Epidemiology of Soil-transmitted Helminths in a Community from Rural Papua New Guinea

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Statement of the Contribution of Others

This thesis contains my original research and contains no material previously published or written by another person unless it has been stated in the text. I have stated the contribution of others, where appropriate, including statistical support and materials provided by other collaborators to facilitate my research that has contributed to this thesis. The results obtained for my Higher Degree Research candidature is the content of my thesis and has not been submitted to qualify for the award or any other degree at any university or institution. I acknowledge that an electronic copy of my thesis will be lodged with the University Library and, subject to the policy and procedures of James Cook University, the thesis will be made available for research and study following the Copyright Act 1968 unless a period of embargo has been approved by the Dean of the Graduate School. I acknowledge that the copyright of all material contained in my thesis resides with the copyright holder(s) of that material. Where appropriate, I have obtained copyright permission from the copyright holder to reproduce material in this thesis.

Nature of assistance	Contribution of others
Supervision and other advisement	This degree was supervised by A/Prof Catherine Rush (Primary Advisor), A/Prof Jeffrey Warner (Secondary Advisor) and A/Prof Wayne Melrose (Secondary Advisor) for the whole duration of this degree.
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JS, CR, JW and WM conceived and designed the study. JS performed the methodology, collated, transcribed and analysed the data, developed the figures and tables, and wrote the manuscript. TIE advised the statistical analyses. CR, JW, WM, and TIE provided feedback on the analysis and results. All authors reviewed the manuscript and provided critical feedback on the content.

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Declaration of Ethics

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Abstract

Background: Soil-transmitted helminths (STHs) are a group of parasitic worms that establish disease in the intestinal tract of humans. STHs include *Ascaris lumbricoides*, *Trichuris trichiura*, hookworm (*Necator americanus* and *Ancylostoma* spp.) and *Strongyloides* spp., and they affect almost one billion people worldwide. In endemic countries, infection with more than one type of helminth infection is common. Typically, STHs are found disproportionately in regions with limited access to clean water and hygienic infrastructure and have poor sanitation practices. STH infections are not generally fatal, but they do contribute to morbidity and can be agents of co-morbidity, making them a significant public health concern in endemic regions.

A few studies have reported the distribution and determinants of STHs, including the two species of *Strongyloides*, *S. stercoralis* and *S. fuelleborni kellyi* present in rural communities of Papua New Guinea (PNG), but an up-to-date prevalence assessment in rural areas has not been done recently. This thesis describes the epidemiology of STHs, including *Strongyloides* within a rural PNG community, using diagnostic tools such as faecal microscopy, quantitative PCR (qPCR), and an immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) for STH diagnosis. A secondary objective was to assess the diagnostic utility of these tools for STH detection.

Materials and Methods: This cross-sectional study was conducted in Balimo, a rural PNG community located in the Delta (Middle) Fly District of the Western Province. Participant information, including demographics and general health, were collected via face-to-face interviews using a structured questionnaire, and faeces and blood were collected from consenting participants. The prevalence of STHs, including *Strongyloides* spp*.,* was assessed by microscopy on 122 preserved faecal samples using unconcentrated and formal ethyl-acetate concentrated (FEAC) techniques for helminth egg/ova and larvae detection. Stool samples were also tested for STHs using a TaqMan qPCR assay, targeting *A. lumbricoides*, *T. trichiura*, *N. americanus*, *Ancylostoma* spp. and *S. stercoralis*. Plasma samples collected from 120 matched participants were used to determine exposure to *S. stercoralis* using an ELISA to detect anti-*Strongyloides* IgG antibodies. *S. f. kellyi* was not directly targeted for serological or molecular methods. However, whether the ELISA used for *S. stercoralis* detection will cross-react with other co-existing *Strongyloides* spp. is unknown. Results from faecal microscopy, qPCR and the anti-*Strongyloides* IgG ELISA were used to determine STH infection prevalence. The proportion of agreement for positive and negative results was determined to assess the agreement between the diagnostic methods (microscopy vs qPCR and IgG ELISA vs qPCR). The Cohen's Kappa (K) statistic was also used to assess the likelihood of agreement between the two diagnostic tools. A multivariable logistics regression analysis was performed to identify links between STH infections,

demographics, and health-related information to determine whether specific characteristics could indicate the odds of STH infections. The strength and directionality of this relationship was reported using an adjusted odds ratio (aOR) with a 95% confidence interval (CI). Statistical inference was made if the *p-value* was < 0.05.

Results: Overall, the prevalence of STH infections in Balimo, Western Province, PNG, was 70.5% (n = 86/122), as determined by a combination of faecal microscopy, qPCR and anti-*Strongyloides* IgG ELISA. Within this cohort, *N. americanus* (52.5%; n = 67/122) was the most frequently detected, followed by *A. lumbricoides* (40.2%; n = 49/122), *Strongyloides* spp. (23%; n = 28/122), *Ancylostoma* spp. (3.3%; n = 4/122), and *T. trichiura* (2.5%; n = 3/122). Further, these results indicated that 38% (n = 46/122) of this cohort are infected with multiple types of STHs, with at least two individuals (1.6%; n = 2/122) infected with four different types of STHs. Of the individuals infected with STHs, 53% (n = 46/86) had multiple STH co-infections, with evidence of frequent overlaps occurring with *N. americanus*, *A. lumbricoides* and/or *Strongyloides* spp..

The total proportion of agreement between faecal microscopy methods and qPCR for any STHs (excluding *Strongyloides* spp.) was 82% and was classified to have "good" agreement, determined by the Kappa statistic (K = 0.64; 95% CI 0.50-0.78%). Faecal microscopy and the anti-*Strongyloides* IgG ELISA indicated greater infection rates for *A. lumbricoides* and *Strongyloides* spp., respectively, compared to qPCR. However, for the detection of hookworm and *T. trichiura* infections, qPCR detected more infections than faecal microscopy methods. Additionally, concentrating the faecal samples using the FEAC technique for microscopic examination improved detection capacity, particularly for *A. lumbricoides*, but was inferior for detecting hookworm or *T. trichiura* in this study.

The multivariable logistics regression analysis revealed that for every year increase in age, the odds of *Strongyloides* spp. infection decreased by 10%. Additionally, higher odds of *N. americanus* infection were found for those who were aged < 40 years (aOR = 7.9; 95% CI: 1.2-51.9; *p-value* = 0.03), lived in a village (aOR = 2.7; 95% CI: 1.0-7.3; *p-value* = 0.04) and had an *A. lumbricoides* co-infection (aOR = 4.7; 95% CI: 1.8-12.8; *p-value* = 0.002). Interestingly, individuals classified as underweight had lower odds of *N. americanus* infection than those with a normal BMI (aOR = 0.04; 95% CI: 0.003-0.7; *p-value* = 0.03). Furthermore, from this analysis, we identified that those experiencing self-reported shortness of breath (SOB) had reduced odds of having any STH (aOR = 0.2; 95% CI: 0.1-0.5; *p-value* = < 0.001), undifferentiated hookworm (aOR = 0.3; 95% CI: 0.1-0.7; *p-value* = 0.005) and *N. americanus* infections (aOR = 0.4; 95% CI: 0.1-1.0; *p-value* = 0.05). Although not statistically significant, this study revealed some notable trends. The prevalence rate of STHs, except for *Ancylostoma* spp.*,* was greater among those residing in a village setting than those in

Balimo town. Furthermore, a greater occurrence of mono- and tri-STH infections was observed for those living in a village. Interestingly, those residing in Balimo town had higher dual- and quad-infection rates.

Conclusion: The prevalence rate of STH infection and co-infections is high within a rural community within PNG. We identified STH-specific associations with age, residency, BMI, co-infections and SOB. It was also found that for the detection of STHs, qPCR could be an advantageous tool to detect hookworm and *T. trichiura* infections within this region, but not for *A. lumbricoides* infection as microscopy was found to be comparable to qPCR findings. Utilising IgG ELISA has a significant capacity to screen populations for strongyloidiasis and identify individuals who require further testing. Multiple wet-mount faecal smears and performing FEAC on preserved samples is more cost-effective, methodologically simpler and does not require sophisticated laboratory infrastructure compared to qPCR, making it an ideal tool in resource-limited regions. The outcomes of this research will help inform potential future public health intervention strategies aimed at reducing the burden of these parasitic worms. Future studies should investigate further the impact of STH infection on health implications such as nutrition and lung-related morbidities and include culturally appropriate questionnaires with sanitation-related variables for a more focused approach to characterising individuals with STH infections.

Table of Contents

List of Tables

List of Figures

Abbreviations

A/Prof: Associate Professor

aOR: adjusted odds ratio

APC: Agar plate culture

AUC: Area under the curve

BDH: Balimo District hospital

BHQ: Black hole quencher

BM: Baermann

BMI: Body mass index

Bp: Base pairs

CDC: Centres for Disease Control

CI: Confidence interval

Cq: Cycle quantification

DALY: Daily adjusted life years

DNA: Deoxyribonucleic acid

ELISA: Enzyme-linked immunosorbent assay

FEAC: Formal ethyl-acetate concentration

GBD: Global burden of Disease

GTC: guanidinium thiocyanate buffer

HIV: Human immunodeficiency virus

IBFQ: Iowan black FQ quencher

IBRQ: Iowa black RQ quencher

IgG: Immunoglobulin G

JCU: James Cook University

LAMP: loop-mediated isothermal amplification assay

LST: Land surface temperature

MS: Microsoft

Mtb: *Mycobacterium tuberculosis*

NTD: Neglected Tropical Disease

OD: Optical density

OR: odds ratio

PCR: Polymerase chain reaction

PNG: Papua New Guinea

QFT-G+: Quanti-FERON-TB gold plus

qPCR: Quantitative PCR

RFU: Relative Fluorescence Unit

ROC: Reporter operating curve

rRNA: Ribosomal Ribonucleic acid

SAF: sodium acetate with 10% formaldehyde

SD: Standard deviation

SE: Standard error

SEA: Southeast Asia

SOB: Shortness of breath

STH: Soil-transmitted helminth

TB: Tuberculosis

VIF: variance of inflation factor

WaSH: Water, sanitation and hygiene

WHO: World Health Organization

WP: Western Pacific

1 Introduction

Soil-transmitted helminths (STHs) are a group of parasitic worms that pose a significant public health challenge, particularly in resource-limited regions. These worms, including *Ascaris lumbricoides*, *Trichuris trichiura*, hookworm (*Necator americanus* and *Ancylostoma* spp.) and *Strongyloides stercoralis*, are primarily transmitted through faecal-contaminated environments, with soil being the primary transmission medium (WHO 2020a). Approximately 1 billion people globally were infected with STHs, and in 2019 contributed to 2090 fatalities (Institute for Health Metrics and Evaluation 2019). While the distribution of conventional STHs has been extensively studied, the burden and prevalence of *S. stercoralis* infections have been relatively overlooked despite its global presence (Buonfrate et al. 2020). Notably, *S. stercoralis* can be fatal, particularly among immunocompromised individuals (Ramanathan & Nutman 2008).

STH distribution is influenced by various parasite-host-environmental interactions governing infection acquisition (Brooker, Clements & Bundy 2006). *A. lumbricoides* and *T. trichiura* infections occur through ingestion of infective eggs/ova via the faecal-oral route, whereas hookworm and *S. stercoralis* infection are acquired through skin or mucosal contact with faecally contaminated soil containing infective larvae. Communities in underserved regions, lacking access to clean water, sanitation and adequate hygiene facilities, experience the highest prevalence rates of STHs due to these transmission dynamics (Echazú et al. 2015). Although STH infections are not generally fatal, they cause significant morbidity, particularly among children (Mehata et al. 2022), women of reproductive age and pregnant women (Aderoba et al. 2015; Garrison et al. 2021; Ness et al. 2020). These infections can result in damage to mucosal tissues in the gut and lungs, leading to nutrient and energy loss due to helminth feeding and migration (Gildner, Cepon-Robins & Urlacher 2022; Loukas, A et al. 2016). STHs, being chronic infections, can persist for many years, influencing local and systematic immunity and potentially impacting susceptibility to other co-infections, such as tuberculosis (TB) and malaria (Mabbott 2018).

Detection of STHs from faecal samples is commonly conducted using microscopy-based techniques (coproscopy). In resource-limited settings, coproscopy is preferred due to its simplicity and affordability, but it exhibits lower sensitivity compared to other advanced techniques, potentially underestimating true prevalence rates (Mbong Ngwese et al. 2020). Direct wet-mount faecal smears, the simplest to perform for STH detection, are less sensitive than centrifugation techniques, which concentrate ova/larvae in a faecal sample (Dana et al. 2020; Yimer et al. 2015; Zeleke et al. 2021).

Regarding the detection of *Strongyloides* spp., microscopy-based identification of larvae in faeces serves as the definitive diagnosis. However, its sensitivity is limited for detecting light-intensity infections due to the complexity of the disease states caused by the worm, which influences the shedding of larvae into faeces (Chankongsin et al. 2020; Hailu et al. 2022). Relying solely on microscopy has likely led to the global underestimation of strongyloidiasis prevalence (Buonfrate et al. 2020). The "gold standard" for detecting *Strongyloides* spp. larvae involves combining multiple culture systems with microscopy, enhancing detection sensitivity compared to microscopy-based methods only (Chankongsin et al. 2020; Forrer et al. 2018). However, this approach is time-consuming, labour-intensive, risks infection, and requires trained personnel, which may limit the utility of these culture techniques in rural and remote laboratories. Serological and molecular methods can offer improved detection sensitivity when reporting strongyloidiasis (Buonfrate et al. 2015) and STH (Miswan, Singham & Othman 2022) infection rates, respectively. However, these methods are not yet standardised and require specialised laboratory capacities, potentially limiting implementation in resource-limited settings (Miswan, Singham & Othman 2022; WHO 2020b). Commercial enzyme-linked immunosorbent assays (ELISA) detecting anti-*Strongyloides* immunoglobulin G (IgG) in serum samples report sensitivities ranging from 89.5 to 91.2% and specificities of 98 to 99% when compared with confirmed strongyloidiasis cases (Bisoffi et al. 2014). Nevertheless, using serological methods has limitations, including difficulties differentiating past and active infections and reported cross-reactivity with other helminths in co-endemic areas (Arifin et al. 2019; Norsyahida et al. 2013). Molecular techniques, such as polymerase chain reaction (PCR) on faecal material, offer improved sensitivity for detecting STHs in endemic settings compared to coprological techniques (Dunn et al. 2020; Llewellyn et al. 2016), but molecular methods have limited utility for detecting *Strongyloides* spp. (Buonfrate et al 2017; Buonfrate et al 2018; Dong et al 2016). Studies combining microscopy and quantitative PCR (qPCR), specifically TaqMan hydrolysis probe assays, have identified more STH cases (Llewellyn et al. 2016; Othman et al. 2020) and can detect lower-intensity infections (Zendejas-Heredia et al. 2021). However, challenges related to cost, lack of methodological standardisation and technical expertise limit its application in resource-limited regions (Easton et al. 2016).

The distribution and determinants of STHs in Papua New Guinea (PNG) in rural communities like Balimo, located in the Western Province, remains poorly understood. Previously published STH surveys dating back to the 1970s to the early 2000s indicate a high prevalence of these worms, with one study predicting that three-quarters of the PNG population nationwide could be infected with

hookworm (Kline et al. 2013). However, the distribution of each worm species across the country is uneven, and the reasons underlying this are unclear.

A more recent survey in 2009 conducted in Goroka, Eastern Highlands, reported that one-third of pregnant women were infected with at least one type of STH, with hookworm being the predominant species causing infections (Phuanukoonnon et al. 2013). In some earlier studies in the Eastern Highlands, STH infection rates reached up to 90% within certain communities, but surveyed community characteristics predisposing the population to infection were unexplored (Shield, Scrimgeour & Vaterlaws 1980). Importantly, STH studies conducted in the Western Province of PNG have not been performed in over two decades, leaving a significant gap in knowledge regarding STH epidemiology in this region.

Given the paucity of STH data and uncertainty as to the impact of evolving improvements in access to public health care services and improved sanitation and hygiene facilities in PNG, a key question arises: What is the current prevalence and epidemiology of STHs in rural communities of the Western Province in PNG and what are the characteristics of this population that might make them more likely to have the infection? Current strategies implemented to alleviate worm burden in PNG include mass distribution of anti-helminthic drugs, and while some communities in PNG are receiving these anti-helminthic drugs, the coverage is considered insufficient for adequate control (Montresor et al. 2020).

This thesis aims to address these knowledge gaps by determining the prevalence and epidemiology of STHs in a rural Western Province, PNG community, explicitly focusing on the Balimo region. Additionally, it aims to identify population characteristics and attributes that may contribute to a greater likelihood of STH infection. The outcomes of this research may provide crucial insights to inform future public health intervention strategies aimed at reducing the burden of these parasitic worms.

The specific Aims of this thesis are:

> Aim 1: Determine the prevalence of STHs and the utility of faecal microscopy and qPCR for STH diagnosis.
>
> Aim 2: Determine the prevalence of *Strongyloides* spp. infection using IgG ELISA and qPCR.
>
> Aim 3: Identify demographic and clinical characteristics associated with STH infections in rural PNG.

2 Soil-Transmitted Helminthiases and Their Historical Occurrence in PNG

2.1 What Are Helminths?

Helminths, or parasitic worms, have been parasitising humans for thousands of years, causing invasive infections (Ledger et al. 2021; Mitchell 2013). Over this time, these worms have evolved unique environmental and host niches for successful reproduction and transmission, as well as establishing complex relationships with the host's physiology and immune system (Jourdan et al. 2018; Loukas, Maizels & Hotez 2021). Various types of helminths that can infect humans are separated into three morphologically distinct groups: Trematodes (Flukes), Cestodes (Tapeworms) and Nematodes (Roundworms) (Castro 2011), as shown in Figure 2.1. Among these, gastrointestinal nematodes, specifically the groups of worms classified as STHs that are outlined by the World Health Organization (WHO) (WHO 2020a), including *S. stercoralis,* are of interest in this review (WHO 2020c). Reported estimates suggest that STH infections, excluding rates of *Strongyloides* spp., affect almost 1 billion people worldwide, primarily in impoverished regions with inadequate sanitation and hygiene (Institute for Health Metrics and Evaluation 2019). The WHO also categorises STHs as neglected tropical diseases (NTDs) because of their widespread distribution and high burden in predominantly marginalised communities worldwide (WHO 2020c). In PNG, the distribution and determinants of STH infections remain unclear as surveys on STHs haven't been published in almost two decades (Bradbury 2021; Kline et al. 2013), but factors such as poor sanitation and limited access to safe water might suggest a high likelihood of infection in this region.

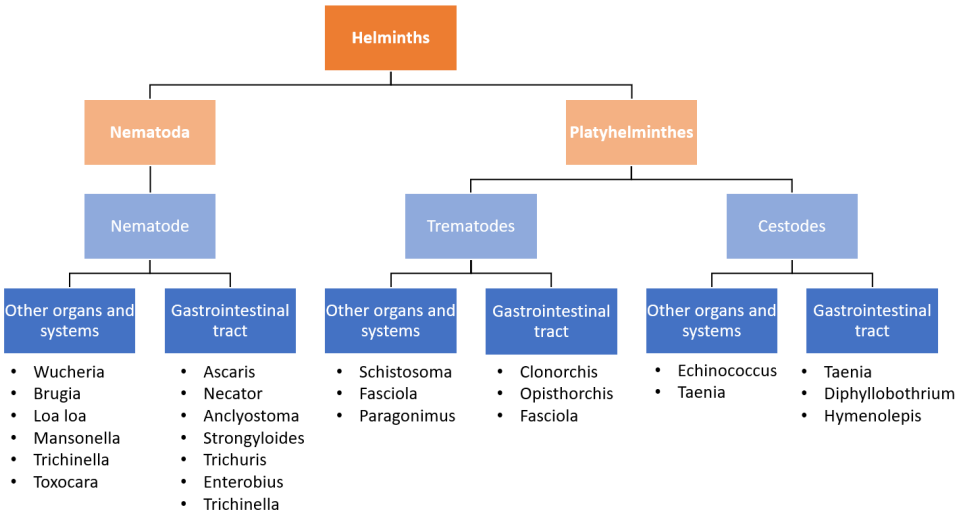

Figure 2.1: Taxonomy of helminths that infect humans. Adult helminths can colonise the gastrointestinal tract or the skin, the lymphatics system, bloodstream or other body organs. "Other organs and systems" encompasses the skin, lymphatics, bloodsteam and other body organs. Adapted from King 2019. RightsLink® Copyrights Clearance centre Inc. and Elsevier has granted permissions of reuse under license number 5613990817675.

This highlights a critical knowledge gap regarding the current epidemiology of STHs in PNG. This narrative review focuses on STHs of public health concern, as outlined by WHO, particularly in the Oceanic and Southeast Asian region (WHO 2020a, 2020c, 2020d). It explores the global distribution of STHs, discussing factors that influence distribution, emphasising the parasite-environmental-host interface paradigm and how the choice of diagnostic tools can impact reported prevalence rates. This review also considers historical helminth surveys conducted in PNG and identifies epidemiological factors that suggest the persistence of these helminths despite a lack of recent survey data.

2.2 STHs of Public Health Concern

Here, we will provide an overview of the global extent of STHs infection, focusing on the prevalence of STHs in the WHO-defined Southeast Asia (SEA) and Western Pacific (WP) regions. Understanding the prevalence of STHs is crucial for delineating their geographical boundaries and ability to sustain infection in specific regions worldwide, particularly in PNG.

STHs are a significant public health concern, given their global distribution and impact on human health. In highly endemic regions, infections with multiple STHs are frequently encountered, potentially exacerbating health issues (Jourdan et al. 2018; Pullan et al. 2014). Although often asymptomatic and associated with low fatality rates, these infections can have long-term consequences on the host. The strategies that STHs deploy to establish infection within the host are why infections can often be sustained chronically and can be life-long. Even after treatment, re-infection can occur if living conditions promote continuous exposure and if there are no behavioural changes that would prevent re-infection (Jia et al. 2012).

Measuring and reporting STH infection-related morbidities can be quantified as daily adjusted life years (DALYs), which describes disease burden (WHO 2020c). The clinical consequences of STH infections vary with each worm and their interaction with the host. It is speculated that the difference between asymptomatic and symptomatic carriage and the extent of morbidity is proportional to the intensity of an individual's worm burden (Loukas, Maizels & Hotez 2021). STHs are linked with various negative health effects, including poor growth and wasting/stunting, impaired physical and cognitive function, with children being particularly prone (Forrer et al. 2017; Garrison et al. 2021; Mehata et al. 2022) and negative impacts on pregnancy outcomes (Aderoba et al. 2015; Ness et al. 2020).

Past public health interventions for STHs have targeted *A. lumbricoides*, hookworm and *T. trichiura*, overlooking the often-co-endemic *Strongyloides* spp. (WHO 2012). However, the latest roadmap by

the WHO now includes strongyloidiasis as a target for control alongside the other STHs (WHO 2020c). Various hookworm species pose a risk to humans in SEA and WP regions, including the anthroponotic species *N. americanus* and *A. duodenale* and the zoonotic species *A. ceylanicum*. Here, we focus on *N. americanus*, *A. duodenale* and human *A. ceylanicum* infections due to their relevance in this region. Although *A. ceylanicum* is not on the official WHO's list of STHs, it has similar treatment strategies as anthroponotic species despite its differing epidemiology (Colella, Bradbury & Traub 2021).

2.3 The Prevalence and Consequence of Incriminated STHs, Particularly in Southeast Asia and Western Pacific Regions

2.3.1 Intestinal Nematode Infections

According to the global burden of disease (GBD) study, data published in 2019 estimated that STHs (excluding strongyloidiasis) infected over 900 million people, with the highest burden reported from Sub-Saharan Africa, Southeast Asia, east Asia and Oceania (Institute for Health Metrics and Evaluation 2019). A meta-analysis by Gilmour, Alene and Clements (2021), including over fifteen thousand participants, found that the overall pooled prevalence of STH infections within minority indigenous communities in the SEA and WP region was 61%. The WP region had higher cumulative prevalence rates of STHs (66%) than the SEA region (30%) (Gilmour, Alene & Clements 2021). However, none of the studies in this meta-analysis included STH surveys from PNG.

A nationwide survey conducted in Fiji revealed that among school-aged students, STH prevalence was 10.5%, with *A. lumbricoides* most frequently detected (Kim et al. 2020). Comparatively, a nationwide survey conducted in the Solomon Islands reported an STH prevalence rate of 63.3%, consisting of samples from adults, with hookworm being the most common type of STH infection (Le et al. 2022). The clear difference between the reported STH prevalence rates could be attributable to the difference in study locations, designs, sample size, diagnostic tools used and whether the nation receives annual mass administration of anti-helminthic drugs.

2.3.2 Ascariasis Prevalence and Burden

The estimated global prevalence of ascariasis was 447 million and caused 0.86 DALYs in 2017 (James et al. 2018; Kyu et al. 2018). Heavy *A. lumbricoides* burden can cause significant illness associated with obstructing the bowels and the bile and pancreatic ducts, which can have fatal consequences (De Silva, Guyatt & Bundy 1997; Magalhães et al. 2021). Additionally, the lung phase of this parasite's lifecycle has also been implicated in causing eosinophilic asthma/pneumonia (Löffler's

syndrome) and exhibiting chronic obstructive lung-like pathologies resulting in overall lower lung function (Chitkara & Krishna 2006; Magalhães et al. 2021). A recent meta-analysis focusing on the global prevalence of ascariasis found that the pooled prevalence of infection was 11% from almost five million surveyed people, corresponding to a predicted estimate of 732 million cases in 2020 (Holland et al. 2022). This analysis then found that in the Melanesia sub-region of Oceania, ascariasis prevalence was reported to be 29% of 1664 pooled participants, corresponding to 11 million people infected in 2020 (Holland et al. 2022). Conversely, another meta-analysis found that from almost twenty-two thousand surveyed indigenous persons from WP and SEA, the cumulative prevalence was 32% (Gilmour, Alene & Clements 2021).

2.3.3 Trichuriasis Prevalence and Burden

The global prevalence of trichuriasis in the GBD 2017 report was estimated at ~290 million people, attributing about 0.23 million DALYs, and cases were predominantly asymptomatic (James et al. 2018; Kyu et al. 2018). Despite its asymptomatic nature, infection with *T. trichiura* establishes infection through partial penetration of the host's large intestines, sequestering nutrients from the host's blood. This partial embedment can result in inflammation of the bowels and cause dysentery syndrome in heavy infections, causing anaemia (Gyorkos et al. 2012) and/or potentially leading to rectal prolapse (Cooper & Bundy 1988; Khuroo, Khuroo & Khuroo 2010). A meta-analytic review published in 2022 has demonstrated that in the Asia-Pacific region, the aggregated prevalence rate of *T. trichiura* infection was 15% of over five million people, and in the SEA region, the prevalence rate was 19% (Badri et al. 2022).

2.3.4 Hookworm Prevalence and Burden

Global estimates of hookworm infection were reported as 229 million in 2017 (James et al. 2018) and were associated with 0.85 million DALYs, and most reported cases were asymptomatic (Kyu et al. 2018). Complicated hookworm infections are frequently linked with moderate and severe anaemia (Brooker, Hotez & Bundy 2008; Gyorkos et al. 2011), causing the greatest amount of blood loss through its ability to adhere to the host's intestines via its cutting plates, resulting in direct tissue damage to the intestinal tissues. Further, hookworm is also attributed to protein malnutrition and hypoalbuminemia (Clements & Addis 2022). During pregnancy, severe hookworm-associated anaemia coupled with demands by the foetus can negatively affect birthweight, contributing to premature birth and maternal mortality (Gyorkos et al. 2011; Ness et al. 2020). Many epidemiological surveys focus on overall hookworm occurrence without considering species diversity, which may limit the effectiveness of intervention strategies (Clements & Addis 2022).

Furthermore, a systematic review found that *N. americanus* was the most prevalent hookworm worldwide, accounting for around 79% of cases, while *Ancylostoma* spp. infections were less common (32%), and *A. ceylanicum* prevalence was greater than *A. duodenale,* found mostly in the Asia-Pacific (Clements & Addis 2022).

2.3.5 Strongyloidiasis Prevalence and Burden

Strongyloidiasis is one of the most neglected tropical parasitic diseases, lacking comprehensive surveys to document its geographic distribution (Luvira et al. 2022). Human infections with *S. stercoralis* have various disease states, ranging from acute and asymptomatic to symptomatic or chronic. Often asymptomatic, when symptoms arise, infections may cause mild gastrointestinal issues (Luvira et al. 2022; Van De et al. 2019). However, its recent inclusion in the list of priority NTDs is warranted by its capacity to cause life-threatening infection, particularly in individuals with weakened immune systems, such as individuals using corticosteroids, which can lead to hyperinfection syndrome and disseminated strongyloidiasis (Chen et al. 2023; Keiser & Nutman 2004; Ramanathan & Nutman 2008). Further, the lung phase of *S. stercoralis*, during severe forms of the infection, has been associated with acute respiratory distress syndrome, acute respiratory failure and pulmonary haemorrhaging (Liu, Hsu & Chang 2009). A systematic review covering studies from 1990 to 2016 reported a global prevalence rate of 8% for strongyloidiasis, estimating that nearly 614 million people were infected in 2017 (Buonfrate et al. 2020). Prevalence rates can vary significantly between geographic regions, depending on the diagnostic tools used (Eslahi et al. 2021). A predictive map model estimated that approximately 2.6 billion people are at risk of strongyloidiasis, with 22% of the population in Oceania at risk (Fleitas et al. 2022). The latter study indicated that around 20% of the population in PNG are at risk of acquiring strongyloidiasis, while an unexpected 26% are at risk in Australia (Fleitas et al. 2022).

The existing literature primarily focuses on *S. stercoralis* infections as the leading cause of strongyloidiasis worldwide. However, occasional infections in humans by other species, such as *S. fuelleborni*, have been reported, though studies on its prevalence are limited. Within *S. fuelleborni*, two sub-species have been identified to also infect humans: *S. f. fuelleborni* mainly infecting non-human primates (Thanchomnang et al. 2019), with spill-over infections documented in Africa and Southeast Asia (Thanchomnang et al. 2017) and *S. f. kellyi* exclusively identified in humans, geographically restricted to PNG. The lifecycle dynamics and virulence factors of *S. f. kellyi* remain unclear, requiring further investigation (Bradbury 2021).

2.3.6 The Extent of STH Polyparasitism and Other Co-infections

Infection with multiple parasites is prevalent in endemic areas, particularly within the Oceanic or WP region. However, despite this, these mixed infections are not often reported in surveys, and the health implications of such co-infections remain poorly understood. A meta-analysis inclusive of 50 peer-reviewed articles on helminth-helminth polyparasitism identified that poly-helminths infections exceeded the prevalence rate of mono-infection helminths by 14.0%, suggesting that helminths-helminths co-infections are common (Donohue, Cross & Michael 2019).

The overview of global and regional estimates of STH infection reveals an uneven geographical distribution of these parasites between communities and countries. This distribution is linked to various factors relating to the environment, parasite factors, and different host physiological, behavioural and cultural factors. Further, the choice of diagnostic tools to assess STH prevalence also plays a crucial role in the reported prevalence rates, as the accuracy of the tool can differ depending on the helminth in question and its application.

2.4 The Parasite-Host-Environmental Interface

STH distribution is influenced by various parasite-host-environmental interactions governing infection acquisition (Figure 2.2) (Brooker, Clements & Bundy 2006). This section will explore the strategies helminths employ to navigate their lifecycle through host encounter, invasion, establishment of infection and persistence. Additionally, it will examine the impact of environmental and host factors on these processes. STHs have direct lifecycles, as an intermediate host is not required. STHs progress through stages of eggs, larvae and adults, but how these helminths progress through their lifecycles is unique. These lifecycles affect their transmission dynamics, host susceptibility and associated morbidity (Castro 2011). Understanding these lifecycles through an epidemiological lens provides a foundation for comprehending the geographical distribution of helminths and whether the conditions in PNG allow for the perpetuation of STHs.

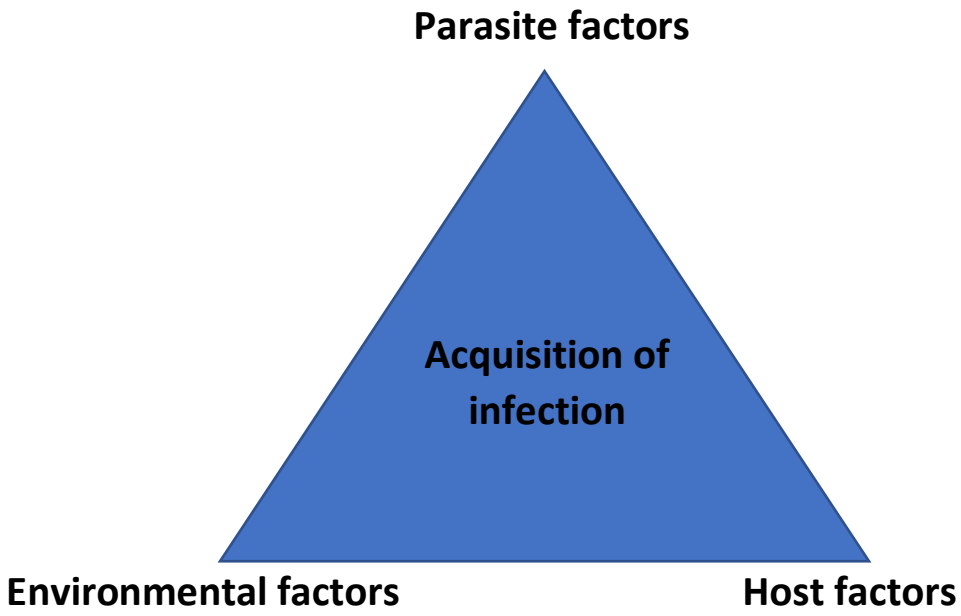

Figure 2.2: Parasite-environment-host interface. The epidemiological triad for the acquisition of helminths.

2.4.1 Parasite-factors STH Lifecycles and Establishment of Infection

A summary of STHs lifecycles is shown in Figure 2.3. This figure demonstrates that STHs have a direct lifecycle, and all have unique interactions with the host for survival. Each section will briefly elaborate on phases of the lifecycles, making special references to STH species.

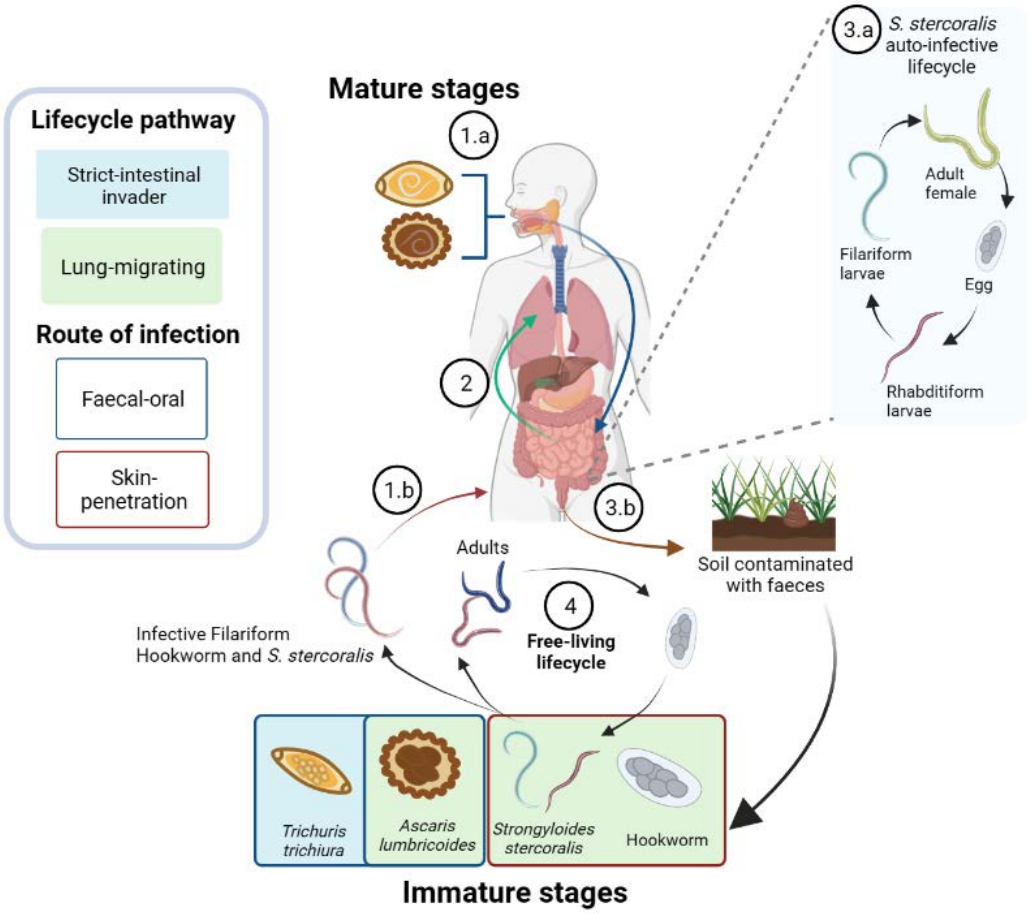

Figure 2.3: Summary of lifecycles for Intestinal STHs.

Encounter and invasion: Infective ova of *Ascaris* and *Trichuris* infect the host via the faecal-oral route **(1.a)**, while infective larvae of *Strongyloides* and hookworm are encountered in the environment and gain entry to the host via skin penetration **(1.b)**. **Establishment of infection**: *Ascaris*, hookworm and *Strongyloides* have lung-migratory pathways as part of their lifecycles **(2)** before establishing infection in the intestines, whereas *Trichuris* remains in the intestines. **Perpetuation of the lifecycle:** Adult female worms of *Strongyloides* release eggs that deposit in the intestines, larvae hatch in the intestines can re-infect the host through mucosal penetration for continuation of the lifecycle **(3.a)** or can be deposited into the environmental through faeces similarly to the ova of *Ascaris*, hookworm and *Trichuris* **(3.b)**. Once excreted from the host via faeces, ova of *Ascaris* and *Trichuris* need time in the environment to mature to become infective. Hookworm ova hatch into larvae in the environment and like *Strongyloides* larvae which are expelled in faeces, begin their free-living lifecycle **(4)**. Hookworm larvae molt once in the environment before becoming infective, while *Strongyloides* also involves a single generation of free-living adults of both sexes, which produce eggs that hatch into larvae that later become infective. Image created in Biorender. Adapted from Gordon et al. 2017, used under Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0/).

2.4.1.1 Host-encounter and Invasion

Typically, helminths have three developmental life cycle stages: Ova (Figure 2.4A), larvae (Figure 2.4B) and mature adult worms (Figure 2.4C). Once developed within the host's gastrointestinal tract, ova or larvae are excreted into the environment through faeces. In the environment, eggs and larvae require a few days to weeks of development before becoming infective. For instance, hookworm ova can hatch within 24 to 72 hours under favourable conditions and begin its free-living lifecycle (Mkandawire et al. 2022). In contrast, *A. lumbricoides* eggs can remain viable and infective in the environment for several years (Senecal et al. 2020). Hosts can encounter these parasites through two routes 1) direct faecal-oral transmission through contaminated hands or ingestion of contaminated food or water or 2) skin or mucosal membrane contact with contaminated soil containing free-living helminth larvae (Figure 2.3) (Brooker, Clements & Bundy 2006; Strunz et al. 2014; Torgerson et al. 2015)

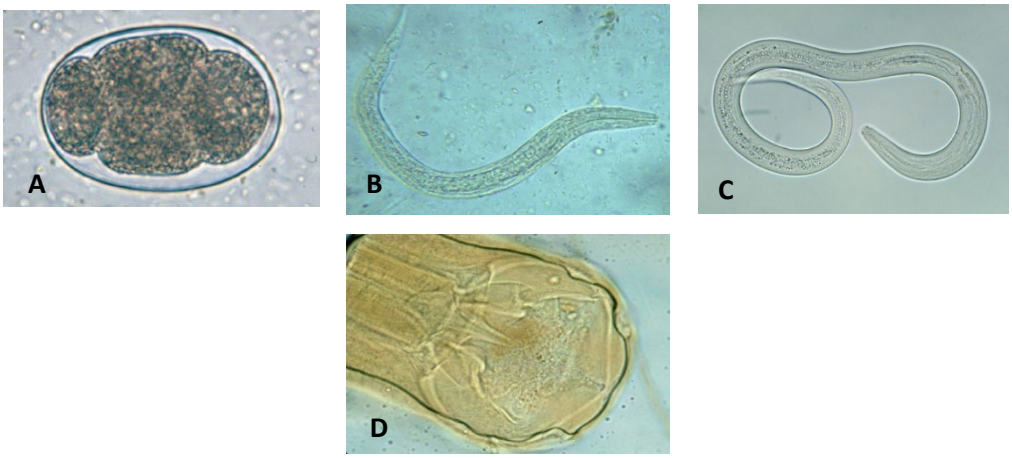

Figure 2.4: Morphological lifecycle stages of helminths, example Hookworm. Ova/egg, measure 250-300µm long and 15-20µm wide (A), rhabditiform (L1) larva (B), infective and adult filiform (L3) worm (C) and buccal cavity of adult worm (*Necator americanus*) (D). All images were sourced and adapted from the CDC / Global Health, Division of Parasitic Diseases and Malaria / CDC, DPDx, Intestinal Hookworm – Public Domain.

A. lumbricoides and *T. trichiura* are acquired via the faecal-oral route and will deposit into the intestines, where they begin their lifecycle phases inside the human host (Else et al. 2020). In contrast, when free-living larvae, *N. americanus* and *Ancylostoma* spp. encounter a host they will attach to the skin and secrete proteolytic enzymes, destroying the cellular components of the skin barrier (Brown et al. 1999; Hotez et al. 1992), where the worms will penetrate and enter host circulation. Similar mechanisms have been reported for *Strongyloides* spp. (Sakura & Uga 2010).

2.4.1.2 Establishment of Infection

After the eggs of *T. trichiura* are deposited within the caecum of the large intestines, they receive signals from the host's microbiota that initiate hatching (Hayes et al. 2010)**.** The immature larvae then penetrate the intestinal epithelial cells of the mucosal membrane to continue their development. As the worm matures, it becomes partially embedded within the intestinal epithelial cells rather than completely intracellular (Jourdan et al. 2018). Under these conditions, female *T. trichiura* will release eggs through faeces into the environment. Given favourable conditions, the infection can persist for years, leading to chronic infection (Duque-Correa et al. 2022).

Tissue-migrating helminths have larvae that traverse through different organs, including the lungs, to complete their lifecycle. For example, after *A. lumbricoides* ova hatch in the small intestines, the larvae penetrate the intestinal walls and enter venous circulation, transporting the larvae to the lungs. From there, they burrow through the alveoli walls, move to the pharynx, and are coughed up, swallowed, and mature in the small intestines. This lifecycle progression is similar for hookworm and *Strongyloides* spp. after larval invasion through the skin or mucosal barriers (Nutman 2017; Ghodeif & Jain 2022). Upon returning to the intestines, hookworm larvae feed on the host's microbiota until reaching maturity as sexually differentiated, blood-feeding worms. Adult female worms can produce between < 1999 to > 4000 eggs per day, exiting the host through faeces into the environment, where the cycle begins again (Ghodeif & Jain 2022; Montresor et al. 1998). In contrast, *S. stercoralis* has a more complex lifecycle in which the infection can be maintained life-long due to its unique auto-infective ability. Once *Strongyloides* larvae return to the intestines from the lungs, they eventually mature into adults. Adult female worms can produce eggs via parthenogenesis, which gives rise to larvae that can either be passed in the stool into the environment and begin their free-living lifecycle or undergo auto-infection, penetrating either the intestinal mucosa or the skin of the perianal region (Lok 2014).

2.4.2 Environmental Factors Influence STH Distribution

Environmental factors like climate and soil properties are critical determinants of STH distribution, as they shape the geographical boundaries where these parasites thrive. These factors influence the transmission dynamics of helminths and the continuation of their lifecycles. Various environmental factors, including temperature, rainfall, vegetation, soil properties, and altitude, impact the development of STH eggs and larvae, which have been reviewed here (Sturrock & Sanchez 2017).

Understanding the relationship between environmental factors and STH prevalence enables the development of geostatistical risk prediction maps. The information from these maps could provide insight into whether the environment in PNG can sustain STH transmission (Magalhães et al. 2011). Previously published literature describing the current understanding of environmental factors and stimuli associated with initiating embryonation and hatching of STHs has been reviewed here (Mkandawire et al. 2022).

2.4.2.1 Temperature

Ambient temperature plays a crucial role in the embryonation of ova. Temperatures below 5℃ or above 37℃ can halt development or lead to egg or larvae mortality (Forman et al. 2021; Harroff et al. 2019; Kim et al. 2012; Udonsi & Atata 1987). Thus, helminths require optimal temperature ranges for development. For *Ascaris* spp. ova, temperatures ranging between 16 to 34℃ were optimal for embryonation and increasing temperatures accelerated development, reaching a threshold of about 37℃, after which resulted in ova degradation (Arene 1986; Kim et al. 2012). A similar observation has been demonstrated for *Trichuris* spp. (Forman et al. 2021). However, increasing temperatures above 30℃ reduced the infectivity and viability of larvae *in vitro*, particularly for *Ascaris* (Arene 1986). Under controlled conditions, larvae viability and infectivity are highest when eggs were embryonated at 22℃ and 26℃ for *Ascaris* and *Trichuris*, respectively (Arene 1986; Forman et al. 2021).

The relationship between temperature and the prevalence of *Ascaris* and *Trichuris* has been demonstrated in some predictive risk mapping models. Areas with land surface temperatures (LST) between 25-33℃, like Africa and the Middle East, show a higher prevalence of STH infection, while infections were less common where LST exceeded 35℃ (Brooker, Clements & Bundy 2006). Studies from Thailand (Chaiyos et al. 2018), Tanzania (Manz et al. 2017; Riess et al. 2013), and Timor-Leste (Wardell et al. 2017) also support the temperature-STH relationship. In contrast, hookworms and *Strongyloides* have free-living lifecycles, with optimal hatching and development occurring between temperatures of 26–30℃ for *N. americanus*. Temperatures below 15℃ and above 35℃ under controlled conditions resulted in arrested development (Udonsi & Atata 1987). The global distribution of hookworm and strongyloidiasis is influenced by annual temperature, similar to the controlled conditions for *N. americanus* mentioned earlier (Fleitas et al. 2022).

2.4.2.2 Soil-Properties, Vegetation (Land Coverage) and Rainfall

Environmental factors such as rainfall, vegetation, soil properties, and pH also play a role in the distribution of STHs. Soil properties, including soil type, pH, moisture, and microbial activity, influence the embryonation of ova and the hatching of larvae (Mkandawire et al. 2022). Soil moisture and humidity affect the viability and development of ova and larvae, with higher humidity accelerating development and lower humidities leading to desiccation (Seamster 1950; Spindler 1929). In predictive risk models, researchers use vegetation indices such as enhanced vegetation index as proxy variables for humidity/soil moisture. Investigators have identified that the risk of STH infection is enhanced in areas with dense vegetation (Aw et al. 2021; Chaiyos et al. 2018). Rainfall also provides insights into STH distribution. High annual rainfall increases the odds of hookworm infection (Fleitas et al. 2022), specifically *N. americanus* infection (Le et al. 2022). In wetter areas, the worms have been found closer to the topsoil when compared to drier areas (Udonsi Nwosu & Anya 1980), and extensive rainfall has been associated with a decreased risk of *A. lumbricoides* infection (Chammartin et al. 2013). Prevalence rates of *Strongyloides* have been modelled to decrease with increasing rainfall rates (Chaiyos et al. 2018), potentially through the immersion of stool in water, inhibiting the development of the larvae in the environment (Anamnart et al. 2013). Soil type and pH also impact STH distribution. Soil with higher porosity, like sandy loam, is conducive to hookworm infection compared to more compact soils (Riess et al. 2013). Acidic soil pH is associated with an increased risk of general STHs, *Strongyloides*, and *Ascaris* infections (Chammartin et al. 2013; Le et al. 2022). These trends align with experimental studies that found optimal hatching of hookworm ova occurs in moderately acidic soils with pH ranging from 4.6 to 9.4, with optimal hatching pH found to be 6.0 (Udonsi & Atata 1987; WHO 2004). Additionally, when *Ascaris* eggs were subjected to alkaline (pH > 9.4) soil, it resulted in lethal outcomes of ova development, which is also supported in other studies (Jensen et al. 2009; Senecal et al. 2020).

