Development and clinical performance of nucleic acid amplification techniques for the diagnosis of Strongyloides stercoralis

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Development and clinical performance of nucleic acid amplification techniques for the diagnosis of *Strongyloides stercoralis*.

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Table of Contents

GLOSSARY

Ab	Antibody
Ag	Antigen
Amplicon	PCR product
bp	Base pair
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
ClustalW2	ClustalW2 multiple sequence alignment tool
cPCR	Conventional PCR
CRS	Composite reference standard
C_t	Cycling threshold
DCP	Department of Clinical Parasitology
DDJB	DNA Databank of Japan
$\Delta\Delta$ C_t	Delta delta C_t
DIS	Disseminated infection syndrome
ELISA	Enzyme-linked immunosorbent assay
EM	Taqman® Environmental master mix 2.0
FECT	Formalin: triton x-100/ ethyl acetate concentration technique for stool microscopy
gfp	Green fluorescent protein of the jellyfish *Aequorea victoria*
GIT	Gastrointestinal tract
HIS	Hyperinfection syndrome
HPA	Health protection agency, now known as Public Health England
HRMC	High resolution melt curve
HS	Hotstart Taq® polymerase
HTD	Hospital for Tropical Diseases
IDEA study	Infectious diseases in Europe and Africa study
LAMP	Loop mediated isothermal amplification
LAMP time	Detection of turbidity produced by amplification of target DNA
$MgCl_2$	Magnesium chloride
PBS	Phosphate Buffered Saline, pH 7.2
PCR	Polymerase chain reaction
PHE	Public Health England

qPCR	Real-time PCR
SDS	Sodium dodecasulphate
S. fuelleborni	*Strongyloides fuelleborni fuelleborni*
S. fuelleborni kellyi	*Strongyloides fuelleborni kellyi*
S. stercoralis	*Strongyloides stercoralis*
SPSs	In-house developed LAMP primer set (inner, outer and loop primers) for the detection of *S. stercoralis* DNA
Stro18S	qPCR primer set and double-labelled probe for the detection of *S. stercoralis* DNA (Stro18S-1530F, Stro18S-1630R and Stro18S-1586T)
UCLH	University College London NHS Foundation Trust
UK	United Kingdom
WTM	Western Travel Medicine
WSs	Published LAMP primer set (inner, outer and loop primers) for the detection of *S. stercoralis* DNA (Watts *et al*., 2014)

LIST OF FIGURES

for target amplicon, only a 97bp internal control amplicon was demonstrated, Lane 17: 97bp internal control and a 145bp anomalous amplicon, Lanes 1 and 18 contain a 100bp DNA marker ladder.

Figure 4.7: qPCR products run on a 2% agarose gel run at 100V for 1.5 hours. Lanes 1, 13: 100bp hyperladder, Lanes 2, 4, 6, 7, 10 and 12: 101bp target amplicon, Lanes 3, 8, 11: Negative, Lanes 5, and 9: 500- 525bp anomalous amplicon. Only one band (target DNA) is generated as the *gfp* primers were not added to the mixture.

Figure 4.8: Mean results of the limit of detection for qPCR (DNA extracted and cloned, using pJET® 1.2 plasmid vector, from a spiked negative stool samples containing 1 *S. stercoralis* larva/ µl). The dilutions 10^{-2} to 10^{-10} were run in triplicate over two different qPCR amplification runs. Black, dark grey and light grey series indicate the triplicate results.

Figure 4.9: Box and Whisper plots for the determination of run cycles for the qPCR protocol. Notch indicates the mean and the whiskers indicate the 25^{th} and 75^{th} percentiles. qPCR HS (Ct) indicates all the C_t values regardless of product size on a 2% agarose gel, qPCR HS (Ct and gel 101) indicates the C_t values of the real-time PCR with a product size of 101 bp.

Figure 4.10: Youden Plot- Determination of cut-off C_t for qPCR positive results. The rectangle represents 2SD (standard deviation) coverage (95% CI: 25.250 to 31.721). Anomalous bands (pale grey square) are found at C_ts >30, No true positive results (clear circle) are found at C_ts >45. Possible true positive results (dark grey square) i.e. samples with very little DNA may be found at C_ts, <40. False positive samples (black square) determined by negative results for the cPCR repeat may also be found at C_ts <45.

Figure 4.11: Comparison of the internal control C_ts from all runs and internal control C_ts after normalisation of the results between runs. Normalisation was carried out so that the internal control data could be assessed between different qPCR runs. (Raw data in grey and normalised data in black).

Figure 4.12: Semi-logarithmic graph for the determination of amplification efficiency (E) in the internal control (*gfp*). A negative slope is obtained if the standard curve is run in the order of most dilute to most concentrated and the trendline is exponential as the graph is semi-logarithmic.

Figure 4.13: Semi-logarithmic graph for the determination of amplification efficiency (E) in target DNA (*S. stercoralis*). The trendline is exponential as the graph is semi-logarithmic.

LIST OF TABLES

Appendix 5: (Table) Business plan for the introduction of a NAAT for the diagnosis of *S. stercoralis* in clinical samples at DCP.

Appendix 6: (Table) *S. stercoralis* DNA detection- raw data for lamp and qpcr assays. Key at end of table.

ABSTRACT

The laboratory diagnosis of *Strongyloides stercoralis* (*S. stercoralis*) at the Department of Clinical Parasitology (DCP) by the routine methods of microscopy and *Strongyloides* culture is not sensitive due to the, usually, low parasite load and intermittent larval excretion of the parasite. Serology (enzyme-linked immunosorbent assay) suffers from a lack of specificity because *Strongyloides* antibodies are known to cross- react with schistosomal, filarial and other helminthic antibodies in serological tests. Moreover, antibody levels are slow to decline after successful treatment therefore serology cannot be used to monitor point of cure. A missed diagnosis of strongyloidiasis in immunocompromised patients or those about to undergo iatrogenic immune suppression may have severe, even fatal, consequences. The disease is poorly studied because of the lack of sensitive, specific and cost-effective tests. Therefore, the decision was made to evaluate and validate nucleic acid amplification techniques (NAATs) for the diagnosis of *S. stercoralis* for use in a well- resourced specialist referral parasitology laboratory. A novel loop mediated isothermal amplification (LAMP) assay was also developed for use in resource- limited regions. The study was conducted over two years (2014-2016) and examined 284 residual diagnostic samples. The cohort was drawn from patients attending a central London western travel medicine (WTM) clinic.

The NAATs chosen for this study were a published real- time PCR (qPCR) assay (ten Hove *et al*., 2009) and a novel LAMP assay. The NAATs were compared to the combined reference standard of microscopy, culture and serology for the diagnosis of *S. stercoralis* in stool samples. The development of the novel LAMP assay for use in resource- limited areas included the investigation of methods for rapid, simple and cost- effective DNA extraction. The qPCR and LAMP assays detect target DNA within areas on either side of the *S. stercoralis* 18S rRNA genome hypervariable region (Hasegawa *et al*., 2009). In this study the LAMP and qPCR assays demonstrated a limit of detection of 10^{-3} and 10^{-4}, respectively for *S. stercoralis* DNA detection in clinical samples. Specificity was determined for the LAMP and qPCR assays to be 100% and 94.83%, respectively and the cost per test was calculated as £4.80 and £8.21, respectively. In this study, persistence of *S. stercoralis* DNA in clinical samples was improved when the samples were stored at -20^{o}C.

While the LAMP assay has a shorter turnaround time and is less costly than qPCR, the superior efficiency of qPCR detection of *S. stercoralis* DNA in clinical samples established that the qPCR assay was a more suitable addition to the diagnostic repertoire at a high- throughput WTM clinic. The LAMP assay showed promise for deployment in resource- limited areas and as a point- of- care test but further work is required to optimise the LAMP assay for these purposes.

This project is dedicated to my parents, my sister and my brother-in-law.

ACKNOWLEDGEMENTS

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Katherine M Bowers

London

September 2017

DECLARATION

I declare that the present work was carried out in accordance with the Guidelines and Regulations of the University of Westminster. The work is original except where indicated by special reference in the text.

The submission as a whole or part is not substantially the same as any that I previously made or am currently making, whether in published form, for a degree, diploma or similar qualification at any university or similar institution.

Until the outcome of the current application to the University of Westminster is known, the work will not be submitted for any such qualification at another university or similar institution.

Any views expressed in this work are those of the author and in no way represent those of the University of Westminster.

Signed: KBowes

Date: 25th September 2017

CHAPTER 1: INTRODUCTION

1.1. *STRONGYLOIDES STERCORALIS*

Strongyloides stercoralis is a soil transmitted helminth infection endemic to tropical, subtropical and temperate climates with poor sanitation and high humidity (Basile *et al*., 2010, Bonn *et al*., 2010, Buonfrate *et al*., 2015, Cimeno and Krowlewiecki, 2014, WHO, 2010).

There are more than 50 species of *Strongyloides*, but only 3 are capable of causing disease in humans: *S. stercoralis*, *Strongyloides fuelleborni fuelleborni* and *Strongyloides fuelleborni kellyi* (WHO, 2010, Requena-Méndez *et al*., 2013, Taylor *et al*., 2014). *S. fuelleborni*, generally infects non-human primates, is rare in humans and causes a sporadic zoonotic disease in Africa. *S. fuelleborni kellyi* is only found in Papua New Guinea and if left untreated can cause fatal "swollen belly" syndrome in new-borns in Papua New Guinea. It is thought to have derived from a local zoonotic source and is now considered a separate species from *S. fuelleborni* on the basis of small subunit ribosomal RNA gene sequences (Dorris *et al*., 2002, Getaneh *et al*., 2010, Taylor *et al*., 2014, Makker *et al*., 2015). Neither *S. fuelleborni* nor *S. fuelleborni kellyi* is thought to cause auto-infection as they shed eggs rather than larvae in faeces (Olsen *et al*., 2009).

Strongyloides stercoralis, the major human pathogen, is capable of causing a disease ranging from asymptomatic or chronic non-specific gastro-intestinal or respiratory symptoms to life-threatening hyperinfection syndrome (HIS). This is due to the unique lifecycle by which the disease can persist in humans for decades due to auto-infection by infectious L3 larvae penetrating the perianal skin or gut wall. This can lead to persistence of infection in immunocompetent hosts or uncontrolled multiplication and invasion of organs outside the gastro-intestinal tract if the patient becomes immunocompromised (WHO, 2010, Requena-Méndez *et al*., 2013). Human strongyloidiasis from pet origin is rare and this is thought to be due to the diversification of *S. stercoralis* into strains adapted to human and non-human hosts (Hasegawa *et al*., 2009). Jaleta *et al*. (2017) described two genetically different types of *S. stercoralis* carried in dogs in Northern Cambodia. One genetic type of *S. stercoralis* was found in both humans and dogs, indicating that dogs may be an important reservoir for zoonotic strongyloidiasis.

Strongyloides stercoralis was first described in 1876 in French soldiers returning from Indochina by Louis Normand and the full life-cycle, pathology and clinical features were described in the 1930's (Schär *et al*., 2013b). Looss, (in the 1900's) after infecting himself and observing the larvae 64 days later, commented that there were still gaps in the knowledge of strongyloidiasis that hampered the

control of the disease (Looss, 1905). This is still the case because of the lack of suitably sensitive and specific diagnostic tests (Taylor *et al*., 2014).

Strongyloides stercoralis is increasingly found in patients attending western travel medicine (WTM) and gastro-intestinal clinics due to the changing patterns of travel, migration and working practices (Gorospe and Oxentenko, 2012, Kramme *et al*., 2011, Libman *et al*., 1993, ten Hove *et al*., 2009). Strongyloidiasis is common in migrants from South East Asia and Africa (Biggs *et al*., 2009) and this trend was also seen in this current study. In developed countries the disease is mainly found in immigrants and returning soldiers (Schär *et al*., 2013b). In previously endemic countries (e.g. Italy and Spain) older individuals may harbour the disease for decades (Requena-Méndez *et al*., 2014). Imported neglected tropical diseases have become an important issue in western travel medicine (ten Hove *et al*., 2009, Whitty *et al*., 2000).

1.2. LIFECYCLE

S. stercoralis has a complicated life-cycle (Figure 1.1) with host-mediated (homogonic) and free-living environmental (heterogonic) life-cycles (Taylor *et al*., 2014).

The stimuli favouring the free-living or parasitic life-cycles are unknown. Shiwaku *et al*. (1988) showed that temperature and faecal dilution have an effect on larval development and Minato *et al*. (2008) demonstrated the development of adult worms at temperatures <15^{o}C and the development of infectious L3 larvae at temperatures > 15^{o}C in *Strongyloides ratti*. A chemical agent is likely involved in the development of *S. stercoralis* larvae (Taylor *et al*., 2014). Siddiqui *et al*. (2000) suggested a parasite receptor that triggers steroid mediated HIS by affecting the development of the parasite.

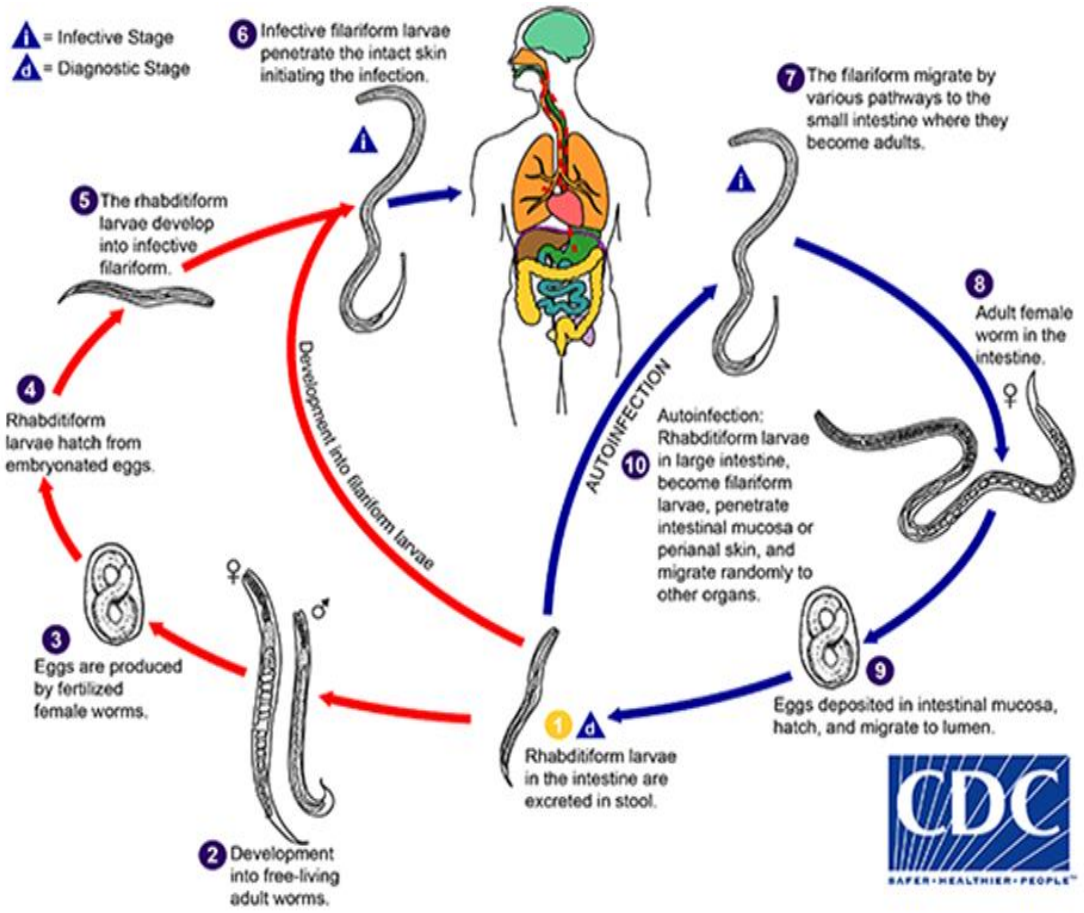


Figure 1.1: Lifecycle of *Strongyloides stercoralis* detailing the parasitic (homogonic) life-cycle (1, 6 - 10) and the free-living (heterogonic) life-cycle (1-6) and the auto- infection cycle (1, 7 and 10) (CDC, n.d.)

Strongyloides stercoralis is a soil- transmitted helminth that can also be transmitted by the faecal-oral route and infected breast milk (Montes *et al*., 2010, Requena- Méndez *et al*., 2013). Infectious filariform larvae (L3) penetrate the skin (mostly the soles of the feet) and travel via the bloodstream to the alveolar spaces. The larvae are expectorated and travel via the trachea to the oesophagus and are swallowed. They become embedded in the lamina propia of the small intestine (duodenum) where they mature into adult worms. All parasitic worms are female (2.2 x 0.5 mm) and reproduce parthenogenically, producing up to 40 embryonated eggs per day. The eggs hatch inside the gut lumen and release rhabditiform larvae (L1) in the faeces (Barros and Montes, 2014, Dorris *et al*., 2002, Ganesh and Cruz, 2011, Mejia and Nutman, 2012, Promma and Songthamwat, 2012).

The rhabditiform larvae (250 µm x 20 µm) may develop into infectious filariform larvae (600 µm x 20 µm) and cause auto-infection by penetrating the lumen of the gut or the perianal skin to continue the infectious cycle causing a disease that may persist for decades (Barros and Montes, 2014, Mejia and Nutman, 2012, Repetto *et al*., 2010) or go directly into the free-living cycle in the soil where they can survive without a mammalian host (Montes *et al*., 2010). Adult males (0.9mm) fertilise the eggs in the environment. There is a possibility that pseudogamic reproduction (where the sperm stimulates the egg cell to produce an embryo but no genetic material is transferred) occurs in *S. stercoralis* or that *S. stercoralis* is a complex of related species or sub species but more work is required in this field (Schär *et al*., 2014).

Research is hampered by the fact that *S. stercoralis* has only one free-living heterogonous life cycle in culture and this makes it difficult to study in a laboratory environment (Olsen *et al*., 2009, Taylor *et al*., 2014)

1.3. MORBIDITY AND MORTALITY

More than half of the infections in immunocompetent people are asymptomatic. Becker *et al*. (2011) showed no difference between infected and non-infected individuals in terms of asymptomatic presentation and Sudarshi *et al*. (2003), in a study carried out at the Hospital for Tropical Diseases (HTD); found that a third of travellers and a third of migrants with confirmed strongyloidiasis were asymptomatic. The development of symptoms appears to be related to the parasite load and immune status (Makker *et al*., 2015, Khieu *et al*., 2013). Chronic diarrhoea is a feature of strongyloidiasis in HIV positive individuals but strongyloidiasis is no longer categorised as an AIDS-defining disease (Montes *et al*., 2010). Chronic clinical manifestations include abdominal discomfort, vomiting, diarrhoea, gastro-intestinal haemorrhage, anorexia, cough, shortness of breath, asthma and a fleeting serpingous urticarial rash at the entry of larvae into the skin and during auto-infection known as *larva currens* (Montes *et al*., 2010). *Larva currens* occurs on the trunk, upper leg and buttocks and moves rapidly at 2-10 cm/ hour. It is a localised allergic response to parasites migrating through the skin. It is indurated, has a red flare and disappears within hours and is pathognomic for strongyloidiasis (Checkley *et al*., 2010, Fischer, 2015, Ganesh and Cruz, 2011). Loeffler's syndrome (fever, malaise, cough, wheezing and shortness of breath) may occur when the larvae are migrating through the lungs in acute or disseminated infection. Larvae are detected in faeces only if the parasite load is high enough to be detected microscopically (Requena-Méndez *et al*., 2013).

Immunocompromised individuals are most at risk of developing severe, life-threatening disease where large numbers of *S. stercoralis* larvae invade multiple organs, frequently involving the

musculoskeletal system (Barros and Montes, 2014, Levenhagen and Costa-Cruz, 2014). Larvae can be found in cerebrospinal fluid, bronchial lavage, sputum, faeces and organs outside of the gastro-intestinal tract (GIT) (Basile *et al*., 2010, Bisoffi *et al*., 2011, Buonfrate *et al*., 2013). The mortality rate for disseminated infection (DIS), found in extra-GIT or respiratory sites, is 85-100% and the mortality rate for HIS where increased numbers of larvae are found in the GIT and lungs is 60-85%. It is easy to diagnose HIS and DIS because of the high parasite numbers but it is difficult to treat (Kassalik and Mönkemüller, 2011). HIS/ DIS may occur 3 months after kidney transplant and carries a mortality of 50%, post-haemopoietic transplant HIS/ DIS may occur immediately after transplantation and has a mortality rate of 85% which may be due to a higher immunosuppressive treatment regime (Roxby *et al*., 2009). Iatrogenic immune suppressive therapy in patients with undiagnosed strongyloidiasis has a fatality rate of up to 87% when corticosteroids are administered. This is not as pronounced with the administration of cyclosporine A immunosuppressive therapy (Mejia and Nutman, 2012, Montes *et al*., 2010, Olsen *et al*., 2012). This may be due to a parasite receptor that triggers development of the L3 infectious larvae (Siddiqui *et al*., 2000). Larval penetration of the gut wall can lead to severe Gram-negative bacterial sepsis, pneumonia and meningitis.

1.4. PREVALENCE AND RISK FACTORS

Strongyloides stercoralis is found in parts of Europe, South Eastern United States, Asia, Africa and Latin America (Becker *et al*., 2015, Bisoffi *et al*., 2011, Bisoffi *et al*., 2013). More recently the parasite has been reported by Taylor *et al*. (2014) in endemic populations in the arid Australian outback associated with faulty or poorly maintained air-conditioning units. The geographical range for the disease is worldwide with the exception of Antarctica (Schär *et al*., 2013a).

Previous prevalence rates of 30-100 million *S. stercoralis* infected individuals were known to be under-estimated (WHO, 2010) and more recent estimates based on serological data has put the prevalence at over 350 million infected individuals (Requena-Méndez *et al*., 2014). There is an under-appreciated economic and public health burden with no Disability Adjusted Life Years (DALYs) for the disease as there are no distinct clinical markers to use (Krowlewiecki *et al*., 2013) and this is impeding the progress of strongyloidiasis control in endemic areas (Becker *et al*., 2011, Glinz *et al*., 2011). Prevalence depends on parasite/ host and environmental interactions so targeted control measures may prevent transmission (Norman *et al*., 2010) and the development of life-threatening disease (Saugar *et al*., 2015). There are no suitable diagnostic tests to determine prevalence and monitor disease control in endemic areas and this has led the World Health Organisation (WHO) to declare *S. stercoralis* a neglected tropical disease (WHO, 2010). Knopp *et al*.

(2008) and Khieu *et al*. (2013) noted that the highest prevalence rate was observed when different parasitological and serological diagnostic methods were combined. Many studies have been performed in endemic areas using different diagnostic methods and study protocols to determine the prevalence of the disease, but these are difficult to compare because of the different testing protocols used (Table 1.1).

Table 1.1: Prevalence and risk of infection: *S. stercoralis*.

AUTHOR	COMMENT	PREVALENCE	RISK FACTOR
Norman *et al*., (2010)	Persistence of strongyloidiasis increases with length of exposure Under-representation from some areas so could not extrapolate to all cases of strongyloidiasis Use of low sensitivity methods	Thailand 62.5%	Prolonged exposure carries a higher risk of infection (migrants/living in endemic areas/visiting friends and relatives/World War II veterans, returning travellers)
Rayan *et al*., (2012)	Different prevalence around the world Dependent on area and monitoring tests, Mainly low sensitivity tests	4-50% worldwide Egypt 1.0-11% Netherlands 10.4%	Living in endemic areas
Schär *et al*. (2013b)	20 year review of community, hospital and migrant studies. Studies in China and India (areas with the highest populations) are scarce and do not cover the whole geographical region 63.3% of studies used low sensitivity methods (community studies) 28.6% used moderate sensitivity methods (hospital studies) 9.9% used high sensitivity tests (PCR) (migrant studies)	South East Asia 17-26% one study as low as 0.02% Ghana 11.6% slightly higher in males Japan 5.5-30.2% (dependent on age) Africa 0.1-91.8% Central and South America 1-75.3% Migrant studies suggest that 10-40% of population in endemic areas is infected	Males and older age group Areas in Europe and the United States of America-farming, mining, migrants and returning soldiers Trend to a higher risk in adults than in children
Makker *et al*., (2015)	Review of *S. stercoralis* prevalence rate in different countries and different diagnostic methods	Kenya 80% Gabon 92% Namibia 99% Dominican Republic 98% Peru 75% PNG: 99%	Immunocompromised or HTLV1 co-infection at risk of severe disease

Reviewing the literature underlined the need for strongyloidiasis to be recognised so that those at risk of developing severe disease can be treated (Gorospe and Oxentenko, 2012, Kramme *et al*., 2011, Libman *et al*., 1993, ten Hove *et al*., 2009). The ability of *S. stercoralis* to cause severe, life-threatening disease in immunocompromised hosts, in both endemic and non-endemic areas, means that missing a diagnosis of strongyloidiasis can have fatal consequences (Barros and Montes, 2014, Levenhagen and Costa-Cruz, 2014)

In an attempted meta-analysis of the global distribution and risk factors Schär *et al*. (2013b) found associations for risk of disease using pooled odds-ratios (OR) with HTLV1 co-infection (OR 2.48, 95% BCI:0.70-9.03), HIV positive (OR 2.17, 95% BCI: 1.18-4.01), alcoholics (OR 6.69, 95%BCI: 1.47-33.8) and patients with malignancies and/or immunocompromising conditions (they were unable to perform the meta-analysis because of the diverse reporting of studies in the literature, nevertheless an association was noted in the studies). An analysis of studies in children could not be performed, but the literature suggests that children have a lower prevalence rate than adults. Norman *et al*. (2010) found that infection with *S. stercoralis* was cumulative in travellers and that single exposures were unlikely to lead to infection. This coupled with the possibility of increased access to warm moist soil due to a change in life-style (child to adult) may suggest a reason for this trend in children.

1.5. DIAGNOSIS

Laboratory diagnosis is important for the detection of asymptomatic disease and a diagnostic test may be employed for more than one purpose. Diagnosis of infectious diseases may be used for:

I. Patient management and treatment follow-up;
II. Screening for asymptomatic diseases;
III. Surveillance;
IV. Monitoring public health intervention;
V. Detection of drug resistance markers (Peeling *et al*., 2007).

It is known that there is a need for more sensitive tests for the diagnosis of strongyloidiasis in clinical samples (Requena-Méndez *et al*., 2013). There are limited methods available for the detection of *S. stercoralis* and these methods lack suitable and adequate sensitivity and specificity (Requena-Méndez *et al*., 2013). The requirement for costly, high maintenance equipment and technical expertise makes these methods unsuitable for use in resource- limited areas (Olsen *et al*., 2009, Requena-Méndez *et al*., 2013). This has led to a lack of knowledge regarding the prevalence and epidemiology of the disease. (WHO, 2010, Requena-Méndez *et al*., 2013).

Diagnosis is problematic as microscopy has a sensitivity of 15-30% using single stool samples (from migrants or travellers) and the formalin: triton x-100/ ethyl acetate concentration technique (FECT) (Requena-Méndez *et al*., 2013). Low larval loads and intermittent secretion make this an insensitive method although the sensitivity can be improved to nearly 100% if 7 stool samples are examined. However, collection of 7 consecutive stool samples is not always feasible. Microscopic expertise is required to correctly identify *Strongyloides* larvae and this may not be available outside of endemic areas and even in endemic areas training may not be available (Figure 1.2). It is important to remember that microscopy detects all parasites and multiple infections are common amongst migrants (ten Hove *et al*., 2009).

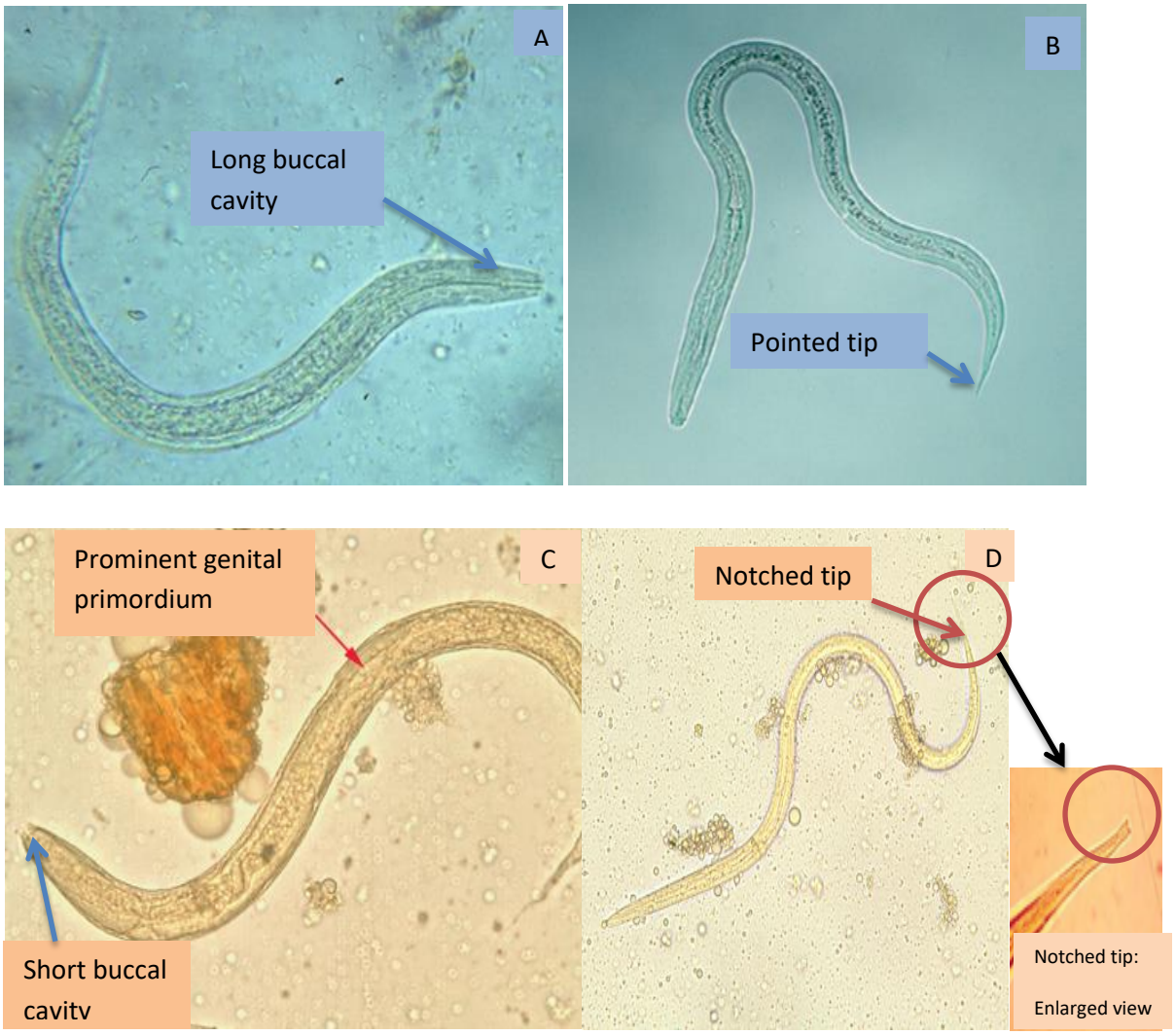


Figure 1.2: The microscopic identification of hookworm. (A: rhabditiform larva-250µm, B: filariform larva-700µm, striated sheath) and *S. stercoralis* larvae (C: rhabditiform larva-250µm, D: filariform larva- 600µm, no sheath). Note the distinguishing features of the buccal cavity (blue arrow), L3

filariform tail (black arrow) and the prominent genital primordium of *S. stercoralis* (red arrow). (Adapted from: CDC, n.d.)

The Baermann technique is recommended for microscopic analysis as it relies on the hydrophilic and thermophilic nature of larvae to exit the stool and collect in the bottom of a warm-water-filled flask. This method is 3.6 to 4 fold more sensitive than FECT (Becker *et al*., 2015) which is used in routine diagnosis at DCP. It is, however, a technique that carries a high risk of laboratory acquired infection and is laborious and time- consuming making it unsuitable for use in a busy diagnostic laboratory (Basuni *et al*., 2011, Becker *et al*., 2015, Biggs *et al*., 2009, Requena-Méndez *et al*., 2013)

Culture of a single stool sample has a sensitivity of 30-70%, using fresh samples (Requena-Méndez *et al*., 2013). The sample size of stool used in this method is up to 10 fold greater than for FECT and this makes it more sensitive than FECT. Sensitivity for culture can also be improved by examining multiple stool samples (Gonzaga *et al*., 2011, Rayan *et al*., 2012, Requena-Méndez *et al*., 2013). Large fresh stool samples (≥ 5 grams; Figure 1.3) are required and the large numbers of viable infectious larvae pose a risk of laboratory acquired infection (Bonn *et al*., 2010).

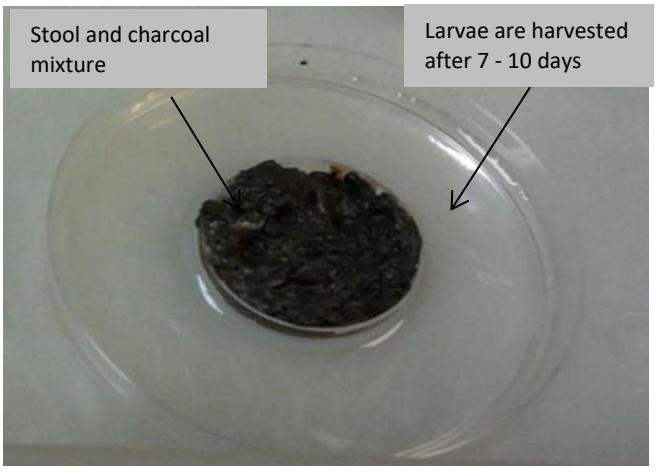


Figure 1.3: In-house *Strongyloides* Charcoal culture

The importance of using fresh stool samples that have not been refrigerated was shown by Inēs *et al*.(2011) when comparing recovery rates of *S. stercoralis* from fresh stools and stools that had been stored at 4^{o}C for 24, 48 and 72 hours. The authors showed a loss of recoverable larvae of up to 50%

after 24 hours. DCP uses an in-house modified charcoal culture method, described in Appendix 1 (Minato *et al*., 2008).

Serology is more effective at detecting strongyloidiasis than parasitological techniques. Animal studies have suggested the role of an innate (where eosinophils serve as the antigen presenting cells for an antibody response) and adaptive immune response (the production of specific IgG and IgE and granulocyte attack to kill the larvae) to *S. stercoralis*. Helminth infections induce the T_H2 cell response and stimulate the regulatory system to avoid tissue damage (Montes *et al*., 2010). Acquired immunity is demonstrated by rising antibody levels, but these antibodies only limit and do not eradicate the disease (Krowlewiecki *et al*., 2013). Antibodies develop 4-12 weeks after infection (Checkley and Sanderson, 2009) but serology is unable to distinguish between past and current infection and is not suitable for use as a test of cure because antibody decay can take up to 12 months. Various methods have been investigated to resolve this issue; a drop in optical density to ≤0.5 post-treatment or a post- and pre-treatment ratio of <0.6 have been used to determine successful cure (Biggs *et al*., 2008). It is also not known whether antibody levels correlate with the level of parasite present in the body (Basuni *et al*., 2011, Biggs *et al*., 2009, Bonn *et al*., 2010, Krowlewiecki *et al*., 2013).

Patients who are immunosuppressed may not develop an antibody response and so a diagnosis of potentially severe disease may be missed (Buonfrate *et al*., 2015). HTLV-1 depresses the T_H2 response and so patients with HTLV-1 and *S. stercoralis* co-infections are susceptible to disseminated infection (Zammarchi *et al*., 2015). Cross-reactions may be seen with other helminth infections, most notably filariasis and schistosomiasis (Requena-Méndez *et al*., 2013). A reason for this may be found in an examination of helminth genomics; a rooted cladogram determined from a maximum likelihood analysis of 18S rRNA from 18 helminth species determined that nematodes (e.g. *Strongyloides* species, filaria, *Ascaris* species) and platyhelminths (e.g. *Schistosoma* species) share a common ancestor (Brindley *et al*., 2009). An enzyme- linked immunosorbent assay (ELISA) is, nonetheless, recommended by WHO (2010) for serological diagnosis of strongyloidiasis. These tests have a specificity of 29-93% and a sensitivity of 73-100% depending on the source and type of antigen used to detect antibodies in the serum, the population studied and the type of immunoglobulins used (Requena- Méndez *et al*., 2013). Sudarshi *et al*. (2003) in a study of travellers and migrants with proven strongyloidiasis determined a sensitivity of 73% in travellers and 98% in migrants. These differences may be due to the length of exposure and possibility of re-infection in migrants (Norman *et al*., 2010).

A combination of methods and multiple stool samples are required to improve the sensitivity of these diagnostic tests for *S. stercoralis* detection (Saugar *et al*., 2015).

Nucleic acid amplification techniques exist for the detection of *S. stercoralis* in clinical samples. The most sensitive method for the detection of *S. stercoralis* DNA, using real-time PCR, in stool samples was shown with the use of primers targeting the 18S rRNA gene (Verweij *et al*., 2009). Therefore, the decision was taken to investigate NAATs for the detection of *S. stercoralis* DNA using primers to target the 18S rRNA gene for this study. This study focussed on the diagnosis of strongyloidiasis in human faecal samples as the aim of the study was to evaluate and validate NAATs for introduction into the diagnostic repertoire in a specialist parasitology referral laboratory.

Processing of stool samples to extract DNA is exacting as the samples contain inhibitors to PCR and *S. stercoralis* has a resistant cuticle that needs to be broken down to release the DNA (Moghaddassani *et al*., 2011, Levenhagen and Costa-Cruz, 2014). DCP uses a modified tissue extraction protocol on stool samples with the Qiagen®QiaSymphony SP magnetic bead based extraction system. This process has a turnaround time of 48 hours, which includes setting up worksheets and templates (Table 1.2). The turnaround time is an important consideration when evaluating and validating new diagnostics tests for addition to, or replacement for, a current diagnostic protocol. Turnaround times have been used as markers for laboratory performance and are associated with clinical outcomes (Hawkins, 2007).

Table 1.2: Comparison of diagnostic methods for the detection of *S. stercoralis* at DCP

METHOD	SENSITIVITY	ADVANTAGES	DISADVANTAGES	PROCESSING TIME
MICROSCOPY (Manser *et al*., 2015)	15-30% (can be improved to 98% plus if 7 stool samples are examined). Very sensitive in hyperinfection syndrome as large numbers of larvae are produced	Rapid, specific method Detects all parasites	Can't detect low larval load or intermittent excretion and it is impractical to collect 7 stool samples from a patient Laborious, risk of laboratory acquired infection in hyperinfection syndrome Specificity and identification relies on well-trained, experienced microscopists as hookworm larvae may be present in older stool samples False negatives as the method is insensitive	One hour
CULTURE (Charcoal agar plate) (Minato *et al*., 2008)	Sensitivity- 30 -70% if more than 1 stool sample is examined	Stool sample size is larger by up to a factor of 10 so culture is more sensitive than microscopy as more of the sample is examined	Laborious, risk of laboratory acquired infection False negative if the stool is not fresh and the method has a low sensitivity if the parasite load is low. Final results are available after 7 – 10 days. This method is only for the detection of *S. stercoralis* larvae, although hookworm larvae may be seen and must be distinguished from *S. stercoralis* larvae	One hour
SEROLOGY (ELISA) (Requena-Méndez *et al*., 2013)	Sensitivity in travellers 73% Sensitivity in migrants 98%	Detects antibodies 4-12 weeks after infection	Will cross-react with other helminthic antibodies e.g. filaria, *Schistosoma* sp., hookworm Immunocompromised patients may not develop an antibody response Can't be used to determine effective therapeutic treatment as the antibodies persist for up to 12 months Can't distinguish between past and current infection	Half a working day
LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) (Notomi *et al*., 2000, Watts *et al*., 2014)	Analytical sensitivity 10^{-3} (at DCP) using stool samples (250µg) spiked with 1 *S. stercoralis* larva/µl	Sensitive and specific Requires less technical expertise than qPCR Can be used in a high-throughput format	In this study LAMP detected slightly more positive cases than microscopy Primers are costly Processing of stool samples is laborious and can take 48 hours Only detects the parasite being investigated	2 days (Preparation of sample for PCR) 1.5 hours (assay and analysis of results)
qPCR (Verweij *et al*., 2009)	Analytical sensitivity 10^{-4} (at DCP)	Sensitive and specific Can be used in a high-throughput format	Primers are costly and expensive automation and thermocyclers are required Stool samples contain PCR inhibitors and so an internal control is required to determine if the samples need to be diluted 1:10 and the assay repeated Processing of stool samples is laborious and can take 48 hours Only detects the parasite under investigation	2 days (Preparation of sample for PCR) 3.5hours (assay and analysis of results)

The development of real-time PCR (qPCR) primers has enabled sensitive detection of *S. stercoralis* in stool samples (Verweij *et al*., 2009, ten Hove *et al*., 2009). Verweij *et al*. (2009) compared primer sets targeting the *cytochrome c* oxidase (*cyto c*), 18S rRNA and *S. stercoralis* specific repeat sequence DNA and determined that the 18S rRNA gene showed superior efficiency to *cyto c* oxidase or *S. stercoralis* specific repeat sequence DNA. ten Hove *et al*. (2009) showed an improvement in the detection rate from 0.1% (number of positives found by routine microscopy) to 0.8% (number of positives found by qPCR) using primers and a probe targeted to the 18S rRNA gene. PCR only detects targeted DNA and in this study 0.5% additional parasites were detected by microscopy. The qPCR assay has a turnaround time of 3.5 hours, post extraction, at DCP (Table 1.2).

The 18S rRNA gene is highly conserved in the *Strongyloides* genus (Hasegawa *et al*., 2009) and exists in various copy numbers, but the exact copy number is not known (Kramme *et al*., 2011). Fitch *et al*. (1995) reported that little is known of the copy number in the Rhabditida order (of which *S. stercoralis* is a member), but a free-living species *Caenorhabditis elegans* has 55 copies of a repeating unit in the genome comprising of one gene each for 18S, 28S and 5.8S RNA. The *Caenorhabditis elegans* 18S gene is similar in length to the *S. stercoralis* 18S gene but has a sequence match of only 69%. Viney and Blaxter (2002) stated that the 18S rRNA gene had proved a useful tool for separating members of the family into different genera, but not necessarily into different species. Later, Hasegawa *et al*. (2009) described four hyper-variable regions in the 18S genome that were able to distinguish between species.

Loop mediated isothermal amplification has become an established nucleic acid amplification technique (NAAT) since it was first described by Notomi *et al*. (2000). LAMP is recommended for use in rural endemic areas due to the isothermal nature of the assay and the requirement for low cost simple equipment (Mori and Notomi, 2009). Lyophilised reagent kits are already available for a number of parasitic diseases e.g. *Trypanosoma cruzi* (Thekisoe *et al*., 2010) and malaria (Polley *et al*., 2013). A LAMP protocol has been published using the 28S rRNA gene of *S. stercoralis* as a target, this study had an analytical sensitivity of 10^{-2} for a single *S. ratti* larva spiked into stool and diluted (Watts *et al*., 2014). LAMP primers to the 18S rRNA gene had been designed at DCP (unpublished, 2011) for use in this study. LAMP has not previously been reported using primers to target the 18S rRNA gene and the LAMP assay has not yet been tested on a statistically significant cohort of clinical samples.

1.6. TREATMENT

Strongyloidiasis is treated with ivermectin 200µg/ kg (Sudarshi *et al*., 2003) and without treatment the infection may last for life (Checkley *et al*., 2010, Feely *et al*., 2010, Moghaddassani *et al*., 2011).

Therefore, complete eradication of *S. stercoralis* is required for the treatment to be effective. The most effective dose for ivermectin is not known because the persistence of antibody means that serology cannot be used for monitoring treatment (Requena- Méndez *et al*., 2013). Drug efficacy trials using new sensitive and specific diagnostic techniques are needed to investigate this (Requena- Méndez *et al*., 2013, Krowlewiecki *et al*., 2013).

Ivermectin binds to the glutamate- gated chloride ion channels in invertebrate nerve and muscle cells and leads to neuro-muscular cell death that affects parasite motility (Biggs *et al*., 2009). Ivermectin is superior to albendazole in terms of safety efficacy and adverse effects, but should be avoided in pregnancy and its use is restricted to children > 3-5 years of age because of the lack of data in these groups (Biggs *et al*., 2009, Ganesh and Cruz, 2011, Pottie *et al*., 2011, Krowlewiecki *et al*., 2013, Requena- Méndez *et al*., 2013, Henriquez-Camacho *et al*., 2016). Ivermectin should not be used in patients with a high *Loa loa* microfilaraemia due to the potential for serious adverse neurological events that occur in microfilaria patients with a high *Loa loa* parasite load (>50 000/ ml) (Boussinesq *et al*., 2003, Pottie *et al*., 2011). *Loa loa* and *S. stercoralis* are known to be endemic in the same areas in parts of Africa (Pottie *et al*., 2011). Prolonged or repeated treatment with ivermectin is indicated in disseminated disease. Albendazole and ivermectin combined therapy may also be used to treat disseminated disease. Resistance to ivermectin has not been seen and this may be due to the fact that larvae are effectively clones of the adult female worm, however, long-term use of ivermectin as a control strategy has not yet been investigated (Ganesh and Cruz, 2011, Henriquez-Camacho *et al*., 2016).

The use of ivermectin at HTD was introduced on the basis of a systematic literature review in 1995. The treatment regime was changed from albendazole 400 mg b.d. (twice daily) for 3–7 days to two doses of ivermectin 200 µg/ kg (Sudarshi *et al*, 2003). The treatment regime for strongyloidiasis at HTD currently consists of 2 doses of ivermectin (200µg/ kg) on day 1 and day 14 for uncomplicated disease and in severe disease ivermectin (200µg/ kg) is administered on day 1, day 2, day 15 and day 16 and continued as necessary. A diagnosis of strongyloidiasis is required for informed treatment and post-treatment monitoring of in-patients and out-patients who are at risk of being infected with *S. stercoralis*. The current laboratory diagnostic strategy suffers from a lack of sensitivity and specificity and the inability to detect the clearance of parasites after treatment. The optimal dosage for treatment of *S. stercoralis* has never been extensively investigated due to the absence of a point of cure test, nor is it known whether the above regimes will eliminate extra-intestinal larvae (Olsen *et al*., 2009, Requena- Méndez *et al*., 2013, Krowlewiecki *et al*., 2013).The

current study will evaluate the potential of NAATs for future use in monitoring successful treatment of strongyloidiasis.

1.7. JUSTIFICATION FOR THE STUDY

Improvement in medical care in the developed world means that there is an increased interest in the diagnosis and detection of *S. stercoralis*, especially in patients that are immunosuppressed (e.g. HTLV1 co-infection, alcoholism, old age or auto-immune diseases) or are to undergo iatrogenic immune suppression by the administration of immune- suppressive drug regimens (Basile *et al*., 2009, Checkley and Sanderson, 2009, Olsen *et al*., 2009, Smith *et al*., 2010, Ganesh and Cruz, 2011, Kassalik and Möntemüller, 2011, Schär *et al*., 2013b).

This has led to the need for reliable and sensitive tests that can be introduced into high-throughput systems to diagnose and follow the disease (ten Hove *et al*., 2009). Daar *et al*. (2002), in a study which sought consensus opinion amongst 28 scientific experts ranked "modified molecular diagnostics for affordable, simple diagnosis of infectious diseases" amongst the most promising biotechnologies for improving health and healthcare in developing countries. New NAATs have shown promise as sensitive and specific methods for the detection of many parasitic diseases. These techniques have revolutionized the diagnosis and treatment of such gastro-intestinal infections as amoebiasis, giardiasis, cryptosporidiosis and microsporidiosis at DCP. It is hoped that the increased sensitivity of NAATs will detect chronic *S. stercoralis* infections (the existence of which may only be shown by later severe infections in immunocompromised patients) and appropriate treatment can be prescribed before immunosuppression leads to severe strongyloidiasis (Basile *et al*., 2010).

Real-time PCR is run in a closed system and obviates the need for gel electrophoresis to visualize the amplification product thereby eliminating the potential for DNA product contamination of the laboratory environment. The results are available within 3.5 hours (post-processing) and amplicon detection and data analysis can be performed automatically using commercial software packages making this technique suitable for high-throughput techniques. This technique is suitable for use in WTM clinics but it is costly and requires a high level of technical expertise which limits the use of this technique in endemic areas. A published qPCR method to detect the 18SrRNA gene of *S. stercoralis* is already available and is being used to detect *S. stercoralis* DNA in clinical samples (ten Hove *et al.*, 2009). The qPCR assay is amenable to a multiplex format to include the detection of other important parasites in WTM clinics (ten Hove *et al*., 2009).

The LAMP assay is an alternative NAAT that is more resistant to inhibition than qPCR and shows great promise as a rapid and simple, yet sensitive, diagnostic technique. LAMP has been developed for use as commercially available kits (Source Eiken, Japan) for the detection of *Trypanosoma cruzi* and malaria in blood samples (Thekisoe *et al*., 2010, Polley *et al*., 2013). The isothermal nature of LAMP means that no sophisticated equipment is required, as the amplification may be run in a simple water-bath or heated block to produce visually identifiable amplification within one hour (post-processing, refer to Table 1.2) or within 1 hour from direct DNA extraction (using manual methods) to visualisation of LAMP product (WHO, 2013, Perera *et al*., 2017). Primers to detect *S. stercoralis* 18S rRNA have been designed by Polley *et al*. at DCP (unpublished, 2011) but have not yet been tested in clinical samples. This technique has the potential to be useful to funded studies carried out in endemic areas.

The follow-up of treatment to determine point of cure is not currently feasible by either microscopy or culture because of the low sensitivities of these methods. Conversely, the follow-up of treatment by ELISA is seldom reliable as the antibody levels may take 6-12 months to decline and, indeed, may never have been positive in immunocompromised patients (Buonfrate *et al*., 2013, Requena-Méndez *et al*., 2013).

These limitations, coupled with the ability of *S. stercoralis* infections to persist for decades in infected individuals make a valid case for the development, evaluation and deployment of new diagnostic methods with improved sensitivity and specificity.

This prospective study has the potential to both identify parasite clearance (post-treatment) and enhance the sensitivity and specificity of the diagnosis of strongyloidiasis. The data from this study can be used to develop a targeted diagnostic and treatment strategy that will benefit the patients and the clinicians charged with patient management at HTD and University College London NHS Foundation Trust (UCLH).

Any tests that subsequently improve the sensitivity and specificity of the routine diagnostic services will provide the data necessary to allow clinicians to improve the clinical management strategy of strongyloidiasis and to determine the persistence of *S. stercoralis* post-treatment in individuals attending at HTD and UCLH. As such, the diagnostics would significantly improve the patient experience at UCLH (Whitty *et al*., 2000, WHO, 2010, Requena- Méndez *et al*., 2013).

Chemotherapy- based control, in addition to improved sanitation, was shown to reduce strongyloidiasis infection risk in a study in rural Cambodia carried out over two years using the

Baermann and Koga agar techniques (Forrer *et al*., 2016). An addition to the diagnostic monitoring array would be useful in establishing an accurate baseline for prevalence in these types of studies.

1.8. AIMS AND OBJECTIVES

The study aimed to clinically evaluate the sensitivity and specificity of qPCR (ten Hove *et al*., 2009) and a novel LAMP assay against existing diagnostic procedures to detect *S. stercoralis* infection. The study also aimed to investigate the development of the LAMP assay for use in endemic areas.

The objective of this study was the development of a "fit for purpose" (CPA standards F1, ISO 15189:2012) (UKAS, n.d., ISO 15189:2012, 2012) diagnostic screening strategy and the introduction of tests to a specialist referral laboratory in the UK that will improve the quality of service supplied to service users and improve patient care in a specialist referral centre by increasing the sensitivity and specificity of detection of *S. stercoralis*.

The primary outcome will be the development, evaluation and validation of new NAATs for use in the *S. stercoralis* diagnostic repertoire. The microscopy, culture and serology techniques were individually validated at DCP when the tests were introduced.

This study also investigated whether LAMP might be a simpler and more rapid assay than qPCR for the sensitive and specific detection of *S. stercoralis* DNA in clinical samples using primers developed at DCP. Simplified methods for DNA extraction suitable for use in resource-limited areas were piloted to determine whether the LAMP assay could be performed on DNA extracted by these methods on stool samples without inhibition of the LAMP reaction.

This will feed back as an improved diagnostic service that clinicians can use to screen patients and determine treatment options. It will also enhance the specialist knowledge- base for a referral centre that can be accessed by outside hospitals.

CHAPTER 2: MATERIALS AND METHODS

Strongyloidiasis is prevalent in humid tropical, sub-tropical and temperate regions with poor sanitation and has the potential to persist undetected in the human host for decades (CDC, n.d.). The potential for severe disease and even death occurs when the human host becomes immunocompromised. This may occur decades after the host has been in an area that is endemic for *S. stercoralis* (Barros and Montes, 2014). The decision was taken to investigate NAATs at DCP as a suitable replacement for the current diagnostic repertoire or as an addition to the service provided. The current study focussed primarily on human strongyloidiasis, but the techniques investigated have been used in other disciplines e.g. veterinary practice to detect *S. stercoralis* (Yang *et al*., 2013, Jaleta *et al*., 2017) and other parasites (Melville *et al*., 2014). The LAMP assay has been used for the detection of micro- organisms in human, livestock and plant diseases (Wong *et al*., 2017). The development of a novel NAAT (LAMP) for use in resource- limited areas was also explored.

Raymaekers *et al*. (2009) describe the verification and validation procedures required by the international quality standard for medical laboratories (ISO 15189:2012, 2012). This chapter describes the study design and the protocols used for the study founded on international quality guidelines. It includes a technical description of the sample preparation and NAATs assessed in the research. The development of a novel LAMP assay using primers designed at DCP (unpublished) is defined and methods for confirmation of the NAAT products are also described. Where methods for NAAT product confirmation were chosen that were not in routine use at DCP, the procedure was described in more detail

The rationale for the choice of statistical analysis used to demonstrate the aims of this study (Chapter 1, Section 1.8) is discussed in Section 2.8.

2.1. PATIENT COHORT

The patient cohort comprised of travellers and migrants (> 18 years. Range in this study 26-90 years) who attended the travel clinic at HTD or patients at UCLH who were being investigated for strongyloidiasis. Patients who were part of the Infectious diseases in Europe and Africa (IDEA) study (Knopp *et al*., 2014) were also included.

2.2. ETHICAL APPROVAL

All human studies are subject to the ethical principles concerning human experimentation. The Declaration of Helsinki (1975) was developed by the World Medical Association (World Medical

Association, 2013) for human research ethics. All research studies pertaining to patients, human volunteers and human material require ethical approval (World Medical Association, 2013). Local ethical approval was sought from the local ethics committee, Clinical Innovation and Research Techniques (CIRT), at UCLH and an ethics approval application was submitted to the National Research Ethics Service (NRES) after local ethical approval was obtained. Ethical approval was granted in October 2014 on the basis of a proportionate review by the Integrated Research Application System (IRAS) Research Office 1406LC for study number 14/0169 and designated the IRAS protocol number 151217.The samples were residual diagnostic samples surplus to diagnostic requirements used for validation of new diagnostics and therefore patient consent was not required.

2.3. STUDY DESIGN

2.3.1. NULL HYPOTHESIS

The null hypothesis states that there is no difference between current diagnostic testing (microscopy, culture and serology) and NAATs (LAMP or qPCR).

2.3.2. COMPOSITE REFERENCE STANDARD

When no "gold standard" exists for diagnostic tests biased accuracy estimates will occur. Bias can be reduced by using a composite reference standard (CRS) as the statistical approach (Baughman *et al*., 2008). This study used the routine diagnosis for strongyloidiasis at DCP (microscopy, culture and serology) as the CRS. Microscopy and culture denoted proven disease and serology denoted probable disease. A positive result in any of the CRS tests was scored as positive. However, statistical analysis was performed in two parts:

I. On the basis of parasitological positivity (proven diagnosis by microscopy and/ or culture).
II. On the basis of the CRS that included serology (probable diagnosis).

This was done to determine the sensitivity and specificity of NAATs against an imperfect CRS. The results of these analyses are discussed in Chapters 3 (LAMP) and 4 (qPCR).

2.3.3. SAMPLE SIZE CALCULATION

A test with a high specificity is required for diagnosis of the disease and a test with high sensitivity is required for screening for disease. Treatment monitoring however requires a test with a high sensitivity and specificity (Kirkwood and Sterne, 1988, Jones and Payne, 1997). The evaluation and validation of NAATs for the diagnosis of strongyloidiasis was performed for screening, diagnosis and treatment monitoring. A sample size for McNemar's test with a power of 90% and a significance level of 5% was chosen to demonstrate the potential of the qPCR and LAMP assays to be used for

diagnosis, screening and treatment monitoring for strongyloidiasis. A sample size of 286 (Figure 2.1) was calculated to give a power of 90% to detect a change between the routine diagnostic repertoire (CRS) and NAATs with a 5% significance level (MedCalc®, n.d.)

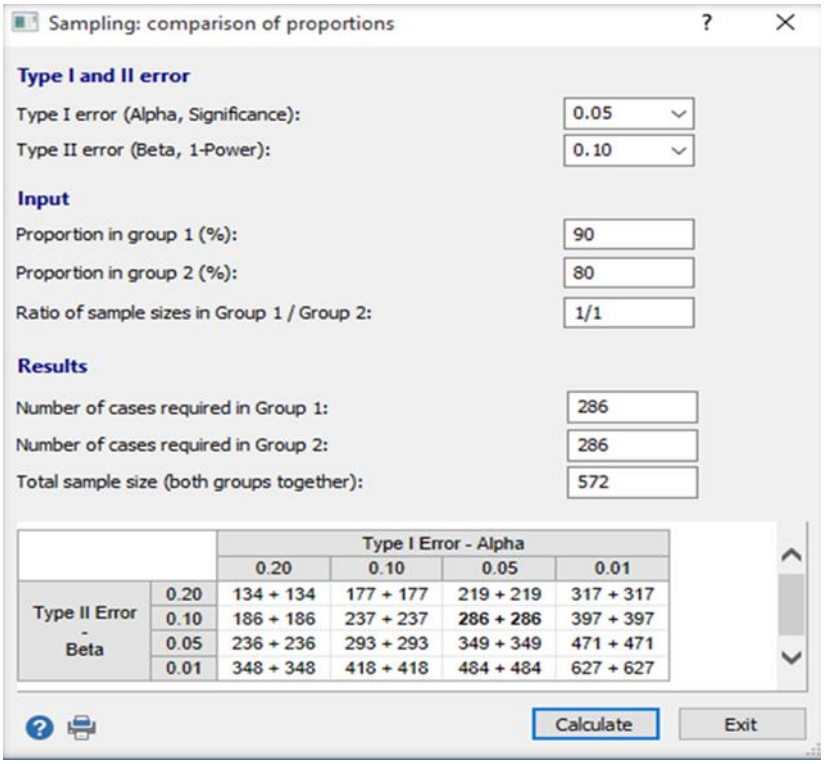


Figure 2.1: Calculation of sample size for comparison of two proportions to detect a difference of at least 10 in a dichotomous dependent variable between two related groups (MedCalc®, n.d.)

