people [30]. This situation challenges the current hookworm paradigm. Despite the global efforts to meet the WHO's 2020 roadmap for eliminating morbidity associated with STH infections, *A. ceylanicum* is not included within this WHO framework [21]. Health informative sites such as the Centers for Disease Control and Prevention (CDC) and the WHO [383] do not include *A. ceylanicum* as the causal agent of human hookworm infection. This is despite a growing body of evidence to demonstrate that its prevalence in certain restricted areas or countries outcompetes that of *A. duodenale* [139].

Importantly, *A. ceylanicum* is a zoonosis and transmissible to humans from animals. This species comprises the predominant hookworm of dogs and cats throughout the Asia Pacific, which act as reservoirs for human infections [30, 144, 342]. This situation contrasts that of *N. americanus* and *A. duodenale*, which are both specific to humans. In 2013 a review by Traub highlighted the history of the reported distribution and overlooked public health significance and impact of *A. ceylanicum* infections in humans in the Asia Pacific [30]. The review hypothesizes that mass anthelmintic programs may contribute to the re-emergence of *A. ceylanicum* infections in humans unless a One Health approach towards its control is undertaken [30]. In this review, we aim to summarize and update information spanning the six years since the last major review by Traub, on the prevalence, distribution and impacts of *A. ceylanicum* on human health.

Here, we conducted a systematic review, searching the publicly available database Google Scholar on Jun 14, 2019 with no restrictions in terms of scientific journal or author for publications in English only. We limited the output to novel original research papers published from 2013 to 2019 using the following search term in the title: “*Ancylostoma ceylanicum*”. Publication titles and abstracts of 70 peer-reviewed articles were screened and relevant papers included in this systematic review. Review articles were excluded with only original research papers included within the context of this mini-review. A list of all included publications can be found in Table 1 and 2.

Table 6.1. Summary of publicly available data on the proportion of hookworm infections attributed to *A. ceylanicum* in humans worldwide published between 2013 and 2019. Systematic review findings are summarized chronologically.

Author and year	Reference	Study country	Diagnostic tools used	Proportion of hookworm positive samples	Type of infection
Koehler et al., 2013	[384]	Australia	PCR-coupled SSCP	18.2% (2/11)	Mono-infection
Phosuk et al., 2013	[309]	Thailand	Agar plate culture + PCR	10% (3/30)	Mono-infection
Inpankaew et al., 2014	[144]	Cambodia	PCR + Sanger sequencing for <i>Ancylostoma</i> spp.	46.0% (57/124) 3.2% (4/124) 1.6% (2/124) 0.8% (1/124)	Mono-infection <i>N. americanus</i> / <i>A. ceylanicum</i> <i>A. duodenale</i> / <i>A. ceylanicum</i> <i>N. americanus</i> / <i>A. duodenale</i> / <i>A. ceylanicum</i>
George et al., 2015	[385]	India	Semi-nested PCR-RFLP	4.9% (2/41)	Mono-infection
Aung et al., 2017	[386]	Myanmar	PCR	27.3% (3/11)	Mono-infection
Bradbury et al., 2017	[387]	Solomon Island	Kato-Katz + PCR	16.7% (11/66) 1.5% (1/66)	Mono-infection <i>N. americanus</i> / <i>A. ceylanicum</i>
Papaiakovou et al., 2017	[388]	Timor-Leste Argentina	Semi-nested PCR-RFLP, PCR	95.5% (21/22 <i>Ancylostoma</i> spp.) 0% (0/8 <i>Ancylostoma</i> spp.)	-
O' Connell et al., 2018	[389]	Thailand (Myanmar refugees)	Semi-nested PCR-RFLP, qPCR	17.4% (83/476 baseline) 4.7% (6/128, follow-up) 0% (0/29, follow-up)	-

Since 2013, *A. ceylanicum* infections in humans continue to be identified across the Asia Pacific, including Thailand, Myanmar, India, Cambodia and the Solomon Islands (Table 6.1, Figure 6.1). The proportion of *A. ceylanicum* infections among all hookworm positive samples was relatively high in some regions (16.7% – 46.0%). Five out of 13 publications describe individual cases of returning travelers or personnel from the Asia Pacific highlighting the relevance of this emerging zoonosis to the field of travel medicine (Table 6.2). The majority of these patients had a severely increased eosinophil count, which is a known immune response to hookworm infections [390]. Symptoms ranged from abdominal pain to weight loss, fever, diarrhea, vomiting and other generalized symptoms (dizziness, tightness of chest, sweats). Interestingly, only one of these clinical cases presented anemia (Table 6.2). Although these were sporadic case reports from developed countries, there is no attention to ascertain morbidity caused on an endemic populations scale. Moreover, these ‘rare’ singular occurrence findings highlight the neglect of other symptomatic or subclinical cases in the country of origin.