2.4.2.3 Altitude and Elevation

Geospatial analyses have demonstrated that the risk of *Strongyloides* spp., hookworm, *Ascaris* and *T. trichiura* infection is decreased with increasing altitudes (Chaiyos et al. 2018; Chammartin et al. 2013; Jember et al. 2022). This decrease in risk at higher altitudes may be due to non-optimal soil temperatures and ambient humidity levels (Jember et al. 2022). Additionally, poorly drained soil was associated with low hookworm infections at higher altitudes with cooler temperatures (Mabaso et al. 2004).

2.4.3 Socioeconomic Factors and Water, Sanitation and Hygiene (WaSH) Circumstances Influence STH Prevalence

Sociodemographic circumstances and behaviours related to WaSH significantly influence the prevalence of STHs (Fleitas et al. 2022). Countries with lower socio-demographic and economic indices are associated with higher STH burden (Brooker, Clements & Bundy 2006). At a regional level, rural regions exhibit higher helminthic infections compared to urban areas (Karagiannis-Voules et al. 2015; Kurscheid et al. 2021; Puhalenthi et al. 2023; Ribado Meñe et al. 2023). Urban settlements demonstrate a lower risk of hookworm infections compared to rural areas (Karagiannis-Voules et al. 2015). Similarly, peri-urban residents have five times higher odds of STH infection than urban dwellers (Ribado et al. 2023). However, in wealthier countries like Australia, higher STH burdens persist in rural regions, particularly in areas with health disparities (Kurcheid et al. 2022).

Access to clean and safe drinking water is a well-established risk factor for STH infections (Campbell et al. 2017; Phillips et al. 2022; Puhalenthi et al. 2023; Strunz et al. 2014). Piped drinking water is protective against STH infection, particularly for *Ascaris* and *Trichuris* (Puhalenthi et al. 2023; Strunz et al. 2014). In contrast, collecting drinking water from streams increases infection risk (Campbell et al. 2017). Barriers to accessing water enhance the risk of hookworm and *Ascaris* infections, potentially due to increased environmental exposure (Phillips et al. 2022). Different water sources have varying impacts on STH infection risk, with borehole or surface water increasing infection risk and water treatments, like boiling water, reducing infection risk (Anegagrie et al. 2021; Campbell et al. 2017; Forrer et al. 2018).

Access to hygienic infrastructure, such as latrines/toilets, plays a crucial role in STH prevalence (Puhalenthi et al. 2023; Strunz et al. 2014; Ziegelbauer et al. 2012). Owning a household toilet/latrine significantly lowers the likelihood of *N. americanus* infection by 60% compared to those without a latrine/toilet (Le et al. 2022). Furthermore, private household latrines/toilets offer greater protection against STH infections than public toilets or open defecation (Halliday et al. 2019; Puhalenthi et al. 2023). Engaging in open defecation doubles the odds of hookworm infection (Assefa, Alemu & Ayehu 2023) and shared latrine usage, coupled with poor hand hygiene, was identified as a risk factor for hookworm and *A. lumbricoides* infection (Phillips et al. 2022).

Occupational exposure, particularly among farmers, significantly contributes to intense hookworm infections, inadequate access to sanitary infrastructure and poor personal hygiene practices at work and home (Agustina et al. 2022; Kurscheid et al. 2021).

Household-level factors influencing STH infection risk include household size, amenities and house infrastructure. Larger household sizes, typically with more than five members, enhance STH infection risk (Assefa, Alemu & Ayehu 2023; Kurscheid et al. 2021). This could be attributed to shared spaces, like household latrines, which could enhance STH exposure when sanitation access and hygienic practices in the household are poor. The absence of electricity is indirectly linked with a higher risk of STH infection, reflecting poor sanitation and limited access to clean water, as water pumps and treatment rely on electricity (Anegagrie et al. 2021; Masaku et al. 2023). Improved flooring, e.g., sealed or covered flooring, has been shown to reduce the odds of hookworm infection by half compared to earthen flooring (Halliday et al. 2019; Kurscheid et al. 2021). One study found limited WaSH-related variables as risk factors for STH infections despite the high prevalence of STHs in the community (Campbell et al. 2017). It was noted that there was poor access to clean and safe drinking water and hygienic/sanitary infrastructure community-wide, which could limit significant associations (Campbell et al. 2017).

Numerous studies show the influence of socioeconomic and demographic factors on STH infections and how these factors interplay with individual behaviours and environmental interactions related to WaSH variables. These factors influence an individual's predisposition to STH infections, including habits like handwashing practices (Anegagrie et al. 2021; Hailu & Ayele 2021; Masaku et al. 2023), wearing shoes (Campbell et al. 2017; Masaku et al. 2023), open defecation (Anegagrie et al. 2021; Campbell et al. 2017; Hailu & Ayele 2021; Masaku et al. 2023) and washing of fruits and vegetables before consumption (da Costa Dantas et al. 2023; Xu et al. 2023). Education and awareness of risk factors related to STH infections significantly influences the risk of STH infections (Hailu & Ayele 2021; Lee et al. 2023; Lim-Leroy & Chua 2020). Lacking formal education may not drive this association, but instead, the awareness and fostering of habitual hygienic behaviour might, as children attending classes related to WaSH were 66% less likely to harbour an STH infection (Puhalenthi et al. 2023). Additionally, the health education intervention "Magic Glasses", trialled in the Philippines, had a modest impact on knowledge and behaviour related to STHs, resulting in a decreased odds of any STH infection in schools with an STH prevalence of < 15% (Mationg et al. 2022). Public health interventions such as the mass administration of anti-helminthic drugs impact the distribution of STHs (Farrell et al. 2018). However, these strategies may not yield long-term success without changing behaviours, targeting environmental reservoirs and interrupting faecal contamination. For example, STH re-infections can occur within three months, with rates ranging from 57% to 94% 12 months post-treatment, without other integrative control strategies (Jia et al. 2012).

2.5 Diagnostics Used to Detect STH and *Strongyloides* spp. Infections Determines Prevalence - What Do You Pick?

Selecting a suitable diagnostic tool for STH and *Strongyloides* spp. detection is crucial for assessing prevalence and evaluating control interventions. Each diagnostic tool has unique sensitivities, specificities, advantages, and disadvantages and will depend on the parasite of interest (Assefa et al. 2014; Hailu et al. 2022; Niguse et al. 2020). Resource-limited regions face challenges in balancing the accuracy and feasibility of implementing diagnostic tools (Mbong Ngwese et al. 2020). Microscopy-based techniques are commonly used to directly examine faecal material for STH eggs, while culture methods for detecting *Strongyloides* larvae are more suitable. Alternatively, serological and molecular methods can offer improved reliability in some cases. Yet, these methods are not standardised and require specialised laboratory capacity, which limits their implementation in resource-constrained laboratory settings. This section provides a brief review of diagnostic tools and discusses limitations.

2.5.1 Microscopy-Based Techniques Using Faecal Specimens for the Detection of STHs

Microscopy-based techniques remains the mainstay of STH detection, as it enables the observation of helminth eggs or larvae in faecal samples (coproscopy). This method is widely used due to its simplicity, affordability, and minimal infrastructure requirements. However, this method has demonstrated lower sensitivities compared to other techniques, such as molecular-based methods and might underreport STH prevalence rates. Direct wet-mount faecal smears are the simplest to perform and require the least resources, yet it demonstrates the lowest sensitivity for detecting STHs, particularly for hookworm and *T. trichiura,* compared to other methods like Kato-Katz (KK), formol ethyl-acetate concentration (FEAC) and flotation techniques (Dana et al. 2020; Yimer et al. 2015; Zeleke et al. 2021). KK, recommended for STH field surveys, uses equipment that is reusable and cost-appropriate for resource-limited regions (WHO 2020a). The ‘gold standard’ approach to KK is examining multiple slides from two stool samples collected on different days (WHO 2019). However, this approach is laborious and lacks standardisation in many field studies, making the comparison of this technique to others challenging. Wet-mount microscopy technique is effective for detecting high-intensity STH infections and has utility in highly endemic areas, but more sensitive methods are needed for detecting hookworm and *T. trichiura* (Dana et al. 2020; Zeleke et al. 2021). Concentration/centrifugation and flotation techniques demonstrate higher sensitivities than single and replicate slides of KK (Glinz et al. 2010; Inpankaew et al. 2014; Niguse et al. 2020; Speich et al. 2014), with flotation techniques demonstrating the highest sensitivities even for detecting low-

intensity infections (Glinz et al. 2010; Niguse et al. 2020; Nikolay, Brooker & Pullan 2014; Zeleke et al. 2021).

Although KK has the advantage of quantifying infection intensities, it requires fresh specimens and is susceptible to rapid sample degradation, particularly for hookworm ova (Bosch et al. 2021; Dacombe et al. 2007). Floatation techniques like FLOTAC offer improved quantification (Cringoli et al. 2010), which correlates well with molecular methods (Clarke et al. 2018; Llewellyn et al. 2016), but this method is relatively more expensive, technically demanding and requires more time and sophisticated equipment, complicating implementation in resource-limited regions (Speich et al. 2010). To enhance the sensitivity of microscopy-based techniques, collecting and examining slides of multiple stool specimens over a few days can be performed, as egg output in faeces can fluctuate day-to-day, but this increases labour and cost (Deka et al. 2021; Liu et al. 2017). Homogenisation of faecal samples is crucial for minimising sample variation and enhances sensitivity for STH detection (Cringoli et al. 2010; Krauth et al. 2012). For hookworm species differentiation, culture systems and microscopy or molecular methods such as sequencing are used in combination, particularly in areas where multiple hookworm species are known to coexist. Speciation is crucial for implementing targeted public health interventions, as some hookworm species are zoonotic (Traub, 2013). However, most surveys use the KK technique only and aggregate the hookworm species due to the morphological similarities of their eggs.

2.5.2 Diagnostic Techniques for Direct Strongyloides spp. Larval Detection in Faecal Specimens

The combined use of the Baermann-Moraes (BM) technique and agar plate culturing (APC) systems, along with microscopy, is currently considered the clinical "gold standard" for detecting *Strongyloides* spp. larvae from faecal material (Buonfrate et al. 2015; Page & Speare 2016). However, challenges arise when relying on only faecal-based methods for *Strongyloides* detection, as parasite load and larvae dissemination into faeces can vary based on the disease stage of *Strongyloides* infection, potentially leading to false negative diagnoses (Buonfrate et al. 2015; Page & Speare 2016). Multiple stool specimens collected over several days are necessary to confirm the absence of *Strongyloides* infection (Knopp et al. 2008). Traditional coproscopy techniques have shown low sensitivities, which underestimates the true prevalence of strongyloidiasis (Chankongsin et al. 2020; Hailu et al. 2022). Compared to other coproscopy methods like FLOTAC, FEAC and KK, which are more suitable for detecting ova of other STHs, APC, such as Koga APC, demonstrates higher sensitivity for detecting *Strongyloides* (Glinz et al. 2010; Hailu et al. 2022; Inês et al. 2011; Steinmann et al. 2007). While the FLOTAC method has demonstrated improved detection of STH ova (Cringoli et al. 2010), its utility for detecting *S. stercoralis* has not been recommended (Bunofrate,

2015; Mutombo et al. 2019). Combining BM and Koga APC tools improves detection sensitivity, enhancing the reliability of prevalence reporting (Buonfrate et al. 2015; Chankongsin et al. 2020). However, conducting these tests in parallel requires time, trained personnel and prompt processing of fresh stool samples with viable larvae (Inês Ede et al. 2011). The use of agar plates can impose a risk of laboratory-acquired infections as larvae are kept until they transform. These culture-based methods have strengths and utility for detecting acute, heavy-intensity infections and hyper-infection disease states of strongyloidiasis (Requena-Méndez et al. 2013) but tend to be less effective for detecting larvae during chronic and light-intensity (Dong et al. 2016; Knopp et al 2014) infections compared to serological-based detection (Arifin et al. 2019; Page & Speare 2016). Nevertheless, coproculture techniques serve as the standard for evaluating serological and molecular-based techniques (Miswan, Singham & Othman 2022).

2.5.3 Serological-based Techniques Using Blood Specimens for the Detection of Strongyloides spp.

In prevalence surveys, immunological blood biomarkers like species-specific antigens or antibodies are frequently used to assess infection exposure. Detecting STH infections through antibody biomarkers is uncommon and not routinely implemented, partly due to cross-reactivity concerns with other co-endemic nematodes (Lamberton & Jourdan 2015). However, there is emerging evidence of their diagnostic potential (Santano et al. 2022). Antibodies to *Strongyloides* spp. derived antigens are useful to detect more chronic stages of the infection in immunocompetent hosts (Arifin et al. 2019; Page & Speare 2016) but cannot easily differentiate present, past or treated infections until 6 to 12 months post successful parasite clearance (Buonfrate et al. 2015; Kearns et al. 2017). Various applications of immunological tools are being investigated for detecting strongyloidiasis alongside parasitological methods. Detection of strongyloidiasis-specific immunoglobulin (Ig) G and other isotypes is typically the target marker, but the target antigen can vary among applications. A hallmark study by Bisoffi et al. (2014), compared five serological-based diagnostic tools, revealing that commercially available ELISAs had sensitivities ranging from 89.5-91.2%. The indirect immunofluorescence assay (IFAT) approach had the highest sensitivity of 93.9% but the lowest specificity of 92.2% due to cross-reactivity with other co-infecting helminths (Bisoffi et al. 2014). Cross-reactivity issues have been reported in other studies (Norsyahida et al. 2013), impacting diagnostic accuracy (Santano et al. 2022). Further in this study, the luciferase immunoprecipitation system using a recombinant protein antigen exhibited excellent specificity but lower sensitivity (Bisoffi et al. 2014). When compared to a composite reference standard comprising of Koga APC and real-time PCR, IFAT demonstrated a sensitivity of 95%, suggesting that serology may be valuable for population screening, while coprological and molecular-based approaches serve as confirmatory

tools for select cases (Buonfrate et al. 2017). The choice of target antigen also influences diagnostics sensitivity (Bisoffi et al. 2014). Commercially available tests using crude (Cr)-antigens show higher sensitivity but lower specificity (Anderson et al. 2014), whilst tests using recombinant NIE antigens offer improved specificity but limited sensitivity (Fradejas et al. 2018). However, the accuracy of clinically validated, commercially available tests can vary significantly between manufacturers, underscoring the need for additional validation to ensure their reliability, particularly in STH co-endemic regions (WHO 2020b). Combining Cr-antigen-based ELISAs and coprological or molecular methods has shown improved accuracy in diagnosing strongyloidiasis (Tamarozzi et al. 2023). The stage of strongyloidiasis impacts the sensitivity of serological methods. For acute infections, seroconversion to IgG can take time after infection, rendering serology unsuitable for ruling out infection. Instead, serial faecal-based tests should be used (Buonfrate et al. 2015). In immunocompromised individuals, the ability to generate IgG can be impacted, thereby increasing false negative diagnoses. In such cases, it would be recommended to use faecal-based culture methods of detection in conjunction (Buonfrate et al. 2015; Luvira et al. 2016). A prototype point-of-care test based on the NIE recombinant protein has shown promise in detecting more infections, particularly in immunocompromised individuals, by targeting IgG4 antibodies (Anuar et al. 2022; Noordin et al. 2022; Tamarozzi et al. 2023).

2.5.4 Molecular-Based Techniques for Detecting STHs and Strongyloides From Faecal Specimens

Nucleic-amplification techniques, including PCR, qPCR and loop-mediated isothermal amplification assay (LAMP), often significantly improve diagnostic sensitivity and specificity for detecting STHs (Miswan, Singham & Othman 2022). Multi-parallel qPCR assays have shown superior sensitivity (98%) compared to traditional techniques like single slide KK (70%) and can detect a higher number of STH cases, particularly for hookworm and *Trichuris* (Benjamin-Chung et al. 2020; Easton et al. 2016). Multiplex qPCR assays can detect nearly twice as many helminth infections compared to the FEAC technique (Othman et al. 2020). These findings align with similar studies that have reported increased sensitivity and detection capabilities compared to microscopy-based techniques (Dunn et al. 2020; Llewellyn et al. 2016).

TaqMan qPCR assays show good correlations with KK and flotation techniques for quantifying infection intensities in high to medium-level STH infections (Cools et al. 2019; Llewellyn et al. 2016; Zendejas-Heredia et al. 2021), highlighting that in highly endemic regions, microscopy-based methods are sufficient as there is high concordance with PCR techniques (Cools et al. 2019). However, for low-intensity infections and areas with low transmission, PCR has an advantage over microscopy as it can detect as few as five eggs per gram (EPG) of faeces for *N. americanus*, *Trichuris*

and *Ascaris* spp., while KK and the flotation technique can only detect down to 50 EPG (Zendejas-Heredia et al. 2021). Despite these advantages, PCR techniques still have limitations in detecting *Strongyloides* spp. infections (Buonfrate et al. 2017; Buonfrate et al. 2018; Dong et al. 2016), that is in part due to low-intensity infections (Knopp et al. 2014), irregular larval output in chronic cases and the small quantity of faecal sample analysed (Buonfrate et al. 2018). Multiplex and multi-parallel qPCR assays are advantageous in detecting multiple helminth species from a single faecal specimen, with automated result interpretation and high throughput capacity (Mbong Ngwese et al. 2020). Nevertheless, their implementation in resource-limited regions is challenging due to the cost, technical requirements, and the need for trained personnel (Easton et al. 2016). Lack of standardisation among these molecular methods, including sample preservation (Ayana et al. 2019; Papaiakovou et al. 2018), storage conditions (Papaiakovou et al. 2018), nucleic acid extraction (Ayana et al. 2019), target sequence regions (Miswan, Singham & Othman 2022; Sharifdini et al. 2015) and the PCR type (Sharifdini et al. 2015) are limitations factors that can significantly impact the accuracy of these diagnostic tools for the detection of STHs and *Strongyloides* spp., ultimately influencing prevalence reporting (Miswan, Singham & Othman 2022).

Alternatively, LAMP assays are a rapid, one-step DNA amplification method that can be performed with minimal equipment in general laboratories (Miswan, Singham & Othman 2022). LAMP assays have demonstrated promising sensitivity and specificity for detecting *Trichuris*, *N. americanus* and *S. stercoralis* (Crego-Vicente et al. 2023; Mugambi et al. 2015; Ngari et al. 2020), with results readable by the naked eye through a colourimetric change that indicates targeted DNA amplification (Rashwan et al. 2017). However, this technique requires DNA extraction, which can be cost-ineffective for some laboratories. Further validation and optimisation efforts are necessary to explore the full utility of LAMP assays in detecting STHs.

2.6 What is Known About the Occurrence of STHs in PNG?

In this section, we will discuss historically published and unpublished literature on surveys and reported patterns of STHs infections in PNG and explore whether there is potential for ongoing transmission of STHs based on our current understanding of the country's environmental and socio-demographic conditions. To accomplish this, we will utilise historical surveys to depict epidemiological trends observed and assess their concurrence with present knowledge and literature.

2.6.1 Geography and Socio-economic Culture of PNG

PNG is an island country in the southwestern Pacific Ocean of the Oceanic region (latitude and longitude of 06° 00 S, 147° 00 E), 160 kilometres north of Australia. It is a country with many islands and occupies the eastern half of the island of New Guinea. The western half is Irian Jaya, a Province of Indonesia. Its location is south of the equator, where the country experiences a humid tropical climate and often experiences high seasonal rainfall. PNG is divided into four regions, 22 Provinces and 89 districts (WHO 2016). The culture in PNG is heterogeneous and linguistically diverse, varying significantly between regions. According to the 2020 Human Development Report and the World Bank, PNG is classified as having a "Medium (level of) human development" and is considered a "lower middle-income country" (UNDP 2020). Over 85% of the population resides in rural or isolated communities (National Statistical Office of Papua New Guinea 2023). Despite an improvement in socioeconomic development indices, it is not equal across regions of PNG (Figure 2.5) (Kitur et al. 2019). The geography and landscape of PNG are heterogeneous, making it challenging to navigate and transport resources and people to other communities. The island is characterised by high island mountain ranges up to an altitude of 4000 meters above sea level, deep valleys, vast and extensive rivers, open plains, thick tropical forests and coast swamps (Attenborough & Alpers 1992).

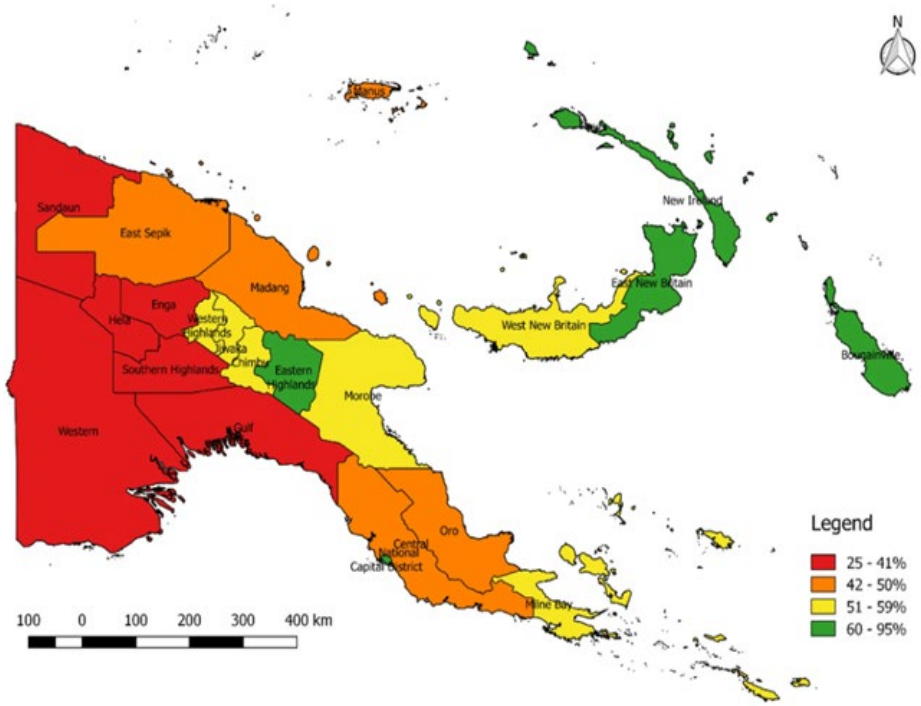

Figure 2.5: A map describing the socio-economic development (%) by provincial states in PNG. Original figure credited to Kitur et al. 2019 (Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0/). The figure legend depicts the range of socio-economic development by Province. The red colour displays Provinces with low levels of development, orange and yellow show moderate and green shows high levels of development.

In particular, the Western Province of PNG is the largest Province and is the closest to Australia's Torres Strait Islands and Indonesia (Figure 2.6). The Western Province has the lowest population density, with vast, unpopulated, isolated areas with a predominantly jungles landscape, separated by rivers, lagoons and estuarine coastal areas. Settlements and villages in this Province cluster around waterways, with the rural population dispersed throughout four districts North, Middle, Delta and South Fly (Fly River Provincial Government Western Province & PNGAus partnership 2022). Balimo is the region's capital district within the Delta Fly district, where the studies included in the thesis are focused. The Balimo town centre is surrounded by a lagoon that the community utilises for the collection of bathing and drinking water, fishing and transportation of resources and individuals between the village and other neighbouring villages (Diefenbach-Elstob et al. 2014).

Figure 2.6: Map of the Western Province showing the north, middle, delta and fly districts. Image credit Fly River Provincial Government Western Province & PNGAus partnership 2022. Distributed under the Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0/).

2.6.2 Challenges Related to Inaccessible Healthcare Infrastructure and Facilities

Given PNG's vast and challenging landscape, inequitable access to health services and the WHO-defined essential services remains an ongoing adversity. However, the country is making continuous efforts towards improving access to healthcare services and quality facilities through coordination and cooperation of community health workers, government health ministries (Government of Papua New Guinea 2021), country partnerships (Fly River Provincial Government Western Province & PNGAus partnership 2022) and technical support from intergovernmental organisations, like WHO (WHO 2016). However, significant gaps still exist, with inequitable access to health care significantly impacting rural and isolated regions that struggle with disproportionately high morbidity and mortalities related to maternal and child health and communicable diseases, with an increasing rise of non-communicable diseases, which indicates that the country is transitioning demographically as well as epidemiologically (Grundy et al. 2019).

2.6.3 Current Accessibility to Improved Water and Sanitation

Access to safe and clean drinkable water, sanitation, and hygiene in PNG exhibits significant disparities between rural and urban areas (WHO 2021). There is a lack of adequate infrastructure and services in rural communities, resulting in limited accessibility. It was reported that PNG is among the ten countries with the lowest access to water, with only 37% of people having access to clean water close to home (Wheeler 2018). In addition, open defecation is notably high in PNG, with approximately 1.4 million people, or at least 16% of the population, practising this behaviour (WHO 2021). However, it is worth noting that some efforts are being made to improve water, sanitation, and hygiene conditions, particularly in certain Provinces and urban regions, as seen in Figure 2.7 (World Bank Group 2017; Government of Papua New Guinea 2021). There is also no evidence to support whether the use of ‘night soil’ (human faeces) as agricultural fertilizer is practised in PNG.

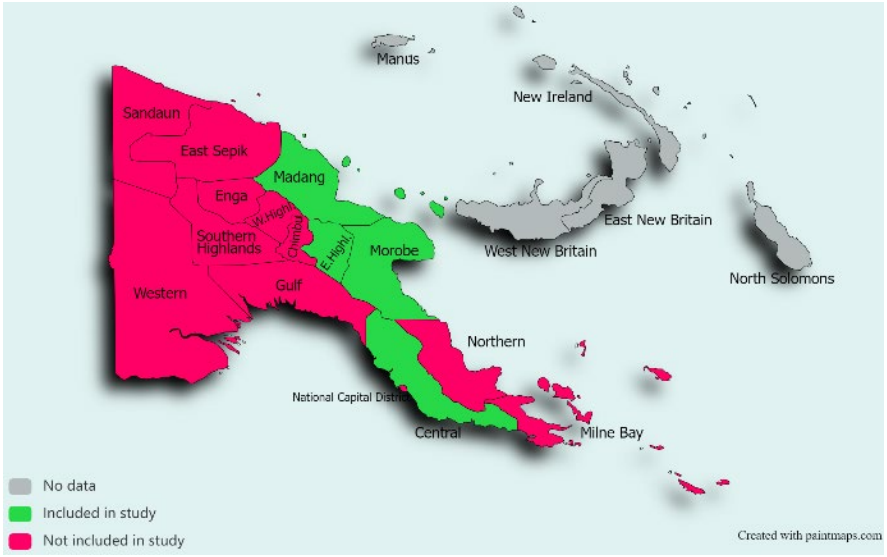

Figure 2.7: A map of PNG showing the improvements to sanitation and hygiene. Information sourced from (World Bank Group 2017).

2.6.4 Historical Evidence of STH Surveys in PNG

The evaluation of STH distribution in PNG has been severely overlooked, potentially due to several factors, including the country's heterogeneous landscape, scattered communities and other endemic and emerging infectious diseases that divert attention and resources. Consequently, the design of effective STH programs and the availability of appropriate diagnostic tools are limited within PNG.

Epidemiological surveys investigating the distribution of STHs in PNG date back to the early 1900s. The first extensive nationwide survey for hookworm was conducted in the 1920s, which revealed a high infection rate of 68% among the sampled population in both the northern (New Guinea) and southern (Papua) parts of PNG. Agriculture labourers had higher infection rates than village residents, while the central Province had lower infection rates (Sweet 1924). Subsequent surveys were conducted during the Second World War until the 1980s, focusing on the impact of parasitic infections on armed forces returning to their homeland (Bradbury & Traub 2016).

A summary of STH surveys conducted from 1979 to the present, focusing on the prevalence of STHs in PNG, is presented in Table 2.1. These surveys relied on traditional coprological-microscopy-based diagnostics to identify and assess the prevalence of STH infection. All STHs of public health concern, including *S. stercoralis,* have been described in PNG (Table 2.1). Also, species such as *S. fuelleborni kellyi* and *A. ceylanicum* have been identified. *S. f. kellyi*, for instance, has been exclusively found in certain parts of PNG. Its discovery was first reported in 1973, but since then, the distribution, clinical consequences and transmission dynamics of this species remains unexplored (Bradbury 2021; Kelly,

Little & Voge 1976). Previous surveys indicate that infection with *S. f. kellyi* is primarily identified in children and rarely in adults (Ashford, Barnish & Viney 1992; Barnish & Ashford 1989), with potential complications ranging from asymptomatic cases to potentially fatal swollen belly syndrome in infants (Ashford, Barnish & Viney 1992).

In addition to these conventional STHs, a zoonotic hookworm *A. ceylanicum* case has been identified in PNG. Although more cases are reported in Southeast Asian regions, cases have also been detected in the Pacific Islands (Colella, Bradbury & Traub 2021; Le et al. 2022). One case was molecularly confirmed in an immigrant worker returning to Japan after working in the agriculture sector on Manus Island for two years (Katanami et al. 2017; Yoshikawa et al. 2018). However, to our knowledge, there are no studies reporting the local occurrence of this worm in PNG, representing another knowledge gap. Hookworm, in many prevalence surveys described here, do not delineate between species. *N. americanus* is described as the dominant species in PNG (Ashford, Hall & Babona 1981), and cases of *A. duodenale* occur but are less frequently identified (Shield & Kow 2013).

A survey conducted in the 1980s (Ashford, Hall & Babona 1981) revealed the uneven distribution of STH infections from 32 isolated villages across six regions in the Western parts of PNG. Hookworm was found in almost every locality, while *Ascaris* and *Trichuris* spp. distribution was common in some areas and absent in others (Ashford, Hall & Babona 1981). Descriptions related to the locations in the latter study suggested that hookworm was more abundant in forested areas, while *Ascaris* and *Trichuris* were common on the coast. These findings were consistent with Shield and Kow's study in 1982 (Shield & Kow 2013), which reported higher STH prevalence in rural regions of the Morobe Province compared to urban areas. Specifically, the prevalence was 67% in rural areas and 56% in urban areas, with *Strongyloides* only identified in urban settings. Within the study, rural coastal areas had lower infection rates compared to rural "uplands" in the forested mountains. *Ascaris* and *Trichuris* were most frequently identified in urban settings and rural coastal regions but were absent in “upland” areas (Shield & Kow 2013). Another study in 1989 (Barnish & Ashford 1989), found hookworm in 44 out of 45 study locations and *Strongyloides* in only 22 locations. However, the authors did not provide information on the location or environmental distribution of these parasites, leaving the abundance of *Strongyloides* in certain areas and its absence in others unexplained (Barnish & Ashford 1989).

Table 2.1: Previous prevalence surveys of STHs in Papua New Guinea from 1979 to 2013. Grouped by Province and then date of STH survey.

Province	District/ Localities	Year of survey	Year of Publication	Number of samples	Cohort characteristics	Diagnostic for STH detection	Total helminth	Hookworm	*Ascaris*	*Trichuris*	*Strongyloides*	Reference
Bougainville	Sipai	2006	Unpublished	435	n.d	Microscopy, standard wet preparation	n.d	40%	2%	0%	n.d	Melrose et al. 2010
	Rorovana	2006	Unpublished		n.d	Microscopy, standard wet preparation	n.d	32%	0%	0%	n.d	
Eastern Highlands	Goroka	1977-1978	1980	312	Adults recruited from Goroka hospital	Microscopy using McMaster egg counting chamber technique	89%	83%	11%	14%	42%	Shield, Scrimgeour & Vaterlaws 1980
		1978	1981	345	Adults recruited from a corrective institution	Microscopy, using McMaster egg counting chamber and Harada Mori filter paper culture technique	90%	88%	88%	16%	n.d	Shield et al. 1981
		2008-2009	2013	201	Recruited pregnant women over the age of 25 years	Microscopy, standard wet preparation	31%	18%	14%	2%	2%	Phuanukoonnon et al. 2013
East New Britain	Kokopo	2006	Unpublished	186	n.d	Microscopy, standard wet preparation	n.d	40%	43%	21%	n.d	Melrose et al. 2010
	Pomio	2006	Unpublished	129	n.d		n.d	10%	1%	0%	n.d	
Gulf	Kanabae	1977	2005	149	infants < 1 week and children up to 15 years	Microscopy	63%	45%	19%	4%	1%	Ashford et al. 1979
	Wabo	1979	1981	69	Infants to adults	Microscopy and Harada Mori filter paper	n.d	78%	25%	3%	42%	Ashford & Babona 1980
	Kapuna			37			n.d	27%	51%	35%	3%	
	Mapaio			16			n.d	44%	19%	19%	0%	
	All			**122**			**n.d**	**58%**	**15%**	**15%**	**25%**	
	Wabo/Delta/Kamea	1981	1981	579	Infants over 1 year to adults up to 45 years	Microscopy using modified volumetric dilution method	n.d	34%	23%	4%	13%	Ashford, Hall & Babona 1981

	Kanabea	1996-1997	2004	179	Children < 5 years	Microscopy using Ashford's modified volumetric dilution method	n.d	9.50%	12%	0%	14.50%	King & Mascie-Taylor 2004
	Opau	2009	Unpublished	376	n.d	Microscopy, standard wet preparation	n.d	63%	7%	1%	n.d	Melrose et al. 2010
Madang	Kar Kar Island, Kebasob	1988	1990	202	All except 3 years old	Microscopy using modified formol-ether sedimentation technique	n.d	81%	10%	1%	n.d	Pritchard et al. 1990
		1988	1993	136	Children over the age of 3 to adults	Microscopy using a modified formal-ether concentration technique	n.d	96%	n.d	n.d	n.d	Quinnell et al. 1993
		1990		123			n.d	79%	n.d	n.d	n.d	
	Yupno	Abstract available only	1994	588	Children and adults	n.d	n.d	59%	n.d	n.d	n.d	Allemann, Bauerfeind & Gyr 1994
	Madang General Provincial Hospital			45	n.d		n.d	82%	n.d	n.d	n.d	
	Kar Kar Island, Kebasob	1996	2001	149	Children over the age of 11 years and adults	Microscopy, using a modified formal-ether concentration technique	n.d	67%	n.d	n.d	n.d	Quinnell et al. 2001
	Bauri, Gumaru, Haven, Mawan, Wasab	1998	2008	945	Children over the age of 4 years and adults	Microscopy using McMaster egg counting chamber technique	n.d	81%	n.d	n.d	n.d	Breitling et al. 2008
		2001					n.d	90%	n.d	n.d	n.d	
Morobe	Lae	1981	1986	272	Children recruited from Lae's Angau Hospital	Microscopy, using McMaster egg counting chamber and Harada Mori filter paper culture technique	n.d	48%	6%	18%	20%	Shield 1986
	Lae	1982	1986	281	Children aged 1-5 years recruited from Lae's Angau Hospital	Microscopy using McMaster's egg counting chamber and Harada and Mori	70%	47%	4%	15%	22%	Shield et al. 1986

						culture plate technique						
	Wasu, rural coastal	1980-1982	2013	178	infants younger than 1 year up to 7 years	Microscopy using McMaster egg counting chamber technique and Harada and Mori plate culture technique	44%	15%	23%	29%	n.d	Shield, & Kow 2013
	Aseki, rural uplands			155	infants younger than 1 year up to 8 years		79%	68%	23%	n.d	29%	
	Aseki, rural uplands, langemar			140	infants younger than 1 year up to 8 years		69%	56%	n.d	n.d	27%	
	Aseki, rural uplands			295	infants younger than 1 year up to 8 years		74%	62%	12%	n.d	28%	
	Lae, Buimo road urban			91	infants younger than 1 year up to 7 years		67%	61%	10%	12%	n.d	
	Lae, Taraka urban			203	infants younger than 1 year up to 10 years		51%	44%	5%	26%	3%	
	Lae, urban			294	infants younger than 1 year up to 10 years		56%	49%	6%	22%	2%	
	Total			**767**	**infants younger than 1 year up to 10 years**		**60%**	**48%**	**12%**	**15%**	**12%**	
New Ireland	Lovangai	2006	Unpublished	386	n.d	Microscopy, standard wet preparation	n.d	53%	0%	2%	n.d	Melrose et al. 2010
	Tanga				n.d		n.d	41%	0%	0%	n.d	
Oro	Saiho	2007	Unpublished	289	n.d	Microscopy, standard wet preparation	n.d	40%	0%	0%	n.d	Melrose et al. 2010
Simbu	Daribi and Karimui plateaux	1981	1987	538	Children and adults	Microscopy, using Ashford's modified volumetric dilution method and Harada and Mori culture plate	n.d	66%	11%	9%	19%	Shield et al. 1987

						technique and serology						
	Yuro, Karimui	Abstract available only	1989	75	Children under the age of 5 years	n.d	n.d	63%	n.d	n.d	56%	Barnish & Harari 1989
West Sepik	n.d	1981	1981	179	Infants over 1 year to adults up to 45 years	Microscopy using modified volumetric dilution method	n.d	28%	0%	1%	0%	Ashford, Hall & Babona 1981
West New Britain	Gloucester	2006	Unpublished	198	n.d	Microscopy, standard wet preparation	n.d	32%	0%	0%	n.d	Melrose et al. 2010
	Vituhu				n.d		n.d	29%	0%	0%	n.d	
Western	Middle Fly (Membo and Erekta)	1947	1979	223	Infants over 1 year to adults older than 15 years	Microscopy	n.d	75%	0%	0%	35%	Knight et al. 1979
	North Fly/Star mountains	1981	1981	265	Infants over 1 year to adults up to 45 years	Microscopy using modified volumetric dilution method	n.d	32%	2%	0%	3%	Ashford, Hall & Babona 1981
	Waiwoi falls	2001	Unpublished	283	All age groups	Microscopy, samples concentrated using formalin ethyl-acetate technique, using Ashford's modified volumetric dilution technique	n.d	49%	38%	4%	2%	Melrose, 2002

n.d. = not described

Although these studies related environmental conditions to STH prevalence in the analysis, they were anecdotal and not thoroughly explored. It is possible that there were quantifiable relationships between the presence of STHs and environmental factors, but such investigations were lacking at the time of these surveys. Some authors of these studies even speculated that the predisposition to hookworm infection might have been linked to a lack of shoe-wearing rather than immunological susceptibility, but these variables were not further investigated (Quinnell et al. 2001). However, as discussed in previous sections, the current literature exhibits parallels to the observations mentioned in the latter study (Quinnell et al. 2001).

In Kanabea, Gulf Province, a small survey focusing on children under five years of age revealed associations between STH infections and various factors (King & Mascie-Taylor 2004). Children residing farther away from the main village, living in smaller households with mothers who lacked formal education, were found to have a higher risk of *Strongyloides* infection (King & Mascie-Taylor 2004). Additionally, houses with tin roofs were found to be protective against *Ascaris* infections, while households with more than six members had a higher risk of hookworm infection (King & Mascie-Taylor 2004). This finding is consistent with the understanding that socio-economic factors like access to water, household size and education play a role in the underpinnings of STHs infections. Similarly, the protective effect of houses with tin roofs against *Ascaris* infections and the higher risk of hookworm infection in larger households resonates with studies emphasising that housing conditions can indicate the level of socioeconomic status that can influence STH prevalence. A survey in the Simbu Province, located in the highlands, found high hookworm infection rates at 66% from over 500 people surveyed. Lower rates were observed for *Strongyloides* spp. (19%) *Ascaris* (11%) and *Trichuris* (9%) in the same region (Shield et al. 1987). This study also found that hookworm prevalence increased with age (Shield et al. 1987), which aligns with the persistent challenges of hookworm and its demographic patterns, as seen in another more recent study (Campbell et al. 2017).

Factors such as household size, education level, access to clean water and sanitation, socio-economic status, and proximity to main villages are known to influence the transmission dynamics of STHs. However, many earlier surveys in PNG did not thoroughly investigate these aspects. Insights into the risk factors within communities were missed by not considering these factors. Further research is needed to bridge this knowledge gap and provide a greater understanding of the social, economic, and environmental determinants of STH infections in PNG.

Studies conducted in specific Provinces of PNG shed light on the prevalence of STH infections over different time points. For example, in Goroka Hospital, the prevalence rates of STHs were high, reaching nearly 90% (Shield, Scrimgeour & Vaterlaws 1980). Thirty years later, a study focusing on pregnant women in the same hospital reported a lower prevalence of STHs of 31% (Phuanukoonnon et al. 2013). The decline in infection rates suggests potential improvements in factors such as access to clean and sanitary environments and better healthcare services, which may have contributed to reducing STH infections.

The Western Province of PNG lacks recent STH studies, creating a significant knowledge gap in its epidemiology. The last STH survey was conducted in 2001 at Waiwoi Falls, Middle Fly district, Western Province, reported that nearly 50% of the surveyed population was infected with hookworm, followed by a third infected with *A. lumbricoides* and less than 5% infected with *T. trichiura* and *Strongyloides* spp. (Melrose 2002). However, the nearby Balimo region lacks STH epidemiology data.

The distribution of anti-helminthic drugs in 2001 primarily targeting *Wuchereria bancrofti*, the worm responsible for lymphatic filariasis in the Asia-Pacific, may have indirectly reduced the burden of STHs. However, the impact on STH distribution in PNG was not demonstrated, as no baseline data were available (WHO 2009). Efforts to control STHs in PNG have been implemented using anti-helminthic drugs since 2010. Nevertheless, recent reports indicate insufficient treatment coverage to effectively reduce the burden of STH infections (Montresor et al. 2020). In 2017, the national coverage of anti-helminthic drugs for at-risk population groups such as school and preschool-aged children was reported as 0% and 8%, respectively (WHO 2020d). Despite some treatment distribution to these groups, reinfection is likely to occur due to the persistence of behaviours and environmental reservoirs of infection that have not been adequately addressed (Jia et al. 2012).

2.7 Integrating Historical Distribution and Current Understanding: The Potential for Sustained Transmission of STHs in PNG

Historical surveys conducted in PNG demonstrate high prevalence rates of STHs, but the current prevalence in specific regions remains unknown due to the lack of recent epidemiological data. Despite improvements in sanitation, hygiene practices, and access to clean drinking water, challenges persist in providing these necessities to isolated and rural areas of the country.

Additionally, while PNG has made progress in healthcare accessibility, resource allocation to vulnerable Provinces remains challenging, particularly in isolated and rural regions. Therefore, an up-to-date assessment of STH prevalence and associated factors in PNG is needed, particularly in the

Western Province. These assessments will inform our understanding of the burden of STH infections and help guide the selection of suitable diagnostic tools for accurate prevalence estimation. Addressing these issues is crucial for developing effective strategies to understand STH distribution and improve the population's health outcomes in PNG.

3 General Materials and Methods

3.1 Study Design and Setting

This cross-sectional study was conducted in collaboration with the Balimo District Hospital (BDH) (Longitude: 142.95981 and Latitude -8.038578) during mid-late January 2020. Participants from this survey were recruited from Balimo Urban, which is situated in the Delta (Middle) Fly District of the Western Province in PNG (Figure 3.1). Balimo Urban is located within the Balimo local-level government area, surrounded by floodplains and has an approximate population of 10,300 individuals (National Statistical Office of Papua New Guinea 2023). This study was in conjunction with broader research within this community that determined the epidemiology of Tuberculosis (TB) and potential drivers of TB susceptibility.

3.2 Participant Recruitment

All community members over 16 years of age were eligible for participation, including patients from the BDH. Participants were recruited through word-of-mouth (convenience sampling). After obtaining written and verbal informed consent, questionnaire-based interviews were conducted face-to-face by myself and another research team member, with a local translator as appropriate. The structured questionnaire was initially purposed for demographic characteristics associated with TB. However, most variables have been used for this study. The questionnaire can be referred to in Appendix A.

3.3 Specimen Collection and Processing

Consenting participants received a sterile plastic container for faecal samples, each with a personal identification number. Approximately one gram (g) of each faecal sample was processed into 3 mL of sodium-acetate and 10% formalin (SAF), and 0.5g of faeces into 1 mL of guanidinium thiocyanate (GTC) within six hours of specimen collection. Details of these buffers are provided in Appendix B. The faeces were mixed well to emulsify the sample within the respective buffers and stored at room temperature. Samples were then transported to James Cook University Townsville for long-term storage of samples at 4℃ for the SAF-preserved faecal samples and -80℃ for the faecal samples in GTC.

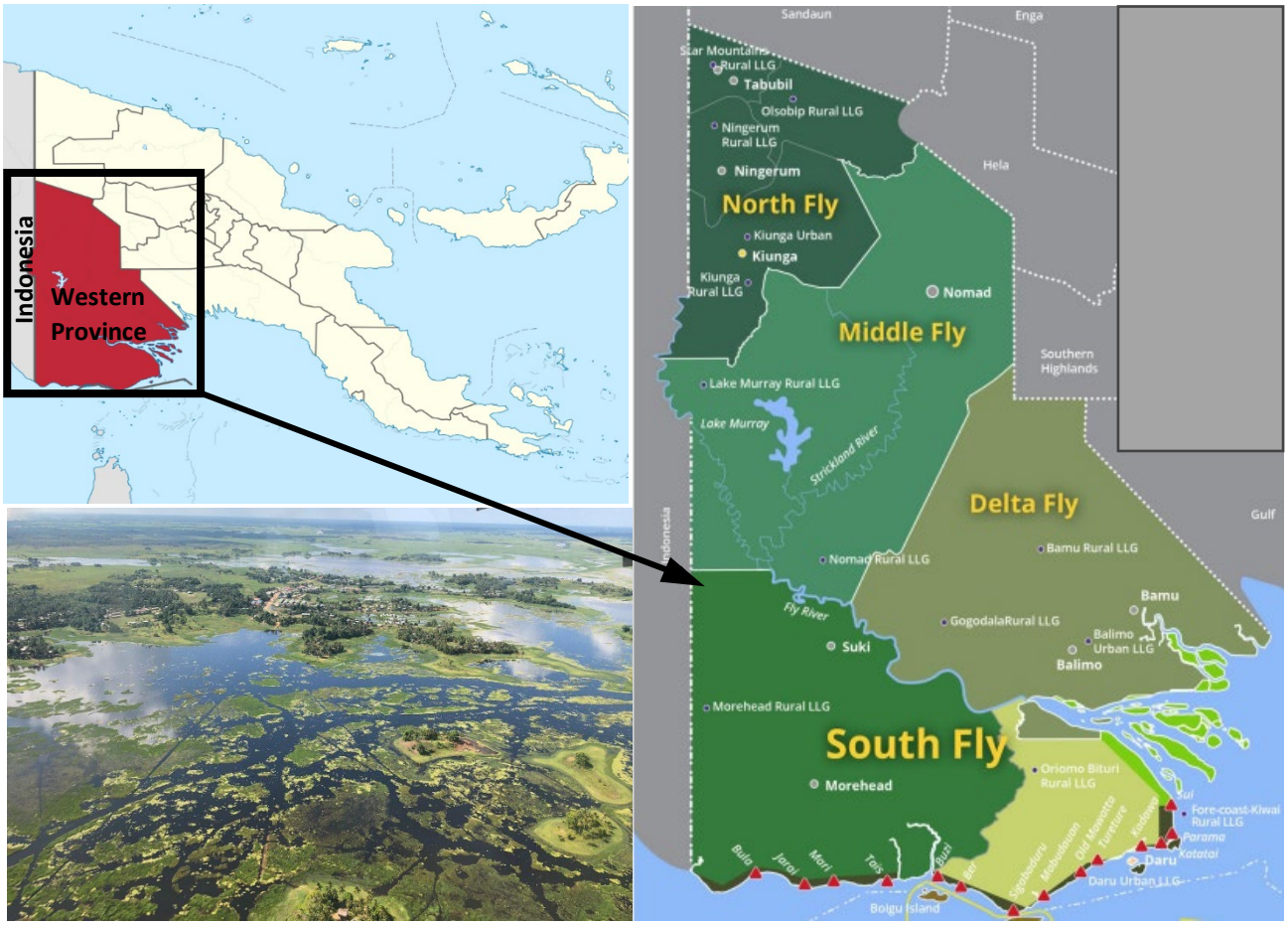

Figure 3.1 Map of Western Province of Papua New Guinea (PNG) showing location of study area, Balimo (red circle). Bottom left most image captures Balimo situated south-east of the Delta Fly District. Image showing the Western Province was adapted from TUBS Wikiwand.com user under the Creative Commons Attribution 3.0. The image displaying the Provincial districts was sourced Fly River Provincial Government Western Province and PNGAus partnership (2022) under the Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0/. Photo credited of Balimo village: A/Prof Jeffrey Warner, used with permission.

3.4 Data Management

Initially, data was recorded on paper copies of the questionnaire. All questionnaire answers were then transcribed to an electronic database, Microsoft Excel (Microsoft® Excel® for Microsoft, version 2304) and checked by me and another research team member. Completed paper copies of the questionnaire were stored securely at BDH and then delivered to JCU Townsville, where documents are archived.