Residual diagnostic samples were collected from 287 patients (over 18 years of age) over a 5 year period (2011- 2016). Patients were investigated for strongyloidiasis at HTD or at UCLH or were part of the IDEA study (Knopp *et al*., 2014). Stool samples stored for the IDEA study (Knopp *et al*., 2014) were included with permission from the IDEA Study Lead Researcher (Dr M Brown, Consultant at HTD). Cross- reactions are known to occur in *Strongyloides* serological assays with filarial and schistosomal antibodies (Requena-Méndez *et al*., 2013) and so samples with positive filarial or schistosomal serology results were excluded.

Stool samples sent for microscopic examination of ova, cysts and other parasites (OCP) and culture, i.e. care as usual, to DCP were analysed by qPCR and LAMP once routine diagnostic screening had been performed.

Serological testing was performed on serum samples at the request of the clinicians overseeing patient care.

The standards for microbial investigations guidelines (HPA UK protocols, 2013) deal with the accuracy and completeness of the study. A flow chart adapted from the guidelines was used to design the protocol for the study (Figure 2.2).

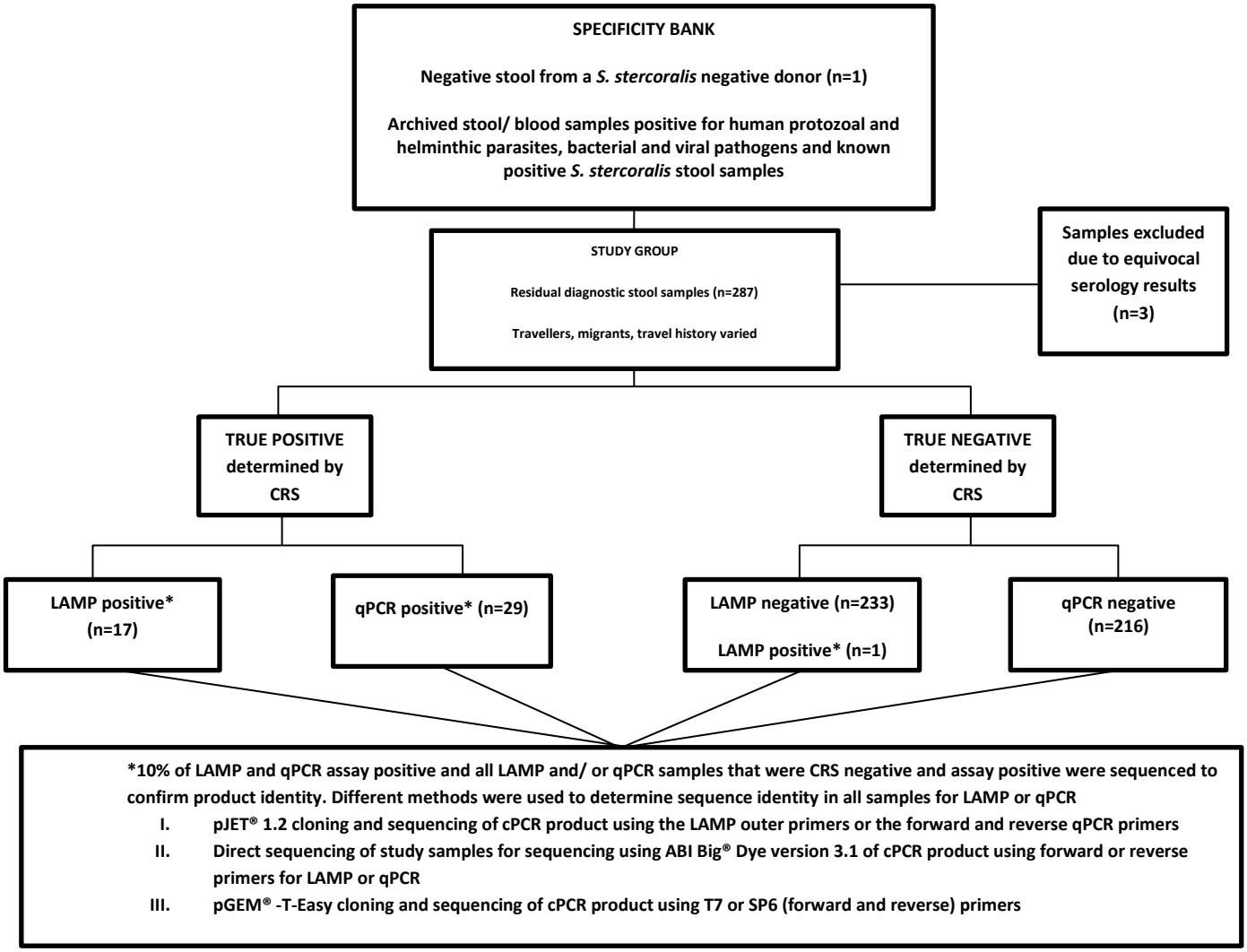


Figure 2.1: Flow chart of the number of true positive and true negative samples determined by the composite reference standard (n=284). Flow chart adapted from HPA UK protocols (2013) for the study design at DCP.

Three samples were excluded from this study. The LAMP and qPCR assays and the microscopy and culture results were negative. Serology was positive (ELISA) for *Strongyloides* and filarial or *Strongyloides* and schistosomal antibodies. These samples were excluded as the possibility of cross-reactions (Brindley *et al*., 2009) in the serological tests could not be determined.

2.3.4. SAMPLE STORAGE

Two aliquots of approximately 200-250 mg of stool (if the original stool sample was large enough) were taken and stored at 4^{o}C (aliquot 1) or -20^{o}C (aliquot 2) until DNA extraction was performed on a Qiagen® Qiasymphony SP using a magnetic bead based extraction system (Halstead *et al*., 2013). Stool samples contain inhibitors to DNA extraction and amplification (Monteiro *et al*., 1997, Murphy *et al*., 2007, Moghaddassani *et al*., 2011). Larger aliquots, whilst this may have increased the sensitivity, were not considered for this study due to the potential for inhibition of the NAATs. Stored samples for investigation by NAATs are usually stored at -20^{o}C as long-term storage without pre-treatment at 4^{o}C is not optimal for DNA survival (Qiagen, n.d.). However, the two storage temperatures were chosen as the IDEA study samples had been collected and stored at these temperatures previously and the study continued this practice for all samples. The introduction of a new test into the diagnostic repertoire at DCP would ideally harmonise with existing laboratory protocols. Diagnostic samples for NAAT identification of other faecal parasites are stored at 4^{o}C, without preservatives, for up to three weeks. Once the DNA had been extracted the DNA was stored at -20^{o}C until qPCR or LAMP was performed, as per existing laboratory protocols.

2.3.5. ANONYMISATION AND DATA STORAGE

The aliquots were entered onto a sample study sheet and identified with a random study number (numbers between 100 and 1200 were randomly allocated using an Excel® spreadsheet) so that the researcher could not match the study number to the original stool sample. A database curator (not the researcher) entered the study number and identification of the stools onto a password protected computer file and the sample code was only broken at the end of the study (April 2016) so that the researcher could perform the study analysis. Data collected for the study analysis included age (years), gender, microscopy result, culture result, *Strongyloides* serology result and a travel history or details of the country of residence. Further data collected for the study included length of time the samples were stored before DNA extraction, the temperature at which the aliquots were stored and the volume of stool deposit that was extracted. Routine diagnostic test results (microscopy, culture and serology) were performed within the departmental turnaround times (Table 2.1) and the results were available to the clinicians as usual.

Table 2.1: Departmental turnaround times at DCP for the current routine diagnosis of *S. stercoralis*

DIAGNOSTIC TEST	TURNAROUND TIME*
Microscopy (ova, cyst and parasite concentration) (Manser *et al.* 2015)	2 working days
***Strongyloides* charcoal culture (Minato *et al*., 2008, Requena-Méndez *et al*., 2013)**	10 working days
Serology (Sudarshi *et al.*, 2003, Bisoffi *et al*., 2013, Requena-Méndez *et al*., 2013)	7 working days
Real-time PCR for faecal Protista infections (ten Hove *et al*., 2007)	8 working days[1]
**** for comparison only**	

*available from: www.uclh.nhs.uk/OurServices/ServiceA-Z/PATH/PATHMICRO/PARA/Pages/Home.aspx

**an established qPCR protocol at DCP included for turnaround time comparison only

[1]Samples, showing possible inhibition, are diluted 10^{-1} and re- tested in the next assay run. This adds an additional 7- 14 days to the turnaround time if the sample requires re- extraction.

2.3.6. NEGATIVE, POSITIVE, NO TEMPLATE AND INTERNAL CONTROLS USED FOR THIS STUDY

UK standards for microbial investigations guidelines, for the development and validation of NAATs for the detection of DNA from clinical samples, recommend that controls are included in all stages of the process (processing, extraction and amplification). This ensures that appropriate DNA has been extracted and added to the reaction to exclude false negative reactions and to eliminate the possibility of false positive reactions from contamination or process failure (HPA UK protocols, 2013).

2.3.6.1. NEGATIVE CONTROL

Five grams of a known negative stool sample (sample from a donor who is *Strongyloides* serology and microscopy negative) was added to 10 ml phosphate buffered saline pH 7.2 (PBS) to form a negative slurry. Aliquots of 250 µl of negative slurry were stored at -20°C to be used as the negative extraction and amplification control.

2.3.6.2. POSITIVE CONTROL

2.3.6.2.1. HARVESTING *S. STERCORALIS* LARVAE FROM A POSITIVE CULTURE

Stool samples for *Strongyloides* culture (Figure 1.3) were performed as per the in-house protocol (Appendix 1). The infectious *S. stercoralis* larvae were harvested by decanting the positive stool culture water into a 50ml centrifuge tube (Source VWR, International), followed by centrifugation (Eppendorf Centrifuge 5702, Source Meadowrose Scientific Ltd.) at 2000 revolutions per minute (rpm) for 5 minutes. The deposit was stored at 4°C and the supernatent was used to continue the culture. This process was repeated until the culture was negative; in this study three harvests were usually obtained. The final pellet was stored at -20°C until required.

2.3.6.2.2. PREPARING THE POSITIVE CONTROL

The pellet stored at -20^{o}C (Section 2.3.6.2.1) was used to prepare aliquots of positive control stool sample as follows: the stored pellet of *S. stercoralis* larvae was thawed to room temperature and reconstituted in 1ml PBS, mixed by vortex (VWR Analog vortex mixer) for 3 seconds and the number of *S. stercoralis* larvae seen in 50 µl under a 22x22 mm coverslip (Source CellPath Ltd.) at x100 magnification (Nikon Eclipse E400) was recorded. The positive culture used throughout this study on the clinical and optimisation samples contained 548 *S. stercoralis* larvae in 50 µl PBS. (50 x 2 = 100, 100 x 10 = 1000 µl i.e. multiplication factor = 20). The positive culture contained 548 x 20 = 10 960 *S. stercoralis larvae* per 1000 µl or 10.96 *S. stercoralis* larvae per µl.

One ml of the positive culture was added to 9 ml of negative slurry (Section 2.3.6.1., this Chapter) to give a positive control containing approximately one *S. stercoralis* larva/ µl. Aliquots of 250 µl of the positive spiked stool sample were stored at -20^{o}C until used as extraction and amplification controls. The extracted DNA from the positive stool controls was tested in the qPCR or LAMP assays at serial 10- fold dilutions of the positive control DNA.

Routine laboratory practice stores samples at 4^{o}C, for up to 3 weeks, without a preservative. A pilot study compared DNA persistence in samples stored at 4^{o}C and -20^{o}C to determine whether sample aliquots for *S. stercoralis* NAAT could be stored using current laboratory protocols. A new positive control was made up to test storage survival time at 4^{o}C and -20^{o}C. The new positive control stool contained 66 larvae in 50µl with a final concentration of 0.1 *S. stercoralis* larvae/ µl. Inēs *et al*. (2011) demonstrated a 50% drop of viable *S. stercoralis* larvae in stool when investigating the effect of storage temperature on the *S. stercoralis* culture result (Section 1.5). This lower concentration of *S. stercoralis* larvae per µl was used to determine a drop in efficiency earlier than a positive control containing a higher concentration of larvae would be able to.

A further pilot study compared the DNA extraction efficiency between manual and automated DNA extraction methods. The extracted DNA was analysed by the LAMP assay to determine whether a DNA extraction method suitable for use in resource- limited areas could be developed.

2.3.6.3. NO TEMPLATE CONTROL

Nuclease free water (Source Thermo Fisher Scientific, UK) was used as the no template control (NTC) for qPCR and LAMP

2.3.6.4. USE OF AN INTERNAL CONTROL

Stool samples contain complex polysaccharides and enzymes that are known to cause inhibition of target cell lysis and nucleic acid degradation or direct inhibition of PCR (Monteiro *et al*., 1997,

Murphy *et al*., 2007, Moghaddassani *et al*., 2011). To monitor qPCR inhibition, an internal control derived from the green fluorescent protein (*gfp*) gene, found in the jellyfish *Aequorea victoria*, which had been incorporated into an *Escherichia coli* (*E. coli*) genome (Source Public Health England- PHE) was used. The internal control (*gfp*) was added to the stool sample before automated DNA extraction was performed to monitor the reliability of nucleic acid extraction, amplification and product detection (Kinson, 2012). The internal control, *gfp*, does not naturally occur in human stool samples and so can be used to detect the presence of inhibitors in human stool samples (Murphy *et al*., 2007).

2.3.7. SPECIFICITY BANK

The specificity bank comprised of 200-250 mg aliquots of positive stool samples, that had been previously stored at -20^{o}C (a DCP collection of positive stool samples), a blood sample containing *Loa loa*, cultures from bacterial human intestinal pathogens and an adenovirus DNA sample. The stool, blood and bacterial culture samples were extracted and the DNA was stored at -20^{o}C until required. Viral, bacterial and other parasitic organisms (Table 2.2) were tested to determine analytical specificity using primers targeted to *S. stercoralis* DNA in the qPCR and LAMP assays. *Strongyloides* species DNA was not available to determine the detection of other *Strongyloides* species DNA using the LAMP or qPCR assays. However, qPCR has been reported to detect other *Strongyloides* species DNA (Requena-Méndez *et al*., 2013, Requena-Méndez *et al*., 2014). In 2013 Sultana *et al*. showed detection of *Strongyloides* DNA using *S. ratti* spiked stools and *S. stercoralis* positive clinical samples. A LAMP assay using primers to target the 18S rRNA gene has not yet been shown to detect *Strongyloides* species DNA. This study was able to obtain *S. stercoralis* DNA but was not able to obtain *Strongyloides* species DNA, although a free-living (non-human pathogen) rhabditiform larva (isolated by microscopy from a diagnostic sample) was included in the specificity bank (Table 2.2). Cross-reactions between *S. stercoralis* LAMP and qPCR primers and free- living rhabditiform larvae were not demonstrated in the current study.

Table 2.2: Human Pathogens (viral n=1, bacterial n= 4, *S. stercoralis* aliquots n=8 and other parasitic species n=20) tested to determine the analytical specificity of the primers targeted to *S. stercoralis* DNA in qPCR and LAMP

	Organism	Pathogen type
1	Adenovirus DNA	Virus*
2	*Campylobacter* sp. NCTC 12850	Bacteria**
3	*Escherichia coli* 0157 NCTC 13126	Bacteria**
4	Peptone water (negative culture control)	Negative culture control**
5	*Shigella sonnei* NCTC1132	Bacteria**
6	*Vibrio cholera* (diagnostic sample)	Bacteria**
7	*Cryptosporidium* sp.	Protista
8	*Cryptosporidium* sp.	Protista
9	*Cryptosporidium* sp.	Protista
10	*Cryptosporidium* sp.	Protista
11	*Cryptosporidium* sp.	Protista
12	*Cyclospora cayetanensis*	Protista
13	*Cystoisospora belli*	Protista
14	*Cystoisospora belli*	Protista
15	Sample containing mixed Protista-*Entamoeba histolytica* , *Entamoeba coli* and *Entamoeba hartmanni*	Protista
16	*E. histolytica*	Protista
17	*E. histolytica*	Protista
18	*E. histolytica*	Protista
19	*E. histolytica*	Protista
20	*E. histolytica*	Protista
21	*Encephalitozoon intestinalis*	Fungi
22	Enterocytozoon bienusi	Fungi
23	Enterocytozoon bienusi	Fungi
24	Enterocytozoon bienusi	Fungi
25	*Giardia lamblia Blastocystis hominis*	Protista
26	*G. lamblia*	Protista
27	*G. lamblia*	Protista
28	*G. lamblia*	Protista
29	*G. lamblia*	Protista
30	*G. lamblia*	Protista
31	*Dicrocoelium dendriticum*	Trematode
32	*Schistosoma mansoni*	Trematode
33	*Bertiella studeri*	Cestode
34	*Taenia saginata*	Cestode
35	Sample containing mixed nematodes-*Ascaris lumbricoides* Hookworm, *T. trichiura*	Nematode
36	Sample containing mixed nematodes-*Ascaris lumbricoides*, Hookworm, *T. trichiura*	Nematode
37	Free-living Rhabditiform larvae (unable to identify further at DCP)	Nematode
38	Hookworm	Nematode
39	Hookworm	Nematode
40	Hookworm	Nematode
41	*Loa loa* in blood	Nematode
42	*Trichostrongylus* sp.	Nematode
43	*Trichuris trichiura*	Nematode
44	*S. stercoralis* culture larvae x1 (various aliquots from the same positive culture- neat or spiked into a negative stool sample)	Nematode
45	Negative stool controls (various aliquots)	Negative stool control

*** supplied by the Virology Department at UCLH**
****Supplied by the Microbiology Department at UCLH**

2.3.8. EXTRACTION USING THE QIAGEN®QIASYMPHONY SP

2.3.8.1. PRE-PROCESSING OF STOOL SAMPLE

The volumes of the stool deposit varied greatly as the amount of supernatant removed depended on the appearance of the stool (Lewis and Heaton, 1997). The volume of type 1 stools was harder to aliquot into smaller samples, while the type 6 and type 7 samples had large volumes of supernatant removed (Table 2.3).

Table 2.3: The effect of appearance of the stool sample on aliquot size in samples used for DNA extraction in this study.

STOOL APPEARANCE	DESCRIPTION	APPROXIMATE* VOLUME SUPERNATENT REMOVED (µl)
Type 1	Separate hard lumps, like nuts (hard to pass)	0-0.1
Type 2	Sausage shaped, but lumpy	0.1-0.3
Type 3	Like a sausage, but with cracks on the surface	0.2-0.4
Type 4	Like a sausage, smooth and soft	0.3-0.5
Type 5	Soft blobs, clear-cut edges, passed easily	0.4-0.7
Type 6	Fluffy pieces, ragged edges, mushy stool	0.5-0.8
Type 7	Watery, no solid pieces, entirely liquid	0.8-0.99

*Approximation by eye only, as stool samples varied greatly by appearance. The length of storage may also have contributed to dehydration of the sample. Adapted from: The Bristol Stool Chart, developed at the University of Bristol, by Lewis and Heaton (1997).

Samples were removed from storage (-20^{o}C) and brought to room temperature. The samples were centrifuged at 13000 rpm (16.2 rcf) for 5 minutes on a MSE Micro Centaur centrifuge. The supernatant, containing potential cell free inhibitors to NAATs, was removed and the deposit was weighed on a balance (Oertling HB63). This procedure also allowed the improved recovery of parasites from watery diarrhoeal samples. Qiagen® DNA tissue lysis buffer (ATL) and proteinase k (Source Thermo Fisher Scientific, UK) were added to the stool deposit to give a dilution of 1 in 2 (approximately) of ATL buffer containing a 10^{-1} volume of proteinase k. Samples were well-mixed and incubated overnight at 56^{o}C. The following day the samples were mixed by vortex and pulse-centrifuged to remove any droplets that might be adhering to the top of the tube lid. The addition of 200µl L6 lysis buffer (Source Thermo Fisher Scientific, UK) and 400µl of an internal control (*gfp*) was added to 200µl of stool sample. The *gfp* internal control (Source LSHTM, UK) was diluted in sterile PBS pH 7.2 to give a 1 in 50 dilution before being added to the L6 buffer and sample mixture.

The sample was then mixed on a Vortex Genie 2 before being placed in the Qiagen® Qiasymphony SP work station.

2.3.8.2. DNA EXTRACTION USING THE QIAGEN®QIASYMPHONY SP WORKSTATION

DNA was extracted on the Qiagen® Qiasymphony SP work station, using magnetic particle-based nucleic acid purification and the tissue extraction program (Kruhøffer *et al*., 2010) from the QIAsymphony®DSP Virus/Pathogen Kit as per the manufacturer's instructions (Qiagen, n.d.). The protocol was modified by the addition of the internal control, *gfp* directly to the samples instead of the carrier RNA mixture. This method was already established at DCP for the extraction of DNA from stool samples. The effectiveness of the method using magnetic particle-based nucleic acid purification to produce DNA with fewer inhibitors present was determined previously for a multiplex stool Protista PCR (Verweij *et al*., 2004, ten Hove *et al*., 2007) in routine use at DCP. In the magnetic particle-based nucleic acid purification method the target DNA (RNA can also be extracted by this method) is lysed and then bound to the magnetic particles, the bound DNA is then washed before being eluted (Halstead *et al*., 2013). The Qiagen® Qiasymphony SP work station is employed for rapid, reliable and high-throughput extraction (up to 96 samples in 4 hours) and is, therefore, useful for routine well-resourced diagnostic settings and was considered suitable for this study.

2.3.8.3. DNA EXTRACTION USING A MANUAL METHOD "BAKE AND SHAKE"

LAMP is less sensitive to inhibition of amplification in blood samples than qPCR due to the use of the *Bst* DNA polymerase (Notomi, 2000, Wong *et al*., 2017) and a method for the direct extraction of DNA from samples has been described for the rapid and easy DNA extraction from sputum, blood and soil samples (WHO, 2013, Perera *et al*., 2017). The PURE® device is a series of interlocking plastic components comprising a heating tube containing lysis buffer, an absorption tube containing absorbent powder to remove inhibitors present in the samples and an injection cap that directly dispenses extracted DNA into reaction tubes in a closed system. The LAMP assay was performed using the rapid ultrapure DNA extraction kit (PURE®) for malaria (Figure 2.3) and a Loopamp- LF 160 (Source Eiken, Japan), a homoeothermic heating and LAMP amplification block with UV lamp, (Figure 2.4). A parallel DNA extraction was performed (PURE® *vs*. Qiagen® Qiasymphony SP). The PURE® method requires a constant power source. Positive stool controls were stored at -20^{o}C until parallel extraction of DNA was performed using the PURE® method or the Qiagen® Qiasymphony SP. *S. stercoralis* has a thick cuticle that may affect the efficiency of DNA extraction by this method (Repetto *et al*., 2010, Levenhagen and Costa-Cruz, 2014) and so some samples were pre-treated to investigate this issue. The samples to be extracted using the PURE®

method were used direct (neat or at a dilution of 1 in 2 in nuclease free water) or pre-treated using one of the following methods before addition to the buffer in the heating tube (figure 2.3 A):

I. pre-incubation at 56^{o}C in ATL plus proteinase k for 2 hours
II. pre-incubation at 56^{o}C in ATL plus proteinase k overnight
III. extreme temperature shock for 5 minutes in liquid nitrogen

Loading sample (60 µl stool sample) was added to PURE® buffer (WHO, 2013, Perera *et al*., 2017) in the heating tubes. The tubes were heated at 75^{o}C for 15 or 30 minutes on a Loopamp- LF 160.The adsorbent tube (Figure 2.3 B) was screwed onto the heating tube and the resultant tube was shaken vigorously to combine the sample with the absorbent powder and remove any inhibitors present in the samples. The ultrapure DNA was delivered (Figure 2.3 C) into a reaction tube which may be used immediately or stored at -20^{o}C. The extracted DNA (Figure 2.3 D) was diluted in a serial 10-fold dilution series to determine the end-point at which DNA could be detected by the LAMP assay. The DNA was used neat or at dilutions made in nuclease free water (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) in the LAMP assay. The positive control used during the study for optimisation of the assay and testing of diagnostic samples contained 1 *S. stercoralis* larva/ µl. Further testing using 10, 20, 50, 80 or 100 µl for the loading sample was performed using PURE® technology to determine the volume of loading sample that is required to eliminate inhibition of the LAMP assay.

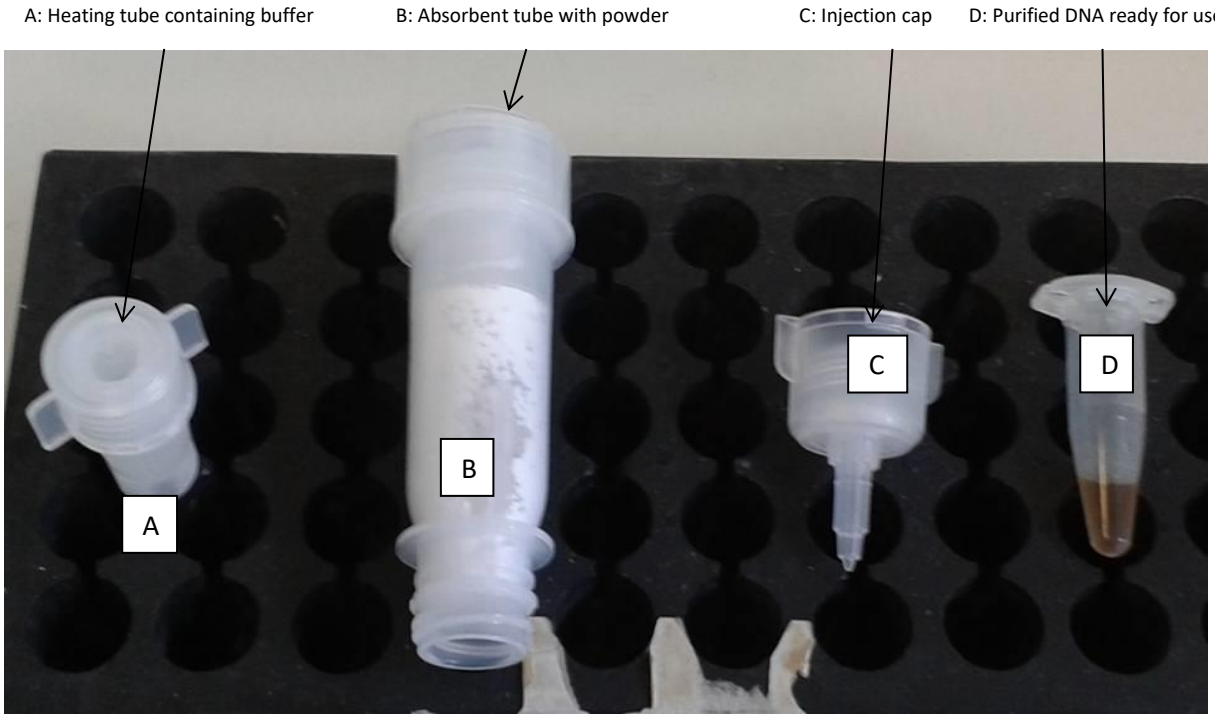


Figure 2.3: PURE® rapid ultrapure DNA extraction kit (Eiken, Japan)

Figure 2.4: Loopamp- LF 160

The Loopamp- LF 160 can perform the DNA extraction, the LAMP assay and direct visualisation of the end- point within one hour (Eiken Chemical Co. Ltd., 2005). In this study results were available within 2 hours.

2.3.8.4 DNA EXTRACTION USING A MANUAL METHOD "BOIL AND SPIN"

A method has been evaluated and described by FIND whereby DNA can be directly extracted from blood (FIND, 2012). This method was investigated in this study for the extraction of DNA from stool using a hot- block at 95^{o}C, a vortex (optional), a micro- centrifuge, a timer and a stable power source (Polley *et al*., 2013).

A loading volume of stool (100, 80, 50, 25 or 10 µl) was added to an equal volume of SDS buffer (400 mM NaCl, 40 mM Tris pH 6.5, 0.4% SDS) in an extraction tube. The sample was mixed by vortex for 10 seconds. Before being placed in a heating block and heated at 95^{o}C for 5 minutes. Overheating may degrade the DNA and reduce the sensitivity of the test (FIND, 2012). The sample was then centrifuged at 10, 200 rpm (9.6 rcf) for 3 minutes. Following this the clear supernatant was transferred to a dilution tube containing 345 µl of nuclease free water and mixed (by pipette ten times or by vortex for 3 seconds). The DNA was used neat or diluted in nuclease free water (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) in the LAMP assay. The extracted DNA was used immediately or stored at -20^{o}C.

2.3.8.5. DETERMINATION OF PERSISTENCE OF DNA IN SAMPLES STORED AT 4°C.

Routine diagnostic samples for DNA extraction at DCP are stored at 4°C for one week before DNA extraction is performed. Some samples requiring re-extraction may be stored at 4°C for up to three weeks. Storage at 4°C without a preservative is less successful for the detection of DNA than storage at -20°C (Qiagen®, 2013).

A pilot study was performed to determine if this protocol could be applied to routine diagnostic samples so that the addition of NAAT testing for *S. stercoralis* DNA could be synchronised into the existing routine work-flow of the laboratory.

Aliquots of 250 µl of positive stool controls (containing 0.1 *S. stercoralis* larvae/ µl) and negative stool controls were stored at 4°C and -20°C for different lengths of time until extraction of DNA was performed using the Qiagen® Qiasymphony SP. A 10- fold dilution series of the extracted DNA was performed and qPCR and LAMP assays were carried out to determine the effect of prolonged storage at 4°C on the persistence of *S. stercoralis* DNA. The qPCR and LAMP assays were performed in parallel on DNA extracted from duplicate samples stored at 4°C or at -20°C.

2.3.9. NUCLEIC ACID AMPLIFICATION TECHNIQUES AND TARGETS CONSIDERED FOR THE DETECTION OF *S. STERCORALIS* DNA IN CLINICAL SAMPLES

The introduction of a method that can detect a low parasite load and determine eradication of *S. stercoralis* post-treatment (point of cure) is urgently required (Requena-Méndez *et al*., 2013).

Based on the, previously mentioned, evidence published by Verweij *et al*. (2009) for the detection of *S. stercoralis* using the 18S rRNA gene in a real-time PCR format the 18S rRNA gene was determined to be a suitable target for this research.

LAMP is available for the detection of parasite DNA (*T. cruzi* and malaria) and has been successfully deployed in endemic areas for these infections (Thekisoe *et al*., 2010, Polley *et al*., 2013). This study investigated the development of LAMP for the detection of *S. stercoralis* DNA in clinical samples using primers, designed at DCP, to detect to detect target DNA in the *S. stercoralis* 18S rRNA gene.

2.4. LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP)

2.4.1. PRIMER DESIGN

Primer design for LAMP is crucial and primers were designed for this study using the software LAMP primer designing software PrimerExplorer V.3 (Eiken Chemical Co. Ltd., 2005). A BLAST (Basic Local Alignment Search Tool) search was performed against the EMBL database on the EBI and NCBI website (NCBI, n.d.) for the *S. stercoralis* 18S rRNA gene. A number of potential primer sets were

obtained (Appendix 2). Figure 2.5 demonstrates the binding sites for the inner and outer primer sets.

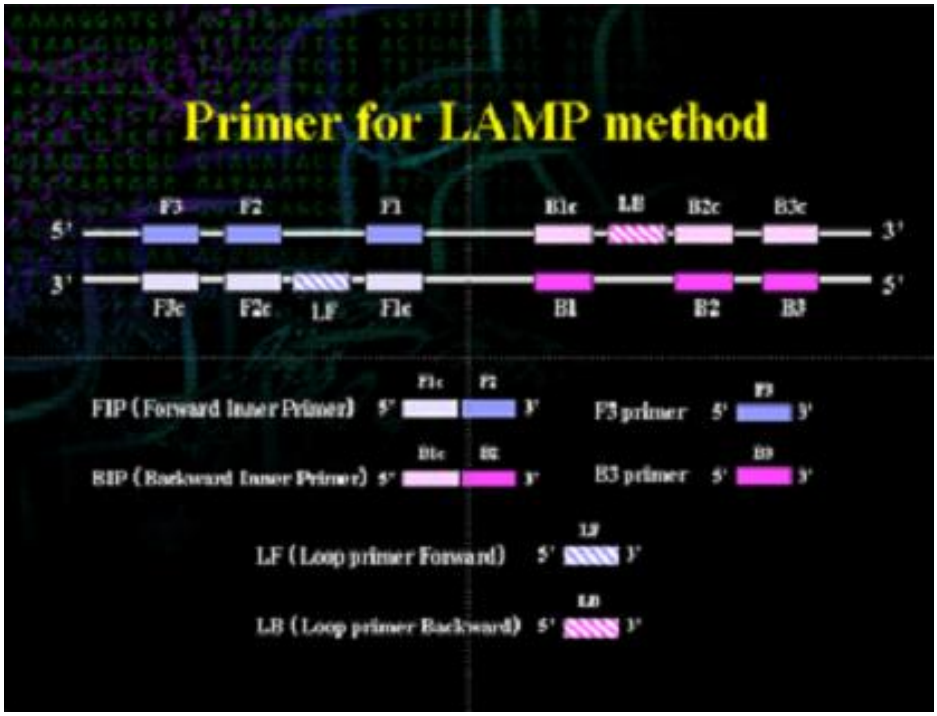


Figure 2.5 LAMP primers and the target DNA binding sites. F3 and B3: Forward and reverse outer primers, FIP (comprised of two segments-F2:F1c) and BIP (comprised of two segments-B2:B1c): Forward and reverse inner primers). http://loopamp.eiken.co.jp/e/lamp/primer/html and "A guide to LAMP primer designing (Primer ExplorerV4)" (Eiken Chemical Co. Ltd., 2005)

LAMP reactions were set up using the protocol supplied by Eiken Chemical Company, Ltd (2005). The LAMP assay was performed in a LA 320C turbidometer and heating block (Source Eiken, Japan) for one hour at 63^{o}C.

The reaction mix contained 20mM Tris-KCl reaction buffer(20 mM Tris-HCl, 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH)_2SO_4$), 0.1% Tween 20, 0.8M Betaine, 25mM each of dNTPs, 40 pmol each of forward and back inner primers, 20 pmol each of forward and back loop primers, 5 pmol each of forward and back outer primers, 8 units/ µl *Bst* DNA polymerase, 2.5 mM NaCl and nuclease free water to make up the volume to 20µl to which was added 5 µl of extracted DNA (Eiken Chemical Co. Ltd., 2005).

A positive result (LAMP time in minutes) was determined by the development of turbidity detected by production of insoluble magnesium pyrophosphate released by the specific binding of the *S. stercoralis* specific outer and inner primers and amplification of DNA at a pre-determined cut-off.

Real-time turbidity was measured by the LA 320C turbidometer and the results were analysed using a program available from the Eiken Chemical Co. Ltd., (2005). Personal communication with Dr van Lieshout regarding the qPCR method (ten Hove *et al*., 2009) noted that *S. stercoralis* DNA (for a qPCR assay) deteriorated with repeated freeze-thaw cycles and this was thought to be the case with the LAMP assay as well. Repeated use of the positive control dilution series was consistently positive at a dilution 10^{-2} but not at 10^{-3}. A consistent loss in the sensitivity of the detection of DNA was demonstrated with the positive control after more than one freeze-thaw cycle and the positive control extracted DNA dilution series was, therefore, kept at 4^{o}C for future use and the problem of deterioration of DNA was mostly eliminated. Whilst the analytical sensitivity was determined as 1 x 10^{-3} *S. stercoralis* larvae/ µl for LAMP assays (Table 3.10), the assays were performed using the control at a dilution of 10^{-2} to eliminate the effect of a possible loss of sensitivity in storage at 4^{o}C.

2.4.2. CONFIRMATION OF LAMP PRODUCT

Conventional PCR (cPCR) was used to generate a product, using the outer LAMP primers, which could be used to determine the sequence of the product and confirm LAMP assay product identity. The master mix consisted of 25 mM $MgCl_2$, 5 mg/ml of bovine serum albumin, 5 pmol each of LAMP forward and back outer primers, 12.5 µl of Hotstart *Taq*® polymerase and water to make up a volume of 20 µl to which was added 5 µl of DNA. The program on a Hybaid thermocycler was: 95^{o}C for 15 minutes followed by 50 cycles of- 95^{o}C for 15 seconds, 60^{o}C for 30 seconds and 72^{o}C for 20 seconds. This was followed by 1 hold cycle of 10^{o}C.

2.5. QUANTITATIVE REAL-TIME PCR (qPCR)

The qPCR assay collects data in real-time so that the amplification and visualisation steps are combined into a single step. This eliminates contamination of the laboratory with amplified DNA as there is usually no need to open the sealed reaction tubes.

A qPCR assay is available for the detection of *S. stercoralis*. Verweij *et al*. (2009) published a method that has been referenced in other publications (Requena- Méndez *et al*., 2013) using primers to the 18S rRNA gene for *S. stercoralis* and a double-labelled probe (Chapter 4, Section 4.3). The samples were run on a Rotagene Q and results were analysed using Rotor-Gene 6 software, version 6.1, Corbett Research (Source Corbett Life Sciences).

2.5.1. PRIMERS AND PROBES

Published primers and a probe for the qPCR assay were chosen (Verweij *et al*., 2009) on the basis of a literature review (Requena- Méndez *et al*., 2013) and the protocol was received from Dr van Lieshout at Leiden University, to ensure that the most up to date protocol was used. An NCBI BLAST

search (NCBI, n.d.) confirmed the sequence (AF 279916) reported by Verweij *et al*. (2009) and displayed a sequence match of 100% for *S. stercoralis*. Verweij *et al*. (2009) reported that the forward primer on the 18S rRNA gene sequence also showed a sequence match of 100% with other *Strongyloides* species.

A qPCR protocol of 95^{o}C for 15 minutes, followed by 55 cycles of 95^{o}C for 15 seconds, 60^{o}C for 30 seconds, and 72^{o}C for 20 seconds was used for the study.

The master mix contained 12.5 µl Hotstart Taq® polymerase, 5 mg/ml BSA, 25 mM $MgCl_2$, 2.5 pmol each of forward and reverse *S. stercoralis* primers, 1.25 pmol of *S. stercoralis* probe, 3.95 pmol each of forward and reverse *gfp* primers, 1.33 pmol *gfp* probe and water to make a final volume of 20 µl to which was added 5µl of DNA.

The product is 101 base pairs (bp) and the sequences of the published primers and probe for qPCR are:

Forward primer Stro18S-1530F 5' -GAATTCCAAGTAAACGTAAGTCATTAGC-3'

Reverse primer Stro18S-1630R 5 ' -TGCCTCTGGATATTGCTCAGTTC-3'

Probe Stro18S-1586T FAM-5 ' -ACACACCGGCCGTCGCTGC-3 ' -BHQ1

2.6. CONFIRMATION OF PCR PRODUCT

All tubes that were qPCR positive were run on a 2% agarose gel with 5µl of Safeview® nucleic acid stain for 1.5 hours at 100V to demonstrate separation of the *S. stercoralis* (101bp) and *gfp* (97bp) bands. Furthermore, the products of LAMP and qPCR were sequenced to confirm the identity of *S. stercoralis* and to confirm target detection where the CRS was negative and LAMP and/ or qPCR was positive. Samples that produced anomalous band sizes were also sent for sequencing reactions to determine the identity of these bands.

A proportion of the positive samples were also amplified using cPCR and the thermo-cycler protocol described for cPCR confirmation of the LAMP product was used. The Stro18S-1530F and Stro18S-1630R primers replaced the LAMP primers. The probe is excluded from this reaction to prevent problems occurring downstream in the sequencing process by remaining probe sequence. The amplified product was run on a 2% agarose gel with 5µl of Safeview® nucleic acid stain for 1 hour at 100V to generate a single band that could be sequenced to confirm product identity.

2.7. SEQUENCING

2.7.1. PREPARATION OF DNA PRODUCT FOR SEQUENCING REACTION (a)

DNA generated by cPCR using Str18S forward (F) and reverse (R) primers (used in qPCR) or LAMP SPSs (Appendix 2) inner forward (F) and back (R) primers was purified and ligated to a pJET® 1.2 plasmid vector using a GeneJET® gel extraction kit (Source Thermo Fisher Scientific, UK). The ligation reaction was then used to transform competent TOP10 *Escherichia coli* (*E. coli*) cells (chemically treated to accept DNA). The ligation reaction and the TOP10 *E. coli* were incubated at 37^{o}C (with shaking at 300 rpm for 1.5 hours) in SOC buffer (Brown, 2006). The transformed *E. coli* was plated out onto LB agar plates containing a 10^{-3} dilution of ampicillin and incubated overnight at 37^{o}C. Four colonies were chosen and incubated overnight in LB broth containing a 10^{-3} dilution of 100 mg/ ml ampicillin. The plasmid DNA was purified using an Invitrogen Quick plasmid minikit (Source Thermo Fisher Scientific, UK). The *Bgl* II restriction enzyme was used to confirm the presence of a cloned insert by gel electrophoresis. DNA concentration was performed on a Nanodrop spectrophotometer at 260 nm. The inserts were sent to Thermo Fisher Scientific, UK for sequencing. The sequence results were used to perform a nucleotide database search (BLASTn) to confirm product identity (NCBI, n.d.).

2.7.2. PREPARATION OF DNA PRODUCT FOR SEQUENCING REACTION (b)

Reactions were set up using cPCR products and primers Stro18S F and R or LAMP outer primers (Forward-F3 and Back- B3).

The DNA was purified using a QIA®quick PCR purification kit (Source Thermo Fisher Scientific, UK). A 2% agarose gel, plus 5µl Safeview® nucleic acid dye (Source NBS Biologicals Limited, UK) was run for one hour at 100V to determine the DNA concentration against a 100bp Hyperladder IV marker (Source Bioline, UK). Hyperladder IV contains known DNA concentrations in the restriction bands. This method was used when a UV spectrophotometer was not available for the estimation of DNA concentration (Figure 2.6).

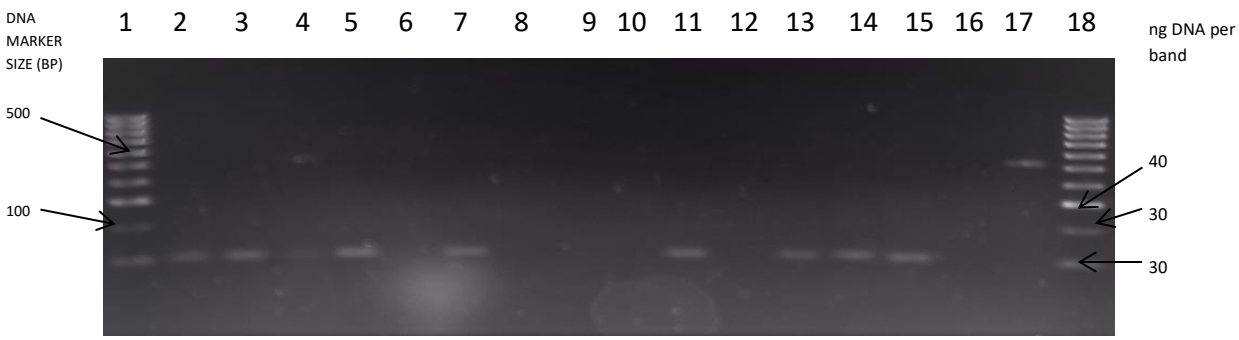


Figure 2.6: DNA concentration using Hyperladder IV after DNA purification. The Hyperladder IV band sizes contain different known concentrations of DNA (Lanes 1 and 18). cPCR product (Lanes 2-5, 7, 11, 13-15). Lane 17 an anomalous band at approximately 500 bp. Negative cPCR (Lanes 6, 8-10, 12 and 16)

The DNA, purified using the QIAquick PCR purification kit, was amplified using single primers, either Stro18S F or Stro18S R or LAMP F3 or LAMP B3 using the protocol supplied for the ABI Big®Dye version 3.1 cycle sequencing kit (Source Thermo Fisher Scientific, UK). The amplicons were sent to The London School of Hygiene and Tropical Medicine (LSHTM) for sequencing on an ABI Prism 310 genetic analyser.

2.7.3. SEQUENCING USING ABI BIG®DYE VERSION 3.1 FOR PERFORMING FLUORESCENCE – BASED CYCLE SEQUENCING REACTIONS USING THE ABI PRISM 310 GENETIC ANALYSER

The ABI Big®Dye version 3.1 cycle sequencing kit terminates the elongation of single stranded DNA by the addition of a fluorescence tagged nucleotide so that a mixture of DNA strands of different lengths is available for sequence analysis. Single primers are used to generate the DNA strands so that only one DNA product is sequenced (Thermo Fisher Scientific Inc., 2016). A laser allows the four nucleotides (thymine, adenine, cytosine and guanine) to radiate different colours of the visible light spectrum (Life Technologies Corp., 2011). The DNA migrates through a capillary with a 50 micron bore and a laser detects any fluorescent tagged nucleotides and transfers the data to a computer for data analysis of the raw data peaks to automatically generate a sequence of the DNA template. An electropherogram of the resulting sequence with the different nucleotides generating four different coloured peaks is produced.

2.7.4. SEQUENCING USING ABI BIG®DYE VERSION 3.1 FOR PERFORMING FLUORESCENCE – BASED CYCLE SEQUENCING REACTIONS USING THE ABI PRISM 310 GENETIC ANALYSER ON CLONED SAMPLES USING pGEM® T- EASY VECTOR SYSTEM TO CLONE THE PCR PRODUCTS

Further sequencing reactions were performed using cloned sequences in the ABI®BigDye version 3.1 for those samples that did not produce an identifiable sequence using the methods described

previously in Section 2.7. Chen *et al*. (2000) confirmed that blunt-edged ligation was less efficient than sticky-ended ligation. A vector that included 3'-T (thymidine) overhangs that improve the efficiency of ligation, by preventing re-circularization of the high-copy number vector and allowing ligation of PCR product, was chosen. TOP10 *E. coli* competent cells were used for the transformation. The vector contains T7 and SP6 RNA polymerase promoters that flank the multiple cloning regions, within the alpha-peptide coding region of the enzyme beta-galactosidase. In this study ampicillin resistance was used to isolate the transformed cells.

The pGEM® T-Easy kit was used as described by the manufacturer (Source Thermo Fisher Scientific, UK). The purified plasmids were restricted using the *EcoR1* restriction enzyme and the presence of DNA inserts was confirmed on a 1% agarose gel. The plasmid DNA was purified using an Invitrogen Quick plasmid minikit (Source Thermo Fisher Scientific, UK). DNA concentration was performed on a Nanodrop spectrophotometer at 260 nm. Samples that contained inserts were amplified using the T7 primer or the SP6 primer to generate a single- stranded product that was sent to LSHTM for sequencing using an ABI Prism 310 genetic analyser.

2.7.5. SEQUENCE ANALYSIS

All sequences generated were analysed using a nucleotide basic local alignment search tool (BLASTn) to determine sequence homology. Where sequence matches to published sequences is determined, a statistical value (E expected) is generated to determine statistical significance of a match, the lower the E value the more significant the sequence similarity is (NCBI, n.d.). The sequences generated from the LAMP and qPCR assays were analysed by the ClustalW2 multiple sequence alignment tool (ClustalW2) to align the *S. stercoralis* sequences with the sequence of the published 18S rRNA genome (Larkin *et al*., 2007)

2.8. STATISTICAL ANALYSIS

Statistical analysis on the comparison of two NAATs was chosen to detect whether the assay was able to exclude the disease with a high sensitivity (a screening test) or to detect the disease with a high specificity (a diagnostic test) (Kirkwood and Sterne, 1988). The use of an imperfect reference test was addressed by the use of statistical analysis methods recommended in the Food and Drug Administration guidelines (FDA, 2007). Statistical analysis was performed only once on each patient, although multiple samples were received on some patients, as repeat samples could not be categorised as new episode/ re-infection, failure of treatment or past infection.

Once the study code was broken the results of the LAMP and qPCR assays were compared with the results for the composite reference standard. The results were separated into samples stored at 4^{o}C

or at -20^{o}C to determine if the final statistical analysis would be performed on the results obtained at both storage temperatures or only at -20^{o}C.

Friedman's non-parametric test can be used when the same parameter (LAMP or qPCR) is measured under different conditions (temperature) on the same subject. This test was performed to confirm the effect of the temperature of sample storage on the LAMP and qPCR assays (Medcalc®, n.d.).

McNemar's test was performed to determine the difference between paired proportions of the composite reference standard and the LAMP or qPCR assay results. This assumes that the sum of the rows equals the sum of the columns when the null hypothesis is true. The definitive tests in the CRS (microscopy and culture) are insufficiently sensitive to enable determination of disease frequency and serology indicates probable disease only. Statistical analysis was performed on CRS result (including serology positive only, which denotes probable disease) or microscopy/ culture result (proven disease) for the determination of non-equivalence of tests using McNemar's test on paired proportions (Medcalc®, n.d.). Fischer's Exact test was used to determine initial statistical significance of the results.

Logistic regression was chosen to determine whether the characteristic of interest would have an effect on the result of the LAMP or qPCR assays (1= positive or 0= negative). Stepwise logistic regression was performed to determine if the storage temperature, aliquot size, length of storage, country of travel/ origin, age (years) or gender had a statistically significant effect on the LAMP or qPCR assay results. (Medcalc®, n.d.). Data was not available to determine the immune status of the patient samples in this study.

Contingency (2x2) tables were used to allow a comparison (plus 95% confidence intervals to determine significance) between two tests. The sensitivity (proportion of true positives detected), specificity (proportion of true negatives detected), positive and negative predictive values (probability that person is infected if they have a positive test or is truly disease-free if they have a negative test) were calculated (Banoo *et al.*, 2007).

Likelihood ratios provide useful clinical information as a positive likelihood ratio >1 indicates a positive result is more likely to occur in those with strongyloidiasis than in those without the disease. A ratio of <1 indicates that a positive result is less likely to occur in those with strongyloidiasis than in those without the disease and the same holds true for the likelihood of a negative result. The greater than 1 the likelihood ratio is, the stronger the association with the

disease, ratios between > 10 and <0.1 provide strong evidence to determine, or exclude, the diagnosis.

However, in this study, quantities such as positive predictive value, negative predictive value, and the positive and negative likelihood ratios must be interpreted with care since the subjects' condition status (as determined by the composite reference standard) is unknown (Kirkwood and Sterne, 1998). The formulae for all the above indices are shown in Table 2.4.

Table 2.4: 2x2 contingency table and associated formulae.

TEST STATUS	TRUE STATUS		TOTAL
	POSITIVE	**NEGATIVE**	
POSITIVE	True positive TP	False positive FP	**TP+FP**
NEGATIVE	False negative FN	True negative TN	**FN+TN**
TOTAL	**TP+FN**	**FP+TN**	**TP+TN+FP+FN**

Sensitivity= TP/(TP+FN)

Specificity= TN/(TN+FP)

Positive predictive value= TP/(TP+FP)

Negative predictive value= TN/(TN+FN)

Positive likelihood ratio= sensitivity/ 1- specificity (probability that that a person who has the disease testing positive over the probability of a person without the disease testing positive)

Negative likelihood ratio= 1-sensitivity/ specificity (probability of the person who has the disease testing negative over the probability of the one who does not have the disease testing negative)

K= (Total number of agreements- expected number of agreements) (total number of observations-expected number of agreements). Value of *K* Strength of agreement- < 0.20= Poor; 0.21 - 0.40= Fair; 0.41 - 0.60= Moderate; 0.61 - 0.80= Good; 0.81 - 1.00= Very good

Prevalence index=([TP-TN])/(TP+TN+FP+FN)

Bias index=([FP-FN])/ (TP+TN+FP+FN)

The Food and Drug Administration (FDA) guidelines for statistical analysis (FDA, 2007) recommends using positive and negative percent agreement for tests without a suitable reference standard and using the overall percent agreement between the reference standard and the test under investigation. When a new test is compared to a non-reference standard rather than to a reference standard, the usual sensitivity and specificity type calculations from the 2x2 table will produce biased estimates of sensitivity and specificity. This is because the non-reference standard is not always correct. However, being able to describe how often a new test agrees with a non-reference standard may be useful. The estimated sensitivity is the proportion of subjects with the condition of

interest (reference standard positive) that are new test positive. Estimated specificity is the proportion of subjects without the condition of interest (reference standard negative) that are new test negative. The differences between sensitivity and percent positive and specificity and percent negative is that the results do not represent whether the subject has the condition of interest as determined by the reference standard and so the results must be interpreted differently. Two commonly used measures are the overall percent agreement and Cohen's kappa. Clinical and analytical sensitivity is discussed in Chapter 3 for the LAMP assay and in Chapter 4 for the qPCR assay.

The sensitivity and specificity data provides information on how often the new test is correct; whereas, percent positive and negative data provides information on how often the new test agrees with a non-reference standard. The simplest measure is overall percent agreement (OPA): the percentage of total subjects where the new test and the non-reference standard agree. The FDA (2007) guidelines state that it is more useful to report a pair of agreement measures, positive percent agreement (PPA) and negative percent agreement (NPA), the overall percent agreement (OPA) will always lie somewhere between the positive percent agreement and the negative percent agreement. The positive percent agreement is the proportion of non-reference standard positive subjects that are new test positive (similar to a sensitivity calculation) (Table 2.5). The limitations are that agreement measured by overall percent agreement or kappa may not be correct as agreement depends on prevalence (relative frequency) of the disease in a specific cohort (pre-test probability). Prevalence is unknown in the current study cohort.

Table 2.5: Calculations required for positive and negative percent agreement

		NON-REFERENCE STANDARD	
NEW TEST		**+**	**-**
	+	A	B
	-	C	D
TOTAL		A+C	B+D
Positive percent agreement (PPA)	Proportion of non-reference standard positive samples where the new test is positive = 100% x A/A+C		
Negative percent agreement (NPA)	Proportion of non-reference standard negative samples where the new test is negative = 100% x D/B+D		
Overall percent agreement (OPA)	Proportion of samples where new test and non-reference standard agree = 100% x (A+D)/(A+B+C+D)		

Cohen's kappa coefficient was performed to determine the level of agreement between the LAMP and qPCR assays with the composite reference standard to give a better indication of concordance as it accounts for agreements due to chance. Interpretation of kappa is influenced by bias (bias index = number of false positive and false negative/ number of observations) and prevalence (prevalence index= true positive - true negative/ number of observations) (Table 2.4). The difference between kappa and maximum obtainable kappa (K_{max}), after marginal possibilities and cell frequencies were adjusted, was used to obtain the greatest possible agreement plus 95% confidence intervals (95% CI) (McHugh, 2012). Kappa assumes independence and factors affecting independence were considered for the interpretation of kappa on factors that could affect independence by stepwise logistic regression analysis, as previously described.

Intraclass correlation and multiple variable clustered graphs were used to demonstrate the influence of the CRS on the LAMP and qPCR assay results. Cicchetti (1994) gives the following guidelines for intraclass correlation interpretation: <0.4 = poor; 0.4-0.59 = fair; 0.6-0.74 = good and 0.75-1.00 = excellent.

The Youden index was used to determine the cut off C_t, at which a qPCR result was a reliable indicator of strongyloidiasis. C_t values of ≥ 40 may be subject to primer dimer and false positive reactions (Caraguel *et al*., 2011).

Box and whisper plots were used to determine the number of cycle runs for the qPCR assay (Medcalc®, n.d.).

All statistical analysis was performed using the Medcalc® statistical program version 16.2.0 (MedCalc®, n.d.) or on an Excel® spreadsheet.

This chapter was designed to meet the requirements for evaluation and validation of new diagnostic tests (ISO 15189:2012, 2012) and to serve as a standard operating protocol for the addition of tests to the diagnostic repertoire in a specialist parasitology diagnostic laboratory (HPA UK protocols, 2013, Bossuyt *et al*., 2015) as required by the aim and outcomes of the current study.

CHAPTER 3: DEVELOPMENT, EVALUATION AND VALIDATION OF A NOVEL LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) ASSAY

3.1 INTRODUCTION

This chapter describes the development of an assay suitable for use in the diagnosis of strongyloidiasis in resource- limited and well- resourced settings for use in the diagnosis of human strongyloidiasis. As previously discussed, development of rapid, accurate and sensitive diagnostics is essential for treating, controlling and eradicating infectious disease (WHO, 2010). This technique has been used for the detection of other parasitic diseases in blood. Perera *et al*. (2017) described the development of a high- throughput assay which was suitable for use in resource- limited areas for the detection of malaria. Stool inhibition of the LAMP assay was investigated in this study as stool has more potential inhibitors than blood and this had the potential to limit the sensitivity of the LAMP assay (Murphy *et al*., 2007). LAMP has also been used in veterinary medicine for the control and cost-effective management of parasites in livestock. Melville *et al*. (2014) described the development of a rapid specific and sensitive LAMP assay for the detection of *Haemonchus contortus* nematode eggs in faecal samples requiring only crude DNA for detection. While it is true that large amounts of *Haemonchus contortus* DNA is available in the faecal samples (Melville *et al*., 2014), the techniques described could also be applied to the detection of *S. stercoralis* DNA in human faecal samples in this study. The detection of *S. stercoralis* DNA in canine stool samples has been previously described by Jaleta *et al*. (2017) using conventional PCR and primers to the hypervariable region of the 18S rRNA gene followed by sequence analysis. This method may be adapted to LAMP using the methods described in the current study for the deployment of a cost-effective technique for control and animal management (Melville *et al*., 2014).

The requirements for verification and validation of new diagnostic techniques is discussed in ISO 15189.2012 standards (UKAS, n.d., ISO 15189:2012, 2012) and in the standards for reporting diagnostic accuracy studies (STARD) guidelines (HPA UK protocols, 2013, Bossuyt *et al*., 2015). STARD guidelines were used to establish and confirm performance characteristics and accuracy measures for the diagnostic assays evaluated in the current study (Bossuyt *et al*., 2015).

Standardisation and quality assurance is required and the study was also designed to detect the risk of bias and sources of variation. For the purposes of this study QADAS 2 guidelines (discussed in Section 6.1) were used to design a protocol to address the lack of quality assurance in studies noted in a systematic review by Whiting *et al*. (2014).