Table 6.2. Summary of *A. ceylanicum* positive clinical cases from 2013 – 2018.

	Yoshikawa <i>et al.</i>, 2018 [391]				Brunet <i>et al.</i>, 2015 [392]	Speare <i>et al.</i>, 2016 [393]	Kaya <i>et al.</i>, 2016 [394]	Ngui <i>et al.</i>, 2014 [395]
Age, Sex	25, Male	47, Male	26, Male	26, Male	33, Male	26, Male	47, Male	58, Female
Country	Malaysia	Laos	India	Papua New Guinea	Myanmar	Solomon Island	Thailand/Laos	Malaysia
Tourist	Yes				Yes	No	Yes	No
Symptoms	Abdominal pain, watery diarrhea			Asymptomatic	Fever, weight loss, dyspnea, abdominal pain, bloody diarrhea	Abdominal pain, peripheral eosinophilia	Intermittent diarrhea, extreme eosinophilia, fever, abdominal pain, watery diarrhea, vomiting	Melena, discolored stool, dizziness, tightness of chest, cold sweats
Eosinophil count/ μ l (range 30-500 or < 6%)	3,000	20,470	7,050	1,570	59%	$6.20 \times 10^9/L$	43%, 74%	1%
Anemia	No	No	No	No	No	No	No	Yes

The inconsistency in diagnostic tools applied to estimate infection prevalence may additionally contribute to this underestimation [255]. In the context of some studies using a traditional microscopy-based gold standard diagnosis tool only hookworm positive samples were further investigated for species-specific infections using molecular tools [144, 387]. Not only does this lead to an underestimation of overall hookworm prevalence [243], but underestimates the proportion of *A. ceylanicum* infections even further [144], owing to the relatively lower egg shedding intensities of *A. ceylanicum* infected individuals compared with *N. americanus* [396].

Despite the increased availability and application of molecular diagnostic assays [243], there remains a lack of large-scale mapping of *A. ceylanicum* in areas other than the Asia Pacific, with a clear lack of available data in regards to infection prevalence and intensity in particular in human populations. This lack of knowledge is partly due to the application of microscopy-based diagnostic tools that are not able to differentiate between hookworm species [397]. Apart from the Asia Pacific, *A. ceylanicum* has also been reported in 3% of canines in Tanzania [398]. To date, the global distribution of cases in humans reflects that of its distribution in dogs. Within the previous six years after the landmark review had been published the focus of STH research has been on molecular biological areas such as genomics, transcriptomics and diagnostics [399]. In correlation, diagnostic tool application is shifting from invasive endoscopy [395] to molecular laboratory-based techniques such as semi-nested PCR-RFLP, qPCR and multiplexed- tandem qPCR [290, 385]. Moving towards these advanced molecular-based diagnostic tools utilizing PCR systems can prove beneficial in increasing general infection prevalence knowledge of individual hookworm species, the species-specific burden they cause and examine effects of co-infections within hosts [231]. Currently however, novel diagnostic techniques are limited to research settings and yet need to be completely transferable

to the endemic field in regards to resources, expertise and simply feasibility in remote settings [228].

As evidently shown within the literature, *A. ceylanicum* continues to present itself as increasingly emergent zoonosis, with a potentially wider than previously assumed geographical distribution spanning from the Pacific Islands to central Africa. For the majority of countries there is a paucity of epidemiological information available, which would serve as the basis for monitoring and diagnosing infections with this zoonosis for both clinical and population settings. Of the 13 epidemiological studies performed on *Ancylostoma ceylanicum* on Google Scholar since 2013 (70 total hits) nearly all utilized molecular-based screening to further identify hookworms to a species level. Traub hypothesized the emergence of *A. ceylanicum* owing to multiple factors, including a widescale mass deworming programs resulting in the disproportional increase of infective a larvae in the environment compared with those of *N. americanus* larvae exacerbated by a lack of an ‘allergy-based’ elimination of any new incoming larvae of ‘unnatural’ hookworms. If this is due to differential benzimidazole drug efficacy for different hookworm species remains unclear.

There is a clear need for further, in-depth investigation of *A. ceylanicum* infection prevalence, intensity and morbidity data in human hosts worldwide. These data will aid in mapping a more detailed picture of soil-transmitted helminth infections, inform governmental agencies about targeted treatment programs (including One Health approaches) and ultimately contribute to an eradication of not only hookworm infections, but helminth infections more broadly.



Figure 1. Known distribution of *A. ceylanicum* in the Asia Pacific to date. Reporting's pre 2013 are highlighted in orange and those from 2013 to date in yellow. Not depicted here are studies of canine *A. ceylanicum* cases from Madagascar, Kenya, Tanzania and South Africa.

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