3.5 Ethics

Approval for this study was obtained from the Middle (now Delta) Fly District Health Service and the Evangelical Church of PNG Health Services. Human research ethics approval was obtained from the

PNG Medical Research Advisory Committee (MRAC 17.02 and 19.21), and the James Cook University Human Research Ethics Committee (HREC approval H6432 and H8015). Written or verbal informed consent was obtained from all study participants. Parental consent was obtained for participants aged 16 to 17 years old.

3.6 Transition to the Next Chapters

Chapter 3 presents the methodology for the collection of data for the study. The following chapters will describe the study's findings using this methodology, plus other methods specific to the corresponding Chapters 4, 5 and 6. Chapter 7 will present the overall discussion, conclusion and recommendations for the future.

4 Prevalence of STH in a Rural PNG Community

4.1 Introduction

STHs include *Ascaris lumbricoides, Trichuris trichiura*, and hookworms (*N. americanus and A. duodenale*) that infect humans. Together, these worms are the most common group of parasites contributing to NTDs, as defined by the WHO (WHO 2020c). Recently, strongyloidiasis was added to the list of NTDs (WHO 2020c) but remains excluded from the WHO's STHs list due to diagnostic and disease management differences. Therefore, in this chapter, *Strongyloides* spp. will be considered separately.

Globally, almost 1 billion people are infected with at least one type of STH infection, and it is not uncommon to find that these individuals are infected with two or more types of STH (World Health Organization 2022). STHs are distributed worldwide, causing 1.97 million DALYs (Institute for Health Metrics and Evaluation 2019). STH infections are not often fatal, but long-term infections can be attributed to significant morbidities, making them a cause of public health concern (World Health Organization 2022). Some studies attribute STH as an important contributor to micronutrient deficiencies (i.e., anaemia) and/or malnutrition, which can lead to stunting, wasting and/or cognitive impairment, particularly in children (Blouin et al. 2018; Deka, Kalita & Hazarika 2022; Mehata et al. 2022; Yentur et al. 2015) and complications related to maternal health, impacting pregnancy outcomes (Aderoba et al. 2015; Bolka & Gebremedhin 2019). Additionally, STHs are implicated in influencing disease outcomes during co-infections with malaria, human immunodeficiency virus (HIV) and TB (Mabbott 2018). These implications may impact the surveillance and management of the disease in regions of the world where the two infections intersect. It is for these reasons STHs are considered a significant public health concern, and it is critical to determine the prevalence and burden of these parasites to inform effective public health interventions.

Transmission of these worms is disproportionally high in low- to middle-income countries with warm, moist tropical and sub-tropical climates, with STH burdens being relatively higher in more marginalised communities (Gilmour, Alene & Clements 2021). Specifically, within indigenous communities of the Western Pacific region, a meta-analysis estimated that the prevalence of STHs was approximately 66% (Gilmour, Alene & Clements 2021). However, this analysis does not include any studies of STH surveys conducted in PNG.

PNG has one of the highest burdens of several critical NTDs in the Oceania region (Kline et al. 2013), and gastrointestinal diseases are caused by both helminths and protozoa. Historical prevalence surveys conducted in PNG have repeatedly described the occurrence of all STHs and other intestinal

helminths. This is supported by historical studies indicating that the STH prevalence rate was > 85% within some isolated, rural villages (Ashford, Hall & Babona 1981; Avery 1946; Bearup & Lawrence 1950; Shield, Scrimgeour & Vaterlaws 1980). However, there is no current data on the distribution and level of burden of STHs, particularly in rural communities in the Western Province of PNG. The previously published surveys have also relied on insensitive detection methods such as microscopy, which could have influenced the prevalence rates reported in these studies.

An absence of a true "gold standard" diagnostic method poses a significant challenge when assessing infection prevalence and level of burden (Nikolay, Brooker & Pullan 2014). Coproscopic-based techniques like wet mount faecal smears, KK, flotation-, and FEAC techniques are relatively simplistic, cost-effective tools used in resource-limited (laboratory personnel and financial) settings like rural regions of PNG. However, these methods can have relatively low sensitivity when compared to other available diagnostic tools, particularly for low-transmission regions and light-intensity infections (Nikolay, Brooker & Pullan 2014). Using tools with poor sensitivities can lead to an underestimation of infection prevalence and could have consequences when reporting the level of burden to determine the extent of control strategies.

Molecular diagnostics such as multiplex qPCRs can detect multiple helminth species simultaneously from a single faecal sample and have repeatedly demonstrated superior sensitivity over coproscopy (Benjamin-Chung et al. 2020; Clarke et al. 2018; Llewellyn et al. 2016; Stracke et al. 2019). In addition, qPCR can determine the intensity of the infection of each worm species. It is postulated that molecular tools will be needed to support the end goals of WHO's roadmap (2021-2030) for the control/elimination of STHs (Papaiakovou, Marina et al. 2021). However, qPCR may have no significant advantage in high transmission regions over conventional microscopy-based techniques (Cools et al. 2019). Further, the economic and operational constraints of qPCR in resource-limited laboratory settings significantly hinder its implementation in rural laboratories.

The overall Aim of the work described in this Chapter was to assess the prevalence of STHs in Balimo PNG, utilising microscopy and qPCR techniques. A further specific Aim was to evaluate the diagnostic utility of these tools for detecting STHs in this community.

4.2 Materials and Methods

Methodology describing the study population, setting, sample processing and ethics have been described in Chapter 3. In total, 122 faecal samples were collected and preserved in SAF for microscopy and separately stored in GTC buffer for TaqMan qPCR assays.

4.2.1 Stool Microscopy for the Detection of STHs

4.2.1.1 Formal-ethyl Acetate Concentration Technique on Faecal Samples

The faecal samples preserved in SAF were concentrated using the FEAC technique, as outlined by Garcia et al. (2018). Briefly, approximately 2-3mL of SAF preserved faecal material was strained through two layers of nylon gauze (provided by the stool examination kit, Kato-Katz equipment, Vestergaard Frandsen) into a 15 mL conical centrifuge tube. Approximately 10mL of 10% formol saline was added to samples and centrifuged for 10 minutes at 500 x g. The supernatant was then discarded. Sediment was re-suspended with 7mL of 10% formal saline then 5mL of ethyl-acetate was added. The sample was vigorously shaken for 30 seconds and centrifuged at 500 x g for 10 minutes. Finally, three of the four resulting layers were decanted, with the pellet re-suspended with a few drops of 10% formal saline and stored at 4℃ until examination.

4.2.1.2 Microscopy Examination of Faecal Samples

All 122 faecal samples were examined for helminths (i.e., ova or larvae). Microscopic examination was performed for both unconcentrated and concentrated faecal specimens to determine whether concentrating the faecal samples improved the detection of ova/larvae. Identification of helminth ova and/or larvae were classified based on the criteria outlined by the WHO bench aids (Bench aids for the diagnosis of intestinal parasites, second edition. Geneve: World Health Organization, 2019). Hookworm classifications include all *Necator* and *Ancylostoma* species, as ova are morphologically indistinguishable. The sizing of helminth ova was estimated using the built-in measurement tool on the Zeiss Apotome confocal microscope (Zeiss Microimaging GmbH, 2006-2010 Carl), using the AxioVision software (Ziess, AxioVs40V version 4.8.20). Microscope calibration was performed on all objective nose pieces using a stage micrometre.

4.2.1.3 Semi-Quantification of Helminth Load in Faecal Samples

Faecal samples were processed into SAF on-site at the BDH laboratory with limited access to a precision balance (weigh scale) to weigh out 1g of the faecal sample, thus it was approximated, resulting in an inaccurate dilution factor to perform direct quantification of health load. To alternatively assess the infection load, we performed systematic semi-qualificative scoring as outlined in Table 4.1, adapted from (Utzinger et al. 2010). Unconcentrated and concentrated faecal samples were assessed with this criterion at the 10× objective (100× magnification), and the entire 22x22mm coverslip was examined. Coverslips were examined by one observer blinded to previous results.

Table 4.1: Semi-quantitative scoring system for helminth ova/larvae observed in concentrated and unconcentrated faecal samples.

Classification of semi-quantitative score	Description informing classification
Negative (0)	no ova/larvae detected in preparation
Scant (1+)	1-3 ova/larvae per preparation
Infrequent (2+)	4-10 ova/larvae per preparation
Moderately frequent	11-20 ova/larvae per preparation
Frequent (4+)	21-40 ova/larvae per preparation
Very frequent (5+)	>40 ova/larvae per preparation

4.2.2 Faecal DNA Extraction and Assessment of Quality

Genomic DNA was extracted from 122 faecal samples stored in GTC using Zymo-Quick-DNA faecal/soil kit microbe miniprep kit (Zymo-research, #D610). DNA was isolated from the faecal sample as per manufacturer's instructions, except that the bead beating step was modified to one cycle, for 45 seconds on the FastPrep-24 (MP Biomedicals, USA) at full speed (6.5 m/s) (Azzopardi et al. 2021). The final eluted DNA sample (100uL) was stored at 4°C prior to analysis. Protein (A260/A280) and salt (A260/A230) contamination, as well as genomic DNA yield, was estimated using the Thermo Scientific NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific Inc, V1.6.198). See Appendix C for sample details.

4.2.3 Determining Amplificability of Extracted Faecal Genomic DNA

Extracted genomic DNA was pre-tested to assess whether extracted faecal samples were amplifiable. Standard PCR was performed for the 16s ribosomal RNA gene (16S-27f: 5'-AGAGTTTGATCMTGGCTCAG-3'; 1391r: 5'-GACGGGCGGTGTGTRCA-3') that achieves a 1.39kbp amplicon. This conserved bacterial gene was selected as the amplification control as all faecal samples contain bacteria. Reactions were performed with 10µL of GoTaq® colourless PCR master mix (Promega, USA #M7132) and 800nM of each primer, using 2µL of sample DNA to amount in a final volume of 20µL. The SimpliAmp Thermal Cycler (AppliedBiosystems) was used for gene amplification using the following conditions: 5 minutes at 95℃, followed by 30 cycles of 95℃ for 5 seconds, 59℃ for 15 seconds and 72℃ for 45 seconds and a final extension at 72℃ for 10 minutes. A positive control for this experiment was genomic DNA extracted from an Australian resident (this positive control was subsequently used as a negative, non-STH endemic control). Other negative controls included in each plate run were a negative water control performed parallel to DNA extraction and non-template control (nuclease-free water and master mix). Gel electrophoresis was performed on the resultant PCR products for each sample using a 1% agarose gel in Tris-acetate buffer for 60 minutes at 80V. Samples without bands on the gel after 60 minutes demonstrate poorly extracted DNA and were either excluded or re-extracted depending on specimen material availability. See Appendix D for details.

4.2.4 Target STH Primer and Probes for TaqMan qPCR assays and Positive Template Controls

Target sequences, primers and probes used for the TaqMan qPCR assays performed in this study are based on previous peer-reviewed publications (Table 4.2). Previously, these STH target primers and probe combinations were multiplexed (Azzopardi et al. 2021), however, for this study, all TaqMan qPCR reactions were performed as a single-plex. All primers and probes for the detection of *A. lumbricoides* were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), and all the other primers/probes were purchased from Integrated DNA Technologies (IDT, Coralville, Iowa, USA). Known positive samples for *N. americanus* and *A. ceylanicum* ova (kindly provided by Prof. Alex Loukas and Dr Luke Becker; James Cook University, Queensland, Australia) and *A. duodenale* positive faecal sample (kindly provided by Patsy A Zendejas Heredia; The University of Melbourne, Victoria, Australia), were used as positive controls in the establishment of PCR conditions. Known positive faecal samples collected in 2019 in a separate Balimo parasite study were used to optimise and validate *Ascaris* spp. and *Trichuris* spp. TaqMan assays. Assessment of positive template control DNA yield and purity are shown in Appendix E.

Table 4.2: Single-plex qPCR of STHs detailing target sequences, primers and probes concentrations.

Target	Primer/ probe	Sequence (5'→3')	Size (bp)	Gene target	Final conc (nM)	Reference
Ascaris lumbricoides	Forward	GTAATAGCAGTCGGCGGTTTCTT	88	ITS1	100	Wiria et al. (2010)
	Reverse	GCCCAACATGCCACCTATTC			100	
	Probe	FAM-TTGGCGGACAATTGCATGCGAT-BHQ1*			50	
Trichuris trichiura	Forward	TTGAAACGACTTGCTCATCAACTT	76	18S	100	Liu et al. (2013)
	Reverse	CTGATTCTCCGTTAACCGTTGTC			100	
	Probe	CY5-CGATGGTACGCTACGTGCTTACCATGG-IBRQ*			50	
Necator americanus	Forward	CTGTTTGTCGAACGGTACTTGC	101	ITS2	100	Verweij et al. (2007)
	Reverse	ATAACAGCGTGCACATGTTGC			100	
	Probe	CY5-CTG[+T]A[+C]TA[+C]G[+C]AT[+T]GTATAC-IBRQ*			50	
Ancylostoma spp.	Forward	CGGGAAGGTTGGGAGTATC	104	ITS1	100	Hii et al. (2018)
	Reverse	CGAACTTCGCACAGCAATC			100	
A. ceylanicum	Probe	HEX-CCGTTC[+C]TGGGTGGC-IBFQ*			50	
A. duodenale	Probe	FAM-TCGTTAC[+T][+G]GGTGACGG-IBFQ*			50	

Bp = Base pairs; Black hole quencher (BHQ), Iowa black RQ quencher (IBRQ), Iowan black FQ quencher (IBFQ). *Modifications made to the probe reporters and/or quenchers. Probe oligotides with [+] denote locked nucleic acids (LNAs).

4.2.5 Validation of STHs TaqMan qPCR assays

Each helminth TaqMan qPCR primer and probe combination was initially validated and optimised if required. A standard curve for each primer and probe combination was performed in triplicate to assess the efficiency, linearity and reproducibility of the TaqMan qPCR primers and probes. Refer to Appendix F to review all TaqMan qPCR assay validation results.

4.2.6 Molecular Detection of STHs Using Validated TaqMan qPCR Assays

All 122 extracted DNA samples, as described, were used in optimal TaqMan qPCR reactions performed using 10 µL of the GoTaq qPCR Probe Master Mix (Promega, USA, #A610A) with 2µL of

stool-derived DNA in a final reaction volume of 20µL. All PCR reactions for samples were conducted in a Hard-Shell® 96-Well PCR Plate (Bio-Rad Laboratories, Inc). Genomic DNA samples were performed singularly for each TaqMan assay. Cycling conditions for the STHs qPCR consisted of the following parameters: 1 cycle of 95°C for 5 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 60 seconds (Azzopardi et al. 2021). The Bio-rad CFX96 thermocycler was used for all qPCR reactions. Various negative controls were included in each optimisation reaction, as previously mentioned. The dilution series for the standard curve, using the appropriate positive template control, was performed in duplicate and was included during the running of each STH target TaqMan qPCR assay. The standard curve was used to set the cycle quantification (Cq) value cut-offs that would indicate sample positivity. Cq values were determined using the Bio-rad CFX manager data analysis software (version 3.1.1517.0823). The Cq value was determined when the fluorescence of the product could be detected above the background signal, which is established by the software as the threshold line. The maximum Cq value considered to indicate a positive sample, regardless of the standard curve, was set at ~36 cycles for all reactions. Signals beyond this value were reported as negative.

4.2.7 Data Analysis

Parasite data generated from faecal microscopy examinations and TaqMan qPCR reactions were transcribed on Microsoft Excel (Microsoft® Excel® for Microsoft, version 2304). Statistical analyses were performed using GraphPad Prism 9 (Prism for Windows, version 9.2.0.332). Frequencies and percentages were used to summarise all microscopy and qPCR results. These findings were used to assess the prevalence rate of STH infections and the diagnostic agreement between microscopy and PCR methods, reported with a 95% confidence interval (CI). Overall, STH prevalence was determined by any positive results that indicated STH infection, as detected by either microscopy and/or qPCR methods. Total observed agreement between microscopy and PCR, i.e., agreement (concordance) or disagreement (discordance) between positive and negative results, was determined and reported as a percentage. Cohen's Kappa agreement statistics were performed to determine whether the probability of agreement between the two tools was not due to chance and was reported with a 95% CI. Continuous Cq data were assessed for normality using the Shapiro-Wilks test. Depending on the outcome of the data normality assessment, the T-test or Mann-Whitney U test was used to assess the mean or median difference between Cq values obtained by hookworm qPCR assays for concordant (microscopy and qPCR positive) and discordant (microscopy negative and qPCR positive) microscopy observations. A *P-value* < 0.05 was deemed statistically different.

4.3 Results

Overall, 122 matched faecal samples preserved in SAF and GTC buffer were assessed for STHs using microscopy and qPCR, respectively. Microscopic evidence of the identification of STH ova is presented in Figure 4.1.

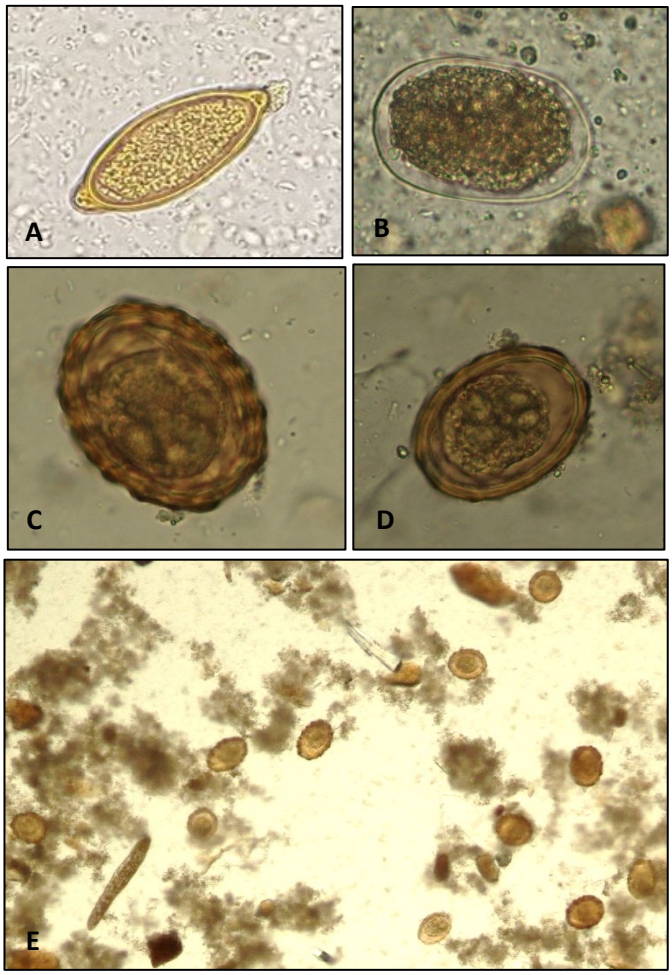

Figure 4.1: STH ova detected in faecal wet mount smears. A) *T. trichiura* (55µm by 23µm). B) Hookworm (60µm by 39µm). C) *A. lumbricoides* with mammillated (cuticle) layer (65µm by 51µm). D) *A. lumbricoides* no cuticle layer (decorticated) (63 by 49 µm). All captured at 400× magnification. E) High intensity *A. lumbricoides* burden, captured at 100× magnification.

4.3.1 Prevalence of STH Infections and Co-infections

Prevalence rates of STH infections and co-infections are presented in Table 4.3 and Figure 4.2, respectively. Stool samples classified as positive for a STH either by microscopy techniques and/or TaqMan qPCR assay was 64.8% (n = 79/122; CI 95%: 55.9-72.7%). The most frequently detected STH, through a combination of all techniques, was hookworm (undifferentiated) at 54.9% (n = 68/122; CI 95%: 46.0-63.5%), then *A. lumbricoides*, 40.2% (n = 49/122; CI 95%: 31.9-49.0%) and *T. trichiura*, 2.6% (n = 3/122; CI 95%: 0.7-7.0%). Using hookworm genus-specific primers and probes in the TaqMan qPCR assays, *N. americanus* was identified to be the dominant hookworm infection in this community, at 52.5% (n = 64/122; 95% CI: 43.7-61.1%), followed by *Ancylostoma* spp., 3.3% (n = 4/122; 95% CI: 1.3-81%). Speciation of *A. ceylanicum* and *A. duodenale* was not achieved, as the previously published *A. duodenale* primer and probe TaqMan qPCR assay appeared to amplify the *A. ceylanicum* positive template control. Therefore, it was concluded that this assay could not reliably be used to speciate samples used in this study. The full rationale for this is provided in Appendix F.

Samples positive for only one type of STH was 32.8%% (n = 40/122; 95% CI: 25.1-41.5%) (mono-infection). When considering co-helminth infections, 27.8% (n = 34/122; 95% CI: 20.7-36.4%) were infected with two helminths (dual-infections), and 4.1% (n = 5/122; 95% CI: 1.8-9.2%) were infected with all three helminths (*A. lumbricoides*, *T. trichiura* and undifferentiated hookworm) (tri-infections). Frequencies described here include co-hookworm infection with *N. americanus* and *Ancylostoma* spp., as determined by qPCR. Participants with dual infections, *A. lumbricoides* and *N. americanus,* were frequently detected together, as demonstrated in Figure 4.2B. *A. lumbricoides* and *T. trichiura* co-infections were less common. Interestingly, when considering differentiated hookworm infections, overlap of infection with *A. lumbricoides*, *N. americanus* and *Ancylostoma* spp. were frequently detected.

Table 4.3 Prevalence rate of STHs in the Balimo region of PNG from 122 participants.

	Positive n (%)	**CI 95%**	**Negative n (%)**	**CI 95%**
Any STH helminth	79 (64.8)	55.9-72.7	43 (35.2)	27.3-44.1
Ascaris lumbricoides	49 (40.2)	31.9-49.0	73 (59.8)	51.0-68.1
Hookworm (unspeciated)	67 (54.9)	46.0-63.5	55 (45.0)	36.5-53.9
N. americanus	64 (52.5)	43.7-61.1	58 (47.5)	38.9-56.3
***Ancylostoma* spp.**	4 (3.3)	1.3-8.1	118 (96.7)	91.9-98.7
Trichuris trichiura	3 (2.5)	0.7-7.0	119 (97.5)	93.0-99.3

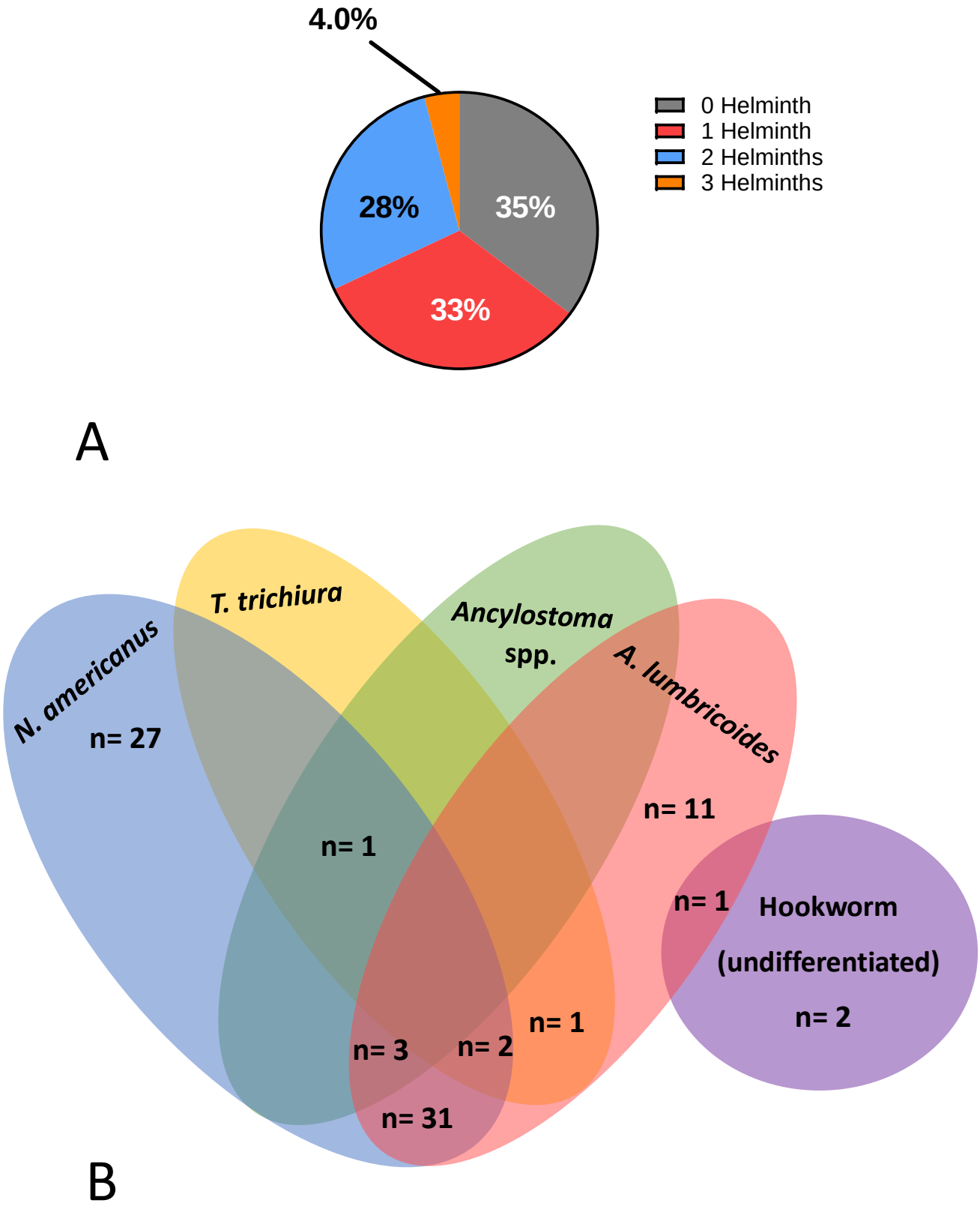

Figure 4.2: STH co-infections (n=79) using results generated from microscopy and qPCR (N= 122). Total negative for STH infection, n= 43. Pie graph depicts total number of different helminths detected in each sample, represented as a percentage (A). Venn diagram represents the overlap of the different types of STH co-infections (B).

4.3.1.1 Semi-quantification of helminth load

Concentrating the faecal samples improved detection sensitivity for both *Ascaris* spp. and hookworm, but not *Trichuris* spp.. See Appendix G for raw data of matched semi-quantitative scoring. However, when considering the samples as unmatched, as summarised in Table 4.4, it was found that concentrating the samples had increased egg load in nearly all participant samples, particularly those with *Ascaris* spp. infection.

Microscopic detection of hookworm ova from faecal specimens was improved by 53% following faecal sample concentration, detecting 12 new undifferentiated hookworm infections. It was found that the intensity of hookworm ova was somewhat comparable to unconcentrated faecal specimen findings, with an improvement in the frequency of samples considered “infrequent” (2+) classification. However, if considering matched data, concentrating the samples did not improve ova detection for eight samples, as these samples were previously assigned “scant” (1+) in the unconcentrated wet preparation and were then classified as negative in the concentrated wet-mount preparations.

When faecal specimens were unconcentrated, *Ascaris* spp. ova load was frequently recorded as 1+ to 2+. Once samples were concentrated, 85% of samples that were positive for *Ascaris* spp. had an improved ova semi-quantitative score, as scores for the unconcentrated faecal samples were frequently recorded as “moderately frequent” (+3) but were then classified as “very frequent” (+5) for the concentrated wet-mount preparations. While concentrating the samples enabled the detection of five new positive samples, four were missed that were previously detected in unconcentrated wet-mount preparations.

Table 4.4: Unmatched semi-quantification of STH ova in participant stool samples by microscopy as determined by unconcentrated vs concentrated wet preparations.

	Microscope direct wet preparation					
	Unconcentrated (n)	**Concentrated (n)**	**Total (n)**	**Additional STH-positive samples when concentrated (n)**	**Missed STH-positive samples when concentrated (n)**	**Samples (%)* with score improvement when concentrated**
Undifferentiated hookworm (total)	16	20	28	12	8	53
Scant (1+)	14	13				
Infrequent (2+)	2	7				
***Ascaris* spp. (total)**	40	43	47	5	4	85
Scant (1+)	17	4				
Infrequent (2+)	11	4				
Moderately frequent (3+)	7	14				
Frequent (4+)	3	6				
Very frequent (5+)	2	15				
***Trichuris* spp. (total)**	1	0	1	0	1	0
Scant (1+)	1	0				

*Determined by whether there was an increase in semi-quantitative score from unconcentrated wet preparations to concentrated wet preparations.

4.3.2 Evaluation of Detection Performance of Microscopy versus qPCR

The diagnostic performance of microscopy and qPCR was evaluated by comparing the prevalence rates obtained by each tool, detailed in Table 4.5. Results of genomic DNA quality and yield are reported in Appendix C, and all 122 samples were amplifiable. Evidence of DNA amplificability is shown by gel electrophoresis results in Appendix D.

It was demonstrated that TaqMan qPCR assays detected more STH infections than microscopy wet-mount observations. See Appendix H for full STH TaqMan qPCR data. Molecular classification of any STH infection detected 1.2 times more than microscopy. Molecular methods detected 2.3 and 3 times greater undifferentiated hookworm and *Trichuris* spp. infections respectively, than microscopy-based methods. However, three *Ascaris* spp. infections that were detected by microscopy were negative by the *Ascaris* spp. TaqMan qPCR assay.

Table 4.5: Comparison of diagnostics tools, microscopy and qPCR for determining STH prevalence.

	Microscopy n^a **(%)**	**TaqMan qPCR** n^b **(%)**	**Ratio (b/a)**
Any STH infection	62 (51)	74 (61)	1.2
A. lumbricoides	47 (38.5)	44 (36)	0.9
Hookworm (undifferentiated)	28 (23)	64 (52)	2.3
T. trichiura	1 (0.8)	3 (2)	3.0

4.3.2.1 Total Observed Agreement and the Kappa Measure of Agreement of Microscopy and qPCR

The diagnostic performance of qPCR versus microscopy was also determined by calculating the total observed agreement of positive and negative results and the Kappa agreement statistics to evaluate the agreement between the two tools (Table 4.6 and Figure 4.3). These results suggest that for the total observed agreement, microscopy and qPCR had an excellent percent agreement for detecting any STH (82.0%), *A. lumbricoides* (94.3%) and *T. trichiura* (98.4%) and modest total observed agreement for undifferentiated hookworm (65.6%). When considering the Kappa agreement statistic, results suggest excellent agreement between *Ascaris* spp. (K = 0.90), good for any STH (K = 0.64), moderate for *Trichuris* spp. (K = 0.49) detection and fair agreement for detecting undifferentiated hookworm (K = 0.33) infection.

It was identified that qPCR detected 17 and 39 more STH and undifferentiated hookworm samples, respectively, than microscopy methods, whereas the qPCR missed five samples that were *Ascaris* positive by microscopy. The prevalence of *Trichuris* spp. was low in this cohort, but qPCR detected two new infections that were missed by microscopy. Overall, five STHs, five *Ascaris* and three undifferentiated hookworm samples were positive by microscopy but negative by qPCR.

Table 4.6: qPCR and microscopy STH prevalence total observed agreement and Kappa agreement

	qPCR (n)	**Microscopy (n)**		**Total observed agreement (%)**	**Kappa* (95% CI)**
		Positive	**Negative**		
Any STH	**Positive**	57	17	82.0	0.64
	Negative	5	43		(0.50-0.80)
A. lumbricoides	**Positive**	42	2	94.3	0.90
	Negative	5	73		(0.79-0.97)
Hookworm (undifferentiated)	**Positive**	25	39	65.6	0.33
	Negative	3	55		(0.19-0.46)
T. trichiura	**Positive**	1	2	98.4	0.49
	Negative	0	119		(-0.11-1.00)

*Kappa (K) agreement level: Poor = K < 0.2; Fair = 0.21-0.40; Moderate = 0.41-0.60; Good = 0.61-0.80; Excellent = 0.81-1.00.

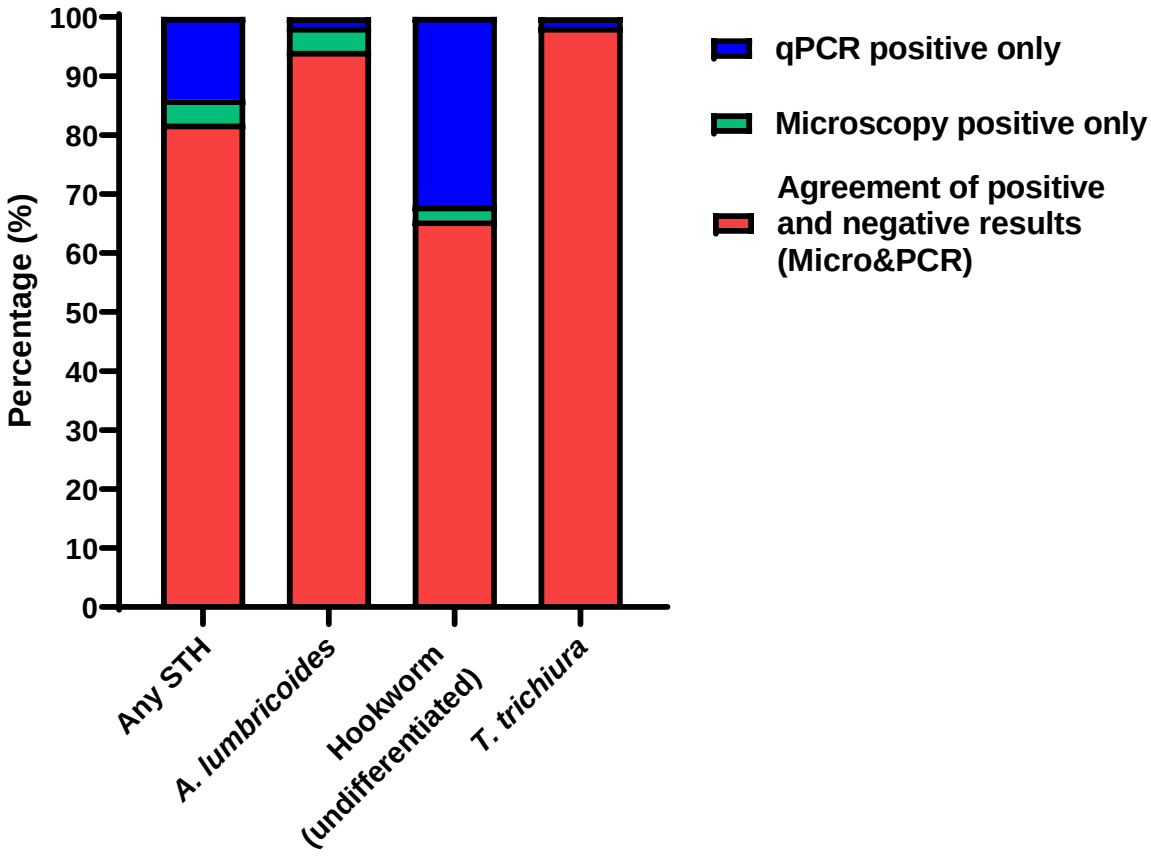

Figure 4.3: Agreement and disagreement between two diagnostic tools, microscopy and qPCR. The agreement between the two tools for positive and negative results is in red, and where the tools disagree in sample positivity are in blue (qPCR positive) and green (microscopy positive).

4.3.2.2 Comparison of Hookworm TaqMan qPCR assay Cq Values with Microscopy

The relationship between hookworm ova observed by microscopy and the Cq values obtained through qPCR was investigated. It was hypothesised that faecal samples where both methods indicated a positive hookworm infection, lower Cq values would be observed, indicating a higher concentration of hookworm DNA and, therefore, a higher hookworm ova burden. Conversely, higher Cq values were expected in samples where no hookworm ova were observed, indicating less hookworm DNA and lower hookworm ova burden.

In Figure 4.4, a large range of Cq values for samples where microscopy and qPCR agreed (i.e., microscopy + / qPCR +). The median Cq value was 26.2 (range: 21.2-36.2), with most samples falling within a range of 23.5 to 28.0. In contrast, samples classified as discordant, with negative microscopy but positive qPCR (i.e., microscopy - / qPCR +), showed a bimodal distribution of Cq values. The median Cq value of 28.2 (range: 22.7-35.7). The difference between the two medians was statistically significant (Mann-Whitney U *p-value* < 0.01), indicating the hookworm ova detected by microscopy tend to be those with the lowest Cq values. Suggesting that qPCR could be more sensitive for detecting low-burden hookworm infections than microscopy.

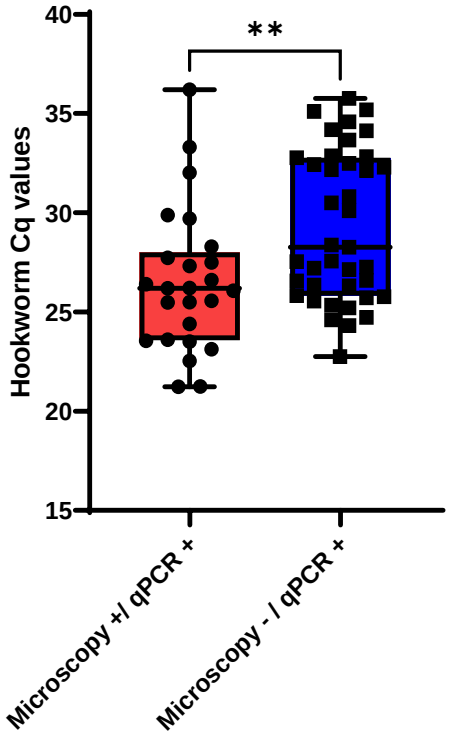

Figure 4.4: Distribution of Hookworm Cq values obtained by qPCR to compare concordant (left) and discordant (right) microscopy observations. Each point represents Cq values for one participant sample. Concordant results were classified as microscopy (+) and qPCR (+) (n= 25), and discordant results were classified as microscopy (-) and qPCR (+) (n= 39). The difference between median values was determined by Mann-Whitney U test, ** indicates statistical significance when *p-value* = < 0.01.

4.3.2.3 Relationship Between Microscopy and qPCR for Determining *Ascaris* spp. Burden

We compared the semi-quantification scores obtained by microscopy with Cq values obtained through qPCR to determine whether the semi-quantification method used in this study could indicate worm burden. The TaqMan qPCR assay, as previously mentioned, can estimate worm burden by indicating the DNA concentration of the genomic target amplified during the reaction in the form of a Cq value. Here, we focused on *A. lumbricoides* findings due to the availability of sufficient data from the systematic scoring of unconcentrated and concentrated samples from the microscopy component of this study that had matched Cq values from qPCR. We expected to observe a decrease in median Cq values as the microscopy semi-quantification score increased, which serves as a proxy for determining the DNA concentration of *Ascaris*. In Figure 4.5, no clear trend is evident between the semi-quantitative microscopy score and Cq values. The median Cq values appear scattered across each of the microscopy grades, making it difficult to discern any pattern. However, a trend between the semi-quantification scoring of concentrated samples and Cq values can be observed. After the "1+" to the “5+” score, median Cq values consistently decrease from 31.1 to 23.3 as the score increases, suggesting that lower scores have higher Cq values, indicating lower concentration of target DNA. In comparison, higher scores with lower Cq values have higher concentrations of target DNA. This finding could suggest that concentrating samples improves the probability of detecting ova in faecal samples and could be used to estimate the level of helminth burden.

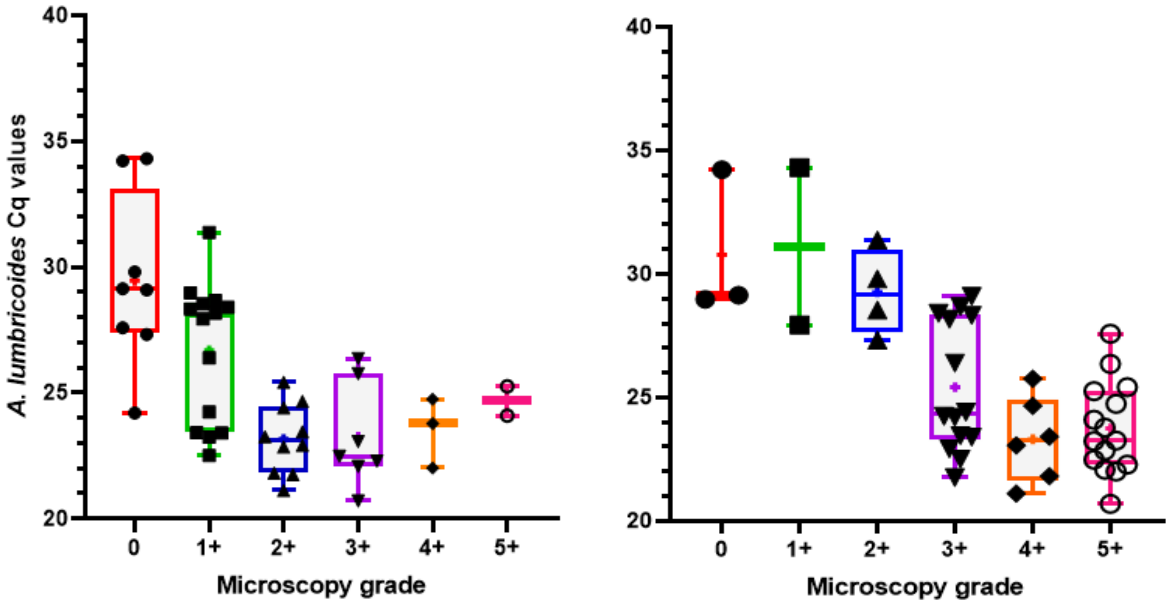

Figure 4.5: Distribution of *A. lumbricoides* Cq values within unmatched unconcentrated (left) and concentrated (right) semi-quantitative classifications of infection intensity determined by microscopy.

4.4 Discussion

Overall, 65% of participants from this study were infected with at least one type of STH as detected by microscopy and qPCR methods. *N. americanus* was the dominant STH infection within this cohort, followed by *A. lumbricoides*, *Ancylostoma* spp*.,* and *T. trichiura*. The most recent STH survey conducted in the Eastern Highlands of PNG, in 2009 found that the prevalence rate in a cohort of pregnant women was 31%, with most helminthiasis aetiologies being hookworm (undifferentiated) followed by *A. lumbricoides* (Phuanukoonnon et al. 2013). A historical survey conducted in the Western Province, in the Nomad District, also reported dominant hookworm infection rates (Usurup & Venture 2000), with another study reporting that *N. americanus* was the main species of hookworm causing human infections in PNG (Shield 1986; Shield et al. 1987; Shield & Kow 2013), similar to our reported findings. Relative to other neighbouring countries, the prevalence rates summarised in this study were similar to those of STHs rates reported in the Solomon Islands (67%) (Le et al. 2022). Furthermore, lower STH infection rates compared to our study were detected in communities from Central Java, Indonesia (Kurscheid et al. 2021).

The WHO's program guidelines state that populations at risk of increased morbidity due to STHs in areas where prevalence exceeds 20% should receive targeted administration of anti-helminthic drugs twice a year (WHO 2020a). However, the coverage rates of these populations in PNG are falling short of the recommended 75% required to interrupt transmission (Montreso et al. 2020). Although this small-scale survey was not designed to target high-risk individuals or provide nationwide prevalence rates, it still revealed STH rates surpassing the threshold in the Balimo region. This suggests that treatment may not be reaching these communities and that the surveyed population of adults could serve as reservoirs for the infection to individuals in at-risk groups.

In the present study, we used molecular methods to distinguish between *N. americanus* and *Ancylostoma* spp. infections. However, we could not specify the two *Ancylostoma* species known to infect humans, namely *A. ceylanicum* and *A. duodenale* (Traub 2013). This limitation may be attributed to the primer and probe combinations used and/or the positive template control used for *A. duodenale* TaqMan qPCR assay validation. Precise reasons for this inability to delineate *Ancylostoma* species are unclear and requires further investigation. The inclusion of *A. ceylanicum* in this parasite detection panel was to identify whether zoonotic cases of hookworm infection are occurring within rural PNG communities, as recent investigations using molecular tools have identified *A. ceylanicum* being common in Southeast Asian regions (Colella, Bradbury & Traub 2021; Gordon et al. 2017) and to our knowledge, only one molecularly confirmed case of *A. ceylanicum* infection was reported in PNG, specifically from Manus Island (Katanami et al. 2017; Yoshikawa et al.

2018). It would be of public health interest to sequence samples that tested positive for *Ancylostoma* spp. by qPCR in this study to differentiate between the species.

The findings of the current study indicate that 43% of individuals positive for an STH were concurrently infected with two helminths, while 6.3% harboured three helminths. Co-infection with *A. lumbricoides* and *N. americanus* was the most frequent, whereas *A. lumbricoides*, *N. americanus* and *Ancylostoma* spp. were the most frequently identified helminths in a tri-co-infection. The presented findings differ from those of a 2019 meta-analysis, which showed a significantly higher occurrence of helminth co-infections than single infections (Donohue, Cross & Michael 2019). The same meta-analysis also indicated that poly-helminth infections negatively impact haemoglobin levels and the prevalence of anaemia, suggesting that co-infections may exacerbate other health issues (Donohue, Cross & Michael 2019). Underlying factors that could contribute to the high levels of STH co-infections identified in this current study could be related to ecological factors of the Balimo region and various community-specific attributes related to WaSH, as identified in other studies (Strunz et al. 2014; Sturrock, Yiannakoulias & Sanchez 2017).

The findings presented in this study indicate that using unconcentrated faecal samples for wet-mount microscopy alone is insufficient for the detection of STHs, similar to findings from other studies (Endris et al. 2013; Hailu & Abera 2015), except for *T. trichiura*. The inability to detect *T. trichiura* in concentrated samples could be attributed to factors such as a low egg output by the worm (Hansen et al. 2016), non-homogeneous samples or limited faecal material used for examination (Bosch et al. 2021; Krauth et al. 2012).

Semi-quantification of helminth ova served as a proxy measure of infection load in the absence of quantitative methods. We observed that unconcentrated samples from individuals with Ascariasis were mainly “scant” to “infrequent” classifications, but concentrating the samples improved detection and semi-quantitative score classification. However, concentrating the samples did not enhance semi-quantitative scoring for hookworm, as some samples previously positive when examined unconcentrated were classified as negative when concentrated. The scoring system used in this study may not be suitable for accurately estimating hookworm infection intensity, as hookworm is less fecund than *Ascaris* (Bethony et al. 2006; Loukas et al. 2016). Improvement for future investigations using microscopy would be to perform the test tube flotation technique, which has shown improved sensitivity and recovery of helminth ova (Zeleke et al. 2021).

A further Aim of the current study was to compare the diagnostic utility of microscopy techniques to that of qPCR. Even when microscopy methods were combined overall, qPCR detected more STH infections. Diagnostic evaluation of microscopy and qPCR in this present study found that qPCR

improved the detection of STHs by 1.2-fold, and agreement of these methods was deemed "good", similar to other studies (Benjamin-Chung et al. 2020; Cools et al. 2019; Llewellyn et al. 2016; Zendejas-Heredia et al. 2021). Improved detection was demonstrated for hookworm and *T. trichiura* but not *A. lumbricoides,* and similar findings have been previously reported (Benjamin-Chung et al. 2020; Meurs et al. 2017). Benjamin-Chung et al. (2020) reported lower rates of *A. lumbricoides* using DNA amplification compared to microscopy techniques. The investigator then revealed through amplicon sequencing that of the discordant samples, none had *A. lumbricoides* (Benjamin-Chung et al. 2020) and indicated that the microscopists potentially misclassified samples. Another potential explanation for this discrepancy could be that the ova of *Ascaris* spp. are environmentally resilient, which are features that protect the embryo (containing DNA) from damage, making it difficult to extract DNA (Amoah et al. 2020). While this explanation is plausible, we would expect similar performance trends for all *A. lumbricoides* TaqMan qPCR assays and a poorer agreement with microscopy findings. Alternatively, other species of *Ascaris* are known to infect man, such as the zoonotic, porcine *A. suum,* which could serve as another plausible reason for the discrepancy (Devi 2022) and as the primer and probe combination used for qPCR does not amplify *A. suum* (Appendix I). To enhance the reliability of prevalence reporting in future investigations, it is recommended to have multiple parasitological examiners for microscopy and develop primer and probe combinations that could speciate anthropogenic from zoonotic *Ascaris* infections.

Consistent with other studies, we also found that more cases of hookworm were detected by qPCR than microscopy (Benjamin-Chung et al. 2020; Cimino et al. 2015; Clarke et al. 2018; Easton et al. 2016). However, unlike some of the above studies, the discordance and poor agreement between the tools seen in this study may not have been entirely due to increased sensitivity for low-intensity infections by qPCR, as identified by Figure 4.4, but is likely related to the ova's rapid degradation if not visually examined promptly by microscopy (Dacombe et al. 2007; Tarafder et al. 2010) and could be attributed to vigorous sample processing by the FEAC technique. Additionally, despite preserving the samples for later examination, prolonged storage can cause progressive reductions in prevalence and intensity estimates, occurring as early as eight days after sample preservation (Barda et al. 2015). Thus, this study design may not be appropriate for an accurate comparison, as microscopy relies on intact morphology of the hookworm ova to make an assessment, while qPCR only requires DNA. Previous STH surveys conducted in PNG have all relied on microscopy as the basis of parasite diagnosis. Most of these studies do not stipulate the time taken from faecal sample acquisition to sample processing and examination. The omission of this detail leads to speculation as to whether the historical prevalence rates of hookworm are higher than what has been reported and whether the intensity of infection has been underestimated.