The criteria chosen for the verification of the assays in the study were analytical sensitivity (the limit of detection), analytical specificity (Saah and Hoover, 1997**)**, LAMP efficiency and assay precision (Reed *et al*., 2002, Salimetrics®, n.d.).

Analytical sensitivity was determined by the lowest detection level for *S. stercoralis* using serial 10-fold dilutions of DNA extracted from a positive stool control containing 1 *S. stercoralis* larva/ µl. Analytical specificity was determined using a specificity bank comprised of known negative stool samples and human viral, bacterial and parasitic pathogens and a free- living rhabditiform larva (Table 2.2). LAMP efficiency was determined by the limit of detection of the method using serial 10-fold dilutions of DNA extracted from a positive stool control or a known concentration of purified DNA.

The LAMP assay is a rapid, sensitive technique that can be used in well-resourced laboratories in a high-throughput or point of care format when an urgent result is required (Wong *et al*., 2017). LAMP can also be transferred to resource-limited areas as the requirement for complex machinery and technical expertise is not as high as that for real-time PCR (Mori and Notomi, 2009). The reaction is described in detail in Section 3.4.

3.2. AIMS

There were three main aims to this part of the study:

1. To develop and evaluate the sensitivity and specificity of a novel LAMP assay and compare to the CRS (this Chapter) and the qPCR assay (Chapter 5) for the detection of *S. stercoralis* DNA from clinical samples.

2. To evaluate the sensitivity and specificity of DNA extraction using the routine method at DCP compared to simplified DNA extraction methods to determine if a simplified method suitable for resource- limited areas could be introduced (this Chapter).

3. To determine the cost and suitability of LAMP and qPCR assays for introduction to the diagnostic NAAT repertoire at DCP (this is discussed further in a comparison of the LAMP and qPCR assays in Chapter 5)

3.3 METHODS FOR THE DEVELOPMENT, EVALUATION AND VALIDATION OF A NOVEL LAMP ASSAY FOR THE DETECTION OF *S. STERCORALIS* DNA IN CLINICAL SAMPLES

This section of the study investigated the development of LAMP to detect *S. stercoralis* DNA using novel primers to target the 18S rRNA gene. LAMP needs to be clinically validated before this technique can be introduced as a diagnostic method (Requena-Méndez *et al*., 2014) and 284 residual diagnostic stool samples from a cohort of returning travellers or migrants from endemic areas were tested were used to determine LAMP performance as a diagnostic technique for the diagnosis of strongyloidiasis. The composite reference standard consisted of microscopy and culture (parasitological definitive diagnosis) and serology (serological probable diagnosis). As previously discussed, a composite reference standard may be used when no "gold standard" exists to eliminate bias in accuracy estimates (Baughman *et al*., 2008). Statistical analysis was performed using an online statistical program (MedCalc®, n.d.).

Optimisation of the LAMP assay and sequence determination of the LAMP assay product was performed to determine whether this assay would be suitable for the detection of *S. stercoralis* DNA in clinical samples. The results of the LAMP assay are presented in Section 3.4.

Mori and Notomi (2009) stated that LAMP is suitable for use in resource- limited areas. The automated DNA extraction method used in this study requires costly equipment and highly trained laboratory staff and is unsuitable for use in resource- limited areas (Minetti *et al*., 2016). To determine the usefulness of LAMP as a screening test in resource-limited areas a pilot study was carried out using the manual method of heating at 75^{o}C for 15 minutes followed by removal of assay inhibitors with absorbent buffer and the rapid extraction of ultrapure DNA for the LAMP assay. DNA extraction was performed in parallel using Qiagen® Qiasymphony SP magnetic bead resonance technology to serve as a comparison control**,** a method which is currently in use at DCP. A manual method to extract DNA using only heating at 95^{o}C for 5 minutes and a centrifuge was also compared to the Qiagen® Qiasymphony SP magnetic bead resonance technique for suitability of use in resource- limited areas. To investigate the issue of stool inhibition of the LAMP assay a range of stool volumes were compared to determine whether the loading volume of stool sample would have an effect on the sensitivity of detection for *S. stercoralis* DNA in clinical samples

The current NAAT protocol for the determination of human protists in stool samples at DCP may require that stool samples are stored for up to 3 week at 4^{o}C. ten Hove *et al*. (2009) recommends the storage of stool samples for *S. stercoralis* DNA extraction at -20^{o}C . Consequently, DNA survival in stool samples stored at 4^{o}C or -20^{o}C was investigated. Aliquots of positive and negative stool control samples were stored at 4^{o}C and at -20^{o}C before DNA extraction and the LAMP assay were

performed. The investigation was performed to determine a storage technique that would be suitable for integration in the current workflow at DCP.

3.4 LOOP- MEDIATED ISOTHERMAL AMPLIFICATION

LAMP, first described by Notomi *et al*. (2000), is recommended for use in monitoring control strategies in resource- limited regions (Figure 3.1). Mori and Notomi (2009) suggest that the technology can also be used in point of care testing in well-resourced laboratories.

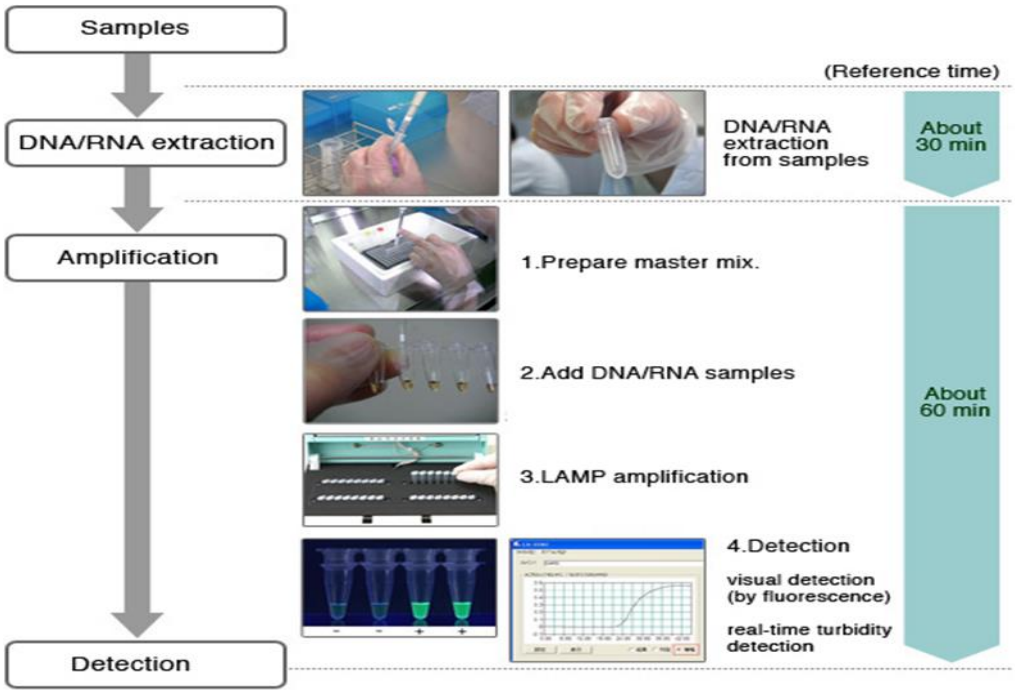


Figure 3.1: Standard procedure for loop-mediated isothermal amplification using blood or microbial cultures* (Eiken Chemical Co. Ltd., 2005).

*The extraction of *S. stercoralis* DNA from stool samples is said to require a more rigorous protocol (Moghaddassani *et al*., 2011, Levenhagen and Costa Cruz, 2014).

The mechanism for LAMP is in two stages: first the production of a stem loop structure that serves as the starting point for the second stage, the cycling amplification stage. The cycling amplification stage is dependent on the strand displacement activity of the *Bst* DNA polymerase. *Bst* DNA polymerase is less susceptible than *Taq* polymerase to PCR inhibition (Notomi, 2000).

The inner primers bind first, the outer primers in a lower concentration bind more slowly. Binding of the outer primer triggers *Bst* DNA polymerase strand displacement and the rest of the primers bind to single stranded DNA. This generates a stem loop structure and starts the cycling

amplification stage. The inner primers bind to the stem loop structure and generate a complementary stem loop structure and an elongated new stem loop structure. The generation of alternating stem loop and complementary stem loop structures forms the elongated product (measured as turbidity due to the release of magnesium pyrophosphate). An animation of the process is available from: www/loopamp.eiken.co.jp/e/lamp/anim.html.

It is a one-step amplification of target DNA with high sensitivity and specificity at one temperature (range usually between 60-65^{o}C) using a water-bath or heating block. A DNA polymerase with strand displacement activity and four to six primers that recognise six to eight distinct regions of the target DNA are required. The production of insoluble magnesium pyrophosphate can be visualised by an attached turbidometer, the use of fluorescent dyes- e.g. calcein® (fluorescence is quenched by the binding of free magnesium ions which is released by the amplification of product) or other intercalating dyes that can be viewed under UV light and turbidity which can also be viewed directly by eye. The turnaround time is shortened to one hour by the fact that temperature ramping and elongation times are eliminated (Mori and Notomi, 2009, Nagamine *et al*., 2001, Njiru, 2012, Polley *et al*., 2013).

The lyophilisation of primers and reagents has led to the development of kits to test various viral, bacterial and other parasitic organisms (e.g. *T. cruzi* and malaria) without the need for a cold chain for storage (Njiru, 2012, Polley *et al*., 2013, Thekisoe *et al*., 2010). This suggests a use for the LAMP assay in rural or resource- limited endemic areas (Eiken Chemical Co. Ltd., 2005).

3.4.1 DEVELOPMENT AND OPTIMISATION OF THE LAMP ASSAY

3.4.1.1. PRIMERS DESIGNED TO DETECT *S. STERCORALIS* DNA FOR LAMP ASSAY

Four primer sets, (St18s:1, St18s:4, St18s:12 (SPSs) and Po18s:299, (Appendix 2) were designed at DCP in 2011 for the detection of *S. stercoralis* DNA targeting the 18S rRNA genome. The primers (Source Eurofins Scientific, UK) were designed using Primer Explorer v3.0 (Eiken Chemical Co. Ltd., 2005). To increase sensitivity and specificity of the LAMP assay, four to six primers are required to target a small segment of the DNA and primer design is restrictive and problematic (Wong *et al*., 2017) and the four resulting primer sets were tested to determine the primer set that was optimal for the detection of *S. stercoralis* DNA. Primer set St18s:12 was chosen for the study on the basis of the results of the LAMP assay using the study protocol (Table 3.1) and was designated primer set SPSs.

In 2014, Watts *et al*. published a LAMP method for the detection of *S. stercoralis* DNA targeting the 28S rRNA genome. A review by Wong *et al*. (2017) found LAMP to be at least 1000 more sensitive

than conventional PCR for the detection of *Strongyloides*, *Necator americanus* and *Trichomonas vaginalis*. The Watts *et al*. (2011) primers were ordered (designated primer set WSs) to establish which primer set (SPSs or WSs) would be the most efficient primer set to use in this study. The results obtained by Watts *et al*. (2014) could not be duplicated in this study. In this study, the published protocol (Watts *et al*., 2014), could not be successfully optimised for the detection of *S. stercoralis* DNA. However, the published primer set (WSs) did successfully detect *S. stercoralis* DNA when used with this study protocol for the LAMP assay. Primer set SPSs demonstrated greater sensitivity than primer set WSs for detection of *S. stercoralis* DNA (Table 3.1).

Table 3.1: Results of primer sets St18s:1, St18s:4, Pol18s:299, SPSs and WSs when run at the optimised reaction temperature of 63^{o}C using the LAMP assay study protocol (I- V). Results for the LAMP assay described by Watts *et al*., 2014 for the primer set WSs using the published protocol (VI).

PRIMER SET AND PROTOCOL USED	LAMP (TIME IN MINUTES) NEGATIVE CONTROL	LAMP (TIME IN MINUTES) POSITIVE CONTROL (DILUTION 10^{-2})
I. 18S rRNA St18s:1 using this study protocol	20.30	16.54
II. 18S rRNA St18s:4 using this study protocol	50.36	23.42
III.18S rRNA St18s:12 (SPSs) using this study protocol	Negative	24.24
IV. 18S rRNA Po18s:299 using this study protocol	18.12	18.3
V. WSs 28S rRNA (Watts *et al*., 2014) using this study protocol	Negative	34.30
VI.WSs 28S rRNA using the published protocol described by Watts *et al*.(2014)	Negative	Negative (repeat assays below limit of detection)

In the primer sets designed at DCP primer set SPSs (III) did not detect amplified product in the negative control, while primer sets St18s:1, St 18s:4 and Pol 18s:299 detected *S. stercoralis* DNA in the negative control. Primer set WSs (V) demonstrated detection of *S. stercoralis* DNA in the positive control only. The LAMP time for primer set WSs was increased when compared to the LAMP time for primer set SPSs. Primer set SPSs detected *S. stercoralis* DNA in the positive stool control with a LAMP time of 24.24 minutes and was therefore chosen for this study (III).

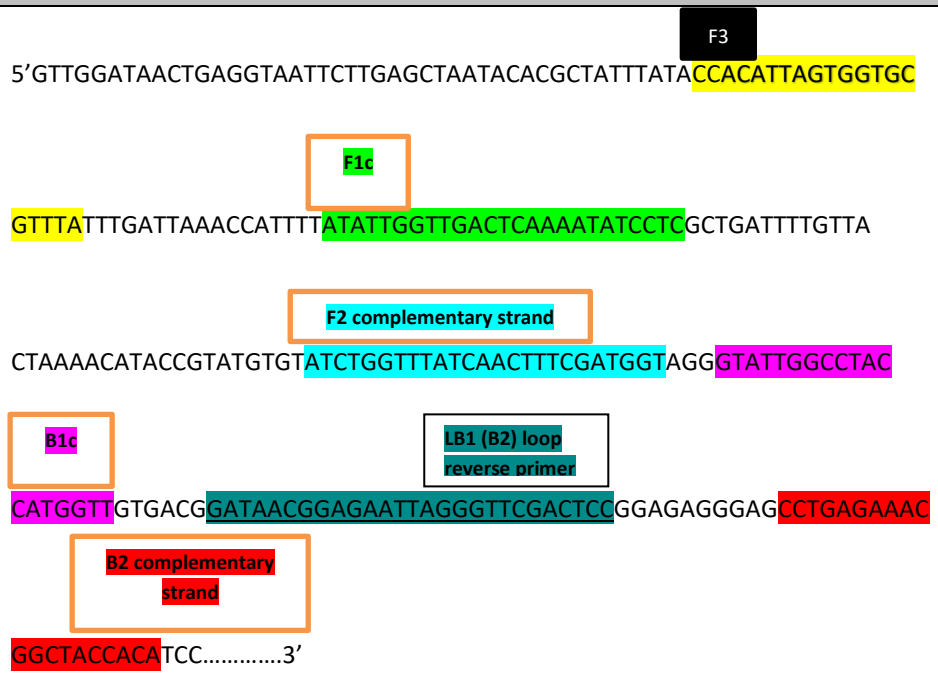
To determine the target site for the primer set chosen, the primer set sequences (Table 3.2a) and a sequence determined by a BLAST search (Table 3.2b) were compared. Table 3.2a shows the primer sequences for primer set 12 (SPSs). The binding sites are colour- coded for F3 (forward outer primer- yellow), B3 (back outer primer- no colour), FIP (forward inner primer- green), BIP (back inner primer- complex primer blue complementary sequence F2 and pink B1c) and LB1 (loop back primer-complementary sequence red, direct sequence in dark green) primers on a sequence from

the NCBI website (BLAST ID: AB453314- *Strongyloides stercoralis* gene for 18S rRNA, partial sequence, host: Pan troglodytes) for the *S. stercoralis* 18S rRNA gene (Table 3.2a and Table 3.2b). The sequence alignment of the LAMP assay product generated by cPCR and the outer primers for BLAST ID: M84229.1 (*Strongyloides stercoralis* 18S ribosomal RNA gene, complete cds) is appended in Appendix 3a. There is an overlap between the product generated by the LAMP assay back primer (B3) and the HVRI and HVRIV hyper variable regions described by Hasegawa *et al*. (2009) (Appendix 3a).

Table 3.2a: Identification of primers Primer set St18s:12 (SPSs) (colour- coded to determine their position on an *S. stercoralis* 18S rRNA gene sequence (Table 3.2b) (ENA|AB453314|AB453314.1)

PRIMER IDENTIFICATION	**PRIMER**	**NUCLEOTIDE SEQUENCE (LENGTH)**
St18s:12:F3	(outer forward primer)	CCACATTAGTGGTCGTTTTA (20)
St18s:12:B3	(outer reverse primer)	CTAAAATTGGGTAATTTTCGCG (22)
St18s:12:FIP (F2:F1c)	(inner forward primer) F1c	ACCATCGAAAGTTGATAAACCAGATATATTGGTTGACTCAAAATATCCTC (50)
St18s:12:BIP (B2:B1c)	Complementary F2 (inner reverse primer) B1c	TGGTAGCTTTCAACTATTTGGTCTATATAACCAACTGAGTTTTATAGGAG GTATTGGCCTACCATGGTTGTGTGGTAGCCGTTTCTCAGG (40)
St18s:12:LB1	Complementary B2 (Loop reverse primer)	CATAACCGGATGGTACCAACACACCATCGGCAAAGAGTCC GATAACGGAGAATTAGGGTTCGACTCC (27)

Table 3.2b: Primer set SPSs- Position of Forward outer primer (F3) Forward inner primers (F2:F1c), reverse inner primer (B2; B1c) and loop reverse primer (LB1)

BLAST ID.	POSITION OF PRIMERS (from Table 2.4 a above)
>ENA|AB453314| AB453314.1 *Strongyloides stercoralis* gene for 18S rRNA, partial sequence, host: Pan troglodytes.	

3.4.2. SODIUM CHLORIDE CONCENTRATION, TEMPERATURE AND TEMPLATE VOLUME OPTIMISATION

The Eiken website (Eiken Chemical Co. Ltd., 2005) describes a protocol for the LAMP assay without the addition of sodium chloride (NaCl). To fully optimise the method for use at DCP, the NaCl concentration, reaction temperature and template volume was investigated. Optimisation of the LAMP assay was performed using a negative stool sample as the negative amplification control and a stool sample spiked with *S. stercoralis* larvae (1 larva/ µl) as the positive amplification control. The concentration of NaCl and the reaction temperature at which *S. stercoralis* DNA was consistently detected in the positive stool control (at dilutions of 10^{-1}, 10^{-2} and 10^{-3}) determined the optimal LAMP assay conditions. Table 3.3 shows the results obtained for the concentration range 0mM to 7.5mM NaCl using the study protocol and primer set SPSs to perform a LAMP assay on a negative stool control and positive stool control dilutions of 10^{-1} to 10^{-3}. A positive result is given in time (minutes) at which turbidity is detected to demonstrate that product had been amplified. 2.5mM NaCl was the most sensitive (rapid) concentration at which *S. stercoralis* DNA was detected.

Table 3.3: Results of LAMP assay using a NaCl concentration curve. A positive result is reported in minutes.

	0mM NaCl	**2.5mM NaCl**	**5mM NaCl**	**7.5mM NaCl**
Negative stool control	Negative	Negative	Negative	Negative
Positive stool control (10^{-1})	21.06	20.0	22.18	25.42
Positive stool control (10^{-2})	24.0	24.72	25.42	25.06
Positive stool control (10^{-3})	27.12	25.0	29.12	36.06

Optimisation of the temperature at which LAMP was performed was determined from the results generated at 60^{o}C, 63^{o}C or 65^{o}C. Table 3.4 shows the results obtained for the temperature range 60^{o}C, 63^{o}C or 65^{o}C using the study protocol and primers SPSs to perform a LAMP assay on a negative stool control and positive stool control dilutions of 10^{-1} to 10^{-3}. A positive result is given in time (minutes) at which turbidity is detected to demonstrate that product had been amplified. The optimum temperature at which to perform LAMP was determined to be 63^{o}C in this study.

Table 3.4: Results of the temperature range optimisation for the LAMP assay (Positive result in minutes)

	60^{o}C	**63^{o}C**	**65^{o}C**
Negative stool control	Negative	Negative	Negative
Positive stool control (10^{-1})	33.0	19.36	30.36
Positive stool control (10^{-2})	46.12	20.06	32.42
Positive stool control (10^{-3})	45.36	23.42	37.54

The Eiken website (Eiken Chemical Co. Ltd., 2005) describes using 12.5 µl of DNA as the template volume. A DNA template range from 2.5 to 12.5 µl was tested to optimise the amount of DNA template to be added to the reaction (Table 3.5).

Table 3.5: Determination of the volume of template DNA for the LAMP assay. (Positive result- LAMP time in minutes)

	2.5 µl template DNA	**5 µl template DNA**	**7.5 µl template DNA**	**10 µl template DNA**	**12.5 µl template DNA**
Negative stool control	Negative	Negative	Negative	Negative	Negative
Positive stool control (10^{-1})	16.18	20.36	23.42	23.3	27.06
Positive stool control (10^{-2})	27.42	21.54	25.36	24.06	29.24

Analysis of the results generated using different DNA template volumes determined that 5µl of DNA template was the optimal volume to use in this LAMP assay. There was a 25% improvement in the detection time at a 10^{-1} dilution and a 24% improvement in the detection time for the 10^{-2} dilution when compared to the detection time for the DNA template volume of 12.5µl.

A smaller DNA template volume (2.5µl) demonstrated an improvement to the detection time of 40% in the 10^{-1} dilution, but an improvement of only 6% was demonstrated for the detection time in the 10^{-2} dilution. This may have been due to very little DNA template present in the reaction (Morrison *et al*., 1998) and indicated that a significant loss of sensitivity to the LAMP assay was likely when using a 2.5µl DNA template. These results were duplicated on repeat LAMP assays (data not shown).

3.4.3 CONFIRMATION OF DETECTION OF *S. STERCORALIS* DNA

To develop the assay confirmation of the sequenced product was performed on DNA amplified from the positive stool control using the inner or outer SPSs LAMP primers in a cPCR reaction (Figure 3.2) to confirm product identity and to generate a product sequence. LAMP product may also be directly sequenced (Saito *et al*., 2005) but this was not performed in this study.

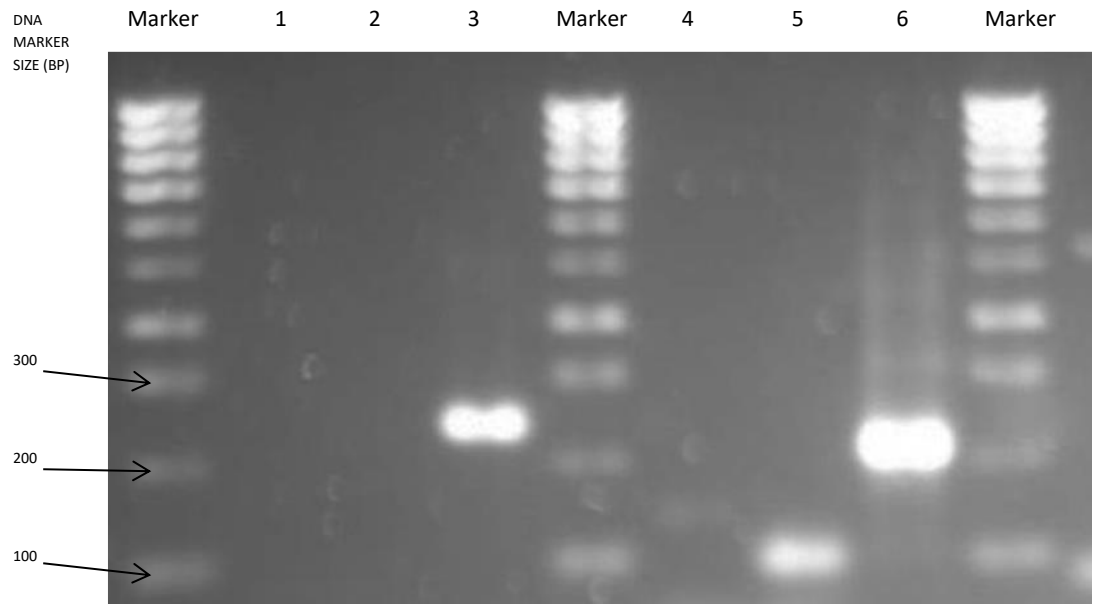


Figure 3.2: Gel electrophoresis (2% agarose run at 100V for 1 hour) of LAMP products generated by cPCR using LAMP SPSs outer or inner primers (Lanes 3 or 6). Lanes 1 and 4 contained the negative control and Lane 2 contained the no template control. Lane 5 shows the cPCR product, generated using qPCR Stro18S primers.

The product was cloned using the pJET® 1.2 plasmid vector kit and sent for sequencing. The cPCR product using the outer primers repeatedly failed to produce an insert with sequence identity. The insert produced using the inner primers failed to give sequence identity on a BLASTn search and could not be used to confirm product identity or to determine the limit of detection of the method. The insert sequence that was generated is shown in Table 3.6 and demonstrated that the pJET® 1.2 plasmid vector method was processed correctly according to the manufacturer's protocol.

Table 3.6: Sequences generated by cPCR from a positive stool control using Fip and Bip (LAMP forward and back inner primers) failed to generate identifiable sequences after cloning and sequencing reactions using the pJET®1.2 plasmid vector

ALIQUOT	SEQUENCE GENERATED	IDENTITY FROM BLAST SEARCH
>LP2	GGCTCGAGTTTTTCAGCAAGATACCATCGAAAGTTGATAAACCAGATA TATTGGTTGACTCAAAATATCCTCCTGAAAAACGGCTACCACACAACC ATGGTAGGCCAATAC	No identity obtained
>LP3	GGCTCGAGTTTTTCAGCAGATGTATTGGCCTACCATGGTTGTGTGGTA GCCGTTTCTCAGGAGGATATTTTGAGTCAACCAATATATCTGGTTTATC AACTTTCGAT	No identity obtained

It is not known why the cPCR product generated by the outer primers failed on two separate occasions to produce an insert using a blunt-ended plasmid vector (pJET® 1.2). The Corning Cellgro troubleshooting guide (Corning Cellgro, 2012) suggests that residual restriction enzyme or phosphatase might inhibit ligation, but this was not the case in this study as all the cPCR products that were sent for ligation had been purified. Another cause for concern is that the cells may have been contaminated allowing the cells to grow in the broth and on the LB agar plate containing ampicillin, but no confirmation was obtained for this in the study. Further investigation found that the ampicillin was in date and used at the correct concentration and so the detection reagent used could not be attributed to the failure of the sequencing reaction to generate a sequence identity. The lack of sequence identity may be due to the sequence of the product or the amount of cloned DNA produced. Table 3.6 demonstrates the sequences obtained and the result of a mega BLAST search using the pJET®1.2 plasmid vector where no sequence identity could be determined.

The DNA product was also sequenced using a direct sequencing method (Section 2.7.2) or by sequencing of the cloned insert using the pGEM® T-Easy plasmid vector, a vector with 3' T overhangs. These methods generated a sequence with matches of 77 to 100% with the *S. stercoralis* 18S rRNA subunit. The samples were sent to LSHTM for sequencing using an ABI Prism 310 genetic analyser. The sequences were analysed using the Seqman® program (Source DNASTAR® Inc., USA). The results of sequencing reactions on the purified product generated by cPCR were used to confirm the product identity for the LAMP assay from the direct sequence reactions (Table 3.7). Table 3.8 shows the sequence identity results using the BLASTn search tool (NCBI, n.d.).

Table 3.7 Sequences generated using an ABI Prism 310 genetic analyser

PRIMER USED	SEQUENCE	NUMBER OF BASE PAIRS (bp)
F3 LAMP forward outer primer	CCTCGCTGANTTTGTTACTAAAACATACCGTATGTGTATCTGGTTTATCAACTTTCGATGGTAG GGTATTGGCCTACCATGGTTGTGACGGATAACGGAGAATTAGGGTTCGACTCCGGAGAGGG AGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGAAA	172
B3 LAMP reverse outer primer	CTAATTCTCCGTTATCCGTCNCNNCNNTGGTAGGTAGGCCAATACCCTACCATCGAAAGT TGATAAACCAGATACACATACGGTATGTTTTAGTAAGAAAATCAGCGAGGATATTTTGAG TCAACCAATATAAAATGGTTTAATCAAATAAACGCACCACTAATGTGG	168

Table 3.8 Sequence identity generated by direct sequencing reactions performed on amplicons generated with LAMP forward outer or LAMP back outer primers using the ABI Big®Dye version 3.1 protocol. Sequence identity was determined using the BLASTn search tool (NCBI, n.d.).

PRIMER USED	PRODUCT	BLAST RESULT	SIZE	E value
Sequence generated using F3 primer and ABI Big®Dye version 3.1 protocol	Product sent for direct sequencing	100% homology to the *Strongyloides stercoralis* gene for 18S small subunit ribosomal RNA, partial sequence. NCBI accession bank numbers: AB923888.1, KF926660.1 , KF926659.1, KF926658.1 and AB453316.1	172bp	8e-47 to 9e-86
Sequence generated using B3 primer and ABI Big®Dye version 3.1 protocol	Product sent for direct sequencing	77% (158/168bp) homology to *Strongyloides* species. AB923888.1, KF926660.1, KF926659.1, KF926658.1 and AB453316.1	168bp	2e-68 to 7e-67

Further confirmation of the amplified product was performed by sequencing of the cloned insert using the pGEM® T-Easy plasmid vector, a vector with 3' T (thymidine) overhangs, to prevent recircularization of the vector and improve ligation of the target. The direct and pGEM® T-Easy

sequences align in the same region of the 18S rRNA genome. The pGEM® T-Easy sequence showed a 99% sequence match (239/240bp) with the forward outer primer (F3) but no sequence identity was found using the back outer primer (B3). Gel electrophoresis of the *EcoRI* digestion product confirmed the presence of the insert before the PCR reactions (using only T7 or SP6 primers) were used to prepare the product for analysis. The T7 forward primer generated the following sequence:

GGGTAATTTTCGCGCCTGCTGCCTTCCTTGGATGTGGTAGCCGTTTCTCAGGCTCCCTCTCCGGAGTCGAACC
CTAATTCTCCGTTATCCGTCACAACCATGGTAGGCCAATACCCTACCATCGAAAGTTGATAAACCAGATACAC
ATACGGTATGTTTTAGTAACAAAATCAGCGAGGATATTTTGAGTCAACCAATATAAAATGGTTTAATCAAATA
AACGCACCACTAATGTGGAAT (240bp).

This was identified on a BLASTn search as *S .stercoralis* with a sequence match of 99% and with an E value of 8e-123 for a sequence size of 239/240bp.

Future transformation reactions will use cPCR and T7 and SP6 as the forward and back primers as a screening method for multiple colonies. A single colony (proven by cPCR to contain the target insert) is subsequently picked from a purity plate (after overnight incubation at 37^{o}C) and placed into LB broth for a further overnight incubation at 37^{o}C. Multiple colonies can be more easily screened using this method. This has the added benefit of confirmation of the presence of the insert before cloning and enzyme digestion is performed.

A final product sequence identity and alignment on the 18S RNA genome for the results of direct (F3 or B3 primers) or pGEM®T-Easy (T7 primer) sequencing reactions was obtained using the BLASTn and ClustalW2 tools (Larkin *et al*., 2007) and is shown in Appendix 3a. The ClustalW2 sequence alignment illustrated that the LAMP and qPCR assay primers do not target the same region of the 18S rRNA genome.

3.5 SENSITIVITY AND SPECIFICITY OF LAMP

The standards for reporting of diagnostic accuracy guidelines recommends validation of a new diagnostic method using analytical sensitivity and specificity to assess the performance and accuracy of new diagnostic methods (Saah and Hoover, 1997, Bossuyt *et al*., 2015).

3.5.1. ANALYTICAL SENSITIVITY

Analytical sensitivity determines the smallest amount of substance that can be detected in an assay. This differs from diagnostic sensitivity, which is the ability to determine disease in the infected population (Saah and Hoover, 1997). Analytical sensitivity was determined by serial 10-fold (10^{-1} to 10^{-7}) dilution of *S. stercoralis* DNA in nuclease free distilled water. *S. stercoralis* DNA was extracted from an aliquot of stool containing 1 *S. stercoralis* larva/ µl.

Dilutions of the positive control diluted in negative stool slurry were well-mixed before DNA extraction was performed to ensure that the stool sample was as homogenous as possible but the results were not consistent. A consistent comparable result was obtained when DNA was serially diluted after extraction from a spiked stool positive control sample and this was the method chosen for the current study. The dilution series also diluted any stool inhibitors of the LAMP assay that may have been present in the DNA aliquots. Table 3.9 demonstrates the results for 10- fold dilution series of the positive control. The sample size of stool extracted is included as it was assumed that the sample size would have an effect on the LAMP results. However, stepwise logistic regression analysis showed that the sample size of stool extracted did not have an effect on the result of the LAMP assay. The results of the statistical analysis are discussed in more detail in Section 3.9.

Table 3.9 Performance of LAMP using serial 10- fold dilutions of DNA extracted from positive control stool samples (Positive LAMP time in minutes)

DILUTION OF DNA FROM POSITIVE STOOL CONTROL	STOOL SAMPLE EXTRACTED (mg) (ALIQUOTS OF SAMPLE CONTAINING 1 *S STERCORALIS* LARVA/ µl)	LAMP RESULT (TIME IN MINUTES) DETERMINED BY THE DEVELOPMENT OF TURBIDITY USING AN LA 320C TURBIDIMETER
10^{-1} Positive stool control (*10-fold dilutions performed on extracted DNA) extracted 25/01/2015	0.08	26.24
10^{-2}		26.54
10^{-3}		39.06
10^{-1} Positive stool control (*10-fold dilutions performed on extracted DNA) extracted 12/02/2015	0.07	30.30
10^{-2}		33.36
10^{-3}		36.06
10^{-1} Positive stool control (*10-fold dilutions performed on extracted DNA) extracted 12/06/2015	0.07	21.06
10^{-2}		24.18
10^{-3}		28.54

A dilution series was performed on the positive stool samples that were extracted with each Qiagen® Qiasymphony SP run and used to monitor the DNA extraction process. The resulting dilution series was also used as an amplification control in the LAMP assays. Table 3.10 shows the

average results obtained from the dilution series of eight positive stool controls that were used in this study and were positive for dilutions 10^{-1} to 10^{-3}.

Table 3.10 Determination of the analytical sensitivity or limit of detection (LOD) using negative stool slurry spiked with L3 (infectious stage) *S. stercoralis* larvae from a positive stool culture- final concentration: 1 *S. stercoralis* larva/µl.

LAMP	**DILUTION SERIES**							***S. STERCORALIS* NEGATIVE STOOL SAMPLE**
	Neat (1 *S. stercoralis* larva/µl)	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	
Average readings-minutes (n=8)	23.4	25.40	27.37	32.38	Negative	Negative	Negative	Negative

Watts *et al*. (2014), using primers targeted to the 28S rRNA gene, found an analytical sensitivity of 10^{-2} (dilution of 1 larva of *S. ratti*/ 50µl of water diluted 1 in 5 in a known negative stool sample). This study showed an analytical sensitivity of 10^{-3} (with a final dilution of 1 larva *S. stercoralis*/ µl diluted in a known negative stool control sample).

The analytical sensitivity for LAMP assays was determined to be 1 x 10^{-3} *S. stercoralis* larvae/ µl, using the limit of detection of an aliquot containing a known concentration of DNA. The amount of DNA in samples (after *EcoRI* digestion) sent for sequencing, using the pGEM®T-EASY plasmid vector, was measured using a Nanodrop spectrophotometer (Source Thermo Fisher Scientific, UK). The Nanodrop spectrophotometer gave a reading of 117.2 ng at a wavelength of 260nm and one serial 10- fold dilution series was performed in duplicate in a LAMP assay. The limit of detection for the LAMP assay was 117.2 x 10^{-9} ng of DNA. Table 3.11 demonstrates the results obtained from duplicate LAMP assays.

Table 3.11 Determination of the LOD using *EcoRI* digested DNA.

LAMP DILUTION	RESULT LAMP ASSAY RUN 1 (TIME IN MINUTES)	RESULT LAMP ASSAY RUN 2 (TIME IN MINUTES)
Neat (1 *S stercoralis* larva/ µl)	12	12
10^{-1}	13	14
10^{-2}	14	15
10^{-3}	17	18
10^{-4}	19	21
10^{-5}	21	27.06
10^{-6}	22	27.36
10^{-7}	25	30.42
10^{-8}	31	32
10^{-9}	44.00	52.12
10^{-10}	Negative	Negative
10^{-11}	Negative	Negative
10^{-12}	Negative	Negative

The values for the LAMP assay (time in minutes) were determined from the analysis of the justification results to determine true positive results. Where the time is reported in whole numbers a turbidity reading was not reported by the LA 320C turbidometer (Source Eiken, Japan) and justification results were used to determine time (minutes) for a true positive result (Figure 3.3). True positive can be determined by a sharp peak and negative product production is determined by wavy lines in the curves and/ or broad peaks and may be the result of non- specific binding or excess magnesium pyrophosphate (Eiken Chemical Co. Ltd., 2005).

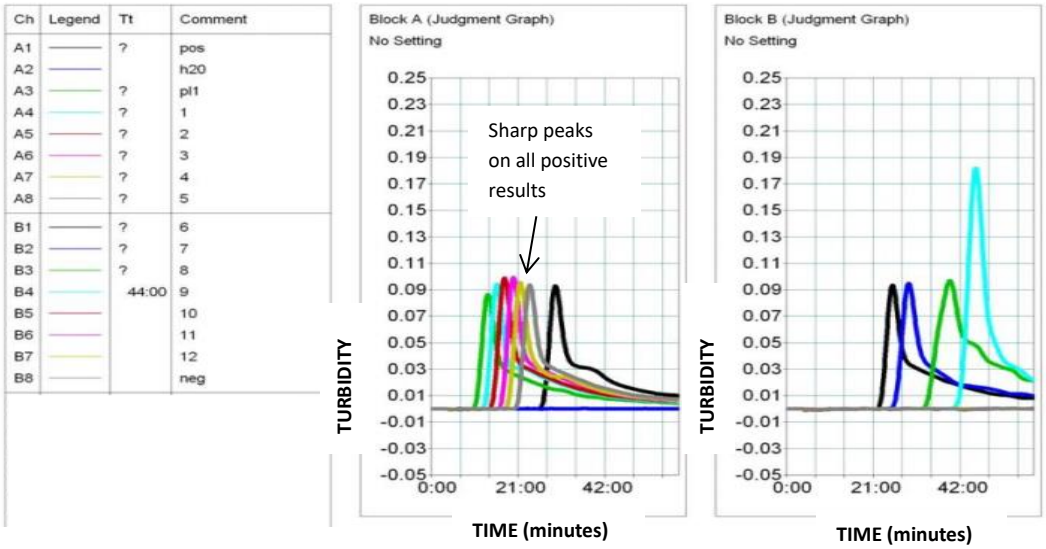


Figure 3.3 Justification results for LAMP assay run 1 to determine true positive results.

A1: Positive stool control 10^{-2} dilution, A2: No template control, A3: LOD sample neat, A4: LOD sample dilution 10^{-1}, A5: LOD sample 10^{-2}, A6: LOD sample 10^{-3}, A7: LOD sample 10^{-4}, A8: LOD sample 10^{-5}, B1: LOD sample 10^{-6}, B2: LOD sample 10^{-7}, B3: LOD sample 10^{-8}, B4: LOD sample 10^{-9}, B5: LOD sample 10^{-10}, B6: LOD sample 10^{-11}, B7: LOD sample 10^{-12}, B8: Negative stool control.

3.5.2. DIAGNOSTIC SENSITIVITY

The determination of diagnostic sensitivity was performed using CRS positive and negative study samples compared with the results of the LAMP assay in a 2x2 table (Jacobson, 1998). Prevalence, which is required for a complete determination of diagnostic sensitivity and specificity, (Baughman *et al*., 2008) is unknown in the patient population attending DCP.

The diagnostic (or clinical) sensitivity was calculated as 23.29% (95% CI: 14.19-34.65%) using the CRS (indicating proven or possible disease) as a comparator. Diagnostic sensitivity was calculated as 46.15% (95% CI: 26.59-66.63%) when using only microscopy and culture results (proven disease) as a comparator. The 95% CI intervals indicate that sensitivity and specificity results are biased (i.e. the interval range is too broad to be significant) and so cannot be interpreted. When using an imperfect reference standard, sensitivity and specificity estimates are biased (Baughman *et al*., 2008) and the FDA recommends using overall percent agreement (OPA) between the current and new tests (FDA, 2007). The positive percent agreement (PPA) for the LAMP assay detection of *S. stercoralis* DNA in clinical samples was determined in this study to be 27.42% with an overall percent agreement (OPA) between current (CRS) and new (LAMP assay) tests of 83.8% (95% CI: 73.5-95.2%). As discussed in Chapter 2, Section 2.8 this result must be interpreted with care as the values indicate only the number of negative or positive agreements between the CRS and the LAMP assay.

3.5.3. ANALYTICAL SPECIFICITY

The ability of an assay to detect only a specific organism/ analyte is termed analytical specificity and is defined as the ability to detect "true" negative samples i.e. those without the disease (Saah and Hoover, 1997). Analytical specificity was determined by the samples in the specificity bank and negative stool control samples. LAMP had 100% specificity as there were no false positive or false negative reactions using the samples in the specificity bank. All known positive *S. stercoralis* samples in the specificity bank using the current stool DNA extraction protocol at DCP were detected. The samples in the specificity bank were processed and analysed using the same protocol that was used for the study samples. The results of the analytical specificity study are shown in Table 3.12.

Table 3.12 LAMP results for viral, bacterial and parasitic human pathogens (total *S. stercoralis* positive samples = 8, total *S. stercoralis* negative samples = 58)

ORGANISM	NUMBER OF ORGANISMS TESTED	LAMP RESULTS (EXTRACTED DNA)
Negative stool	8	Negative (8)
Positive *S. stercoralis* stool control	8	Positive (8)
Rhabditiform free-living larvae NOT *S. stercoralis*	1	Negative (1)
Nematodes	11- *Ascaris lumbricoides* (2), *Trichuris trichiura*(2), hookworm (5), *Trichostrongylus* sp. (1), *Loa loa* (1)	Negative (11)
Trematodes	2- *Dicrocoelium dendriticum* (1), *Schistosoma mansoni*(1)	Negative (2)
Cestodes	2- *Bertiella studeri* (1), *Taenia saginata*(1)	Negative (2)
Intestinal protists/ intracellular fungi	29- *Cryptosporidium* sp. (5), *Cystoisospora belli* (2), *Cyclospora cayetanensis* (1), *Entamoeba histolytica/dispar* (6), *Entamoeba hartmanni* (1), *Entamoeba coli* (1), *Giardia lamblia* (6), *Blastocystis hominis* (1), *Enterocytozoon bienusi* (5), *Encephalitozoon intestinalis* (1)	Negative (29)
Bacteria	4- *Campylobacter jejuni* (1), *Shigella sonnei* (1), *Escherichia coli O157* (1), *Vibrio cholera* (1)	Negative (4)
Viruses	1- Adenovirus (1)	Negative (1)

3.5.4. DIAGNOSTIC SPECIFICITY

Diagnostic specificity was determined using the results of the LAMP assay and the CRS for the study samples in a 2x2 contingency table (Jacobson, 1998).

The specificity was calculated as 99.57% (95% CI: 97.63-99.99%) using the CRS as a comparator and 97.86% (95% CI: 95.39-99.21%) when using microscopy and culture as a comparator. The negative percent agreement (NPA) is used when there is no "gold standard" and was calculated to be 99.55% (FDA, 2007).

3.6. EVALUATION OF THE LAMP ASSAY FOR THE DETECTION OF *S. STERCORALIS* DNA FROM CLINICAL SAMPLES

The sample size for this study was calculated for a comparison of two proportions (McNemar's test) to show a difference of at least 10 in the row and column totals of a 2x2 table, for the *null* hypothesis to be disproven (Jacobson, 1998). Test performance was evaluated using McNemar's test, overall percent agreement and Cohen's kappa co-efficient (MedCalc®, n.d.). The reproducibility and reliability was evaluated on samples that were positive for LAMP and qPCR or negative for LAMP and positive for qPCR. Furthermore a proportion of the negative samples were re-tested with no additional positive samples detected.

With the exception of a failing batch of primers (these results were excluded and the LAMP assay was repeated using a fresh set of primers) only two inconsistent results were obtained. The inconsistent results were generated from template DNA at a dilution of 10^{-1} and these are thought to be due to the presence of very little DNA in the samples (Table 3.13).

Table 3.13 Reproducibility of LAMP assay for the detection of *S. stercoralis* DNA in clinical samples in samples stored at -20^{o}C reported as the results of the LAMP assay (i.e. positive or negative).

LAMP POSITIVE qPCR POSITIVE (LAMP RESULTS)	LAMP POSITIVE qPCR POSITIVE REPEAT LAMP ASSAY (LAMP RESULTS)	LAMP POSITIVE qPCR POSITIVE REPEAT LAMP ASSAY USING DNA AT A DILUTION OF 10^{-1} TO DETERMINE IF ANY INHIBITION OF THE ASSAY IS PRESENT (LAMP RESULTS)	LAMP NEGATIVE qPCR POSITIVE (LAMP RESULTS)	LAMP NEGATIVE qPCR POSITIVE REPEAT LAMP ASSAY (LAMP RESULTS)	LAMP NEGATIVE qPCR POSITIVE REPEAT LAMP ASSAY USING DNA AT A DILUTION OF 10^{-1}TO DETERMINE IF ANY INHIBITION OF THE ASSAY IS PRESENT (LAMP RESULTS)
7 out of 9 samples positive	9 out of 9 samples positive	1 out of 2 samples positive*.	24 out of 24 negative	24 out of 24 negative	12 out of 12 samples negative

*The positive sample was negative in 2 out of 3 LAMP assays. The LAMP time (minutes) was 52.24 indicating that there was very little DNA present in that sample.

The LAMP assay in these samples was tested at a dilution of 10^{-1} as the qPCR assay indicated that possible inhibitors were present in these samples. Any samples that were found by qPCR (Chapter 4) to have possible inhibitors present (internal control C_t raised above the run mean + 2SD) were diluted 10^{-1} and a repeat LAMP and qPCR assay was performed. Low levels of DNA in faecal aliquots may result in false negative results in the LAMP assay (Morrison *et al*., 1998) and a determination of the effect of stool inhibition on low levels of DNA was investigated and discussed in Section 3.7.

All of the samples that were LAMP assay positive also tested positive by qPCR (Chapter 4). Analysis of the results of the gel electrophoresis of the qPCR products showed that no samples with anomalous bands on qPCR were positive for *S. stercoralis* DNA in the LAMP assay (Table 3.14).

Table 3.14: Results of the LAMP assay positive or negative compared with qPCR assay positive and CRS (microscopy, culture and serology) results in samples stored at -20°C. Total number of samples n=284.

NUMBER OF SAMPLES	REFERENCE STD RESULT (CRS)	LAMP ASSAY RESULT- NEGATIVE OR POSITIVE RANGE (minutes)	qPCR ASSSAY POSITIVE RANGE (C_t)	NUMBER OF ANOMALOUS BANDS ON A 2% AGAROSE GEL FOR qPCR PRODUCT
1	CRS negative	40.12 (n=1)	27.15	0
7	Microscopy/ culture positive	20-52.24 (n=7)	19.5-27.49	0
4	Serology positive	27.24-58.42 (n=4)	24.06-26.42	0
6	Full CRS positive	24.06-53 (n=6)	14.98-26.78	0
18	CRS negative	Negative (n= 18)	27.49-35.7	5*
2	Microscopy/ culture positive	Negative (n= 2)	27.49-31.84	0
5	Serology positive	Negative (n= 5)	31.29-40.05	0
2	Full CRS positive	Negative (n= 2)	28.47-33.89	0

*Repeat qPCR assays generated 2 negative results (Original qPCR C_ts 38.15-39.5). Final total 3 anomalous bands detected by qPCR in this study.

In this study the LAMP assay failed to detect *S. stercoralis* DNA in a number of qPCR positive samples (where C_t was >31.46). All qPCR assay positive and LAMP assay negative results were repeated and no new positive LAMP samples were detected. The cut-off for detection of *S. stercoralis* DNA by the LAMP assay was a qPCR C_t of ≤31.46 (in samples stored at 4°C or -20°C) also indicating that low concentrations of *S. stercoralis* DNA will not be detected by the LAMP assay (Table 3.15). Clinical samples are not homogenous samples and small aliquots taken from clinical samples for analysis may not necessarily detect the parasite (Monteiro *et al*., 1997). Also very little DNA present in samples for molecular analysis may not be detected (Morrison *et al*., 1998). The target site for the 18S rRNA genome was found to be different for the LAMP and qPCR assays (Appendix 3a) and this may have contributed to the less sensitive detection of *S. stercoralis* DNA with the LAMP assay.

Table 3.15: Maximum, minimum, mean and standard deviation values for the LAMP (time in minutes) and qPCR (C_t) assays. Samples stored at 4°C or -20°C.

	MINIMUM	MAXIMUM	MEAN	STANDARD DEVIATION
qPCR (C_t)	14.98 (LAMP= 29.54)	31.46 (LAMP= 49.36)	31.49	6.75
LAMP (minutes)	23.36 (qPCR C_t = 20.73)	58.42 (qPCR C_t = 26.42)	36.71	9.65

While this determines that there is very little improvement over current methods for proven disease available in the field (microscopy and culture combined), the decrease in time taken to diagnose cases of strongyloidiasis (LAMP= one hour, microscopy with a sensitivity of up to 50% = one hour but may contain infectious L3 larvae in cases of hyperinfection and culture with a sensitivity of up to 70% = 7 to 10 days, may contain infectious L3 larvae) makes this a useful assay for deployment to endemic areas. In addition, this has the added benefit of limiting laboratory staff to exposure of infectious L3 larvae that may be abundant in culture techniques.

Precision between the LAMP amplification runs was tested from the results generated by the positive control (at a dilution of 10^{-2}) in amplification runs using the study protocol. A standard curve of the positive control DNA at dilutions of 10^{-1}, 10^{-2} and 10^{-3} was used during optimisation runs for the LAMP assay and so could not be used to calculate percentage coefficient of variation (% CV). The % CV for the 10^{-2} dilution of the positive control was 9.7% over 16 consecutive amplification runs. The % CV should be <15% between runs and <10% within runs (Reed *et al*., 2002, Salimetrics®, n.d.) and the LAMP assay was shown to have an acceptable level of precision (9.7% between runs) for a diagnostic assay.

3.7 PERSISTENCE OF *S. STERCORALIS* DNA AT STORAGE TEMPERATURES OF 4°C AND -20°C

As previously discussed (Section 3.3) faecal samples for *S. stercoralis* molecular testing should be stored at -20°C before DNA extraction if no preservative (e.g equal volume of ethanol) is used. The LAMP assay was assessed for the persistence of DNA with short-term storage at 4°C without a preservative as this would impact the routine workflow at DCP. To investigate the decreased sensitivity in the detection of *S. stercoralis* in samples stored at 4°C and -20°C a pilot study was set up to determine the short-term persistence of DNA in samples stored at 4°C or -20°C. This was performed to confirm that *S. stercoralis* DNA in clinical sample aliquots could safely be stored at 4°C for a short-term without a decrease in sensitivity in the detection of *S. stercoralis* DNA.

Table 3.16 shows the results obtained using a positive control stool sample stored at 4°C or -20°C and tested at various time intervals. A positive control stool sample containing 0.1 *S. stercoralis* DNA/ µl was used in this study so that a drop in efficiency of the LAMP assay could be determined within the pilot study time- frame. The DNA was extracted using the Qiagen® Qiasymphony SP and the extracted DNA was stored at -20°C until a LAMP assay could be performed. The DNA was diluted in a 10- fold dilution series and *S. stercoralis* DNA was detected in the dilution range 10^{-1} to 10^{-4}.

Table 3.16: Results of the survival study at storage temperatures of 4°C and -20°C

DATE PLACED IN STORAGE	EXTRACTED BY QIAGEN® QIASYMPHONY SP	DATE TESTED BY LAMP ASSAY	4°C NEGATIVE STOOL CONTROL	4°C POSITIVE STOOL CONTROL	-20°C NEGATIVE STOOL CONTROL	-20°C POSITIVE STOOL CONTROL
			LAMP ASSAY	LAMP ASSAY (final dilution positive)	LAMP ASSAY	LAMP ASSAY (final dilution positive)
29-12-2016	Week 1	15-04-2017	Negative	Neat	Negative	10^{-4}
	Week 2	15-04-2017	Negative	Negative	Negative	10^{-3}
	Week 3	15-04-2017	Negative	Negative	Negative	10^{-3}
	Week 4	15-04-2017	Negative	Negative	Negative	10^{-3}
	Week 5	15-04-2017	Negative	Negative	Negative	10^{-3}
	Week 8	17-04-2017	Negative	10^{-1}	Negative	10^{-3}
	Week 12	17-04-2017	Negative	10^{-1}	Negative	10^{-2}

The results from the DNA persistence pilot study demonstrated that storage at 4°C was not recommended, even for short-term storage, and samples requiring a LAMP assay for detection of *S. stercoralis* DNA in clinical samples must be stored at -20°C.

Discrepant results demonstrated in the LAMP assay for some of the 10^{-1} dilutions of aliquots that had been stored at 4°C before DNA extraction was performed, may be attributed to poor quality DNA (the DNA was extracted on separate Qiagen® Qiasymphony SP runs) or very small amounts of DNA present in the sample when DNA may not always be detected in each assay (Morrison *et al*., 1998). These results do not alter the conclusion reached from the pilot study that storage at 4°C is sub- optimal for the detection of *S. stercoralis* DNA in the LAMP assay. Storage at -20°C showed some deterioration of the stored DNA and samples requiring a LAMP assay for the detection of *S. stercoralis* DNA must be tested as soon as possible.

While this is not difficult to arrange in busy routine well-resourced laboratories this must be considered when choosing an assay for epidemiological monitoring and control studies as it may not be possible to test the samples in the field in a timely manner. It may also not be possible to store the samples at -20°C in resource- limited areas. Further work is required to determine if storage in DNA preservative or on FTA cards (Source Sigma Aldridge) would eliminate the effect of sub- optimal storage. FTA cards contain chemicals to lyse cells, denature proteins and protect nucleic acids from damage by nucleases, oxidation or UV damage (Mullen *et al*., 2009) and may improve the detection of *S. stercoralis* DNA in the LAMP assay.

3.8 INVESTIGATION OF METHODS FOR DNA EXTRACTION THAT ARE SUITABLE FOR USE WITH LAMP ASSAYS IN RESOURCE-LIMITED AREAS

Automated DNA extraction is expensive and requires complex technology and high technical expertise and, as such, is not suitable for use in resource- limited areas. The LAMP assay is reported to be more resistant to inhibition than the qPCR assay (Notomi, 2000) and so manual DNA extraction methods were examined for the suitability of LAMP assay deployment in resource-limited areas. Manual methods of DNA extraction for use in the LAMP assay to detect blood parasites have been described that are suitable for use in resource- limited areas (FIND, 2012, Lucchi *et al*., 2016, Perera *et al*., 2017).

Strongyloides stercoralis has a thick cuticle that may inhibit successful DNA extraction using methods that do not lyse the parasite (Moghaddassani *et al*., 2011, Levenhagen and Costa Cruz, 2014). Gasser *et al*. (1993) demonstrated a reduced yield of DNA from *Trichostrongylus* sp. worms due to the thick cuticle, nevertheless sufficient DNA was extracted using a homogenisation method. The pilot study, therefore, included pre- treatment methods to determine whether lysis of the thick cuticle of *S. stercoralis* would improve the manual extraction of DNA from stool samples.

Aliquots of negative and positive stool control samples were stored at 4^{o}C and -20^{o}C until DNA extraction was performed using the PURE® method (Source Eiken, Japan), the “boil and spin” method (FIND, 2012) or the Qiagen® Qiasymphony SP automated DNA extraction method.

Using the magnetic bead resonance DNA extraction method, in this study, purified DNA was extracted that was suitable for use in the LAMP assay. To investigate manual methods of DNA extraction, 60 µl of positive control stool samples (containing 1 *S. stercoralis* larvae/ µl) and negative control stool samples, stored at -20^{o}C were extracted by the manual method with pre-treatment of the sample before manual DNA extraction by one of the following methods:

I. Incubation with a 1 in 2 dilution of the Qiagen® tissue lysis buffer (ATL) containing a 1 in 50 dilution of proteinase k (Source Thermo Fisher Scientific, UK) at 56^{o}C for 2 hours;
II. Incubation with a 1 in 2 dilution of the Qiagen® tissue lysis buffer (ATL) containing a 1 in 50 dilution of proteinase k (Source Thermo Fisher Scientific, UK) at 56^{o}C overnight;
III. Extreme freeze-thaw in liquid nitrogen for 5 minutes.

The positive stool control was also analysed without pre- treatment:

I. Neat;
II. At a dilution of 1 in 2 in nuclease free water, for the manual PURE® DNA extraction methods.

A parallel automated DNA extraction was performed on the Qiagen® Qiasymphony SP (the comparison control) (Section 2.3.8.3). Negative stool controls were extracted using the same conditions (FIND, 2012, Perera *et al*., 2017).

In this study the aliquots were diluted in a 10- fold dilution series in nuclease free water and stored at -20^{o}C until the LAMP assay could be performed using the study protocol.

Inhibition (shown in red) was determined by comparison of the LAMP time of the DNA extracted by the automated method and the LAMP time for the different treatment methods for the DNA extracted by the manual method. Where the LAMP time for the manual extraction method was greater than the LAMP time for the automated DNA extraction method plus a 1.96 standard deviation, sample inhibition of the LAMP assay was suspected. Where the standard deviation could not be calculated (too few results) sample inhibition of the LAMP assay was suspected when the LAMP time for the manual DNA extraction method was more than 3 minutes greater than the LAMP time for the automated DNA extraction method.

Table 3.17 shows the results obtained using negative and positive stool controls (containing 1 *S. stercoralis* larvae/ µl). The positive stool sample DNA was diluted in a 10- fold dilution series and the LAMP assay was performed using the study protocol.

Table 3.17: Comparison of LAMP times for the manual DNA extraction method- PURE® technology (Source Eiken, Japan) and the automated Qiagen® Qiasymphony SP DNA extraction method. Results in red indicate possible inhibition of the assay. Underlined results indicate that DNA degradation may have occurred as a result of the extreme temperature pre- treatment method.