Selecting a suitable diagnostic tool for a rural setting such as Balimo in PNG depends on the context of the survey and the worms being detected. The findings in this study suggest that microscopy could be the more feasible approach to performing a baseline community-wide assessment of STH infection rates within this community. Unlike qPCR, microscopy methods are simple and do not require advanced laboratory infrastructure. Our study showed that microscopy techniques were suitable for detecting *A. lumbricoides*, as qPCR provided no diagnostic advantage. Similar conclusions have been reached in other studies, demonstrating that qPCR and coproscopy detection capabilities in medium-high prevalence settings are comparable (Cools et al. 2019; Knopp et al. 2014). Conversely, microscopy alone may not effectively detect hookworm or *T. trichiura* within this community, as qPCR identified slightly more positive cases than microscopy, but the infection rate was low (below 3%).

Limitations of this study include our inability to speciate the *Ancylostoma* positive samples detected by qPCR, which is important for public health reasons due to the differing epidemiological and clinical characteristics of *A. duodenale* and *A. ceylanicum* (Jonker et al. 2012). Additionally, examining multiple faecal samples per person and spacing the time of collections apart would improve the detection rates of microscopy-based methods (Knopp et al. 2008; Liu et al. 2017). Future studies should consider a design that allows for an accurate enumeration of STHs to estimate the level burden (Azzopardi et al. 2021; Llewellyn et al. 2016; Zendejas-Heredia et al. 2021), as higher infection intensities are associated with worse STH-related morbidities (Loukas, Maizels & Hotez 2021), which is important to report when designing suitable public health interventions. Applying molecular-based methods to detect STHs is a strength of this study design, nevertheless, it is important to consider the limitations associated with this technique. For example, protocol variations influence sensitivity and specificity (Miswan, Singham & Othman 2022). Here, our qPCR assays did not include internal amplification controls to verify sample contamination/inhibition, which might have unsuspectingly increased the risk of false-negative qPCR results. We attempted to circumvent this by amplifying the 16s gene that is ubiquitous in all human faecal samples. Prolonged storage could have also influenced our DNA yield in this study (Clarke et al. 2018). Another strength of this study design is the use of multiple diagnostic tools to establish a baseline STH prevalence rate within Balimo, suggesting that the prevalence rates described here are likely to approach the true prevalence of STHs present. Utilising multiple diagnostics tools has led to important conclusions about the extent of human hookworm infection and high levels of co-STH infections that are present in rural PNG. To increase the impact of this study, it is recommended to expand the survey community-wide and include the detection of intestinal protozoan parasites, as these diseases commonly coexist and are of public health concern (Donohue, Cross & Michael 2019). Furthermore,

since a baseline for helminth prevalence has been established, it is possible to investigate the potential impact of STHs on other prevalent comorbidities in PNG, such as TB, which has known implications (Mabbot 2018).

During separate microscopic investigations involving the detection of other intestinal protozoa using iron-haematoxylin with modified acid-fast stain (methods not described here), a helminth larva was unexpectedly observed in an unconcentrated faecal smear (Figure 4.6). Typically, helminth larvae are not present in faecal preparations, apart from *S. stercoralis*, unless there is a delay when fixing the sample that allows the hatching of hookworm eggs to take place. However, the significant shrinkage of the larva and other artefacts made morphological identification and differentiation of similar larvae, e.g., hookworm, challenging. Therefore, it was suggested that for detecting *Strongyloides* spp., a more sensitive diagnostic tool like IgG ELISA may be utilised in conjunction with qPCR methods.

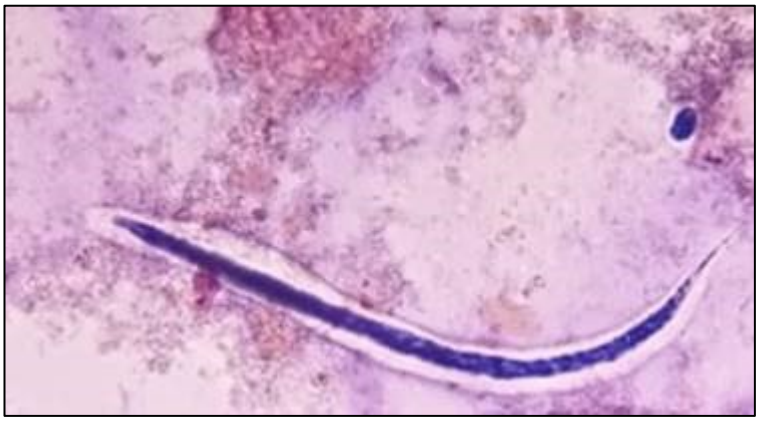

Figure 4.6: Query *Strongyloides* spp. helminth captured in an unconcentrated, stained faecal smear, at 100x magnification. Stain is iron haematoxylin with modified acid-fast.

4.5 Conclusion

In conclusion, this study highlights the high prevalence of STH infections and co-infections in a rural community in Balimo, Western Province, PNG. We found that qPCR did not offer a diagnostic advantage over microscopy for *Ascaris* detection, but concentrating faecal samples improved the detection capacity. However, qPCR was more effective in detecting hookworm and *Trichuris* infections. Selecting an appropriate diagnostic tool is crucial to avoid underestimating infection prevalence. Baseline prevalence rates are important for programmatic control efforts. Future investigations should encompass multiple communities and include the detection of intestinal protozoa. Furthermore, understanding the impact of STHs on co-existing conditions like TB could significantly inform disease management strategies moving forward.

5 Prevalence of *Strongyloides* spp. Infection Using IgG ELISA and qPCR

5.1 Introduction

It is estimated that *Strongyloides* spp. infects over 600 million people globally (Buonfrate et al. 2020). Clinical presentations of strongyloidiasis can range from acute and asymptomatic to chronic and symptomatic (Tamarozzi et al. 2019), and weakened immunity can result in uncontrolled parasite replication (hyperinfection), leading to visceral dissemination, which can have fatal consequences (Keiser & Nutman 2004; Lam et al. 2006; Tamarozzi et al. 2019), making it a significant concern to public health (WHO 2020c). *Strongyloides* infection is acquired through skin penetration from infective filariform larvae that have contaminated the environment through contact with infected faeces. Therefore, transmission is maintained where sanitation and hygiene practices and infrastructure are inadequate (Nutman 2017).

S. stercoralis is the most frequently reported species infecting humans, but there are another two sub-species within *S. fuelleborni* capable of causing human infection, but their prevalence is under-assessed, and infection risk is geographically restricted (Nutman 2017). *S. f. fuelleborni* mainly infects non-human primates (Thanchomnang et al. 2019), but spill-over infection has been reported in Africa and Southeast Asia (Thanchomnang et al. 2017), while *S. f. kellyi* has been exclusively reported to parasitise humans in PNG (Ashford, Barnish & Viney 1992). The prevalence of strongyloidiasis in PNG is considered high (Ashford, Barnish & Viney 1992; Kline et al. 2013). However, no recent reports exist on the current disease occurrence, particularly in isolated, rural communities in the Western Province. Since the discovery of *S. f. kellyi* in 1973, much of its distribution, clinical consequences and reservoir of infection have been unexplored (Bradbury 2021; Kelly, Little & Voge 1976). From previous studies, *S. f. kellyi* has been described as a childhood infection (< 15 years), with the prevalence decreasing into adulthood (Ashford, Barnish & Viney 1992; Barnish & Ashford 1989). Historical survey data have demonstrated that both *S. stercoralis* and *S. f. kellyi* have various levels of endemicity in multiple Provinces (Ashford, Hall & Babona 1981; Ashford et al. 1979; King & Mascie-Taylor 2004; Shiel 1986; Shield et al. 1987). Previous large-scale survey data of the disease was decades ago and consisted of only faecal examination results, which has been demonstrated as an insensitive indicator of disease (Campo, Gutiérrez & Cardona Arias 2014).

Strongyloidiasis is definitively diagnosed by detecting larvae in stool samples, typically detected using traditional parasitological tools. These tools rely on a combination of culture systems and microscopy to find larvae in stool samples (coproculture). These tools can often be insensitive, laborious (methodologically and technically demanding) and require multiple stool specimens (Chan

& Thaenkham 2023). Accurate diagnosis could require numerous diagnostic tests, which poses significant challenges for poorer-resourced regions (Chan & Thaenkham 2023; Hailu et al. 2022). Asymptomatic and/or chronic infections, resulting in low to intermittent output of larvae into faeces, also compromise the sensitivity of these parasitological methods (Kaminsky, Reyes-García & Zambrano 2016; Nutman 2017).

Other diagnostic tools include immunoassays and nucleic acid amplification methods like stool PCR platforms (Buonfrate et al. 2018; Costa et al. 2021). Immunoassays, e.g., ELISA, offers a more sensitive approach, ranging between 70-100% (Bisoffi et al. 2014), but may lead to false-positive readings due to potential cross-reactivity of other helminth infections, particularly in endemic regions (Arifin et al. 2019) and false-negative results may arise in acute infections, mature aged individuals and immunocompromised patients (Arifin et al. 2019; Luvira et al. 2016). Taking the molecular approach to detect *S. stercoralis* has shown some improved detection sensitivity compared to coproculture methods (Chankongsin et al. 2020; Hailu et al. 2022), but these findings are inconsistent, particularly in low-intensity infections (Dong et al. 2016; Knopp et al. 2014), which can be common for different disease stages like acute, chronic and asymptomatic infections (Buonfrate et al. 2015). Despite high throughput, routine use of molecular tools in limited-resource settings may not be feasible economically or logistically, and the lack of standardisation between molecular methods significantly influences the reproducibility of results (Buonfrate et al. 2018; Chan & Thaenkham 2023). It could be argued that the same applies to adopting serological-based diagnostic methods, similar to coprological methods. Selecting the appropriate diagnostic tool to determine strongyloidiasis is important, particularly for resource-limited areas. The selected tool should have a reasonable degree of detection capacity without being too costly, laborious or require significant technical upkeep.

This Chapter aims to determine the prevalence of strongyloidiasis in a rural PNG community using IgG ELISA and qPCR for diagnosis. In addition, this Chapter will evaluate the utility of these tools in determining infection.

5.2 Materials and Methods

5.2.1 Study Design, Setting, Participants and Ethics

Information regarding this chapter's study design, setting, population and ethical considerations has already been described in Chapter 3. The total number of matched plasma and stool samples used for this study was 120, with two unmatched faecal samples.

5.2.2 Stool Collection and Microscope Examinations

Faecal specimens used in this chapter are the same samples from Chapter 4. For methodology regarding stool sample processing and strategies for classifying and examining parasites, see Chapter 4.

5.2.3 Blood Specimen Collection and Processing

In conjunction with TB investigations, blood was drawn from participants into four separate heparin, QuantiFERON-TB Gold Plus (QFT-G+) tubes (QIAGEN, United States of America). Briefly, the QFT-G+ is an interferon-gamma release assay used to determine exposure to *Mycobacterium tuberculosis* (Mtb), the bacterial pathogen that causes TB. Two of the four tubes are coated with Mtb-specific peptide antigens that stimulate memory T lymphocytes to produce interferon-gamma. The other two tubes are controls; one is coated with mitogen, used as a positive control, and the final only contains heparin, used as the negative control, referred to as the "nil" tube. QFT-G+ blood samples were incubated for 16 hours at 37℃ before separating the plasma from the red blood cells. Plasma was stored at 4℃ in Balimo PNG and then at -80℃ after being delivered to JCU, Townsville, Australia. Resultant plasma from the nil tube of QFT-G+ was used for strongyloidiasis serology. Previous work from our group (data not shown) has determined that antibody integrity is maintained throughout the sample processing and storage procedures required for the QFT-G+ assay. Therefore, samples can be used for further serological work.

5.2.4 Strongyloidiasis Serodiagnosis Using IgG ELISA

Plasma samples of 120 participants were used to determine *Strongyloides* spp. seroreactivity. A commercial IVD *Strongyloides* Serum Antibody Detection Microwell ELISA testing kit (DRG Instruments GmbH, Marburg, Germany) was used and performed per the manufacturer's instructions. This assay detects anti-IgG *Strongyloides* spp. antibodies using an ELISA plate coated with *S. stercoralis* somatic antigens. The ELISA plates were read using the SPECTROstar Nano (BMG

Labtech). In this study, an absorbance reading, as determined by the SPECTROstar Nano data analysis software (MARS version 3.33), of ≥ 0.4 optical density (OD) units indicated a positive result; OD units between 0.2-0.4 were deemed intermediate, while an OD unit of ≤ 0.19 was considered negative. Cut-off ranges were adopted from laboratory standards in Western Australia (Shield et al. 2021).

5.2.5 DNA Extraction of Faecal Samples

Methods regarding stool sample collection, processing and extraction of genomic DNA have been described in Chapter 4. We are using the same faecal samples, whereby methods for assessing sample amplificability have already been described in Appendix C.

5.2.6 TaqMan qPCR Testing of Faecal Sample for S. stercoralis

S. stercoralis specific primers (forward: 5'-GAATTCCAAGTAAACGTAAGTCATTAGC-3'; reverse: 5'-TGCCTCTGGATATTGCTCAGTTC-3') and probe (5'-HEX-ACACACCGGCCGTCGCTGC-BHQ1-3') were used, based off sequences described by Pilotte et al. (2016), with modifications made to the 5' fluorescent reporter and 3' quencher. Preliminary trials were done to validate published assay parameters, see Appendix J for the full workflow. The outcomes of the results described in the appendices informed the qPCR assay used in this current study. As previously described, all 122 extracted DNA faecal samples were tested for the presence or absence of *S. stercoralis* infection using a TaqMan qPCR platform. Reactions were performed with 10µL of GoTaq qPCR Probe Master Mix (Promega, USA, #A610A), 200nM of each primer, 100nM of probe and 2µL of sample DNA to amount to a final volume of 20µL. Cycling conditions for this assay consisted of the following parameters: 1 cycle of 95℃ for 5 minutes, followed by 40 cycles of 95℃ for 10 seconds, and 60℃ for 60 seconds (Azzopardi et al. 2021). The Bio-rad CFX96 thermocycler was used for all TaqMan qPCR reactions. A PCR-confirmed sample of *S. stercoralis* was used as a positive template control (kindly provided by Dr Richard Bradbury from Federation University, Melbourne), in addition to various negative controls, including a non-endemic faecal sample, non-template control and a water sample that was prepared in parallel to DNA extraction as in last Chapter. The dilution series for the standard curve, using the *S. stercoralis* positive template control, was performed in duplicate and was included during the running of the *S. stercoralis* TaqMan qPCR assay. The standard curve was used to set the Cq value cut-offs that would indicate sample positivity. Cq values were determined as per Chapter 4.

5.2.7 Data Analysis

All statistical analyses in this chapter were performed using GraphPad Prism 9 (Prism for Windows, version 9.2.0.332). The IgG ELISA and qPCR results are summarised as frequencies and percentages, reported with a 95% CI. Participants were matched based on the reported results from each diagnostic tool to assess agreement between qPCR and serology. Ratios were calculated to compare the frequency of *Strongyloides* spp. detected with qPCR and by ELISA. Total observed agreement between diagnostics was determined using percentages of total positive and negative samples whereby both tools agree. The Cohen's Kappa agreement statistic was performed to provide insight into whether the probability of agreement between the two tools is genuine and not due to chance, and 95% CI was reported.

5.3 Results

5.3.1 Prevalence of Strongyloidiasis and Diagnostic Tool Evaluation

The overall prevalence of strongyloidiasis, when combining positive results obtained by IgG ELISA and qPCR, was 23.3% (n = 28/122, 95% CI 16.4-31.2%), as described in Table 5.1. The IgG ELISA identified 5.4 times more *Strongyloides* spp. infections than qPCR. Total IgG positive samples, which indicates current or previous strongyloidiasis, was 22.5% (n = 27/120; 95% CI 15.9-30.8%). Results by the TaqMan qPCR suggest that 4.2% (n = 5/122, 95% CI 1.8-9.2%) of this cohort have detectable *S. stercoralis* DNA in their faecal samples. The complete dataset for IgG serodiagnosis and TaqMan qPCR is present in Appendix K.

Table 5.1: Diagnostic comparison of IgG ELISA and qPCR for *Strongyloides* spp. detection.

***Strongyloides* spp. infection status**	**Methods of detection**		**Ratio (a/b)**	**Combined total of methods**	
	IgG ELISA n [a] (%)	**qPCR n [b] (%)**		**n (%)**	**95% CI**
Positive	27 (22.5)	5 (4.2)	5.4	28 (23.3)	16.4-31.2
Negative	93 (77.5)	117 (95.8)	0.8	94 (76.7)	68.8-83.6

Total observed agreement and Kappa agreement statistic was used to assess the diagnostic performance between IgG ELISA and qPCR, as seen in Table 5.2. Molecular and serological methods agreed with 80% of the total positive and negative results. However, the Kappa agreement statistic suggests poor agreement (K = 0.2). Only one qPCR-positive sample was missed by the IgG ELISA. This participant was found to have an OD unit between 0.2-0.4 and was classified as intermediate and, therefore, grouped with the negatives, see Appendix J.

Table 5.2: Matched qPCR and IgG ELISA results (n = 120) for the detection of *Strongyloides* spp. and agreement statistics.

	IgG ELISA (n)		**Total observed**	**Cohen's Kappa**
qPCR (n)	**Positive**	**Negative**	**agreement (%)**	**(95% CI)**
Positive	4	1	80.0	0.2
Negative	23	92		(0.01-0.40)

Kappa (K) agreement level classifications poor (K = < 0.2), fair (K = 0.21-0.40), moderate (K = 0.41-0.60), good (K = 0.61-0.80), excellent (K = 0.81-1.00).

5.4 Discussion

The current study found that 23% of participants were considered positive for strongyloidiasis through a combination of IgG ELISA and qPCR results. The prevalence rate observed in this study could be regarded as high, yet it aligns with rates reported in other neighbouring countries (Buonfrate et al. 2020; Schär et al. 2013). In Southeast Asia, estimates of strongyloidiasis were reported to be about 10-15% (Buonfrate et al. 2020), but a nationwide survey conducted in Cambodia reported a prevalence of 30.5% in rural populations (Forrer et al. 2019), higher than the rates reported in the current study. In contrast to our current study, the study conducted in Cambodia (Forrer et al. 2019) used a combination of culture systems in addition to PCR, which could result in greater sensitivity and, thus, a higher reported prevalence rate (Chankongsin et al. 2020). Strongyloidiasis has been described in historical studies in PNG, highlighting the high endemicity of the disease (Bearup & Lawrence 1950). Other PNG-based studies also demonstrate a scattered distribution of the infection across different geographical areas (Ashford & Babona 1980). For example, research conducted in the 1980s within the six localities in the Purari-Kikori delta area, Western PNG, reported a *Strongyloides* spp. prevalence rate of 1% in children aged 6-15 years only (Ashford, Hall & Babona 1981). In contrast, the infection rates from other isolated communities in the Kerema area, Gulf Province, diagnosed by traditional parasitological tools, are similar to the findings presented in this study, which was found to be approximately 25% (Ashford & Babona 1980). However, since this time, no other studies have examined the distribution of *S. stercoralis* nor the closely related species *S. f. kellyi*, which is understood to be highly prevalent among infants and young children (Barnish & Ashford 1989).

Here, we found that utilising an ELISA, a serological-based diagnostic tool, revealed more positive cases than qPCR, consistent with other published studies (Buonfrate et al. 2017; Chankongsin et al. 2020; Robertson et al. 2017; Swan, Phan & McKew 2022). The discordance observed between the current study's serological and molecular diagnostic tools may be attributed to their differences in sample type requirements and detection targets. For example, the qPCR detects DNA from faecal material, while the ELISA uses the blood biomarker IgG for detection. The limitations associated with molecular techniques, such as low sensitivity and poor detection of low-intensity infections, have been highlighted in previous studies (Buonfrate et al. 2018). Other studies have similarly reported an inferior capacity of qPCR to detect the low-intensity infections common during chronic *Strongyloides* spp. infections, compared to coproculture methods that used multiple stool specimens (Knopp et al. 2014; Sultana et al. 2013). Variability of results demonstrated in these studies, including this current study, could also be related to methodological differences in sample preservation, DNA extraction

procedures, PCR platforms, primer and probe combinations and reaction conditions, which are all known to influence assay accuracy (WHO 2020b). The primer and probe combinations used in this current study were originally designed by Pilotte et al. (2016), which has not been through rigorous clinical validation compared to the more commonly used genus-specific assay by Verweij et al. (2009). However, a WHO report has recommended the former assay due to its reported species specificity (WHO 2020b).

Further, it is important to acknowledge that another species of *Strongyloides* spp. might exist within the Balimo region, but its presence has not yet been molecularly confirmed. The presence of *S. f. kellyi* may potentially contribute to the discrepancy observed between the diagnostic tools, as the primer and probe combination targets a hypervariable region of the genome that is specific for *S. stercoralis* (Pilotte et al. 2016) and not *S. f. kellyi*. To strengthen the findings from this study, it would be beneficial to validate the species of *Strongyloides* present by sequencing the PCR products from positive samples. This could have implications for health-related outcomes, given that the distribution and consequences of *S. f. kellyi* infection in adults are unknown (Bradbury 2021). Perhaps adults in this setting serve as a reservoir of infection for newborns, infants and young children who are vulnerable to complications associated with this species (Ashford, Barnish & Viney 1992; Bradbury 2021).

Using serology-based tools as the basis of strongyloidiasis diagnosis is contentious, and various factors could contribute to the disagreement between qPCR results (Buonfrate et al. 2015). Previous studies highlight that this method of diagnosis can exaggerate strongyloidiasis prevalence due to potential cross-reactivity with other co-existing helminths and the inability to delineate between present and past (treated) infections. Without treatment, it is speculated that strongyloidiasis can be sustained chronically within the host through its unique lifecycle ability of “autoinfection” (Van De et al. 2019), and the treatment required for *Strongyloides* is different from what is administered for other STH infections. If treatment for strongyloidiasis has occurred, it has been demonstrated that antibody titres decrease over 6 to 12 months, usually resulting in seronegative status (Buonfrate et al. 2015; Kearns et al. 2017). Treatment for strongyloidiasis is not occurring in rural communities like Balimo, suggesting that if *Strongyloides* infections are present within this community, they are chronic. Furthermore, anti-*Strongyloides* spp. IgG antibodies typically appear six weeks after initial exposure and have been shown to remain elevated during chronic infections (Arifin et al. 2019; Atkins et al. 1997), making it a suitable biomarker for detecting infection exposure. Another factor that could contribute to exaggerated prevalence rates attributable to serological-based diagnostics is the potential cross-reactivity with other co-endemic helminth species. Cut-off values used in this study were adopted from laboratories based in an endemic location within Australia (Shield et al.

2021) and ensure that only strong positive seroreactions are considered when determining prevalence rates, which may, to some extent, circumvent cross-reactive false positives. Nonetheless, this decision enhances the risk of false-negative interpretations, such as the singular discrepancy of an intermediate serology result, which was classified as negative in this study, and a positive qPCR result (Table 5.2). Antibody cross-reactivity between filaria and *Strongyloides* spp. antigens have also been described (Bisoffi et al. 2014; Norsyahida et al. 2013), and *Wuchereria bancrofti*, a causative agent of filariasis, is endemic in parts of PNG, albeit distribution is uneven (Graves et al. 2013) and its occurrence in the Balimo region is unclear.

In addition to causing direct pathology in the gastrointestinal tract, infections with *S. stercoralis* have also been implicated in impacting the clinical outcomes of TB. In TB-helminth co-endemic communities, the presence of *S. stercoralis* impacts the protective TB immunity of individuals (Anuradha et al. 2017; George et al. 2015) and can alter disease manifestations (Kumar et al. 2020; Sikalengo et al. 2018). This may lead to complications in patient management (Elias et al. 2008; Neto et al. 2009; Resende Co et al. 2007).

This study was the first to use both serological and molecular methods for detecting strongyloidiasis in PNG and contributes to our understanding of the disease in this rural setting. Selecting an appropriate diagnostic tool to obtain prevalence data from surveys on strongyloidiasis is essential for eliminating this disease as a public health concern (WHO 2020a, 2020c). Regardless, it is apparent that enhancing the reliability of strongyloidiasis diagnosis requires the simultaneous use of various parasitological tools (Bisoffi et al. 2013), which could strain laboratories in endemic, resource-limited areas. In the absence of reliable tools in rural settings like Balimo, serology can play a role in population screening, with molecular or conventional parasitological detection techniques potentially serving as a confirmatory method if resources permit (Napier et al. 2017).

5.5 Conclusion

The current study found that the prevalence of strongyloidiasis in Balimo, Western Province, PNG, was 23%. This prevalence rate was informed by serology and molecular methods. It was found that the anti-*Strongyloides* IgG ELISA indicated more positive infections than the TaqMan qPCR. The use of the ELISA in this setting could hold potential for screening large populations, whilst qPCR could have a confirmatory role. Selecting a suitably sensitive, economically sustainable and logistically feasible diagnostic tool for detecting strongyloidiasis in resource-limited settings has important implications for future public health control programs.

6 Epidemiology of STHs, Including *Strongyloides* spp. in a Rural Community in PNG

6.1 Introduction

Soil-transmitted helminthiasis, including strongyloidiasis, are linked strongly to poverty-associated risk factors. STH infections are considered the litmus test of faecal-contaminated environments, as soil contaminated with faeces is the main facilitator of STH infections. Worms like *A. lumbricoides* and *T. trichiura* are acquired orally by ingesting infective ova from contaminated fomites, food and water (Echazú et al. 2015). In contrast, hookworm and *Strongyloides* spp. are acquired through skin contact with contaminated soil (Echazú et al. 2015). Therefore, transmission is sustained in regions where promiscuous defecation and walking barefoot persist, and people have limited access to adequate sanitary infrastructure, safe and clean drinking water and accessible healthcare services (WHO 2020d). Thus, high levels of STH endemicity are predominantly described in low and middle-socioeconomic communities.

Collectively these worms have been described to be the cause and consequence of stunting economic growth and re-enforcing poverty through adversely affecting productivity (Kyu et al. 2018), pregnancy outcomes, and the nutritional and cognitive development of children (Aderoba et al. 2015; Blouin et al. 2018; Bolka & Gebremedhin 2019; Pabalan et al. 2018; Taghipour et al. 2020), which is why they have been classified as a significant public health concern (WHO 2020a). It has also been reported that these helminths can impact the outcome of other overlapping infections (Mabbott 2018), and the degree of morbidity associated with STH infections is proportional to the intensity of worms infecting the host (Campbell et al. 2016; Gyorkos et al. 2011; Turner et al. 2008) and can be worsened if an individual is infected with multiple parasites (Donohue, Cross & Michael 2019). Strongyloidiasis has various clinical presentations. For example, in immunocompromised hosts, infection with *Strongyloides* can be fatal but could otherwise be asymptomatic in immunocompetent hosts, and if symptoms do arise, they can be non-specific, having various skin, respiratory and gastrointestinal-related manifestations (Luvira et al. 2022; Ramanathan & Nutman 2008; Tamarozzi et al. 2019). Infection with *Strongyloides*, alike other STHs, can contribute to a significant range of morbidities, including negatively influencing infant outcomes, wasting and stunting in children (Forrer et al. 2017; Paltridge & Traves 2018).

Control strategies recommended by the WHO currently aim to reduce global STH morbidity levels (WHO 2020c). Periodic mass drug administration campaigns are the mainstay of control and generally target at-risk population groups that could be adversely affected by infection, such as school-aged children and women of reproductive age (WHO 2020c). Decisions regarding

implementing control programs and resource mobilisation rely on findings from epidemiological surveys. By determining demographic characteristics and assessing infection prevalence, it becomes possible to identify who are most at risk of harbouring STHs, enabling a more targeted approach to control. In turn, this would optimise the use of already limited resources and improve the efficiency of disease management.

The results described in Chapter 4 revealed that nearly 65% of participants from Balimo, Western Province in PNG, are infected with at least one type of STH, excluding *Strongyloides* spp.. Chapter 5 details the prevalence rate of *Strongyloides* spp. as ~23%. However, these rates do not describe the characteristics of these individuals and their respective STH infection status.

The primary Aim of Chapter 6 was to identify the demographic and some clinical characteristics of participants that might determine STH infection status in a rural community from PNG. A further Aim was to describe the cumulative prevalence of STHs, including *Strongyloides* spp. in Balimo, using the different diagnostic tools described in previous Chapters. Understanding the community prevalence of all types of STHs and the respective characteristics of individuals infected with these worms highlights at-risk population groups that could benefit from future public health interventions.

6.2 Materials and Methods

6.2.1 Study design, Setting and Participant Recruitment

Details regarding the study design, setting, participant recruitment and ethics were described in Chapter 3.

6.2.2 Participant Interviews Using Structured Questionnaire

Eligible community members of the Balimo region participated in face-to-face questionnaire-based interviews. Demographic and various clinical information was obtained from participants via a structured questionnaire. The original questionnaire used for this study is available in Appendix A. The structured questionnaire was initially purposed for demographic characteristics and clinical variables associated with TB. However, variables considered for this study have been identified as potential predictor variables for STH infections, such as age and biological sex. Anthropogenic measurements such as height and weight were collected via the questionnaire to calculate crude body mass index (BMI), which was used to indirectly measure nutritional status in this study. Employment status was selected as a proxy variable that could indirectly indicate daily activities, level of exposure to the environment and accessibility to improved assets. Similarly, education level may be an indirect measure of values and beliefs regarding awareness surrounding the behaviour of sanitation and hygiene practices. Clinical variables included self-reported cough (lasting longer than two weeks) and shortness of breath (SOB), given that some helminths identified in previous Chapters have lung migratory pathways.

6.2.3 Parasitological Methods to Detect STHs, Including Strongyloides spp.

Details regarding the methodology of parasite detection have been described in Chapters 4 and 5.

6.2.4 Data Analysis

All statistical analyses described in this Chapter were performed using IBM SPSS Statistics (v. 29.0). Graphs used to visualise data were curated in GraphPad Prism (GraphPad Prism 9.5.1.733) and Microsoft® Excel® (Version 2304 Build 16.0.16327.20200). To summarise general cohort characteristics, frequencies and percentages of categorical variables were calculated and reported with a 95% CI. Gaussian (normal) distribution was assessed for continuous variables, including Age (years) and BMI (kg/m^2) using the Shapiro-Wilks method, and data was summarised as mean or median and variation using $\pm$ standard deviation or interquartile range, depending on the data normality assessment outcome. The complete data set of cohort characteristics used for all analyses is available in Appendix L.

The following variables obtained through the questionnaire were transformed into categorical variables to summarise the overall demographic characteristics of this cohort as frequencies and percentages. Age was obtained as a continuous variable and was transformed into dichotomous variables < 40 years and > 41 years. Age was grouped based on the central measure of tendency. Biological sex was analysed as male and female. Calculated body mass index (based on height and weight) was analysed as a continuous variable and was also transformed into categories underweight (≤ 18.5 kg/m^2), normal (18.6-24.9 kg/m^2) and overweight (≥ 25 kg/m^2). Resident localities were classified as either living in “Balimo town” or a village (a community outside Balimo town). Employment types were simplified to employed and unemployed statuses. Education was documented as grade level but was simplified into none/primary or secondary/tertiary. Additional yes/no parameters included in the analysis were whether the participant had SOB and experienced a cough. These transformed variables were used for further inferential analysis. Categorised demographic variables were stratified by STH infection status and type, summarised as frequencies and prevalence, reported with 95% CI.

Crude associations between predictors (independent variables; socio-demographic and clinical categories) and the outcome (dependent variable; helminth infectivity status) were assessed using a binary (univariable) logistic regression model. Variables included in the univariable logistics regression were presented with an odds ratio (OR) and reported with standard error (SE) and a 95% CI. Variables considered in the multivariable model were pre-selected based on their clinical importance (age (continuous) and sex), and other candidate variables were retained if *p-value*s were

≤ 0.25 in the univariable analysis. An assessment of multicollinearity for the variables was done using the variance of inflation factor (VIF) before producing the final multivariable model. An independent variable VIF > 5 was removed from the final model. Goodness-of-fit of the data was assessed using several inferential tools. Briefly, Tjurs R squared was used to determine the variation in the outcome variable that could be explained by the predictor variables in the model. Reporter-operating curve (ROC) and area under the curve (AUC) were also applied, and the models with values under 0.5 were deemed to have no predictive value, while values over 0.5 were considered models with sufficient predictive value. Hosme-Lemeshow hypothesis tests indicate the difference between the expected and observed values of the model, and *p-value*s > 0.5 indicates no difference, meaning there's a good fit of the modelled data. The final multivariable model presents an adjusted odds ratio (aOR) estimate, reported with SE and a 95% CI. The inference of statistical significance was based on a *p-value* of ≤ 0.05.

6.3 Results

6.3.1 Study Population Characteristics

A total of 122 participants were included in this study, submitted a faecal sample for examination and completed questionnaire responses. Females formed most of this cohort, 66.4% (n = 81/122; 95% CI 25.8-42.7%) and are considered over-represented in this study relative to the nation's census (National Statistical Office of Papua New Guinea 2023). In this cohort, younger individuals (< 40 years old) were under-represented at 31.4% (n = 38/122) compared to 68.6% (n = 83/122) who were aged > 41 years, with the average age being 46 years ($\pm$ 14.1 years). According to the 2022 population census, young people in PNG who were < 25 years old make up 58% of the general demographic (National Statistical Office of Papua New Guinea 2023). In this study, 61.5% (n = 75/122; 95% CI 52.6-69.6%) had residency located in the district capital of the Delta Fly, Balimo, whilst the remainder were located outside Balimo in other villages (38.5%; 95% CI: 30.4-47.4%). Based on crude height and weight measurements, 54.2% (n = 64/118; 95% CI: 45.3-63%) have a normal BMI (18.6-24.9 kg/m^2), with the average being 24.01 ($\pm$ 4.3), while 39.8% (n = 47/118; 95% CI: 31.5-48.9%) and 6.0% (n = 7/118; 95% CI 3-11.8%) are considered overweight (≥ 25 kg/m^2) and underweight (≤ 18.5 kg/m^2), respectively. Participants classified as 'overweight' were higher in Balimo town (49%; n = 35/71) compared to participants living in a village (26%; n = 12/47).

6.3.2 Combined Prevalence of STH Infections, Including Strongyloides spp.

The total prevalence of STHs, including *Strongyloides* spp., is 70.5% (n = 86/122; 95% CI 61.9-77.9%). An overall prevalence rate was informed by a combination of results obtained from 122 microscopy and qPCR faecal samples and 120 plasma samples by an anti-*Strongyloides* spp. IgG ELISA, as described in Chapters 4 and 5, respectively. The most frequently detected helminth was *N. americanus* (52.5%; 95% CI 43.7-61.1%), followed by *A. lumbricoides* (40.2%; CI 95% 31.9-49.0%), *Strongyloides* spp. (23%; 95% CI 16.4-31.2%), *Ancylostoma* spp. (3.3%; 95% CI 1.3-8.1%) and *Trichuris* spp. (2.5%; 95% CI 0.7-7.0%).

6.3.3 Stratification of Cohort Characteristics by Different Species of STH Infections, Including Strongyloides spp.

Prevalence rates of STH infections were stratified by demographic characteristics obtained through a questionnaire, as seen in Table 6.1. Participants aged < 40 years and living in a village had a higher rate of any STH infection, more specifically, *A. lumbricoides*, *Strongyloides* spp., *T. trichiura*, undifferentiated hookworm or *N. americanus,* compared to those aged > 41 years and living in Balimo town. In contrast, the opposite trend was demonstrated for *Ancylostoma* spp. infection. For any STH infection, including *A. lumbricoides*, *Strongyloides* spp., *T. trichiura*, undifferentiated hookworm, *N. americanus* and *Ancylostoma* spp.*,* males had a higher prevalence rate than females. Participants classified as underweight had a greater rate of any STH infection, including *A. lumbricoides*, *Strongyloides* spp., and *T. trichiura,* compared to those with either normal or overweight classifications. However, this data is skewed due to the small sample representation. In addition, those classified as having a normal BMI had a higher rate of undifferentiated hookworm, specifically, *N. americanus* or *Ancylostoma* spp. infection than those classified as underweight or normal.

Table 6.1: STH prevalence by species, stratified by sex, age group, location and BMI.

Factor	Variables	Number analysed n (%)	Any STH % (95% CI)	*A. lumbricoides* % (95% CI)	*T. trichiura* % (95% CI)	*Strongyloides* spp. % (95% CI)
All participants		122 (100)	70.5 (61.9-77.9)	40.2 (31.9-49.0)	2.5 (0.7-7.0)	23 (16.4-31.2)
Age	**≤ 40**	38 (31.4)	81.6 (66.6-90.8)	52.6 (37.3-67.5)	5.3 (0.9-17.3)	28.9 (17-44.8)
	≥ 41	83 (68.6)	65.0 (54.3-74.4)	33.7 (24.5-44.4)	1.2 (0.06-6.5)	19.3 (12.2-29.0)
Sex	**Male**	41 (33.6)	73.2 (58.1-84.3)	41.5 (27.8-56.6)	4.9 (0.9-16.1)	29.3 (17.6-44.5)
	Female	81 (66.4)	69.1 (58.4-78.1)	39.5 (29.6-50.4)	1.2 (0.06-6.7)	19.8 (12.5-29.7)
Location	**Balimo town**	75 (61.5)	64.0 (52.7-73.9)	33.3 (23.7-44.6)	1.3 (0.1-7.2)	20.0 (12.5-30.4)
	Village	47 (38.5)	80.9 (67.5-89.6)	51.1 (37.2-64.7)	4.3 (0.8-14.2)	27.7 (16.9-41.8)
BMI categories (kg/m^2)	**Normal, (18.6-24.9)**	64 (54.2)	75.0 (63.2-84)	42.2 (30.9-54.4)	3.1 (0.6-10.7)	26.6 (17.3-38.5)
	Underweight, (≤ 18.5)	7 (6)	85.7 (48.7-99.3)	85.7 (48.7-99.3)	14.3 (0.7-51.3)	57.1 (25.0-84.2)
	Overweight/ Obese, (≥ 25)	47 (39.8)	61.7 (47.4-74.2)	31.9 (20.4-46.2)	0 (0-7.6)	14.9 (7.4-27.7)

Table 6.1: STH prevalence by species, stratified by sex, age group, location and BMI (continued).

Factor	Variables	Number analysed n (%)	Hookworm (undifferentiated) % (95% CI)	*N. americanus* % (95% CI)	*Ancylostoma* spp. % (95% CI)
All participants		122 (100)	54.9 (46.0-63.5)	52.5 (43.7-61.1)	3.3 (1.3-8.1)
Age	**≤ 40**	38 (31.4)	68.4 (52.5-80.9)	68.4 (52.5-80.9)	2.6 (0.1-13.5)
	≥ 41	83 (68.6)	48.2 (37.8-58.8)	44.6 (34.4-55.3)	3.6 (1.0-10.1)
Sex	**Male**	41 (33.6)	53.6 (38.7-67.9)	32.8 (22.6-45.0)	4.9 (0.9-16.1)
	Female	81 (66.4)	55.6 (44.7-65.9)	67.2 (55.0-77.4)	2.5 (0.4-8.6)
Location	**Balimo town**	75 (61.5)	46.7 (35.8-57.8)	44.0 (33.3-55.3)	4.0 (1.1-11.1)
	Village	47 (38.5)	68.0 (53.8-79.6)	66.0 (51.7-77.8)	2.1 (0.1-11.1)
BMI categories (kg/m^2)	**Normal, (18.6-24.9)**	64 (54.2)	64.0 (51.8-74.7)	64.0 (51.8-74.7)	4.7 (1.3-12.9)
	Underweight, (≤ 18.5)	7 (6.0)	57.2 (25.0-84.2)	57.1 (25.0-84.2)	0 (0-35.4)
	Overweight/ Obese, (≥ 25)	47 (39.8)	40.4 (27.6-54.7)	34.0 (22.2-48.3)	2.1 (0.1-11.1)

6.3.4 Stratification of Cohort Characteristics by Types of STH Infections

To further analyse types of STH infections within this cohort, we examined demographics stratified by those with no, single, or multiple types of STHs infections, as presented in Table 6.2. Figure 6.1A & B visually presents the proportion of participants harbouring one or multiple STH infections and the overlap of STH infections, respectively. Participants who were considered negative for helminth infection were 29.5%. Most negative classifications were females, those over 41 years, who lived in Balimo town and were classified as overweight. Here, it was found that 32.8% were infected with only one type of helminth, predominantly *N. americanus* (Figure 6.1A&B). In contrast, 37.7% were infected with multiple helminths, with the most frequent overlap observed for *A. lumbricoides* and *N. americanus* (Figure 6.1B). All participants positive for *Ancylostoma* spp. were also positive for *N. americanus*, see Chapter 4, but were grouped as undifferentiated hookworm in this study. The percentage of participants infected with two, three or four STHs was 23.8%, 12.3% and 1.6%, respectively (Figure 6.1A). Those aged < 40 years and were male had higher rates of dual-, tri- and quad-STH infections, while mono-infections were greatest for females and those aged > 41 years. Participants living in a village had greater rates of mono- and tri-STH co-infections than participants from Balimo town, whereas higher rates of dual- and quad-STH infections were found in participants from Balimo town. When considering BMI, it was found that rates of dual- and quad-STH co-infections were highest for those with a normal BMI. Moreover, those classified as underweight and overweight had higher rates of tri- and mono-STH co-infections, respectively.

Table 6.2: Stratification of population demographics in relation to STH co-infections.

Factor	Variables	Number analysed n (%)	Negative for helminth infection % 95% CI	Mono-infection % 95% CI	Dual infection % 95% CI	Tri-infection % 95% CI	Quad-infection n (%) 95% CI
All participants		122 (100)	29.5 (22.1-38.1)	32.8 (25.1-41.5)	23.8 (17.1-32.1)	12.3 (7.6-19.3)	1.6 (0.3-5.8)
Age	**$\leq$ 40**	38 (31.4)	18.4 (9.2-33.4)	31.6 (19.1-47.5)	26.3 (15.0-42.0)	21.1 (11.1-36.3)	2.6 (0.1-13.5)
	$\geq$ 41	83 (68.6)	34.9 (25.6-45.7)	33.7 (24.5-44.4)	22.9 (15.2-33.0)	7.2 (3.4-14.9)	1.2 (0.1-6.5)
Sex	**Male**	41 (33.6)	26.8 (15.7-42.0)	31.7 (19.6-47.0)	24.4 (13.8-39.3)	14.6 (6.9-28.4)	2.4 (0.1-12.6)
	Female	81 (66.4)	30.9 (21.9-41.6)	33.3 (24.0-44.1)	23.5 (15.6-33.8)	11.1 (6.0-19.8)	1.2 (0.1-6.7)
Residency	**Balimo Town**	75 (61.5)	36.0 (26.1-47.3)	32.0 (22.5-43.2)	25.3 (16.9-36.2)	4.0 (1.1-11.1)	2.7 (0.5-9.2)
	Village	47 (38.5)	19.1 (10.4-32.5)	34.0 (22.2-48.3)	21.3 (12.0-34.9)	25.5 (15.3-39.5)	0.0
BMI categories (kg/m^2)	**Normal, (18.6-24.9)**	64 (54.2)	25.0 (16.0-36.8)	31.3 (21.2-43.4)	25.0 (16.0-36.8)	15.6 (8.7-26.4)	3.1 (0.6-10.7)
	Underweight, ($\leq$ 18.5)	7 (6.0)	14.3 (0.7-51.3)	14.3 (0.7-51.3)	14.3 (0.7-51.3)	57.1 (25.0-84.2)	0.0
	Overweight/ Obese, ($\geq$ 25)	47 (39.8)	38.3 (25.8-52.6)	36.2 (24.0-50.5)	23.4 (13.6-37.2)	2.1 (0.1-11.1)	0.0

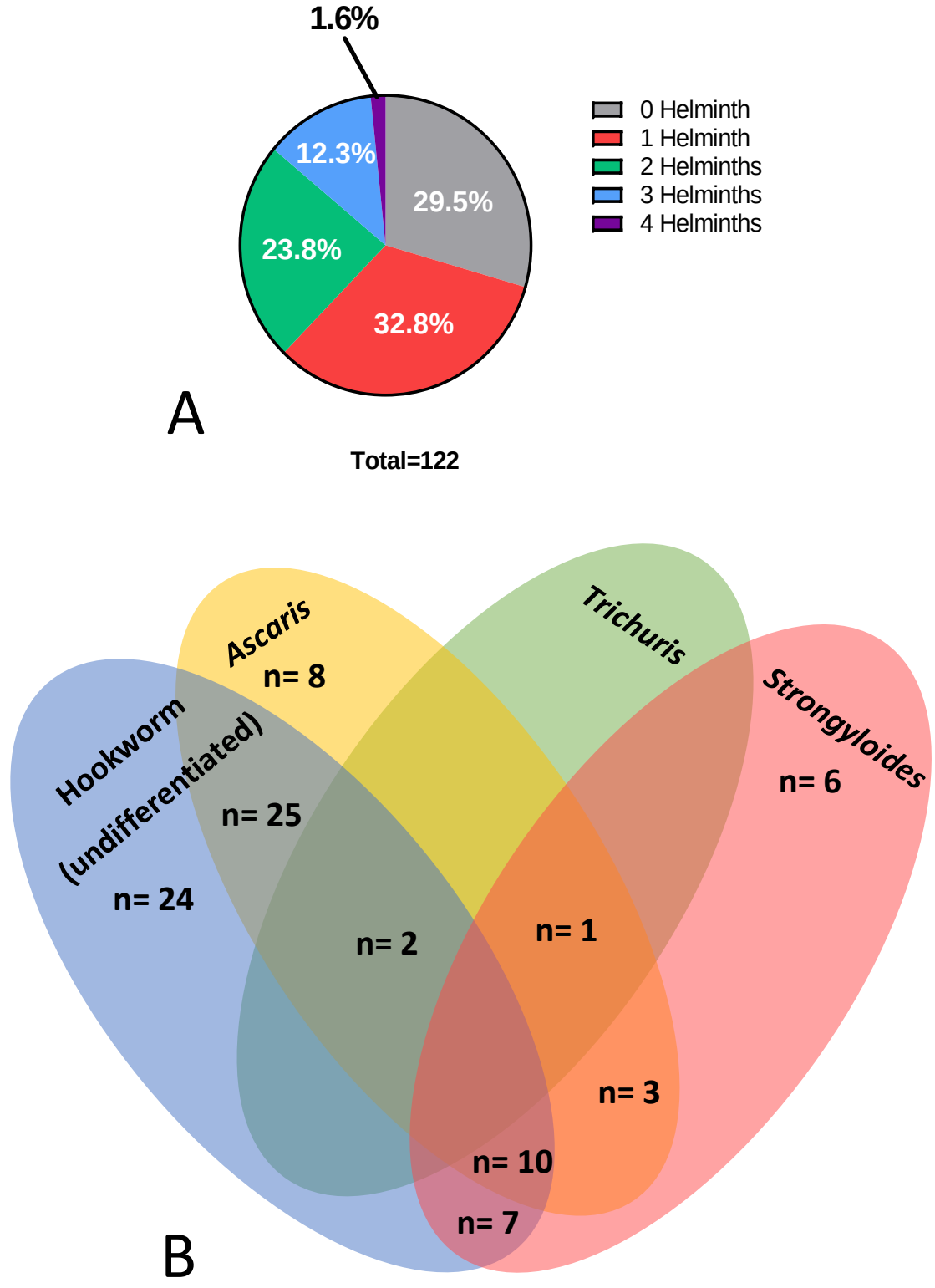

Figure 6.1: STH Polyparasitism. Pie graph depicting total number of parasites detected in each sample represented as a percentage, n= 122 (A), curated in Graphpad Prism. Venn diagram that represents polyparasitism detected in the 86 faecal samples that were positive for at least one STH, using results generated from microscopy, qPCR and serology (B), curated in Microsoft® Excel®.

6.3.5 Determining Associations Between Cohort Characteristics and STH Infections Using Univariable Logistics Regression

Potential predictor variables that could aid in identifying population characteristics that could inform STH infection status are described in Table 6.3. For the logistics regression analysis, prevalence rates of STHs below 20% were not included in the analysis due to the small sample representation. Thus, *T. trichiura* and *Ancylostoma* spp. were not included in the regression analysis.

It was found that participants from a village had greater odds of infection for any STH infection (OR = 2.4; 95% CI: 1.00-5.64; *p-value* = 0.05), particularly *A. lumbricoides* (OR = 2.1; 95% CI: 1.00-4.40; *p-value* = 0.05), undifferentiated hookworm (OR = 2.4; 95% CI: 1.14-5.23; *p-value* = 0.02) and *N. americanus* (OR = 2.5; 95% CI: 1.16-5.25; *p-value* = 0.02), compared to participants living in Balimo town. Compared to those > 41 years, those aged < 40 years had increased odds of *A. lumbricoides* (OR = 2.2; 95% CI: 1.00-4.77; *p-value* = 0.05), undifferentiated hookworm (OR = 2.3; 95% CI: 1.04-5.23; *p-value* = 0.04) and *N. americanus* (OR = 2.7; 95% CI: 1.20-6.05; *p-value* = 0.02) infection. In comparison, no change in odds for age in years was found for *Strongyloides* spp. (OR = 1.0; 95% CI: 0.94-0.99; *p-value* = 0.03). Moreover, for every kg/m^2 increase in BMI, there is a decrease in the odds of infection for any STH (OR = 0.9; 95% CI: 0.82-0.99; *p-value* = 0.03), *Strongyloides* spp. (OR = 0.9; 95% CI: 0.760.96; *p-value* = 0.01), undifferentiated hookworm (OR = 0.9; 95% CI: 0.82-0.98; *p-value* = 0.02) and *N. americanus* (OR = 0.9; 95% CI: 0.80-0.96; *p-value*= 0.004). This trend for decreased odds of infection concerning those classified as overweight is also demonstrated for undifferentiated hookworm (OR = 0.7; 95% CI: 0.18-0.83; *p-value* = 0.02) and *N. americanus* (OR = 0.3; 95% CI: 0.13-0.64; *p-value* = 0.002) infection. Interestingly, those who responded to the questionnaire about experiencing SOB have decreased odds of having any STH infection (OR = 0.3; 95% CI: 0.12-0.62; *p-value* = 0.002), particularly undifferentiated hookworm (OR = 0.3; 95% CI: 0.13-0.61; *p-value* = < 0.001) and *N. americanus* (OR = 0.3; 95% CI: 0.16-0.72; *p-value* = 0.004) compared to those who don't experience SOB. Participants who had *A. lumbricoides* infection had increased odds of also having undifferentiated hookworm (OR = 4.4; 95% CI: 1.98-9.84; *p-value* = < 0.001) and *N. americanus* (OR = 4.5; 95% CI: 2.02-9.81; *p-value* = < 0.001) co-infections.

Table 6.3: Univariable logistics regression analysis of socio-demographic characteristics and STH infections.

Group categories	Any STH infection				*A. lumbricoides*				*Strongyloides* spp.			
	OR	**95% CI**	**Std error**	***p-value***	**OR**	**95% CI**	**Std error**	***p-value***	**OR**	**95% CI**	**Std error**	***p-value***
Male sex [a]	1.2	0.52-2.81	0.427	0.65	1.1	0.51-2.33	0.390	0.84	**0.6**	**0.25-1.42**	**0.442**	**0.24***
Age group < 40 [b]	**2.4**	**0.97-6.47**	**0.478**	**0.07***	**2.2**	**1.00-4.77**	**0.399**	**0.05****	**1.7**	**0.70-4.15**	**0.453**	**0.24***
Age (years)	**1.0**	**0.95-1.00**	**0.015**	**0.17***	**1.0**	**0.95-1.01**	**0.014**	**0.11***	**1.0**	**0.94-0.99**	**0.016**	**0.03****
Residency, village [c]	**2.4**	**1.00-5.64**	**0.442**	**0.05****	**2.1**	**1.00-4.40**	**0.381**	**0.05****	1.5	0.65-3.59	0.436	0.33
No/primary education [d]	0.8	0.37-1.80	0.403	0.62	1.2	0.55-2.40	0.374	0.71	1.1	0.49-2.66	0.431	0.81
Unemployed [i]	0.8	0.36-1.90	0.427	0.65	0.8	0.37-1.70	0.389	0.55	0.9	0.37-2.21	0.451	0.79
BMI Underweight [f]	2.0	0.22-17.90	1.118	0.54	**8.2**	**0.94-72.33**	**1.109**	**0.06***	**3.7**	**0.75-18.19**	**0.815**	**0.11***
BMI Overweight [f]	0.5	0.24-1.22	0.416	0.31	0.6	0.29-1.41	0.402	0.27	**0.5**	**0.18-1.28**	**0.498**	**0.15***
BMI (continuous)	**0.9**	**0.82-0.99**	**0.048**	**0.03****	1.0	0.88-1.05	0.044	0.35	**0.9**	**0.76-0.96**	**0.059**	**0.01****
Shortness of breath (SOB)	**0.3**	**0.12-0.62**	**0.415**	**0.002****	0.7	0.34-1.57	0.387	0.43	0.6	0.25-1.54	0.469	0.30
Cough (persisted > 2 weeks)	0.8	0.38-1.91	0.410	0.68	0.8	0.36-1.67	0.388	0.52	0.8	0.33-1.96	0.457	0.62
A. lumbricoides co-infection	-	-	-	-	-	-	-	-	**2.0**	**0.87-4.78**	**0.436**	**0.10***
T. trichiura co-infection	-	-	-	-	ǂ	ǂ	ǂ	ǂ	1.7	0.15-19.52	1.244	0.67
Strongyloides spp. co-infection	-	-	-	-	**2.0**	**0.87-4.78**	**0.436**	**0.10***	-	-	-	-
Hookworm (undifferentiated)	-	-	-	-	**4.4**	**1.98-9.84**	**0.409**	**<0.001****	1.7	0.69-3.96	0.445	0.26
N. americanus co-infection	-	-	-	-	**4.5**	**2.06-10.09**	**0.403**	**<0.001****	**1.9**	**0.79-4.49**	**0.445**	**0.16***
Ancylostoma spp. co-infection	-	-	-	-	**4.7**	**0.47-46.52**	**1.170**	**0.15***	**11.2**	**1.11-111.96**	**1.176**	**0.04****

Std error: Standard error
ǂ: Could not fit a logistics regression. Either due to perfect separation or because one or more of the predictors are linearly dependent.
*: *p-value* $\leq$ 0.25
**: *p-value* $\leq$ 0.05
OR: odds ratio

Reference categories:
[a] Sex, Female
[b] Age group > 41 years
[c] Residency, Balimo town
[d] Secondary education
[i] Employed
[f] BMI normal

Table 6.3: Univariable logistics regression analysis of socio-demographic characteristics and STH infections (continued).

Group categories	Hookworm (undifferentiated)				*N. americanus*			
	OR	**95% CI**	**Std error**	***p-value***	**OR**	**95% CI**	**Std error**	***p-value***
Male sex [a]	0.9	0.44-1.97	0.385	0.84	0.9	0.44-1.97	0.384	0.846
Age group < 40 [b]	**2.3**	**1.04-5.23**	**0.412**	**0.04****	**2.7**	**1.20-6.05**	**0.413**	**0.02****
Age (years)	1.0	0.96-1.01	0.013	0.29	**1.0**	**0.96-1.01**	**0.013**	**0.18***
Residency, village [c]	**2.4**	**1.14-5.23**	**0.389**	**0.02****	**2.5**	**1.16-5.25**	**0.386**	**0.02****
No/primary education [d]	1.0	0.48-2.04	0.370	0.97	1.0	0.51-2.16	0.368	0.90
Unemployed status [e]	1.1	0.51-2.30	0.385	0.84	1.1	0.51-2.29	0.384	0.85
BMI Underweight [f]	0.7	0.15-3.64	0.807	0.72	0.7	0.15-3.64	0.807	0.72
BMI Overweight [f]	**0.7**	**0.18-0.83**	**0.395**	**0.02****	**0.3**	**0.13-0.64**	**0.403**	**0.002****
BMI (continuous)	**0.9**	**0.82-0.98**	**0.046**	**0.02****	**0.9**	**0.80-0.96**	**0.048**	**0.004****
Shortness of breath (SOB)	**0.3**	**0.13-0.61**	**0.394**	**< 0.001****	**0.3**	**0.16-0.72**	**0.390**	**0.004****
Cough (persisted > 2 weeks)	0.8	0.40-1.78	0.378	0.66	0.9	0.41-1.80	0.377	0.68
A. lumbricoides co-infection	**4.4**	**1.98-9.84**	**0.326**	**< 0.001****	**4.5**	**2.02-9.81**	**0.403**	**< 0.001****
T. trichiura co-infection	1.7	0.15-18.83	1.239	0.68	1.8	0.16-20.83	1.238	0.62
Strongyloides spp. co-infection	1.7	0.69-3.96	0.445	0.26	1.9	0.79-4.49	0.445	0.16*
Hookworm (undifferentiated)	-	-	-	-	-	-	-	-
N. americanus co-infection	-	-	-	-	-	-	-	-
Ancylostoma spp. co-infection	-	-	-	-	ǂ	ǂ	ǂ	ǂ

Std error: Standard error

ǂ: Could not fit a logistics regression. Either due to perfect separation or because one or more of the predictors are linearly dependent.

*: *p-value* $\leq$ 0.25

**: *p-value* $\leq$ 0.05

OR: odds ratio

Reference categories:

[a] Sex, Female

[b] Age group > 40 years

[c] Residency, Balimo town

[d] Secondary education

[i] Employed

[f] BMI normal

6.3.6 Determining Associations Between Socio-Demographic Characteristics and STH Infections Using Multivariable Logistics Regression Analysis

Candidate variables identified in the univariable logistics regression for predicting various STH infections were applied in an adjusted model, as presented in Table 6.4. Diagnostics for collinearity did not indicate the need for removing or adjusting any variables. The goodness-of-fit models and predictive value assessments were satisfactory but could be improved. Appendix M has the full assessment of model appropriateness for each STH model used in this analysis.

After controlling for covariates, the adjusted model revealed that for every year increase in age, the odds of *Strongyloides* spp. infection decreased by 10% (aOR = 0.9; 95% CI 0.89-0.99; *p-value* = 0.03). Additionally, the adjusted model for *N. americanus* indicates higher odds of infection for those aged < 40 years (aOR = 7.9; 95% CI: 1.21-51.90; *p-value* = 0.03). When re-grouping the age categories through further separation of those aged > 40 years old, as seen in Figure 6.2, we observe a downward trend of the *Strongyloides* spp. prevalence rate, reaching 0% for those > 64 years of age. In contrast, for *N. americanus*, there is no obvious trend for age distribution across these categories, but infection rates appear to be higher in those < 39 and > 64 years old when compared to those aged between 40 and 63 years old.

Living in a village was associated with an almost three times higher odds of undifferentiated hookworm (aOR = 2.7; 95% CI: 1.04-7.13; p-value = 0.04) and *N. americanus* (aOR = 2.7; 95% CI: 1.04-7.27; *p-value* = 0.04) infection, compared to residing in Balimo town, as depicted in Figure 6.3. Underweight individuals had lower odds of *N. americanus* infection than those with a normal BMI (aOR = 0.04; 95% CI: 0.003-0.67; *p-value* = 0.03), as shown in Figure 6.3. After controlling for covariates found in the univariable analysis, those who reported experiencing SOB had 80% decreased odds of having STH infection (aOR = 0.2; 95% CI: 0.08-0.52; p-value = < 0.001). This trend was similar for those with undifferentiated hookworm (aOR = 0.3; 95% CI: 0.10-0.67; p-value = 0.005) and *N. americanus* infection (aOR = 0.4; 95% CI: 0.14-0.98; *p-value* = 0.05), as seen in Figure 6.3. There was a 4.1-fold increase in the odds of having an undifferentiated hookworm infection for those co-infected with *A. lumbricoides* (aOR = 4.1; 95% CI: 1.66-10.16; *p-value* = 0.002). Similarly, a vice-versa trend was observed between *A. lumbricoides* and undifferentiated hookworm (aOR = 4.7; 95% CI: 1.74-12.59; *p-value* = 0.002). A similar trend was found for those infected with *N. americanus,* indicating enhanced odds of *A. lumbricoides* co-infection (aOR = 4.7; 95% CI: 1.75-12.78; *p-value* = 0.002).

Table 6.4: Multiple logistics regression of socio-demographic characteristics and STH infections.

Group categories	Any STH infection				*Ascaris* spp.				*Strongyloides* spp.			
	aOR	**95% CI**	**Std error**	***p-value***	**aOR**	**95% CI**	**Std error**	***p-value***	**aOR**	**95% CI**	**Std error**	***p-value***
Male sex [a]	1.1	0.43-3.08	0.506	0.79	0.9	0.34-2.15	0.470	0.74	1.8	0.69-4.89	0.500	0.22
Age group < 40 [b]	2.5	0.48-13.41	0.848	0.27	1.7	0.34-8.0	0.802	0.53	0.2	0.03-1.32	0.939	0.10
Age (years)	1.0	0.94-1.05	0.027	0.77	1.0	0.95-1.05	0.025	0.94	**0.9**	**0.89-0.99**	**0.030**	**0.03****
Residency, village [c]	2.7	0.97-7.24	0.512	0.06	1.4	0.58-3.44	0.453	0.44	-	-	-	-
BMI Overweight [d]	-	-	-	-	1.0	0.42-2.58	0.461	0.93	2.3	0.40-13.37	0.894	0.35
BMI Underweight [d]	-	-	-	-	7.9	0.69-90.78	1.244	0.10	1.2	0.10-13.62	1.243	0.89
BMI (continuous)	1.0	0.86-1.07	0.056	0.46	-	-	-	-	0.8	0.64-1.05	0.126	0.11
Shortness of breath (SOB)	**0.2**	**0.08-0.52**	**0.478**	**< 0.001****	-	-	-	-	-	-	-	-
A. lumbricoides co-infection	-	-	-	-	-	-	-	-	-	-	-	-
Strongyloides spp. co-infection	-	-	-	-	1.4	0.50-3.82	0.519	0.53	-	-	-	-
Hookworm (undifferentiated) co-infection	-	-	-	-	**4.1**	**1.66-10.16**	**0.463**	**0.002****	1.6	0.57-4.70	0.538	0.36
N. americanus co-infection	-	-	-	-	-	-	-	-	1.5	0.52-4.39	0.545	0.45

Std error: Standard error
**: *p-value* $\leq$ 0.05
aOR: adjusted odds ratio

Reference categories:
[a] Sex, Female
[b] Age group > 40 years
[c] Residency, Balimo town
[d] BMI normal

Table 6.4: Multiple logistics regression of socio-demographic characteristics and STH infections (continued).

Group categories	Hookworm (undifferentiated)				*N. americanus*			
	aOR	**95% CI**	**Std error**	***p-value***	**aOR**	**95% CI**	**Std error**	***p-value***
Male sex [a]	0.7	0.25-1.72	0.494	0.39	0.5	0.18-1.40	0.526	0.19
Age group < 40 [b]	3.1	0.98-9.79	0.588	0.06	**7.9**	**1.21-51.90**	**0.959**	**0.03****
Age (years)	-	-	-	-	1.0	0.97-1.09	0.029	0.30
Residency, village [c]	**2.7**	**1.04-7.13**	**0.490**	**0.04****	**2.7**	**1.04-7.27**	**0.497**	**0.04****
BMI Overweight [d]	0.9	0.19-4.50	0.813	0.91	0.6	0.12-3.32	0.842	0.59
BMI Underweight [d]	0.1	0.004-1.01	1.383	0.06	**0.04**	**0.003-0.67**	**1.418**	**0.03****
BMI (continuous)	0.9	0.71-1.10	0.111	0.28	0.9	0.71-1.11	0.116	0.3
Shortness of breath (SOB)	**0.3**	**0.10-0.67**	**0.484**	**0.005****	**0.4**	**0.14-0.98**	**0.499**	**0.05****
A. lumbricoides co-infection	**4.7**	**1.74-12.59**	**0.505**	**0.002****	**4.7**	**1.75-12.78**	**0.508**	**0.002****
Strongyloides spp. co-infection	-	-	-	-	1.6	0.48-5.31	0.613	0.45

Std error: Standard error
**: *p-value* $\leq$ 0.05
aOR: adjusted odds ratio

Reference categories:
[a] Sex, Female
[b] Age group > 40 years
[c] Residency, Balimo town
[d] BMI normal

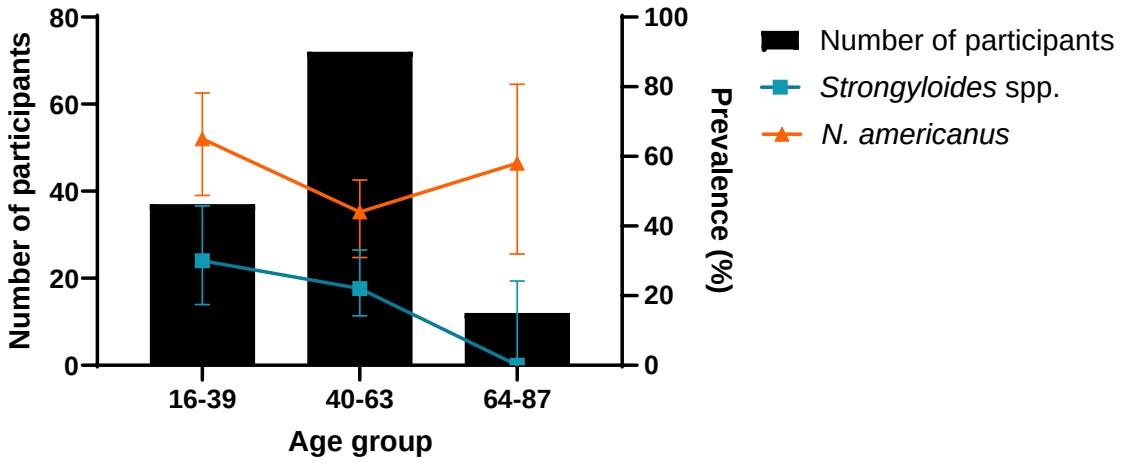

Figure 6.2: Prevalence of *N. americanus* and *Strongyloides* spp. distributed by age categories. Error bars report a 95% CI.

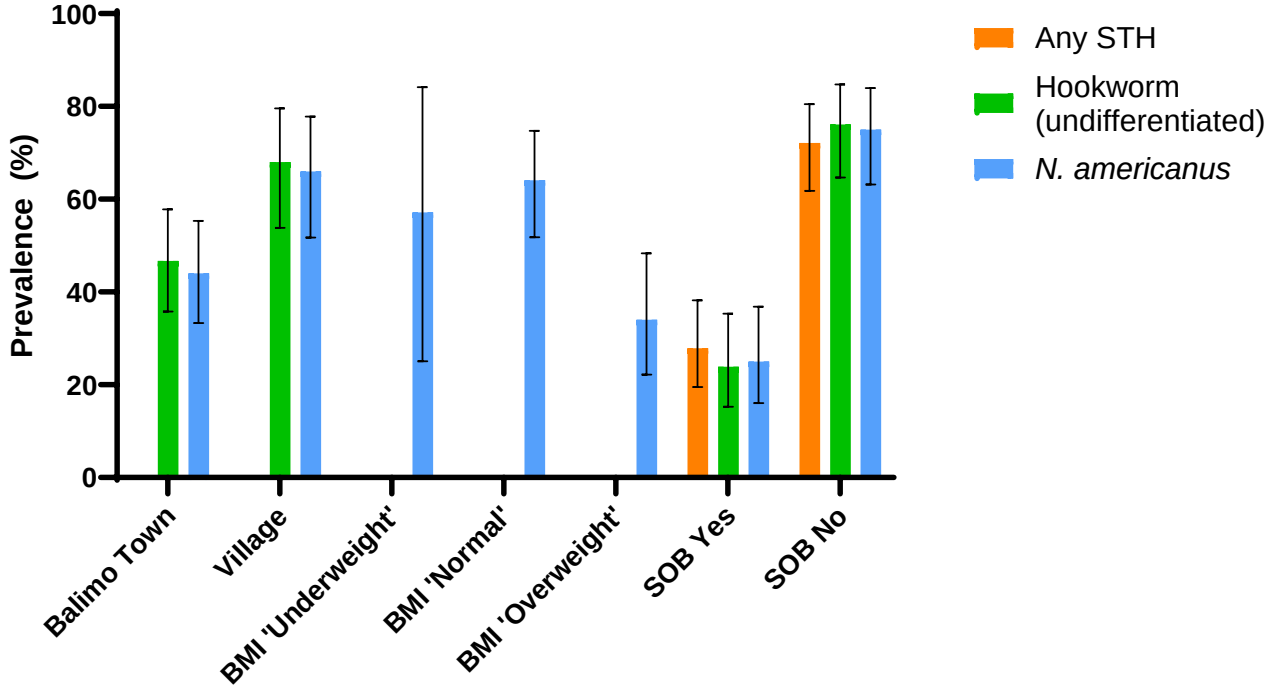

Figure 6.3: Prevalence distribution of any STH undifferentiated hookworm and *N. americanus* by candidate variables from multivariable logistics regression. Helminth infection is stratified by Balimo (n = 75) and village (n = 47); body mass indices (BMI) as categories (underweight, (n = 7) normal (n = 64) and overweight (n = 47)); and shortness of breath (SOB) by 'yes' (n = 45) or 'no' (n = 77) categories. Error bars report a 95% CI.

6.4 Discussion

At the time of this study, there was limited published data on the prevalence of STHs, particularly in rural communities in the Western Province of PNG. Further, there was a lack of epidemiological information regarding the demographic and clinical characteristics of individuals with these infections in this region. This current study aimed to provide an overall prevalence rate of STHs, including *Strongyloides* spp., in the rural Balimo region of PNG. Using this prevalence data, we determined the characteristics of individuals with STHs to identify potential determinants of STH infection within this community.

Overall, the prevalence rate of STH infections from this community in the Balimo region was 70.5%. This prevalence rate was determined from results generated by a combination of diagnostic tools described in previous chapters. *N. americanus* was the most frequently detected helminth, followed by *A. lumbricoides*. According to our results, almost 38% of participants are infected with more than two different types of STHs, the most frequent co-infection being the overlap occurring for *A. lumbricoides* and hookworm (undifferentiated). Contextualisation of these findings have been discussed in previous Chapters, but the reported prevalence rate in this community was higher than combined prevalence rates found in indigenous minority communities in the Southeast Asian and Western Pacific regions, reported at ~61.4% (Gilmour, Alene & Clements 2021).

In the current study, age was considered an indicator of specific STH infections, with a greater proportion of participants from < 40 years of age having *A. lumbricoides*, *Strongyloides* spp., *T. trichiura* and *N. americanus* infections compared to those > 41 years of age. A similar trend was also observed for those with multiple concurrent STH infections. Once controlling for covariates in the final logistics regression model, the results suggest enhanced odds of *N. americanus* infection for those < 40 years, compared to those > 41 years of age. Furthermore, for *Strongyloides* spp. we found a 10% reduction in the odds of infection for every year increase in age.

A study conducted on Karkar Island, PNG, almost two decades ago found that the prevalence of hookworm infection increased with age and was not sex-dependent, reaching a peak in the 16–20-year group (Pritchard et al. 1990). Furthermore, a recent survey conducted in Ghana, Africa, found that younger age groups have a significantly higher hookworm infection rate than older age groups (Adu-Gyasi et al. 2018), consistent with the findings presented in the current study. Interestingly, our findings are inconsistent with other studies published in Southeast Asian regions, whereby hookworm prevalence was found to peak in the 20-29 age group and remain high across older age groups (Dunn et al. 2016; Kache et al. 2020). The prevalence of *Strongyloides* spp. infection has been

demonstrated in other studies to increase with age (Forrer et al. 2018; Van De et al. 2019), which is contradictory to the results from the current study. It is speculated that without treatment for strongyloidiasis, it can be sustained chronically within the host through its unique lifecycle ability of "autoinfection", leading to life-long infection (Van De et al. 2019). Thus, finding an inverse relationship between *Strongyloides* infection rates and age is unexpected, considering treatment for strongyloidiasis within this community does not occur. An observable trend also demonstrated that participants aged > 64 years did not have strongyloidiasis in this cohort. It is plausible that an insufficient sample size could influence this result. However, we must also consider that the ability to mount or sustain a memory immune response could be compromised in aging populations, potentially causing a decrease in IgG titres influencing the anti-*Strongyloides* IgG ELISA results in this study. A similar trend for a reduction in *Strongyloides* spp. IgG titres and increased age has been described (Atkins et al. 1997), but this study did not elaborate on these findings. Alternatively, in PNG, two species of *Strongyloides* spp. can parasitise humans, *S. stercoralis* and *S. f. kellyi*. Historical studies done in PNG have reported a decreasing trend for strongyloidiasis and intensity of infection with increasing age, particularly for *S. f. kellyi* (Barnish & Ashford 1989; King & Mascie-Taylor 2004; Shield et al. 1987). However, whether the IgG ELISA used to detect strongyloidiasis can also detect other coexisting STHs and Strongyloides species is not known. Further investigations are required to understand this relationship in this community. Additionally, issues with assay cross-reactivity with other co-existing helminth species could also occur, resulting in false positives, as discussed in previous Chapters.

It was found in this study that the prevalence rate of STHs, except for *Ancylostoma* spp., was greater for those residing in a village than in Balimo town. Furthermore, a greater proportion of mono- and tri-STH infections were observed for those living in a village. Interestingly, those residing in Balimo town had higher dual- and quad-infection rates. It was identified in the adjusted logistics regression model that those living in a village were associated with higher odds of undifferentiated hookworm and *N. americanus* infection compared to those living in Balimo town. The overarching trend of lower STH prevalence in urban-like areas (Balimo town) and higher rates in rural/remote type areas (villages) has already been established in the literature and is consistent with this current study's observations (Abera et al. 2013; Alelign, Degarege & Erko 2015; Kattula et al. 2014; Silver et al. 2018; Witek-McManus et al. 2021). However, categorising communities as urban, peri-urban or rural alone is insufficient. Instead, investigations now focus on identifying specifics on housing infrastructure and environmental and behavioural risk factors within at-risk communities to inform targeted control measures. In the Balimo region, villagers with subsistence lifestyles have increased interactions with their environment, potentially explaining their higher hookworm infection rates.

Factors such as walking barefoot (Jiraanankul et al. 2011), poor sanitation and hygiene (Punsawad et al. 2018), an agricultural or animal-related occupation (Agustina et al. 2022), inaccessibility to latrines (Jin et al. 2021; Le et al. 2022) and limited access to safe sources of drinking water have all been linked to STH infections (Chin et al. 2016; Zeynudin et al. 2022) and polyparasitism (Elyana et al. 2016). This current study did not explore the variables mentioned previously to assess differences in village and town dynamics, but future investigations should incorporate questions related to water, sanitation and hygiene, which are suitable for this community in PNG to gain additional insights into potential risk factors for STH infection.

It was revealed in the adjusted logistics regression model that underweight individuals had lower odds of *N. americanus* infection, suggesting that hookworm-related infections are not responsible for those classified as underweight in this cohort. Previous studies have suggested a potential association between STH infection and undernutrition/malnutrition, particularly in children, but causal relationships have not been established (Cossa-Moiane et al. 2022; Deka, Sangeeta, Kalita & Hazarika 2022; Guan & Han 2019; Rajoo et al. 2017). Conversely, emerging evidence shows that STH infections may have minimal to no impact on nutritional status (Campbell et al. 2016; Djuardi et al. 2021; Fauziah et al. 2022). However, the findings of this study should be interpreted with caution due to the small sample size and the use of an indirect measure of nutritional status. The temporal causation of being underweight cannot be determined based on the current study design.

Interestingly, in the final regression model, individuals reporting SOB had decreased odds of STH infection, specifically hookworm (undifferentiated) and *N. americanus*. This indicates that there is potentially an alternative explanation of the causes of SOB in this community and could be related to other intersecting community morbidities such as TB (Diefenbach-Elstob et al. 2018) and obstructive pulmonary disorders (Wang et al. 2022). Nevertheless, the results obtained from this study could potentially give rise to speculation that individuals infected with hookworm may have a reduced likelihood of experiencing symptoms of SOB. Lung migrating helminths like *Ascaris*, *Strongyloides*, and hookworm are known to cause some lung-related pathologies, including eosinophilic asthma/pneumonia or Loeffler's syndrome, exhibiting chronic obstructive lung-like pathologies resulting in overall lower lung function. Some cases of strongyloidiasis can cause acute respiratory failure and pulmonary haemorrhaging (Chen et al. 2023; Chitkara & Krishna 2006; Magalhães et al. 2021). Previous meta-analyses have inferred that hookworm infection was strongly associated with a reduction in asthma prevalence proportional to infection intensity (Leonardi-Bee et al. 2006). However, it remains a contentious topic (Arrais et al. 2021). An accurate assessment of infection intensity was not possible with this current study design but should be considered in future investigations as the extent of certain morbidities caused by STHs are often found to be proportional

to infection intensity (Campbell et al. 2016; Gyorkos et al. 2011; Turner et al. 2008). It is recommended in future investigations to also include quantifiable, objective measures of SOB, such as spirometry, rather than relying on self-reported cases.

Descriptions of the rate of STH co-infections are neglected in epidemiological investigations (Chin et al. 2016). Complications associated with multiple parasite infections are being increasingly investigated in the literature, which has been shown to influence immunity (M'Bondoukwé et al. 2022), potentially impacting other co-morbidities such as TB (Donohue, Cross & Michael 2019). Overlap of *A. lumbricoides* and *N. americanus* co-infection were significantly associated in this study. Given the differing transmission dynamics of these worms, this finding indicates faecal-contaminated environments (Echazú et al. 2015).

This Chapter explored the characteristics of a cohort in the Balimo region of PNG with STH infection. However, there are certain limitations to acknowledge within this study. Firstly, the sample size was small, and increasing it would enhance the statistical power and reliability of the findings. The multivariable models showed a weak relationship between independent variables (demographic and clinical characteristics) and the outcome variables (STH infection), explaining less than 20% of the variation. This suggests the existence of unaccounted-for factors or variables that were not included in this study but could be considered in future studies. Including relevant variables such as retrospective environmental data and water, sanitation, and hygiene-related behaviours could increase the explained variation in the model, as these variables have shown strong correlations to STH infection (Campbell et al. 2017; Le et al. 2022; Magalhães et al. 2011). Additionally, the demographic composition of the participants may not represent the broader population, and recruiting representative groups would improve the generalisability of our findings. Selecting an appropriate statistical model is critical to identifying potential risk factors accurately, and alternative techniques like Bayesian networks could capture complex relationships and improve the model's predictive value (Aw et al. 2021). Assessing micronutrient biomarkers from blood samples could provide more meaningful data on nutritional status, considering their known associations with helminth infections (Arinola et al. 2015; Mehata et al. 2022). These associations have also been identified in previous STH surveys in PNG (Shield et al. 1986; Shield et al. 1981) and should be considered for future investigations.

6.5 Conclusion

The primary aim of this chapter was to identify the demographic and clinical characteristics of individuals with STH infections and identify potential determinants of infection. The overall prevalence of STH infection in Balimo, Western Province, PNG, was 70.5%, with almost 38% of this cohort infected with multiple types of STHs. Age, village residence, *A. lumbricoides* co-infection, BMI and SOB were associated with *N. americanus* infection, while age was significantly associated with *Strongyloides* spp. infection. Understanding the baseline prevalence and characteristics of individuals at risk of STH infections can inform targeted public health interventions. Future studies should investigate further the impact of STH infection on health implications such as nutrition and lung-related morbidities and include culturally appropriate questionnaires with sanitation-related variables for a more focused approach to characterising individuals with STH infections.

7 General Discussion

The data presented in this thesis investigated the epidemiology of soil-transmitted helminthiasis in Balimo, Western Province, PNG. Prevalence data was obtained using established diagnostic tools and included microscopy and qPCR to detect STH infection and serology to determine the presence of strongyloidiasis within this community. Additionally, we compared the utility of these diagnostic tools for STH detection.

The prevalence rate of STHs in this cohort of participants from PNG (70.5%) is among the highest reported in the WHO-defined Western Pacific region, *N. americanus* was the most frequently detected, followed by *A. lumbricoides, Strongyloides* spp., *Ancylostoma* spp., and *T. trichiura.* These elevated rates within a small subset of this community perhaps reveals extensive community-wide worm burden, but their impacts on health outcomes in this community were unexplored in this study. It is important to consider the health implications of helminths as they contribute significantly to the global disease burden, causing approximately 1.97 million DALYs per year (Institute for Health Metrics and Evaluation 2019). STHs can negatively impact maternal health and pregnancy outcomes (Aderoba et al. 2015; Arinola et al. 2015; Bolka & Gebremedhin 2019) and impact the nutritional health of children (Mehata et al. 2022), which can adversely affect childhood development, including physical growth and cognitive development (Blouin et al. 2018; Deka, Kalita & Hazarika 2022).

Additionally, our study found that nearly 38% of this cohort have helminth co-infections, with at least two individuals infected with four different types of STHs. The most frequent overlaps of STHs infections were found with *N. americanus*, *A. lumbricoides* and/or *Strongyloides* spp.. Helminth co-infections have been found to amplify infection-related morbidities such as anaemia and micronutrient deficiencies (Donohue, Cross & Michael 2019). In addition, there is evidence to suggest that infection with STHs may influence the outcome of other concurrent infections, including malaria, HIV and TB (Mabbott 2018), all of which are significant public health concerns in PNG (Cleary et al. 2021; Diefenbach-Elstob et al. 2019; Pham et al. 2022). A meta-analysis also identified an additive effect related to the extent of these morbidities in individuals infected with multiple types of helminths, which could be proportional to increasing helminth load (Donohue, Cross & Michael 2019). However, the findings regarding these interactions are conflicting and complex in the literature, and it is unknown whether STHs play a role in influencing disease outcomes in PNG.

The high STH burden may allude to public health issues associated with poor access to healthcare services, compromised water sources and poor sanitation and hygiene practices, which are known to influence STH prevalence (Campbell et al. 2017; Echazú et al. 2015; Ercumen et al. 2019; Le et al.

2022). These factors were not investigated in this study, although factors related to WaSH have been previously highlighted in PNG as significant issues in other studies (Horwood, Barrington & Greenhill 2013). While this small-scale study did not target at-risk individuals such as children and women of reproductive age, the high prevalence rate exceeds the WHO's recommended STH threshold rate of communities to administer anti-helminthic drugs. Emphasising a need to conduct nationwide surveys and implement effective intervention strategies, advocating for improved infrastructure and access to healthcare and sanitation facilities (WHO 2017, 2020a).

Additionally, our findings identified several relationships between STH infection status and the demographic characteristics of this cohort. The multivariable logistics regression model identified that for every year older a person is, the less likely they are to be indicative of strongyloidiasis in this cohort. Individuals with a low BMI and were aged < 40 years had reduced odds of having *N. americanus* infection. If an individual was infected with hookworm (specifically *N. americanus*), they also had a greater likelihood of having *A. lumbricoides* co-infection and vice versa. Although this data is exploratory, it identifies characteristics that could be targeted for future health surveys and subsequent public health interventions. Importantly, it may also provide insight into individuals who demonstrate protective characteristics, allowing programmatic intervention designers to harness practices that could alleviate infection within this community. Unexpectedly, a relationship was identified between STH infection and a decrease in self-reported SOB. This finding could have implications regarding the lung health of individuals within this community. Hookworms, *A. lumbricoides,* and *S. stercoralis* all require lung migration to facilitate the completion of their lifecycle, providing a plausible mechanism for mechanical disruption to lung tissue that could influence normal physiological and immunological function (Weatherhead et al. 2020). Currently, there is limited research surrounding the implications of lung-migrating helminths, particularly regarding whether helminths enhance susceptibility to other concurrent respiratory pathogens within endemic communities (Yap & Gause 2018) or attribute to the development of various respiratory-related morbidities such as asthma, emphysema or chronic obstructive pulmonary disorders in humans (Weatherhead et al. 2020). In contrast, there is extensive research investigating the therapeutic benefits of helminth infections on allergies, including allergic asthma (Logan et al. 2018), and treatment for various inflammatory autoimmune diseases, including inflammatory bowel disease, Crohn's disease and multiple sclerosis (Loukas, Maizels & Hotez 2021).

In resource-limited settings, selecting a suitable diagnostic tool appropriate for prevalence surveys is critical. The chosen tool should be sensitive enough to accurately assess infection prevalence and be cost-effective, requiring minimal resources, infrastructure and sophisticated equipment (Mbong Ngwese et al. 2020). As a secondary objective to this thesis, our current study found that

microscopy-based techniques and IgG serodiagnosis were suitable for detecting *A. lumbricoides* and *Strongyloides* spp., respectively, which was found superior to qPCR methods. Conversely, qPCR demonstrated advantages over microscopy in detecting hookworm and *T. trichiura* infections. Previous research in medium to high STH prevalence settings has shown comparable prevalence rates between molecular methods and coproscopy (Cools et al. 2019; Knopp et al. 2014), demonstrating no advantage to using molecular-based tools**.** In Balimo, given that the prevalence rate of STHs is so high, these tools were comparable. Economically, harnessing microscopy and serological-based methods for STH and *Strongyloides* spp. detection, respectively, would be most appropriate. However, in settings with low prevalence rates and lower transmission, typically after repeated rounds of mass administration of anthelminthic drugs, more sensitive methods like molecular-based tools may be necessary (Nikolay, Brooker & Pullan 2014). Based on this study's findings, conducting community-wide assessments of STH infection through coproscopic-based methods, with minor adjustments to sample processing procedures, would be the most practical and economical approach if future surveys were undertaken in this region. Additionally, serodiagnosis of strongyloidiasis can play a role in screening the population and identify individuals who require further confirmatory testing. Although implementing PCR to detect STHs in routine diagnostic practice in rural communities like Balimo and other provincial hospitals is currently not feasible, we included PCR in our study to ensure a robust detection technique to assess the prevalence and to evaluate the potential future diagnostic value of PCR in this setting.

This study used a comprehensive approach combining epidemiological investigations, data collection using multiple diagnostic methods, and evaluating the practicality of these diagnostic tools from samples derived from a rural and resource-limited laboratory setting. This approach generated sound data to thoroughly assess the prevalence and determinants of STH infections within a subset of the population in the Balimo region of the Western Province in PNG. Moreover, we provide an up-to-date snapshot of STH prevalence that sheds light on factors influencing individual infection likelihood. It is worth noting that this is the first STH study conducted in a rural community in the Western Province of PNG in over two decades to report its findings. The findings highlight the high burden of STH infections faced by the community in Balimo, which underpins existing public health challenges. Using multiple diagnostic tools to detect STH infection in this study allowed reliable determination of prevalence rates close to reaching the true prevalence within this cohort. Furthermore, this study was the first to report on using molecular-based methods such as qPCR to assess STH prevalence in PNG. The qPCR TaqMan assays and target DNA sequences used for the main STHs (excluding *Strongyloides* spp.) have been validated (Azzopardi et al. 2021; Llewellyn et al.

2016), widely accepted within the field and used in numerous studies (Clarke et al. 2018; Le et al. 2022; Vaz Nery et al. 2019)

A limitation of this study is the small sample size and limited geographical scope, which restricts the generalisability of the findings to the wider community and hinders predictive estimates of STH distribution in the region. Convenience sampling introduced a potential bias, affecting the relationships observed, as it is non-probability based. Furthermore, the demographic characteristics of the sampled cohort were also not representative of the national population, again limiting the generalisability of the infection determinants used in the analysis. The logistics regression model may not have accounted for all confounding factors, introducing potential bias and impacting the interpretation of results. Some variables used in the logistics regression model were subjective and prone to errors, such as self-reported SOB and crude measurements of BMI, respectively.

Examining only one faecal specimen limits the sensitivity of microscopy, influencing the performance of the diagnostic tools evaluated in this study. Detecting low-intensity helminth infections or helminths with intermittent shedding can be challenging to detect with one specimen (Liu et al. 2017). Another shortcoming of the study design was the delay in sample processing and examination of faecal specimens. It may have had a profound impact on the visibility of hookworm ova in faecal material, as hookworm ova is prone to degradation (Dacombe et al. 2007), which would have affected the accuracy of the diagnostic evaluations made. The inclusion of coproculture methods to detect *Strongyloides* spp. would have strengthened our study design and facilitated a better comparative assessment to understand the discrepancies between coprological-based methods, serology and qPCR. Particularly given that coproculture allows for the cultivation and identification of *Strongyloides* spp. larvae from faecal material. If other species were present within this community, they may have been identified via this technique.

Our study is the first to report the prevalence of STHs from a rural community in PNG in almost two decades, revealing a high prevalence and high burden of STHs in PNG, highlighting the importance of performing a similar study in other regions to assess the external validity of this study. Expanding the survey to encompass the entire community would enhance the reliability and generalisability of the results. This could include building upon local capacity and engaging other communities to partake in the survey, which could allow for analysis of spatial distribution and regional variation of STHs within PNG. Such extensions to this study could also identify geographical and cultural differences that have been shown to influence infection distribution (Owada et al. 2018; Vaz Nery et al. 2019) and could also include targeted questionnaires with culturally grounded questions about lifestyle and values surrounding WaSH-related parameters and the inclusion of local environmental data (i.e.,

rainfall, vegetation coverage, soil type, etc) (Campbell et al. 2017; Le et al. 2022). Including these variables would also enrich the predictive power of the logistics regression models, strengthening the identification of risk factors related to the outcome of STH infections. Understanding these factors is necessary to design impactful and effective targeted interventions (WHO 2020c).

It is recommended that future studies explore the health implications of STHs infections using objective and quantifiable measurements. Relying on self-reported clinical symptoms like SOB and crude measurements of biometric factors like BMI may lead to imprecise results. Objective measures could include spirometry to assess lung function, providing valuable insights into the potential impact of STH infections on respiratory health. Furthermore, assessing various nutritional blood biomarkers associated with STH infections can help elucidate the relationship between helminth infections and nutritional health outcomes. Incorporating more accurate methods to quantify helminth infection intensity, such as improved techniques like qPCR for enumerating ova in faecal material, similar to what has been performed previously (Azzopardi et al. 2021; Zendejas-Heredia et al. 2021). Enumerating helminth infection intensity would allow for exploration of STH burden and health implications, which is essential to understanding whether these infections contribute to poor health outcomes in this community.

An unexplored aspect in this community was the prevalence of other intestinal parasites, such as protozoa. Human intestinal protozoan diseases (i.e., giardiasis, entamoebiasis, blastocystosis and cryptosporidiosis) are likely to overlap as both STH and protozoa share a faecal-oral transmission dynamics and predisposing infection risk factors related to WaSH (Efstratiou, Ongerth & Karanis 2017). It is estimated that intestinal protozoan infections impact 3.5 billion people worldwide, with outbreaks causing 1.7 billion cases of diarrhoea each year, classifying them as significant concerns to public health (Efstratiou, Ongerth & Karanis 2017). Previously published literature has described the presence of intestinal protozoa in PNG (Ashford & Atkinson 1992; Phuanukoonnon et al. 2013), but alike STHs, information regarding the distribution of infection is unknown and underrecognized. Given the extent of STH burden within this community, it would be recommended to assess the burden of protozoa in conjunction with STHs, which would simultaneously contribute to understanding the various aetiologies of childhood diarrhoea in PNG (Horwood & Greenhill 2013).

Now that we have described the epidemiology of STH in Balimo, Western Province, in PNG, identifying the ramifications of STH infections within this community will be the next pertinent step. Particularly concerning the overlap of STHs and other chronic infections of significant public health concern, such as TB, which is known to have a high presence in PNG and the Balimo region

(Diefenbach-Elstob et al. 2018; Diefenbach-Elstob et al. 2019). Indicating that not only are both infections likely to co-exist within PNG, but they are also likely to occur within the same individual.

Humans have had close interactions with helminths for hundreds of thousands of years. As a result of these close interactions, helminths have evolved specialised environmental and host niches that allow for their perpetuation and chronic persistence within their hosts. This ability for helminths to sustain chronic infection within the host is attributable to its molecular arsenal of excretory/secretory products that manipulate the host's immune response and dampen down immune mediators required to clear the infection. This molecular manipulation or modulation induced by helminths has been linked to influencing disease outcomes of various viral, bacterial and other parasitic infections (Mabbott 2018). There is evidence that helminths can weaken protective immunity, achieved through activation of a tightly controlled type-2 and regulatory immune responses, resulting in a downregulation of pro-inflammatory type-1 immunity. A robust pro-inflammatory type-1-immune response is critical for the control of Mtb, the causative agent of TB. There is evidence to suggest that helminth-induced immune system modulation during concurrent helminth-Mtb infection can dampen protective defences, which would usually limit an individual's progression to active TB disease (Anuradha et al. 2017; George et al. 2013; George et al. 2015; Li et al. 2015).

Furthermore, studies have indicated that STHs might reduce TB vaccine efficacy (Elias et al. 2008; Elias et al. 2001), prolong anti-TB treatment (Resende Co et al. 2007) and affect the accuracy of serological-based diagnostics that are used to determine TB infection status (Wassie et al. 2013). Taken together, these implications could potentially impact the surveillance and management of TB disease where these two infections intersect. Conversely, other studies imply that certain helminth infections may protect against developing TB disease through controlling Mtb replication inside host cells (Franke et al. 2014; O'Shea et al. 2018; Wong et al. 2019) and limiting lung damage, reducing disease severity (Abate et al. 2015; Mhimbira et al. 2017; Santos et al. 2019). The conflicting data highlights the complexity and importance of understanding host-parasite-TB interactions. Understanding the epidemiological and immunological links between concurrent STH and TB infections could have significant implications on how these infections are managed in the future and whether STHs or other intestinal parasites should be treated in conjunction with TB therapies.

7.1 Conclusion

The data presented in this thesis indicates that the prevalence of STHs in this rural PNG community was 70.5%, with high rates of STH co-infections. The most frequently detected worm was hookworm, with *N. americanus* and *A. lumbricoides* identified as the most frequent co-infection. In this study, we also identified species STH-specific associations with age, BMI, helminth co-infections and SOB. Describing the epidemiology of STHs is fundamental to informing potential future public health measures. This information can serve as a foundation for public health authorities to strategically implement community-wide surveys and targeted interventions such as deworming programs, health education campaigns, improved sanitation practices and access to clean and safe water. It was also found that for the detection of STHs, qPCR could be an advantageous tool to detect hookworm and *Trichuris* spp. infection within this region, but not for *Ascaris* spp. infection as microscopy was found to be comparable to qPCR findings. However, implementing qPCR in rural areas depends on resources and laboratory capacities. Utilising IgG serology has a significant capacity to screen populations for strongyloidiasis and identify individuals who require further testing. Multiple wet-mount faecal smears and performing FEAC on preserved samples is more cost-effective, methodologically simple and does not require sophisticated laboratory infrastructure compared to qPCR, making it ideal in resource-limited regions. In future studies, it would be of interest to expand the detection of intestinal parasites to include protozoa and investigate the potential health implications associated with STH infection, particularly regarding the potential overlap and influence that STHs have on TB within this community in rural PNG.