SAMPLES	ATL PLUS PROTEINASE K INCUBATED 2 HOURS AT 56°C HEATED AT 75°C 15 MINUTES 60µl	ATL PLUS PROTEINASE K INCUBATED OVERNIGHT AT 56°C HEATED AT 75°C 15 MINUTES 60µl	FREEZE-THAW IN LIQUID NITROGEN HEATED AT 75°C 15 MINUTES 60µl	FREEZE-THAW IN LIQUID NITROGEN HEATED AT 75°C 30 MINUTES 60µl	DIRECT MANUAL DNA EXTRACTION HEATED AT 75°C 15 MINUTES 60µl	DIRECT MANUAL DNA EXTRACTION HEATED AT 75°C 30 MINUTES 60µl	DIRECT MANUAL DNA EXTRACTION (SAMPLE DILUTED 1 IN 2 IN PBS PH 7.2 HEATED AT 75°C 15 MINUTES 60µl	AUTOMATED DNA EXTRACTION (COMPARISON CONTROL)
Negative stool	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Positive stool control (neat)	Negative	Negative	Negative	Negative	Negative	Negative	Negative	31.18
Positive stool control 10^{-1}	Did not reach threshold	Did not reach threshold	38.54	35.36	40.18	34.36	39.30	33.18
Positive stool control 10^{-2}	26.3	31.24	32.3	28.06	35.42	43.48	44.3	42.42
Positive stool control 10^{-3}	34.24	37.3	Negative	Negative	47.24	53.06	Negative	42.42
Positive stool control 10^{-4}	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative

The results demonstrated that inhibition of the LAMP assay occurred when large sample volumes were used, as shown by the reduced numbers of samples showing inhibition in the LAMP assay when the samples were diluted. Extreme temperature (freeze- thaw in liquid nitrogen) pre-treatment of samples was unsuitable for the extraction of *S. stercoralis* DNA using PURE® technology. Pre- treatment of the stool samples with ATL plus proteinase k incubated at 56°C (overnight or 2 hours) improved the detection of *S. stercoralis* DNA. This may be due to improved

lysis of the larval cuticle recommended by Moghaddassani *et al*. (2011) and Levenhagen and Costa Cruz (2014) for the DNA extraction of whole parasites.

These results indicated that DNA manual extraction may be performed on neat stool samples with ATL and proteinase k pre-treatment but further modification of the method is required as assay inhibition was demonstrated when using large sample volumes.

Therefore, a further study was performed to determine the effect of the stool loading sample volume on the manual DNA extraction method. Negative and positive stool control samples, without pre-treatment, were extracted using decreasing volumes of stool loading sample: 100, 80, 50, 20 and 10 µl. These results generated by the LAMP assay are shown in Table 3.18.

Table 3.18: Effect of inhibition on the LAMP assay using the PURE® manual DNA extraction method. Results in red indicate possible inhibition of the LAMP assay. All negative stool control samples were negative in the LAMP assay.

LAMP ASSAY (POSITIVE RESULTS IN MINUTES)	100 µl	80 µl	50 µl	20 µl	10 µl	AUTOMATED DNA EXTRACTION (COMPARISON CONTROL)
Positive stool control (neat)	Negative	Negative	Negative	Negative	30.24	25
Positive stool control 10^{-1}	Negative	32.18	26.12	26	28.3	23.2
Positive stool control 10^{-2}	29.42	28	26.48	26.42	24.36	24.36
Positive stool control 10^{-3}	28.36	25	26.36	24	27.06	24.36

The determination of inhibition of the LAMP assay is reported in red, using the same criteria for the determination of inhibition as previously described. A loading volume of 10 µl detected *S. stercoralis* DNA (extracted from an aliquot containing 1 *S. stercoralis* larva/ µl) in the neat DNA and the DNA dilutions 10^{-1} to 10^{-3}.

A loading volume of 10 µl detected *S. stercoralis* DNA in the neat DNA and the DNA dilutions 10^{-1} to 10^{-3} demonstrating that a smaller loading sample volume is required for this technique. Inhibition was demonstrated in the 10^{-3} dilution for this volume. This may be due to the fact that 10 µl only contains a small amount of DNA template and this will affect the sensitivity of detection of *S. stercoralis* DNA in the LAMP assay (Morrison *et al.*, 1998). There is a corresponding decrease in sensitivity of the LAMP assay when small loading volumes are used. This study concluded that, in

the present format, PURE® DNA extraction on untreated stool samples was unsuitable for the LAMP assay.

A simpler method ("boil and spin") requiring only a vortex, a centrifuge, a stable power supply and heating at 95°C was also assessed for efficiency of DNA extraction for use in the LAMP assay. This was previously described for the malaria LAMP assay for detection of protist parasites in blood (Polley *et al*., 2013). The results of the "boil and spin" method investigated for the suitability of use in extracting DNA in endemic areas are shown in Table 3.19.

Table 3.19: Effect of inhibition on the LAMP assay using the "boil and spin" manual DNA extraction method. Results in red indicate possible inhibition of the LAMP assay. (* indicates small amount of DNA template)

LAMP ASSAY (POSITIVE RESULTS IN MINUTES)	100 µl	80 µl	50 µl	25 µl	10 µl	AUTOMATED DNA EXTRACTION (COMPARISON CONTROL)
Negative	Negative	Negative	Negative	Negative	Negative	Negative
Positive stool control (neat)	Negative	Negative	Negative	42.5	Negative	27.0
Positive stool control 10^{-1}	41.12	33.06	34.42	34.24	34.24	32.18
Positive stool control 10^{-2}	36.0	42.3	35.0	35.0	41.18*	34.0
Positive stool control 10^{-3}	32.0	Negative	33.0	43.0*	42.0*	38.12

Positive (containing 1 *S. stercoralis* larva/ µl) and negative stool controls were emulsified in SDS buffer (Section 2.3.8.4) before DNA extraction was performed. Inhibition of the assay was calculated using the same criteria previously mentioned. Inhibition is shown in red in Table 3.19.

The results demonstrated that a loading volume of 25 µl was optimal for this manual DNA extraction method. A smaller loading sample of 10 µl showed possible inhibition of the LAMP assay when compared to the comparison control (DNA extraction using the automated Qiagen® Qiasymphony SP). The loading sample is small and the increased LAMP time may be due to the fact that very little *S. stercoralis* DNA template was present in this sample (denoted by * in Table 3.19). Nevertheless, this method for extraction of DNA for use in the LAMP assay shows promise for use in resource- limited areas after further optimisation of the stool sample storage conditions has been completed. The SDS extraction buffer is a relatively inexpensive reagent and is easy to prepare and store at room temperature making this a suitable reagent to use in resource- limited areas.

The LAMP assay detected *S. stercoralis* DNA extracted using the manual or automated methods investigated in this study (Figure 3.4)

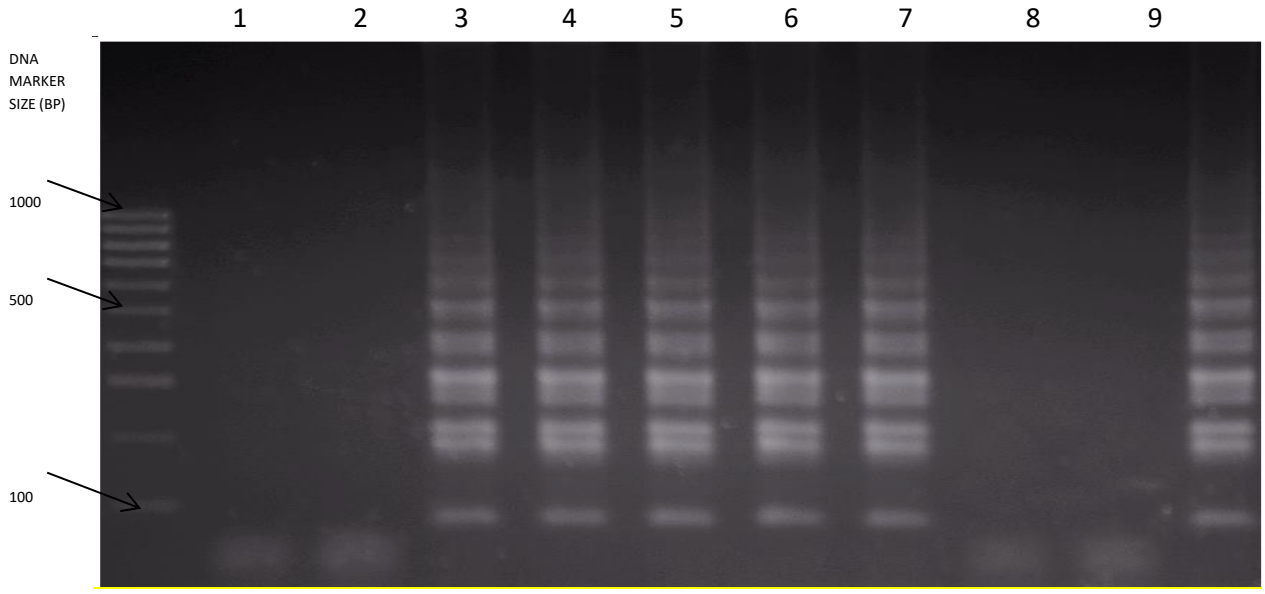


Figure 3.4: Gel electrophoresis of LAMP assay products (positive study samples, positive stool control PURE® manual DNA extraction method and the automated Qiagen® Qiasymphony SP DNA extraction method). Lane 1: negative stool control, Lane 2: no template control, Lanes 3, 4: study samples, Lanes 5, 6: PURE® DNA (manual) dilutions 10^{-1} (Lane 5) and 10^{-2} (Lane 6), Lane 7: Qiagen® Qiasymphony SP DNA (automated) dilution 10^{-2}, Lanes 8, 9: negative study samples.

The LAMP assay also detected DNA extracted directly from cultured larvae. The data is not shown as optimisation of the assay was performed on stool samples spiked with *S. stercoralis* larvae.

3.9 STATISTICAL ANALYSIS OF LAMP

287 stool samples that had been stored at 4°C and -20°C before DNA extraction were tested by the LAMP assay. Only one sample per patient was included in the statistical analysis for this study. Duplicate samples were excluded using the criterion that statistical analysis was performed using the first sample only (the diagnostic sample). The results of the repeat samples could not be determined to be due to failed treatment, active disease or persistent antibody (serology result as part of the CRS) and so were removed from the analysis of the data. The microscopy and culture assays in the CRS were performed from the same sample that was sent for storage at 4°C or -20°C. Standard McNemar's test for the comparison of proportions and diagnostic sensitivity and

specificity calculations were carried out (MedCalc®, n.d.) and the results are recorded in Table 3.20. The sensitivity results were poor for the LAMP assay as an imperfect reference standard was used that denoted proven disease or probable disease and was subject to sensitivity and specificity bias (Baughman *et al*., 2008). The overall percent agreement between the CRS and the new test when using an imperfect reference standard was used (FDA, 2007). This is shown in Table 3.20.

Table 3.20: McNemar's test and 2x2 contingency table results for the LAMP assay and overall percent positive results (including 95% CI) for significance of results

STATISTICAL TEST		**CRS AND LAMP -20°C**		**MICRO/CULTURE AND LAMP -20°C**	
			95%CI		**95%CI**
McNemar's test	**Difference**	9.06%	6.07-9.76%	2.61%	-0.96-4.98%
	Probability	p=<0.0001		p=0.1153	
Diagnostic test 2x2 table	**Sensitivity**	23.29%	14.19-34.65%	46.15%	26.59-66.63%
	Specificity	99.57%	97.63-99.99%	97.86%	95.39-99.21%
	AUC	0.61	0.56-0.67	0.72	0.67-0.77
	Positive likelihood ratio	54.26	7.35-400.78	21.54	8.81-52.65
	Negative likelihood ratio	0.77	0.68-0.87	0.55	0.39-0.79
	Disease Prevalence (from CRS results)	23.86%	19.19-29.04%	8.50%	5.63-12.2%
	PPV	94.44%	72.71-84.96%	66.67%	40.99-86.66%
	NPV	80.56%	75.51-84.96%	95.14%	91.98-97.32%
FDA recommendation when using an imperfect reference standard (FDA, 2007)	**Positive percent agreement PPA (instead of sensitivity)**	27.4%	-	-	-
	Negative percent agreement NPA (instead of specificity)	99.6%	-	-	-
	Overall percent agreement OPA (between CRS and new test)	83.8%	73.5-95.2%	-	-

McNemar's test showed a significant difference between the CRS and the LAMP assay, but no significant difference was shown when using microscopy/ culture as a comparator. The sensitivity

calculations were not valid as the 95% CI was too large to demonstrate a significant value. The 95% CI for specificity between 97.63-99.99% (using the CRS as a comparator) and 95.39-99.21% (using microscopy/ culture as a comparator) and an NPA of 99.6% indicate that the LAMP assay is a useful diagnostic test. This indicates that there is a high probability that disease is present when a positive result is found. The area under the ROC curve (AUC) demonstrated that the LAMP assay is less sensitive than the CRS or serology but is a fair test for the diagnosis of strongyloidiasis. The OPA confirms that there is a good overall percent agreement between the CRS and the LAMP assay.

Further statistical analysis was carried out on those samples where the travel history was known (Asia n= 73 and Africa n=73). Analysis could not be performed in those with a known travel history to other geographical areas (e.g. Mediterranean) as the numbers were too low for accurate analysis. A comparison of LAMP and the CRS or LAMP and microscopy/ culture positive only for samples with a travel history in Asia (Table 3.21) or Africa (Table 3.22) demonstrated the effect of serological results on the comparison of the usefulness of the LAMP assay when using an imperfect composite reference standard.

Table 3.21: Intraclass correlation of: LAMP and microscopy/ culture positive only or LAMP and CRS in samples with a travel history to Asia.

	LAMP *VS.* MICROSCOPY/ CULTURE POSITIVE		**LAMP *VS.* CRS**	
		95% CI		**95% CI**
Single measures (degree of consistency among measurements)	0.52	0.413-0.68	0.29	0.144-0.441
Average measures (Reliability of averages of kappa ratings)	0.793	0.68-0.87	0.55	0.335-0.703

Table 3.22: Intraclass correlation of: LAMP and microscopy/ culture positive only or LAMP and CRS in samples with a travel history to Africa.

	LAMP *VS*. MICROSCOPY/ CULTURE POSITIVE		**LAMP *VS*. CRS**	
		95% CI		**95% CI**
Single measures (degree of consistency among measurements)	0.416	0.258-0.570	-0.0104	-0.131-0.138
Average measures (Reliability of averages of kappa ratings)	0.681	0.511-0.799	-0.03186	-0.531-0.324

In Tables 3.21 and 3.22 the effect of removal of the serological result alters the interpretation of the usefulness of LAMP as an effective screening tool from good to excellent (Table 3.21) and poor to good (Table 3.22) (Cicchetti, 1994). Serology is a screening diagnostic test used in the current repertoire at DCP and this, along with the percentage of positives detected by both the LAMP assay and serology, demonstrates the unsuitability of LAMP as a replacement screening test at DCP. LAMP has a low PPA and cannot rule out the presence of disease in all cases, the false negative rate is not suitable for first- line diagnostic screening for disease in a condition where severe disease or even death may occur in immunocompromised patients (Pewsner *et al*., 2004, Pottie *et al*., 2011). As demonstrated in Table 3.23 the percentage of positive LAMP reactions in samples stored at -20^{o}C was 6.7 % and that for serology was 26 %.

Table 3.23: Percentage positive of the total number of samples detected by LAMP, CRS, serology only or microscopy/ culture. (Data obtained for samples stored at -20^{o}C).

ASSAY	**PERCENTAGE POSITIVE**
CRS % positive	27%
Microscopy % positive	4.80%
Culture % positive	3.90%
Serology % positive	26%
LAMP assay % positive	6.70%

While some of the serology positive results may be due to the persistence of antibody there may well be serology positive samples that indicate active disease.

The consequences in immunocompromised patients of a missed diagnosis (Pottie *et al*., 2011), necessitates the retention of serology as a diagnostic test. Serology is difficult to interpret as a positive result reflects past or current disease and may indicate persistent antibodies after successful treatment. A negative result may indicate no disease, early disease or a compromised immune response. WHO (2010) for this reason recommends a range of tests for the diagnosis and monitoring of *S. stercoralis* infections.

Microscopy positive samples did not necessarily have cultures performed and this is shown in the reduced percentage positive result obtained for the *Strongyloides* cultures, even though culture is a more sensitive method than microscopy for the detection of *S. stercoralis*. Statistical analysis was therefore, in all data analysis divided into two parts: (I) full CRS and (II) microscopy/ culture positive only.

The purpose of this chapter was to ascertain the analytical sensitivity and specificity of a novel LAMP assay in order to assess the suitability for diagnostic assay of strongyloidiasis in human faecal samples in both resource- limited and well-resourced settings.

This study concluded that the LAMP assay demonstrated a high probability of disease when the LAMP assay is positive. The LAMP assay, which is superior to microscopy and culture (Table 3.23) for the detection of strongyloidiasis (in samples stored at -20^{o}C) cannot replace serology in this study, although the LAMP assay may be useful as an additional test to determine disease when current routine tests are negative in those patients that are at risk of severe disease.

The LAMP assay in the current format shows a limited use in the high- throughput diagnosis of strongyloidiasis in well- resourced settings, but it has the potential to be useful in point of care testing for urgent results (Mori and Notomi, 2009). The LAMP assay also has the potential to be useful in resource- limited areas once the appropriate sample storage conditions have been determined. The LAMP assay has been developed for the detection of other parasitic diseases in blood (Thekisoe *et al*., 2010, Polley *et al*., 2013, Perera *et al*., 2017) and this study will provide a platform for the development of LAMP assays for the detection of stool parasites.

Further development is, therefore, indicated to determine the protocol that will allow the sensitivity (10^{-3}) for the detection of *S. stercoralis* DNA in clinical samples to approach the limit of detection sensitivity (117.2 x 10^{-9} ng DNA) seen in the detection of *S. stercoralis* target DNA in samples with a known concentration of purified DNA. This is discussed further in Section 6.2.

While this study was focussed on the evaluation and validation of a diagnostic test for the detection of a parasitic disease in humans, LAMP assays have been described for the detection of parasites in

veterinary medicine (Savan *et al*., 2005, Jaleta *et al*., 2017). Parasitic infection in production animals carries an increased financial cost to the producers of reduced productivity, increased treatment costs and loss of livestock and is responsible for a reduced quality of life for the animals (Perry and Randolph, 1999).The outcomes from this study may be used to develop further assays that will provide simple and cost effective diagnostic assays that may be used in the diagnosis and management of parasitic diseases in animals in resource- limited or well- resourced areas (Jaleta *et al*., 2017). This is discussed further in Chapter 6.

CHAPTER 4: EVALUATION AND VALIDATION OF REAL-TIME PCR (qPCR)

4.1. INTRODUCTION

Basuni *et al*. (2011) described a multiplex real- time PCR for the detection of *Ancylostoma* sp., *Necator americanus*, *A. lumbricoides* and *S. stercoralis* with detection of low levels of DNA in faecal samples in microscopy negative samples. This indicated that a real- time PCR would be a suitable candidate for the detection of *S. stercoralis* DNA in faecal samples at DCP.

Verweij *et al*., 2009 published a method for the detection of *S. stercoralis* DNA using a qPCR assay targeting the 18S rRNA genome. The qPCR assay uses specific primers and a fluorescent double-labelled internal probe to detect target amplicons that can be analysed and possibly quantitated. This reaction can be developed for a multiplex format (using multiple primers and probes in a single reaction mix) (Basuni *et al*., 2011, Verweij and Stensvold, 2014). A multiplex real-time PCR has also been developed to detect *Ancylostoma duodenale*, *Necator americanus* and *Oesophagostomum bifurcum* by Verweij *et al*. (2006) demonstrating that real-time PCR detection of *S. stercoralis* may be used in a high- throughput multiplex format (Basuni *et al*., 2011). Basuni *et al*. (2011) found a limit for the number of target sites that can be detected in a multiplex format with a decrease in sensitivity of the assay if five target sites were used. As described above by Verweij *et al*. (2006), the qPCR assay is useful for detecting parasite DNA for up to three target sites with the current technology available at DCP.

The product is detected when the amplification fluorescent intensity rises above the background fluorescence intensity, the threshold level can be determined by the user, and is termed the cycling threshold (C_t). Detection of DNA starts during the exponential phase (Figure 4.1) (Wong and Medrano, 2005).

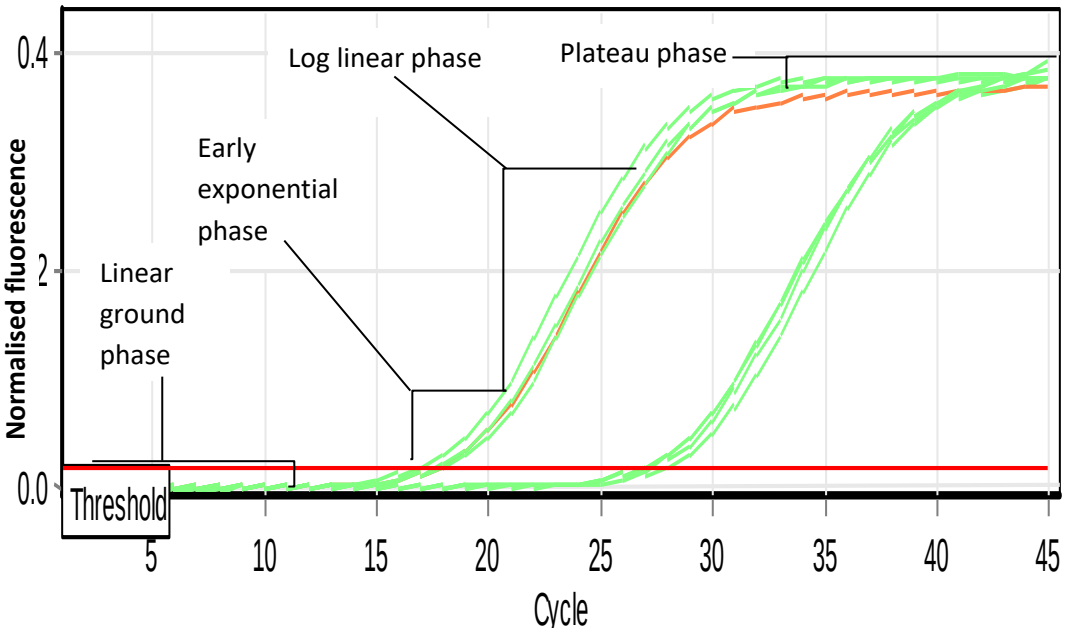


Figure 4.1: Real-time exponential amplification curve

There are a variety of detection methods for qPCR:

I. Melting curve analysis (MC) can only be performed when the fluorophore remains bound to the amplified DNA. The level of fluorescence with SYBER®Green fluorophore increases when bound to double-stranded DNA (dsDNA). Applying a melting curve to the amplified product immediately after the thermal cycling profile will generate a melting curve for single-stranded DNA that is specific to the amplicon and can distinguish primer dimers and contaminating nucleotides from amplified DNA. It can also be used to detect point mutations when a high resolution melting curve is analysed. (Life Technologies Corporation, 2012)

II. Attachment of a short specific probe to the amplicon causes the fluorescent dye to be separated from a fluorescent quencher allowing amplification of specific DNA to be detected (Wong and Medrano, 2005).

III. Self-quenching labelled primers, where the secondary structure of the primers reduces fluorescence to a minimum until the primer has bound to target DNA. This process is not independent of primer binding so gel electrophoresis is required to ensure that a single product has been produced (Wong and Medrano, 2005). Refer to Figure 4.2 for a simplified guide to DNA detection methods.

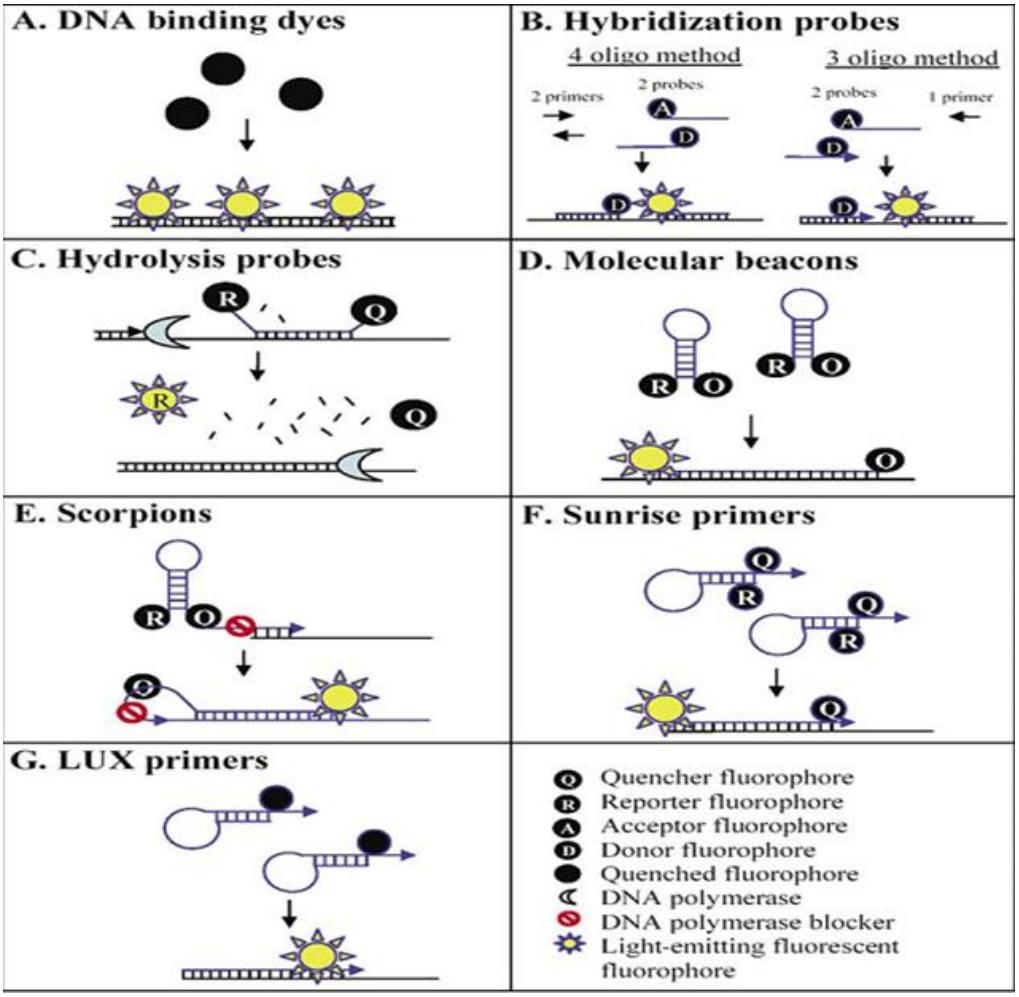


Figure 4.2: Detection of amplicons produced by real-time PCR using DNA binding dyes (A), short specific probes that bind to the amplicon and release fluorescence by hydrolysis or inactivation of a fluorescent quencher (B,C,D,E,F), self-quenched labelled primers, does not require a quencher, but does require gel electrophoresis to ensure a single product has been amplified (G). (Source: Image from Wong and Medrano, 2005).

Once the thermal cycling protocol was completed statistical analysis of the run was performed using an analysis package supplied by the manufacturer (Figure 4.3).

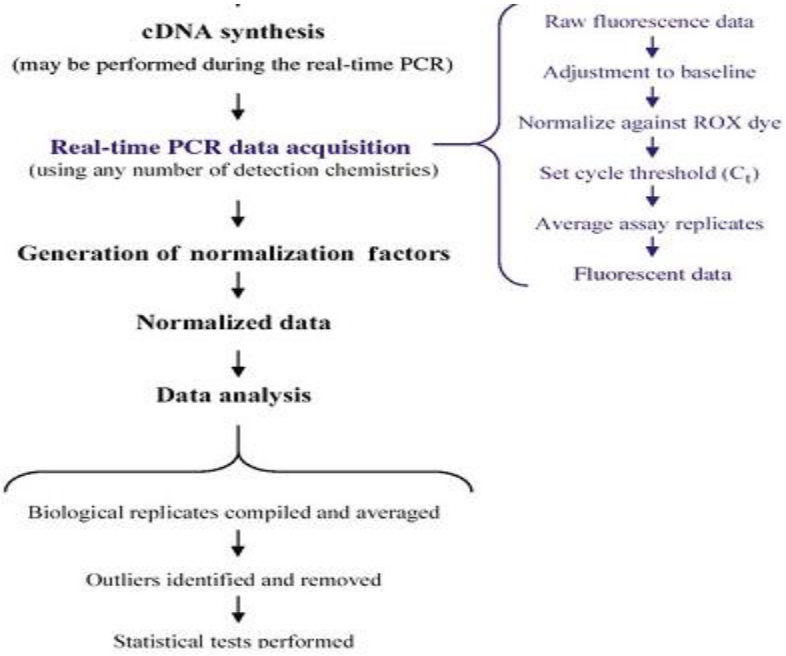


Figure 4.3: Generalised real-time PCR protocol (Wong and Medrano, 2005)

Generally accepted figures for C_ts during analysis of the qPCR assay run are that C_ts < 29 are strong positive reactions indicating large amounts of target DNA in the sample; C_ts of 30-37 are indicative of moderate amounts of target DNA in the sample and C_ts between 38-40 indicate minimal amounts of target DNA in the sample or environmental contamination (Caraguel *et al*., 2011).

The use of an internal probe is expensive (Verweij and Stensvold, 2014). High resolution melting curve analysis (HRMC) can also be performed but an internal control cannot be used as it is limited by the number of fluorescent channels available (Wittwer *et al*., 2003). Two reactions are performed for melt curve analysis (MC) one reaction using the specific target primers and another reaction using the extraction and amplification control (*gfp*) primers. HRMC cannot be performed on a Rotagene 3000 (available at DCP) but a melting curve analysis (MC) to determine the potential use of melting curves was performed at DCP. A pilot study was carried out to determine the potential of MC analysis to be used to detect *S. stercoralis* amplicons in an MC format for information only. This technique was not chosen for this study as the standards for microbial investigations guidelines (HPA UK protocols, 2013) strongly recommend the use of an internal control to monitor the process from extraction to amplification in the UK. However, MC and HRMC (Wittwer *et al*., 2003) may be useful in future studies to detect geographical differences in *S. stercoralis* strains or possible treatment- resistant strains and the results demonstrated that this method has the potential for use in epidemiological and resistance monitoring studies.

Optimisation of the method and the reaction mix was performed for the evaluation and validation of this assay for the detection of *S. stercoralis* DNA in clinical samples at DCP. A comparison between Hotstart ® *Taq* polymerase master mix (HS) and the Taqman® environmental master mix 2.0 (EM) was performed (Source Thermo Fisher Scientific, UK). The published protocol by Verweij *et al*. (2009) recommends the use of HS master mix. However, Environmental® master mix (EM) (Source Thermo Fisher Scientific, UK) is in use at DCP for a multiplex qPCR for Protista. The Environmental® master mix is used for DNA extracted from stool samples for qPCR as it is more resistant to PCR inhibition than the HS master mix (Minogue *et al*., 2014). A composite reference standard and samples from viral, bacterial and other parasitic human pathogens and 284 clinical samples from patients being investigated at UCLH and HTD for strongyloidiasis were used to validate the assay. Chapter 3 describes the validation procedure for LAMP and this was also applied to the qPCR assay.

The analytical verification of this method used analytical sensitivity, analytical specificity and qPCR efficiency (determined by statistical analysis of the qPCR runs) using Rotagene software (Source Corbett Life Sciences). Statistical analysis was performed using an online statistical program (MedCalc®, n.d.) as previously described (Section 2.8).

4.2. AIMS OF THE STUDY

There were two main aims of this part of the study:

1. To determine the sensitivity and specificity of a published real-time assay and comparison to the CRS (this Chapter) and a novel LAMP assay for the detection of *S. stercoralis* DNA from clinical samples (Chapter 5).
2. To determine the cost of LAMP and qPCR for introduction of a NAAT to the diagnostic repertoire at DCP (Chapter 5, Section 5.3)

4.3. OPTIMISATION OF REACTION MIX

The method was optimised for use by the comparison of HotStart®Taq polymerase (HS) or TaqMan® Environmental master- mix 2.0 (EM). The EM was tested as this master- mix is said to be more resistant to inhibition (Minogue *et al*., 2014) than the HS master mix. Using DNA extracted from a negative stool control and a positive stool control (1 *S. stercoralis* larva/ µl) with a 10- fold dilution series and used neat and 10^{-1} to 10^{-5} plus or minus bovine serum albumin (BSA). BSA may be added to minimise stool inhibition. The reactions were run using the study protocol. Reaction mixtures were also set up in HS or EM (plus or minus added $MgCl_2$).

To further optimise HS and EM, a $MgCl_2$ curve was performed (dilution ranges: 0, 5, 10, 15, 20 mM and 0, 3, 3.5, 4, 4.5 mM $MgCl_2$) in each of the reactions. $MgCl_2$ is required as a cofactor for thermostable polymerase activity (Qiagen, n.d.). Primers and probe concentration curves were also run using HS and EM. The results demonstrated similar cycling thresholds (C_ts) for HS and EM but the amplitude of the fluorescence was lower with EM than with HS (Figure 4.4) when run in the optimised study protocol. There was a positive reaction using HS in the dilution series from 10^{-1} to 10^{-6}. A positive reaction was obtained, using EM, in the dilution series from 10^{-1} to 10^{-5} (a 10- fold difference in sensitivity).

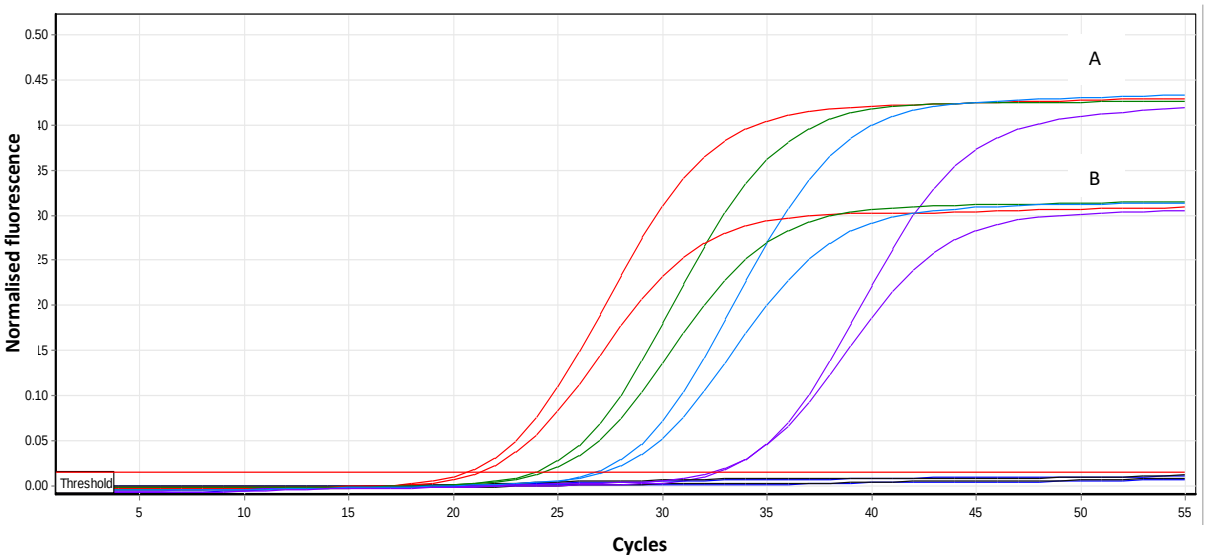


Figure 4.4: Comparison of HotStart®Taq polymerase (HS) and TaqMan® Environmental master- mix 2.0 (EM) using a ten-fold dilution series of the positive control DNA showing the difference in fluorescent amplitude between HS and EM (10^{-1} = red, 10^{-2} = green, 10^{-3} = blue, 10^{-4} = purple), there was very little difference between the C_t values for the two master-mixes. The straight red line indicates the cycling threshold, A= fluorescence amplitude for HS, B= fluorescence amplitude for EM.

A pilot study was performed with the SYBR®Green master mix to determine if identification by melting temperature could be a feasible option for future detection of geographical variation in *S. stercoralis* strains or detection of treatment resistant strains. Melt-curve analysis depends on the melting temperature at which double-stranded DNA with the incorporated intercalating dye becomes single-stranded DNA and releases the intercalating dye. A graph of change of fluorescence *vs.* temperature produces a characteristic melt curve that is affected by the sequence of the

product, the reagents, presence of inhibitors and low concentrations of DNA (which may give rise to non-specific products). The melting temperature is determined to be the point at which there is 50% dissociation of double stranded DNA. Using DNA extracted from a negative stool control and a positive stool control at dilutions ranging from: neat to 10^{-5}, the reactions were run using the study protocol. Two reactions were set up using either the Stro18 forward and reverse primers to detect *S. stercoralis* DNA or *gfp* forward and reverse primers to detect the internal control. The internal control was added to the positive and negative stool samples prior to DNA extraction and was used to monitor the extraction process and the amplification reactions. Melting temperature peaks ranged from: 75 – 77.5°C with a peak at 76.3°C (*gfp*) and 78.8-80.5°C with a peak at 79.75°C (*S. stercoralis*) (Figure 4.5). The addition of BSA showed a dampening effect on the melting curve- i.e. the amplitude of fluorescence was reduced (data not shown).

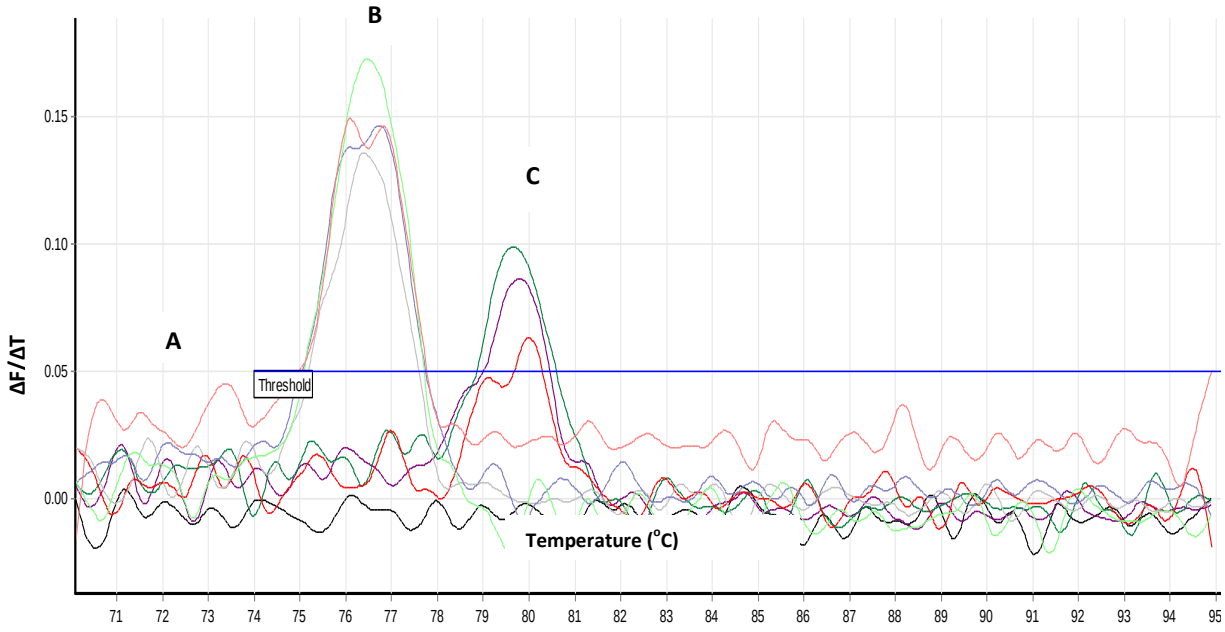


Figure 4.5: Melt curve analysis of qPCR *S. stercoralis* melt curves (green) and *gfp* amplification control (orange) showing a fluorescent peak at 80°C with the intercalating dye SYBR®Green. The *gfp* reaction is shown in grey and the Stro18S reactions are shown as negative stool control (black), positive stool control at dilutions of 10^{-1} (green), 10^{-2} (purple) and 10^{-3} (red). A= primer dimers or insufficient DNA, B= *gfp* peak, C= *S. stercoralis* peak.

The threshold for the MC analysis has been set at 0.05 and any fluorescence below the threshold (with a melting temperature <74°C) may be due to primer dimer formation as shown in Figure 4.5 A or different length amplicons generated from a diluted DNA template. The double- peaks in Figure

4.5 B and C may be due to the ability of SYBR®Green to bind to any double stranded DNA to produce a detectable fluorescence. In high dilutions with very little detectable DNA shortened amplification products may arise and different melting temperatures may be observed. This distinction is also useful in HRMC analysis for single-point mutation detection with the generation of distinct peaks with different T_m (Wittwer *et al*., 2003). However, in this case, further optimisation of the method is required so that light infections with very little *S. stercoralis* DNA may be reliably detected. This study demonstrated that this method can be used to detect *S. stercoralis* DNA, but requires further optimisation and no further work was performed using the SYBR®Green master mix as this method was investigated for information only.

4.3.1. FINALISATION OF THE STUDY PROTOCOL

HS was chosen as the DNA polymerase for the study and optimisation for the primers and probes showed no adjustments were necessary from the published protocol (Verweij *et al*., 2009). The only difference to the published protocol was the choice of 55 cycles rather than published 50 cycles so that the final cycle number for an assay in use at DCP could be determined at the end of the study. A qPCR assay is already available at DCP in a multiplex format for protest parasites (ten Hove *et al*., 2007) with a run protocol of 45 cycles and harmonisation of the qPCR assays would be expedient for new methods deployed in the routine laboratory. Analysis of the qPCR assay (Table 4.1) was interpreted from the results generated by the internal control, the negative stool control, the positive stool control (at a dilution of 10^{-3}) and the no template control (nuclease free water). The positive control was only run at a full dilution range of neat and 10^{-1} to 10^{-6} when testing a new positive extraction control to ensure that efficient DNA extraction was performed.

Table 4.1: Interpretation of the qPCR assay at DCP.

TEST TUBE		RUN CONTROL TUBE			INTERPRETATION
SAMPLE	**INTERNAL CONTROL (*gfp*)**	**NEGATIVE STOOL CONTROL**	**POSITIVE STOOL CONTROL**	**NO TEMPLATE CONTROL**	
Positive	Positive	Negative	Positive	Negative	Positive, a repeat qPCR assay using neat DNA and DNA at a 10^{-1} dilution will be performed if the sample *gfp* C_t is above the mean *gfp* C_t for the run
Positive	Negative	Negative	Positive	Negative	Possible positive result, validation of the run will require a repeat qPCR at a 10^{-1} dilution to determine if inhibition of the qPCR assay is present. Re- extraction of the sample may be required.
Negative	Positive	Negative	Positive	Negative	Negative
Negative	Negative	Negative	Positive	Negative	Repeat sample at a dilution of 1in10 to determine if inhibition of the qPCR assay has occurred. Re- extraction of the sample may be required.
Negative/ Positive	Negative	Positive	Positive	Negative/ Positive	Failed run
Negative/ Positive	Positive	Positive	Positive	Negative/ Positive	Failed run
Negative/ Positive	Negative	Negative	Negative	Negative/ Positive	Failed run
Negative/ Positive	Positive	Negative	Negative	Negative/ Positive	Failed run

4.3.1.1. CHALLENGE OF qPCR AND THE DECISION FOR GEL ELECTROPHORESIS OF ALL qPCR PRODUCTS

Once the qPCR method was optimised a set of residual diagnostic samples, from routine qPCR for protist and microsporidial (intracellular fungi, formerly protists) parasites, was used to challenge the method before the start of the study (n=20). Three unexpected positive results were obtained from samples of patients who were not being investigated for strongyloidiasis and so no routine diagnostic results for *S. stercoralis* were available. Repeated qPCR runs were negative, this may have been due to contamination of the DNA in the assay but further information regarding

strongyloidiasis risk in these patients was unavailable. On this basis it was decided that all qPCR positive results would be further investigated. A European committee comprised of experts in the field of molecular and routine diagnostics has ruled that qPCR tubes, as a closed system, must not be opened and run on an agarose gel as this could result in DNA product contamination of the laboratory (Personal communication with Dr van Lieshout). However, in light of the unexpected results it was decided to perform gel electrophoresis on all tubes that were qPCR positive in a separate PCR product laboratory. Very little DNA is released as droplets in this procedure and it is unlikely that significant DNA contamination of an electrophoresis gel in a designated PCR product room will occur. A sodium hypochlorite solution and a UV lamp (15 minutes) were used to clean all work spaces and no anomalous results were attributed to the gel electrophoresis of qPCR product.

All qPCR positive samples were run on a 2% agarose gel for one and half hours at 100V to allow for distinction between the *S. stercoralis* product band and the internal control *gfp* band. The published band size for *S. stercoralis* is 101 base pairs (bp) (Verweij *et al*., 2009) and the internal *gfp* control is *97*bp (Murphy *et al*., 2007, Kinson, 2012). All DNA samples that were positive in the qPCR assay were also tested by conventional PCR (cPCR) using only the specific Stro18S forward and reverse primers (to generate single bands that could be used for sequencing reactions) and the resulting products were viewed after gel electrophoresis on a 2% agarose gel run for one hour at 100V. Three study samples showed anomalous bands approximately 145bp or 500- 525bp and the identity of these bands was investigated by sequencing of the cPCR product. The cPCR product was purified using a QIAquick PCR clean-up kit (Source Thermo Fisher Scientific, UK). The purified product was sequenced using one or more of the following methods:

I. Sequence reaction of the pJET® 1.2 plasmid vector insert;
II. Direct sequencing of cPCR product using ABI Big®Dye version 3.1 chemistry on an ABI Prism 310 automated sequencer (Source Applied Biosystems, UK) using the forward or reverse Stro18S primers;
III. Sequence of the plasmid vector insert generated by pGEM®T-Easy plasmid vector. A single *contig* was compiled for each product using the Seqman® program (DNASTAR).

The resultant sequences were identified using a nucleotide BLAST search against the EMBL database on the NCBI website (NCBI, n.d.).

4.4 SEQUENCING OF THE cPCR PRODUCT TO CONFIRM *S. STERCORALIS*

The positive control was cloned and sequenced and the product was confirmed to be *S. stercoralis* with a 96% sequence match using the pJET® 1.2 plasmid vector. The sequence results of some of

the positive study samples with no anomalous results or bands are shown in Table 4.2 and the sequence alignment on an NCBI BLAST reference strain M84229.1 is shown in Appendix 3a.

Table 4.2: Sequences generated from the cPCR product of the positive stool control and positive study samples, using pJet® 1.2 plasmid vector, generated a 111bp or 121bp segment with sequence matches to *S. stercoralis* 18S ribosomal RNA gene Accession number M84229.1 ranging from 93 – 100% and a 96% sequence match to *Strongyloides* species

ID/ STUDY NUMBER	ACCESSION NUMBER	SEQUENCE	SIZE OF SEGMENT (bp)	SEQUENCE MATCH M84229.1	E VALUE
Positive control (pJET1.2)	M84229.1	GGCTCGAGTTTTTCAGCAAGATGAATTCCAAGT AAACGTAAGTCATTAGCTTACATTGATTACGTC CCTGCCCTTTGTACACACCGCCCGTCGCTGCCC GGAACTGAGCAATATCCAGAGG	121	96%	1e-47
858 (pJET1.2)	M84229.1	ATGGCTCGAGTTTTTCAGCAAGATTGCCTCTGG ATATTGCTCAGTTCCGGGCAGCGACGGGCGGT GTGTACAAAGGGCAGGGACGTAATCAATGTAA GCTAATGACTTACG	111	100%	8e-40
184 (pJET1.2)	M84229.1	GGCTCGAGTTTTTCAGCAGATTGCCTCTGGATA TTGCTCATTTCCAGGTAACGACGGGCGGTGTG TAGAAAGGGCAGGGACGTAATCAATGTAAGCT AATGACTTACGTTTACTTGGAATTCA	111	95%	3e-39
635 (pJET1.2)	M84229.1	GGATGGCTCGAGTTTTTCAGCAAGATTGNCTCT GGATATTGCTCAGTTCCGGGTAACGACGGGNG NTGTGTAGAAAGGGCAGGGACGTGATCAATG TAAGCTAATGACTTACGTTTACTTGGAATTCA	111	93%	2e-37
622 (pJET1.2)	M84229.1	TGGCTCGAGTTTTTCAGCAAGATTGCCTCTGGA TATTGCTCAGTTCCGGGCAGCGACGGGCGGTG TGTACAAAGGGCAGGGACGTAATCAATGTAAG CTAATGACTTACGTTTACTTGGAATTCA	111	100%	1e-47

Anomalous bands at 145bp (Figure 4.6) and 500-525bp (Figure 4.7) were cloned but failed to generate a cloned insert for sequencing reactions twice using the pJET® 1.2 plasmid vector and a direct sequencing method was chosen in an attempt to sequence and identify these products.

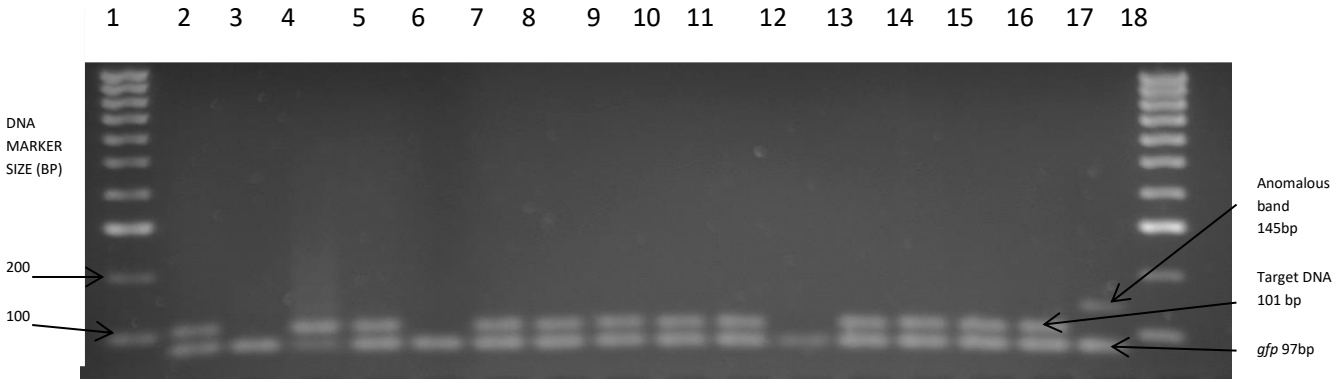


Figure 4.6: qPCR products run on a 2% agarose gel run at 100V for 1.5 hours. Lanes 2, 4, 5, 7- 11, 13- 16: positive 101bp target amplicon and a 97bp internal control, Lanes 3, 6, 12: negative for target amplicon, only a 97bp internal control amplicon was demonstrated, Lane 17: 97bp internal control and a 145bp anomalous amplicon, Lanes 1 and 18 contain a 100bp DNA marker ladder.

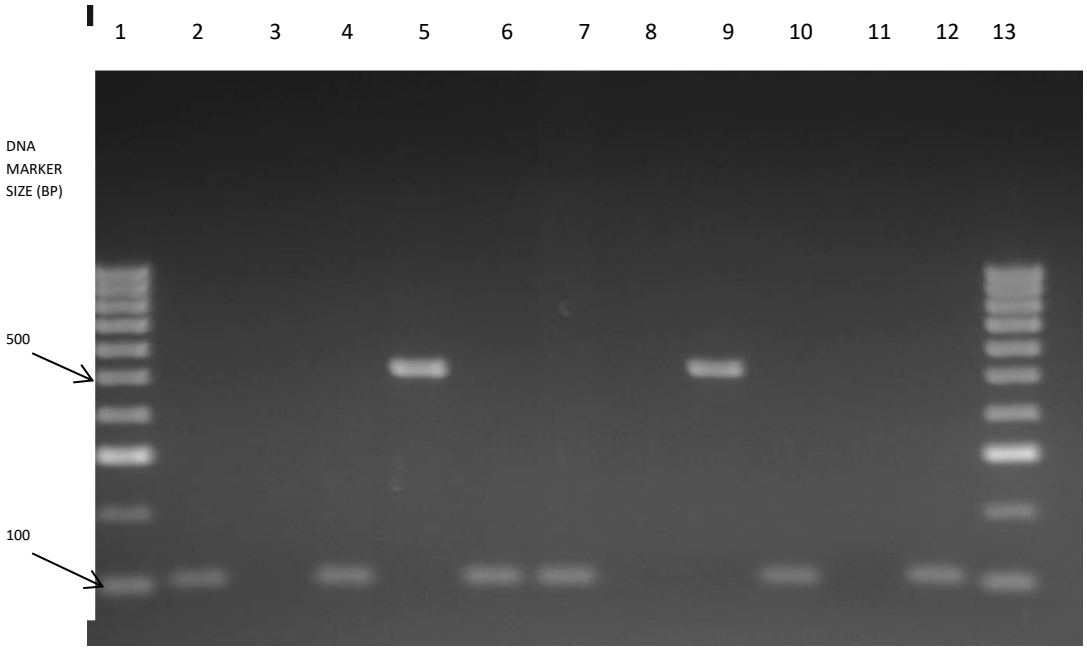


Figure 4.7: cPCR products run on a 2% agarose gel run at 100V for 1.5 hours. Lanes 1, 13: 100bp hyperladder, Lanes 2, 4, 6, 7, 10 and 12: 101bp target amplicon, Lanes 3, 8, 11: Negative, Lanes 5, and 9: 500- 525bp anomalous amplicon. Only one band (target DNA) is generated as the *gfp* primers were not added to the mixture.

The cPCR products were sent for sequencing to confirm product identity, either by using blunt-end plasmid vector pJET® 1.2, “sticky end” plasmid vector (pGEM® T-Easy) or direct sequencing.

Watts *et al*., 2016 stated that samples containing hookworm or *Blastocystis hominis* have been reported to amplify product with species specific primers that generated non-target bands on electrophoresis. There have, however, been no reports in the literature regarding the identity of these anomalous bands. Samples containing *B. hominis* were tested by LAMP and qPCR but no non-target bands were detected in this study. One stool containing hookworm was positive with the qPCR in only 1 out 3 assay runs and the qPCR was thought to have been contaminated for the one positive result. Direct sequencing was performed on the cPCR amplicons that generated anomalous bands to identify the product. Samples that were qPCR positive but CRS negative were determined to be anomalous results and were also sent for direct sequencing.

The results of sequencing reactions on the product generated by cPCR of the positive control stool sample (ON2, ON4, 2H2, 2H4) were used to confirm the product identity for the qPCR reactions. The results from the sequence reactions generated from the purified cloned DNA are listed in Table 4.3. The sequences generated from DNA extracted from the positive stool control containing 1 *S. stercoralis* larva/µl showed a 100% sequence match to *S. stercoralis* M84229.1 on a BLAST search of nucleotide identity on the NCBI website (NCBI, n.d.). ON2 was used to perform the limit of detection study and Table 4.3 shows the amount of DNA present in the purified DNA digests, measured on a Nanodrop spectrophotometer at 260nm.

Table 4.3: Sequences generated using the pJET®1.2 plasmid vector from a positive stool control containing 1 *S. stercoralis* larva/ µl.

ID	DNA ng	SEQUENCE RESULTS	BLAST ID	E values
>ON2	154.4	GGCTCGAGTTTTTCAGCAAGATGAATTCCAAGTAAACGTAAGTCATTA GCTTACATTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTGC CCGGAACTGAGCAATATCCAGAGG	AB923888.1	7e-46
>ON4	176.6	GGCTCGAGTTTTTCAGCAAGATGAATTCCAAGTAAACGTAAGTCATTA GCTTACATTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTGC CCGGAACTGAGCAATATCCAGAGG	AB923888.1	7e-46
>2H4	127.7	GGCTCGAGTTTTTCAGCAAGATGAATTCCAAGTAAACGTAAGTCATTA GCTTACATTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTGC CCGGAACTGAGCAATATCCAGAGG	AB923888.1	7e-46
>2H2	128.2	GGCTCGAGTTTTTCAGCAAGATGAATTCCAAGTAAACGTAAGTCATTA GCTTACATTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTGC CCGGAACTGAGCAATATCCAGAGG	KF926662.1	5e-17

Study samples with an anomalous results (qPCR positive when the CRS was negative) or band sizes different to the published product size of 101bp (anomalous bands) were run on ABI Big®Dye v3.1 for direct sequencing to determine identity of anomalous results or anomalous bands (Appendix 4).

This failed to generate identifiable sequences so the samples were cloned using the pGEM®T-Easy vector system protocol and sent to the LSHTM for sequencing. Only one of the 500- 525bp bands produced a sequence with a 100% sequence match to *S. stercoralis* AJ558163.1 (37bp) with the T7 primer and a sequence with 99% sequence match to *S. stercoralis* M84229.1 (90bp) with the SP6 primer. The E value was 3E-11 (for T7 primer) and 8E-40 (for the SP6 primer) indicating that there is only a small probability that this sequence would occur in other species. The 500- 525bp anomalous band size produced by the qPCR assay was probably *S. stercoralis* DNA. Three anomalous band sizes were detected during this study and only one anomalous band showed sequence matches to *S. stercoralis*. It is possible that there was very little DNA available and the sequencing reaction was unable to generate a sequence identification for the anomalous bands where no sequence identity was obtained. It may be that a new species of *S. stercoralis* is detected by real-time PCR or that the primers are detecting hypervariability in this region. However, care must be taken to interpret this data as the sequence matches for the 500bp query were only 37 or 90bp. Jaleta *et al*. (2017) examined *S. stercoralis* species infecting canines and humans in rural villages in Northern Cambodia, using comparison of mitochondrial sequences and whole genome analysis of the 18S DNA, found that hypervariability in the HVR1 region (Hasegawa *et al*., 2009) of the genome does not indicate different species, rather that *S. stercoralis* is more variable in this region than other nematodes. However, they do state that this does not exclude the existence of a cryptic *Strongyloides* species. Further work is required to confirm the findings in this study.

This confers a false positive rate (probability of receiving an incorrect positive test) of 0.8% to the qPCR assay (5 anomalous bands detected in the qPCR assay of 610 samples). The study was designed to detect a difference between the CRS and the NAAT with a power of 90% and a probability of 5%. As previously discussed, the development of an assay is designed with a power of 80-95% and a probability of 5-20%. The choice of power and probability depends on the use of the test (Jones and Payne, 1997). The qPCR assay requires a high specificity if it is to be used as a diagnostic test and a high sensitivity if it is to be used as a screening test. A false negative result could have severe consequences for the patient and in a disease such as strongyloidiasis the detection of a false positive would have a less severe impact than a missed diagnosis. The patient would have a range of tests (microscopy and serology) to confirm the result and a clinical decision would determine whether further testing or treatment was required.