8 References

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Appendix A Questionnaire

CONFIDENTIAL
Date:
Interviewers Name:

Sample Number:
Spirometry: Yes/No

Patient Details			**TB Register Number:**
Name:			Home Village:
Year of Birth:	If unknown adult/chid	Height:	Current residence:
Sex: M/F	Religion:	Weight:	
Employment:			Mothers Clan:
Education:			Fathers Clan:

Questionnaire	
1. Presence and # of BCG scars	Y# /N
2. Are you sick?	Y/N – go to over the page if Y
3. Are you a TB patient?	Y/N – go to over the page if Y
4. Have you ever been a TB patient in the past?	Y/N
5. Does anyone in your household have TB?	Y/N
6. Do you have a cough?	
7. How long have you had the cough?	Y/N
8. Do you have SOB?	Y/N
9. Have you ever worked in a logging camp? Which camps? When(relevant years and length of employment)?	Y/N
10. Do you smoke? 11. Have you ever smoked? If yes a)How many cigarettes/day? b)Homemade/Purchased cigarettes? c)How long have you been smoking/quit for?	Y/N Y/N
12. Do you currently chew buai/betel nut?	Y/N
13. What do you use to cook food? If fire : a) What fuel do you use? Wood/Other: b)Where is the fire? Inside/Outside/ Under the house/Away from the house c)Are you the main cook for your family? Yes/No d)Are the kids around the fire? Always/Mostly/Sometimes/Occasionally/Never	Fire/Gas Stove/ Kerosene

Clinical information to be obtained from TB register	
Reason for participation:	TB Patient (on treatment)/TB suspect (no treatment)/Past patient/ Never had known TB/Other:
Current health status:	Well/Unwell
If TB patient or suspect: If Pulmonary If Extrapulmonary	Pulmonary/Extrapulmonary Smear +ive/-ive Site of infection:
Current TB Patient:	
Date of Diagnosis:	
Date treatment started:	
Treatment type:	DOTS/ Loose
Treatment category:	Category 1/Category 2
Duration of Treatment so far:	
Has there been any improvement? If Yes/Some: a) How much? b) How soon after starting treatment?	Yes/No/Some
Past TB Patient	
Date of Diagnosis:	
Type of previous TB diagnosis: If Pulmonary If Extrapulmonary	Pulmonary/Extrapulmonary Smear +ive/-ive Site of infection:
Treatment type:	DOTS/ Loose
Treatment category:	Category 1/Category 2
Were there any treatment interruptions? If Yes, how often? If yes, how long?	Yes/No
Was treatment completed?	Yes/No
Was treatment successful?	Yes/No

Appendix B Reagent Recipes

Sodium-acetate and 10% formaldehyde

To make 1 litre (1000 mL)

- Sodium Acetate…… 15.0 g
- Formalin, 5%..... 40.0 mL
- Acetic acid 2%…… 20.0 mL
- Deionised water…… 925.0 mL

Guanidinium thiocyanate buffer

To make 1 litre (1000 mL) of 4M Guanidinium thiocyanate buffer (GTC)

- Guanidinium thiocyanate …… 472.75 g
- 0.1M Tris hydrochloric acid (HCl) (pH 6) …… 550 mL
- 0.5M Ethylenediaminetetraacetic acid (EDTA) …….. 50mL
- Triton-X-100 …… 30mL
- Diethyl pyrocarbonate (DEPC)-treated water ……~ 180 mL to get to 1000 mL total volume

Appendix C DNA Yield and Purity from DNA Extraction of 122 Faecal Samples

In total, 122 faecal samples stored in GTC were extracted for genomic DNA. Each extracted DNA sample was screened for sample amplifiability by targeting the 16s ribosomal RNA gene. The amplicon size is estimated to be 1.4kbp, demonstrated in Appendix D.

Table C.1: DNA yield and purity of 122 extracted DNA faecal samples

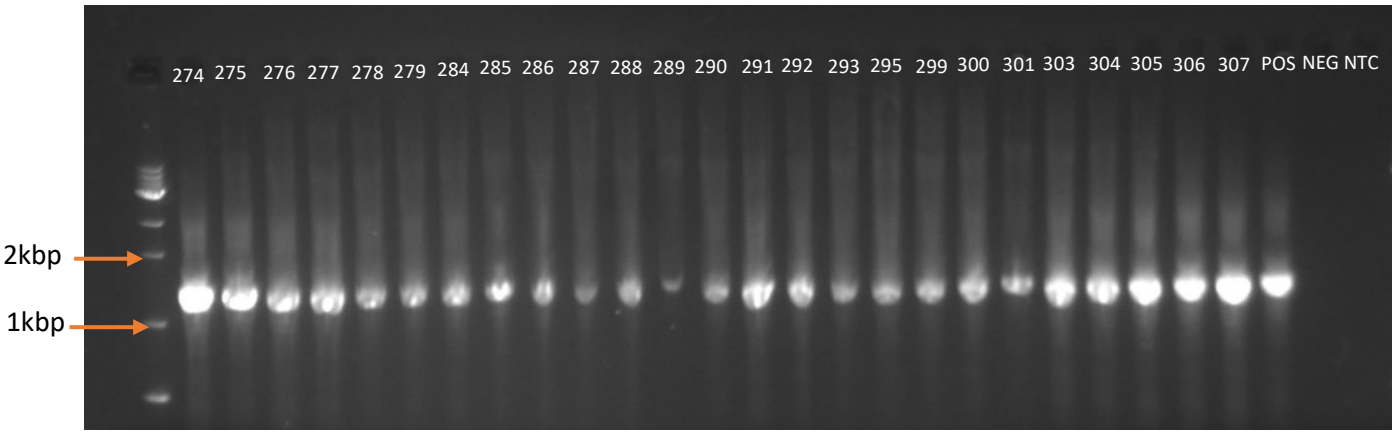
Sample ID	Date DNA extracted	Mass of beads (g)	A60/A280	A260/A230	DNA yield (ng/µL)
274	16-03-2022	0.31	1.8	2.15	125.5
275	16-03-2022	0.27	1.87	1.66	58
276	16-03-2022	0.3	1.87	2.12	178.8
277	16-03-2022	0.3	1.88	1.87	125.3
278	16-03-2022	0.3	1.88	2	105.9
279	16-03-2022	0.25	1.85	2.12	118.3
284	16-03-2022	0.3	1.88	2.14	186
285	17-03-2022	0.32	1.96	1.98	107.9
286	17-03-2022	0.26	2.01	1.77	75.3
287	17-03-2022	0.27	1.92	2.07	209.9
288	17-03-2022	0.28	1.9	1.74	99.4
289	17-03-2022	0.31	2.02	1.5	45.3
290	17-03-2022	0.27	1.8	1.35	57.4
291	17-03-2022	0.26	1.88	1.9	147.5
292	17-03-2022	0.26	1.88	2.18	202.2
293	17-03-2022	0.26	1.88	2.13	225.3
295	17-03-2022	0.3	1.86	2.17	153
299	17-03-2022	0.28	1.87	2.13	189.8
300	17-03-2022	0.3	1.86	1.84	131.2
301	17-03-2022	0.3	1.79	0.86	17.7
303	17-03-2022	0.28	1.89	2.13	221.8
304	17-03-2022	0.3	1.97	1.87	128.9
305	17-03-2022	0.28	1.89	1.65	115.3
306	17-03-2022	0.34	1.92	1.88	247.1
307	17-03-2022	0.25	1.92	2.04	199.1
308	17-03-2022	0.3	1.92	2.09	182.3
309	17-03-2022	0.35	1.95	1.7	130.8
310	17-03-2022	0.3	1.9	1.83	148.2
311	17-03-2022	0.32	1.88	2.0	130.1
312	17-03-2022	0.28	1.87	1.96	111.3
313	17-03-2022	0.31	1.91	1.86	141.3
314	18-03-2022	0.32	1.79	1.42	20.4
316	18-03-2022	0.27	1.95	1.36	30.2
317	18-03-2022	0.27	1.88	1.6	155.5
318	18-03-2022	0.32	1.86	2.16	153.7
319	18-03-2022	0.31	1.83	1.49	62.8
320	18-03-2022	0.31	1.87	2.14	160.8
321	18-03-2022	0.32	1.84	2.06	117.5

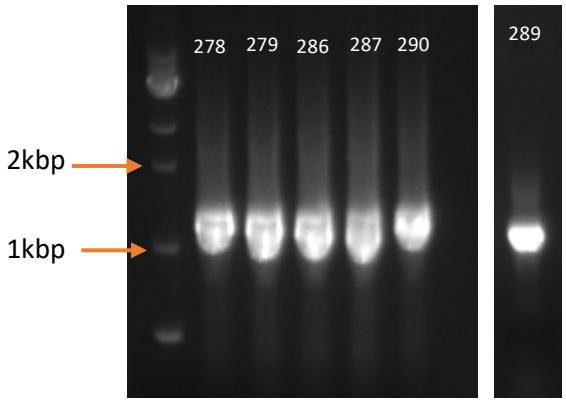
322	18-03-2022	0.29	1.86	2	94.7
323	18-03-2022	0.29	1.87	1.82	159.3
324	18-03-2022	0.29	1.85	1.61	102
325	18-03-2022	0.3	1.88	1.67	171.5
326	18-03-2022	0.3	1.86	1.95	166.1
327	18-03-2022	0.35	1.81	1.43	58.6
328	18-03-2022	0.3	1.76	0.24	4.7
329	18-03-2022	0.33	1.86	1.11	52.3
330	18-03-2022	0.26	1.79	0.33	14.7
331	19-03-2022	0.33	1.84	1.26	63.1
332	19-03-2022	0.35	1.88	1.85	142.2
333	19-03-2022	0.33	1.86	1.72	154.3
334	19-03-2022	0.32	1.89	1.9	225.3
335	19-03-2022	0.33	1.87	1.21	50.6
336	19-03-2022	0.27	1.9	1.95	126.1
337	19-03-2022	0.26	1.89	2.05	136.6
338	19-03-2022	0.29	1.98	1.6	239.3
339	19-03-2022	0.28	1.93	1.99	129.4
340	19-03-2022	0.29	1.96	1.85	164.2
341	19-03-2022	0.29	1.9	1.87	154.2
342	19-03-2022	0.33	1.88	1.75	123.5
345	20-03-2022	0.29	1.92	1.81	145.1
347	20-03-2022	0.28	1.91	1.79	240.4
348	20-03-2022	0.35	1.85	1.98	83.2
349	20-03-2022	0.29	1.86	2.27	128.6
350	20-03-2022	0.31	1.91	2.17	121.8
351	20-03-2022	0.32	1.88	1.47	81.4
352	20-03-2022	0.26	1.87	1.99	42.8
355	20-03-2022	0.31	1.89	2.22	202.8
356	20-03-2022	0.28	1.93	1.04	26.4
357	20-03-2022	0.33	1.87	1.91	134.3
358	20-03-2022	0.26	1.86	2.2	204.2
359	20-03-2022	0.3	1.86	2.14	180.2
360	20-03-2022	0.33	1.88	2.21	176.6
361	20-03-2022	0.28	1.87	2.19	170.6
363	20-03-2022	0.26	1.87	2.08	121.2
364	20-03-2022	0.27	1.88	2.22	226.0
365	21-03-2022	0.32	1.92	2.22	189.7
366	21-03-2022	0.29	1.87	1.71	100.3
367	21-03-2022	0.28	1.91	1.79	96.2
368	21-03-2022	0.25	1.9	2.23	194.2
369	21-03-2022	0.33	1.87	2.13	150.7
370	21-03-2022	0.31	1.86	2.03	61.8
371	21-03-2022	0.35	1.88	2.28	145.5

376	21-03-2022	0.28	1.91	2.1	171.3
377	21-03-2022	0.34	1.88	2.04	148.1
379	21-03-2022	0.29	1.85	1.1	22.3
382	21-03-2022	0.28	1.85	1.2	78
383	21-03-2022	0.26	1.86	1.89	58.1
384	21-03-2022	0.25	1.84	1.71	70.3
385	21-03-2022	0.28	1.87	2.16	164.4
386	21-03-2022	0.27	1.86	2.24	176.7
387	21-03-2022	0.29	1.85	2.05	105.7
388	21-03-2022	0.27	1.87	2.07	103.7
389	21-03-2022	0.32	1.87	2.1	140.5
390	21-03-2022	0.34	1.86	2.09	105.3
391	22-03-2022	0.35	1.87	2.19	148.9
392	22-03-2022	0.34	1.89	1.98	152.8
393	22-03-2022	0.29	1.87	2.18	146.8
394	22-03-2022	0.3	1.86	2.16	129
395	22-03-2022	0.31	1.88	2.1	181.5
396	22-03-2022	0.32	1.89	1.71	125.2
397	22-03-2022	0.33	1.88	2.06	172.4
398	22-03-2022	0.35	1.88	2.12	145.3
399	22-03-2022	0.32	1.89	1.83	133.7
400	22-03-2022	0.24	1.77	1	26.5
401	22-03-2022	0.33	1.91	1.56	101.7
403	22-03-2022	0.26	1.88	1.97	131.4
404	22-03-2022	0.35	1.86	1.84	111.9
405	22-03-2022	0.28	1.88	2	150.6
406	22-03-2022	0.26	1.88	2	158.6
407	22-03-2022	0.34	1.88	1.93	134
408	22-03-2022	0.26	1.88	2.23	204.8
409	23-03-2022	0.3	1.89	2.2	215.7
410	23-03-2022	0.2	1.88	2.18	154.1
411	23-03-2022	0.32	1.88	2.05	159.8
413	23-03-2022	0.32	1.88	1.89	198.7
414	23-03-2022	0.28	1.89	2.11	194.1
415	23-03-2022	0.34	1.88	1.99	149.2
416	23-03-2022	0.32	1.89	1.94	160.4
419	23-03-2022	0.25	1.88	2.12	183.9
421	23-03-2022	0.31	1.89	1.71	73.7
449	23-03-2022	0.34	1.88	1.96	136.6
450	23-03-2022	0.32	1.83	1.59	41.4

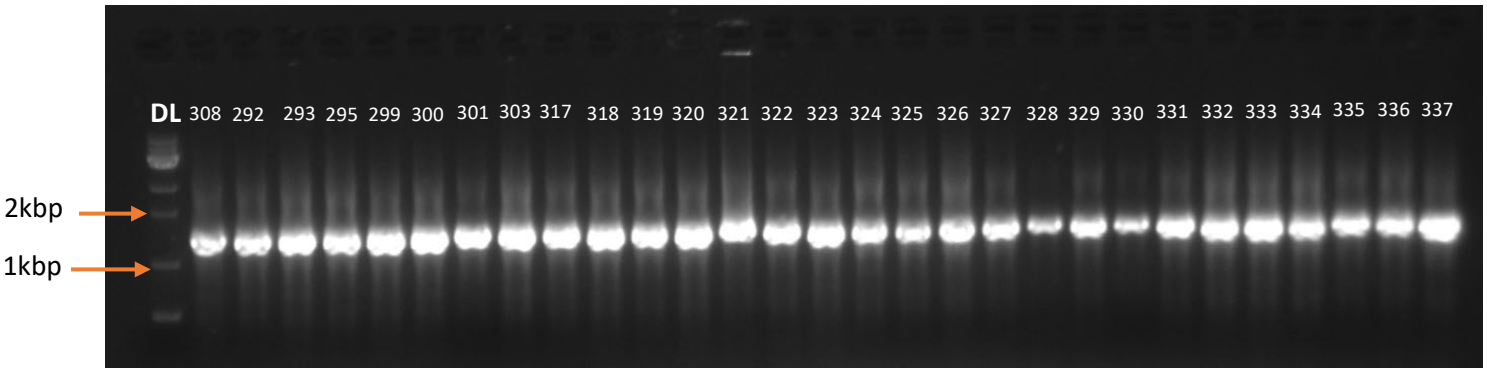
Appendix D Faecal Sample Amplifiability Through 16s PCR Amplification

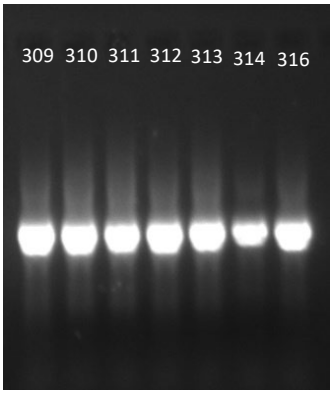
Overall, all samples were amplifiable. Thick and intense bands were noted for nearly all samples, indicating amplification of highly abundant bacterial DNA (perhaps diluting the extracted DNA before PCR would have improved band resolution for appropriate sizing). A few samples had to be re-run on gels, resulting in greater band intensity comparable to other bands. All reactions were in control.

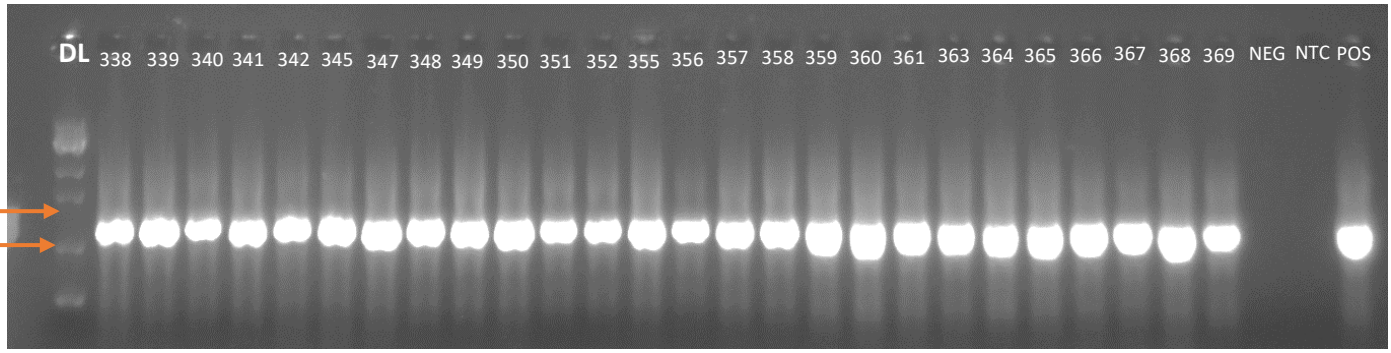


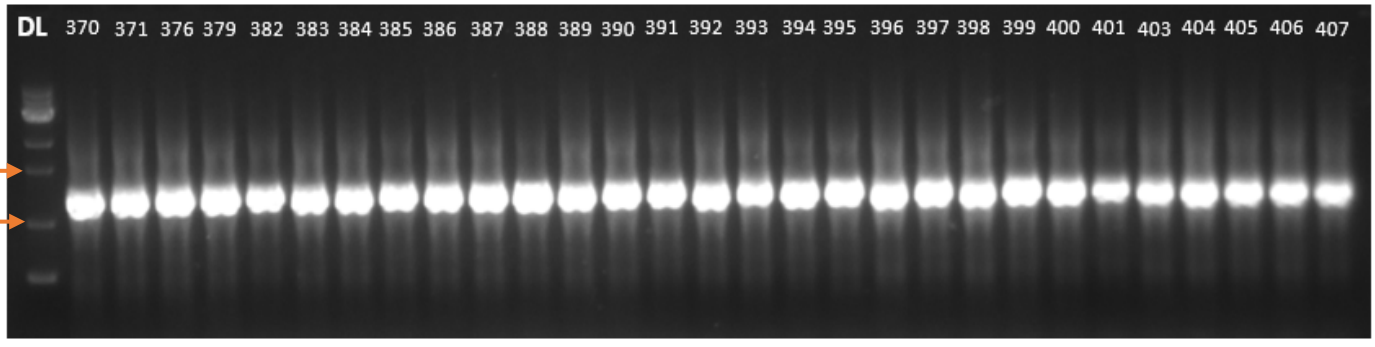


Re-do gel electrophoresis for samples 278, 279, 286, 290 and 289.


DL 308 292 293 295 299 300 301 303 317 318 319 320 321 322 323 324 325 326 327 328 329 330 331 332 333 334 335 336 337
2kbp
1kbp


309 310 311 312 313 314 316


DL 338 339 340 341 342 345 347 348 349 350 351 352 355 356 357 358 359 360 361 363 364 365 366 367 368 369 NEG NTC POS
2kbp
1kbp


DL 370 371 376 379 382 383 384 385 386 387 388 389 390 391 392 393 394 395 396 397 398 399 400 401 403 404 405 406 407
2kbp
1kbp

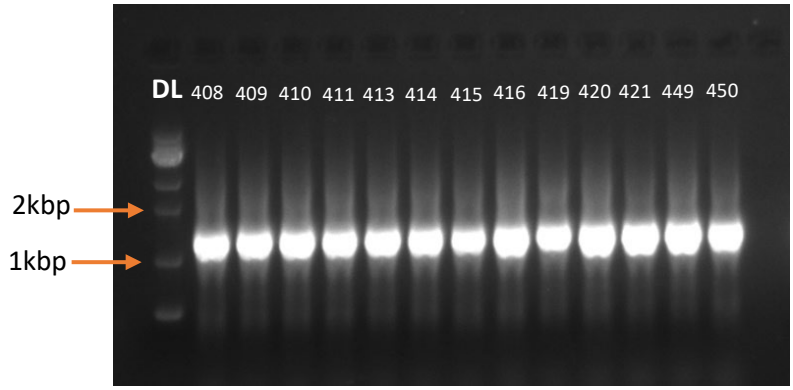


Figure D.1: Gel electrophoresis of 122 genomic DNA samples, extracted from faecal material showing amplification of the 16s rRNA gene. Amplicon size is approximately 1.4Kbp. DL : DNA ladder

Appendix E Protocol of DNA Extraction for Positive Template Controls and Analysis of DNA Yield and Purity

Dr. Luke Becker and Prof. Alex Loukas provided human faecal specimens containing known positive *N. americanus* and *A. ceylanicum* from James Cook University, Cairns campus, Queensland, Australia. Samples were fresh and frozen at -20℃ until ready for extraction. Zymo-Quick-DNA faecal/soil kit microbe miniprep kit (Zymo-research, #D610) was used to extract from the faecal sample as per manufacturer's instructions, except that the bead beating step was modified to one cycle, for 45 seconds on the FastPrep-24 (MP Biomedicals, USA) at full speed (6.5 m/s) (Azzopardi et al. 2021).

Table E.1: DNA yield and protein and salt contamination of positive template controls

Target	**Date of extraction**	**Bead mass (g)**	**DNA yield (ng/ µL)**	**A260/A280**	**A230/A260**
A. lumbricoides	03-03-22	0.3	124.2	1.87	2.2
T. trichiura	03-03-22	0.3	124.2	1.87	2.2
N. americanus	12-07-22	0.32	37.4	1.86	1.46
A. ceylanicum	12-07-22	0.37	7.9	1.79	0.56

Appendix F Validation and Optimisation of STH TaqMan qPCR Assays

A standard curve for each primer and probe combination was performed in triplicate to assess the efficiency, linearity and reproducibility of the TaqMan qPCR primers and probes. The generation of a standard curve for each STH target had at least six serial dilutions of 1:10 of the template controls. Non-template control was included in all reactions.

F.1 *Ascaris lumbricoides*

Reagent	Final concentration	Dilution factor	volume for 1 reaction (µL)
2X GoTaq qPCR Master Mix	1X	2	10
Forward primer (10 µM)	0.1µM (100nM)	100	0.2
Reverse primer (10 µM)	0.1µM (100nM)	100	0.2
FAM Probe (10µM)	0.05µM (50nM)	200	0.1
DNAse-free water			7.5
DNA template			2
Total volume			20

Cycling conditions	
95 °C, 5 min	x1
95 °C, 10 s 60 °C, 60 s	}x40

Plate layout			
	1	**2**	**3**
A	Neat	Neat	Neat
B	10^-1	10^-1	10^-1
C	10^-2	10^-2	10^-2
D	10^-3	10^-3	10^-3
E	10^-4	10^-4	10^-4
F	10^-5	10^-5	10^-5
G	10^-6	10^-6	10^-6
H	NTC	NTC	NTC

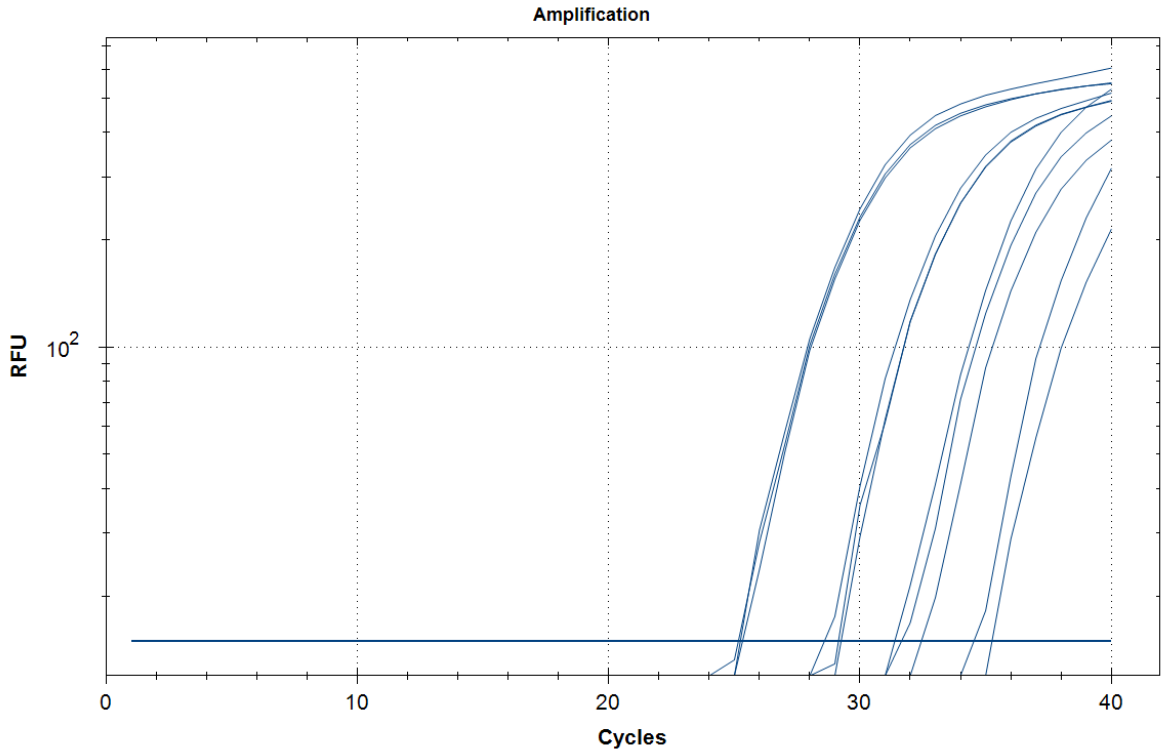


Figure F.1: Amplification curve for the establishment of a standard curve for *A. lumbricoides* TaqMan qPCR assay, performed in triplicate, using positive template control, sample 237. The amplification curve demonstrates a four ten-fold serial dilution of *A. lumbricoides* positive template control. Baseline threshold was set at 20% of the total RFU. RFU was set to log-scale. RFU, relative fluorescence unit.

Table F.1: Raw data for *A. lumbricoides* standard curve

Sample dilution	Cq*	Mean Cq* ($\pm$SD)	Cycle difference based on averages
Neat (Sample # 237)	25.22	25.33 (0.12)	
	25.45		
	25.32		
10^-1	29.16	29.11 (0.20)	3.78
	29.28		
	28.89		
10^-2	31.98	32.16 (0.53)	3.05
	32.76		
	31.74		
10^-3	35.28	35.07 (0.30)	2.91
	34.86		
	N/A		

*Cq: cycle value

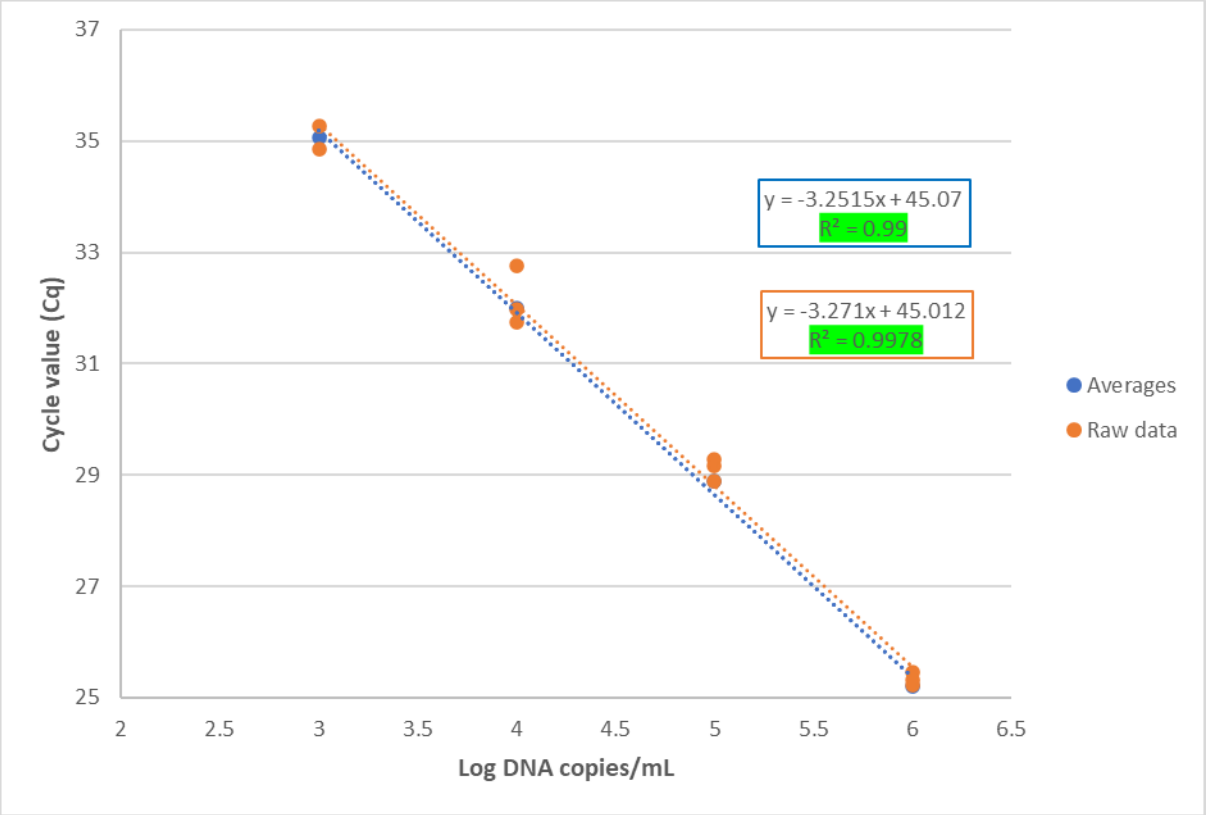


Figure F.2: Assessment of *A. lumbricoides* TaqMan qPCR assay efficiency. Linear regression plotting Cq values obtained by qPCR against dilution series.

Determination of PCR efficiency (E)

Linear line equation describing the y-intercept in Figure F.2 informs the gradient and/or slope of the line required to determine % PCR efficiency.

y = -3.271x + 45.012

Slope (m) = -3.271

$$E = 10^{-1/m}$$

$$E = 10^{-1/-3.271}$$

$$E = 2.022$$

Percent (%) PCR efficiency (E%)

$$E\% = (E - 1) \times 100$$

$$E\% = 102.2$$

Therefore, **PCR efficiency is 102.2%**

Table F.2: Criteria for assessment of TaqMan qPCR assay performance for *A. lumbricoides* (adapted from Promega PCR: Guidelines for validating a qPCR assay)

Does the singleplex reaction for this target fit the following criteria	Acceptable?
Cq Values	
• Are Cq values roughly 3-3.3 different from each dilution?	Acceptable
Reproducibility	Excluding dilution 10^-2
• Are standard deviations within each assay below 0.5 Cq?	Acceptable
Linearity	
• Is the r^2 value greater than 0.98?	Acceptable
Efficiency	
• Is efficiency for the reaction between 90-110%?	Acceptable

F.2 *Trichuris trichiura*

Before determining *T. trichiura* TaqMan qPCR assay efficiency, we determined whether the positive template control used for this assay was suitable. This involved including various other samples (from previous field study) known to contain other STHs, such as hookworm, *A. lumbricoides* and intestinal protozoa.

Initial trial

Reagent	Final concentration	Dilution factor	volume for 1 reaction (µL)
2X GoTaq Probe qPCR Master Mix	1X	2	10
Forward primer (10 µM)	0.1 µM (100nM)	100	0.2
Reverse primer (10 µM)	0.1 µM (100nM)	100	0.2
Cy5 Probe (10 µM)	0.05µM (50nM)	200	0.1
DNAse-free water			7.5
DNA template			2
Total volume			20

Cycling conditions	
95 °C, 5 min	x1
95 °C, 10 s 60 °C, 60 s	}x40

Table F.3: Outcome of an initial trial of *T. trichiura* TaqMan qPCR assay and positive template control.

Sample	Cycle value (Cq)	Known
237	27.76	Pos
419	31.76	Pos
256	N/A	Neg
257	N/A	Neg
270	N/A	Neg
Neg H	N/A	Neg
NegW	N/A	Neg
NTC	N/A	Neg

NegH, Healthy negative control; NegW, DNA extraction water control.

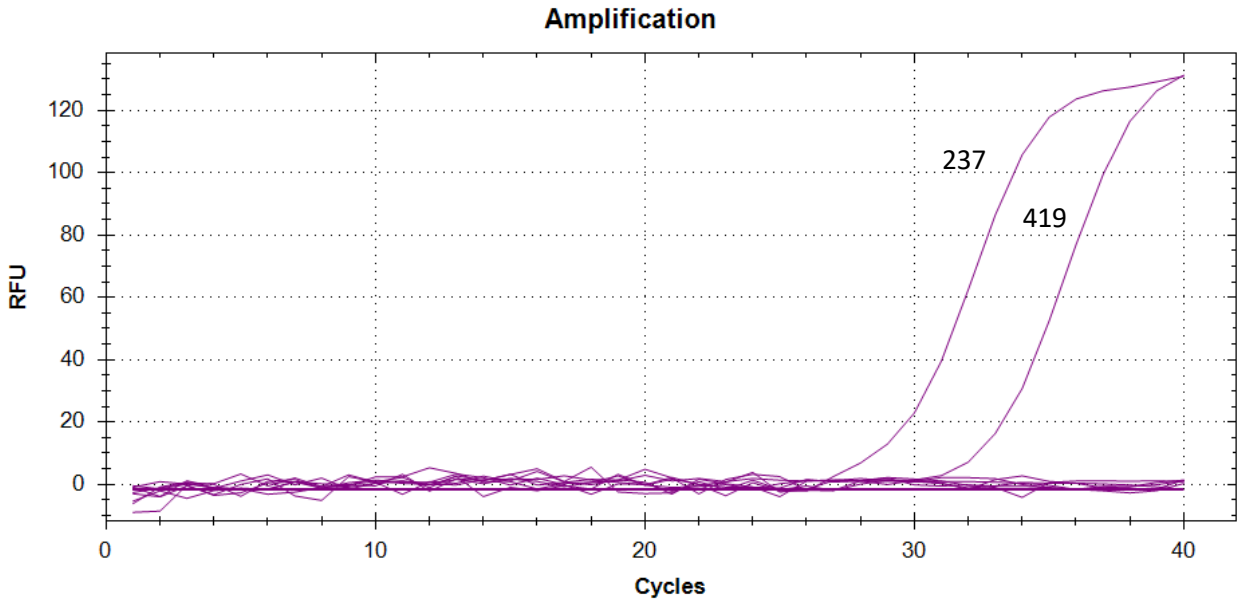


Figure F.3: Amplification curve to trial *T. trichiura* positive template control. RFU, relative fluorescent unit. Known positive DNA templates derived from samples 237 and 419.

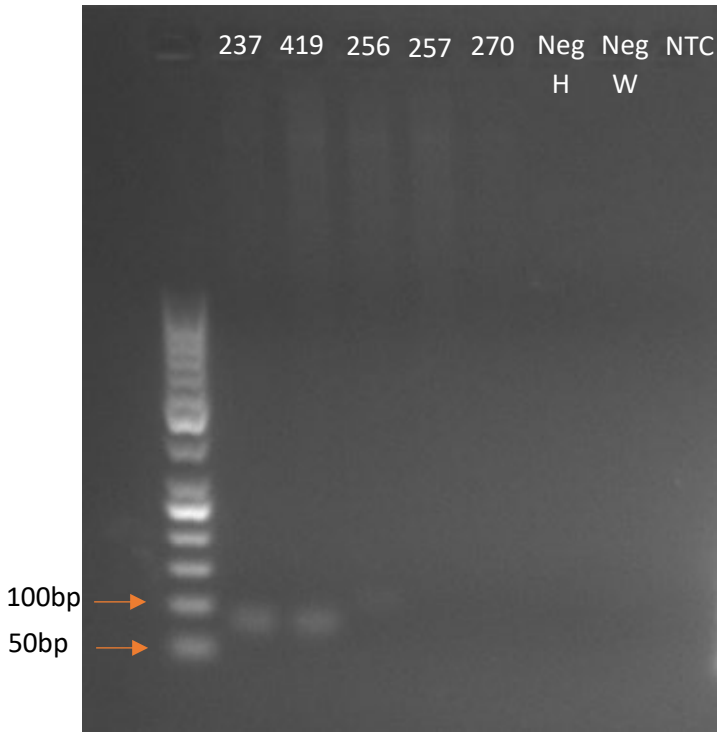


Figure F.4: Gel electrophoresis of initial PCR trial for *T. trichiura*. Amplicon size is approximately 76bp for *T. trichiura*.

Conclusion: Sample 237 is a suitable positive template control to detect *T. trichiura*.

Standard curve

Reagent	Final concentration	Dilution factor	volume for 1 reaction (µL)
2X GoTaq Probe qPCR Master Mix	1X	2	10
Forward primer (10 µM)	0.1 µM (100nM)	100	0.2
Reverse primer (10 µM)	0.1 µM (100nM)	100	0.2
Cy5 Probe (10 µM)	0.05µM (50nM)	200	0.1
DNAse-free water			7.5
DNA template			2
Total volume			20

Cycling conditions	
95 °C, 5 min	x1
95 °C, 10 s 60 °C, 60 s	}x40

Plate layout			
	1	**2**	**3**
A	Neat	Neat	Neat
B	10^-1	10^-1	10^-1
C	10^-2	10^-2	10^-2
D	10^-3	10^-3	10^-3
E	10^-4	10^-4	10^-4
F	10^-5	10^-5	10^-5
G	10^-6	10^-6	10^-6
H	NTC	NTC	NTC

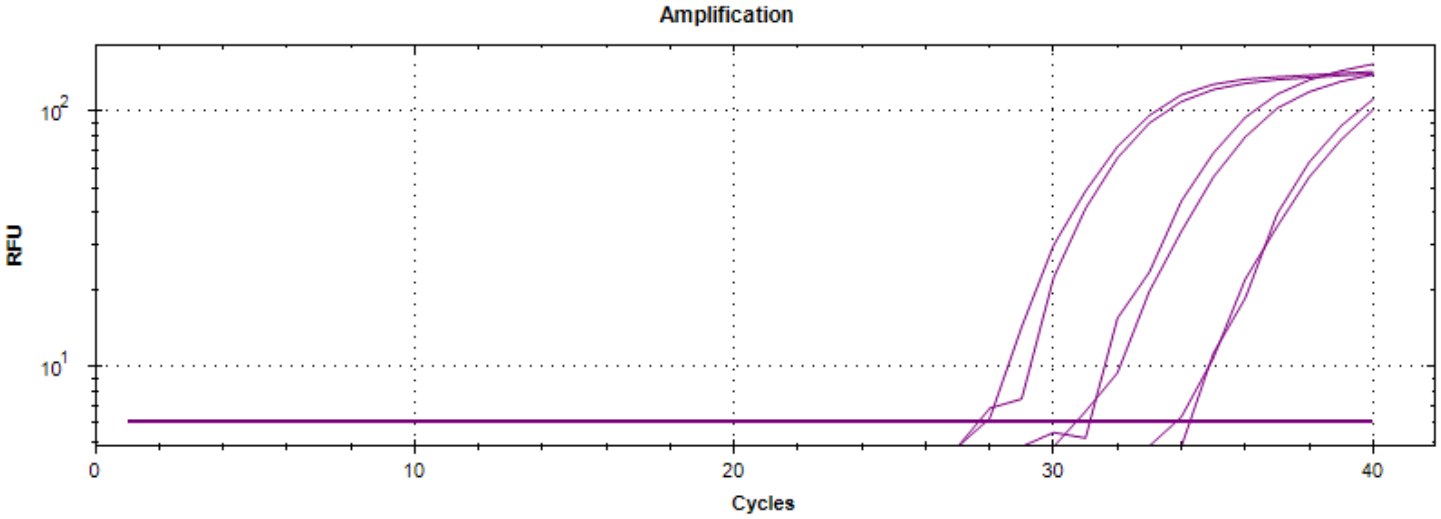


Figure F.5: Amplification curve for the establishment of a standard curve for *T. trichiura* the TaqMan qPCR assay. Performed in triplicate, using positive template control sample 237 containing *T. trichiura*. The amplification curve demonstrates a three ten-fold serial dilution of *T. trichiura* positive template control. Baseline threshold was set at 20% of total RFU. RFU was set to log-scale. RFU, relative fluorescence unit.

Table F.4: Raw data for *T. trichiura* standard curve

Sample dilution	Cycle value (Cq)	Mean Cq (±SD)	Difference between cycles using averages
Neat	27.85 27.94	27.9 (0.06)	
10^-1	30.89 31.08	31.0 (0.13)	3.09
10^-2	33.81 34.2	34.0 (0.28)	3.02

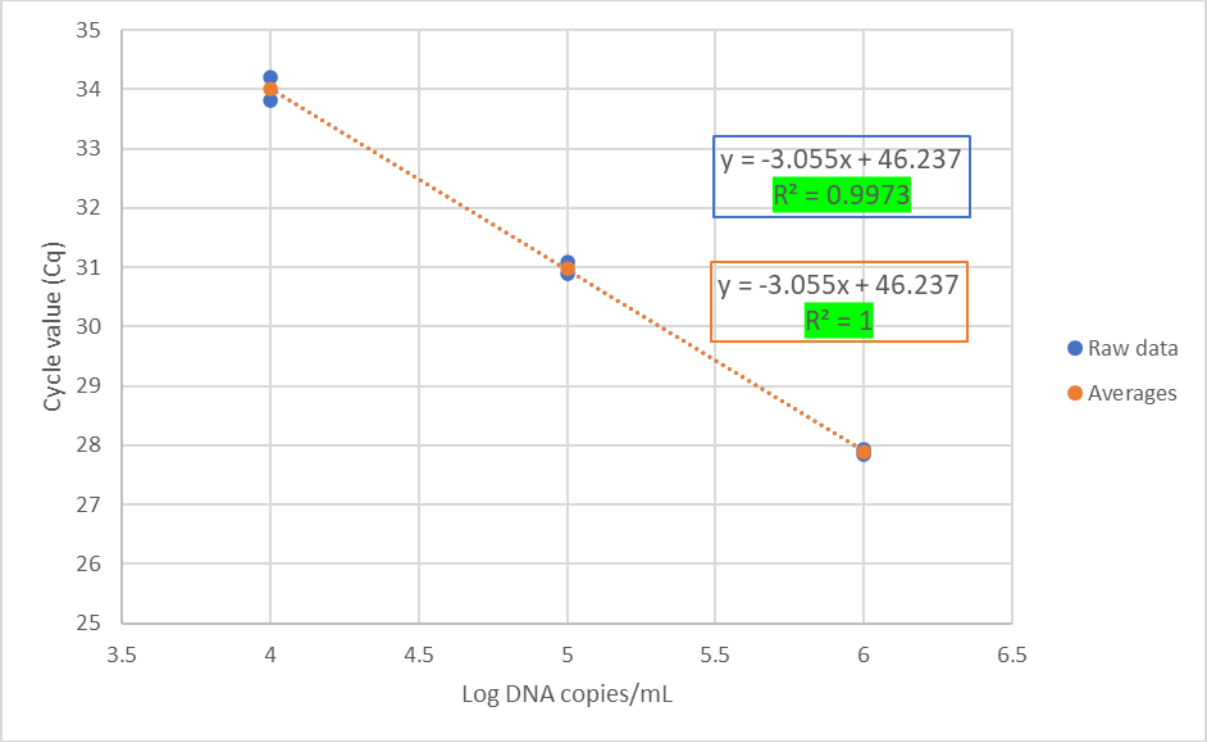


Figure F.6: Assessment of *T. trichiura* TaqMan qPCR assay efficiency. Linear regression plotting Cq values obtained by qPCR against dilution series.

Determination of PCR efficiency (E)

The linear line equation describing the y-intercept in Figure F.6 informs the gradient and/or slope of the line required to determine % PCR efficiency.

y = -3.055x + 46.237

Slope (m) = -3.055

$$E = 10^{-1/m}$$

$$E = 10^{-1/-3.055}$$

$$E = 2.125$$

Percent (%) PCR efficiency (E%)

$$E\% = (E - 1) \times 100$$

$$E\% = 112.5$$

Therefore, PCR efficiency is 112.5%

Table F.5: Criteria for assessment of the TaqMan qPCR assay performance for *T. trichiura* (adapted from Promega PCR: Guidelines for validating a qPCR assay)

Does the singleplex reaction for this target fit the following criteria	Acceptable?
Cq Values • Are Cq values roughly 3-3.3 different from each dilution?	Acceptable
Reproducibility • Are standard deviations within each assay below 0.5 Cq?	Acceptable
Linearity • Is the r^2 value greater than 0.98?	Acceptable
Efficiency • Is efficiency for the reaction between 90-110%?	Acceptable

Conclusion: This TaqMan qPCR assay is acceptable for assessing the presence/absence of infection but would be unsuitable for quantification. Further optimisation of this assay to fit the accepted criteria would improve its use for quantification. Sequencing control products would also improve the confidence of assay validation.

F.3 *Necator americanus*

Reagent	Final concentration	Dilution factor	volume for 1 reaction (µL)
2X GoTaq Probe qPCR Master Mix	1X	2	10
Forward primer (10 µM)	0.1 µM (100nM)	100	0.2
Reverse primer (10 µM)	0.1 µM (100nM)	100	0.2
Cy5 Probe (10 µM)	0.05µM (50nM)	200	0.1
DNAse-free water			7.5
DNA template			2
Total volume			20

Cycling conditions	
95 °C, 5 min	x1
95 °C, 10 s 60 °C, 60 s	}x40

Plate layout			
	1	**2**	**3**
A	Neat	Neat	Neat
B	10^-1	10^-1	10^-1
C	10^-2	10^-2	10^-2
D	10^-3	10^-3	10^-3
E	10^-4	10^-4	10^-4
F	10^-5	10^-5	10^-5
G	10^-6	10^-6	10^-6
H	NTC	NTC	NTC

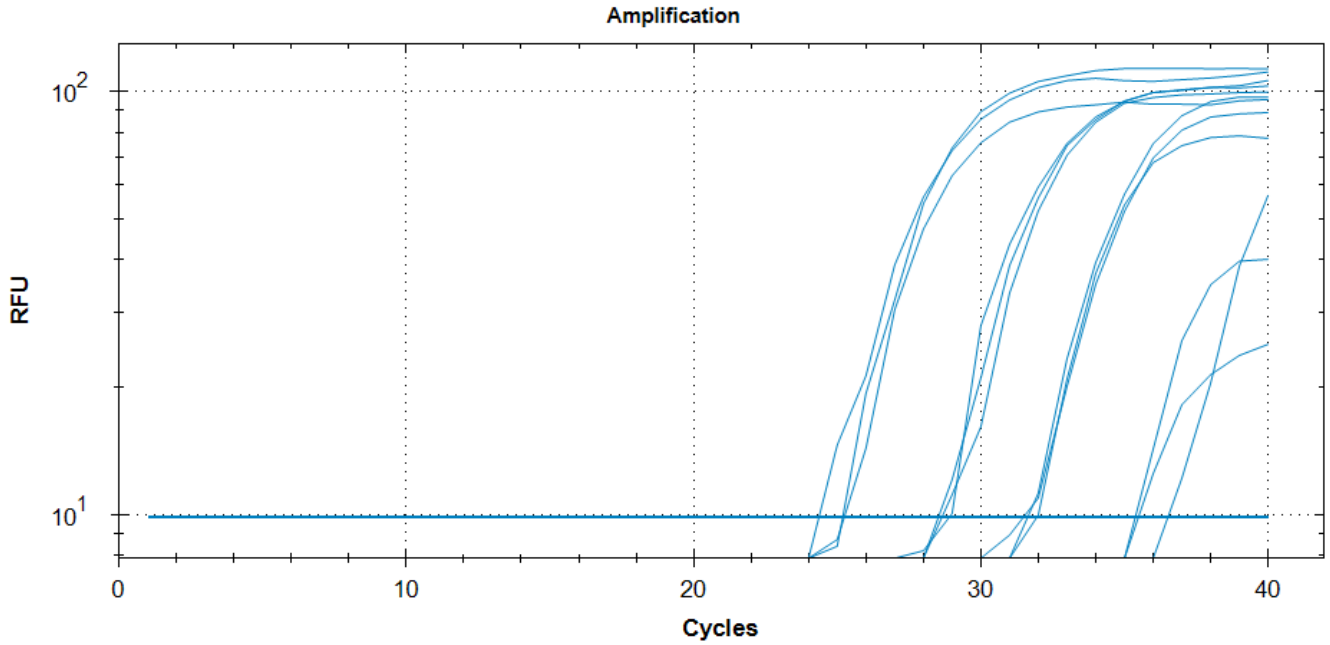


Figure F.7: Amplification curve for the establishment of a standard curve for *N. americanus* TaqMan qPCR assay, performed in triplicate, using positive template control. The amplification curve demonstrates a four ten-fold serial dilution of *N. americanus* positive template control. Baseline threshold was set at 20% of total RFU. RFU was set to log-scale. RFU, relative fluorescence unit.

Table F.6: Raw data of Cq values obtained by the TaqMan qPCR for *N. americanus*

Sample dilution	Cycle value (Cq)	Average Cq (±SD)	Difference between cycles
Neat	24.48	24.94 (0.40)	
	25.20		
	25.13		
10^-1	28.89	28.74 (0.15)	3.80
	28.73		
	28.59		
10^-2	31.74	31.73 (0.26)	2.99
	31.46		
	31.99		
10^-3	36.50	35.82 (0.60)	4.09
	35.42		
	35.55		

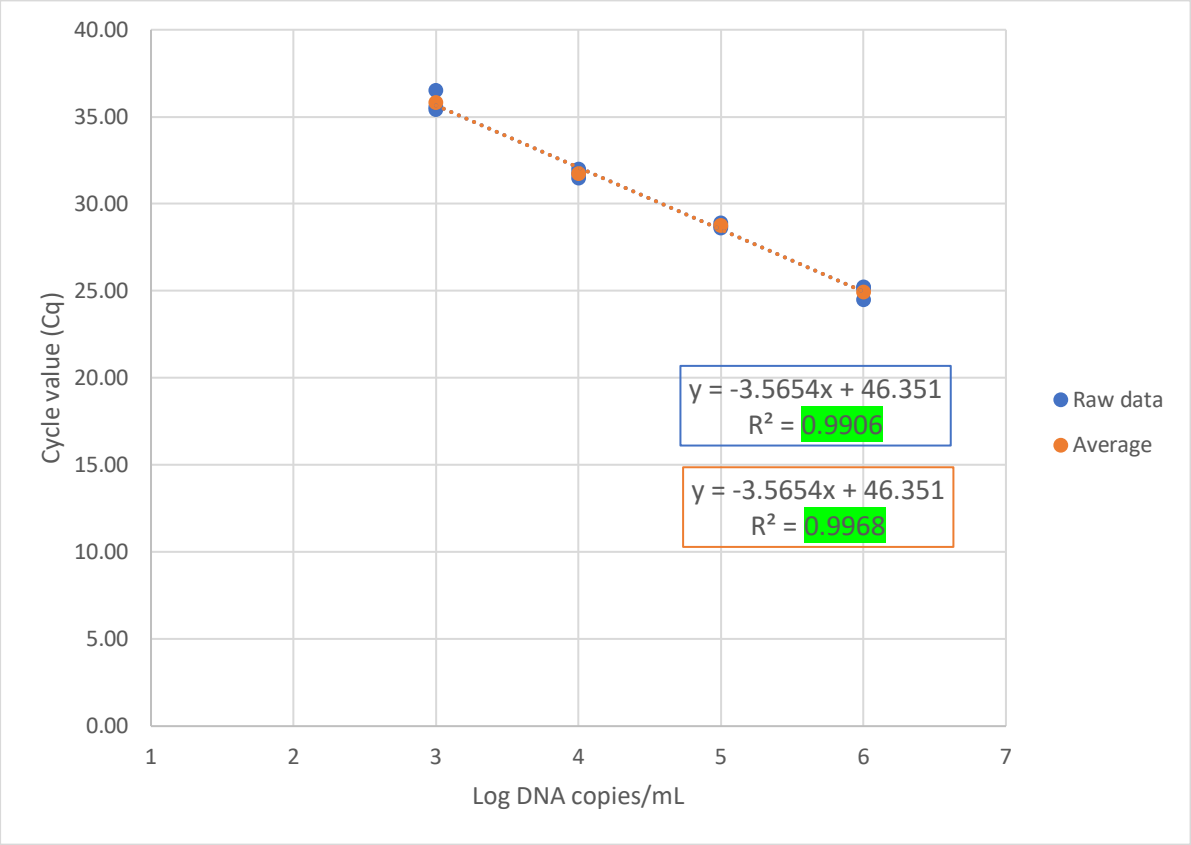


Figure F.8: Assessment of *N. americanus* TaqMan qPCR assay efficiency. Linear regression plotting Cq values obtained by qPCR against dilution series.

Determination of PCR efficiency (E)

The linear line equation describing the y-intercept in Figure F.8 informs the gradient and/or slope of the line required to determine % PCR efficiency.

y = -3.5654x + 46.351

Slope (m) = -3.5654

$$E = 10^{-1/m}$$

$$E = 10^{-1/-3.5654}$$

$$E = 1.907$$

Percent (%) PCR efficiency (E%)

$$E\% = (E - 1) \times 100$$

$$E\% = 90.75$$

Therefore, PCR efficiency is 90.75%

Table F.7: Criteria for assessment of the TaqMan qPCR assay performance for *N. americanus* (adapted from Promega PCR: Guidelines for validating a qPCR assay)

Does the singleplex reaction for this target fit the following criteria	Acceptable?
Cq Values • Are Cq values roughly 3-3.3 different from each dilution?	Acceptable However, slope is close to 3.3
Reproducibility • Are standard deviations within each assay below 0.5 Cq?	All except 10^-3
Linearity • Is the r^2 value greater than 0.98?	Acceptable
Efficiency • Is efficiency for both reactions between 90-110%?	Acceptable

F.4 *Ancylostoma ceylanicum*

Reagent	Final concentration	Dilution factor	volume for 1 reaction (µL)
2X GoTaq Probe qPCR Master Mix	1X	2	10
Forward primer (10 µM)	0.1 µM (100nM)	100	0.2
Reverse primer (10 µM)	0.1 µM (100nM)	100	0.2
HEX Probe (10 µM)	0.05µM (50nM)	200	0.1
DNAse-free water			7.5
DNA template			2
Total volume			20

Cycling conditions	
95 °C, 5 min	x1
95 °C, 10 s 60 °C, 60 s	}x40

Plate layout			
	1	**2**	**3**
A	Neat	Neat	Neat
B	10^-1	10^-1	10^-1
C	10^-2	10^-2	10^-2
D	10^-3	10^-3	10^-3
E	10^-4	10^-4	10^-4
F	10^-5	10^-5	10^-5
G	10^-6	10^-6	10^-6
H	NTC	NTC	NTC

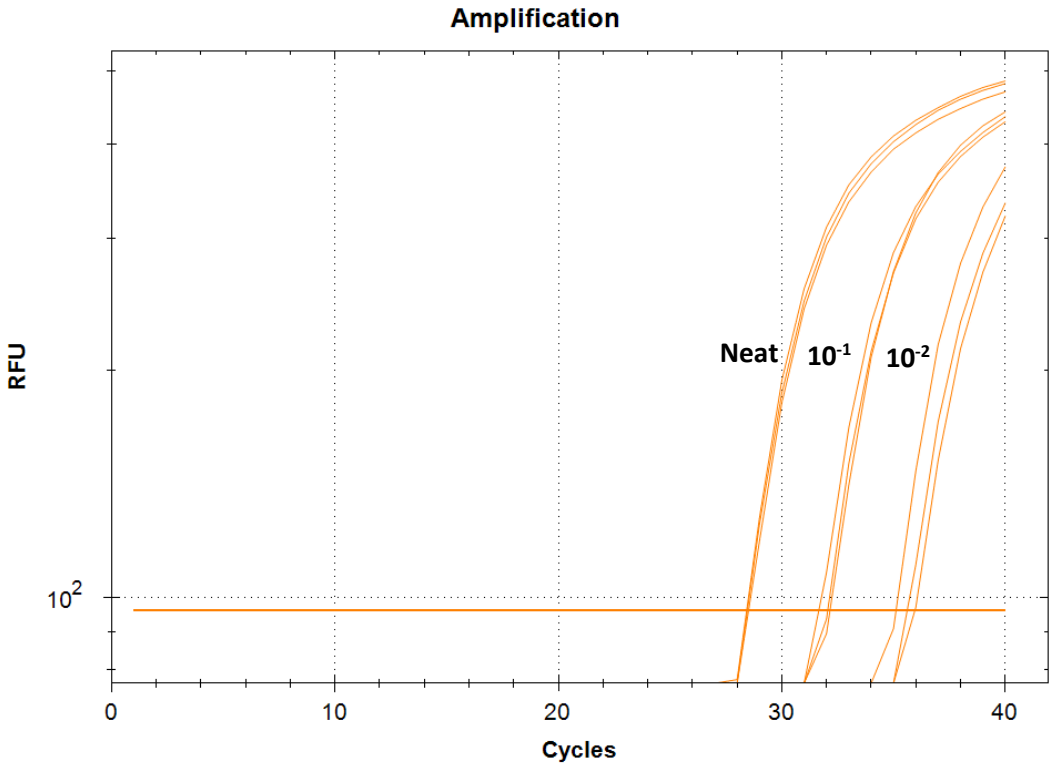


Figure F.9: Amplification curve for the establishment of a standard curve for *A. ceylanicum* TaqMan qPCR, in triplicate, using positive template control. The amplification curve demonstrates a two-fold serial dilution of *A. ceylanicum* positive template control. Baseline threshold was set at 20% of total RFU, RFU was set to log-scale. RFU, relative fluorescence unit.

Table F.8: Raw data of Cq values obtained by TaqMan qPCR for *A. ceylanicum.*

Serial dilution	Cq	Mean (±SD)	Difference between cycles
Neat	28.42	28.43 (0.066)	
	28.50		
	28.37		
10^-1	31.72	31.96 (0.22)	3.53
	32.04		
	32.13		
10^-2	35.98	35.59 (0.45)	3.62
	35.69		
	35.09		
10^-3	0	0	
	0		
	0		

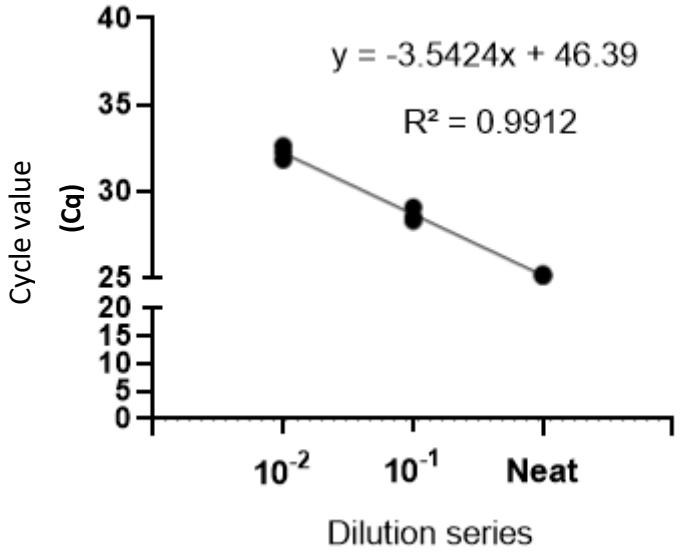


Figure F.10: Cq values obtained from TaqMan qPCR in relation with two ten-fold dilution series (three-point) standard curve of *A. ceylanicum*.

Determination of PCR efficiency (E)

The linear line equation describing the y-intercept in Figure F.10 informs the gradient and/or slope of the line required to determine % PCR efficiency.

y = -3.5783x + 49.89

Slope (m) = -3.573

$$E = 10^{-1/m}$$

$$E = 10^{-1/-3.5783}$$

$$E = 1.90$$

Percent (%) PCR efficiency (E%)

$$E\% = (E - 1) \times 100$$

$$E\% = 90.3$$

Therefore, PCR efficiency is 90.3%

Table F.9: Criteria for assessment of the TaqMan qPCR assay performance for *A. ceylanicum* (adapted from Promega PCR: Guidelines for validating a qPCR assay)

Does the singleplex reaction for this target fit the following criteria	Y or N
Cq Values	Acceptable
• Are Cq values roughly 3-3.3 different from each dilution?	However, slope is close to 3.3
Reproducibility	Y
• Are standard deviations within each assay below 0.5 Cq?	
Linearity	Y
• Is the r^2 value greater than 0.98?	
Efficiency	Y
• Is efficiency for both reactions between 90-110%?	

F.5 *Ancylostoma duodenale*

Encountered issues with A. duodenale primer and probe combination

During the validation of the *A. duodenale* TaqMan qPCR assay, it was of interest to determine specificity. Thus, in preliminary experiments, we decided to run positive control *A. duodenale* and known positive *A. ceylanicum* sample with various negative controls as previously described. Below are the reaction conditions:

Reagent	Final concentration	Dilution factor	volume for 1 reaction (µL)
2X GoTaq Probe qPCR Master Mix	1X	2	10
Forward primer (10 µM)	0.4 µM (200nM)	25	0.8
Reverse primer (10 µM)	0.4 µM (200nM)	25	0.8
FAM Probe (10 µM)	0.2µM (100nM)	50	0.4
DNAse-free water			6
DNA template			2
Total volume			20

Cycling conditions	
95 °C, 5 min	x1
95 °C, 10 s 60 °C, 60 s	}x40

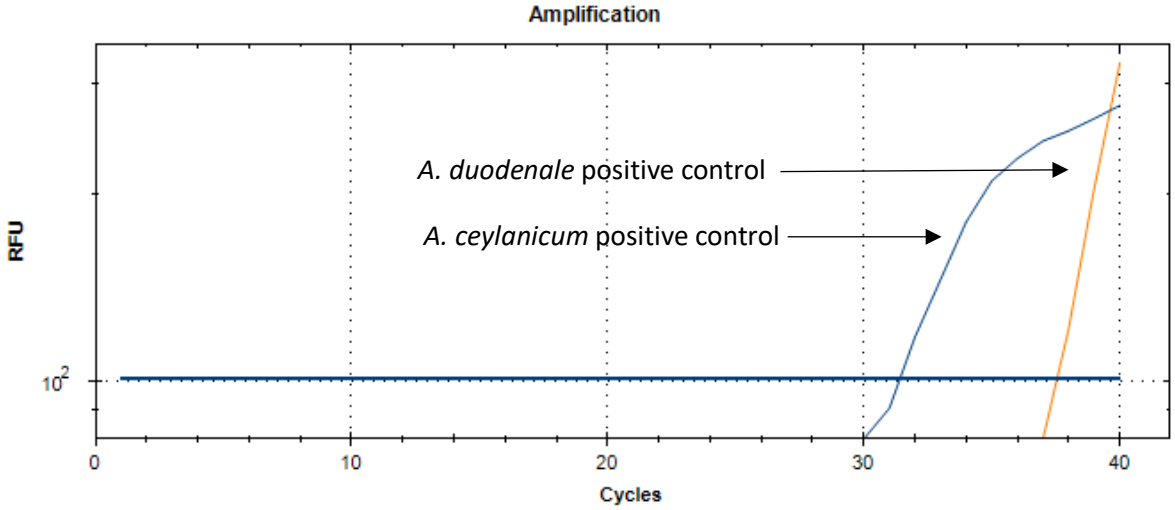


Figure F.11: Annotated amplification curve (log-scale) of preliminary outcomes of *A. duodenale* primer and probe TaqMan qPCR assay. *A. ceylanicum* positive control (Blue line) at a Cq of 31.4, *A. duodenale* positive control (orange line) at a Cq of 37.6. Baseline threshold (thick horizontal blue line) is 101.22 relative fluorescence unit (RFU). All negative controls were as expected.

Conclusion: Something was amplified in the *A. duodenale* sample, but the sample had a low DNA concentration. The resultant PCR products of that sample were used in an optimisation reaction. Most notably, *A. duodenale* primers and probes reacted with *A. ceylanicum* positive DNA template control, as demonstrated through signal generation detected by the qPCR, indicating a positive reaction.

Determining the optimal dilution of the PCR product

Same reaction conditions above. The suspected *A. duodenale* PCR product was serially diluted 1/100, 1/1000, and 1/10000.

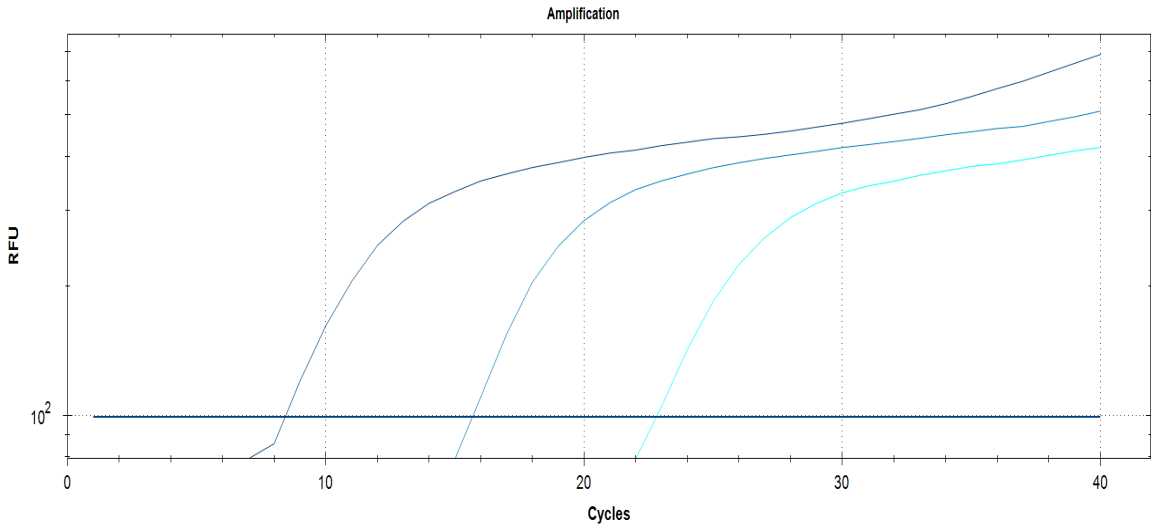


Figure F.12: Amplification curve (log-scale) of PCR products of *A. duodenale* to determine working dilutions. 1/100 dilution (dark blue line) Cq of 8.4, 1/1000 dilution (Blue line) Cq at 15.70 and 1/10000 (light blue line) Cq at 22.9.

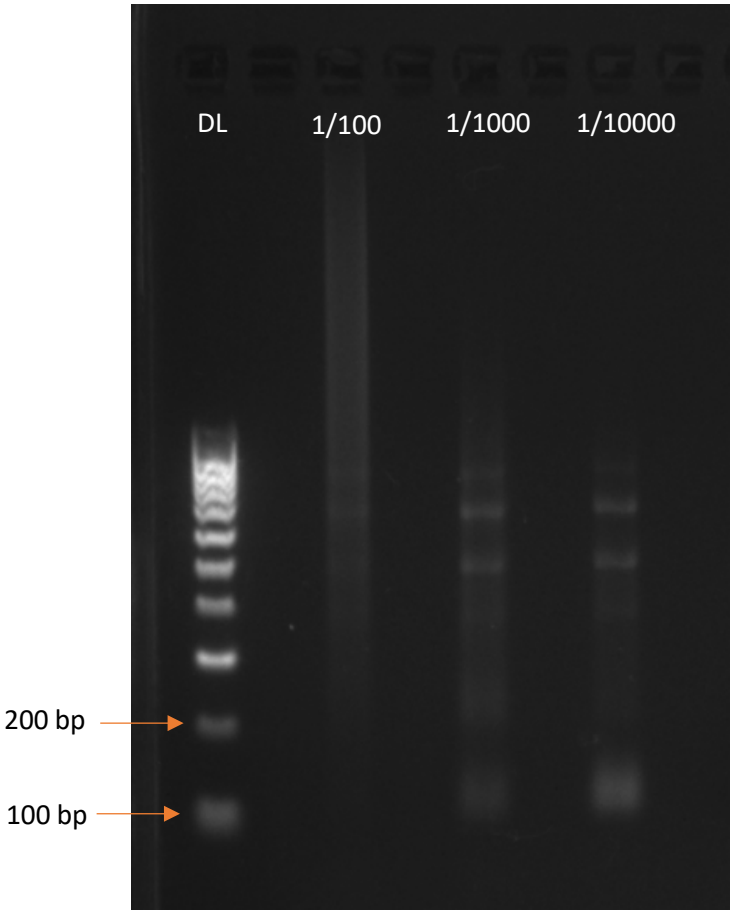


Figure F.13: Gel electrophoresis of the TaqMan qPCR assay for *A. duodenale* dilution of PCR products. Target region for *Ancylostoma* spp. is ~101bp. DNA ladder (DL) produces bands ranging from 10kbp to 100bp.

Conclusion: Combining the results generated from the amplification curve and the gel, it was decided that a dilution of 1/10000 was to be used to optimise this reaction, given that there is also a substantial degree of non-specific binding in this assay.

Optimisation of the annealing/extension temperature of the A. duodenale primer and probe for qPCR

Using the same reaction conditions as previously. We optimised the annealing/extension temperature by performing a temperature gradient to reduce non-specific binding. The gradient consisted of five temperatures 61.4, 60.9, 60, 58.3 and 56.3℃. PCR product of suspected *A. duodenale* 1/10000 dilution as the DNA template.

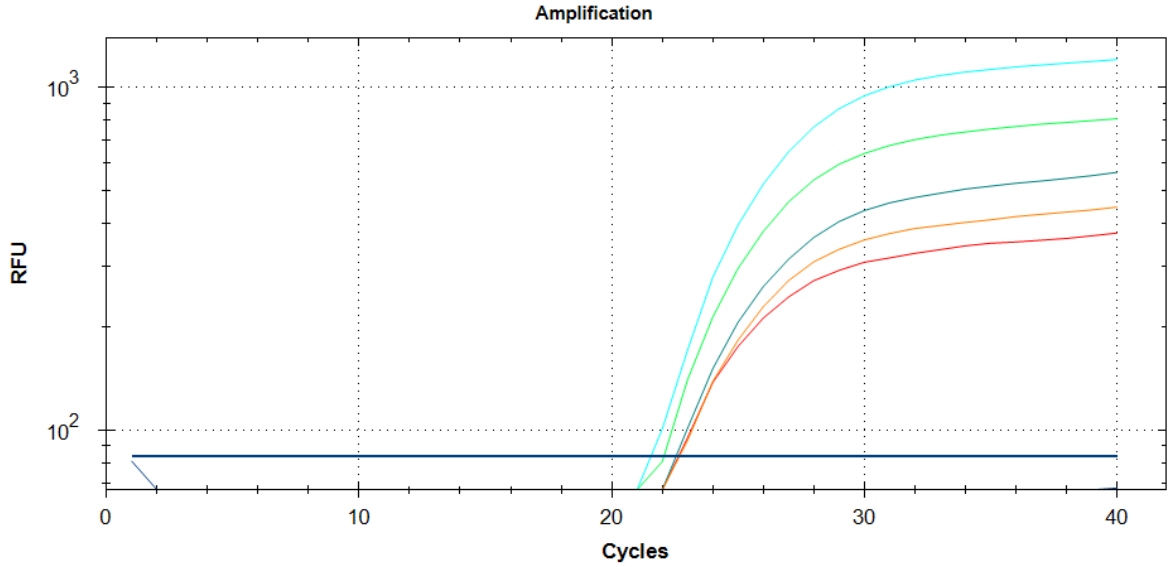


Figure F.14: Amplification curve generated by qPCR of the annealing/extension temperature gradient for *A. duodenale* TaqMan qPCR assay optimisation. 61.4℃ (red line) Cq 22.7; 60.9℃ (orange line) Cq 22.8; 60.0℃ (Dark green line) Cq 22.5; 58.3℃ (light green line) Cq 22.05; 56.3℃ (blue line) Cq 21.60. Baseline threshold at 83.60 relative fluorescence unit (RFU). Scale was transformed into log linear.

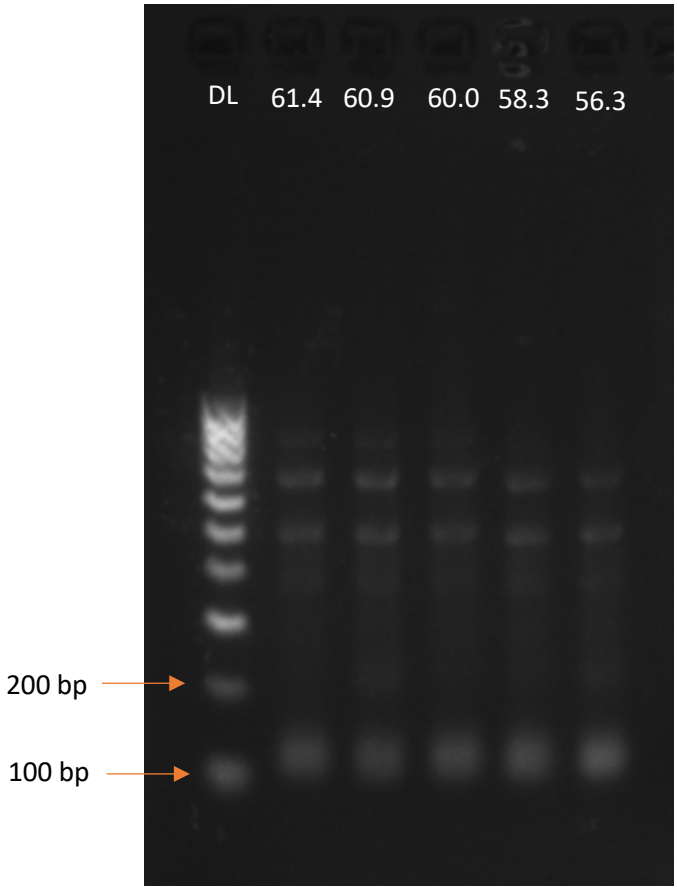


Figure F.15: Gel electrophoresis of qPCR for *A. duodenale* annealing/extension temperature optimisation reactions. Target region for *Ancylostoma* spp. is ~101bp. DNA ladder (DL) produces bands ranging from 10kbp to 100bp.

Overall conclusion: Ultimately, the qPCR amplification curve above suggests that 56.3℃ is the optimal annealing/extension temperature for this *A. duodenale* assay. However, when referring to the gel, none of the trialled temperatures in the gradient reduced the non-specific binding. Therefore, it is likely that the initial reaction whereby amplification was seen may have been contamination and that the positive control for *A. duodenale* cannot be used to optimise and validate this assay. In conjunction with this observation, if we could optimise this reaction, we would not be able to reliably validate whether the reaction detects *A. duodenale* or *A. ceylanicum* based on the cross-reactivity demonstrated in the initial trial. For these reasons, it was concluded that for the purposes of this study, any samples considered positive either by the *A. duodenale* and/or *A. ceylanicum* qPCR assays were overall classified as *Ancylostoma* spp. positive.

This set of primers and probes for *A. duodenale* and *A. ceylanicum* was developed based on the sequence accession numbers EU344797.1 (Gene bank) and DQ831517;DQ780009 (Gene bank), respectively.

Name	% Pairwise I...	Description
EU344797 A. duodenale alignment	100.0%	Alignment of 4 sequences: EU344797.1, Acylostoma Forward, A. duodenale, Acylostoma Reverse (reversed)
EU344797 A. ceylanicum alignment	94.3%	Alignment of 4 sequences: EU344797.1, Acylostoma Forward, A. ceylanicum, Acylostoma Reverse (reversed)
DQ780009 A. duodenale alignment	94.5%	Alignment of 4 sequences: DQ780009, Acylostoma Forward, A. duodenale, Acylostoma Reverse (reversed)
DQ780009 A. ceylanicum alignment	100.0%	Alignment of 4 sequences: DQ780009, Acylostoma Forward, A. ceylanicum, Acylostoma Reverse (reversed)

EU344797.1 – This sequence is *A. duodenale*

DQ780009 – This sequence is A. ceylanicum

Figure F.16: Sequence alignment of *A. duodenale* gene of interest to *A. ceylanicum* probe.

Figure F.17: Sequence alignment of *A. ceylanicum* gene of interest to *A. duodenale* probe

Appendix G Matched Semi-quantitative Scoring of Unconcentrated Versus Concentrated Samples.

Table G.1: Matched microscopy observations of STH ova for semi-quantitative scoring of unconcentrated vs concentrated detected in faecal samples.

Sample No.	*Ascaris* spp. score		Hookworm score		*Trichuris* spp. score	
	Unconcentrated	Concentrated	Unconcentrated	Concentrated	Unconcentrated	Concentrated
274	2+	4+	0	0	0	0
275	0	0	0	0	0	0
276	2+	0	0	0	0	0
277	0	0	0	0	0	0
278	0	0	0	0	0	0
279	5+	5+	0	0	0	0
284	0	0	0	0	0	0
285	0	0	0	0	0	0
286	1+	3+	0	0	0	0
287	4+	5+	0	0	0	0
288	1+	3+	0	0	0	0
289	2+	5+	0	0	0	0
290	0	0	0	0	0	0
291	0	0	0	0	0	0
292	0	0	0	0	0	0
293	0	0	0	2+	0	0
295	0	0	0	0	0	0
299	0	0	0	0	0	0
300	0	0	0	1+	0	0
301	1+	3+	0	0	0	0
303	1+	1+	0	0	0	0
304	0	0	0	0	0	0
305	2+	4+	0	0	0	0
306	0	0	0	0	0	0
307	1+	3+	1+	1+	0	0
308	0	0	0	0	0	0

309	0	0	0	0	0	0
310	0	1+	0	0	0	0
311	1+	3+	0	1+	0	0
312	2+	4+	0	0	0	0
313	0	0	0	0	0	0
314	0	0	0	0	0	0
316	0	0	0	0	0	0
317	0	0	0	0	0	0
318	0	0	0	0	0	0
319	0	1+	0	0	0	0
320	3+	5+	0	0	0	0
321	0	0	0	0	0	0
322	0	0	0	0	0	0
323	0	0	0	0	0	0
324	0	0	1+	2+	0	0
325	0	0	0	1+	0	0
326	0	0	0	0	0	0
327	0	0	0	1+	0	0
328	1+	1+	0	0	0	0
329	0	0	0	0	0	0
330	0	0	0	0	0	0
331	0	0	0	0	0	0
332	1+	0	0	0	0	0
333	2+	3+	0	0	0	0
334	0	0	0	1+	0	0
335	0	0	0	0	0	0
336	0	0	0	0	0	0
337	0	3+	0	0	0	0
338	0	0	0	0	0	0
339	4+	5+	0	0	0	0
340	1+	5+	0	0	0	0
341	3+	5+	0	1+	0	0

342	0	0	0	0	0	0
345	0	0	0	0	0	0
347	0	0	0	0	0	0
348	2+	5+	0	0	0	0
349	0	0	0	0	0	0
350	1+	3+	0	0	0	0
351	0	0	0	0	0	0
352	2+	3+	0	0	0	0
355	3+	4+	0	2+	0	0
356	0	2+	0	0	0	0
357	0	0	0	0	0	0
358	2+	3+	2+	2+	0	0
359	0	0	0	0	0	0
360	0	0	0	0	0	0
361	0	0	0	0	0	0
363	0	0	0	1+	0	0
364	3+	5+	0	0	0	0
365	0	0	0	0	0	0
366	0	0	0	0	0	0
367	0	0	0	0	0	0
368	0	5+	0	0	0	0
369	0	0	0	0	0	0
370	0	0	0	0	0	0
371	0	0	1+	2+	0	0
376	0	0	0	0	0	0
377	0	0	0	1+	0	0
379	0	0	0	0	0	0
382	2+	3+	1+	0	0	0
383	1+	4+	0	0	0	0
384	1+	4+	0	0	0	0
385	0	0	0	0	0	0
386	2+	5+	1+	0	0	0

387	0	0	0	0	0	0
388	0	0	0	0	0	0
389	0	0	1+	0	0	0
390	0	3+	0	0	0	0
391	3+	5+	1+	0	0	0
392	0	0	0	0	0	0
393	1+	3+	0	0	0	0
394	0	0	0	0	0	0
395	0	0	0	0	0	0
396	0	0	0	0	0	0
397	0	0	0	0	0	0
398	0	0	0	0	0	0
399	0	0	0	0	0	0
400	1+	0	0	0	0	0
401	0	0	0	1+	0	0
403	3+	5+	1+	2+	0	0
404	0	0	0	0	0	0
405	0	0	0	0	0	0
406	2+	5+	1+	1+	0	0
407	0	0	1+	1+	0	0
408	1+	2+	1+	0	0	0
409	4+	5+		1+	0	0
410	0	0	0	0	0	0
411	0	0	0	0	0	0
413	0	0	1+	0	0	0
414	1+	2+	0	0	0	0
415	1+	3+	0	2+	0	0
416	0	0	1+	0	0	0
419	0	2+	0	0	1+	0
421	1+	0	0	0	0	0
449	0	0	0	0	0	0
450	0	0	2+	0	0	0

Appendix H STH TaqMan qPCR Cycle Quantification (Cq) Data

Table H.1: Raw data of qPCR Cq values for STH detection

Sample number	*A. lumbricoides*	*T. trichiura*	*N. americanus*	*Ancylostoma* spp.
274	21.1	0	26.3	0
275	0	0	0	0
276	0	0	0	0
277	0	0	30.8	0
278	0	0	35.8	0
279	25.3	0	0	0
284	0	0	0	0
285	0	0	0	0
286	26.4	0	28.3	0
287	22.0	0	32.9	0
288	28.2	0	26.6	0
289	22.8	0	0	0
290	0	0	0	0
291	0	0	0	0
292	0	0	0	0
293	0	0	28.3	0
295	0	0	0	0
299	0	0	0	0
300	0	0	32.0	0
301	28.7	0	27.2	35.3
303	27.9	0	0	0
304		0	0	0
305	24.7	0	0	0
306		0	0	0
307	23.4	0	24.4	27.4

308	0	0	38.2	0
309	0	0	0	0
310	0	0	32.5	0
311	22.5	0	36.2	0
312	21.8	0	24.7	0
313	0	0	0	0
314	0	0	0	0
316	0	0	0	0
317	0	0	0	0
318	0	0	0	0
319	34.3	0	0	0
320	22.1	28.2	26.6	0
321	0	0	0	0
322	0	0	0	0
323	0	0	34.1	0
324	0	0	23.1	0
325	0	0	26.2	0
326	0	0	0	0
327	0	0	25.5	0
328	0	0	0	0
329	0	0	0	0
330	0	0	0	0
331	0	0	30.5	0
332	29.0	0	32.3	0
333	23.5	0	27.1	0
334	0	0	29.7	0
335	0	0	24.6	24.6
336	0	0	0	0
337	29.1	0	34.6	0
338	0	0	0	0

339	23.8	0	0	0
340	23.2	0	35.1	0
341	20.7	0	0	0
342	0	0	25.5	0
345	0	0	27.5	0
347	0	0	35.2	0
348	25.4	0	25.3	0
349	0	0	0	0
350	24.2	0	27.3	0
351	0	0	0	0
352	21.8	0	24.3	0
355	23.1	0	21.2	0
356	27.3	29.6	0	0
357	0	0	0	0
358	24.4	0	26.4	0
359	0	0	0	0
360	0	0	32.1	0
361	0	0	0	0
363	0	0	27.7	0
364	22.1	0	32.4	0
365	0	0	0	0
366	0	0	0	0
367	0	0	0	0
368	27.0	0	22.7	25.1
369	0	0	0	0
370	0	0	0	0
371	0	0	23.6	0
376	0	0	39.3	0
377	0	0	0	0
379	0	0	0	0

382	22.5	0	26.2	0
383	23.0	0	26.4	0
384	25.3	0	0	0
385	**38.3**	0	25.8	0
386	23.7	0	23.5	0
387	0	0	25.7	0
388	0	0	0	0
389	34.0	0	27.5	0
390	23.7	0	25.2	0
391	22.0	0	25.5	0
392	28.7	0	32.8	0
393	27.9	0	27.6	0
394	0	0	0	0
395	0	0	0	0
396	0	0	0	0
397	0	0	32.2	0
398	0	0	0	0
399	0	0	33.7	0
400	0	0	0	0
401	0	0	0	0
403	26.0	0	29.9	0
404	0	0	0	0
405	0	0	28.4	0
406	22.9	0	23.5	0
407	0	0	25.6	0
408	31.0	0	21.2	0
409	24.3	0	27.3	0
410	0	0	30.1	0
411	0	0	34.2	0
413	0	0	26.6	0

414	28.1	0	0	0
415	28.0	0	22.5	0
416	0	0	33.3	0
419	29.5	31.9	32.8	0
421	0	0	25.8	0
449	0	0	35.5	0
450	0	0	26.1	0

Cq values > 36 were deemed negative.

Appendix I Specificity of *A. lumbricoides* primer and probe sequences

Here, I wanted to assess the specificity of the primer and probe combinations used for the *A. lumbricoides* TaqMan qPCR assay against other potentially co-endemic zoonotic *Ascaris* species, like *A. suum*. To do this I took an *in-silico* approach using Blast Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Genious Prime ® (v. 2022.0.2) tool.

Workflow:

Firstly, forward and reverse primers were blast searched using 'Primer-Blast'. In the Primer Pair Specificity Checking parameters section, the Database selected was 'nr' and the organism search was restricted to *Ascaris* (taxid: 6251), *Ascaris lumbricoides* (taxid: 6252) and *Ascaris suum* (taxid: 6253). A multiple sequence alignment to primers and probe were performed for the following sequences:

Accession number	**Definition**
OQ825951.1	*Ascaris suum* isolate P-402 internal transcribed spacer 1 and 5.8S ribosomal RNA gene, partial sequence.
OQ825950.1	*Ascaris suum* isolate P-187 internal transcribed spacer 1 and 5.8S ribosomal RNA gene, partial sequence.
OQ825949.1	*Ascaris suum* isolate P-28 internal transcribed spacer 1 and 5.8S ribosomal RNA gene, partial sequence.
ON678068	*Ascaris lumbricoides* isolate A4 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence.
OQ539679	*Ascaris lumbricoides* isolate NM-59, NM-68 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.
OQ778747	*Ascaris lumbricoides* isolate RNR3 internal transcribed spacer 1, partial sequence.

These sequences were download into Genious Prime® tool and were aligned to the primers and probe selected for *A. lumbricoides* detection in this study. Figure I.1 demonstrates that the primer and probe combinations selected for detecting *A. lumbricoides* in this study are specific for *A. lumbricoides* and not *A. suum*, as the probe is not binding to the aligned sequences.

Figure I.1: Multiple sequence alignment of *Ascaris* primer and probes used for the TaqMan qPCR assay used in this study against sequences originating from *A. lumbricoides* and *A. suum*. *A. lumbricoides* accession numbers: ON678068, OQ539679 and OQ778747 and *A. suum* accession numbers: OQ825951.1, OQ825950.1 and OQ825949.1.

Appendix J *S. stercoralis* Preparation of Positive Template Control, Assessment of Sample Quality and Amplificability and Validation of TaqMan qPCR Assay

J.1 Positive Template Control Pre-treatment Prior to DNA Extraction

A faecal specimen known to contain *S. stercoralis* were kindly provided by Dr Richard Bradbury (Federation University, Australia). DNA extraction from these samples was processed similarly to other faecal samples, as previously described, except for a pre-treatment step before extraction to remove 70% ethanol. Pre-treatment step involved two washes with 0.85% sterile saline, centrifuging samples at 16, 000g for 3 minutes and resuspending the pelleted product in nuclease-free water before proceeding with extraction.

J.2 Assessment of Quality and Amplificability of Genomic DNA Extracted from Positive Template Control

To determine the amplifiability of the *S. stercoralis* positive template control, the extracted DNA was pre-tested by conventional PCR. Positive and negative controls were used, as previously described. Conventional PCR was performed to amplify the 16s rRNA gene using cycling conditions already outlined in Appendix D. Subsequently, the PCR products were run on a 1.5% agarose gel, and the presence or absence of bands was assessed. Samples without bands on the gel after 60 minutes demonstrate sample inhibition and would be re-extracted depending on specimen material availability.

Genomic DNA yield from the positive template control containing *Strongyloides* spp. was ~5.5ng/µL, protein contamination (A260/A280) was considered poor at 1.32, as was salt contamination (A260/A230), 0.31. Together, these values indicate high protein and DNA contamination, but the sample is amplifiable as the 16s rRNA gene has been amplified, as demonstrated in Figure I.1 through gel electrophoresis. Subsequently, it was of interest to investigate whether the *Strongyloides* spp. primers and probes would work in a simple trial containing the positive template control and various negative controls.

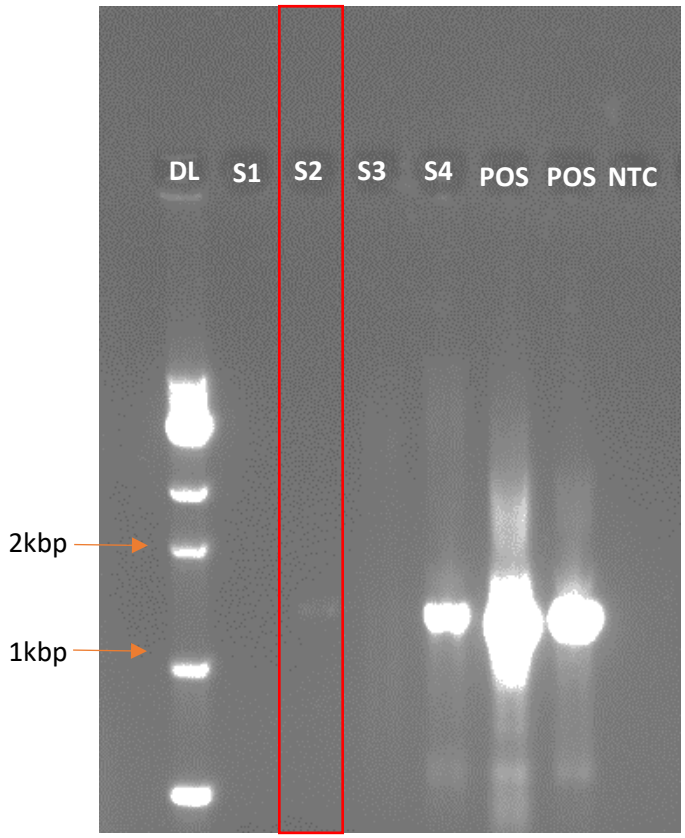


Figure J.1: Gel electrophoresis demonstrating the amplification of the 16s rRNA gene at ~1.4kbp. This PCR product and gel assess sample amplificability of the *S. stercoralis* positive template control (lane S2). Other lanes labelled S1, S3 and S4 are irrelevant to this project. DL: DNA ladder that produces bands from 100bp to 10kbp. POS: positive control and NTC: non-template control.

J.2.1 Trial of S. stercoralis Positive Template Control

Prior to generating a standard curve, we wanted to run a preliminary trial to determine whether *S. stercoralis* positive template control would amplify using primer and probe combinations adopted for this investigation. If successful, it would be of interest to purify this product to enhance the concentration of available DNA for subsequent reactions. This trial reaction was performed with 10 µL of GoTaq qPCR Probe Master Mix (Promega, USA, #A610A), 200nM of each primer, 100nM of probe and 2µL of sample DNA to amount to a final volume of 20µL. Various negative controls were included in this trial, including NTC and negative healthy control. Cycling conditions for the qPCR consisted of the following parameters: 1 cycle of 95℃ for 5 minutes; followed by 40 cycles of 95℃ for 10 seconds, and 60℃ for 60 seconds (Azzopardi, 2021). The Bio-rad CFX96 thermocycler was used for all qPCR reactions. The PCR products ran a 1.5% agarose gel in tris acetate buffer for 60 minutes at 80Vs.

While there was minimal evidence of hits for detecting *S. stercoralis* from the qPCR, something amplified towards the end of the reaction seen in I.2. Resultant PCR products were run on a 1.5% agarose gel at 85V for 60 minutes.

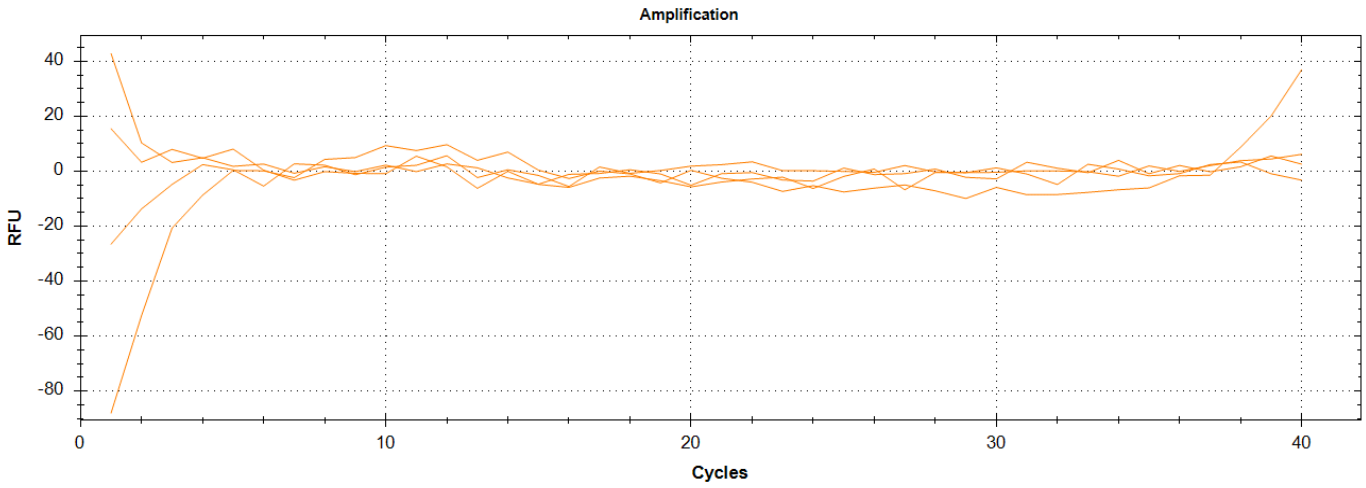


Figure J.2: Amplification curve of the TaqMan qPCR for *S. stercoralis*, using positive template control.

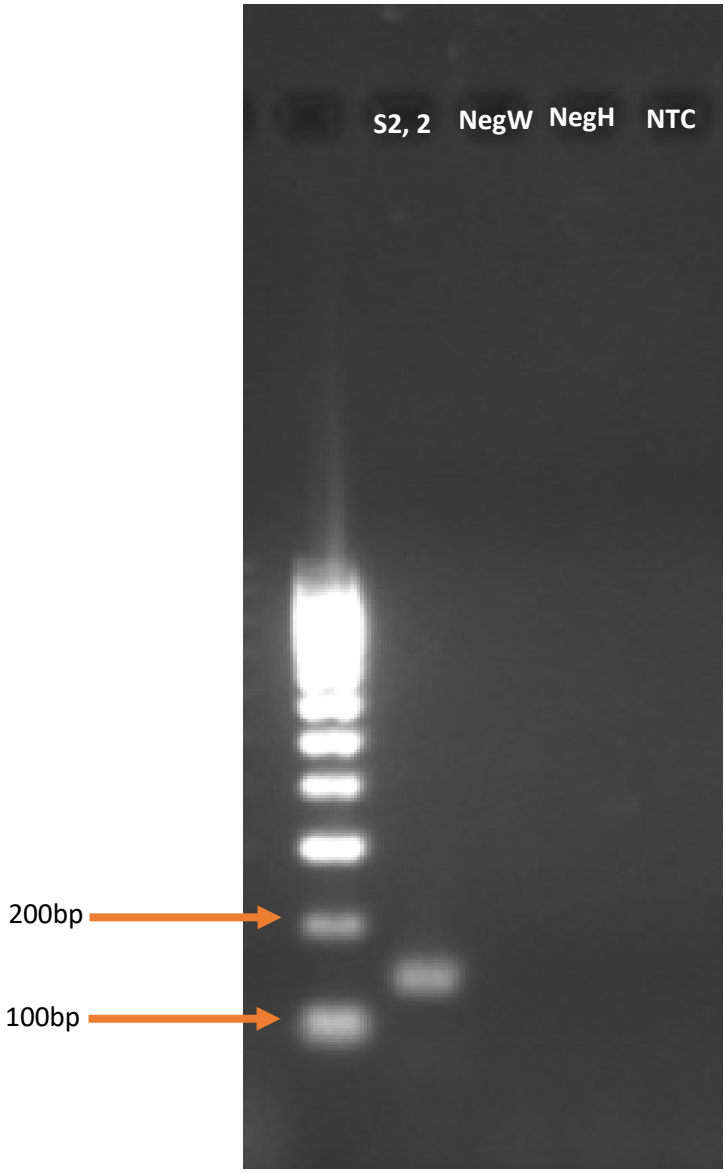


Figure J.3: Gel electrophoresis of a 1.5% agarose gel that suggests the amplification of a product size that is between 100-200bp.

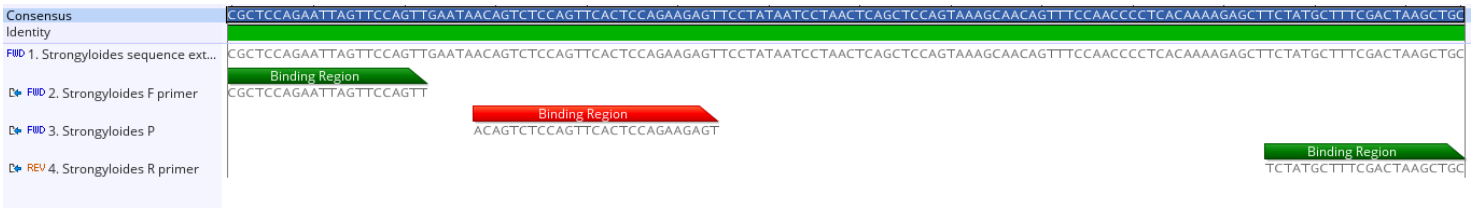


Figure J.4: Alignment of *Strongyloides* stercoralis highly repetitive gene sequence and primers and probes combination (Accession number: AY028262.1). Total amplicon region in this image is 136bp.