4.5 SENSITIVITY AND SPECIFICITY OF qPCR

4.5.1 ANALYTICAL SENSITIVITY

Analytical sensitivity was determined by the serial 10- fold dilution of DNA extracted from positive control stool samples (1 *S. stercoralis larva*/ µl) as previously described in Section 3.5 (Saah and Hoover, 1997). The analytical sensitivity, or limit of detection (LOD) for the number of *S. stercoralis* larvae/ µl, was determined as 10^{-4} for qPCR (Table 4.4 and Table 4.5). Stepwise logistic regression did not show any effect on the qPCR results using the criterion of sample size of stool extracted. The variation in the sample size of stool extracted has been previously discussed in Section 2.3.8.1.

Table 4.4 Performance of qPCR using serial 10- fold dilutions of DNA extracted from positive control stool samples (Positive result in C_t)

DILUTION OF DNA FROM POSITIVE STOOL CONTROL	STOOL SAMPLE EXTRACTED (mg)	qPCR RESULT (C_t)
10^{-1} Positive stool control (*10-fold dilutions performed after DNA extraction) extracted on 25/01/2015	0.143	25.04
10^{-2}		20.74
10^{-3}		24.01
10^{-4}		26.93
10^{-5}		32.82
10^{-6}		34.83
10^{-7}		Not done
10^{-1} Positive stool control (*10-fold dilutions performed after DNA extraction) extracted on 12/06/2015	0.024	28.15
10^{-2}		31.19
10^{-3}		35.81
10^{-4}		33.53
10^{-5}		Negative
10^{-6}		Negative
10^{-7}		Not done
10^{-1} Positive stool control (*10-fold dilutions performed after DNA extraction) extracted on 20/12/2015	0.040	28.15
10^{-2}		31.19
10^{-3}		35.81
10^{-4}		46.93
10^{-5}		Negative
10^{-6}		Negative
10^{-7}		Negative
10^{-1} Positive stool control (*10-fold dilutions performed after DNA extraction) extracted on 23/01/2016	0.016	16.22
10^{-2}		19.42
10^{-3}		23.04
10^{-4}		29.2
10^{-5}		33.33
10^{-6}		Negative
10^{-7}		Negative

Table 4.5: Determination of the analytical sensitivity or limit of detection (LOD) using negative stool slurry spiked with L3 *S. stercoralis* larvae from a positive stool culture- final concentration: 1 *S. stercoralis* larva/µl.

PCR	DILUTION SERIES							*S. STERCORALIS* NEGATIVE STOOL SAMPLE
	Neat (1 larva/µl) (n=13)	10^{-1} (n=13)	10^{-2} (n=13)	10^{-3} (n=13)	10^{-4} (n=13)	10^{-5} (n=5)	10^{-6} (n=13)	
Average readings- C_t	20.97	23.63	26.26	31.57	33.516667	35.846667	0	Negative

The positive control was not consistently positive at a final dilution of 10^{-5} so the analytical sensitivity for this assay was chosen as 10^{-4} *S. stercoralis* larvae/ µl.

The LOD was also determined by dilution of purified DNA with a concentration of 154.4 ng. The concentration was determined by a Nanodrop spectrophotometer. To determine the limit of detection by this method cPCR was performed on positive stool control DNA. The resulting product was purified using the GeneJET® gel extraction kit and ligated into TOP10 *E. coli* using cloning vector pJET® 1.2 and the resulting clone was sent for sequencing (Source Bioscience, UK). The resultant sequences were identified using a BLAST search against the EMBL database on the EBI and NCBI website (NCBI, n.d.). The sequence results showed a 100% sequence match to *S. stercoralis* and a 93-98% sequence match with *Strongyloides* species. Once identity had been confirmed one of the clones was chosen for the study (ON 2) and a serial 10- fold dilution range of 10^{-1} to 10^{-20} was tested in triplicate on two separate qPCR runs. An LOD of 154.4 x 10^{-9} ng was demonstrated (Figure 4.8). The qPCR assay was negative, in triplicate, for the neat DNA. The qPCR assay was positive in only 1 out 3 samples for the 10^{-1} dilution and this may be due to the fact that excess DNA was present in these samples and stochastic inhibition of primer binding had occurred.

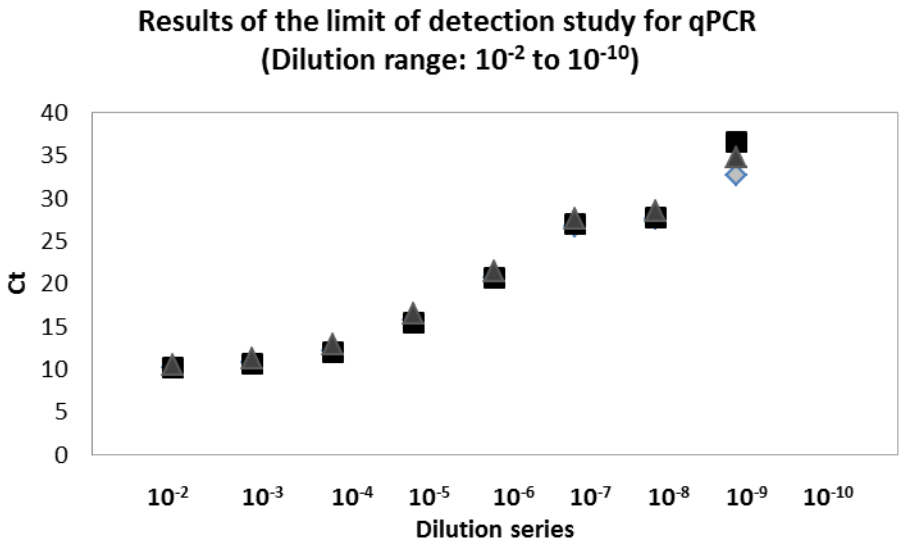


Figure 4.8: Results of the limit of detection for qPCR (DNA extracted and cloned, using pJET® 1.2 plasmid vector, from a spiked negative stool samples containing 1 *S. stercoralis* larva/ µl). The dilutions 10^{-2} to 10^{-10} were run in triplicate over two different qPCR amplification runs. Black, dark grey and light grey series indicate the triplicate results. The 10^{-10} dilution was negative in triplicate runs of the qPCR assay.

Table 4.6: The limit of detection (LOD) of the qPCR. Results are expressed as the C_t of the qPCR amplification runs

POSITIVE STOOL DNA: NEAT	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
0	38.61	10.19	10.79	12.22	15.77	20.67	26.8	27.69	32.77	0
0	44.59*	10.2	10.71	12.04	15.5	20.71	27.04	27.84	36.59	0
0	0	10.55	11.33	13.01	16.59	21.42	27.65	28.58	34.82	0

* C_t= 44.59 was determined in this study to be an equivocal result.

A consistent positive result was obtained in dilutions 10^{-2} to 10^{-9}. No reactions were seen in the dilutions 10^{-10} to 10^{-20}.

4.5.2 ANALYTICAL SPECIFICITY

Analytical specificity was determined by the samples in the specificity bank (previously described in Section 3.5 for the LAMP assay) and qPCR was 94.83% specific (Saah and Hoover, 1997). Viral, bacterial, protist and other helminthic human stool pathogens formed the specificity bank to determine whether the qPCR assay would detect only *S. stercoralis* target DNA. Three samples in the specificity bank were positive for qPCR *S. stercoralis* DNA which was confirmed by gel

electrophoresis. The specificity bank samples were completely anonymised prior to the start of the study (stored positive samples for research purposes) and no further action could be taken (Table 4.7).

Table 4.7: qPCR results for viral, bacterial and parasitic human pathogens (total *S. stercoralis* positive samples =8, total *S. stercoralis* negative samples =58)

ORGANISM	NUMBER OF ORGANISMS TESTED	qPCR RESULTS (EXTRACTED DNA)
Negative stool	8	Negative (n=8)
Positive *S. stercoralis* stool control	8	Positive (n=8)
Rhabditiform free-living larvae NOT *S. stercoralis*	1	Negative (n=1)
Nematodes	11- *Ascaris lumbricoides* (2), *Trichuris trichiura*(2), hookworm (5), *Trichostrongylus* sp. (1), *Loa loa* (1)	Negative (n=11)
Trematodes	2- *Dicrocoelium dendriticum* (1), *Schistosoma mansoni*(1)	Negative (n=2)
Cestodes	2- *Bertiella studeri* (1), *Taenia saginata*(1)	Negative (n=2)
Intestinal protista/ intracellular fungi	29- *Cryptosporidium* sp. (5), *Cystoisospora belli* (2), *Cyclospora cayetanensis* (1), *Entamoeba histolytica/dispar* (6), *Entamoeba hartmanni* (1), *Entamoeba coli* (1), *Giardia lamblia* (6), *Blastocystis hominis* (1), *Enterocytozoon bienusi* (5), *Encephalitozoon intestinalis* (1)	Negative (n=26) **qPCR POSITIVE**: 1 x *C. belli* C_t= 35.11 1 x *E. intestinalis* C_t= 35.31 1x hookworm C_t= 44.56
Bacteria	4- *Campylobacter jejuni* (1), *Shigella sonnei* (1), *Escherichia coli O157* (1), *Vibrio cholera* (1)	Negative (n=4)
Viruses	1- Adenovirus (1)	Negative (n=1)

Positive results obtained with the protist pathogens with C_ts of 35.11 and 35.31 were repeated and the results were negative in 2 consecutive qPCR assays. The positive hookworm sample had a C_t of 44.56 and C_ts of > 40 are considered equivocal in this study. In the case of a diagnostic sample a 10^{-1} dilution of re- extracted DNA would be performed in the qPCR assay or a repeat sample would be requested. The repeat qPCR for this sample was negative in 2 consecutive runs. It is thought that contamination of the qPCR reaction tube occurred. The specificity of qPCR was determined to be 94.83% because of the false positive results obtained. This is discussed further in Section 6.4.

4.5.3 DIAGNOSTIC SENSITIVITY

Diagnostic sensitivity was determined using the results of qPCR and the CRS for the study samples in a 2x2 contingency table (Jacobson, 1998). Prevalence is unknown for strongyloidiasis in the patient population attending DCP and larger sample sizes are required for a complete determination of prevalence. The diagnostic sensitivity was determined to be 39.73% (95% CI: 28.45-51.86%) using the CRS (indicating proven or possible disease) as a comparator and 74.07% (95% CI: 53.72-88.89%) when using only microscopy and culture results (proven disease). When

using an imperfect reference standard sensitivity and specificity estimates are biased (Baughman *et al*., 2008) and the FDA recommends using overall percent positive agreement (OPA) between the old and new tests (FDA, 2007). The positive percent agreement for the qPCR assay for the detection of *S. stercoralis* DNA in clinical samples was determined, in this study, to be 40.32 % with an overall percent agreement (OPA) between old and new tests of 80.63% (95% CI: 70.5-91.8).

4.5.4 DIAGNOSTIC SPECIFICITY

Diagnostic specificity was determined using the results of qPCR and the composite reference standard for the study samples in a 2x2 table (Jacobson, 1998). The specificity was calculated as 92.70% (95% CI: 88.58-95.69%) using the CRS as a comparator and 90.68% (95% CI: 86.64-93.82%) when using microscopy and culture as a comparator. The negative percent agreement (NPA) is used to replace specificity estimation when there is no "gold standard" and was calculated to be 91.89% (FDA, 2007).

The qPCR assay sensitivity and specificity estimates were biased due to lack of a suitable "gold standard" and overall percent agreement was chosen to eliminate some of the bias (FDA, 2007). The diagnostic overall percent agreement between the CRS and qPCR demonstrated that qPCR is a suitable diagnostic test that can be included in a diagnostic repertoire consisting of serology and qPCR. Microscopy is a very insensitive test that will nevertheless detect parasites other than *S. stercoralis*. Until the future introduction of multiplex NAATs or microarrays (Requena-Méndez *et al*., 2014) for the diagnosis of parasitic diseases microscopy cannot be excluded from a routine diagnostic repertoire. The future use of culture as a diagnostic test for strongyloidiasis, however, may require the removal of this test from the laboratory. Although it is more sensitive than microscopy, it entails a biological risk to laboratory staff from infectious L3 larvae and is not more sensitive than combined serology and qPCR.

4.6 DETERMINATION OF THE OPTIMUM NUMBER OF RUN CYCLES AND POSITIVE CUT-OFF VALUES

Once all the samples and controls had been tested the results of the positive samples and controls were entered into the MedCalc® statistical program to generate Box and Whisper plots with the whiskers indicating the 25^{th} and 75^{th} percentile to determine the cut-off for the number of cycles required to detect all cases of *S. stercoralis*. The cut-off was determined to be 50 cycles (if all qPCR positive results were recorded i.e. this included band sizes different to 101bp shown in Figure 4.9). The published protocol (ten Hove *et al*., 2009, Verweij *et al*., 2009,) has a cycling run of 50 cycles. Using only qPCR positives with a band size of 101bp the cut-off was determined to be 45 cycles and this is the same number of amplification cycles as for the multiplex protist qPCR protocol already deployed in the routine laboratory.

The final number of run cycles for this study was chosen as 45 cycles as this identified all the samples containing a 101bp product when run on a 2% agarose gel at 100V for 1.5 hours. The qPCR HS plot (C_t) was generated from the cycling thresholds (C_ts) of all positive qPCR results, regardless of the product size visualised on a 2% agarose gel. The qPCR HS plot (C_t and gel 101) was generated from the C_ts of all positive qPCR results that had a product that generated a band of 101bp on gel electrophoresis.

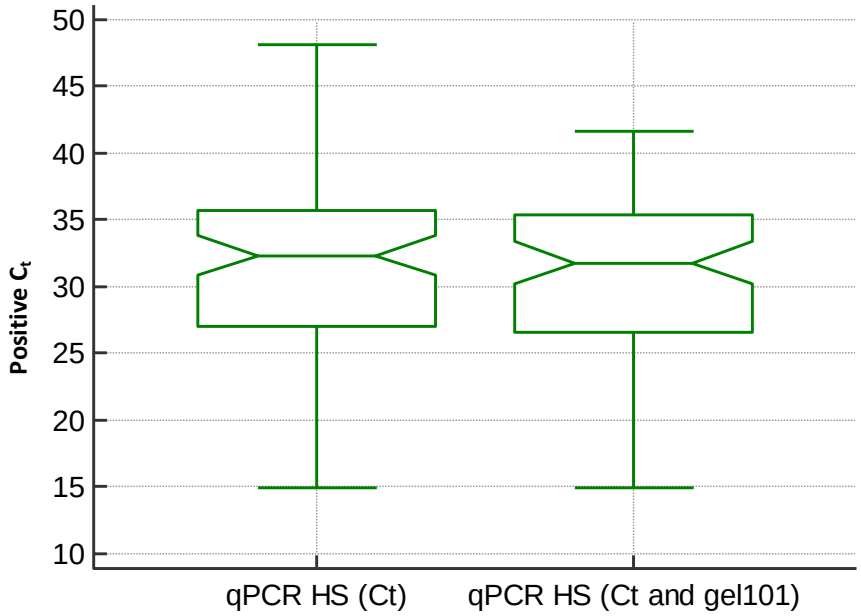


Figure 4.9: Box and Whisper plots for the determination of run cycles for the qPCR protocol. Notch indicates the mean and the whiskers indicate the 25th and 75th percentiles. qPCR HS (Ct) indicates all the C_t values regardless of product size on a 2% agarose gel, qPCR HS (Ct and gel 101) indicates the C_t values of the real-time PCR with a product size of 101 bp.

The results from sequence identity were used to determine the number of amplification cycles that would be used in a diagnostic test. All anomalous band sizes were detected in samples where the composite reference standard (CRS) was negative and the qPCR C_ts ranged from 35.13 to 48.16. Two out of five anomalous bands were negative on repeat cPCR assays and, of those that were still positive, only one (the 500- 525bp band) gave a partial sequence identity and was identified as *S. stercoralis*. This sample had a C_t of 35.29 and would be detected by a qPCR assay with 45 amplification cycles. The results of the repeat qPCR and interpretation of the results is discussed in Appendix 4.

Caraguel *et al*. (2011) recommend the Youden Index, amongst others, to determine the limit of detection of the qPCR assay. A qPCR protocol of >35 cycles has limitations in that primer dimers and

false positive reactions occur at around 40 cycles. However Caraguel *et al*. (2011) do admit that the determination of the limit of detection cut-off does depend on the consequences of a missed diagnosis. In Figure 4.10 a cut-off of 35 cycles would miss true positive results (clear circles). The results of the study determined that samples with a C_t of <40 will be considered positive and samples with a C_t ≥40 will be considered equivocal and a repeat sample will be requested.

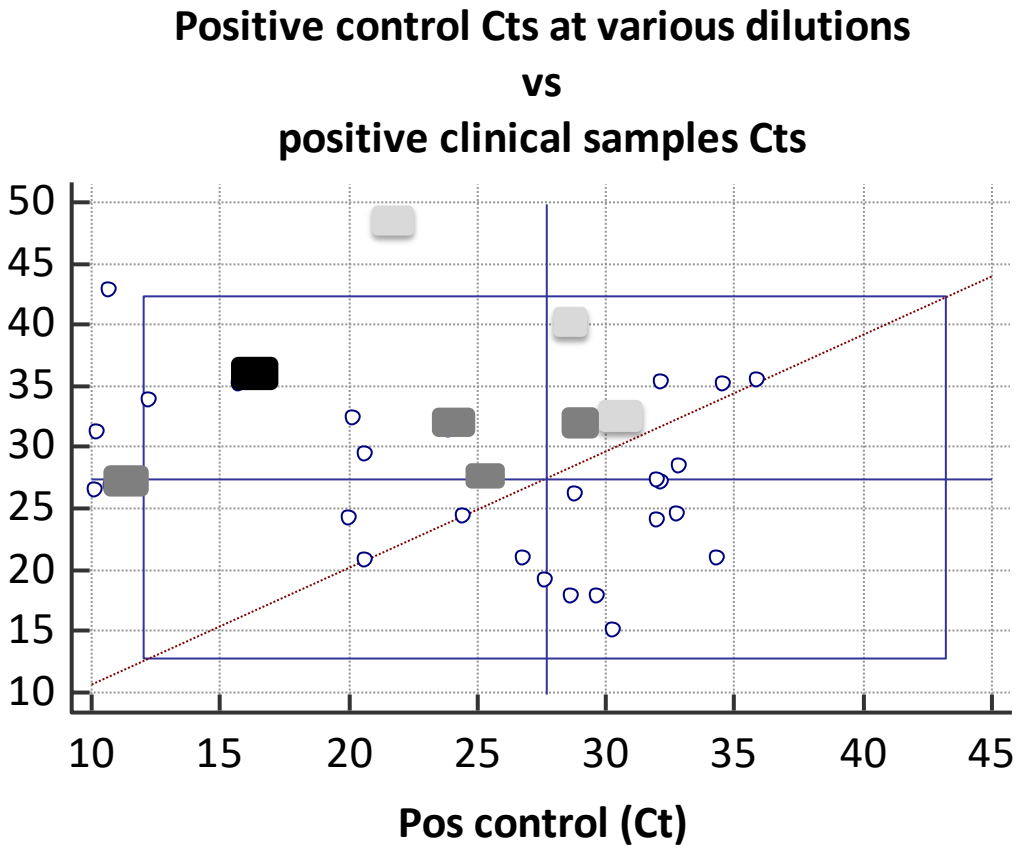


Figure 4.10: Youden Plot- Determination of cut-off C_t for qPCR positive results. The rectangle represents 2SD (standard deviation) coverage (95% CI: 25.250 to 31.721). Anomalous bands (pale grey square) are found at C_ts >30, No true positive results (clear circle) are found at C_ts >45. Possible true positive results (dark grey square) i.e. samples with very little DNA may be found at C_ts, <40. False positive samples (black square) determined by negative results for the cPCR repeat may also be found at C_ts <45.

Therefore the study determined that 45 amplification cycles were suitable for the qPCR assay and this would be a suitable introduction into the routine diagnostic workflow already in use at DCP. As has been previously discussed, missing a diagnosis of strongyloidiasis can have severe consequences (Pottie *et al*., 2011). Although C_ts >30 may be due to primer dimers (Caraguel *et al*.,

2011), the size of the anomalous bands on gel electrophoresis (145bp and 500- 525bp) does not indicate the formation of primer dimers (Vallone and Butler, 2004). In proven cases of strongyloidiasis very low DNA concentrations have been detected with a C_t >30 in this study.

The NAAT assays for *S. stercoralis* detection may be required to detect very small amounts of DNA in cases where there has been a relevant travel history, even after a long time-scale, in patients who are about to undergo immune-suppressive therapy or are otherwise immunocompromised. A highly sensitive test with a low limit of detection is susceptible to contamination therefore, a limit of detection cut- off value of C_t = 40 was chosen. Any samples, with an acceptable internal control value, with a C_t >40 are determined to have given an equivocal result. Verweij and Stensvold (2014) stated that a sample demonstrating a C_t value above the limit of detection value is not necessarily negative and so cannot be determined to be negative.

A limit of detection cut-off of C_t=40 does detect those samples with anomalous bands and would miss true positive samples with very little target DNA, however, there were very few of those samples in this study (n=8, Minimum C_t= 40.05, maximum C_t=48.16) One sample (266) in this study with a C_t=48.16 would not have been detected (Appendix 4). This sample also generated a product in the cPCR assay that could not be sequenced as the concentration of DNA was too low.

The qPCR assay C_t range was: minimum = 14.98, maximum = 40.05 (n=1) for true positives and minimum = 27.15 and maximum = 48.16 for equivocal positive results as determined by the CRS. Therefore, the qPCR assay protocol was established at 45 cycles with a limit of detection cut-off at $C_t \geq 40$. A cautionary note will be added to C_t results ≥ 40:

- Equivocal result obtained by real-time PCR in this sample, please send a repeat sample if clinically indicated.

4.7 VALIDATION OF THE INTERNAL CONTROL

Once all the samples and controls had been tested the results of the internal control (*gfp*) were normalised (sample *gfp* C_t x [*gfp* mean C_t of all runs/sample run mean]) (Figure 4.11). All the results were used to generate a mean and standard deviation in a Microsoft Excel® spreadsheet. Any samples that had an internal control C_t reading >*gfp* mean C_t for the run +1.96SD (mean + 2.04) were diluted 10^{-1} and the qPCR assay was repeated to determine whether any inhibition of the qPCR has occurred. Inhibition of qPCR is confirmed if the *gfp* reading of the 10^{-1} diluted sample C_t > mean + 5.2 in the repeated sample run.

Thirty three samples out of a total number of 610 reactions (this includes duplicate samples and samples stored at 4°C or -20°C) had a *gfp* reading > run mean+ 2.04 and required a 10^{-1} dilution before the qPCR was repeated (i.e. only 5.4% of samples required further testing).

The repeated 10^{-1} dilution results were the same as the initial qPCR results (i.e. positive or negative) for all samples tested. The low number of samples requiring 10^{-1} dilution to investigate the presence of inhibition of qPCR and the fact that there is very little difference between the C_ts before and after normalisation demonstrates that the extraction and amplification methods used in this study are suitable for the detection of *S. stercoralis* DNA in clinical samples for the qPCR assay.

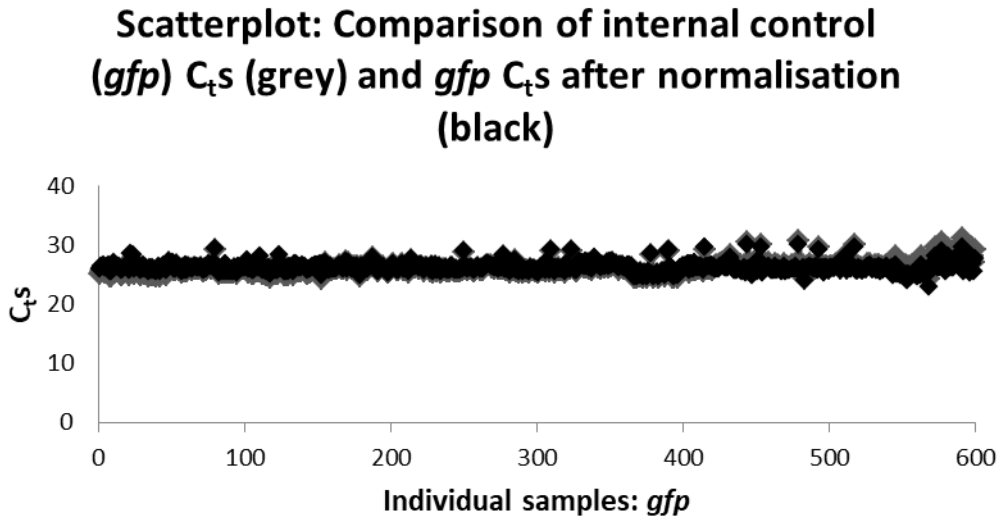


Figure 4.11: Comparison of the internal control C_ts from all runs and internal control C_ts after normalisation of the results between runs. Normalisation was carried out so that the internal control data could be assessed between different qPCR runs. (Raw data in grey and normalised data in black).

4.8 FINAL qPCR PROTOCOL FOR THE DETECTION OF *S. STERCORALIS* DNA IN CLINICAL SAMPLES

All positive and negative stool controls, specificity bank samples and anonymised study samples (stored at 4°C or -20°C) were tested using the study protocol. The internal control was validated and all samples that gave a mean *gfp* reading greater than the run mean + 2.04 were diluted 10^{-1} (in nuclease-free water) and the qPCR assay was performed. Any 10^{-1} diluted samples with a *gfp*

reading greater than the run mean + 5.2 were determined to contain inhibitors for qPCR and would have been excluded from the study, however, no samples showed inhibition to qPCR after 10^{-1} dilution and no alteration to the qPCR result (positive or negative) was demonstrated as previously discussed. This was performed on raw data to determine whether the individual sample contained inhibitors.

Analytical verification was determined by analytical sensitivity (limit of detection) and analytical specificity was performed using samples in the specificity bank. Amplification efficiency affects the cycling threshold, which is the point where the amplification curve crosses the threshold line and is set above the baseline and within the exponential growth region of the curve. So investigating amplification efficiency is part of the analytical verification of a qPCR assay. $C_n = C_i \times (1 + E)^n$, where Ci = initial copy number, Cn = copy number at cycle n, n = number of cycles, E = efficiency of target amplification. When E=1 (maximum efficiency) $C_n = C_i x 2^n$ (two-fold increase at each cycle), the quantity of product generated at each cycle decreases when E is decreased, thus delaying the amplification plot and affecting quantitation using this assay. The C_t is inversely proportional to the amount of starting nucleic acid in the amplification tube. The recommended E should lie between 90-110% (Life Technologies, 2011).

Rotagene recommend that the results are reported as delta delta C_t ($\Delta\Delta C_t$) by normalising the sample results to the sample reporter gene (internal control) and also to the calibrator (in this study the positive control at a dilution of 10^{-3}) so that comparable results are obtained across the different qPCR assay runs. The reporter gene and the gene of interest must have the same amplification efficiency for $\Delta\Delta C_t$ analysis. The formula for the calculation of amplification efficiency is: [10(-1/slope of the trendline)]-1. The correlation coefficient (R^2) should be ≥ 0.99 and the closer the slope (M) is to -3.32 the closer the amplification efficiency is to 100%. (Qiagen, n.d.). The amplification efficiencies for the internal control and the target gene were performed (Figures 4.12 and 4.13). E for the internal control and the target gene was calculated from the results of five consecutive qPCR runs where the positive control DNA had been diluted in a 10-fold dilution series. The mean of the C_t readings for the internal control and target DNA was calculated and this was used to determine the slope and correlation co-efficient from a semi-logarithmic graph to allow the amplification efficiency to be calculated on an Excel® spreadsheet. To determine the amplification efficiencies for the internal control and the target of interest a dilution series of 10^{-1} to 10^{-3} was chosen. While amplification efficiency for some of the 10^{-4} dilutions could be determined for *S. stercoralis*, the effect of dilution on the internal control meant that matching *gfp* C_t values were not always available or that the result was not in the exponential part of the curve. Therefore a

comparison of amplification efficiencies could only be performed using those dilutions where the C_t was demonstrated in the exponential part of the curve for the internal control and the target DNA. Figures 4.12 and 4.13 exhibit similar values for E and demonstrated that *gfp* was a suitable internal control for use in this qPCR assay.

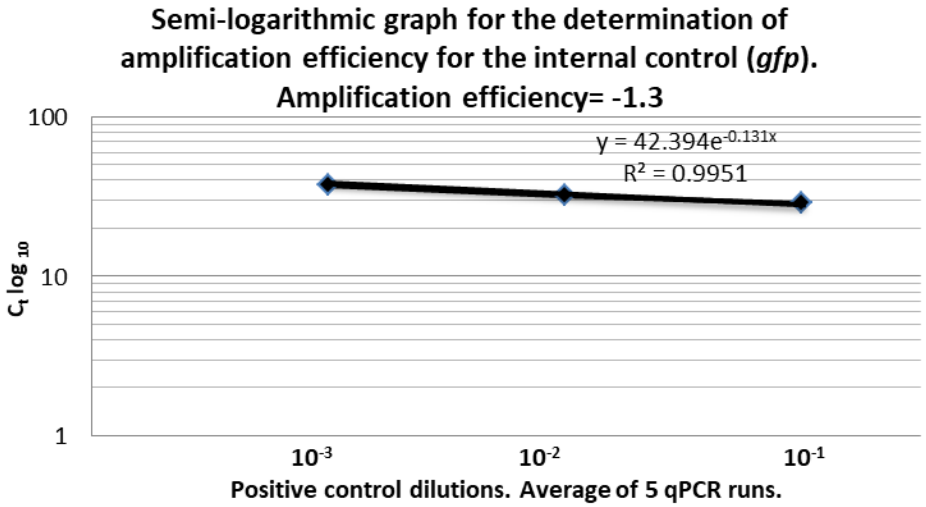


Figure 4.12: Semi-logarithmic graph for the determination of amplification efficiency (E) in the internal control (*gfp*). A negative slope is obtained if the standard curve is run in the order of most dilute to most concentrated and the trendline is exponential as the graph is semi-logarithmic.

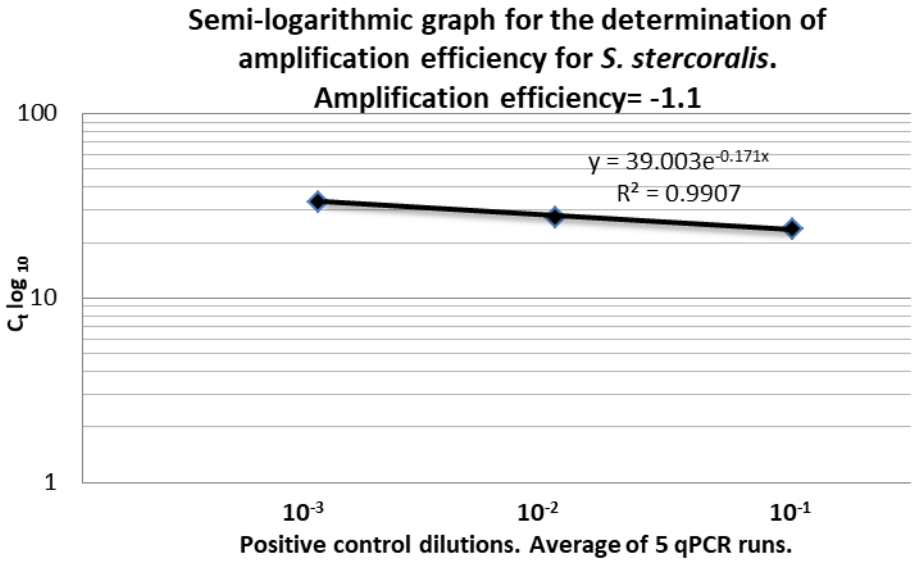


Figure 4.13: Semi-logarithmic graph for the determination of amplification efficiency (E) in target DNA (*S. stercoralis*). The trendline is exponential as the graph is semi-logarithmic.

Absolute Δ C_t (another method to confirm amplification efficiency) determines the copy number of the target template and is used with a standard curve. However, this method was not used in this study as the internal control of each sample is compared to the internal control run mean plus the standard deviation (2SD) to detect sample inhibition or qPCR variation in the individual tube. The internal control (*gfp*) can be used to compare the qPCR assay within and between runs as it has been previously shown to be stable between different qPCR runs (Figure 4.9).

Clinical samples are complex environments consisting of microbial flora, faecal matter and PCR inhibitors and this affects the amplification efficiency of the assay (Moghaddassani *et al*., 2011, *Monteiro et al*., 1997, Murphy *et al*., 2007). Degradation of DNA is determined by copy number variations which will affect amplification efficiency but not the correlation coefficient (R^2) values (Dhanasekaran *et al*., 2010). Three parameters are important to determine quantification parameters: PCR efficiency, copy number variance and the correlation coefficient, and will reflect variations in pipetting and target DNA degradation (Dhanasekaran *et al*., 2010).

Amplification efficiency can be performed using the Rotagene statistical analysis package (Source Corbett Life Sciences) and Figure 4.14 demonstrates the amplification efficiency calculated from a study qPCR assay run for the 10- fold dilution series of purified DNA from a positive stool control (standard curve) and clinical samples. The qPCR standard curve produced an amplification efficiency of 1.14 with a correlation coefficient R^2= 0.651 and a slope of -3.028 (Figure 4.14).

Amplification efficiency for the qPCR assay for clinical samples.

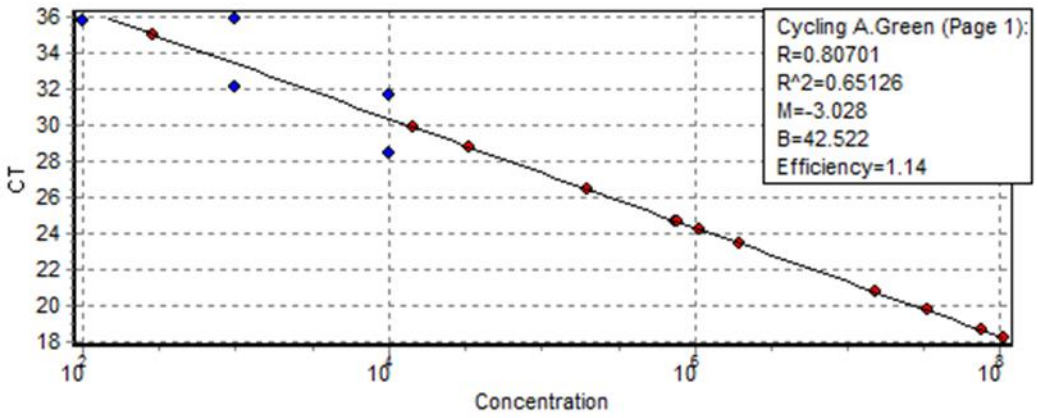


Figure 4.14: Amplification efficiency determined by qPCR standard curve (blue), clinical samples (red)

To determine the effect of the variation on precision between the different qPCR amplification runs the percentage co-efficient of variation (% CV) was determined from 10- fold dilution series on different aliquots of the positive control (one incubated for 2 hours at 56^{o}C and one incubated overnight at 56^{o}C during the extraction protocol). The analysis was performed on three consecutive qPCR runs and the % CV was calculated as the standard deviation divided by the mean and multiplied by 100. An F test to compare standard deviations was performed to see if there was any difference between the standard deviations of the samples incubated for two hours or incubated overnight at 56^{o}C.

The % CV between runs and between different incubation times ranged from 0.3 to 3.5% i.e. <5% variation. As previously discussed in Chapter 3, % CV should be <15% for inter- assay runs and <10% for intra- assay runs (Reed *et al*., 2002, Salimetrics, n.d.). The F Test,a comparison of the standard deviations, produced no statistically significant difference in the results. The p values for the target DNA ranged from: 10^{-1} dilution (p=0.87), the 10^{-2} dilution (p=0.92) and the 10^{-3} dilution (p=0.439) between the samples incubated at 56^{o}C for 2 hours or overnight. The internal control p values were: 10^{-1} dilution (p=0.39), the 10^{-2} dilution (p=0.33) and the 10^{-3} dilution (p=0.899) between the samples incubated at 56^{o}C for 2 hours or overnight. This finding determined that overnight incubation at 56^{o}C in tissue lysis buffer can be performed in two hours as there was no statistically significance difference in the qPCR assay results between the two methods.

4.9 PERSISTENCE OF *S. STERCORALIS* DNA AT STORAGE TEMPERATURES OF 4^{o}C AND -20^{o}C

As previously described for the LAMP assay (Section 3.7), the qPCR assay was assessed for the persistence of short-term storage at 4^{o}C without a preservative as this would impact the routine workflow at DCP where neat stool aliquots for NAAT testing are stored at 4^{o}C (without a preservative) for up to 3 weeks. To investigate the decreased sensitivity in the detection of *S. stercoralis* in samples stored at 4^{o}C and -20^{o}C a pilot study was set up to determine the short-term persistence of DNA in samples stored at 4^{o}C or -20^{o}C. Table 4.8 shows the results obtained using a positive control stool sample containing 0.1 *S. stercoralis* larvae/ µl, stored at 4^{o}C or -20^{o}C in a 10-fold dilution series and tested at various time intervals. The DNA was extracted using a Qiagen® Qiasymphony SP and the extracted DNA was stored at -20^{o}C until a qPCR assay could be performed. The DNA was diluted in a 10-fold dilution series and the dilution range was 10^{-1} to 10^{-5}.

Table 4.8: Results of the survival study at a storage temperature of 4°C and a storage temperature of -20°C

DATE PLACED IN STORAGE	EXTRACTED BY QIAGEN® QIASYMPHONY SP	DATE TESTED BY qPCR ASSAY	4°C NEGATIVE STOOL CONTROL qPCR ASSAY	4°C POSITIVE STOOL CONTROL qPCR ASSAY (final dilution positive)	-20°C NEGATIVE STOOL CONTROL qPCR ASSAY	-20°C POSITIVE STOOL CONTROL qPCR ASSAY (final dilution positive)
29-12-2016	Week 1	01/04/2017	Negative	10^{-3}	Negative	10^{-4}
	Week 2	01/04/2017	Negative	10^{-3}	Negative	10^{-4}
	Week 3	01/04/2017	Negative	10^{-3}	Negative	10^{-4}
	Week 4	01/04/2017	Negative	10^{-2} (C_t 30.91)	Negative	10^{-4}
	Week 5	01/04/2017	Negative	10^{-3}	Negative	10^{-5} (C_t 38.7). Repeat 10^{-4}
	Week 8	17/04/2017	Negative	10^{-3}	Negative	10^{-4}
	Week 12	17/04/2017	Negative	10^{-3}	Negative	10^{-4}

As previously discussed in the survival study for the LAMP assay, the results from the DNA persistence pilot study show that storage at 4°C is not recommended even for short-term storage and samples requiring a qPCR assay for detection of *S. stercoralis* DNA in clinical samples must be stored at -20°C. There is a 10- fold decrease in the sensitivity of detection of *S. stercoralis* DNA in samples stored at 4°C *cf*. samples stored at -20°C.

An anomalous result was seen in the week 4 sample stored at 4°C and tested on the 1st April 2017 where the lowest positive result was detected in a dilution of 10^{-2} when all other samples stored at 4°C detected *S. stercoralis* DNA at a dilution of 10^{-3}. This may be attributed to poor quality DNA or there may have been a dilution error in the dilution series. An anomalous result was also seen in the sample (week 5) stored at -20°C. Although the final dilution that was positive was 10^{-5} (a dilution increase of 10- fold), the C_t of 38.7, which is just before the C_t cut-off of 40, indicates the presence of a small amount of DNA. This sample was repeated on the 17th April 2017 and gave a final positive dilution of 10^{-4}. This does not alter the conclusion reached from the pilot study that storage at -20°C showed no deterioration of the DNA stored for up to 12 weeks.

4.10 STATISTICAL ANALYSIS

The study code was broken after all study samples had been analysed and statistical analysis was performed. The tests in the CRS were performed from the same sample that was sent for storage at 4°C or -20°C. Standard McNemar's test for the comparison of proportions and diagnostic sensitivity and specificity calculations were performed (MedCalc®, n.d.) and the results were recorded in Table 4.9. The sensitivity results were poor for the qPCR assay as an imperfect low sensitivity and low specificity composite reference standard was used. The CRS denoted proven disease or probable

disease and is, therefore, subject to bias in the sensitivity and specificity estimations (Baughman *et al*., 2008). The FDA guidelines (FDA, 2007) suggest reporting the overall percent agreement between the CRS and the new test when using an imperfect reference standard (Table 4.9).

Table 4.9: McNemar's test and 2x2 contingency table results for the qPCR assay and overall percent positive results (including 95% CI to determine significance)

STATISTICAL TEST		**CRS AND qPCR -20^{O}C**		**MICRO/CULTURE AND qPCR -20^{O}C**	
			95%CI		**95%CI**
McNemar	**Difference**	8.97%	3.72-13.32%	6.21%	2.39-8.85%
	Probability	p=0.0007		p=0.0013	
Fischer's exact	**Significance level**	p=<0.000001		p=<0.000001	
Diagnostic test 2x2	**Sensitivity**	39.73%	28.45-51.86%	74.07%	53.72-88.89%
	Specificity	92.70%	88.58-95.69%	90.68%	86.64-93.82%
	AUC	0.66	0.61-0.71	0.82	0.78-0.86
	Positive likelihood ratio	5.44	3.18-9.32	7.95	5.18-12.20
	Negative likelihood ratio	0.65	0.54-0.79	0.29	0.15-0.54
	Disease Prevalence (from the CRS)	23.86%	19.19-29.04%	8.82%	5.9-12.98%
	PPV	63.04%	47.55-76.79%	43.48%	28.93-58.89%
	NPV	83.08%	77.96-87.43%	97.31%	94.53-98.91%
FDA recommendation when using an imperfect reference standard (FDA, 2007)	**Positive percent agreement PPA (instead of sensitivity)**	40.32%	-	-	-
	Negative percent agreement NPA (instead of specificity)	91.9%	-	-	-
	Overall percent agreement OPA (between CRS and new test)	80.63%	70.5-91.8%	-	-

As previously discussed in Section 3.6, this study determined that the use of an imperfect reference standard achieved sensitivity and positive predictive values that could not be accurately assessed. The qPCR assay showed suitable specificity and negative predictive values. The negative percent agreement and the overall percent agreement values indicate that the qPCR assay will detect the presence of disease with a high probability, there was also a suitable negative predictive value determined for this test. A note of caution must be made as the negative predictive values are related to disease prevalence and this could not be determined in this study.

The overall percent agreement (OPA) will always lie between the negative and positive percent agreement, care must be taken with interpretation of the results as OPA does not differentiate between the agreement of the negatives and the agreement of the positives with an imperfect reference standard. Percent agreement can be used in conjunction with Cohen's kappa where the expected and observed results are expressed as a fraction of the maximum difference (FDA, 2007) (Table 4.10).

Table 4.10: Cohen's kappa tables for qPCR results when the CRS is negative (0), when the microscopy/ culture only is positive (1), when the serology only is positive (2) and when all the CRS tests are positive (3)

		qPCR RESULTS				
CRS RESULTS		**0**	**1**	**2**	**3**	**TOTAL**
Negative	**0**	206	4	31	2	243 (85.6%)
Microscopy/ culture positive	**1**	11	15	10	0	36 (12.7%)
Serology positive	**2**	5	0	0	0	5 (1.8%)
Full CRS positive	**3**	0	0	0	0	0
TOTAL		222	19	41	2	284
		-78.20%	-6.70%	-14.40%	-0.70%	

Kappa assumes independence this gave a weighted kappa of 0.229 with a standard error of 14.617 (95% CI: -1.0 – 1.0). There was an 85.6% agreement between the qPCR result and the CRS when the CRS is negative. When the microscopy was positive there was only a 12.7% agreement and there was little agreement between serology positive and qPCR. The lack of agreement between microscopy/ culture positive samples and qPCR is due to the fact that there were very few positive samples and qPCR sample volumes are much smaller than the volumes used for stool cultures. Verweij *et al*., 2009 noted a similar effect with qPCR negative and Baermann test positive samples. The low agreement between qPCR positive and serology positive samples is due to the fact that serology may be positive in cases where there is no active strongyloidiasis.

Positive predictive values for the qPCR assay cannot be determined as the disease prevalence is unknown in this study cohort, however negative predictive values show that qPCR can be used as a screening test as the detection of true negatives was above 80%. Jones and Payne (1997) stated that the evaluation of a new test aims for a power (sensitivity) of 80 to 95% and a probability (specificity) of 5 to 10%. A negative predictive value of >80% is an acceptable figure for a screening test.

As with the data described for the LAMP assay missing a true positive sample carries severe consequences for the patient (Pottie *et al*., 2011). However, a false positive, in the protocol for this study would be considered in the context of serology, qPCR and a clinical decision on whether repeat samples are required or if treatment is indicated.

Likelihood ratios are clinically important in the determination of how many times more or less likely the patient will have a positive or negative result in a patient with strongyloidiasis than in a patient without strongyloidiasis. When the positive likelihood ratio >1, it indicates that a positive result is more likely in a patient with strongyloidiasis than in a patient without the disease and the same holds true for a negative qPCR result in a patient without strongyloidiasis. A positive qPCR assay, in patients with strongyloidiasis, is 7.95 times more likely than a negative result when using proven disease as a comparator. This indicates that qPCR assays may be used for treatment monitoring. The same is not true for a negative qPCR result as a negative microscopy or culture result does not necessarily mean that the patient does not have strongyloidiasis due to the low sensitivity of these methods.

The purpose of this chapter was to ascertain the analytical sensitivity and specificity of a published qPCR assay to assess the suitability for diagnostic assay of strongyloidiasis in human faecal samples in a high- throughput specialist diagnostic well- resourced setting.

The conclusions from this part of the study were that qPCR is a useful adjunctive test to the diagnostic repertoire, but cannot fully determine cases of absence of disease. It is, however, a suitable diagnostic test with a specificity of >90%.

A discussion of published work for other parasites using qPCR is found in Chapter 6.

CHAPTER 5: ANALYSIS OF PATIENT DEMOGRAPHICS AND SAMPLE CONDITIONS AND COMPARISON OF LAMP AND qPCR RESULTS

5.1 INTRODUCTION

The data generated from this study was analysed after the study code had been broken and the results were separated into samples stored at 4°C or -20°C. The composite reference standard (CRS) divided the 284 samples into 17 true positive and 233 true negative for the LAMP assay and 29 true positive and 216 true negative samples for the qPCR assay as shown in Table 5.1.

Table 5.1: True positive (sensitivity) and true negative (specificity) diagnostic samples: determined by the CRS.

REFERENCE TEST		CRS DETERMINATION OF TRUE POSITIVE AND TRUE NEGATIVE	NUMBER OF SAMPLES POSITIVE: LAMP	NUMBER OF SAMPLES POSITIVE: qPCR
Microscopy and culture (proven cases)*	**Serology (probable cases)***			
P	**P**	TP (8) The negative LAMP assay results may be due low levels of DNA target in the sample[1].	6 (75%)	8 (100%)
P	**N**	TP (12). Serology negative, microscopy and/or culture positive may be due to early diagnosis, before the development of an antibody response or the lack of an antibody response in immunocompromised individuals	5 (41.7%)	8 (66.7%)
N	**P**	TP (42) (probable cases, positive results may be due to post treatment persistence of antibody)	4 (9.5%)	9 (21.4%)
N	**N**	TN (222)	1 (0.45%)	9 (4.1%, excluding the 2 anomalous bands 3.2%) 9 positive samples, two have an anomalous band that could not be identified

*P= positive N= negative TP= true positive TN= true negative

[1] qPCR: C_ts: 33.89, 28.47. (LAMP remained negative on repeated assays)

These results were used for the statistical analysis of the LAMP and qPCR assays to determine sensitivity, specificity, negative predictive values and overall percent agreement of the LAMP or qPCR assay with the CRS.

Patient demographics and sample quality were examined using stepwise logistic regression to determine if there was any effect on the detection of *S. stercoralis* DNA by the LAMP or qPCR assays by patient demographics (gender, patient age in years and country of travel or origin) or the sample conditions (length of storage, temperature of storage and aliquot size). Stepwise logistic regression was chosen to determine the effect of multiple variables on the LAMP or qPCR assays. Graphical analysis of the individual variables is demonstrated by percentage positive of the total number of samples in the study. The y axis maximum unit was set at 100% unless the effect was too small to be determined at this level and the y axis was adjusted accordingly.

Statistical analysis was performed (MedCalc®, n.d.) and the results compared between LAMP and qPCR to determine the efficiency and suitability of the assays for the detection of strongyloidiasis in a WTM clinic or in the field.

To further determine the suitability of the use of qPCR and LAMP in a WTM clinic a costing analysis was performed, the results of a further costing analysis determined the suitability of the LAMP assay for use in resource-limited areas. The introduction of a novel NAAT for the diagnosis of strongyloidiasis into the diagnostic repertoire at DCP require**s** a business plan (Appendix 5) and the full cost and suitability of the test chosen is discussed in Section 5.3, this Chapter).

5.2 PATIENT DEMOGRAPHICS

5.2.1 GENDER

There was no significant difference between the number of females and males in this study for the samples stored at 4^{o}C or -20^{o}C and very little difference was detected between the number of positive samples detected in the male and female groups in this study. Not all samples that were stored at 4^{o}C (n= 285) were also stored at -20^{o}C (n= 284). Stepwise logistic regression showed no difference for gender in the detection of *S. stercoralis* DNA by LAMP or qPCR, but this may be due to the small numbers of positive samples detected in this study. There were 53% (n=285) and 52% (n=284) males in the samples stored at 4^{o}C or -20^{o}C, respectively and 47% (n=285) and 48% (n=284) females in the samples stored at 4^{o}C or -20^{o}C, respectively. There was a consistent increase in the efficiency of detection of *S. stercoralis* DNA in samples stored at -20^{o}C by both the LAMP and qPCR assays. The results are demonstrated in Figure 5.1 by the percentage of total number of samples with positive results for the LAMP and qPCR assays.

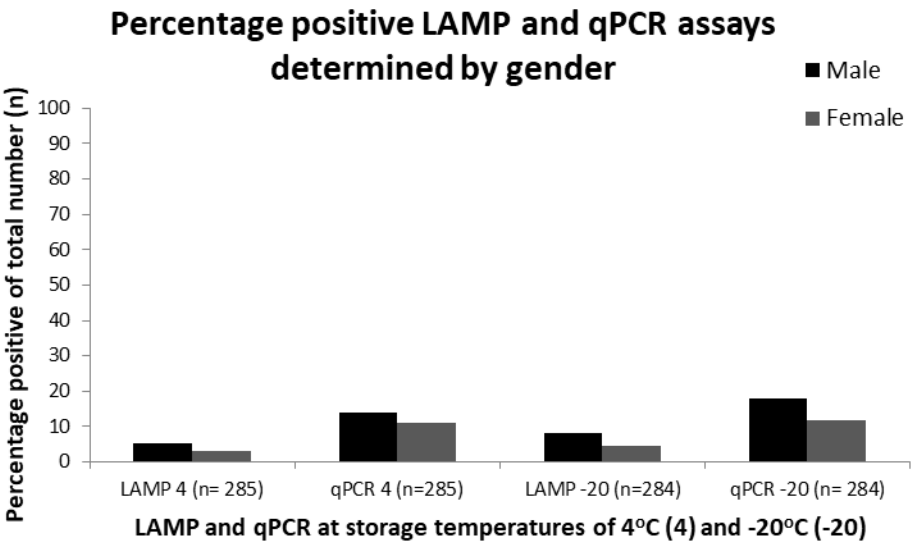


Figure 5.1 Percentage positive of total for LAMP and qPCR assays determined by gender in samples stored at 4°C or -20°C.

5.2.2. AGE (YEARS)

The majority of patients that attended the HTD travel clinic were in the 20 to 50 year age group. The patient population in this study is biased towards patients (including the "worried well") who seek post-travel advice with or without gastro-intestinal symptoms. Figure 5.2 shows the percentage of LAMP and qPCR positive results for the total number of samples in the different age groups in the samples stored at 4°C or -20°C. Stepwise logistic regression showed no significance in the age of patient for the detection of *S. stercoralis* DNA by LAMP or qPCR assays in the samples stored at 4°C. The analysis did show a small effect in qPCR assay detection of *S. stercoralis* DNA in the samples stored at -20°C (Odds ratio= 0.7128 that the qPCR assay will be positive, 95% CI: 0.5800 to 0.8759). A larger set of positive samples might be able to further detect a significant difference in the detection of *S. stercoralis* DNA by the LAMP assay in samples stored at 4°C or -20°C or by the qPCR assay in samples stored at 4°C, but this was not possible in this study due to the low number of positive results. The findings are a reflection of unavoidable patient bias in the study cohort due to patient demographics. The number of study samples found in each of the age groups is shown in Table 5.2. The study samples were stored at 4°C or -20°C but not all of the study samples were stored in duplicate, therefore the study analysis was performed on the samples stored at 4°C or at -20°C. Both sets of study samples generated data with a sufficient power for statistical analysis.

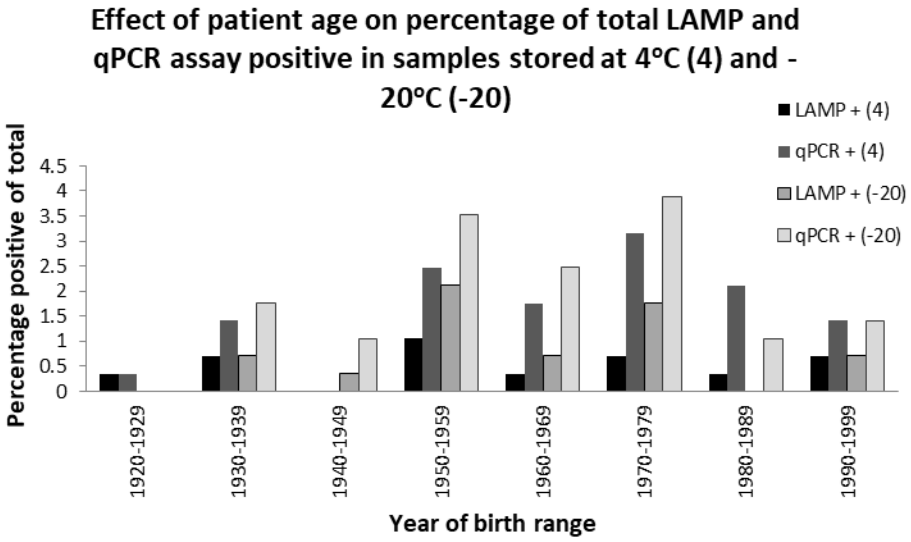


Figure 5.2: The effect of patient age on percentage of total LAMP and qPCR assay positive in samples stored at 4°C or -20°C.

Table 5.2: Number of study samples in the different age groups in samples stored at 4°C or at -20°C.

AGE RANGE (YEARS)	STORAGE AT 4°C	STORAGE AT -20°C
1920-1929	4	4
1930-1939	12	11
1940-1949	21	22
1950-1959	35	34
1960-1969	61	60
1970-1979	61	60
1980-1989	59	60
1990-1999	32	33
TOTAL	**285**	**284**

5.2.3. COUNTRY OF TRAVEL/ RESIDENCE

In the majority of samples travel history was not able to be determined or the travel history was unclear as many countries had been visited (worldwide). Where the travel history was known the most frequent countries visited were in Africa and Asia. Biggs *et al*. (2009) has previously noted that strongyloidiasis is common in migrants from Africa and South East Asia. Stepwise logistic regression showed no significance in the country travelled to, or originated from, for the detection of *S. stercoralis* DNA by the LAMP or qPCR assays in samples stored at 4°C or -20°C. However, the

efficiency of the detection of *S. stercoralis* DNA was improved in the samples stored at -20°C. The difficulty in ascribing a geographical region to samples from patients at HTD has been noted before (Sudarshi *et al*., 2003) as travel history may be complicated or not documented. The percentage positive LAMP and qPCR assay results of the total number of samples stored at 4°C or -20°C is shown in Figure 5.3. It must be noted that there were very small numbers of samples with a travel history to the Caribbean, Europe and the Middle East and the analysis of these categories must be interpreted with care. A comparison of data was only performed for LAMP (Figures 3.21 and 3.22) for those samples with a travel history to Africa (n=73, all samples) or Asia (n=73, all samples) and demonstrates the effect of serology results on the CRS. Anomalous band sizes were detected in this study but could not be explained as being due to geographical differences as the low number of positive results in the LAMP and qPCR assays and the complicated travel histories made this finding unsuitable for statistical analysis. The comparison of data to demonstrate the effects of the CRS on the qPCR assay is presented in Table 4.10. The total number of positive samples by geographical region in the study samples stored at 4°C or -20°C is shown in Table 5.3.

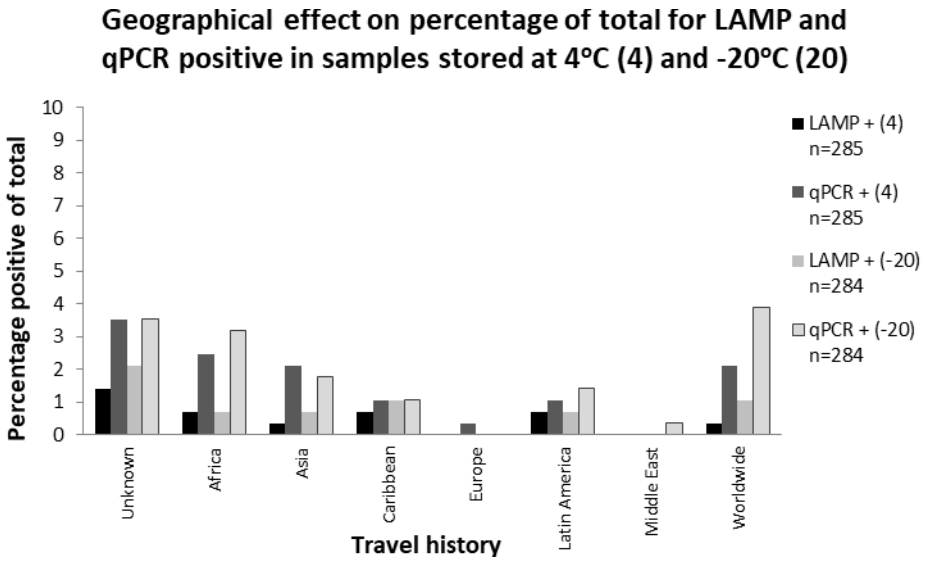


Figure 5.3: Geographical effect on percentage of total LAMP and qPCR assay positive in samples stored at 4°C or -20°C.