The band present in Figure I.3 is between 100-200bp in lane 1, labelled S2, 2. It is presumed that this is the amplicon of *S. stercoralis*. However, the legitimate size of this amplicon was not stipulated by the original authors (Pilotte et al. 2016). Utilising NCBI's gene bank, a sequence of *S. stercoralis* was identified, accession number AY028262.1. This sequence was downloaded and imported into Geneious Prime® 2022.0.2 (https://www.geneious.com) and aligned to the primer and probe sequences to identify the pairwise identity and size of the amplicon. The resultant qPCR amplicon size was found to be ~136bp as seen in Figure I.4. To increase the DNA concentration of the positive template, we cleaned up the PCR product from this experiment.

J.2.2 PCR product clean-up of S. stercoralis positive template control

To enhance the concentration of *Strongyloides* spp. DNA to be used as a positive template control the PCR product amplified in the trial was purified using a PCR clean-up system. The Wizard® SV Gel and PCR Clean-up system (Promega Corporation, WI) was used. PCR clean-up of amplified PCR products was performed as per manufacturer's instructions. Product DNA yield and quality were assessed using the Thermo Scientific NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific Inc, V1.6.198.).

Table J.1: Outcome of PCR clean up, estimating quantity and quality.

Target	DNA yield (ng/µL)	A260/A280	A230/A260
Strongyloides stercoralis	13.8	1.63	0.72

J.3 Optimisation of *S. stercoralis* Primer and Probe for TaqMan qPCR Assay; Optimising for Concentration and Annealing/Extension Temperature

We assessed two primer and probe concentrations and various annealing/extension temperatures to determine optimal reaction conditions. The purified PCR product containing *S. stercoralis* template control was diluted 1:10000. Previously published works suggested using 0.1uM of forward and reverse primers and 0.05uM of probe, so this concentration was compared to doubling the concentrations to 0.2uM forward and reverse primers and 0.1uM of probe. Annealing/extension temperature was trailed from 57.3 to 63.8℃, increasing the temperature by 6.5℃ increments. The outcome of this optimisation reaction is presented in Table I.2.

Table J.2: Optimisation of reaction conditions of primers and probes for *S. stercoralis* TaqMan qPCR assay.

	Cq values	
Temperature gradient (℃)	**High concentration of primers (0.2uM) and probe (0.1uM)**	**Low concentration of primers (0.1uM) and probe (0.05uM)**
63.8	19.66	21.74
63.5	19.34	21.06
62.7	19.45	19.93
61.4	19.68	20.04
59.9	19.28	19.40
58.6	18.50	19.12
57.7	19.13	19.26
57.3	18.32	19.13

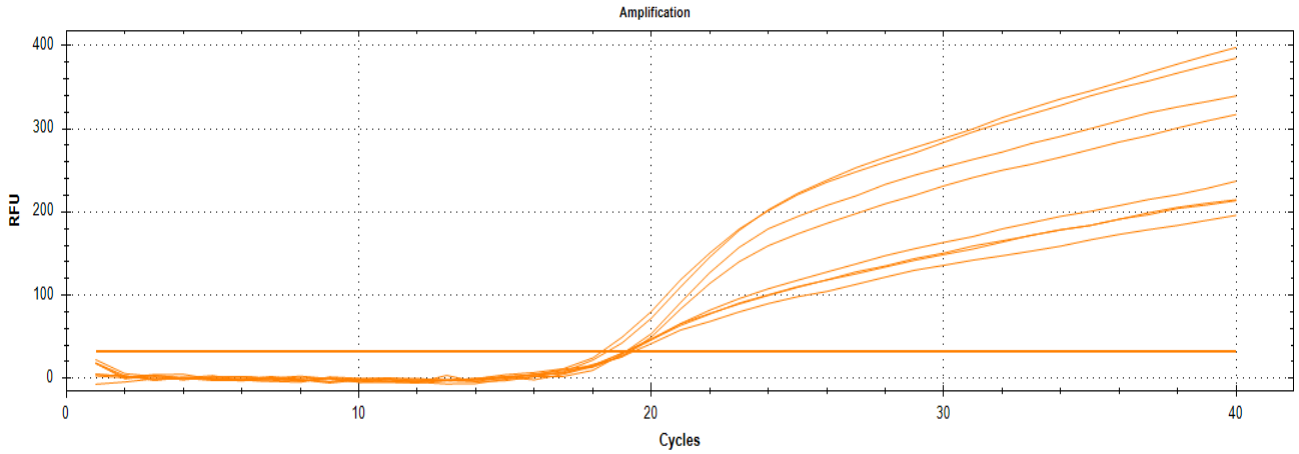


Figure J.5:Amplification curve of samples with temperature gradients 53.8 to 63.8°C, which had the best Cq-values, to compare high vs low concentrations of primer and probe combinations for *S. stercoralis* qPCR assay optimisation.

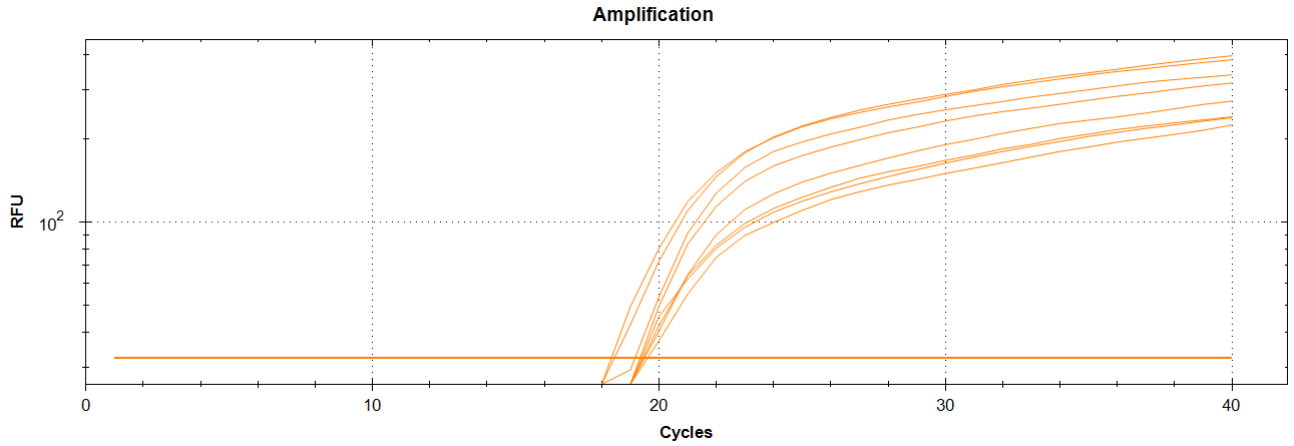


Figure J.6: Amplification curve of samples from temperature gradient 53.8 to 63.8°C for the high concentration primer (200nM) and probe (100nM) combination.

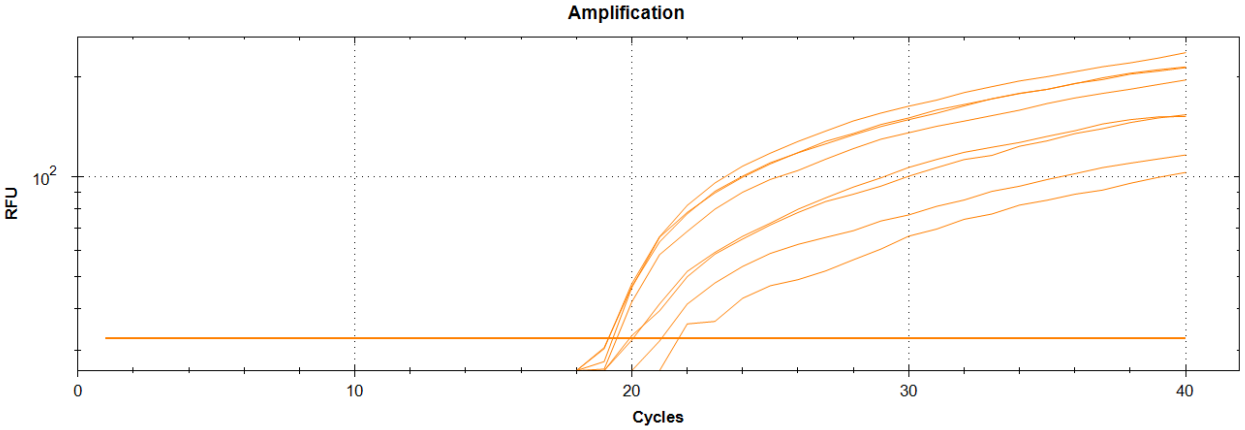


Figure J.7: Amplification curve of samples from temperature gradient 53.8 to 63.8℃ for the low concentration primer (100nM) and probe (50nM) combination.

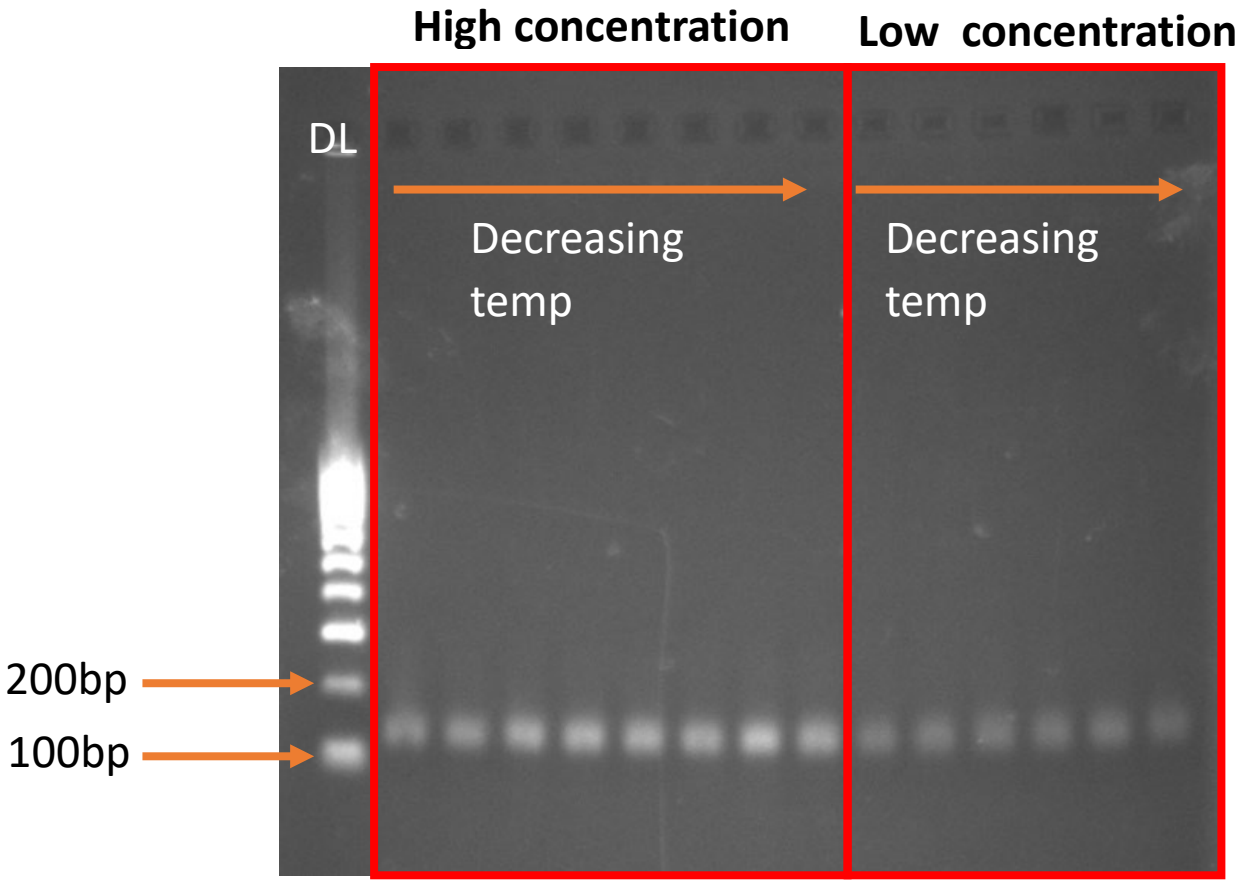


Figure J.8: Gel electrophoresis of optimisation reactions for *S. stercoralis* TaqMan qPCR assay, run on a 1.5% agarose gel. Gel is separated by high vs low concentration of primers/probes and each well (from left to right) contains PCR products from temperature gradients from 53.8 to 63.8℃.

Outcome of optimisation reactions

- High concentrations of primer and probe are more efficient.
- Plateauing of the qPCR curve is better with an increase in primer/probe concentration. Might represent *S. stercoralis* sequence variation, whereby the probe is not quite specific
- Decreasing temperature below 60 degrees also decreases Cq values. Indicating greater specificity

Therefore, reaction conditions that will be used to test field samples include using 200nM of forward and reverse primer and 100nM of probe. Optimal annealing/extension temperature was determined to ~60℃. While 57 also appeared to have low Cq-values, it was of interest to multiplex other various primer and probe combinations in future, and it is optimal to keep all annealing/extension temperature similar.

J.4 Validation of *S. stercoralis* qPCR

Standard Curve

Table J.3: Summary of raw data from the standard curve generated for *S. stercoralis*.

Sample dilution	Cq value	Average Cq (±)	Difference between cycles
Neat	22.31	22.75 (±0.402)	3.80
	22.86		
	23.09		
10^-1	26.48	26.55 (±0.15)	4.37
	26.72		
	26.45		
10^-2	30.14	30.92 (±0.804)	4.68
	30.86		
	31.75		
10^-3	35.25	35.60 (±0.72)	
	35.12		
	36.42		
10^-4	0	0	
	0		
	0		

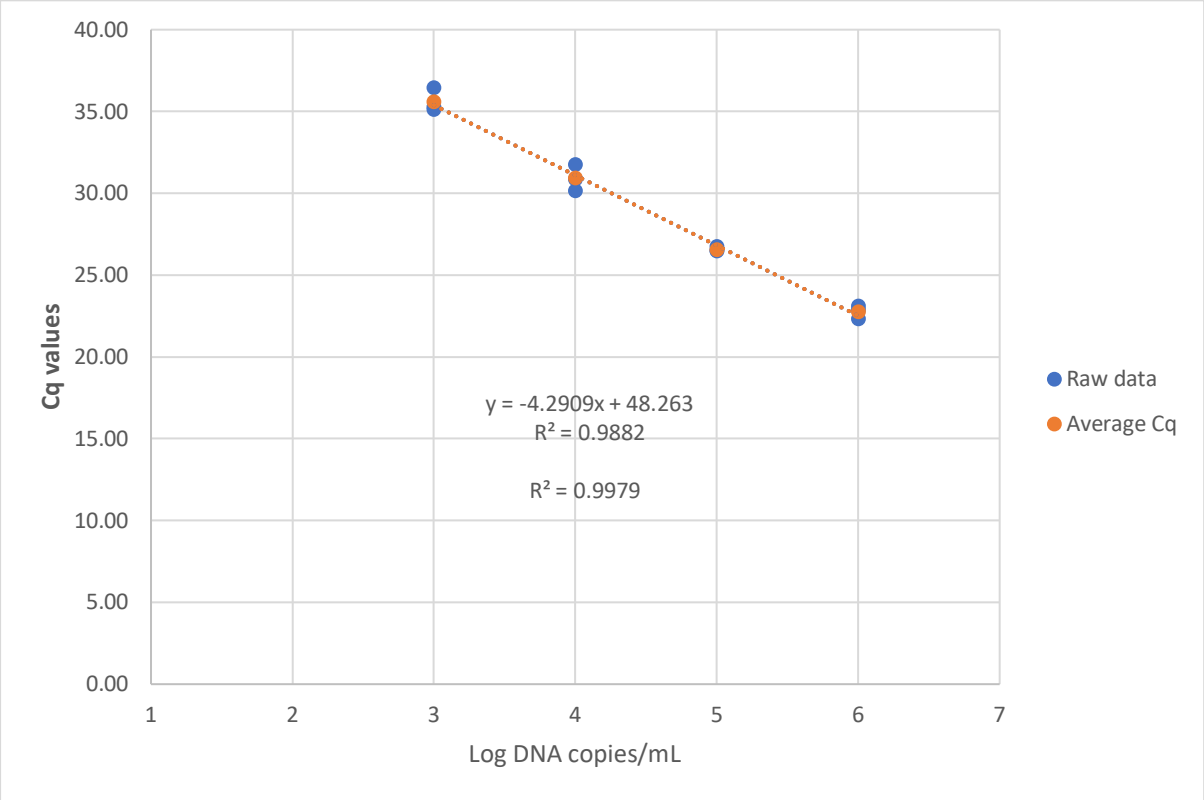


Figure J.9: Cq values obtained from the TaqMan qPCR assay in relation with a 4-fold dilution series (four-point) standard curve of *S. stercoralis*.

Calculation for determining primer and probe efficiency: S. stercoralis

y = -4.2909x + 48.263

Slope (m) = -4.2909

$$E = 10^{-\frac{1}{m}} \quad (1)$$

Equation 1: Part 1 of 2, to determine qPCR assay efficiency

$$E = 10^{-1/-4.2909}$$

$$E = 1.7102$$

Percent (%) PCR efficiency (E%)

$$E\% = (E - 1) \times 100 \quad (2)$$

Equation 2: Part 2 of 2, to determine qPCR assay efficiency

$$E\% = 71.02$$

Therefore, PCR efficiency is 71.0%

Table J.4: Criteria for assessment of TaqMan qPCR assay performance for *S. stercoralis* (adapted from Promega PCR: Guidelines for validating a qPCR assay)

Does the singleplex reaction for this target fit the following criteria	Acceptable?
Cq Values • Are Cq values roughly 3-3.3 different from each dilution?	No
Reproducibility • Are standard deviations within each assay below 0.5 Cq?	Mostly acceptable
Linearity • Is the r^2 value greater than 0.98?	Acceptable
Efficiency • Is efficiency for both reactions between 90-110%?	No, but efficiency is moderately successful

This TaqMan qPCR assay would not be ideal to use for quantification. Further optimisation of this assay to fit the accepted criteria would improve its use for quantification. Sequencing control products would also improve confidence of assay validation.

Appendix K Data for the Anti-*Strongyloides* IgG ELISA and TaqMan qPCR Assay for the Detection of Strongyloidiasis

K.1 Anti-*Strongyloides* IgG ELISA using Plasma Samples

Total IgG positive response to *S. stercoralis* somatic antigens was 22.5% (n = 27/120; 95% CI 16-31%). It was found that 44.2% (n = 53/120) of this cohort was considered intermediate for seroreactivity to *S. stercoralis* antigens, whereas 33.3% (n = 40/120) were deemed negative. For the final analysis, intermediate results were grouped with negative results.

Table K.1: Data of IgG ELISA *Strongyloides* spp. seroreactivity from 120 participant plasma samples.

Sample number	OD unit	OD results interpretation with Intermediate readings	Final OD results interpretation
274	0.254	Intermediate	Negative
275	0.128	Negative	Negative
276	1.433	Positive	Positive
277	0.168	Negative	Negative
278	0.133	Negative	Negative
279	0.166	Negative	Negative
284	0.094	Negative	Negative
285	0.125	Negative	Negative
286	0.112	Negative	Negative
287	0.233	Intermediate	Negative
288	0.136	Negative	Negative
289	0.228	Intermediate	Negative
290	0.13	Negative	Negative
291	0.237	Intermediate	Negative
292	0.167	Negative	Negative
295	0.116	Negative	Negative
299	0.161	Negative	Negative
300	0.102	Negative	Negative
301	2.321	Positive	Positive
303	0.176	Negative	Negative
304	0.234	Intermediate	Negative
305	0.459	Positive	Positive
306	0.246	Intermediate	Negative
307	1.142	Positive	Positive
308	0.235	Intermediate	Negative
309	0.213	Intermediate	Negative
310	0.368	Intermediate	Negative
311	0.338	Intermediate	Negative
312	0.446	Positive	Positive
313	0.24	Intermediate	Negative
314	0.229	Intermediate	Negative
316	0.48	Positive	Positive

317	0.13	Negative	Negative
318	0.793	Positive	Positive
319	0.214	Intermediate	Negative
320	0.365	Intermediate	Negative
321	0.327	Intermediate	Negative
322	0.268	Intermediate	Negative
323	0.155	Negative	Negative
324	1.932	Positive	Positive
325	2.388	Positive	Positive
326	0.452	Positive	Positive
327	0.485	Positive	Positive
328	0.173	Negative	Negative
329	0.177	Negative	Negative
330	0.427	Positive	Positive
331	0.291	Intermediate	Negative
332	0.183	Negative	Negative
333	0.19	Negative	Negative
334	0.277	Intermediate	Negative
335	0.712	Positive	Positive
336	0.204	Intermediate	Negative
337	0.194	Negative	Negative
338	0.267	Intermediate	Negative
339	0.317	Intermediate	Negative
340	0.259	Intermediate	Negative
341	0.239	Intermediate	Negative
342	0.357	Intermediate	Negative
345	0.248	Intermediate	Negative
347	0.245	Intermediate	Negative
348	0.249	Intermediate	Negative
349	0.834	Positive	Positive
350	0.193	Negative	Negative
351	0.169	Negative	Negative
352	0.791	Positive	Positive
355	0.218	Intermediate	Negative
356	0.402	Positive	Positive
357	0.414	Positive	Positive
358	0.293	Intermediate	Negative
360	0.201	Intermediate	Negative
361	0.24	Intermediate	Negative
363	0.165	Negative	Negative
364	0.114	Negative	Negative
365	0.282	Intermediate	Negative
366	0.114	Negative	Negative
367	0.362	Intermediate	Negative

368	0.259	Intermediate	Negative
369	0.195	Negative	Negative
370	0.3	Intermediate	Negative
371	1.224	Positive	Positive
376	0.3	Intermediate	Negative
377	0.215	Intermediate	Negative
379	2.544	Positive	Positive
382	0.145	Negative	Negative
383	0.541	Positive	Positive
384	0.141	Negative	Negative
385	0.239	Intermediate	Negative
386	0.433	Positive	Positive
387	0.346	Intermediate	Negative
388	0.239	Intermediate	Negative
389	1.089	Positive	Positive
390	0.164	Negative	Negative
391	0.401	Positive	Positive
392	0.134	Negative	Negative
393	0.25	Intermediate	Negative
394	0.284	Intermediate	Negative
395	0.134	Negative	Negative
396	0.255	Intermediate	Negative
397	0.204	Intermediate	Negative
398	0.267	Intermediate	Negative
399	0.145	Negative	Negative
400	0.236	Intermediate	Negative
401	0.106	Negative	Negative
403	0.324	Intermediate	Negative
404	0.178	Negative	Negative
405	0.276	Intermediate	Negative
406	0.556	Positive	Positive
407	0.121	Negative	Negative
408	0.144	Negative	Negative
409	0.269	Intermediate	Negative
410	1.333	Positive	Positive
411	0.14	Negative	Negative
413	0.166	Negative	Negative
414	0.295	Intermediate	Negative
415	1.168	Positive	Positive
416	0.144	Negative	Negative
419	0.266	Intermediate	Negative
421	1.005	Positive	Positive
449	0.3	Intermediate	Negative
450	0.347	Intermediate	Negative

OD = optical density

Table K.2: Summary of raw data from anti- *Strongyloides* spp. IgG assay

	Positive	Intermediate	Negative
All participants (N=120)	**Frequency n (%)**		
	27 (22.5)	53 (44.2)	40 (33.3)

K.2 *S. stercoralis* Detection in Faecal Samples by TaqMan qPCR

Table K.3: Cq values obtained by qPCR targeting *S. stercoralis* in 122 faecal samples.

Sample number	qPCR Cycle quantification	Result Interpretation
274	0	Negative
275	0	Negative
276	34.3	Positive
277	0	Negative
278	0	Negative
279	0	Negative
284	0	Negative
285	0	Negative
286	0	Negative
287	0	Negative
288	0	Negative
289	0	Negative
290	0	Negative
291	0	Negative
292	0	Negative
293	0	Negative
295	0	Negative
299	0	Negative
300	0	Negative
301	0	Negative
303	0	Negative
304	0	Negative
305	0	Negative
306	0	Negative
307	0	Negative
308	0	Negative
309	0	Negative
310	0	Negative
311	0	Negative
312	0	Negative
313	0	Negative
314	0	Negative
316	0	Negative
317	0	Negative
318	0	Negative
319	0	Negative
320	0	Negative
321	0	Negative
322	0	Negative
323	0	Negative

324	35.4	Positive
325	28.0	Positive
326	0	Negative
327	0	Negative
328	0	Negative
329	0	Negative
330	0	Negative
331	0	Negative
332	0	Negative
333	0	Negative
334	0	Negative
335	0	Negative
336	0	Negative
337	0	Negative
338	0	Negative
339	0	Negative
340	0	Negative
341	0	Negative
342	0	Negative
345	0	Negative
347	0	Negative
348	0	Negative
349	0	Negative
350	0	Negative
351	0	Negative
352	33.6	Positive
355	0	Negative
356	0	Negative
357	0	Negative
358	0	Negative
359	0	Negative
360	0	Negative
361	0	Negative
363	0	Negative
364	0	Negative
365	0	Negative
366	0	Negative
367	0	Negative
368	0	Negative
369	0	Negative
370	0	Negative
371	0	Negative
376	0	Negative
377	0	Negative

379	0	Negative
382	0	Negative
383	0	Negative
384	0	Negative
385	0	Negative
386	0	Negative
387	0	Negative
388	0	Negative
389	0	Negative
390	0	Negative
391	0	Negative
392	0	Negative
393	0	Negative
394	0	Negative
395	0	Negative
396	0	Negative
397	0	Negative
398	0	Negative
399	0	Negative
400	0	Negative
401	0	Negative
403	0	Negative
404	0	Negative
405	0	Negative
406	0	Negative
407	0	Negative
408	0	Negative
409	32.0	Positive
410	0	Negative
411	0	Negative
413	0	Negative
414	0	Negative
415	0	Negative
416	0	Negative
419	0	Negative
421	0	Negative
449	0	Negative
450	0	Negative

Cycle quantification value > 36 were deemed negative.

Appendix L Characteristics of Balimo cohort used for analysis

Table L.1: Raw continuous and categorical data used for data analysis.

Sample No.	Gender	Age	<40?	BMI	BMI categorical	Residency	Employed/ Unemployed	Level of Education	Cough?	SOB?
274	Male	42	No	-	-	Balimo town	Employed	Primary	No	No
275	Male	34	Yes	-	-	Balimo town	Employed	Secondary	Yes	No
276	Male	60	No	22.11	Normal	Balimo town	Employed	Primary	No	Yes
277	Female	36	Yes	-	-	Balimo town	Unemployed	Secondary	No	No
278	Female	55	No	25.49	Overweight	Balimo town	Employed	Secondary	No	No
279	Female	62	No	27.89	Overweight	Village	Unemployed	Primary	Yes	Yes
284	Female	58	No	25.09	Overweight	Balimo town	Employed	Secondary	Yes	Yes
285	Female	55	No	28.96	Overweight	Balimo town	Unemployed	Primary	Yes	No
286	Female	57	No	30.15	Overweight	Balimo town	Unemployed	Primary	No	No
287	Female	56	No	30.75	Overweight	Balimo town	Unemployed	Primary	Yes	Yes
288	Female	46	No	29.31	Overweight	Balimo town	Unemployed	Primary	No	No
289	Female	26	Yes	31.45	Overweight	Balimo town	Unemployed	Secondary	No	No
290	Female	44	No	28.44	Overweight	Balimo town	Unemployed	Secondary	No	No
291	Female	37	Yes	28.44	Overweight	Balimo town	Unemployed	Secondary	Yes	No

292	Male	34	Yes	26.98	Overweight	Balimo town	Unemployed	Secondary	No	Yes
293	Male	48	No	21.76	Normal	Balimo town	Unemployed	Primary	Yes	No
295	Female	48	No	24.58	Normal	Balimo town	Employed	Secondary	No	Yes
299	Female	49	No	22.19	Normal	Balimo town	Unemployed	Secondary	Yes	Yes
300	Female	35	Yes	29.16	Overweight	Balimo town	Unemployed	Secondary	Yes	Yes
301	Female	42	No	20.76	Normal	Balimo town	Unemployed	Primary	No	No
303	Female	43	No	37.08	Overweight	Balimo town	Employed	Primary	No	Yes
304	Female	47	No	22.91	Normal	Balimo town	Employed	Secondary	No	Yes
305	Female	51	No	26.10	Overweight	Balimo town	Unemployed	-	Yes	No
306	Female	53	No	26.40	Overweight	Balimo town	Unemployed	Primary	No	No
307	Male	31	Yes	23.71	Normal	Balimo town	Unemployed	Secondary	Yes	Yes
308	Female	41	No	22.55	Normal	Balimo town	Unemployed	Primary	No	Yes
309	Male	36	Yes	24.73	Normal	Balimo town	Unemployed	Secondary	Yes	Yes
310	Female	30	Yes	21.16	Normal	Balimo town	Unemployed	Secondary	No	No
311	Female	56	No	22.76	Normal	Balimo town	Employed	Secondary	No	No
312	Female	40	Yes	23.77	Normal	Village	Unemployed	Secondary	No	No
313	Male	68	No	20.27	Normal	Balimo town	Unemployed	Primary	Yes	No
314	Female	48	No	25.73	Overweight	Balimo town	Employed	Secondary	Yes	Yes
316	Female	53	No	20.30	Normal	Balimo town	Unemployed	-	No	No

317	Male	66	No	22.68	Normal	Balimo town	Employed	Primary	No	No
318	Male	49	No	23.49	Normal	Village	Employed	Secondary	Yes	Yes
319	Male	28	Yes	19.80	Normal	Balimo town	Unemployed	Secondary	Yes	Yes
320	Female	16	Yes	19.73	Normal	Village	Unemployed	Primary	Yes	No
321	Female	47	No	28.60	Overweight	Village	Employed	Primary	Yes	Yes
322	Male	68	No	18.94	Normal	Balimo town	Unemployed	Primary	No	No
323	Female	86	No	19.44	Normal	Balimo town	Employed	Primary	No	No
324	Male	28	Yes	22.02	Overweight	Balimo town	Employed	Secondary	No	No
325	Female	24	Yes	19.84	Normal	Balimo town	Unemployed	Secondary	No	No
326	Female	52	No	25.32	Overweight	Balimo town	Unemployed	Primary	No	No
327	Female	21	Yes	20.37	Normal	Balimo town	Unemployed	Secondary	Yes	No
328	Female	60	No	30.68	Overweight	Balimo town	Employed	Secondary	No	Yes
329	Female	61	No	24.50	Normal	Village	Employed	None	Yes	Yes
330	Male	63	No	29.26	Overweight	Village	Employed	Secondary	No	No
331	Male	39	Yes	26.78	Overweight	Village	Employed	Secondary	Yes	No
332	Male	68	No	26.46	Overweight	Village	Employed	Primary	Yes	No
333	Male	40	Yes	24.75	Normal	Village	Employed	Secondary	Yes	No
334	Female	54	No	19.48	Normal	Balimo town	Employed	Secondary	Yes	Yes
335	Male	46	No	23.74	Normal	Village	Employed	Primary	No	Yes

336	Male	34	Yes	26.26	Overweight	Balimo town	Employed	Secondary	No	Yes
337	Female	36	Yes	23.98	Normal	Balimo town	Employed	Secondary	No	Yes
338	Male	56	No	30.05	Overweight	Balimo town	Unemployed	Secondary	No	Yes
339	Female	54	No	21.53	Normal	Balimo town	Unemployed	Secondary	No	No
340	Female	23	Yes	24.68	Normal	Balimo town	Employed	Secondary	No	No
341	Female	41	No	32.11	Overweight	Balimo town	Unemployed	Secondary	No	No
342	Female	57	No	31.73	Overweight	Balimo town	Unemployed	Secondary	No	No
345	Male	37	Yes	23.80	Normal	Balimo town	Unemployed	Secondary	No	No
347	Female	68	No	28.05	Overweight	Balimo town	Unemployed	Primary	No	No
348	Female	28	Yes	21.19	Normal	Balimo town	Unemployed	Secondary	No	No
349	Female	42	No	20.87	Normal	Balimo town	Unemployed	Primary	No	No
350	Female	28	Yes	19.61	Normal	Balimo town	Unemployed	Secondary	No	No
351	Female	52	No	22.94	Normal	Balimo town	Unemployed	Primary	No	No
352	Female	50	No	22.35	Normal	Balimo town	Unemployed	Primary	No	No
355	Female	51	No	21.16	Normal	Balimo town	Unemployed	Primary	No	No
356	Male	37	Yes	17.07	Malnourished	Balimo town	Employed	Primary	Yes	Yes
357	Male	23	No	26.71	Overweight	Balimo town	Employed	Secondary	No	No
358	Female	70	No	24.57	Normal	Balimo town	Employed	Primary	No	No
359	Male	60	No	23.92	Normal	Village	Unemployed	Secondary	No	Yes

360	Female	62	No	19.69	Normal	Balimo town	Unemployed	Primary	Yes	No
361	Female	51	No	25.76	Overweight	Balimo town	Employed	Secondary	No	No
363	Female	60	No	23.50	Normal	Balimo town	Unemployed	Primary	No	No
364	Female	68	No	27.84	Overweight	Balimo town	Employed	Primary	No	No
365	Female	63	No	28.85	Overweight	Balimo town	Unemployed	Primary	No	Yes
366	Female	52	No	29.99	Overweight	Balimo town	Unemployed	Secondary	No	No
367	Male	56	No	20.59	Normal	Balimo town	Unemployed	-	No	Yes
368	Female	54	No	26.14	Overweight	Balimo town	Unemployed	Secondary	No	No
369	Female	19	Yes	16.52	Malnourished	Balimo town	Unemployed	Primary	Yes	Yes
370	Female	24	Yes	30.77	Overweight	Balimo town	Unemployed	Secondary	Yes	Yes
371	Male	59	No	26.74	Overweight	Balimo town	Unemployed	Secondary	Yes	No
376	Female	47	No	37.62	Overweight	Balimo town	Unemployed	Secondary	No	Yes
377	Male	49	No	26.16	Overweight	Balimo town	Employed	Secondary	Yes	No
379	Female	51	No	25.46	Overweight	Balimo town	Unemployed	Primary	Yes	Yes
382	Female	52	No	23.49	Normal	Village	Unemployed	Secondary	No	No
383	Female	18	Yes	17.13	Malnourished	Village	Employed	Primary	No	Yes
384	Male	17	Yes	20.72	Normal	Village	Unemployed	Secondary	No	No
385	Female	22	Yes	20.16	Normal	Village	Unemployed	Secondary	No	No
386	Female	48	No	19.43	Normal	Village	Unemployed	Primary	No	No

387	Male	20	Yes	20.83	Normal	Village	Unemployed	Secondary	Yes	Yes
388	Female	52	No	20.43	Normal	Village	Unemployed	Primary	No	No
389	Female	54	No	21.94	Normal	Village	Unemployed	Secondary	No	No
390	Female	33	Yes	28.62	Overweight	Village	Unemployed	Secondary	No	Yes
391	Female	32	Yes	24.14	Normal	Village	Unemployed	Secondary	No	No
392	Male	51	No	23.56	Normal	Village	Employed	Secondary	Yes	Yes
393	Male	64	No	22.09	Normal	Village	Unemployed	Primary	No	No
394	Male	41	No	23.87	Normal	Village	Employed	Primary	No	Yes
395	Female	63	No	30.00	Overweight	Village	Unemployed	Primary	Yes	No
396	Female	57	No	27.74	Overweight	Village	Unemployed	Primary	No	No
397	Male	59	No	18.97	Normal	Village	Unemployed	Secondary	Yes	No
398	Female	58	No	19.27	Normal	Village	Unemployed	Primary	Yes	Yes
399	Female	56	No	25.06	Overweight	Village	Unemployed	Primary	Yes	Yes
400	Male	72	No	27.68	Overweight	Village	Unemployed	Secondary	Yes	No
401	Female	72	No	26.06	Overweight	Village	Unemployed	Primary	No	No
403	Female	34	Yes	15.82	Malnourished	Village	Unemployed	Primary	Yes	Yes
404	Female	42	No	20.89	Normal	Village	Unemployed	Primary	No	Yes
405	Female	51	No	21.85	Normal	Village	Unemployed	Primary	No	Yes
406	Male	37	Yes	21.64	Normal	Village	Unemployed	Primary	No	No

407	Male	68	No	19.33	Normal	Village	Unemployed	Primary	Yes	No
408	Male	50	No	20.06	Normal	Village	Unemployed	Primary	Yes	Yes
409	Male	-	-	22.71	Normal	Village	Unemployed	Primary	Yes	Yes
410	Female	50	No	22.04	Normal	Village	Unemployed	Primary	No	No
411	Female	52	No	24.80	Normal	Village	Unemployed	Primary	No	No
413	Male	53	No	25.44	Overweight	Village	Unemployed	Primary	Yes	Yes
414	Female	36	Yes	14.88	Malnourished	Village	Employed	Secondary	No	No
415	Female	34	Yes	12.57	Malnourished	Village	Employed	Secondary	No	No
416	Female	47	No	19.14	Normal	Village	Employed	Secondary	No	No
419	Male	44	No	23.76	Normal	Village	Employed	Primary	No	No
421	Male	33	Yes	15.66	Malnourished	Village	Employed	Secondary	Yes	No
449	Female	57	No	26.87	Overweight	Balimo town	Unemployed	Primary	No	No
450	Female	36	Yes	-	-	Balimo town	Employed	Secondary	No	No

Appendix M Assessment of Multicollinearity and Goodness-of-fit for Multivariable Logistics Regression Models

M.1 STH Multivariable Logistics Regression Model Assessment

Collinearity

Variable [Reference level]	VIF	R2 with other variables
Intercept		
Biological sex[Male]	1.088	0.08053
Age	2.849	0.6490
<40?[Yes]	2.888	0.6537
Body Mass Index	1.173	0.1476
Residency [Village]	1.103	0.09325
SOB?[Yes]	1.054	0.05163

Goodness-of-fit

Area under the ROC curve	
Area	0.7645
Std. Error	0.04459
95% confidence interval	0.6771 to 0.8519
P value	<0.0001

Negative predictive power (%)	62.50
Positive predictive power (%)	75.25

Pseudo R squared	**Value**	**Interpretation**
Tjur's R squared	0.1694	Regression model explains relatively small amount of variation of the data. Limited predictive power

Hypothesis tests	**Statistic**	**P value**	**Interpretation**
Hosmer-Lemeshow	6.682	0.5713	Selected model is correct

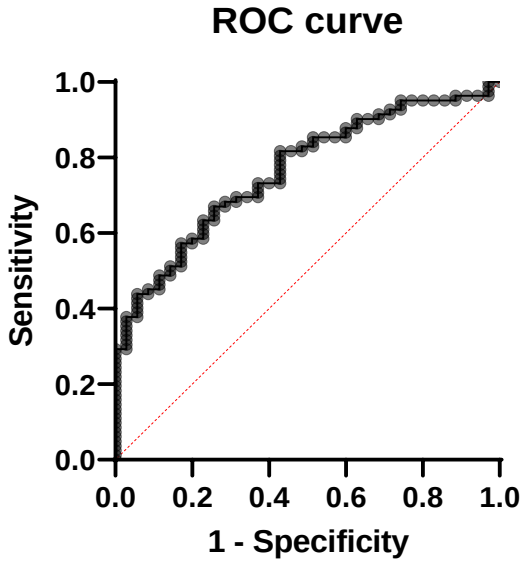


Figure M.1: Reporter operating curve (ROC) to test goodness-of-fit for the model of predictor variables of any STH infection.

M.2 A. lumbricoides multivariable logistics regression model assessment

Collinearity

Variable [Reference level]	VIF	R2 with other variables
Intercept		
Biological sex[Female]	1.120	0.1070
Age	2.960	0.6621
<40?[Yes]	3.229	0.6903
BMI categorical[Overweight]	1.173	0.1472
BMI categorical[Malnourished]	1.302	0.2322
Residency [Village]	1.164	0.1405
Hookworm co-infection [Yes]	1.174	0.1482
Strongyloides spp. co-infection [1]	1.119	0.1065

Goodness-of-fit

Area under the ROC curve	
Area	0.7286
Std. Error	0.04667
95% confidence interval	0.6371 to 0.8200
P value	<0.0001

Negative predictive power (%)	68.83
Positive predictive power (%)	57.50

Pseudo R squared	Value	Interpretation
Tjur's R squared	0.1790	Regression model explains relatively small amount of variation of the data. Limited predictive power

Hypothesis tests	Statistic	P value	Interpretation
Hosmer-Lemeshow	6.052	0.6414	Selected model is correct

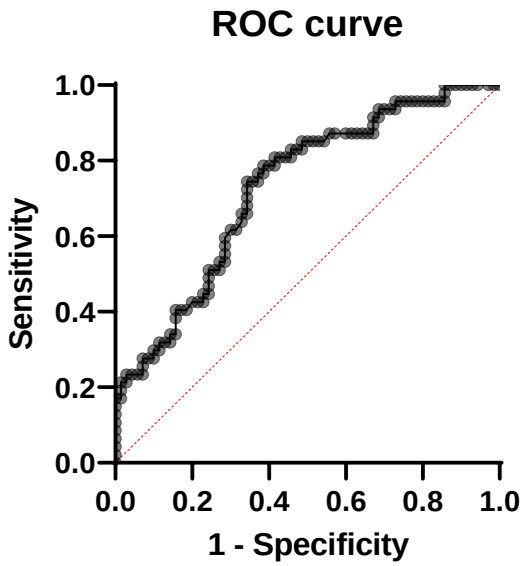


Figure M.2: Reporter operating curve (ROC) to test goodness-of-fit for the model of predictor variables of *A. lumbricoides* infection.

M.3 *Strongyloides* spp. Multivariable Logistics Regression Model Assessment

Collinearity

Variable [Reference level]	VIF	R2 with other variables
Intercept		
Biological sex[Female]	1.074	0.06891
Age	2.867	0.6511
<40?[Yes]	3.206	0.6880
Body Mass Index	3.958	0.7474
BMI categorical[Overweight]	3.086	0.6760
BMI categorical[Malnourished]	1.833	0.4546
A. lumbricoides co-infection[Yes]	1.282	0.2200
N. americanus co-infection[Yes]	1.340	0.2537

Goodness-of-fit

Area under the ROC curve	
Area	0.7465
Std. Error	0.04963
95% confidence interval	0.6492 to 0.8438
P value	0.0001

Negative predictive power (%)	79.44
Positive predictive power (%)	50.00

Pseudo R squared	**Value**	**Interpretation**
Tjur's R squared	0.1340	Regression model explains relatively small amount of variation of the data. Limited predictive power

Hypothesis tests	**Statistic**	**P value**	**Interpretation**
Hosmer-Lemeshow	12.64	0.1249	Selected model is correct

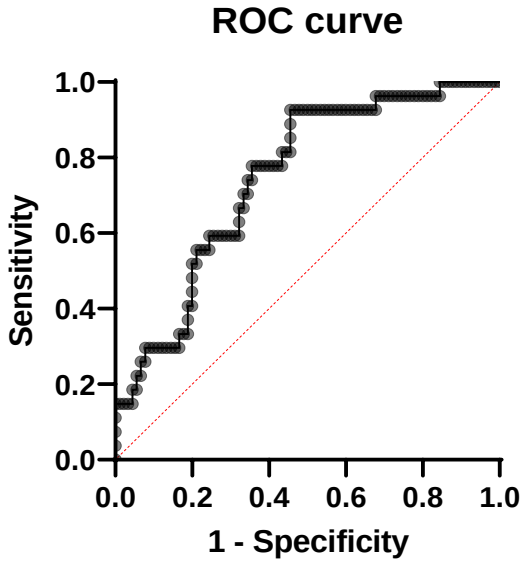


Figure M.3: Reporter operating curve (ROC) to test goodness-of-fit for the model of predictor variables of *Strongyloides* spp. infection.

M.4 Hookworm (undifferentiated) Multivariable Logistics Regression Model Assessment

Collinearity

Variable [Reference level]	VIF	R2 with other variables
Intercept		
Biological sex[Male]	1.091	0.08358
<40?[Yes]	1.243	0.1954
Body Mass Index	4.109	0.7566
BMI categorical[Overweight]	3.154	0.6830
BMI categorical[Malnourished]	1.850	0.4594
Residency [Village]	1.137	0.1207
SOB?[Yes]	1.118	0.1056
A. lumbricoides co-infection[Yes]	1.186	0.1569

Goodness-of-fit

Area under the ROC curve	
Area	0.8166
Std. Error	0.03997
95% confidence interval	0.7382 to 0.8949
P value	<0.0001

Negative predictive power (%)	75.93
Positive predictive power (%)	79.37

Pseudo R squared	Value	Interpretation
Tjur's R squared	0.2972	Regression model explains relatively small amount of variation of the data. Limited predictive power

Hypothesis tests	Statistic	P value	Interpretation
Hosmer-Lemeshow	12.32	0.1374	Selected model is correct

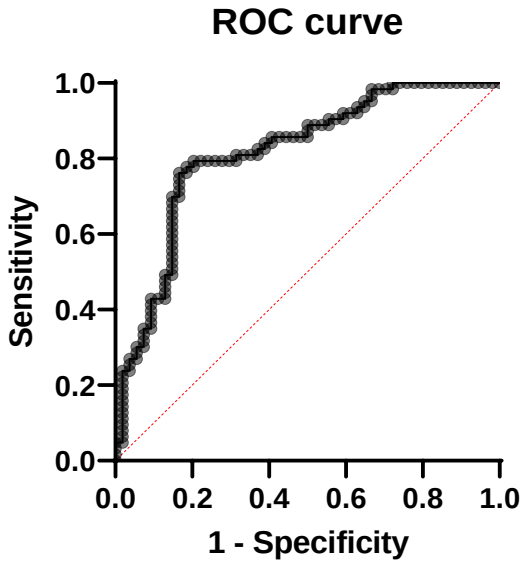


Figure M.4: Reporter operating curve (ROC) to test goodness-of-fit for the model of predictor variables of Hookworm (undifferentiated) infection.

M.5 *N. americanus* Multivariable Logistics Regression Model Assessment

Collinearity

Variable [Reference level]	VIF	R2 with other variables
Intercept		
Biological sex[Male]	1.138	0.1214
Age	3.047	0.6718
<40?[Yes]	3.196	0.6871
Body Mass Index	4.186	0.7611
BMI categorical[Overweight]	3.199	0.6874
BMI categorical[Malnourished]	1.862	0.4631
Residency [Village]	1.139	0.1223

SOB?[Yes]	1.173	0.1476
A. lumbricoides co-infection[Yes]	1.197	0.1644
Strongyloides spp. co-infection [Yes]	1.178	0.1514

Goodness-of-fit

Area under the ROC curve	
Area	0.8260
Std. Error	0.03760
95% confidence interval	0.7523 to 0.8997
P value	<0.0001

Negative predictive power (%)	75.00
Positive predictive power (%)	75.41

Pseudo R squared	**Value**	**Interpretation**
Tjur's R squared	0.3173	Regression model explains relatively small amount of variation of the data. Limited predictive power

Hypothesis tests	**Statistic**	**P value**	**Interpretation**
Hosmer-Lemeshow	13.56	0.0940	Selected model is correct

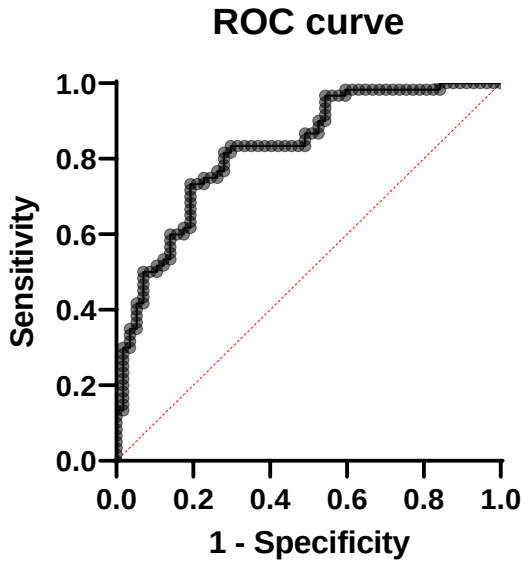


Figure M.5: Reporter operating curve (ROC) to test goodness-of-fit for the model of predictor variables of *N. americanus* infection.