Table 5.3: Number of positive samples in each geographical region and the number of LAMP or qPCR positive assays in samples stored at 4°C or -20°C.

GEOGRAPHICAL REGION	TOTAL NUMBER 4°C	LAMP POSITIVE 4°C	qPCR POSITIVE 4°C	TOTAL NUMBER - 20°C	LAMP POSITIVE -20°C	qPCR POSITIVE -20°C
UNKNOWN	**57**	4	10	**57**	6	10
AFRICA	**65**	2	7	**68**	2	9
ASIA	**69**	1	6	**69**	2	5
CARIBBEAN	**8**	2	3	**8**	3	3
EUROPE	**8**	0	1	**7**	0	0
LATIN AMERICA	**17**	2	3	**16**	2	4
MIDDLE EAST	**2**	0	0	**2**	0	1
WORLWIDE	**59**	1	6	**57**	3	11

5.3 SAMPLE CHARACTERISTICS

The quality of the samples, stored at 4°C or -20°C before DNA extraction, was investigated to determine if this had an effect on the detection of *S. stercoralis* DNA by the LAMP or qPCR assays. The percentage of the total number of samples that were LAMP or qPCR positive was compared to aliquot size, length of storage and storage temperature.

5.3.1. LENGTH OF STORAGE

Samples were collected and stored at 4°C or -20°C (without preservatives) from 2011- 2016. Those samples collected in 2015-2016 were stored for a period of less than 6 months before DNA extraction. The extracted DNA was stored at -20°C until the LAMP or qPCR assay was performed. DNA extraction on samples stored from 2011- 2014 was carried out from January 2015. Stepwise logistic regression did not demonstrate a statistically significant effect on the detection of *S. stercoralis* DNA by the length of time the samples were stored at 4°C or -20°C by LAMP or qPCR. The largest number of samples in the study was collected in 2015- 2016 and this is demonstrated in Figure 5.4. The y axis maximum was set at 10% to demonstrate the effect of length of storage at 4°C or at -20°C on the low number of positive samples obtained in this study.

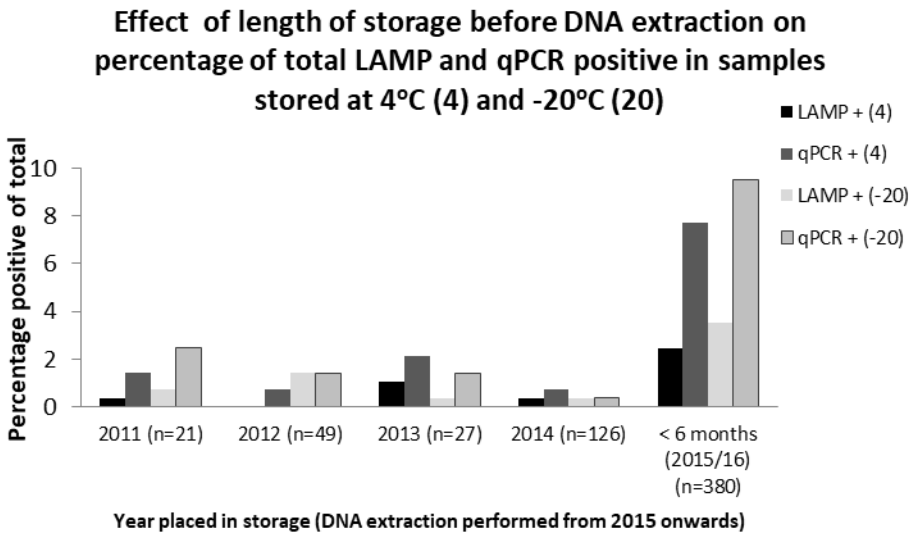


Figure 5.4: Effect of length of storage at 4°C or -20°C before DNA extraction on the percentage of total LAMP and qPCR assay positive.

5.3.2. ALIQUOT SIZE

Martins and De Paula (2015) suggested that the difference in the sensitivity for the detection of *S. stercoralis* DNA in true positives samples (determined in this study by composite reference standards of culture and microscopy only) between studies may be due to the different sizes of stool aliquots that were extracted. To determine if aliquot size would have an effect on this study this factor was included in a stepwise logistic regression and demonstrated no significant difference in the detection of *S. stercoralis* DNA. The aliquot size for DNA extraction varied due to consistency and hydration of the samples (Lewis and Heaton, 1997) and Figure 5.5 shows the percentage of the total number of samples with LAMP and qPCR positive results in the different aliquot sizes. The y axis has a maximum unit of 6% to demonstrate the effect that a low number of positive samples has on the LAMP and qPCR assays.

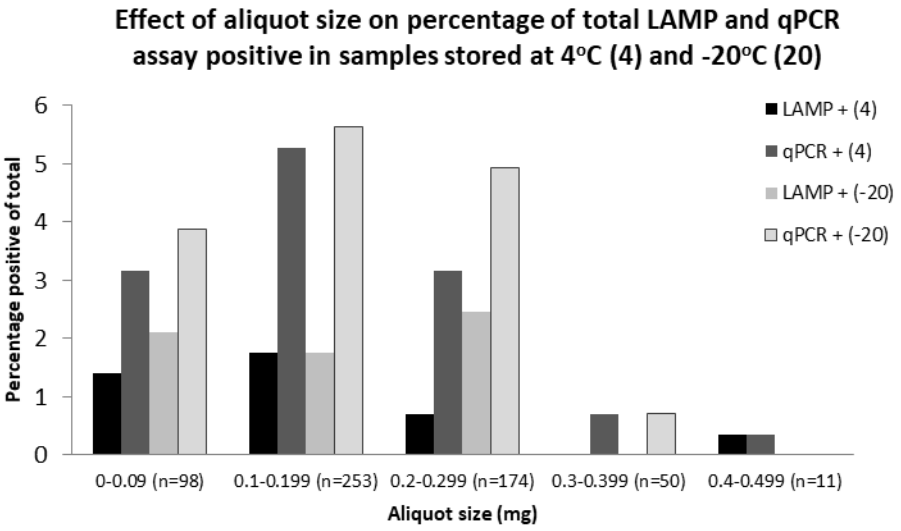


Figure 5.5: Effect of aliquot size on percentage of total LAMP and qPCR assay positive in samples stored at 4°C or -20°C.

5.3.3. STORAGE TEMPERATURE

Samples for this study were aliquoted and stored at 4°C or -20°C before DNA extraction. Samples stored at 4°C should be kept in ethanol to preserve the DNA or stored neat at -20°C (ten Hove *et al.*, 2009). This was not done during this study as the IDEA study samples were collected and stored in the laboratory at DCP before the start of this study. The current laboratory protocol for detection of DNA in stool samples states that samples are stored at 4°C without preservative (up to 3 weeks) until DNA extraction is performed.

Integration of a NAAT into the current workflow required an assessment of the effect of storage temperature. To determine the effect that sub-optimal storage would have on the efficiency of the LAMP and qPCR assays to detect *S. stercoralis* DNA this storage method for the aliquots was maintained for all samples. The effect was measured after the study had been completed.

The aliquots stored at 4°C were given a different study number to the aliquots stored at -20°C so that the effect of storage temperature could not be linked to the LAMP or qPCR results until the study code was broken. There was an increase in the percentage positive LAMP and qPCR results for the total number of samples stored at -20°C in comparison to the total number of positive samples stored at 4°C before DNA extraction. Stepwise logistic regression did not demonstrate a significant difference between the LAMP or qPCR assay results in the samples stored at 4°C or -20°C but the number of positive samples in all groups was small and this necessitates that the analysis

must be interpreted with care. Figure 5.6 shows the effect of storage temperature on the detection of *S. stercoralis* DNA in clinical samples.

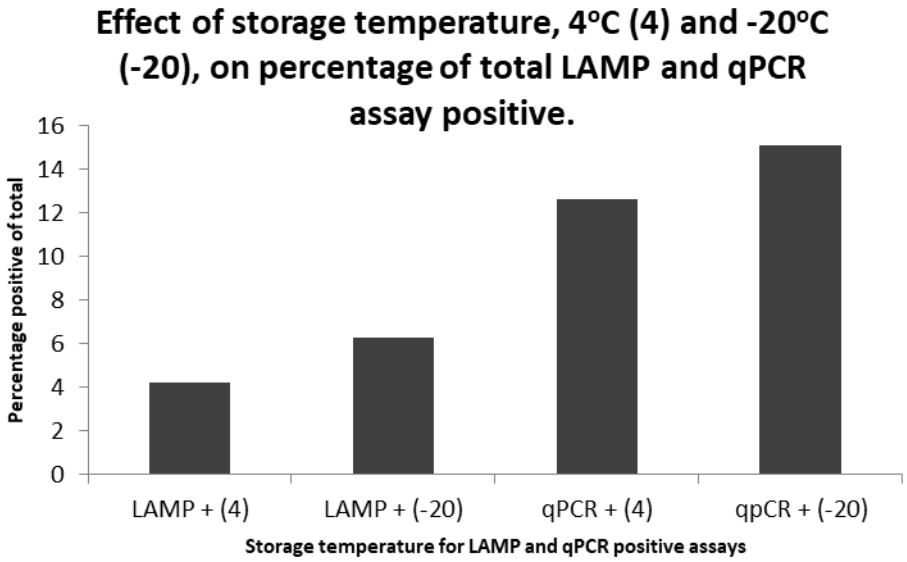


Figure 5.6: The effect of storage temperature (4°C or -20°C) before DNA extraction on percentage of total LAMP and qPCR assay positive.

The results were analysed using Friedman's test (with a significance level of 0.05), with the normalised C_t values or LAMP time in minutes as the dependent variable and temperature as the independent variable (Medcalc®, n.d.) (Table 5.4).

Table 5.4: Friedman's non-parametric test for the difference between samples stored at 4°C or -20°C in the detection of *S. stercoralis* DNA using LAMP or qPCR assays.

	LAMP AND qPCR (4°C)	**LAMP AND qPCR (20°C)**	**LAMP (4°C and -20°C)**	**qPCR (4°C and -20°C)**
P (significance P<0.05)	0.00026	0.00371	0.00007	0.00098

Friedman's test demonstrated a significant difference between the samples stored at 4°C and -20°C. The samples were analysed by LAMP and qPCR assays for temperature (4°C or -20°C) and for NAAT (LAMP or qPCR) to detect any differences between the samples.

The decrease in sensitivity of both the LAMP and qPCR assays in samples stored at 4°C and the significant difference demonstrated by Friedman's test decided the final statistical analysis of LAMP and qPCR assays for those samples stored at -20°C only. Friedman's test also demonstrated

significant difference in the detection of *S. stercoralis* DNA by the LAMP or qPCR assays in samples stored at the same temperature (4°C or -20°C).

A separate study investigating the role of NAATs in determining point of cure after treatment is under way. The samples for this study are being stored at -20°C only before DNA extraction is performed.

5.4 COMPARISON OF LAMP AND qPCR TO ASSESS THE SUITABILITY OF THE ASSAYS FOR DETECTION OF *S. STERCORALIS* DNA IN CLINICAL SAMPLES

5.4.1. COMPARISON OF LAMP AND QPCR POSITIVE RESULTS AT STORAGE TEMPERATURES OF 4°C OR -20°C

Determination of LAMP positive results when the qPCR is positive was performed on all study samples tested to determine the maximum C_t (related to decreasing amounts of target DNA in the sample) at which a LAMP positive result will be obtained. A comparison of all the results for LAMP and qPCR (including duplicates) at 4°C and -20°C (n=610) demonstrated that a LAMP positive result was obtained when qPCR $C_t \leq 31.46$ (mean $C_t = 23.25$ at which a LAMP positive result is obtained). No samples were qPCR negative and LAMP positive. Figure 5.7 demonstrates the comparison of LAMP and qPCR positive results in a notched Box and Whisper plot (MedCalc®, n.d.).

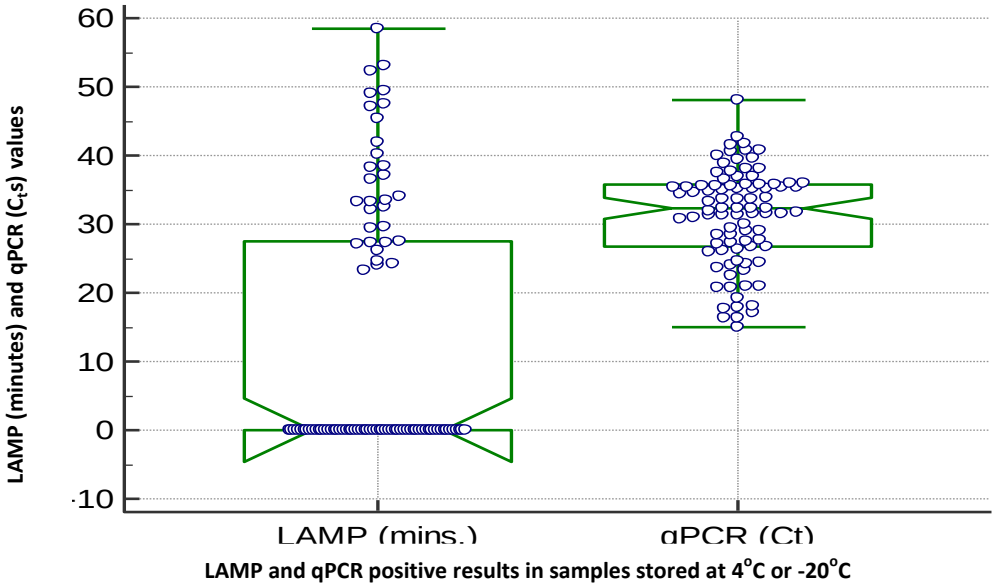


Figure 5.7: Box and Whisper Plot for the comparison of LAMP positive (minutes) *vs*. qPCR positive (C_t). (qPCR: n=90 positive, LAMP: n=31 positive i.e. LAMP was positive in only 34% of positive qPCR samples)

5.4.2. THE EFFECT OF SEROLOGY ON THE COMPOSITE REFERENCE STANDARD

The persistence of antibody, even after successful treatment, is known and may only denote probable or past disease (Requena-Méndez *et al*., 2013). The composite reference standard was deconstructed into the individual tests. The number of positive results (LAMP or qPCR) was expressed as a percentage of the total number of samples for the composite reference standard, microscopy/ culture or serology. The percentage of positive samples (from the total number of samples assayed) for the LAMP and qPCR assays was determined. The results were analysed for the 4°C and -20°C stored samples separately to demonstrate the difference in sensitivity of the NAATs (Figure 5.8).

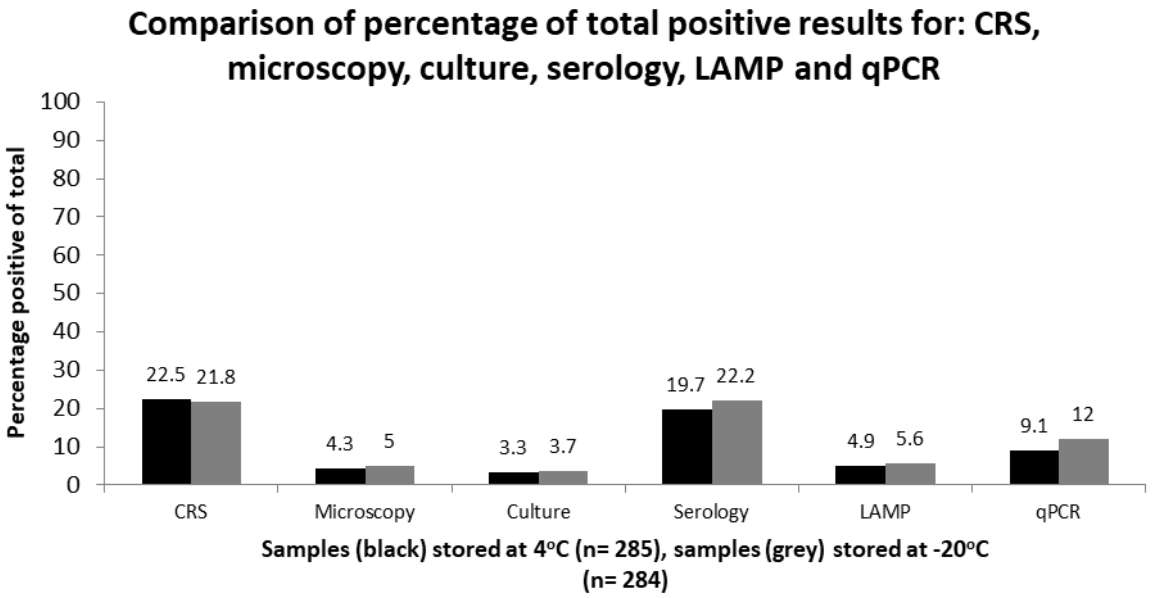


Figure 5.8: Comparison of percentage of total positive results for the combined reference standard (microscopy, culture and serology) or microscopy, culture or serology positive and LAMP or qPCR positive.

As noted previously not all samples were stored both 4°C and at -20°C so the data generated for microscopy, culture and serology differs slightly. Nevertheless significant results can be determined from the data generated. In the study samples the LAMP assay demonstrated an increase of only 0.6 % in detection of *S. stercoralis* DNA when compared to microscopy percentage positive (definitive diagnosis) in samples stored at 4°C and at -20°C. The qPCR assay showed an increase of 4.8 % in the detection rate for samples stored at 4°C and an increase of 7 % in the detection rate for samples stored at -20°C when compared to microscopy percentage positive.

5.4.3. COMPARISON OF TEST PERFORMANCE, TURNAROUND TIME AND COST BETWEEN LAMP AND qPCR

Minetti *et al*. (2016) highlighted that there are three main obstacles to the uptake of diagnostic tests:

I. The standard method to use for quality standards and agreement on the best approach;
II. Cost of advanced equipment, training and reagents;
III. Result interpretation regarding the use of molecular data to inform decision making by the development of suitable guidelines (Figure 6.1).

The standard methods and study design have been previously discussed in Chapters 2, 3 and 4 and result interpretation has been previously discussed in Chapters 3 and 4. .

A comparison of the cost to the laboratory and the time taken to perform LAMP and qPCR was performed to determine the effect of the introduction of a NAAT into the routine diagnostic laboratory workflow (Table 5.5).

Table 5.5: Comparison of test performance, turnaround time, analytical sensitivity (positive stool control in a 10-fold dilution series), analytical specificity and cost between LAMP and qPCR

	LAMP	**qPCR**
Turnaround time: Processing (time taken)	2.5 days. Processing for LAMP may be reduced to 30 minutes using a manual DNA extraction method (e.g. PURE®)	2.5 days
Turnaround time: Testing (time taken for assay and analysis)	1.5 hours	3.5 hours
Analytical sensitivity (spiked positive stool samples)	1 *S. stercoralis* larva x 10^{-3}/ µl	1 *S. stercoralis* larva x 10^{-4}/ µl
Analytical sensitivity (purified DNA)	117.2 x 10^{-9} ng of DNA	154.4 x 10^{-9} ng of DNA
Analytical Specificity (*cf*. Specificity bank)	100	97
% NAAT positive when serology is positive (n=101) (in samples stored at 4°C and -20°C)	5.94	17.8
% NAAT positive when microscopy/ culture is positive(n=53) (in samples stored at 4°C and -20°C)	37.7	64.1
Cost per test (including equipment, reagents and staff)	£20.18	£44.09

Analytical sensitivity and specificity (previously calculated for the LAMP and qPCR assays) and the limit of detection for the assays were included to determine the most suitable assay for the introduction into the diagnostic repertoire. The turnaround time was determined from the time taken to process the sample, perform the assay and analyse the data.

The department required a business plan (Appendix 5) to be completed which included a costing analysis. The cost was calculated using the time taken by a senior member of staff to perform the assays and the middle scale of the salary range for a senior member of staff (£10.25 per hour). DCP is a specialist referral laboratory and all specialist staff members are senior biomedical scientists with specialist parasitology training and knowledge. Processing of samples for DNA extraction was not added to the calculation for staff time as the extraction is an automated process. LAMP costs are 54% less than the costs for qPCR and the results for LAMP can be obtained from extracted DNA

within 1.5 hours rather than the 2.5 hours for qPCR post- processing. The business plan for assays at DCP must be adjusted for use in different settings where staffing and running costs in WTM or endemic areas will be unrelated (European Commission Directorate-General Education and Culture, 2006).

There was no difference in analytical sensitivity and the analytical specificities were greater than the 80% lower limit described by Jones and Payne (1997) for a new diagnostic test. A new diagnostic test ideally has a sensitivity of 100% and specificity of 100%, however, this is not the case in biological systems where inhibition and sampling can influence the result and a probability of a false positive of 5-10% and a power of 80-90% to detect true negative results is chosen to determine the usefulness of new diagnostic test.

Diagnostic sensitivity and specificity, (determined from the results of the patient study, excluding serial and duplicate samples in the samples stored at -20^{o}C) was 23.29% (95% CI: 14.19-34.65%) for sensitivity and 99.57% (95% CI: 97.63-99.99%)for specificity with the LAMP assay; 39.73% (95% CI: 28.45-51.86%) for sensitivity and 92.70% (95% CI: 88.58-95.69%) for specificity with the qPCR assay, using the CRS as the reference standard. A sensitivity of 46.15% (95% CI: 26.59-66.63%) and a specificity of 97.86% (95% CI: 95.39-99.21%) was obtained for the LAMP assay and a sensitivity of 74.07% (95% CI: 53.72-88.89%) and a specificity of 97.86% (95% CI: 86.64-93.82%) was obtained for the qPCR assay using microscopy/ culture positive as the reference standard.

The area under the ROC curve (AUC) is a measure of the ability of the assay (LAMP or qPCR) to distinguish the true population from the area under the curve and the 95% CI is the interval in which the true population is found in the area under the ROC curve. The closer the AUC is to 1 the better the test is to distinguish between the two groups. The p value determines the significance value for the null hypothesis, when p> 0.05 there is no difference between the two tests. The AUC for the LAMP assay (using the CRS as the reference standard) is 0.61 (p=<0.0001) and for the qPCR assay it is 0.66 (p=<0.0001). The AUC assay (using microscopy/ culture positive as the reference standard) for the LAMP is 0.72 (p=<0.0001) and for the qPCR assay it is 0.82 (p=<0.0001). The null hypothesis for this study was, therefore, rejected.

Disease prevalence was not known in this study cohort so positive predictive value was not able to be interpreted. The negative predictive value (NPV) or true negative value using the CRS as a reference standard for the LAMP assay was 83.08% (95% CI: 75.51-84.96%) and for the qPCR assay it was 80.56% (95% CI: 77.96-87.43%). Using microscopy/ culture positive as the reference standard the NPV for the LAMP assay was 95.14% (95% CI: 91.98-97.32%) and for the qPCR assay it was

97.31% (95% CI: 94.53-98.91%). When serology was negative (determined as part of the CRS) LAMP detected one extra positive result and qPCR detected four extra positive results, the LAMP positive (40.12 minutes) result was also qPCR positive (C_t 27.15) with a 101bp product identified on the agarose gel. This may be due to early infection where the specific antibodies have not yet developed or an immunocompromised condition where the antibody levels may not develop at all (Requena-Méndez *et al*., 2013). There was no further information in the case notes regarding this patient. This case was also microscopy and culture negative, indicating that the larval load was below the limit of detection for both microscopy and culture.

Where the CRS was determined to be positive by serology only- LAMP was positive in 6.3% of cases and qPCR was positive in 18.75% of cases. This result is difficult to interpret as specific antibodies are known to persist up to 12 months after successful treatment. Nevertheless the risk to the patient may be significant if a false negative result is recorded and NAATs cannot, at present, replace serology as a screening test for strongyloidiasis.

LAMP detected 0.45% and qPCR detected 1.8% of cases when all the tests in the reference standard were negative. NAATs will therefore be a useful addition to the diagnostic repertoire for strongyloidiasis at DCP as they may detect strongyloidiasis cases in individuals where the CRS is negative.

The qPCR assay, which requires DNA extracted from clinical stool samples using a labour and time intensive method to minimise the effect of inhibition on qPCR and is 54% more costly than LAMP, was determined to be an improved method for the detection of *S. stercoralis* DNA in clinical samples compared with the LAMP assay.

This study concluded that the introduction of a qPCR assay into the diagnostic laboratory workflow was superior to the introduction of a LAMP assay. Furthermore the qPCR assay is suitable for deployment into the workflow of a high- throughput automated diagnostic laboratory where existing technology is available.

The same extracted DNA samples were used for the LAMP and qPCR assays and so the difference in the detection of target DNA and in the analytical measures might be attributed to the target alignment on the 18S rRNA genome (Section 6.4, Appendix 3a). The LAMP assay is 10- fold less sensitive than the qPCR assay and may not be able to detect all cases of strongyloidiasis. LAMP is reported to be more sensitive than cPCR (Notomi, 2000) but this was not demonstrated in this study.

There was no difference in the limit of detection between the LAMP and qPCR assays when a limit of detection (LOD) assay run was performed on purified DNA from a cloned insert of the cPCR product. The effect of stool inhibition of the LAMP assay was excluded when the limit of detection was performed on cloned purified DNA. The LAMP assay had an LOD of 117.2 x 10^{-9} ng of DNA and the qPCR assay had an LOD of 154.4 x 10^{-9} ng of DNA. This is discussed further in Section 6.4.

A separate costing analysis was performed for the cost of the LAMP assay for use in resource-limited areas. A method of rapid ultrapure DNA extraction was tested for use without the need for technical expertise and costly automated equipment and reagents. A simpler manual DNA extraction method was also investigated that requires only a hot block, a centrifuge (the “boil and spin” method). The cost of staff and equipment was not included in the costing analysis because epidemiological control and monitoring studies will be funded in a different way to well-resourced WTM departments (European Commission Directorate-General Education and Culture, 2006, FIND, 2012) (Table 5.6). This is discussed in Section 6.5. The price for DNA extraction was calculated from the cost of reagents for the study when they were ordered.

Table 5.6: Calculation of the cost per LAMP test using automated or manual DNA extraction.

REAGENTS AND CONSUMABLES	Qiagen® DNA EXTRACTION (192 tests per kit)	EIKEN CHEMICAL CO., LTD. PURE® DNA EXTRACTION (90 tests per kit)	BOIL AND SPIN MANUAL DNA EXTRACTION
DNA extraction per test	3.02* (price in 2014)	2.81* (price in 2017)	0.7*
LAMP per test	0.7	0.7	0.7
Consumables	1.08	1.08	1.08
Turnaround time	2 days	1.5 hours	1.5 hours
Equipment, staff and running costs	Not included	Not included	Not included
TOTAL per test	£4.80	£4.59	£2.48

*The cost is reduced when large numbers of samples are processed (Eiken Chemical Co. Ltd., 2005)

There is very little difference in cost using the prices quoted by the manufacturers of the PURE® and automated DNA extraction methods (Eiken Chemical Co. Ltd., 2005, Qiagen, 2013). The savings will occur in the elimination of the need for high cost and maintenance equipment and the requirement for highly trained staff.

LAMP can be set up in a high throughput format which greatly reduces the cost of the assay (Perera *et al*., 2017). FIND negotiates the costs for studies in endemic areas on an individual basis (Eiken Chemical Co. Ltd., 2005, European Commission Directorate-General Education and Culture, 2006, FIND, 2012). This study demonstrated that the simple "boil and spin" method with further optimisation would prove a very useful simple cost- effective tool for use in resource- limited settings.

5.5 COMPARISON OF REPRODUCIBILITY FOR LAMP AND qPCR ASSAYS USING PAIRED SAMPLES STORED AT 4^{o}C AND -20^{o}C AND A POSITIVE PAIRED STOOL SERIES

Two patients had a series of stool samples stored in 2012 (archived stools not collected as part of the study so no details were available) only 1 patient was positive. The positive samples were examined as an evaluation of the replication of results where duplicate samples were stored at 4^{o}C and -20^{o}C and a series of samples was kept (Table 5.7).

Table 5.7: Positive stool series: LAMP and qPCR results in samples stored at 4°C and -20°C

SAMPLE	COMPOSITE REFERENCE STANDARD	LAMP + (-20)	qPCR + (-20)	LAMP + (4)	qPCR + (4)
1	Microscopy positive	0	34.61	0	33.57
2	Microscopy positive	49.36	31.46	0	31.29
3	Microscopy positive	0	33.65	0	35.56
4	Microscopy positive	0	30.84	0	31.84
	MEAN	Not applicable	32.64	Not applicable	33.065
	STANDARD DEVIATION	Not applicable	1.78	Not applicable	1.93

The LAMP assay detected the target DNA in only one sample (sample 2) with a C_t of 31.46 (stored at 4°C). This sample was also stored at – 20°C with a qPCR C_t of 31.29 where the LAMP assay was negative. The qPCR positive (C_t of 31.29) and LAMP assay negative sample was stored at 4°C for three years before DNA extraction was performed.

The C_t is proportional to the amount of target DNA present in the sample. The LAMP assay detected the target DNA at the limit of detection (when qPCR $C_t \leq 31.46$) and may indicate that insufficient target DNA was present in the rest of the samples for detection by the LAMP assay (Morrison *et al*., 1998). As previously discussed the storage of samples for the LAMP assay, before DNA extraction, was sub- optimal and this could have influenced the results seen. No conclusions about the replication of results for the LAMP assay could be determined, however, the qPCR assay showed good replication of results in a series of duplicate samples. As for the LAMP assay, improved performance was demonstrated by the qPCR assay in samples stored at -20°C.

These results support the previously discussed DNA survival study for the LAMP and qPCR assays which demonstrated that samples for *S. stercoralis* DNA detection must be stored at -20°C and tested as soon as possible. A further study into the optimal storage conditions of stool samples for the LAMP assay is required.

The purposes of this chapter were to assess the effect of external parameters on the LAMP and qPCR assays and to compare the suitability and cost of the two assays for diagnosis of strongyloidiasis in human faecal samples in well- resourced and resource- limited settings.

The LAMP assay has the potential to be deployed in a suitable format for use in resource- limited areas, however further work is required to optimise sample storage and manual DNA extraction methods before this assay can be recommended.

The qPCR assay is suitable for use in a high- throughput busy WTM setting, but is too costly for, and requires resources that are not available in, resource- limited areas. One step qPCR kits for use with

purified DNA templates are available (Qiagen, n.d.) with a reaction time shortened to one hour making the introduction of qPCR assays for multiple parasite targets a feasible option for a specialist parasitology referral laboratory.

CHAPTER 6: DISCUSSION, CONCLUSIONS AND FURTHER RESEARCH

This study focussed on the development of a novel LAMP assay for use in resource- limited settings and the evaluation and validation of the LAMP and qPCR assays for the diagnosis of strongyloidiasis in a specialist parasitology referral diagnostic laboratory. There is a need for the development of suitably sensitive and specific tests (Requena-Méndez *et al*., 2013) that would be useful as an addition to, or a replacement for, the current diagnostic repertoire at DCP.

The introduction of NAATs for some tropical diseases that are suitable for use in resource- limited areas has already been reported in the literature e.g. LAMP diagnostics for malaria (Polley *et al*.,2013, Perera *et al*., 2017) and for the detection of *T. cruzi* (Thekisoe *et al*., 2010). At DCP the introduction of qPCR for the detection of microsporidia (a group of parasitic intracellular fungi) (Polley *et al*., 2011) and a multiplex protist qPCR for the detection of *E. histolytica*, *G. lamblia/ intestinalis* and *Cryptosporidium* sp. (ten Hove *et al*., 2007) has enhanced the diagnosis of these organisms. Multiplex qPCR is available for the detection of other nematode human pathogens. Verweij *et al*. (2006) described a multiplex qPCR for the detection of *Ancylostoma duodenale*, *Necator americanus*, and *Oesophagostomum bifurcum* in faecal samples. These reports indicated that LAMP and qPCR assays could be successfully developed for the detection of *S. stercoralis.* Future development of these assays to detect parasitic infections in multiplex formats (Iseki *et al*., 2007, Verweij *et al*., 2006) will enhance the diagnostic throughput for the molecular detection of intestinal parasites. Jaleta *et al*. (2017) concluded that molecular diagnosis of *S. stercoralis* is important as the clinical outcomes of different species is not yet known and may have a role to play in the treatment options for strongyloidiasis in humans and canines.

The aim of this study was, therefore, to evaluate and validate nucleic amplification tests for the detection of *S. stercoralis* in clinical samples in a well- resourced specialist parasitology reference laboratory and to develop a LAMP assay that could be used for the diagnosis of strongyloidiasis in resource- limited areas with appropriate sensitivity and specificity estimations. The main objective was the development of a “fit for purpose” (CPA standards F1, ISO 15189:2012) (UKAS, n.d., ISO 15189:2012, 2012) diagnostic screening strategy for introduction into the testing repertoire of a specialist parasitology referral laboratory to enhance clinical care for strongyloidiasis.

6.1 EVALUATION CRITERIA

Peeling *et al*. (2007) discussed the problems associated with evaluating diagnostics. This study was designed to address the potential shortcomings that were highlighted in that report (Table 6.1).

Table 6.1: Problems that may be encountered when performing diagnostic test evaluation: the design of this study to address these potential problems. (Adapted from Peeling *et al*., 2007)

POTENTIAL PROBLEM	THIS STUDY
Evaluation in an inappropriate study group	This study examined a cohort of patients being investigated for strongyloidiasis at HTD and UCLH. While HTD is not in an endemic setting some bias in the choice of samples was introduced by the fact that patients were being investigated for strongyloidiasis due to previous exposure in an endemic setting. This was unavoidable for this patient cohort.
Evaluation in an inappropriate setting	A higher number of false positive than true positive results would be expected in a low prevalence setting e.g. HTD. Overall percent agreement was used to determine the suitability of the assays as this is independent of prevalence in the current and new assays.
Inappropriate purpose	This study was used to develop and investigate NAATs for detection of strongyloidiasis in asymptomatic patients. The appropriate statistical analysis was chosen to investigate the tests for this use (McNemar's test for comparison of paired data and overall percent agreement)
Inappropriate reference standard	This was an unavoidable problem as there are no sensitive and specific tests that can be used as a "gold standard" however, a search of the literature found that a composite reference standard (CRS) could be used to eliminate bias in sensitivity estimations. A best possible CRS was chosen for this study and the CRS was broken down into two parts (a) the use of the full CRS that includes a test that indicates probable disease and (b) the use of the CRS with only those tests that indicate proven disease. LAMP (a novel assay developed at DCP) was compared to qPCR, which is a published method (Verweij *et al*., 2009), to overcome the limitations of the lack of a suitable "gold standard"
Inadequate sample size	An appropriate sample size was calculated with a significance level of 0.05 and a power of 0.10 to detect a difference of at least ten in the rows and columns of a 2x2 table using McNemar's test for the comparison of paired data.
Lack of blinding	All samples were blinded at the start of the study by a database curator, not the researcher, and the code was broken after the study was completed
Assessing the quality of an evaluation trial	Quality assurance was assessed by STARD (HPA UK protocols, 2013, Bossuyt *et al*., 2015), CPA and ISO 15189.2012 standards (UKAS, n.d., ISO 15189:2012, 2012) in this study

The study examined samples from patients (returning travellers and migrants) who were being investigated for strongyloidiasis on the basis of travel history, symptomology or screening of patients with compromised immunity and a relevant travel history. Spectrum bias was, therefore, unavoidable and overstated the sensitivity and specificity estimations (Pewsner *et al*., 2011). Nevertheless, the assays evaluated in this study are to be deployed for use in patients with a high index of suspicion for strongyloidiasis and the study cohort was deemed to be appropriate. The limitations encountered in this study for the analysis of the data are discussed in Sections 2.8, 3.9 and 4.10. The NAAT assays (detection of specific target DNA) were compared to microscopy/ culture (detection of whole parasite) and serology (detection of specific antibodies) in this study.

The selection of an appropriate assay must be considered in the context of utilisation of the assay and consequences of the disease (Caraguel *et al*., 2011).

WHO (2013) described the selection requirements for a diagnostic test as:

I. Screening for the disease requires a high sensitivity to confirm that the individual is free of disease;
II. Diagnosing the disease in symptomatic patients requires a test with high specificity;
III. Monitoring treatment requires a test with suitable sensitivity and high specificity. Culture with a sensitivity of only 70% (Requena-Méndez *et al*., 2013) and a requirement for untreated parasites is unsuitable for treatment monitoring and is not sensitive enough to detect low levels of parasite present in the stool samples.

The sensitivity of a test is the true positive rate of a test and specificity is the true negative rate of a test. These parameters are independent of the disease prevalence and together determine the diagnostic power of the test (Pewsner *et al*., 2011). A negative result in a test with high sensitivity is useful for ruling out those who do not have the disease (a screening test) however this test will produce false positive results so it is not a useful test for diagnostic purposes. A positive result in a test with high specificity will determine a high probability of the disease (a diagnostic test) (Pewsner *et al*., 2011).

In a low prevalence setting (e.g. HTD) a higher false positive to true positive results ratio may be detected than in endemic settings (Peeling *et al*., 2007). However, in a chronic disease, such as strongyloidiasis, where the subsequent consequences of a missed diagnosis may be fatal (Pottie *et al*., 2011, Barros and Montes, 2014, Levenhagen and Costa-Cruz, 2014) the selection of a test with a low level of false positive results may be deemed appropriate (Caraguel *et al*., 2011). At DCP,

microscopy, culture and serology form part of the diagnostic repertoire and the decision to treat patients on the basis of only a positive qPCR assay would require clinical input.

6.2 DEVELOPMENT AND EVALUATION OF A NOVEL LAMP PCR FOR THE DETECTION OF *S. STERCORALIS* DNA IN CLINICAL SAMPLES IN RESOURCE- LIMITED AND WELL- RESOURCED SETTINGS

Isothermal amplification was developed and evaluated in this study as it has a shorter turnaround time than cPCR and qPCR and is a simple and cost- effective assay. Moreover, the equipment required was already available in the laboratory at DCP for research purposes. Wong *et al*. (2017), in a review of LAMP assays, noted that LAMP has successfully been used as a diagnostic tool for human, livestock and plant diseases. DCP is a high- throughput, busy diagnostic specialist parasitology referral laboratory. LAMP has been shown to be suitable for adaptation to a high-throughput format (Perera *et al*., 2017, Wong *et al*., 2017) and the rapid turnaround time has made it suitable for the development of an individual point of care test format for urgent samples (Njiru, 2012). However, the rigorous protocol required for DNA extraction from stool samples requires adaptation to a more rapid and simple method of DNA extraction before LAMP can be useful for a point of care test format or for use in a resource- limited setting.

One aim of this study was to develop a novel LAMP assay for the detection of *S. stercoralis* DNA in clinical samples for use, in both, resource- limited and well- resourced settings. Novel LAMP assay primers were designed and evaluated for the detection of *S. stercoralis* DNA (Chapter 3). Raw data is found in Appendix 6. Sequencing of the cPCR product, generated using the forward and back outer LAMP primers, confirmed the detection of a 240bp sequence of the target DNA with 99% sequence match to *S. stercoralis* (Section 3.4, Appendix 3a). No non-specific DNA was detected by the LAMP assay. Based on the findings in this part of the study (Chapter 3), the LAMP assay was determined to be a potentially suitable screening test for the diagnosis of strongyloidiasis (Pewsner *et al*., 2011). Analysis of sample storage temperature demonstrated a 0.6% increase (in samples stored at 4^{o}C or -20^{o}C) in the detection of *S. stercoralis* when evaluated against microscopy/ culture. The study findings revealed that, when the LAMP assay was evaluated against serology, 6.3% of serology positive samples were LAMP assay positive (Table 5.5). Serology may be positive in cases where active disease is not present (Requena-Méndez *et al*., 2013), nevertheless, a missed diagnosis can have severe consequences (Pottie *et al*., 2011) and the LAMP assay is unsuitable as a standalone diagnostic test (Section 3.8). Further evaluation of short- term DNA persistence and rapid manual DNA extractions methods highlighted the limitations of the assay.

As previously stated storage of neat stool samples at -20^{o}C is recommended for efficient *S. stercoralis* DNA extraction (ten Hove *et al*., 2009). Current standard operating procedures at DCP entail storage of clinical samples for up to three weeks at 4^{o}C before DNA extraction is performed for routine NAAT testing. To determine the standardisation of the LAMP assay required to harmonise with existing standard operating procedures at DCP the samples were evaluated for DNA persistence at storage temperatures of 4^{o}C and -20^{o}C. In this study, short- term persistence of DNA in stored samples was superior at a storage temperature of -20^{o}C but did demonstrate deterioration of *S. stercoralis* DNA, even at -20^{o}C (Table 3.16). Fresh samples are required and this current format is not suitable for the introduction of a NAAT into the diagnostic repertoire at DCP (a busy well –resourced reference laboratory) where samples may arrive in the post and be several weeks old. This storage condition is also unsuitable in endemic areas (which may be resource-limited) where samples may be stored for a period of time before the LAMP assay can be performed. Alternative sample storage before DNA extraction is required and further work is planned to evaluate the storage of samples in ethanol at 4^{o}C and sample storage on FTA cards (Section 3.7).

To determine the suitability of the LAMP assay for use in resource- limited areas, or in a point of care test format, rapid simplified manual methods for purified DNA extraction were examined. This study used an automated DNA extraction method (Qiagen® Qiasymphony SP) that requires expensive, high maintenance equipment that is not suitable for use in resource- limited areas. The LAMP assay is said to be less sensitive to assay inhibition than PCR as it uses a *Bst* polymerase rather than a *Taq* polymerase (Notomi, 2000) so methods for direct manual extraction of DNA suitable for use in the LAMP assay were investigated. Rapid, simple and cost-effective manual DNA extraction methods have been developed for use in endemic areas (WHO, 2013, Perera *et al*., 2017) and this study evaluated the use of two rapid, simple manual DNA extraction methods that require only a constant power supply, a heating block and a centrifuge (Section 3.7). At DCP, the PURE® DNA extraction system was simple and easy to use but required more stages, and cost 54% more than, the “boil and spin” method (Table 5.4). DNA from both manual DNA extraction methods demonstrated inhibition of the LAMP assay when evaluated against the automated DNA extraction method (Tables 3.19 and 3.20). The demonstration of inhibition of the LAMP assay, in this study, when using large sample volumes for the manual extraction of DNA may indicate a possible reason for the reduced sensitivity of the LAMP assay when compared to the qPCR assay for the detection of *S. stercoralis* DNA in clinical samples. This study tested stool aliquots ranging from 0.01 to 0.5 mg. In the current study inhibition was demonstrated in samples > 10µg (0.001mg).

This study recommends the “boil and spin” method as a rapid, simple and cost-effective method that is easy to use and suitable for use in resource- limited settings. However, further work on sample loading volumes and reproducibility is required to address the issue of inhibition of the LAMP assay before this method can be deployed in endemic areas or used in a point of care test format.

The current study demonstrated the feasibility of a LAMP assay to detect *S. stercoralis* in clinical samples. The use of LAMP for the detection of *S. stercoralis* DNA in clinical samples using novel primers designed to target the 18S rRNA gene has not yet been reported in the literature. This section of the study will be published after further optimisation of storage conditions for clinical samples and the manual DNA extraction methods has been completed.

6.3 REAL-TIME PCR (qPCR) FOR THE DETECTION OF *S. STERCORALIS* DNA IN CLINICAL SAMPLES IN A HIGH- THROUGHPUT SPECIALIST PARASITOLOGY REFERRAL LABORATORY.

This part of the study focused on the evaluation and validation of a published qPCR (Verweij *et al*., 2009, ten Hove *et al*., 2009) suitable for high-throughput processing in a well-resourced laboratory setting. The development of a protocol that would harmonise with existing work practices was also investigated. The qPCR assay was optimised for use at DCP and analytical sensitivity and specificity was performed using known negative and spiked positive stool samples and a bank of DNA from viral, bacterial and other parasitic organisms that may also be found in diagnostic stool samples received at DCP for strongyloidiasis investigation. Raw data from the study samples is found in Appendix 6.

Sequence analysis confirmed the detection of the target 101bp DNA with a sequence match of 100% to *S. stercoralis* and a sequence match of 93- 98% to *Strongyloides* species (Section 4.4). Anomalous results were detected, where the CRS was negative and the qPCR assay was positive. This finding may be due to increased sensitivity of the qPCR assay when compared to the microscopy/ culture result or to the serology result which may not be positive during acute infection or in immunocompromised individuals (Suddarshi *et al*., 2003). Repeat qPCR and cPCR assays were negative in some cases (Appendix 4) or were proven to be *S. stercoralis* by sequence analysis. It could not be established whether the negative repeated assays were due to small quantities of DNA in the sample (Morrison *et al*., 1998, Minogue *et al*., 2014), primer dimers or whether theoriginal tubes had been contaminated due to environmental contamination (Caraguel *et al*., 2011). The DNA extraction method and the master mix and sample loading were all automated procedures and only one instance of environmental contamination occurred after the limit of detection evaluation (Figure 4.8) had been performed. The environmental contamination

was determined to be at the master mix and sample loading stage as the repeat qPCR assay (after laboratory decontamination) did not demonstrate contamination in any of the repeated study samples. Once the qPCR assay had been optimised for use in the study (Section 4.3) residual diagnostic sample DNA from the microsporidia and Multiplex protist qPCR assays, from patients not being investigated for strongyloidiasis, was used to challenge the qPCR assay. These samples (n=20) were completely anonymised so no reference to patient data was available. A positive qPCR assay result was detected in three of the samples but no further action could be taken. It was, therefore, decided to perform gel electrophoresis on the products from the qPCR assay, including those samples in the specificity bank that were completely anonymised. Non-specific DNA was detected in three of the samples in the specificity bank. Two of the samples with C_ts of 35.11 and 35.13 (Section 4.5.2) were negative in two consecutive qPCR assays. Caraguel *et al*. (2011) suggest the reason for this may be due to primer dimer formation or environmental contamination. One sample with a C_t of 44.56 was detected in this study and the sample was microscopy positive for hookworm ova (Table 4.7). The target DNA band of 101bp was confirmed by gel electrophoresis. Hookworm and *S. stercoralis* may be found in the same geographical regions (CDC, n.d.) and this sample may have contained low levels of detectable *S. stercoralis* DNA or this result may indicate environmental contamination (Morrison *et al*., 1998, Caraguel *et al*., 2011). However, a cut-off C_t of >40 was determined in this study for qPCR positive results and C_ts >40 were classified as equivocal results. In diagnostic samples the generation of an equivocal result would require clinical input and a repeat sample would be requested. The qPCR assay was repeated twice more and generated negative results both times. It is thought to be more likely that the qPCR result was due to environmental contamination in this case (Caraguel *et al*., 2011).

The findings from the gel electrophoresis of the products from the qPCR assay for the study samples generated three anomalous qPCR products (one 145bp and two 500- 525bp- different patients). Only one of the qPCR products (500- 525bp) was identified. Multiple sequence alignment analysis (Larkin *et al*., 2007) demonstrated alignment with the reference strain *Strongyloides stercoralis* 18S ribosomal RNA gene, complete cds (Appendix 3b). However the products generated small fragments that aligned with the reference strain (M84229.1) from a 500bp query with Evalues of 3E-11 (39bp) and 8E-40 (90bp). While it may be possible that the detection of a 500bp product indicated a region of hypervariability (Jaleta *et al*., 2017), or a different strain, this result must be interpreted with care. This assay may, therefore, detect *S. stercoralis* from different geographical regions, but it was not possible to determine this as the number of positive samples with anomalous identifiable bands in the study was too low for data analysis to be performed.

Confirmation of the identity of the 500bp sequence is required before the presence of anomalous bands in the qPCR assay, which has not yet been reported in the literature, can be published to expand the knowledge base for the detection of *S. stercoralis* DNA. Further work is required to determine if there is a significant difference in the sequence of the DNA products to determine whether the samples contain a new species of *S. stercoralis* able to infect humans. Jaleta *et al*. (2017) demonstrated that *S. stercoralis* is more variable in the HVR I and HVR IV (Hasegawa *et al*., 2009) regions of the 18S rRNA gene than other nematodes and determined that this might not, therefore, indicate the presence of a cryptic *Strongyloides* species capable of infecting humans.

Where the sequence analysis confirmed the identity of *S. stercoralis* after repeated qPCR assays (Appendix 4) it was concluded that qPCR detected low levels of *S. stercoralis* DNA and indicates that further study into the use of the qPCR assay for treatment monitoring would be applicable as the disease was, detected by this assay. It was determined by this study to be 7.95 times more likely for *S. stercoralis* DNA to be present when the qPCR assay result is positive. The increased sensitivity of the qPCR assay may, therefore, be used to monitor treatment in studies designed to determine the exact dose of ivermectin required to eliminate strongyloidiasis in a patient. The findings in this study determined that the qPCR assay is a suitable screening test for the diagnosis of strongyloidiasis (Pewsner *et al*., 2011), but cannot replace serological diagnosis as only 21.4% of serology positive samples were qPCR assay positive (Table 5.5). The limitations regarding the use of serology (probable disease) as part of the CRS have been previously discussed (Requena-Méndez *et al*., 2013). This qPCR assay is suitable for deployment to well- resourced laboratories, with available expert advice, in a high- throughput format and will transform the diagnosis and clinical care of strongyloidiasis at DCP.

Similar to the LAMP assay, a DNA persistence study was performed for samples stored at 4^{o}C and

-20^{o}C. This determined harmonisation of standard operating procedures between the qPCR assay and current molecular assays for stool parasites at DCP. The findings confirmed that stool samples for *S. stercoralis* DNA detection by the qPCR assay must be stored at -20^{o}C before DNA extraction is performed.

6.4 COMPARISON OF LAMP AND qPCR ASSAYS FOR THE DETECTION OF *S. STERCORALIS* IN CLINICAL SAMPLES

This study investigated the application of LAMP and qPCR assays for the detection of *S. stercoralis* DNA in clinical samples. The cohort demographics and the quality of the samples used in this study were investigated for the effect that study sample demographics or storage conditions would have on NAATs deployed for use on fresh clinical samples or in epidemiological study conditions

(Sections 5.1 and 5.2). As demonstrated in Chapter 3, further experiments are required to optimise sample storage conditions before the LAMP assay can be deployed to diagnostic settings (Section 5.3.1). An improved performance for the qPCR assay *cf*. the LAMP assay in the detection of *S. stercoralis* DNA in clinical samples was consistently demonstrated in this study (Sections 3.8 and 4.10). An exception for this was the demonstration of similar limits of detection for the LAMP and qPCR assays on purified DNA (Table 5.5) and the reasons for this may be attributed to sub- optimal storage of neat stool and/ or inhibition of the LAMP assay. A further reason for the difference in detection of *S. stercoralis* DNA between the LAMP and qPCR assays may be due to the fact that the primers detect different target regions of the 18S rRNA gene. Clustal W2 multiple sequence alignment (Larkin *et al*., 2007) was used to determine the target regions of the 18S rRNA gene for the LAMP and qPCR assays. The results are shown in Appendix 3a.

Demonstration of stool inhibition for the LAMP assay was determined in this study by the evaluation of rapid manual DNA extraction (Notomi, 2000, Perera *et al*., 2017) and the automated Qiagen® Qiasymphony SP magnetic bead resonance technology. The automated DNA extraction technology had been previously validated for extraction of DNA from stool samples at DCP. The effect of inhibition was diminished or excluded from the LAMP assay when the DNA was diluted before use or smaller loading stool samples were used. Sub- optimal storage of stool samples for the LAMP and qPCR assays was demonstrated in this study for those samples stored neat at 4^{o}C and for the LAMP assay in those samples stored neat at -20^{o}C before DNA extraction. The reason for the improved performance of the qPCR assay was believed to be due to the sub- optimal storage of stool samples for the LAMP assay, LAMP assay inhibitors present in the DNA extracted from stool or a different target DNA detection site in the *S. stercoralis* genome for the LAMP assay. Multiple sequence alignment analysis demonstrated that the LAMP and qPCR primers target different regions in the *S. stercoralis* 18S rRNA gene (Appendix 3a). The similar limit of detection for purified DNA, suggested that further optimisation of the stool sample storage before DNA extraction and the DNA sample loading volume for rapid manual DNA extraction are required before the LAMP assay can be deployed for the detection of *S. stercoralis* DNA in clinical samples.

6.5 THE INTRODUCTION OF A qPCR ASSAY FOR THE DETECTION OF *S. STERCORALIS* IN CLINICAL SAMPLES

The information provided in this section forms part of a business plan (Appendix 5) that will be presented to the UCLH board of directors for the introduction of a qPCR assay for the detection of *S stercoralis* DNA in clinical samples into DCP. The algorithm described in Figure 6.1 is recommended for the diagnosis of strongyloidiasis at DCP

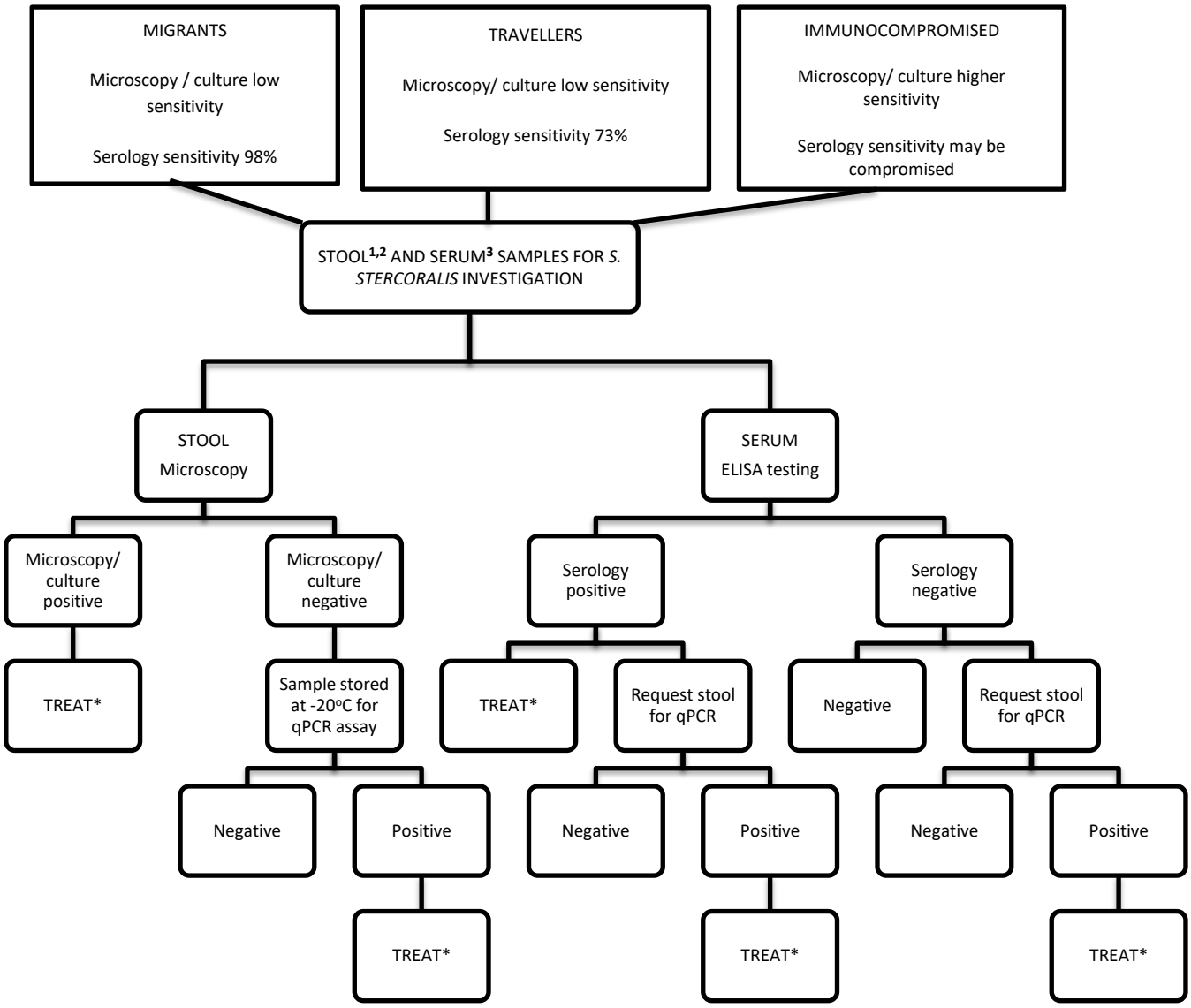


Figure 6.1: An algorithm for the use of the qPCR assay in the routine diagnostic laboratory at DCP. Potential for future use of qPCR to monitor treatment*

[1] Stool samples for culture and FECT to be processed immediately. Stool samples that are processed after 24 hours are stored at room temperature for culture or 4°C for FECT

[2] Stool samples for qPCR: store an aliquot at -20°C immediately

[3] Serum samples may be stored at 4°C or at -20°C

This demonstrates the use of the qPCR assay when microscopy and culture are negative and a qPCR assay can be requested when the clinician has determined that there is a risk of strongyloidiasis for the patient. Clinical input is required when the serological assay is positive and a clinical decision must be made to treat the patient or request qPCR confirmation. Clinical input is also required

when the microscopy, culture and/ or serological assay is negative when the qPCR assay is positive. A clinical decision can be made to request qPCR confirmation and will depend on the clinical degree of suspicion for strongyloidiasis.

Microscopy will still be an integral part of the diagnostic repertoire as this assay detects all parasites found in stool samples. The continued inclusion of the culture assay will be evaluated six months after the introduction of the qPCR assay for relevance to the diagnostic repertoire. The culture assay remains an infection risk and the removal of this test from the diagnostic repertoire will positively impact on the workload and the health and safety of laboratory staff.

Research and development for more sensitive detection methods is continuously being performed in the department. The removal of a time- consuming test (culture) will allow the introduction of more sensitive and specific assays for parasite detection to be introduced into a busy, high-throughput routine diagnostic department. A further improvement to the turnaround time of the Qiagen® Qiasymphony SP was investigated for the qPCR assay (Section 4.8), and was also demonstrated for the LAMP assay (Section 3.8). The current protocol requires stool samples to be incubated overnight in ATL buffer and proteinase k at 56^{o}C. Parallel DNA extraction was performed on multiple positive and negative control stool samples and no significant difference between those samples incubated at 56^{o}C for 2 hours or overnight was demonstrated. This determines that the automated DNA extraction method may be shortened to 1.5 days as opposed to the current protocol of 2.5 days. This suggests that other qPCR assays (e.g. microsporidia and Multiplex protist qPCR assays) may also be investigated for shorter turnaround times and will allow the further introduction of novel assays into the diagnostic repertoire at DCP.

The cost analysis calculation is described in Section 5.3 and the comparison of the LAMP and qPCR assays, in Table 5.5, describes the factors included into the decision to introduce the qPCR assay into DCP. A running cost of £44.09 per qPCR assay was calculated. This amount is heavily subsidised as the equipment and consumables are already available at DCP. The cost of staff is also subsidised as the qPCR assay can easily harmonise into existing standard operating procedures and automated DNA extraction for the *S. stercoralis* qPCR assay will be performed alongside DNA extraction for microsporidia and Multiplex protist qPCR assays. Removal of the equipment and staff costs provides a cost per qPCR assay of £8.21.

A cost analysis of the LAMP assay was performed to calculate the running costs of the assay in well-resourced and resource- limited areas (Table 5.5). FIND (2012), in reference to a report on the sustainability of international cooperation in the field of higher education and vocational training

(European Commission Directorate-General Education and Culture, 2006), determined that funding will be different in resource- limited areas where involvement of local government and non-governmental agencies is required to maintain funding for projects in these areas. FIND (2012) therefore, will supply reagents and kits with costing determined on an individual basis. Projects in these areas may require the supply of equipment (e.g. Loopamp- LF160, Figure 2.4) and this will be funded and supplied on an individual basis. The cost of staff and training is also performed based on the individual need of the projects. The costing analysis performed in this study was therefore performed on the cost of the reagents published (FIND, 2012) only. Nevertheless, based on this analysis the LAMP assay using the "boil and spin" method for rapid manual DNA extraction (FIND, 2012) will be a suitable candidate for deployment to resource- limited areas for the detection of *S. stercoralis* DNA in clinical samples once further optimisation experiments have been completed. Based on the costing analysis (Table 5.5) the LAMP assay is potentially suitable for point of care testing in well- resourced settings. The LAMP assay may also be designed in a high- throughput format (Perera *et al*., 2017) and this format is suitable for both well- resourced high throughput and resource- limited settings.

6.6 CONCLUSIONS

The conclusions of this study were that the LAMP assay may be suitable for use in endemic areas after further optimisation has been performed. In this study LAMP assay inhibition was reduced by using a low sample loading volume for manual DNA extraction methods (FIND, 2012, Perera *et al*., 2017) but the sensitivity of the assay was also reduced. Additional experiments are required to determine the optimal storage conditions for samples and to further develop manual DNA extraction methods. Further work is planned to investigate storage conditions and manual DNA extraction methods for the LAMP assay, but this is beyond the timescale for this professional doctorate degree (Appendix 7) and will be completed within a further study investigating the use of the qPCR assay for post- treatment monitoring. Once the LAMP assay has been optimised for suitability of use in the field the potential exists for the manufacturing of a lyophilised kit version (Wong *et al*., 2017).

The qPCR assay is a suitable diagnostic test for use in well- resourced areas that meets the molecular diagnostic test standards of an internal control and quantification capability that can be used to detect disease and monitor therapy follow up (Kramme *et al*., 2011). The qPCR assay is suitable for introduction as an adjunctive test that requires clinical input into assay requesting and interpretation of results. This assay cannot be used as a first- line test but it is suitable for detecting cases of strongyloidiasis in patients who are about to undergo iatrogenic immunosuppressive

therapy where a missed diagnosis can have severe consequences. Statistical analysis of data demonstrated that the qPCR assay is suitable for treatment monitoring in conjunction with serology.

Detection of *S. stercoralis* is not only a problem in humans. Reports of *S. stercoralis* infection amongst canines (Yang *et al*., 2013) have highlighted a need for a rapid cost-effective method for use in veterinary medicine as well (McNally *et al*., 2013, Sudhakara and Sivajothi, 2017). Dillard *et al*. (2007) reported on an outbreak in a Finnish kennel which led to the death of a 10- week old puppy from a naturally acquired *S. stercoralis* infection.

Molecular methods for the detection of human parasites in stool samples may also be used in veterinary medicine. There are published reports of the techniques described in this study being used in veterinary medicine (Yang *et al*., 2013, Melville *et al*., 2014, Jaleta *et al*., 2017). Sudhakara and Sivajothi (2017) reviewed methods for detection of parasites in veterinary medicine and noted the increased sensitivity and number of molecular techniques that are available. Diagnosis in veterinary medicine is important to the successful control of veterinary diseases which have an economic impact due to loss of the animal or reduced food production. The most common application of molecular diagnosis in veterinary medicine is the investigation of herd health to determine disease strategies (Sudhakara and Sivajothi, 2017). McNally *et al*. (2013) described a method for the extraction of DNA from stool samples and a multiplex quantitative PCR to detect *Haemonchus*, *Trichostrongylus* and *Telodorsagia* infections in sheep. The techniques described in the current study could also be used for veterinary medicine and a LAMP assay useful for resource-limited areas would be beneficial to the diagnosis of strongyloidiasis in animal husbandry. New primers designed to target *Strongyloides* sp., rather than *S. stercoralis*, would be required for the utilisation of this method in veterinary medicine as the *Strongyloides* sp. found in animals is usually different to the *Strongyloides* sp. found in humans (Jaleta *et al*., 2017). Humans have been shown to be susceptible to infection with certain strains of *S. stercoralis* that infect canines (Jaleta *et al*., 2017). There is, therefore, a use for the introduction of molecular techniques for epidemiological studies in areas where animals and humans are exposed to parasites. Jaleta *et al*. (2017) recommended that humans and associated infected dogs are treated together to eliminate the risk of potential zoonotic transfer.

6.7 FUTURE RESEARCH

A new study has been given ethical approval at DCP to investigate the usefulness of the qPCR assay in monitoring treatment for strongyloidiasis and determination of the correct dosage of ivermectin

in cases of chronic infection and hyperinfection syndrome. This work will be carried out after the conclusion of this professional doctorate degree and collection of samples has already begun.

Further work is planned to determine the optimal storage temperature for the LAMP assay samples, it is thought that storage of samples under optimal conditions will improve the performance of the LAMP assay for use in endemic areas. Manual DNA extraction methods suitable for use with the LAMP assay in endemic regions require further development and this will also be performed after the completion of the Professional doctorate degree.

Recently, cell-free DNA defined as DNA fragments found extracellularly in different body fluids has been investigated for the sensitive diagnosis of disease. The origin and distribution is unclear but it is being used with increasing success as a diagnostic biomarker (Weerakoon and McManus, 2016). Lodh *et al*. (2016) published a study where *S. stercoralis* DNA was detected in urine samples. Further work by Lodh *et al*. (2016) is planned to determine the sensitivity and specificity of this technique. Urine may be easily collected in larger volumes than stool samples and contains fewer inhibitors to NAATs and may be a suitable method for the detection of *S. stercoralis* DNA. This would be useful in determining where *S. stercoralis* DNA could be detected in humans and may be able to further the understanding of this parasite and its unique life-cycle. An investigation of cell-free DNA was not performed in this study as no urine was saved on patients entered into the study. However, this study will be continued and a request to the treatment monitoring study co-ordinator has been put forward for the investigation of urine samples on patients where this is available. Demmerdash *et al*. (1995) demonstrated schistosomal antigens in urine and serum that could be detected by monoclonal antibodies. It would be beneficial to perform a pilot study to determine whether *S. stercoralis* cell- free DNA is present in serum or plasma and can be detected by qPCR.

REFERENCES

Banoo, S., Bell, D., Bossuyt, P., Herring, A., Mabey, D., Poole, F., Smith, P.G., Sriram, N., Wongsrichanalai, C., Linke, R., O'Brien, R., Perkins, M., Cunningham, J., Matsoso, P., Nathanson, C.M., Olliaro, P., Peeling, R. W. and Ramsay, A. (2007). Evaluation of diagnostic tests for infectious diseases: general principles. *Nature Reviews Microbiology*. **S21-S31**. [online]. Available from: www.nature.com. [Accessed 6th March 2017].

Barros, N. and Montes, M. (2014). Infection and hyperinfection with *Strongyloides stercoralis*: Clinical presentation, etiology of disease, and treatment options. *Curr. Trop. Med. Rep*. **1**, 223-228.

Basile, A., Simzar, S., Bemtow, J., Antelo, F., Shitaba, P., Peng, S. and Craft, N. (2010). Disseminated *Strongyloides stercoralis*: Hyper infection during medical immunosuppression. *Journal of the American Academy of Dermatology*. **63** (5), 896-902.

Basuni, M., Muhi, J., Othman, N., Verweij, J. J., Ahmad, M., Miswan, N., Rahumatullah, A., Abdul Aziz, F., Zainudin, N. S. and Noordin, R. (2011). A pentaplex real-time polymerase chain reaction assay for detection of four species of soil-transmitted helminths. *American Journal of Tropical Medicine and Hygiene*. **84**:338-343.

Baughman, A.L., Bisgard, K.M., Cortese, M.M., Thompson, W.W., Sanden, G.N. and Strebel, P.M. (2008). Utility of composite reference standards and latent class analysis in evaluating the clinical accuracy of diagnostic tests for pertussis. *Clinical and Vaccine Immunology*. **15**(1), 106-114.

Becker, S.L., Sieto, B., Silué, K.D., Adjossan, L., Koné, S., Hatz, C., Kern, W.V., N'Goran, E.K. and Utzinger, J. (2011). Diagnosis, clinical features, and self-reported morbidity of *Strongyloides stercoralis* and hookworm infection in a co-endemic setting. *PLoS Neglected Tropical Diseases*. **5**(8), e1292. [online]. Available from: www.plosntds.org. [Accessed 16th June 2013]

Becker, S.L., Piraisoody, N., Kramme, S., Marti, H., Silué, K.D., Panning, M., Nickel, B., Kern, W.V., Herrmann, M., Hatz, C.F., N'Goran, E.K. Utzinger, J. and von Müller, L. (2015). Real-time PCR for detection of *Strongyloides stercoralis* in human stool samples from Côte d'Ivoire: diagnostic accuracy, inter-laboratory comparison and patterns of hookworm co-infection. *Acta Tropica*. [online].Available from: www.journals.elsevier.com/Acta-Tropica. [Accessed 19th July 2015].

Biggs, B., Caruana, S., Mihrshabi, S., Jolley, D., Leydon, J., Chea, L. and Nuon, S. (2009). Short report: Management of chronic Strongyloidiasis in immigrants and refugees: Is serological testing useful? *American Journal of Tropical Medicine and Hygiene*. **80** (5), 788-791.

Bisoffi, Z., Buonfrate, D., Anghben, A., Boscolo, M., Anselmi, M., Marocco, S., Monteiro, G., Gobbo, M., Bisoffi, G. and Gobbi, F. (2011). Randomized clinical trial on ivermectin versus thiabendazole for the treatment of strongyloidiasis. *PLoS Negl Trop Dis*. **5**(7), e1254. [online]. Available from : www.plos.ntds.org [Accessed: 26th December 2013].

Bisoffi, Z., Buonfrate, D., Montresor, A., Requena-Méndez, A., Muňoz, J., Krolewiecki, A.J., Gotuzzo, E., Mena, M.A., Chiodini, P.L., Anselmi, M., Moreira, J. and Albonico, M. (2013). *Strongyloides stercoralis*: A plea for action. *PLoS Negl Trop Dis*. **7**(5). E2214. [online].Available from: www.plos.ntds.org. [Accessed 26th December 2013].

Bon, B., Houze, S., Talabani, H., Magne, D., Belkadi, G., Develoux, M., Senghor, Y., Chandenier, J., Anselle, T. and Hennequin, C. (2010). Evaluation of a rapid enzyme-linked immunosorbent assay for diagnosis of strongyloidiasis. *Journal of Clinical Microbiology*. **48**(5), 1716-1719.

Bossuyt, P.M., Reitsma, J.B., Bruns, D.E., Gatsonis, G.A., Glasziou, P.P., Irwig, L., Lijmer, J.G., Moher, D., Drummond, R., de Vet, H.C.W., Rifai, N., Golub, R.M., Altman, D.G., Hooft, L., Korevaar, D.A., Cohen, J.F. (2015). STARD 2015: an updated list of essential items for reporting diagnostic accuracy studies. *BMJ*. **351**. [online]. Available from: www.bmj.com. [Accessed 29th December 2015].

Boussinesq, M., Gardon, J., Gardon-Wendel, N. and Chippaux, J. P. (2003). Clinical picture, epidemiology and outcome of *Loa*-associated serious adverse events related to mass ivermectin treatment of onchocerciasis in Cameroon. *Filaria Journal*. **2** (Suppl 1), S4. [online]. Available from: filariajournal.com. [Accessed 26th August 2013].

Brindley, P.J., Mitreva, M., Ghedin, E. and Lustigman, S. (2009). Helminth genomics: The implications for human health. *PLoS Neglected Tropical Diseases*. **3**(10).[online]. Available from: www.plosntds.org. Accessed: 17th December 2017.

Brown, T.A. (2006). *Gene cloning and DNA analysis*. 5th Edition. Oxford: Blackwell Publishing Ltd. Chapter 3, page 30.

Buonfrate, D., Requena-Méndez, A., Angheben, A., Muňoz, J., Gobbi, F., Van Den Ende, J. and Bisoffi, Z. (2013). Severe strongyloidiasis: a systematic review of case reports. *BMC Infectious Diseases*. **13**(78), 1471-2334.

Buonfrate, D., Sequi, M., Mejia, R., Cimino, R.O., Krowlewiecki, A. J., Albonico, M., Degani, M., Tais, S., Angheben, A., Requena-Mendes, A., Muňos, J., Nutman, T.B. and Bisoffi, Z. (2015). Accuracy of five serological tests for the follow up of *Strongyloides stercoralis* infection. . *PLoS Negl Trop Dis.* Feb 10th. **9**(4), E0003491. [online]. Available from www.plosone.org. [Accessed 27th June 2015].

Caraguel, C. G. B., Stryhn,H., Gagne´, N., Ian R. Dohoo, I. R. and Hammell, K.L. (2011) Selection of a cut-off value for real-time polymerase chain reaction results to fit a diagnostic purpose: analytical and epidemiologic approaches. *J Vet Diagn Invest* **23**:2–15.

CDC (Centers for Disease Control and Prevention) (n.d.). A- Z index. [online]. Available from: dpd.cdc.gov. [Accessed 21st June 2013].

Checkley, A. M. and Sanderson, F. (2009). The investigation of eosinophilia. *Medicine*. **38**(1), 30 – 35.

Checkley, A.M., Chiodini, P.L., Dockrell, D.H., Bates, I., Thwaites, G.E., Booth, H.L., Brown, M., Wright, S.G., Grant, A.D., Mabey, D.C., and Whitty, C.J.M. (2010). Eosinophilia in returning travellers and migrants from the tropics: UK recommendations for investigation and initial management. *Journal of Infection*. **60**, 1-20.

Chen, L., Trujillo, K., Sung, P. and Tomkinson, A. E. (2000). Interactions of the DNA Ligase IV-XRCC4 Complex with DNA Ends and the DNA-dependent Protein Kinase. *J. of Biolog. Chem*. **275**(34), 26196 –26205.

Cicchetti, D.V. (1994). Guidelines, criteria and rules of thumb for evaluating normed and standardized assessment instruments in psychology. *Psychological Assessment*. **6**(4), 284-290.

Cimeno, R.O. and Krowlewiecki, A. (2014). The epidemiology of human strongyloidiasis. *Curr. Trop. Med. Rep*. **1**, 216-222.

Corning Cellgro. (2012) Troubleshooting: transformation. © 2012 Corning Incorporated Printed in U.S.A. 3/12 POD CLS-CG-TS-382. [online]. Available from: cellgro.com/ . [Accessed 14th March 2017].

Daar, A.S., Thorsteinsdóttir, H., Martin, D.K., Smith, A.C., Nasti, S. and Singer, P.A. (2002). Top ten biotechnologies for improving health in developing countries. *Nature Genetics*. **32**, 229-232.

Demerdash, Z.A., Mohamed, S.H., Shaker, Z.A., Hassan, S.I., el Attar, G.M., Abadeer, N.I. and Mansour, M.M. (1995). Detection of circulating schistosome antigens in serum and urine of schistosomiasis patients and assessment of cure by a monoclonal antibody. *J. Egypt Soc. Parasitol*. **25**(2), 471- 484.

Dhanasekaran S., Doherty T.M., Kenneth J., TB Trials Study Group.(2010). Comparison of different standards for real-time PCR-based absolute quantification. *J Immunol Methods*. **31**, 354(1-2), 34-39. [online]. Available from: www.ncbi.nlm.nih.gov. [Accessed 20th May 2016].

Dillard, K.J., Saari, S.A.M. and Antila, M. (2007). *Strongyloides stercoralis* infection in a Finnish kennel. *Acta Veterinaria Scandinavica*. **49**(37). [online]. Avaiable from: https://actavetscand.biomedcentral.com/articles. [Accessed: December 2017].

Dorris, M., Viney, M.E. and Blaxter, M. (2002). Molecular phylogenetic analysis of the genus *Strongyloides* and related nematodes. *International Journal for Parasitology*. **32**, 1507-1517

Eiken Chemical Co. Ltd. (2005) Eiken genome site. Principle of LAMP. [online]. ©Copyright EIKEN CHEMICAL CO., LTD., 2005. [online]. Available from: loopamp.eiken.co.jp. [Accessed September 2012].

Erali, M, and Wittwer, C.T. (2010). High Resolution Melting Analysis for gene scanning." *Methods.* **50**(4), 250–261. [online]. Available from: www.ncbi.nlm.nih.gov. [Accessed 23rd October 2016]

European Commission Directorate-General Education and Culture. (2006) Sustainability of international cooperation projects in the field of higher education and vocational training. Handbook on sustainability. ©Copyright, European Communities, 2006. [online]. Available from: www.ec.europa.eu . [Accessed 29th January 2017].

FDA US food and drug administration (2007). Statistical Guidance on Reporting Results from Studies Evaluating Diagnostic Tests. [online]. Available from: www.fda.gov/ . [Accessed 11th February 2017].

Feely, N.M., Waghorn, D. J., Dexter, T., Gallen, I. and Chiodini, P. (2010). Case report. *Strongyloides stercoralis* hyperinfection: difficulties in diagnosis and treatment. *Anaesthesia*. **65**, 298–301.

FIND (Foundation for innovative new diagnostics). (2012). Manual of standard operating procedures for malaria LAMP: Foundation for innovative new diagnostics. [online]. Available from: www.finddiagnostics.org. [Accessed: May 2017].

Fischer, N. (2015). *Strongyloides* "larva currens" following high dose dexamethasone for upper airway burns: a case report and brief review of the literature. *Trop. Med. And Surg*. **3**(1) [online]. Available from: www.esciencecentral.org. [Accessed 25th October 2015]

Fitch, D.A., Bugaj-Gaweda, B. and Emmens, S.W. (1995). 18S ribosomal gene phylogeny for some Rhabditidae related to *Caenorhabditis*. *Mol. Biol. Evol*. **12**(2), 346-358.

Forrer, A., Khieu, V., Schindler, C., Schär, F., Marti, H., Char, M. C., Muth, S. and Odermatt, P. (2016). Ivermectin treatment and sanitation effectively reduce *Strongyloides stercoralis* infection risk in rural communities in Cambodia. *PLoS Neglected Tropical Disease*. **10**(8), e0004909. [online]. Available from: www.plos.ntds.org. [Accessed: 11th March, 2017].

Ganesh, S. and Cruz Jr., R. (2011). Strongyloidiasis A Multifaceted Disease. *Gastroenterology and Hepatology*. **7**(3), 194 -196.

Gasser, R.B., Chilton, N.B., Hoste, H. and Beveridge, I. (1993). Rapid sequencing of rDNA from single worms and eggs of parasitic helminths. *Nucleic Acids Reasearch*. **21** (10), 2525-2526.

Gene Codes Corporation. (2017). Free download: DNASTAR. ©Copyright 2017, Gene Codes Corporation. [online]. Available from: www.dna.genecodes.com. [Accessed May 2015].

Getaneh, A., Medhin, G. and Shimelis, T. (2010). *Cryptosporidium* and *Strongyloides stercoralis* infections among people with and without HIV infection and efficiency of diagnostic methods for *Strongyloides* in Yirgalem Hospital, southern Ethiopia. *BMC Research Notes*. **3**(90). [online]. Available from: www.biomedcentral.com. [Accessed September 2012].

Glinz, D., Silué, K. D., Knopp, S., Lohourignon, L. K., Kouassi, P. Y., Steinmann, P., Rinaldi, L., Cringoli, G., N'Goran, E. K. and Utzinger, J. (2011). Comparing Diagnostic accuracy of Kato- Katz, Koga agar plate, ether- concentration and FLOTAC for *Schistosoma mansoni* and soil- transmitted helminths. *PLoS Neglected Tropical Diseases*. **84**(2), 338 -343.

Gonzaga, H. T., Ribeiro, V. S., Feliciano, N. D., Manhani, M. N., Silva, D. A. O., Ueta, M. T. and Costa-Cruz, J. M. (2011). IgG avidity in differential serodiagnosis of human strongyloidiasis active infection. *Immunology Letters*. **139** (1-2), 87 -92.

Gorospe, E. C. and Oxentenko, A. S. (2012). Nutritional consequences of chronic diarrhoea. *Best practice and Research Clinical Gastroenterology*. **26**, 663-675.

Halstead, F.D., Lee, A.V., Couto-Parada, X., Polley, S.D., Ling, C., Jenkins, C., Chalmers, R.M., Elwin, K., Gray, J.J., Iturriza-Gómara, M., Wain, J., Clark, D.A., Bolton, F.J. and Manuel, R.A.: Olympics GI Group. (2013). Universal extraction method for gastrointestinal pathogens. *J. Med. Microbiol*. **62**(10), 1535-1539.

Hasegawa, H., Hayashida, S., Ikeda, Y. and Sato.H. (2009). Hyper-variable regions in 18S rDNA of *Strongyloides* spp. As markers for species-specific diagnosis. *Parasitology Research*. **104**, 869-874.

Hawkins, R.C. (2007). Laboratory turnaround time. *Clin Biochem Rev*. **28** [online]. Available from: www.ncbi.nlm.nih.gov. [Accessed 28th December 2017]

Henriquez-Camacho, C., Gotuzzo, E., Echevarria, J., White, A.C., Terashima, A., Samalvides, F., Pérez-Molina, J.A. and Plana, M. N. (2016). Ivermectin versus albendazole or thiabendazole for *Strongyloides stercoralis* infection (Review). The Cochrane Library, **1**, 1-50. [online]. Available from: www.thecochranelibrary.com [Accessed 6th January 2016].

Hotez, P.J., Molyneux, D.H., Fenwick, A., Kumaresan, J., Ehrlich Sachs, S., Sachs, J. D. and Savioli, L. (2007) Control of neglected tropical diseases. *New Engl. J. of Med*. **357**, 10. [online]. Available from: www.nejm.org. [Accessed 21st Oct 2016]

HPA UK protocols. (2013). UK standards for microbiology investigations. *Standards Unit, Microbiology Services Division*. [online]. Available from: www.hpa.org.uk/SMI. [Accessed June 2013].

Hu, M, Chilton, N.B., Gasser, R.B. (2003). The mitochondrial genome of *Strongyloides stercoralis* (Nematoda) – idiosyncratic gene order and evolutionary implications. *International Journal for Parasitology*. **33**(12), 1393-1408

Inēs, E. de J., Souza, J. N., Santos, R. C., Souza, E. S., Santos, F. L., Silva, M. L. S., Silva, M.P., Teixeira, M. C. A. and -Soares, N. M. (2011). Efficacy of parasitological methods for the diagnosis of *Strongyloides stercoralis* and hookworm in faecal specimens. *Acta Tropica***. 120,** 206 – 210.

Iseki, H., Alhassan, A., Ohta, N., Thekisoe, O. M. M., Yokoyama, N., Inoue, N., Nambota, A., Yasuda, J. and Igarashi, I. (2007). Development of a multiplex loop- mediated isothermal amplification (mLAMP) method for the simultaneous detection of bovine *Babesia* parasites.

J. of Microbiolog. Methods. **71**, 281- 287.[online]. Available from : www.sciencedirect.com. [Accessed: December 2017].

ISO 15189:2012. (2012). Medical laboratories- requirements for quality and competence. [online]. Available from: www.iso.org/standard. [Accessed 28th Feb 2017]

Jacobson, R.H. (1998). Principles of validation of diagnostic assays for infectious diseases. *Rev. Sci. Tech*. **17**. 469-486

Jaleta, T.G., Zhou, S., Bemm, F.M., Schär, F., Khieu, V., Muth, S., Odermatt, P., Lok, J.B. and Streit, A. (2017). Different but overlapping populations of *Strongyloides stercoralis* in dogs and humans- dogs as a possible source for zoonotic strongyloidiasis. *PLOS Neglected Trop. Dis*. **11**(8). [online]. Available from: www.plosntds.org. [Accessed December 2017].

Jones, R. and Payne, B. (1997). Medical research: study design, analysis and reporting in *Clinical investigations and statistics in laboratory medicine*. Cambridge: ACB Venture Publications. Chapter 4, pages 124- 185.

Kassalik, M., and Mönkemüller, K. (2011) *Strongyloides stercoralis* hyperinfection syndrome and disseminated disease. *Gastroenterology and Hepatology*. **7**(11), 766-768.

Khieu, V., Schär, F., Marti, H., Sayasone, S., Duong, S., Muth, S. and Odermatt, P. (2013). Diagnosis, treatment and risk factors of *Strongyloides stercoralis* in schoolchildren in Cambodia. *PLoS Neglected Tropical Diseases*. **7**(2). E2035. [online]. Available from: www.plosntds.org. [Accessed 30th March 2014].

Kinson, R. (2012). Quality of *gfp* as an internal control. Internal Audit of the use of an internal control for real-time PCR at the Department of Clinical Parasitology. Available from: UCLH intranet: g://parasitology/audit meetings and minutes. [Accessed 12th December, 2012]

Kirkwood, B.R. and Sterne, J.A.C (1998). Calculation of required sample size in *Essential medical statistics*. 2nd Edition. ©Copyright, Blackwell Science Ltd. Oxford: Blackwell Publishing Ltd. Chapter 35, pages 413- 428.

Knopp, S., Mgeni, A.F., Khamis, S., Steinmann, P., Stothard, R., Rollinson, D., Marti, H. and Utzinger, J. (2008). Diagnosis of soil-transmitted helminths in an era of preventative chemotherapy: effect of multiple stool samples and use of different diagnostic techniques. *PLoS Neglected Tropical Diseases*. November. **2**(11), e331. [online]. Available from: www.plosntds.org. [Accessed 21st September 2012].

Kramme, S., Nissen, N., Soblik, H., Erttmann, K., Tannich, E., Fleischer, B., Panning, M. and Brattig, N. (2011). Novel real-time PCR for the universal detection of *Strongyloides* species. *Journal of Medical Microbiology*. **60**, 454- 458.

Krowlewiecki, A.J., Lammie, P., Jacobson, J., Gabrielli, A., Levecke, B., Socias, E., Arias, L.M., Sosa, N., Abraham, D., Cimino, R., Echazú, A., Crudo, F., Vercruysse, J. and Albonico, M. (2013). A public health response against *Strongyloides stercoralis*: Time to look at soil-transmitted helminths in full. *PLOS Neglected Tropical Diseases*. **7**(5), e2165. [online]. Available from: www.plosntds.org. [Accessed 6th August 2013].

Kruhøffer, M., Voss, T., Beller, K., Scherer, M., Cramer, J., Deutschmann, T., Homberg, C., Schlumpberger, M. and Lenz, C. (2010). Evaluation of the QIAsymphony SP workstation for magnetic particle-based nucleic acid purification from different sample types for demanding downstream applications. *Journal for the Association of Laboratory Automation*. [online]. Available from: www.la.sagepub.com. [Accessed 13th Apr 2016]

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J. and Higgins, D. G. (2007). ClustalW version 2 (ClustalW2) at EMBL-EBI. Clustal W and Clustal X version 2. *Bioinformatics*. **23** (21), 2947-2948.

Levenhagen, M.A. and Costa-Cruz, J.M. (2014). Update on immunologic and molecular diagnosis of human strongyloidiasis. *Acta Tropica*. **135**, 33-43

Lewis, S.J. and Heaton, K.W. (1997). Stool form scale as a useful guide to intestinal transit time. *Scand. J. Gastroenterology*. **32**(9), 920-924.

Libman, M. D., MacLean, J. D. and Gyorkos, T. W. (1993). Screening for Schistosomiasis, Filariasis and Strongyloidiasis among expatriates returning from the Tropics. *Clinical Infectious Diseases*. **17**, 353- 359.

Life Technologies Corporation. (2011) PCR handbook. © 2011 Life Technologies Corporation. [online]. Available from: www.gene-quantification.com. [Accessed 24th July 2016]

Lodh, N., Caro, R., Sofa, S., Scott, A., Krowlewiecki, A and Schiff, C. (2016) Diagnosis of *Strongyloides stercoralis*: detection of parasite-derived DNA in urine. *Acta Tropica*. **163**, 9-13. [online]. Available from: www.journals.elsevier.com/Acta-Tropica. [Accessed 16th Jan 2017]

Looss, A. *(*1905). Die Wanderung der *Ancylostoma duodenale* und *Strongyloides* Larven von der haut nach dem Darm. *Comptes Rendus du Sixième Congrès Internationale de Zoologie, Berne, Switzerland*, p. 255-233.

Lucchi, N. W., Gaye, M., Dialo, M. A., Goldman, I. F., Ljolje, D., Deme, A.D., Badiane, A., Ndiaye, Y., D., Barnwell, J. W., Udhayarkumar, V. and Ndiaye, D. (2016). Evaluation of the *Illumigene* malaria LAMP: a robust molecular tool for malaria parasites. *Nature*. [online]. Available from: www.nature.com. [Accessed 16th December 2016].

Makker, J., Balar, B., Niazi, M. and Daniel, M. (2015) Strongyloidiasis: A case with acute pancreatitis and a literature review. *World J. Gatroenterol*. **21**(11), 3367-3375.

Manser, M., Saez, A. and Chiodini, P.L. (2015) Poster: Faecal Parasitology: Concentration technology really does matter! [online]. Available from: www.apacor.com/pdf/NEQAS Evaluation_Reference EECMID. [Accessed 14th April 2016]

Marti, H. and Koellaz, J. C. (1993). Multiple stool examinations for ova and parasites and rate of false-negative results. *J. Clin. Micro*. **31** (11), 3044-3045. [online]. Available from: www. Jcm.asm.org. [Accessed 20th May 2017].

McHugh, M. L. (2012). Interrater reliability: The Kappa statistic. *Biochemia Medica* **22**(3), 276–282.

McNally, J., Callan, D., Andronicos, N., Bott, N. and Hunt, P. W. (2013). DNA based methodology for the quantification of gastrointestinal nematode eggs in sheep faeces. *Veter. Paras*., **198** (Issues 3-4), 325- 335.

MedCalc®. (n.d.) a free statistical software program, version 16.2.0. Software Instruction Manual. (MedCalc Software, Ostende, Belgium). ©Copyright, 1993-2016 MedCalc Software bvba. [online]. Available from: www.medcalc.org. [Accessed 2014].

Mejia, R. and Nutman, T.B. (2012). Screening, prevention and treatment for hyperinfection syndrome and disseminated infections caused by *Strongyloides stercoralis*. *Curr Opin Infect Dis*. **25**(4), 458-463.

Melville, L., Kenyon, F., McElarney, I., Demeler, J. and Skuse, P. (2014). Development of a loop-mediated isothermal amplification (LAMP) assay for the sensitive detection of *Haemonchus contortus* eggs in ovine faecal samples. *Vet Parasitology* **206**(3-4), 308-312. [online]. Available from: j.vetpar.2014.10.022. [Accessed: December 2017].

Minato, K., Kimura, E., Shintoku, Y. and Uga, S. (2008). Effect of temperature on the development of free-living stages of *Strongyloides ratti*. *Parasitology Research*. **102**, 315-319

Minogue, T.D., Rachwal, P.A., Trombley Hall, A., Koehler, J.W. and Weller, S.A. (2014). Cross-institute evaluations of inhibitor-resistant PCR reagents for direct testing of aerosol and blood samples containing biological warfare agent DNA. *Applied Environmental Microbiology*. **80**(4), 41322-41329. [online] . Available from: www.aem.asm.org. [Accessed 24th April 2016].

Moghaddassani, H., Mirhendi, H., Hosseini, M., Rokni, M.B., Mowlavi, G. and Kia, E. (2011). Molecular diagnosis of *Strongyloides stercoralis* infection by PCR detection of specific DNA in human stool samples. *Iranian Journal of Parasitology*. **6**(2), 23 – 30.

Monteiro, L., Bonnemaison, D., Vekris, A., Petry, K.G., Bonnet, J., Vidal, R., Cabrita, J. and Mégraud, F. (1997). Complex polysaccharides as PCR inhibitors in feces: *Helicobacter pylori* model. *J. Clin. Microbiol*. **35**(4), 995-998.

Montes, M., Sawhney, C. and Barros, N. (2010). *Strongyloides stercoralis*: there but not seen. *Current Opinions in Infectious Diseases*. **23**, 500-504.

Mori, Y. and Notomi, T. (2009). Loop-mediated isothermal LAMP: a rapid, accurate and cost-effective diagnostic method for infectious diseases. *J. Infect Chemother*. **15**, 62-69

Morrison, T.B., Weiss, J.J. and Wittwer, C.T. (1998). Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. *Biotechniques*. **24**(6), 954-958, 960,962.

Mullen, M.P., Howard, D.J., Powell, R. and Hanrahan, J.P. (2009). A note on the use of FTA™ technology for storage of blood samples for DNA analysis and removal of inhibitors. *Irish J. of Agricultural and Food Res*. **48**, 109-113.

Murphy, N. M., McLaughlin, J, Ohai, C. and Grant, K. A. (2007). Construction and evaluation of a microbiological positive process internal control for PCR-based examination of food samples for *Listeria monocytogenes* and *Salmonella enteritica*. *Int. J of food Microbiol*. **120**, 110-119.

Nagamine, K., Watanabe, K., Ohtsuka, K., Hase, T. and Notomi, T. (2001). Letter to the editor. Loop-mediated isothermal amplification reaction using a nondenatured template. *Clin. Chem*. **47**(9), 1742-1743.

NCBI. National Center for Biotechnology Information. (n.d.). Basic local alignment search tool BLAST. [online]. Available from: www.ncbi.nlm.nih.gov. [Accessed April 2014].

Njiru, Z.K. (2012). Loop-mediated isothermal amplification technology: towards point of care diagnostics. *PLoS Neglected Tropical Diseases*. **6** (6). E1572. [online]. Available from: www.plosntds.org. [Accessed March 2015].

Norman, F., Pérez de Ayala, A., Pérez-Molina, J., Monge-Maillo, B., Zamarrón, P. and López-Vélez, R. (2010). Neglected Tropical Diseases outside the Tropics. *PLoS Neglected Tropical Diseases*. **4**(7), e762. [online]. Available from: www.plosntds.org. [Accessed 21st September 2012]

Notomi, T., Okayame, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. and Hase, T. (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acid Research*. **28**(12). E63. [online]. Available from: www.ncbi.nlm.nih.gov. [Accessed December 2014]

Olsen, A., van Lieshout, L., Marti, H., Polderman, T., Polman, K., Steinmann, P., Stothard, R., Thybo, S., Verweij, J. J. and Magnussen, P. (2009). Review Article. Strongyloidiasis- the most neglected of the neglected tropical diseases? *Transactions of the Royal Society of Tropical Medicine and Hygiene*. **103**, 9667- 972.

Peeling, R.W., Smith, P.G. and Bossuyt, P.M.M. (2007). A guide for diagnostic evaluations. *Nature Rev. Microb*.**4** (9 Suppl.) S2-S6.

Perera, R .S., Ding, X. C., Tully, F., Oliver, J., Bright, N., Bell, D., Chiodini, P. L., Gonzalez, I. J. and Polley, S.D. (2017). Development and clinical performance of high-throughput loop-mediated isothermal amplification for the detection of malaria. *PLoS One*, **12** (2). [online]. Available from: www.plosntds.org. [Accessed 23rd April 2017].

Perry, B.D. and Randolph, F. (1999). Improving the assessment of the economic impact of parasitic diseases and of their control in production animals. *Vet. Paras*. **84** (Iss. 3-4), 145-168.

Polley, S.D., Boadi, S., Watson, J., Curry, A. and Chiodini, P.L. (2011). Detection and species identification of microsporidial infection using SYBR Green real-time PCR. *Journal of Medical Microbiology*. **60**, 459-466.

Polley, S.D., González, I.J., Mohamed, D., Daly, R., Bowers, K., Watson, J., Mewse, E., Armstrong, M., Gray, C., Perkins, M., Bell. D., Kanda, H., Tomita, N., Kubota, Y., Mori, Y., Chiodini, P.L. and Sutherland, C.J. (2013). Clinical evaluation of a loop-mediated amplification kit for diagnosis of imported malaria. *Journal of Infectious Diseases*. [online]. Available from: www.jid.oxfordjournals.org/ . [Accessed 21st June 2013]

Pottie,K., Greenaway, C., Feightner, J., Welch, V., Swinkels, H., Rashid, M., Narasiah, L., Kirmayer, L. J., Ueffing, E., MacDonald, N.E., Hassan, G., McNally, M., Khan, K., Buhrmann, R., Dunn, S., Dominic, A., McCarthy, A.E., Gagnon, A.J., Rousseau, C., Tugwell, P. and co-authors of the Canadian Collaboration for Immigrant and Refugee Health. (2011). Evidence-based clinical guidelines for immigrants and refugees. *Canadian Medical Assoc. Journal(CMAJ)*. **183**(12), E824-E867. [online]. Available from: www.cmaj.ca. [Accessed January 2013]

Promma, K. and Songthamwat, D. (2012). The 18S ribosomal DNA sequence of *Strongyloides stercoralis* in Thailand. *The Joint International Tropical Medicine Meeting (JITMM 2011) Proceedings*. **1**, 1-8.

Qiagen (n.d.) Kit inserts for extraction, purification and ligation of DNA. ©Copyright Qiagen, 2013-2016. [online]. Available from: www.qiagen.com. [Accessed 2013]

Qiagen (2013). DNA protocols and Applications. ©Copyright Qiagen, 2013-2015. [online]. Available from: www.qiagen.com. [Accessed 11th April 2016].

Raymaekers, M., Smets, R., Maes, B. and Cartuywels, R. (2009). Checklist for optimisation and validation of real-time PCR assays. *J of Clin. Lab. Analysis*. **23**, 145-151.

Rayan, H.Z., Soliman, R.H and Galal, N.M (2012). Detection of *Strongyloides stercoralis* in fecal samples using conventional parasitological techniques and real-time PCR: A comparative study. *PUJ*. **5**(1), 27-34.

Reed, G.F., Lynn, F. and Meade, B. D. (2002). Use of coefficient of variation in assessing variability of quantitative assays. *Clin. and Diag. Laboratory Immunology*. **9** (6), 1235- 1239.

Repetto, S.A., Durán, P.A., Lasala, M.B. and González-Cappa, S.M. (2010). High rate of strongloidosis infection out of endemic area, in patients with eosinophilia and without risk of exogenous infection. *American Journal of Tropical Medicine and Hygiene*. **82**(6), 1088-1093.

Requena-Méndez, A., Chiodini, P., Bisoffi, Z., Buonfrate, D., Gotuzzo, E. and Muñoz, J. (2013). The laboratory diagnosis and follow up of strongyloidiasis: a systematic review. *PLoS Neglected Tropical Diseases*. **7**(1). [online]. Available from: www.plosntds.org. [Accessed 31st May 2013].

Requena-Méndez, A., Buonfrate, D., Bisoffi, Z. and Muñoz Gutiérrez, J. (2014). Advances in the diagnosis of human strongyloidiasis. *Current Trop. Med.Rep.***1**, 207-215.

Roxby, A.C., Gottlieb, G.S. and Limaye, A.P. (2009). Strongyloidiasis in transplant patients. Immunocompromised hosts. *Clinical Infectious Diseases*. **49**, 1411-1423.

Saah, A.J. and Hoover, D.R. (1997). "Sensitivity" and "specificity" reconsidered: The meaning of these terms in analytical and diagnostic settings. *Annals of Internal Medicine*. **126**(1). 91-94.

Saito, R., Misawa, Y., Moriya, K., Koike, K., Ubukata, K. and Okamura, N. (2005). Development and evaluation of a loop-mediated isothermal amplification assay for rapid detection of *Mycoplasma pneumonia*.*J. Med. Microbiol*.**54**, 1037-1041.

Salimetrics®. (n.d.) Inter- and intra- assay coefficients of variability. No copyright. [online]. Available from: www.salimetrics.com/ . [Accessed 17th March 2017].

Saugar, J.M., Merino, F.J., Martín-Rabadán, P., Fernández-Soto, P., Ortega, S., Gárate, T. and Rodríguez, E. (2015). Application of real-time PCR for the detection of *Strongyloides* spp. in clinical samples at a reference center in Spain. *Acta Tropica*. **142**, 20-25

Savan, R., Kono, T., Itami, T. and Sakai, M. (2005). Loop- mediated isothermal amplification: an emerging technology for detection of fish and shellfish pathogens. *J. Fish Dis*. **28**, 573- 581.

Schär, F., Odermatt, P., Khieu, V., Panning, M., Duong, S., Muth, S., Marti, H. and Kramme, S. (2013a). Evaluation of real-time PCR for *Strongyloides stercoralis* and hookworm as diagnostic tool in asymptomatic schoolchildren in Cambodia. *Acta Tropica*. **126**, 89-92.

Schär, F., Trostdorf, U., Giardina, F., Khieu, V., Muth, S., Marti, H., Vounatsou, P. and Odermatt, P. (2013b). *Strongyloides stercoralis*. Global distribution and risk factors. *PLOS Neglected Tropical Diseases*. July, **7**(7). [online]. Available from www.plosntds.org. [Accessed 6th August 2013].

Schär, F., Guo, L., Streit, A., Khieu, V., Muth, S., Marti, H. and Odermatt, P. (2014). *Strongyloides stercoralis* genotypes in humans in Cambodia. *Parasitology International*. **63**, 533-536.

Shiwaku, K., Chigusa, Y., Kadosaka, T. and Kaneko, K. (1988). Factors influencing development of free-living generations of *Strongyloides stercoralis*. *Parasitology*. **97**(Pt 1), 129-138.

Siddiqui, A.A., Stanley, C.S., Berk, S.L. and Skelly, P.J. (2000). A cDNA encoding a nuclear hormone receptor of the steroid/thyroid hormone-receptor superfamily from the human parasitic nematode *Strongyloides stercoralis*. *Parasitology Research*. **86**(1). 24-29.

Smith, P.J., Theis, B., McCartney, S. and Brown, M. (2011). Helminths: an unrecognised disease burden prevalent among migrants in the gastroenterology clinic. *Frontline Gastroenterology*. **2**, 124-129.

Sudarshi, S., Stümpfle, R., Armstrong, M., Ellman, T., Parton, S., Krishnan, P., Chiodini, P. L. and Whitty, C. (2003). Clinical presentation and diagnostic sensitivity of laboratory tests for *Strongyloides stercoralis* in travellers compared with immigrants in a non- endemic country. *Travel Medicine and International Health*. **8**(8), 728 – 732.

Sudhakara, R. B. and Sivajothi, S. (2017). Proteomics and genomics in veterinary parasitology- a diagnostic tool. *J. Molecul. Biomarkers and Diagnosis*. **8** (6), 6.

Sultana, Y., Jeoffreys, N., Watts, M. R., Gilbert, G.L. and Lee, R. (2013). Real-time polymerase chain reaction for detection of *Strongyloides stercoralis* in stool. *Am. J. Trop Med Hyg*. **88**(6), 1048-1051.

Taylor, M. J., Garrard, T.A., O'Donahoo, F.J. and Ross, K.E. (2014). Human strongyloidiasis: identifying knowledge gaps, with emphasis on environmental control. *Research and Reports in Tropical Medicine*. **5**, 55-63.

ten Hove, R., Schuurman, T., Kooistra, M., Möller, L., van Lieshout, L. and Verweij, J.J. (2007). Detection of diarrhoea- causing protozoa in general practice patients in The Netherlands by multiplex real-time PCR. *Clin. Microbiol Infect*. **13**, 1001-1007.

ten Hove, R. J., Esbroeck, M., Vervoort, T., Ende, J., van Lieshout, L. and Verweij, J. J. (2009). Molecular diagnostics of intestinal parasites in returning travellers. *European Journal of Clinical Microbiology and Infectious Diseases*. **28**(9), 1045 – 1053.

Thekisoe, O.M.M., Rodriguez, C.V., Rivas, F., Coronel-Servian, A.M., Fukumoto, S., Sugimoto, C., Kawazu, S. and Inoue, N. (2010). Detection of *Trypansoma cruzi* and *T. rangeli* infections from *Rhodnius pallescens* bugs by loop-mediated isothermal amplification (LAMP). *American Journal of Tropical Medicine and Hygiene*. **82**(5), 855-860.

Thermo Fisher Scientific Inc. (2016) Kit inserts. ©Copyright 2016 Thermo Fisher Scientific Inc. [online]. Available from: corporate.thermofisher.com. [Accessed March 2016].

UKAS. (n.d.) Medical laboratory accreditation (ISO 15189). ©Copyright, United Kingdom Accreditation Service.[online]. Available from: www.ukas.com. [Accessed 30th May 2017].

Vallone, P.M. and Butler, J.M. (2004). Autodimer: a screening tool for primer dimer and hairpin structures. *Biotechniques*. **37**, 226- 231.

Vasoo, S. and Pritt, B.S. (2013). Molecular diagnostics and parasitic disease. *Clin Lab Med* **33**, 461–50.

Verweij, J.J., Blange, R.A., Templeton, K., Schinkel, J., Brienen, E.A.T., van Rooven, M.A.A., van Lieshout, L. and Polderman, A.M. (2004). Simultaneous detection of *Entamoeba histolytica*, *Giardia lamblia* and *Cryptosporidium parvum* in fecal samples by using real-time PCR. *J. Clin. Microb*. **42**(3), 1220-1223.

Verweij, J.J., Brienen, E.A.T., Ziem, J., Yelifari, L., Polderman, A.M. and van Lieshout, L. (2006). Simultaneous detection and quantification of *Ancylostoma duodenale*, *Necator americanus*, and *Oesophagostomum bifurcum* in fecal samples using Multiplex Real-Time PCR. Am. J. of Trop. Med. And Hyg. 77(4). [online]. Available from: http://www.ajtmh.org. Accessed: December 2017.

Verweij, J.J., Canales, M., Polman, K., Ziem, J., Brienen, E.A.T., Polderman,. A.M., and van Lieshout, L. (2009). Molecular diagnosis of *Strongyloides stercoralis* in faecal samples using real-time PCR. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. **103**, 342-346.

Verweij, J. J. and Stensvold, C.R. (2014) Molecular testing for clinical diagnosis and epidemiological investigations of intestinal parasitic investigations. *Clin. Microbiol. Reviews*. **27**(2), 371-418.

Wang, J. and Stamey, J.D. (2010). A Bayesian algorithm for sample size determination for equivalence and non-inferiority test. *Journal of Applied Statistics*. **37**(10), 1749-1759.

Watts, M.R., James, G., Sultana, Y., Ginn, A.N., Outhred, A. C., Kong, F., Verweij, J.J., Ireldell, J.R., Chen, S.C-A. and Lee, R. (2014). A loop-mediated isothermal amplification (LAMP) assay for *Strongyloides stercoralis* in stool that uses a visual detection method with SYTO-82 fluorescent dye. *American Journal of Tropical Medicine and Hygiene*. **90**(2), 306-311.

Watts, M.R., Robertson, G and Bradbury, R.S. (2016). The laboratory diagnosis of *Strongyloides stercoralis*. *Microbiology Australia*. [online]. Available from: www.researchgate.net/. [Accessed 29th May 2016]

Weerakoon, K. G. and McManus, D.P. (2016) Cell-free DNA as a diagnostic tool for human parasitic infections. *Trends in Parasitology*. **32** (5), 378-391.

Whiting, P.F., Ruties, A.W.S., Westwood, M.E., Mallett, S., Deeks, J.J., Reitsma, J.B., Leeflang, M.M.G., Sterne, J.A.C., Bossuyt, P.M.M and the QUADAS-2 Group. (2011) QUADAS-2: A revised tool for the quality assessment of diagnostic accuracy studies. *Ann. Internal Med*. **155**. 529-536.

Whitty, C.J.M., Carroll, B., Armstrong, M., Dow, C., Snashall, D., Marshall, T. and Chiodini, P.L. (2000). Utility of history, examination and laboratory tests in screening those who return to Europe from the tropics for parasitic infection. *Tropical Medicine and International Health*. **5**(11), 818-823.

WHO. (2010). Research Priorities for Helminth Infections. [online]. ©Copyright WHO, 2010. WHO Technical Report Series. 972. [online]. Available from: apps.who.int/ . [Accessed 23rd June 2013]

WHO. (2013). The use of a commercial isothermal amplification assay (TB-LAMP) for the detection of tuberculosis. *Expert group meeting report*. [online]. Available from: www.who.int. [Accessed 23rd April 2017].

Wittwer, C., Reed, G. H. ,Gundry, C. N., Vandersteen, J. G. and Pryor, R. J. (2003). High-resolution genotyping by amplicon melting analysis using LC Green. *Clinical Chemistry.* **49** (6), 853–860

Wong, M.L. and Medrano, J.F. (2005). Real-time PCR for mRNA quantification. *Biotechniques* **39**(1), 1-11.*World Medical Association (2013). "Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects". JAMA **310** (20), 2191–2194.*

World Medical Association. (2013). WMA Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects. ©Copyright WMA 2017. *WMA*, 2013. [online]. Available from: www.wma.net/. [Accessed March 2014].

Yang, M., Gebeyehu, E.B., Kwon, O. and Kwak, D. (2013). Treatment of naturally acquired *Strongyloides stercoralis* infection in a dog with ivermectin. *J. Animal and Plant Sciences*. **23**(1), 337-339.

Zammarchi, L., Montagnani, F., Tordini, G., Gotuzzo, E., Bisoffi, Z., Bartoloni, A. and De Luca, A. (2015). Case Report: Persistent strongyloidiasis complicated by recurrent meningitis in an HTLV seropositive Peruvian migrant resettled in Italy. *Am. J. Trop. Med. Hyg*. **92**(6), 1257–1260.

APPENDICES

APPENDIX 1: *S. STERCORALIS* MODIFIED "IN- HOUSE" CHARCOAL CULTURE METHOD (MINATO *ET AL.*, 2008)

The in-house modification of the charcoal culture requires up to 5 g of faeces to be mixed with an equal volume of charcoal in a small petri-dish lid that has been fixed to the bottom of a medium sized petri-dish. The bottom of the medium-sized petri-dish is covered in enough water to reach the bottom of the lip of the small petri-dish but does flow into the small petri-dish. The lid of the medium-sized petri-dish is applied and the culture is incubated at 23^{o}C for 10 days. The culture water is examined weekly under an inverted microscope (200x magnification), but may be examined more frequently. Distinctive *S. stercoralis* larvae must be differentiated from hookworm larvae which may appear early in the incubation period.

APPENDIX 2: PRIMER SETS DESIGNED FOR LOOP- MEDIATED ISOTHERMAL AMPLIFICATION USING PRIMEREXPLORER V.3 (EIKEN CHEMICAL CO. LTD., 2005)

eurofins mwg|operon

Oligonucleotide Synthesis Report

Mr. David Manser
Clinical Parasitology
HTD

Order ID: 2241834
Customer ID: 946342
Your Order ID (PO#): UCLH086026

Order Date: 17/08/2010
Lab No.: 2294
No. of Oligos: 83/83

Eurofins MWG Operon
Anzingerstraße 7a
D- 85560 Ebersberg

No.	Oligo Name	Sequence (5' -> 3')	Yield [OD]	Yield [µg]	Yield [nmol]	Concentration [pmol/µl]	Vol. for 100pmol/µl	Tm [°C]	MW [g/mol]	GC-Content	Synthesis Scale	Purification	Modification	Barcode IDO	QC Report
1	st18s:1:F3	AAAGTCTTTCGGTTCCGG (18)	6.3	183	33.2	-	332	53.7	5505	50 %	0.01 µmol	HPSF	-	012132852	-
2	st18s:1:B3	AACTAAGAACGGCCATG C (18)	6.3	165	29.9	-	299	53.7	5501	50 %	0.01 µmol	HPSF	-	012132853	-
3	st18s:1:FIP	CAAATTAAGCCGCAGGCT CCAGTATGGTTGCAAAGC TGAA (40)	10.2	275	22.3	-	223	72.5	12338	47.5 %	0.01 µmol	HPSF	-	012132854	-
4	st18s:1:BIP	CTCAACACGGGAAAACTC ACCCCACTAAATCATGAA AGAGCTATC (45)	9.9	263	19.2	-	192	73.1	13728	44.4 %	0.01 µmol	HPSF	-	012132855	-
5	st18s:1:LF1	GTGCCCTTCCGTCAATTC CT (20)	5.7	180	30.0	-	300	59.4	5994	55 %	0.01 µmol	HPSF	-	012132856	-
6	st18s:1:LB	CGGACACTATAAGGATTG ACAGATT (25)	7.9	207	26.9	-	269	59.7	7714	40 %	0.01 µmol	HPSF	-	012132857	-
7	st18s:1:LF2	GTGCCCTTCCGTCAATTC C (19)	7.2	226	39.8	-	398	58.8	5690	57.9 %	0.01 µmol	HPSF	-	012132858	-
8	St18s:4:F3	GGAAGTATGGTTGCAAA GC (19)	5.8	152	25.7	-	257	54.5	5916	47.4 %	0.01 µmol	HPSF	-	012132859	-
9	St18s:4:B3	TCGCTCGTTATCGGAATC (18)	6.0	177	32.3	-	323	53.7	5465	50 %	0.01 µmol	HPSF	-	012132860	-
10	St18s:4:FIP	CGGGTGAGTTTTCCCGT GTTGAATTGACGGAAGG GCAC (38)	11.2	318	26.9	-	269	74.9	11805	55.3 %	0.01 µmol	HPSF	-	012132861	-
11	St18s:4:BIP	GCCGGACACTATAAGGA TTGACCGAACTAAGAACG GCCATG (41)	11.0	294	23.2	-	232	74.4	12661	51.2 %	0.01 µmol	HPSF	-	012132862	-

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Oligonucleotide Synthesis Report

No.	Oligo Name	Sequence (5' -> 3')	Yield [OD]	Yield [µg]	Yield [nmol]	Concentration [pmol/µl]	Vol. for 100pmol/µl	Tm [°C]	MW [g/mol]	GC-Content	Synthesis Scale	Purification	Modification	Barcode IDO	QC Report
12	St18s:4:LB	TCTTTCATGATTTAGTGG TTGGTGG (25)	9.7	283	36.6	-	366	59.7	7739	40 %	0.01 µmol	HPSF	-	012132863	-
13	St18s:4:LF	AAATTAAGCCGCAGGCTC CA (20)	5.6	150	24.6	-	246	57.3	6095	50 %	0.01 µmol	HPSF	-	012132864	-
14	St18s:4:LB	GCTCTTTCATGATTTAGT GGTTGGT (25)	8.0	236	30.7	-	307	59.7	7699	40 %	0.01 µmol	HPSF	-	012132865	-
15	St18s:12:F3	CCACATTAGTGGTGCGTT TA (20)	5.2	148	24.2	-	242	55.3	6122	45 %	0.01 µmol	HPSF	-	012132866	-
16	St18s:12:B3	CTAAAATTGGGTAATTTT CGCG (22)	7.8	214	31.6	-	316	54.7	6764	36.4 %	0.01 µmol	HPSF	-	012132867	-
17	St18s:12:Fip	ACCATCGAAAGTTGATAA ACCAGATATATTGGTTGA CTCAAAATATCCTC (50)	9.0	239	15.6	-	156	70.2	15343	34 %	0.01 µmol	HPSF	-	012132868	-
18	St18s:12:BIP	GTATTGGCCTACCATGGT TGTGTGGTAGCCGTTTCT CAGG (40)	9.8	290	23.5	-	235	74.6	12355	52.5 %	0.01 µmol	HPSF	-	012132869	-
19	St18s:12:LF	CATACGGTATGTTTTAGT AACAAAATCAGC (30)	15.0	397	43.1	-	431	61.3	9213	33.3 %	0.01 µmol	HPSF	-	012132870	-
20	St18s:12:LB1	GATAACGGAGAATTAGG GTTCGACTCC (27)	8.0	216	25.8	-	258	65.0	8348	48.1 %	0.01 µmol	HPSF	-	012132871	-
21	St18s:12:LB2	GAGAATTAGGGTTCGACT CCGGAGAG (26)	5.6	151	18.6	-	186	66.4	8100	53.8 %	0.01 µmol	HPSF	-	012132872	-
22	Po18s:299:F3	GCTGAATTTGCTTATTTT GAAGA (23)	8.3	228	32.2	-	322 360 nicht	53.5	7083	30.4 %	0.01 µmol	HPSF	-	012132873	-
23	Po18s:299:B3	CTCGTTATACATATCAGT GTAGC (23)	9.0	252	36.0	-	360	57.1	7013	39.1 %	0.01 µmol	HPSF	-	012132874	-
24	Po18s:299:BI P	TAGAGGAACGATGTGTGT CTAACAGTGCAGCCTAGT TCATCT (42)	12.1	335	25.8	-	258	72.4	12968	45.2 %	0.01 µmol	HPSF	-	012132875	-

APPENDIX 3a: CLUSTAL W2 MULTIPLE SEQUENCE ALIGNMENT OF LAMP AND QPCR ASSAY PRODUCTS (LARKIN *ET AL*., 2007)

Grey= Reference sequence: M 84229 (*Strongyloides stercoralis* 18S ribosomal RNA gene, complete cds**)**

Yellow= LAMP amplicon sequence.

Red= hypervariable region sequence (Hasegawa *et al*., 2009).

Blue= qPCR amplicon sequence.

Identities are normalised by aligned length.

```
184           ------------------------------------------------------------
635           ------------------------------------------------------------
858           ------------------------------------------------------------
622           ------------------------------------------------------------
HVRIII        ------------------------------------------------------------
B3LAMP        ------------------------------------------------------------
HVRI          ------------------------------------------------------------
HVRIV         ------------------------------------------------------------
qPCR          ------------------------------------------------------------
ON2           ------------------------------------------------------------
F3LAMP        ------------------------------------------------------------
M84229.1      ACCTGGTTGATCCTGCCAGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTA
HVRII         ------------------------------------------------------------

184           ------------------------------------------------------------
635           ------------------------------------------------------------
858           ------------------------------------------------------------
622           ------------------------------------------------------------
HVRIII        ------------------------------------------------------------
B3LAMP        ------------------------------------------------------------
HVRI          ------------------------------------------------------------
HVRIV         ------------------------------------------------------------
qPCR          ------------------------------------------------------------
ON2           ------------------------------------------------------------
F3LAMP        ------------------------------------------------------------
M84229.1      AGTATAAACAAATTCATACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATAGTTTAT
HVRII         ------------------------------------------------------------

184           ------------------------------------------------------------
635           ------------------------------------------------------------
858           ------------------------------------------------------------
622           ------------------------------------------------------------
HVRIII        ------------------------------------------------------------
B3LAMP        ------------------------------------------CTAAT-------------
HVRI          ------------------------------------------------------------
HVRIV         ------------------------------------------------------------
qPCR          ------------------------------------------------------------
ON2           ------------------------------------------------------------
F3LAMP        ------------------------------------------------------------
M84229.1      TTGATGGTTTCTTGCTACATGGATAACTGTGGTAATTCTAGAGCTAATACATGCTKAAAA
HVRII         ------------------------------------------------------------
```

```
184          ----------------------------------------------------------
635          ----------------------------------------------------------
858          ----------------------------------------------------------
622          ----------------------------------------------------------
HVRIII       ----------------------------------------------------------
B3LAMP       ----------------------------------------------------------
HVRI         ----------------------------------------------------------
HVRIV        ----------------------------------------------------------
qPCR         ----------------------------------------------------------
ON2          ----------------------------------------------------------
F3LAMP       ----------------------------------------------------------
M84229.1     GCCCCGACTTCTGGAAGGGGTGTATTTATTAGATAAAAAACCAATGACTTCGGGCTCCTT
HVRII        ----------------------------------------------------------

184          ----------------------------------------------------------
635          ----------------------------------------------------------
858          ----------------------------------------------------------
622          ----------------------------------------------------------
HVRIII       ----------------------------------------------------------
B3LAMP       ----------------------------------------------------------TCTCC
HVRI         ----------------------------------------------------------
HVRIV        ----------------------------------------------------------
qPCR         ----------------------------------------------------------
ON2          ----------------------------------------------------------
F3LAMP       ----------------------CCTCGCTGANTTTGTTACTAAAACATACCGTATGTGT
M84229.1     GGTGATTCATAATAACTTCTCGAATCGCATGGCCTTGCGCCGGCGATGCTTCATTCAAAT
HVRII        ----------------------------------------------------------

184          ----------------------------------------------------------
635          ----------------------------------------------------------
858          ----------------------------------------------------------
622          ----------------------------------------------------------
HVRIII       ----------------------------------------------------------
B3LAMP       GTTATCCGTCNCNNCNNTGGTAGGTAGGCCAATACCCTACCATCGAAAGTTGATAA----
HVRI         ----------------------------------------------------------
HVRIV        ----------------------------------------------------------
qPCR         ----------------------------------------------------------
ON2          ----------------------------------------------------------
F3LAMP       ATCTGGTTTATCAACTTTCGATGGTAGGGTATTGGCCTACCATGGTTGTGACGGATAACG
M84229.1     TTCTGCCCTATCAACTTTCGATGGTAGGATAGTGGCCTACCATGGTATCAACGGGTAACG
HVRII        ----------------------------------------------------------

184          ----------------------------------------------------------
635          ----------------------------------------------------------
858          ----------------------------------------------------------
622          ----------------------------------------------------------
HVRIII       ----------------------------------------------------------
B3LAMP       ----------------------------------------------------------
HVRI         ----------------------------------------------------------
HVRIV        ----------------------------------------------------------
qPCR         ----------------------------------------------------------
ON2          ----------------------------------------------------------
F3LAMP       GAGAATTAGGGTTCGACTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAG
M84229.1     GGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAG
HVRII        ----------------------------------------------------------
```

```
184          ------------------------------------------------------------
635          ------------------------------------------------------------
858          ------------------------------------------------------------
622          ------------------------------------------------------------
HVRIII       ------------------------------------------------------------
B3LAMP       ------------------------------------------------------------
HVRI         ------------------------------------------------------------
HVRIV        ------------------------------------------------------------
qPCR         ------------------------------------------------------------
ON2          ------------------------------------------------------------
F3LAMP       GCAGCAGGCGCGAAA-------------------------------------------
M84229.1     GCAGCAGGCGCGAAAATTACCCAATTTTAGTTAAAAGAGGTAGTGACGAAAAATGACAAC
HVRII        ------------------------------------------------------------

184          ------------------------------------------------------------
635          ------------------------------------------------------------
858          ------------------------------------------------------------
622          ------------------------------------------------------------
HVRIII       ------------------------------------------------------------
B3LAMP       ------------------------------------------------------------
HVRI         ------------------------------------------------------------
HVRIV        ------------------------------------------------------------
qPCR         ------------------------------------------------------------
ON2          ------------------------------------------------------------
F3LAMP       ------------------------------------------------------------
M84229.1     CAAATATTATTATTAATATTTGGATTGAAAATCTTCAAGTTTAAATMACCTTGTTGGTAA
HVRII        ------------------------------------------------------------

184          ------------------------------------------------------------
635          ------------------------------------------------------------
858          ------------------------------------------------------------
622          ------------------------------------------------------------
HVRIII       ------------------------------------------------------------
B3LAMP       ----------------------------------------ACC--------AGATACACATAC
HVRI         ------------------------------------------------------------
HVRIV        ------------------------------------------------------------
qPCR         ------------------------------------------------------------
ON2          ------------------------------------------------------------
F3LAMP       ------------------------------------------------------------
M84229.1     AGGAAAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATACCAGCTTTCCAAGTGCATAAAA
HVRII        ------------------------------------------------------------

184          ------------------------------------------------------------
635          ------------------------------------------------------------
858          ------------------------------------------------------------
622          ------------------------------------------------------------
HVRIII       ------------------------------------------------------------
B3LAMP       GG--------------------------------------------------------
HVRI         ------------------------------------------------------------
HVRIV        ------------------------------------------------------------
qPCR         ------------------------------------------------------------
ON2          ------------------------------------------------------------
F3LAMP       ------------------------------------------------------------
M84229.1     TGATTGTTGTGGTTAAAAAGCTCGTAGTTGGATTATAAAGATTGTATAATGAGCATCTTG
HVRII        ------------------------------------------------------------
```

```
184          ------------------------------------------------------------
635          ------------------------------------------------------------
858          ------------------------------------------------------------
622          ------------------------------------------------------------
HVRIII       ------------------------------TTATTATAATTAGANNNNAATAATATAAT
B3LAMP       ------------------------------------------------------------
HVRI         ----------AACCATTTTNAT----------------------------------------
HVRIV        ------------------------------------------------------------
qPCR         ------------------------------------------------------------
ON2          ------------------------------------------------------------
F3LAMP       ------------------------------------------------------------
M84229.1     GATGTTATTTAATCATTATCATCTTATATTTTTATTATATTAGAAATAATATAATAACTG
HVRII        --TGTTATTTAATCAT-------------------------------------------


184          ------------------------------------------------------------
635          ------------------------------------------------------------
858          ------------------------------------------------------------
622          ------------------------------------------------------------
HVRIII       NAACT-------------------------------------------------------
B3LAMP       ------------------------------------------------------------
HVRI         ------------------------------------------------------------
HVRIV        ------------------------------------------------------------
qPCR         ------------------------------------------------------------
ON2          ------------------------------------------------------------
F3LAMP       ------------------------------------------------------------
M84229.1     TCACTTTGAATAAATCAGAGGGTTTAAACCAGACATTATATGTTTGTATGGTCTAGCATG
HVRII        ------------------------------------------------------------


184          ------------------------------------------------------------
635          ------------------------------------------------------------
858          ------------------------------------------------------------
622          ------------------------------------------------------------
HVRIII       ------------------------------------------------------------
B3LAMP       ------------------------------------------------------------
HVRI         ------------------------------------------------------------
HVRIV        ------------------------------------------------------------
qPCR         ------------------------------------------------------------
ON2          ------------------------------------------------------------
F3LAMP       ------------------------------------------------------------
M84229.1     GAATAACACTATAGAAAAATTTAGTGTGGTTTCACTTAATTTTTCATGATTAATAGGAAC
HVRII        ------------------------------------------------------------


184          ------------------------------------------------------------
635          ------------------------------------------------------------
858          ------------------------------------------------------------
622          ------------------------------------------------------------
HVRIII       ------------------------------------------------------------
B3LAMP       ------------------------------------------------------------
HVRI         ------------------------------------------------------------
HVRIV        ------------------------------------------------------------
qPCR         ------------------------------------------------------------
ON2          ------------------------------------------------------------
F3LAMP       ------------------------------------------------------------
M84229.1     AAACGGGGGCATTCGTATCGCTACGTTAGAGGTGAAATTCTTGGACCGTAGCGAGACGTC
HVRII        ------------------------------------------------------------


184          ------------------------------------------------------------
```

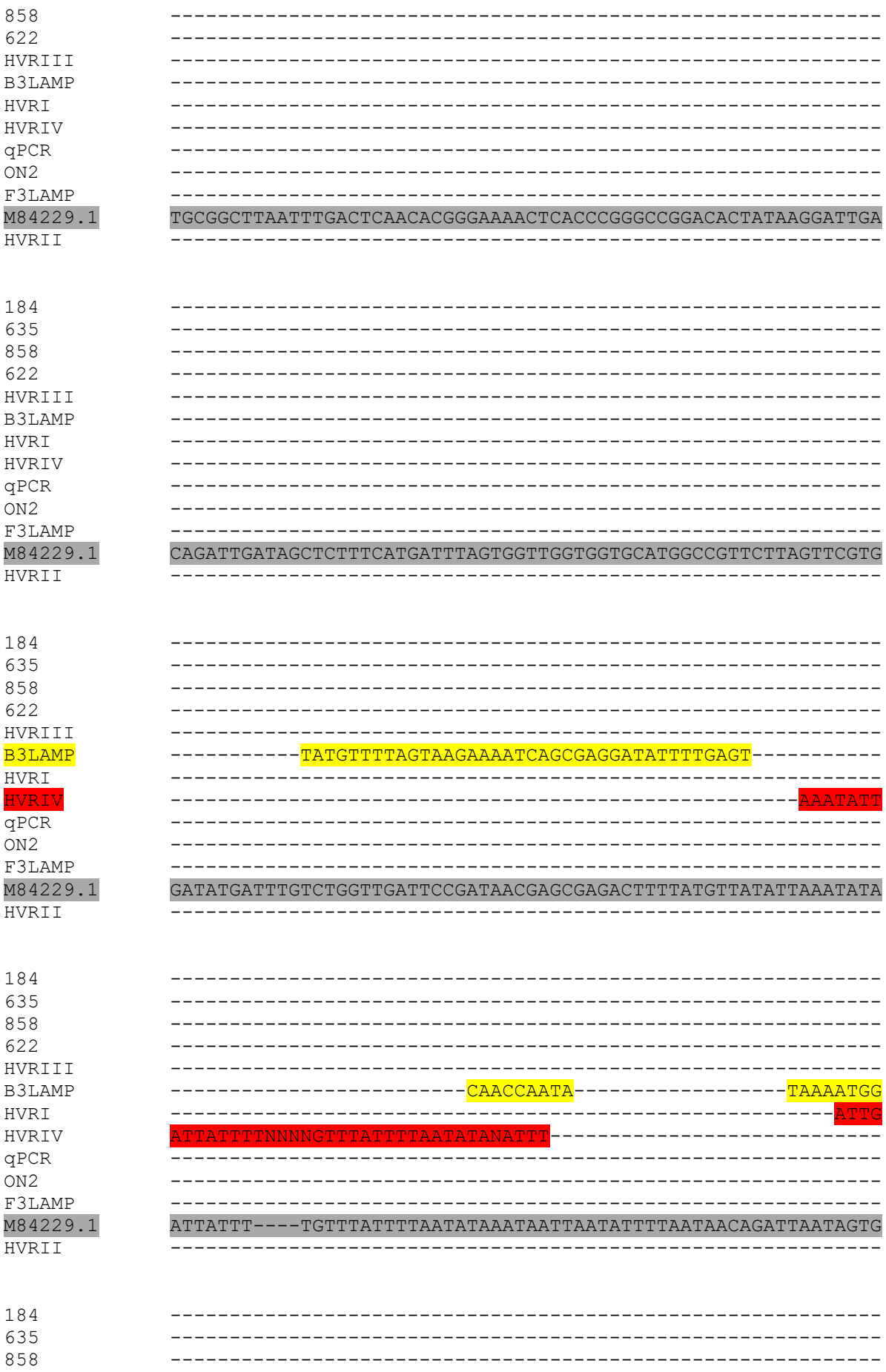
```
635          ------------------------------------------------------------
858          ------------------------------------------------------------
622          ------------------------------------------------------------
HVRIII       ------------------------------------------------------------
B3LAMP       ------------------------------------------------------------
HVRI         ------------------------------------------------------------
HVRIV        ------------------------------------------------------------
qPCR         ------------------------------------------------------------
ON2          ------------------------------------------------------------
F3LAMP       ------------------------------------------------------------
M84229.1     CTACTGCGAAAGCATTTGCCAAGAATGTTTTCATTAATCAAGAACGAAAGTTAGAGGTTC
HVRII        ------------------------------------------------------------

184          ------------------------------------------------------------
635          ------------------------------------------------------------
858          ------------------------------------------------------------
622          ------------------------------------------------------------
HVRIII       ------------------------------------------------------------
B3LAMP       ------------------------------------------------------------
HVRI         ------------------------------------------------------------
HVRIV        ------------------------------------------------------------
qPCR         ------------------------------------------------------------
ON2          ------------------------------------------------------------
F3LAMP       ------------------------------------------------------------
M84229.1     GAAGGCGATCAGATACCGCCCTAGTTCTAACCGTAAACTATGCCTACTAGATGTATGAAT
HVRII        ------------------------------------------------------------

184          ------------------------------------------------------------
635          ------------------------------------------------------------
858          ------------------------------------------------------------
622          ------------------------------------------------------------
HVRIII       ------------------------------------------------------------
B3LAMP       ------------------------------------------------------------
HVRI         ------------------------------------------------------------
HVRIV        ------------------------------------------------------------
qPCR         ------------------------------------------------------------
ON2          ------------------------------------------------------------
F3LAMP       ------------------------------------------------------------
M84229.1     TATTAGTTATAATTATTTATGCATCTTCTCGGAAACGAAAGTCTTTCGGTTCCGGGGGAA
HVRII        ------------------------------------------------------------

184          ------------------------------------------------------------
635          ------------------------------------------------------------
858          ------------------------------------------------------------
622          ------------------------------------------------------------
HVRIII       ------------------------------------------------------------
B3LAMP       ------------------------------------------------------------
HVRI         ------------------------------------------------------------
HVRIV        ------------------------------------------------------------
qPCR         ------------------------------------------------------------
ON2          ------------------------------------------------------------
F3LAMP       ------------------------------------------------------------
M84229.1     GTATGGTTGAAAAGCTGAAACTTAAAGGAATTGACGGAAGGCACCACCAGGAGTGGAGCC
HVRII        ------------------------------------------------------------

184          ------------------------------------------------------------
635          ------------------------------------------------------------
```


858
622
HVRIII
B3LAMP
HVRI
HVRIV
qPCR
ON2
F3LAMP
M84229.1 TGCGGCTTAATTTGACTCAACACGGGAAAACTCACCCGGGCCGGACACTATAAGGATTGA
HVRII
184
635
858
622
HVRIII
B3LAMP
HVRI
HVRIV
qPCR
ON2
F3LAMP
M84229.1 CAGATTGATAGCTCTTTCATGATTTAGTGGTTGGTGGTGCATGGCCGTTCTTAGTTCGTG
HVRII
184
635
858
622
HVRIII
B3LAMP -----TATGTTTTAGTAAGAAAATCAGCGAGGATATTTTGAGT-----
HVRI
HVRIV AAATATT
qPCR
ON2
F3LAMP
M84229.1 GATATGATTTGTCTGGTTGATTCCGATAACGAGCGAGACTTTTATGTTATATTAAATATA
HVRII
184
635
858
622
HVRIII
B3LAMP CAACCAATA TAAAATGG
HVRI ATTG
HVRIV ATTATTTTNNNNGTTTATTTTAATATANATTT
qPCR
ON2
F3LAMP
M84229.1 ATTATTT----TGTTTATTTTAATATAAATAATTAATATTTTAATAACAGATTAATAGTG
HVRII
184
635
858

```
622          ------------------------------------------------------------
HVRIII       ------------------------------------------------------------
B3LAMP       TTTAATCAAAT-AAACGCACCACTAA----T--GTGG----------------------
HVRI         GTT-------------------------------------------------------
HVRIV        ------------------------------------------------------------
qPCR         ------------------------------------------------------------
ON2          ------------------------------------------------------------
F3LAMP       ------------------------------------------------------------
M84229.1     TTTAACTATTTGAGAGAGAGCGATAACAGGTCTGTGATGCCCTTAGATGTCCGGGGCTGC
HVRII        ------------------------------------------------------------

184          ------------------------------------------------------------
635          ------------------------------------------------------------
858          ------------------------------------------------------------
622          ------------------------------------------------------------
HVRIII       ------------------------------------------------------------
B3LAMP       ------------------------------------------------------------
HVRI         ------------------------------------------------------------
HVRIV        ------------------------------------------------------------
qPCR         ------------------------------------------------------------
ON2          ------------------------------------------------------------
F3LAMP       ------------------------------------------------------------
M84229.1     ACGCGCGCTACAATGTAGTGATCATTATGTTCCTGTTTAGAGATAAATGGGTAAACATTG
HVRII        ------------------------------------------------------------

184          ------------------------------------------------------------
635          ------------------------------------------------------------
858          ------------------------------------------------------------
622          ------------------------------------------------------------
HVRIII       ------------------------------------------------------------
B3LAMP       ------------------------------------------------------------
HVRI         ------------------------------------------------------------
HVRIV        ------------------------------------------------------------
qPCR         ----------------------------GGCTCGAGTTTTTCAGCAAGATGAATTCCAA
ON2          ----------------------------GGCTCGAGTTTTTCAGCAAGATGAATTCCAA
F3LAMP       ------------------------------------------------------------
M84229.1     AAAACATTACGTAACTGGGAGTGAAAATTGCAATTATTTTTCATGAACGAGGAATTCCAA
HVRII        ------------------------------------------------------------

184          ----------------GGCTCGAGTTTTTCAGCAGATTGCCTCTGGATATTGCTCATTT
635          ------------GGATGGCTCGAGTTTTTCAGCAAGATTGNCTCTGGATATTGCTCAGTT
858          --------------ATGGCTCGAGTTTTTCAGCAAGATTGCCTCTGGATATTGCTCAGTT
622          ---------------TGGCTCGAGTTTTTCAGCAAGATTGCCTCTGGATATTGCTCAGTT
HVRIII       ------------------------------------------------------------
B3LAMP       ------------------------------------------------------------
HVRI         ------------------------------------------------------------
HVRIV        ------------------------------------------------------------
qPCR         GTAAACGTAAGTCATTAGCTTACATTGATTACGTCCCTGCCCTTTGTACACACCGCCCGT
ON2          GTAAACGTAAGTCATTAGCTTACATTGATTACGTCCCTGCCCTTTGTACACACCGCCCGT
F3LAMP       ------------------------------------------------------------
M84229.1     GTAAACGTAAGTCATTAGCTTACATTGATTACGTCCCTGCCCTTTGTACACACCGCCCGT
HVRII        ------------------------------------------------------------

184          CCAGGTAACGACGGGCGGTGTGTAGAAAGGG---CAGGGACGTAATCAATGTAAGCTAAT
635          CCGGGTAACGACGGGNGNTGTGTAGAAAGGG---CAGGGACGTGATCAATGTAAGCTAAT
858          CCGGGCAGCGACGGGCGGTGTGTACAAAGGG---CAGGGACGTAATCAATGTAAGCTAAT
622          CCGGGCAGCGACGGGCGGTGTGTACAAAGGG---CAGGGACGTAATCAATGTAAGCTAAT
```

```
HVRIII      ------------------------------------------------------------
B3LAMP      ------------------------------------------------------------
HVRI        ------------------------------------------------------------
HVRIV       ------------------------------------------------------------
qPCR        CGCTGCCCGGAACTGAGCAATATCCAGAGG----------------------------
ON2         CGCTGCCCGGAACTGAGCAATATCCAGAGG----------------------------
F3LAMP      ------------------------------------------------------------
M84229.1    CGCTGCCCGGAACTGAGCAATATCCAGAGGCAGGAAGAGATGTAATAAATTT-------T
HVRII       ------------------------------------------------------------

184         GACTTACGTTTACTTGGAATTCA-----------------------------------
635         GACTTACGTTTACTTGGAATTCA-----------------------------------
858         GACTTACG--------------------------------------------------
622         GACTTACGTTTACTTGGAATTCA-----------------------------------
HVRIII      ------------------------------------------------------------
B3LAMP      ------------------------------------------------------------
HVRI        ------------------------------------------------------------
HVRIV       ------------------------------------------------------------
qPCR        ------------------------------------------------------------
ON2         ------------------------------------------------------------
F3LAMP      ------------------------------------------------------------
M84229.1    TAATTTTTTTTATATTAAATCCTTCCAATCGCTGTTGTTTGAACCGGGCAAAAGTCGTAA
HVRII       ------------------------------------------------------------

184         ------------------------------------
635         ------------------------------------
858         ------------------------------------
622         ------------------------------------
HVRIII      ------------------------------------
B3LAMP      ------------------------------------
HVRI        ------------------------------------
HVRIV       ------------------------------------
qPCR        ------------------------------------
ON2         ------------------------------------
F3LAMP      ------------------------------------
M84229.1    CAAGGTTTTCGTAGGTGAACCTGCAGAAGGATCATCA
HVRII       ------------------------------------
```

APPENDIX 3b: CLUSTAL W2 MULTIPLE SEQUENCE ALIGNMENT OF ANOMALOUS QPCR ASSAY PRODUCT (LARKIN *ET AL.*, 2007)

Grey= Reference sequence: M 84229 (*Strongyloides stercoralis* 18S ribosomal RNA gene, complete cds**).**

Green= Nucleotide sequence match for the anomalous qPCR product (F- forward and B- back direct sequencing outer primer product, pGEM- product generated by the pGEM® T-Easy kit).

```
F207qPCR      ------------------------------------------------------------  0
pGEM          ------------------------------------------------------------  0
M84229.1      ACCTGGTTGATCCTGCCAGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTA  60
B207qPCR      ------------------------------------------------------------  0

F207qPCR      ------------------------------------------------------------  0
pGEM          ------------------------------------------------------------  0
M84229.1      AGTATAAACAAATTCATACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATAGTTTAT  120
B207qPCR      ------------------------------------------------------------  0

F207qPCR      ------------------------------------------------------------  0
pGEM          ------------------------------------------------------------  0
M84229.1      TTGATGGTTTCTTGCTACATGGATAACTGTGGTAATTCTAGAGCTAATACATGCTKAAAA  180
B207qPCR      ------------------------------------------------------------  0

F207qPCR      ------------------------------------------------------------  0
pGEM          ------------------NNNNNNNNNNNNNNCNNNNNNNNNNGCGGCNGCNGNNNNNNA  42
M84229.1      GCCCCGACTTCTGGAAGGGGTGTATTTATTAGATAAAAAACCAATGACTTCGGGCTCCTT  240
B207qPCR      ------------------------------------------------------------  0

F207qPCR      ------------------------------------------------------------T  1
pGEM          TTGNNTTCCNAGTAAACGTANNNNNNNNNNNNNNNNNNCAGACNACNACTTCGTACCAT  102
M84229.1      GGTGATTCATAATAACTTCTCGAATCGCATGGCCTTGCGCCGGCGATGCTTCATTCAAAT  300
B207qPCR      ------------------------TCGTATGCCACGGTG--------------------  15

F207qPCR      TCGAGCAGCTTCNGGGTGCCNAGATGGAAGAGTTGAAG------------------CTTG  43
pGEM          NNNNNNNNNTTCAGGGTGCCAAGATGGAAGAGTCGAAG------------------CTTG  144
M84229.1      TTCTGCCCTATCAACTTTCGATGGTAGGATAGTGGCCTACCATGGTATCAACGGGTAACG  360
B207qPCR      ------------------------------------------------------------  15

F207qPCR      AGGA-----------AATCAAGAACAAGNTCATGAAGAATGCCCAGGAG------CGAGA  86
pGEM          AGGA-----------AATCAAGAACAAGATCATGAAGAATGCCCAGGAG-------CAGA  186
M84229.1      GGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAG  420
B207qPCR      ------------------------------------------------------------  15

F207qPCR      ACATCATCTCCGATCA-TACCAAGATTGATGTACA---------TACCAAGGTAGCCAAC  136
pGEM          ACATCATCTCCGATCA-TACCAAGATTGATGTACA---------TACCAAGGTAGCCAAC  236
M84229.1      GCAGCAGGCGCGAAAATTACCCAATTTTAGTTAAAAGAGGTAGTGACGAAAAATGACAAC  480
B207qPCR      ------------------------------------------------------------  15

F207qPCR      GCCACC------GATGCATCAGGTAAGAACATCTTCAACTATGATGTAGATGTATCTTAT  190
pGEM          GCCACC------GATGCATCAGGTAAGAACATCTTCAACTATGATGTAGATGTATCTTAT  290
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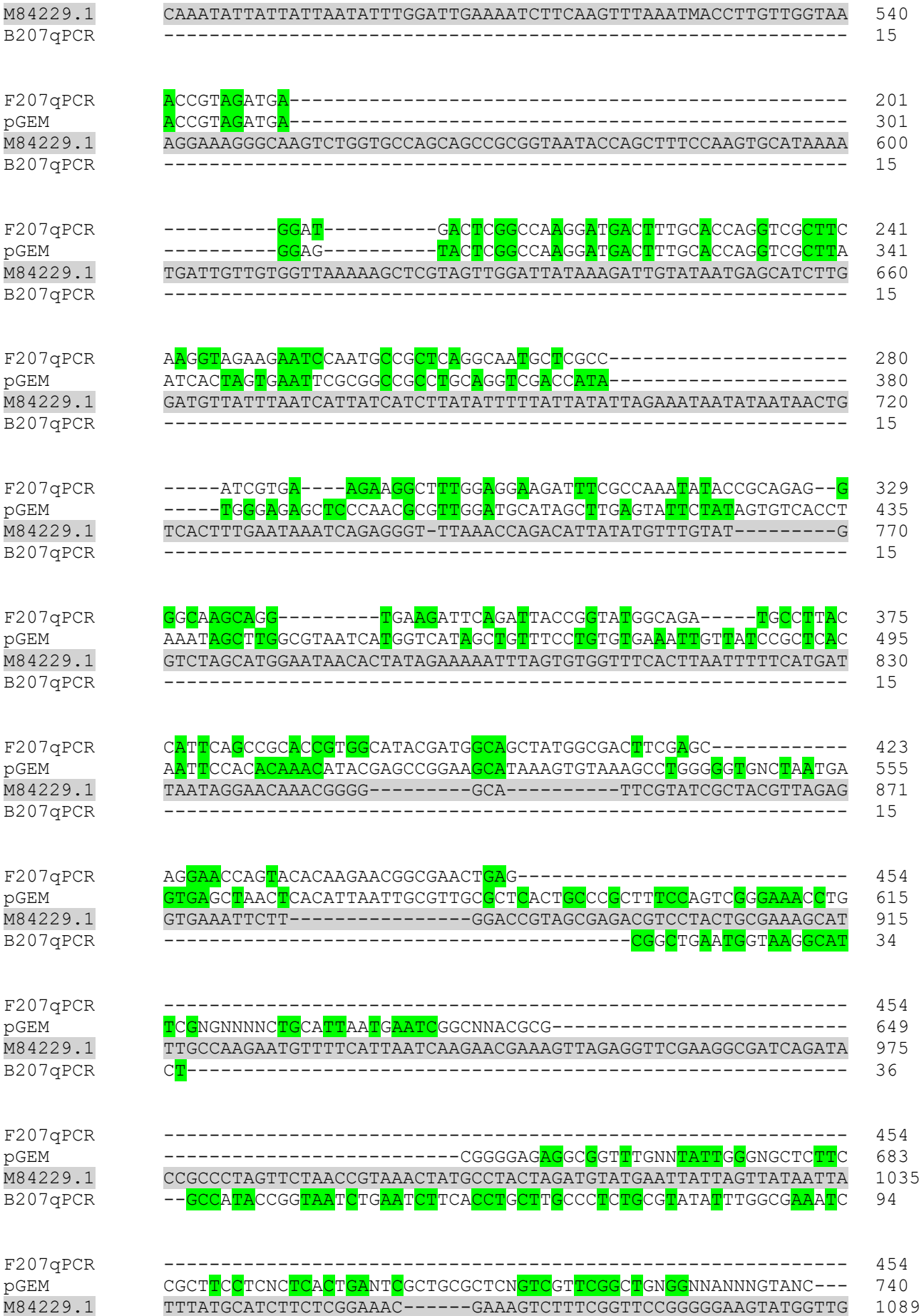
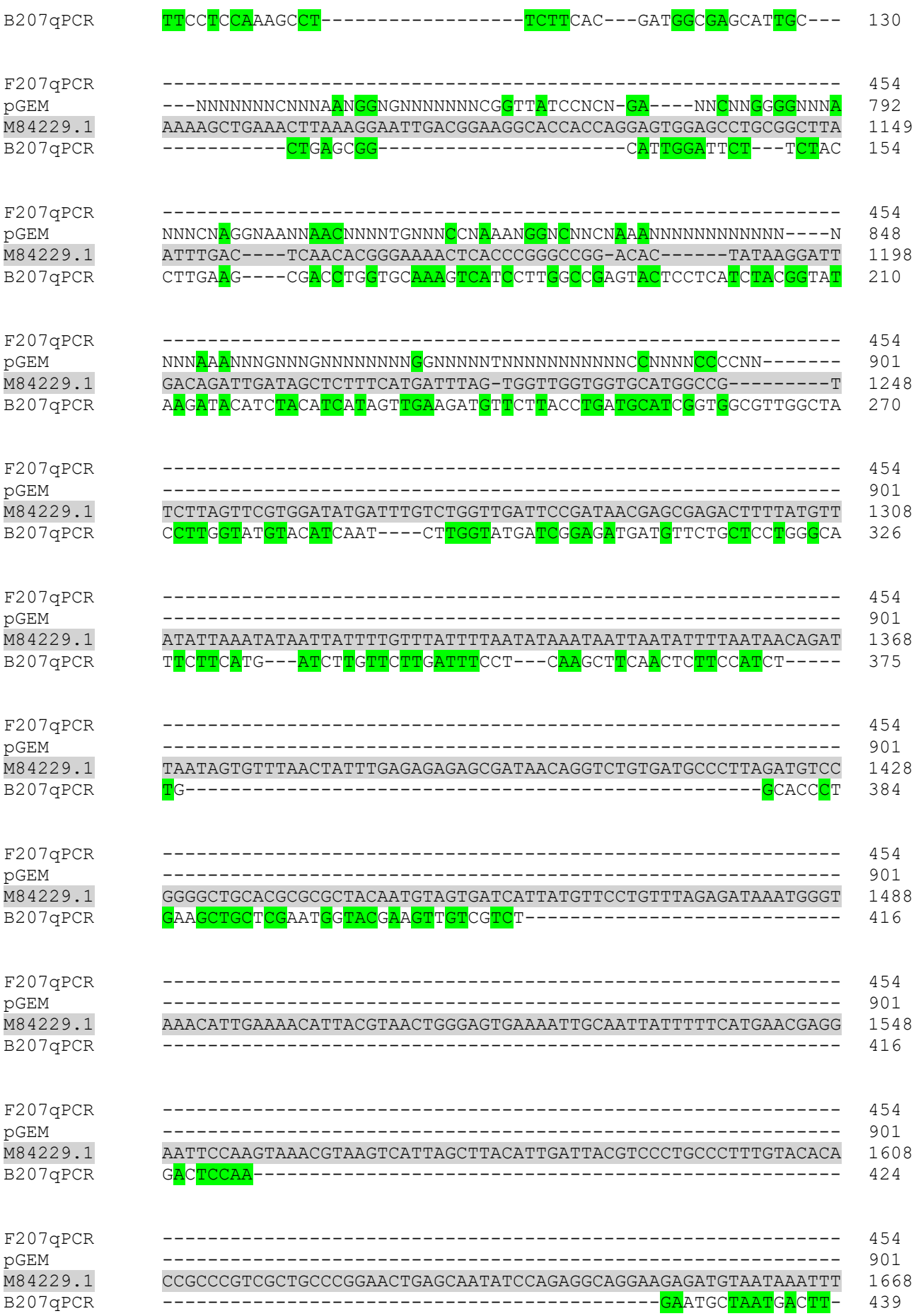

M84229.1 CAAATATTATTATTAATATTTGGATTGAAAATCTTCAAGTTTAAATMACCTTGTTGGTAA 540
B207qPCR ---------- 15
F207qPCR ACCGTAGATGA---------- 201
pGEM ACCGTAGATGA---------- 301
M84229.1 AGGAAAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATACCAGCTTTCCAAGTGCATAAAA 600
B207qPCR ---------- 15
F207qPCR ----------GGAT----------GACTCGGCCAAGGATGACTTTGCACCAGGTCGCTTC 241
pGEM ----------GGAG----------TACTCGGCCAAGGATGACTTTGCACCAGGTCGCTTA 341
M84229.1 TGATTGTTGTGGTTAAAAAGCTCGTAGTTGGATTATAAAGATTGTATAATGAGCATCTTG 660
B207qPCR ---------- 15
F207qPCR AAGGTAGAAGAATCCAATGCCGCTCAGGCAATGCTCGCC---------- 280
pGEM ATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATA---------- 380
M84229.1 GATGTTATTTAATCATTATCATCTTATATTTTTATTATATTAGAAATAATATAATAACTG 720
B207qPCR ---------- 15
F207qPCR -----ATCGTGA----AGAAGGCTTTGGAGGAAGATTTCGCCAAATATACCGCAGAG--G 329
pGEM -----TGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCT 435
M84229.1 TCACTTTGAATAAATCAGAGGGT-TTAAACCAGACATTATATGTTTGTAT---------G 770
B207qPCR ---------- 15
F207qPCR GGCAAGCAGG---------TGAAGATTCAGATTACCGGTATGGCAGA-----TGCCTTAC 375
pGEM AAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCAC 495
M84229.1 GTCTAGCATGGAATAACACTATAGAAAAATTTAGTGTGGTTTCACTTAATTTTTCATGAT 830
B207qPCR ---------- 15
F207qPCR CATTCAGCCGCACCGTGGCATACGATGGCAGCTATGGCGACTTCGAGC---------- 423
pGEM AATTCCACACAAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGGGTGNCTAATGA 555
M84229.1 TAATAGGAACAAACGGGG---------GCA----------TTCGTATCGCTACGTTAGAG 871
B207qPCR ---------- 15
F207qPCR AGGAACCAGTACACAAGAACGGCGAACTGAG---------- 454
pGEM GTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTG 615
M84229.1 GTGAAATTCTT----------GGACCGTAGCGAGACGTCCTACTGCGAAAGCAT 915
B207qPCR ----------CGGCTGAATGGTAAGGCAT 34
F207qPCR ---------- 454
pGEM TCGNGNNNNCTGCATTAATGAATCGGCNNACGCG---------- 649
M84229.1 TTGCCAAGAATGTTTTCATTAATCAAGAACGAAAGTTAGAGGTTCGAAGGCGATCAGATA 975
B207qPCR CT---------- 36
F207qPCR ---------- 454
pGEM ----------CGGGGAGAGGCGGTTTGNNTATTGGGNGCTCTTC 683
M84229.1 CCGCCCTAGTTCTAACCGTAAACTATGCCTACTAGATGTATGAATTATTAGTTATAATTA 1035
B207qPCR --GCCATACCGGTAATCTGAATCTTCACCTGCTTGCCCTCTGCGTATATTTGGCGAAATC 94
F207qPCR ---------- 454
pGEM CGCTTCCTCNCTCACTGANTCGCTGCGCTCNGTCGTTCGGCTGNGGNNANNNGTANC--- 740
M84229.1 TTTATGCATCTTCTCGGAAAC------GAAAGTCTTTCGGTTCCGGGGGAAGTATGGTTG 1089


B207qPCR TTCCTCCAAAGCCT----------TCTTCAC---GATGGCGAGCATTGC--- 130
F207qPCR ---------- 454
pGEM ---NNNNNNNCNNNAANGGNGNNNNNNNCGGTTATCCNCN-GA----NNCNNGGGGNNNA 792
M84229.1 AAAAGCTGAAACTTAAAGGAATTGACGGAAGGCACCACCAGGAGTGGAGCCTGCGGCTTA 1149
B207qPCR ----------CTGAGCGG----------CATTGGATTCT---TCTAC 154
F207qPCR ---------- 454
pGEM NNNCNAGGNAANNAACNNNNTGNNNCCNAAANGGNCNNCNAAANNNNNNNNNNNN----N 848
M84229.1 ATTTGAC----TCAACACGGGAAAACTCACCCGGGCCGG-ACAC------TATAAGGATT 1198
B207qPCR CTTGAAG----CGACCTGGTGCAAAGTCATCCTTGGCCGAGTACTCCTCATCTACGGTAT 210
F207qPCR ---------- 454
pGEM NNNAAANNNGNNNGNNNNNNNNGGNNNNNTNNNNNNNNNNNCCNNNNCCCCNN------- 901
M84229.1 GACAGATTGATAGCTCTTTCATGATTTAG-TGGTTGGTGGTGCATGGCCG---------T 1248
B207qPCR AAGATACATCTACATCATAGTTGAAGATGTTCTTACCTGATGCATCGGTGGCGTTGGCTA 270
F207qPCR ---------- 454
pGEM ---------- 901
M84229.1 TCTTAGTTCGTGGATATGATTTGTCTGGTTGATTCCGATAACGAGCGAGACTTTTATGTT 1308
B207qPCR CCTTGGTATGTACATCAAT----CTTGGTATGATCGGAGATGATGTTCTGCTCCTGGGCA 326
F207qPCR ---------- 454
pGEM ---------- 901
M84229.1 ATATTAAATATAATTATTTTGTTTATTTTAATATAAATAATTAATATTTTAATAACAGAT 1368
B207qPCR TTCTTCATG---ATCTTGTTCTTGATTTCCT---CAAGCTTCAACTCTTCCATCT----- 375
F207qPCR ---------- 454
pGEM ---------- 901
M84229.1 TAATAGTGTTTAACTATTTGAGAGAGAGCGATAACAGGTCTGTGATGCCCTTAGATGTCC 1428
B207qPCR TG----------GCACCCT 384
F207qPCR ---------- 454
pGEM ---------- 901
M84229.1 GGGGCTGCACGCGCGCTACAATGTAGTGATCATTATGTTCCTGTTTAGAGATAAATGGGT 1488
B207qPCR GAAGCTGCTCGAATGGTACGAAGTTGTCGTCT---------- 416
F207qPCR ---------- 454
pGEM ---------- 901
M84229.1 AAACATTGAAAACATTACGTAACTGGGAGTGAAAATTGCAATTATTTTTCATGAACGAGG 1548
B207qPCR ---------- 416
F207qPCR ---------- 454
pGEM ---------- 901
M84229.1 AATTCCAAGTAAACGTAAGTCATTAGCTTACATTGATTACGTCCCTGCCCTTTGTACACA 1608
B207qPCR GACTCCAA---------- 424
F207qPCR ---------- 454
pGEM ---------- 901
M84229.1 CCGCCCGTCGCTGCCCGGAACTGAGCAATATCCAGAGGCAGGAAGAGATGTAATAAATTT 1668
B207qPCR ----------GAATGCTAATGACTT- 439

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F207qPCR      ------------------------------------------------------------  454
pGEM          ------------------------------------------------------------  901
M84229.1      TTAATTTTTTTTATATTAAATCCTTCCAATCGCTGTTGTTTGAACCGGGCAAAAGTCGTA  1728
B207qPCR      ------------------------------------------------------------  439

F207qPCR      ------------------------------------  454
pGEM          ------------------------------------  901
M84229.1      ACAAGGTTTTCGTAGGTGAACCTGCAGAAGGATCATCA  1766
B207qPCR      ------------------------------------  439
```

APPENDIX 4: TABLE OF RESULTS OBTAINED FOR THE SAMPLES WITH ANOMALOUS RESULTS (CRS NEGATIVE) OR ANOMALOUS BANDS ON GEL ELECTROPHORESIS.

STUDY NUMBER	STORAGE TEMP.	ORIGINAL C_T	GEL BAND SIZE	CPCR	GEL BAND SIZE	SEQUENCE RESULT	STORAGE TEMP.	DUPLICATE SAMPLE (study number)	TRAVEL HISTORY
296	-20°C	38.14	101	Negative	N/A	N/A	4°C	Negative (289)	Latin America
1054	4°C	40.59	101	Negative	N/A	N/A	N/A	No duplicate study sample	Bangladesh
368	-20°C	31.36	101	Positive	101	*S. stercoralis* 99% homology	4°C	Negative (497)	Mauritius
266	-20°C	48.16	500	Positive	500	Insufficient DNA	N/A	No other samples	Worldwide
331	-20°C	35.7	101	Negative	N/A	N/A	4°C	Negative (873)	Worldwide
1087	-20°C	36.56	101	Negative	N/A	N/A	4°C	Negative (363)	Worldwide
838	-20°C	35.2	101	Negative	N/A	N/A	4°C	Negative (1064)	Worldwide
200	-20°C	36.9	101	Negative	N/A	N/A	4°C	Negative (310)	Unknown
756	-20°C	35.52	101	Negative	N/A	N/A	-20°C/ 4°C	Negative (716, 165 1097, 639,369)*[1]	Africa
1127	-20°C	42.74 = equivocal result	101	Positive	101	Insufficient DNA, no sequence identity obtained	4°C	Positive (1196) 40.7= equivocal result. Anomalous band on gel electrophoresis*[2]	Africa
146	4°C	24.06 (LAMP also Positive for this sample)	101	Positive	101	No sequence identity obtained	-20°C	Positive (358) 32.29 cPCR Positive 101bp gel band *[2]	Africa
994	4°C	35.84	Anomalous band	Positive	101	Insufficient DNA	-20°C	(231) 39.5 cPCR Negative*[2]	Latin America
800	4°C	26.76 (1in10 repeat 30.36 LAMP also Positive)	101	Positive	101	264 *S. stercoralis* 94% homology	-20°C	Positive (264) 27.15 101bp gel band. LAMP also Positive *[2]	Brazil
786	4°C	35.4	101	Positive	101	Insufficient DNA	-20°C	Negative (415) *[3]	Worldwide
677	4°C	38.91	Anomalous band	Negative	N/A	N/A	-20°C	Positive (770) 38.15 anomalous band cPCR Negative*[4]	Worldwide
748	4°C	34.43	101	Negative	N/A	N/A	-20°C	Negative (199) *[4]	Bangladesh
214	4°C	34.26	101	Negative	N/A	N/A	-20°C	Negative (450) *[4]	Unknown
1169	4°C	35.57	Anomalous band	Negative	N/A	N/A	-20°C	Negative (388) *[4]	Spain
318	4°C	41.6	101/ 125 Anomalous band	Positive	Double band	Insufficient DNA	-20°C	Negative (999) *[4]	Unknown
1042	-20°C	36.92	101	Negative	N/A	N/A	4°C	Negative (413) *[4]	Worldwide
120	-20°C	35.3	101	Negative	N/A	N/A	4°C	Negative (823) *[4]	Africa
1002	4°C	37.53	101	Positive Repeat Negative	101/500 N/A	N/A	-20°C	Negative (241) *[5]	Bangladesh
176	4°C	35.99	101	Positive	101	Insufficient DNA	-20°C	Negative (1120) - *[5]	Worldwide
725	-20°C	41.67	Anomalous band 150bp	Positive	101	Insufficient DNA	4°C	39.62 (213) *[5]*	Worldwide
209	-20°C	35.4	101	Positive	101	*S. stercoralis* 100% homology	4°C	Negative (479) *[5]	Israel
171	-20°C	35.13 (1in10 31.5)	101	Positive	101	*S. stercoralis* 100% homology	4°C	Negative (1016) *[5]	Africa
207	-20°C	35.29	101/ 500	Positive	500	*S. stercoralis* 100% homology- short sequence	4°C	Negative (265) *[5] anomalous band no sequence identity obtained	Morocco
213	-20°C	39.62	101/ 500	Positive	101	Insufficient DNA	4°C	Positive (725 41.67 101/ 500 insufficient DNA for sequencing)*[6]	Worldwide

*[1] 369 and 756 were received on the same date. False positive possibly due to contamination, no product generated by cPCR

*[2] True positive confirmed by cPCR

*[3] True positive confirmed by cPCR, discrepancy may be due to sampling error

*[4] False positive result, no product generated by cPCR

*[5] Possible true positive with very little DNA present

*[6] True positive and an anomalous band. Very little DNA present

APPENDIX 5: BUSINESS PLAN FOR THE INTRODUCTION OF A NAAT FOR THE DIAGNOSIS OF *S. STERCORALIS* IN CLINICAL SAMPLES AT DCP

NON-DISCLOSURE AND CONFIDENTIALITY AGREEMENT AND PROPERTY RIGHTS	This report is confidential and is the property of the University College London Hospitals NHS Foundation Trust (UCLH). All intellectual property rights belong to UCLH
CONTACT INFORMATION	Researcher: Katherine M Bowers (BMS band 7) katherine.bowers@uclh.nhs.uk Scientific Lead: Dr Spencer Polley spencer.polley@uclh.nhs.uk Clinical Lead: Prof. P Chiodini peter.chiodini@uclh.nhs.uk
EXECUTIVE SUMMARY	The introduction of a NAAT for the detection of *S. stercoralis* DNA in clinical samples with improved sensitivity and specificity to the current diagnostic repertoire on the basis of a prospective study performed at the Department of Clinical Parasitology for the fulfilment of a Professional Doctorate degree (with the University of Westminster) This study is submitted for approval to the UCLH board of Directors
BACKGROUND JUSTIFICATION FOR THE STUDY	Strongyloidiasis is caused by *Strongyloides stercoralis* (*S. stercoralis*) and is characterised by an array of symptoms ranging from asymptomatic to chronic non-specific gastro-intestinal problems in immunocompetent individuals and severe, potentially fatal, hyperinfection syndrome in immunocompromised individuals. The diagnosis of strongyloidiasis at the Department of Clinical Parasitology (DCP) is subject to low sensitivity because of a frequently low larval load and intermittent excretion of the parasite and low specificity where the antibodies are known to cross react with other helminthic parasites. Nucleic acid amplification techniques are available for the diagnosis of strongyloidiasis with reported improved sensitivity and specificity. A qPCR assay was investigated as part of a study for the fulfilment of a Professional Doctorate degree at the University of Westminster and a report is submitted here for approval
PRODUCT REVIEW	This study determined the following parameters for the qPCR assay: Analytical sensitivity: 100% Analytical specificity: 94.83% Limit of detection: 10^{-4} *S. stercoralis* larvae/ µl Overall percentage agreement with the composite reference standard: 80.63% (95% CI: 70.5-91.8) Precision: 0.3% within run (acceptable value < 10 % within run) Cost per test £8.21
SERVICES	The introduction of this test would offer an improved diagnostic service to users of DCP for the diagnosis of strongyloidiasis. This test is not available to routine diagnostic laboratories and as such would improve the service offered by DCP Further research is being carried out on the use of the qPCR assay for the determination of an appropriate treatment strategy using improved technology which was not available at UCLH under the current diagnostic testing regime. This will be of benefit to patient care and improve clinical decision making
MARKETING PLAN	The provision of advanced technology by DCP to UCLH and referred samples from around the UK would improve the service available to UCLH by the introduction of a test that is not available elsewhere in the UK The cost of the test is presented to UCLH for developing a pricing analysis and includes turnaround time and reagents required. The costing analysis does not include running costs as the diagnostic service at UCLH has been privatised and the joint venture will determine the running costs. All equipment required is already available for diagnostic use
GOALS AND STRATEGIES	The goal is to introduce an improved diagnostic test for the diagnosis of strongyloidiasis The success of the new NAAT will be seen by the uptake of an improved diagnostic test for strongyloidiasis. *S. stercoralis* is increasingly being identified in Western travel medicine and the risk of severe disease in immunocompromised individuals indicates that this test will be of benefit to patients at UCLH and to other hospitals around the UK. The future plan is to develop this assay for the monitoring of treatment and to design an algorithm that can be used for an appropriate treatment strategy

APPENDIX 6: *S. STERCORALIS* DNA DETECTION- RAW DATA FOR LAMP AND QPCR ASSAYS. KEY AT END OF TABLE

SOURCE	STUDY NUMBER	LENGTH OF STORAGE CODE	REF STD CODE	TRAVEL CODE	GENDER CODE	AGE CODE	LAMP (TIME IN MINS)	LAMP CODE	qPCR HS (C_t)	1:10 RPT	RE-RUN	GEL BAND (bp)	qPCR CODE
IDEA 4°C	115	4	2	2	0	6	Negative	0	Negative				0
IDEA 4°C	117	1	2	7	0	4	Negative	0	33.65			101	1
IDEA 4°C	119	1	0	7	1	6	Negative	0	Negative				0
IDEA 4°C	125	3	1	0	1	1	47.12	1	28.97			101	1
IDEA 4°C	130	0	0	0	0	6	Negative	0	Negative				0
IDEA 4°C	141	3	1	3	0	2	24.24	1	22.49			101	1
IDEA 4°C	156	1	0	2	1	5	Negative	0	Negative				0
IDEA 4°C	157	4	0	1	0	7	Negative	0	Negative				0
IDEA 4°C	158	4	2	5	1	6	Negative	0	Negative				0
IDEA 4°C	161	3	0	2	1	5	Negative	0	Negative				0
IDEA 4°C	164	2	1	2	0	4	Negative	0	Negative				0
IDEA 4°C	165	2	0	1	0	8	Negative	0	Negative				0
IDEA 4°C	168	1	0	1	0	6	Negative	0	Negative				0
IDEA 4°C	172	1	0	0	0	6	Negative	0	Negative				0
IDEA 4°C	181	3	0	1	0	7	Negative	0	Negative				0
IDEA 4°C	196	4	2	2	1	3	Negative	0	Negative				0
IDEA 4°C	263	0	2	7	0	6	Negative	0	Negative				0
IDEA 4°C	285	4	0	1	0	7	Negative	0	Negative				0
IDEA 4°C	334	3	2	1	0	4	Negative	0	30.07			101	1
IDEA 4°C	352	1	0	2	1	7	Negative	0	Negative				0
IDEA 4°C	359	2	0	0	0	5	Negative	0	Negative				0
IDEA 4°C	363	3	0	7	1	6	Negative	0	Negative				0
IDEA 4°C	364	3	0	0	0	5	Negative	0	Negative				0
IDEA 4°C	383	1	0	1	1	4	Negative	0	Negative				0
IDEA 4°C	402	4	0	2	0	4	Negative	0	Negative				0
IDEA 4°C	408	3	2	0	0	2	Negative	0	Negative				0
IDEA 4°C	413	1	0	7	1	4	Negative	0	Negative				0
IDEA 4°C	419	1	0	1	0	7	Negative	0	Negative				0
IDEA 4°C	470	3	2	2	0	5	Negative	0	Negative				0
IDEA 4°C	497	1	0	1	0	2	Negative	0	Negative				0
IDEA 4°C	515	3	0	7	0	6	Negative	0	Negative				0
IDEA 4°C	528	2	0	2	0	2	Negative	0	Negative				0
IDEA 4°C	536	3	0	0	0	2	Negative	0	Negative				0
IDEA 4°C	576	3	1	2	0	6	Negative	0	Negative				0
IDEA 4°C	593	1	0	1	0	5	Negative	0	Negative				0
IDEA 4°C	666	3	0	1	0	1	Negative	0	Negative				0
IDEA 4°C	683	3	2	1	0	2	Negative	0	Negative				0
IDEA 4°C	684	4	2	2	0	5	Negative	0	Negative				0
IDEA 4°C	686	3	2	4	1	4	Negative	0	Negative				0
IDEA 4°C	699	3	1	0	0	5	Negative	0	Negative				0

SOURCE	STUDY NUMBER	LENGTH OF STORAGE CODE	REF STD CODE	TRAVEL CODE	GENDER CODE	AGE CODE	LAMP (TIME IN MINS)	LAMP CODE	qPCR HS (C_t)	1:10 RPT	RE-RUN	GEL BAND (bp)	qPCR CODE
IDEA 4°C	792	3	2	3	0	4	Negative	0	Negative				0
IDEA 4°C	716	2	2	1	0	1	Negative	0	Negative				0
IDEA 4°C	810	2	0	3	0	1	Negative	0	Negative				0
IDEA 4°C	813	1	3	1	0	4	Negative	0	Negative				0
IDEA 4°C	828	3	0	2	0	5	Negative	0	Negative				0
IDEA 4°C	840	4	1	2	0	4	Negative	1	29.09			101	1
IDEA 4°C	842	0	0	1	0	5	Negative	0	Negative				0
IDEA 4°C	858	1	2	1	1	4	32.18	1	23.63			101	1
IDEA 4°C	893	3	1	2	0	6	Negative	0	Negative				0
IDEA 4°C	894	1	0	2	1	7	Negative	0	Negative				0
IDEA 4°C	896	4	2	2	0	5	Negative	0	35.25			101	1
IDEA 4°C	923	3	0	0	1	5	Negative	0	Negative				0
IDEA 4°C	939	3	0	2	0	3	Negative	0	Negative				0
IDEA 4°C	951	3	2	1	1	6	Negative	0	Negative				0
IDEA 4°C	970	3	2	2	0	4	Negative	0	Negative				0
IDEA 4°C	1007	3	0	0	0	5	Negative	0	Negative				0
IDEA 4°C	1024	4	0	2	1	5	Negative	0	Negative				0
IDEA 4°C	1054	3	0	2	0	8	Negative	0	40.59			101	1
IDEA 4°C	1060	2	0	1	1	4	Negative	0	Negative				0
IDEA 4°C	1081	4	2	7	1	5	Negative	0	Negative				0
IDEA 4°C	1097	2	0	1	0	8	Negative	0	Negative				0
IDEA 4°C	1121	3	1	2	0	6	Negative	0	37.76			101	1
IDEA -20°C	111	2	0	0	1	7	Negative	0	Negative				0
IDEA -20°C	135	4	2	2	0	5	Negative	0	Negative				0
IDEA -20°C	136	3	0	1	0	1	Negative	0	Negative				0
IDEA -20°C	142	3	1	3	0	2	24.06 (21.2.16)	1	17.76			101	1
IDEA -20°C	143	3	2	2	0	5	Negative	0	Negative				0
IDEA -20°C	148	2	0	2	0	2	Negative	0	Negative				0
IDEA -20°C	151	2	0	1	1	4	Negative	0	Negative				0
IDEA -20°C	153	1	0	1	1	4	Negative	0	Negative				0
IDEA -20°C	228	3	0	7	0	6	Negative	0	Negative	Negative 5.3.16			0
IDEA -20°C	229	0	0	1	0	5	Negative	0	Negative				0
IDEA -20°C	244	3	0	1	0	7	Negative	0	Negative				0
IDEA -20°C	266	1	0	7	0	6	Negative	0	48.16			500	2
IDEA -20°C	274	0	0	0	0	6	Negative	0	Negative				0
IDEA -20°C	282	1	0	2	1	5	Negative	0	Negative				0
IDEA -20°C	315	1	0	1	0	7	Negative	0	Negative				0
IDEA -20°C	327	0	2	7	0	6	Negative	0	Negative				0
IDEA -20°C	333	4	0	1	0	7	Negative	0	Negative				0
IDEA -20°C	368	1	0	1	0	2	Negative	0	31.36			101	1
IDEA -20°C	369	2	2	1	0	1	Negative	0	Negative				0
IDEA -20°C	387	4	2	2	0	5	Negative	0	Negative				0
IDEA -20°C	424	3	2	0	0	2	Negative	0	31.55			101	1

SOURCE	STUDY NUMBER	LENGTH OF STORAGE CODE	REF STD CODE	TRAVEL CODE	GENDER CODE	AGE CODE	LAMP (TIME IN MINS)	LAMP CODE	qPCR HS (C_t)	1:10 RPT	RE-RUN	GEL BAND (bp)	qPCR CODE
IDEA -20°C	473	4	0	1	0	7	Negative	0	Negative				0
IDEA -20°C	494	3	0	2	1	5	Negative	0	Negative				0
IDEA -20°C	532	2	1	2	0	4	38.48	1	27.37			101	1
IDEA -20°C	549	1	0	7	1	6	Negative	0	Negative				0
IDEA -20°C	561	3	0	0	0	5	Negative	0	Negative				0
IDEA -20°C	569	3	0	1	0	7	Negative	0	Negative				0
IDEA -20°C	601	3	0	2	0	3	Negative	0	Negative				0
IDEA -20°C	605	3	1	2	0	6	Negative	0	28.47			101	1
IDEA -20°C	607	1	2	7	0	4	58.42(21.2.16)	1	26.42			101	1
IDEA -20°C	608	1	0	1	0	5	Negative	0	Negative				0
IDEA -20°C	625	1	0	0	0	6	Negative	0	Negative				0
IDEA -20°C	639	2	0	1	0	8	Negative	0	Negative				0
IDEA -20°C	669	4	0	2	1	5	Negative	0	Negative				0
IDEA -20°C	729	3	2	1	0	2	Negative	0	Negative				0
IDEA -20°C	756	2	0	1	0	8	Negative	0	35.52			101	1
IDEA -20°C	790	4	2	2	1	3	Negative	0	Negative				0
IDEA -20°C	810	2	0	3	0	1	Negative	0	Negative				0
IDEA -20°C	829	4	0	2	0	4	Negative	0	Negative				0
IDEA -20°C	845	2	0	0	0	5	Negative	0	Negative				0
IDEA -20°C	860	3	2	3	0	4	Negative	0	Negative				0
IDEA -20°C	898	4	2	2	0	6	Negative	0	Negative				0
IDEA -20°C	931	1	0	1	0	6	Negative	0	Negative				0
IDEA -20°C	936	1	2	1	1	4	33.48	1	20.9				1
IDEA -20°C	950	4	1	2	0	4	33.30 (21.2.16)	1	24.58			101	1
IDEA -20°C	955	3	1	2	0	6	Negative	0	Negative				0
IDEA -20°C	960	3	0	2	0	5	Negative	0	Negative				0
IDEA -20°C	980	4	2	5	1	6	Negative	0	Negative				0
IDEA -20°C	997	1	0	2	1	7	Negative	0	Negative				0
IDEA -20°C	1022	1	2	1	0	4	Negative	0	33.3			101	1
IDEA -20°C	1042	1	0	7	1	4	Negative	0	36.92			101	1
IDEA -20°C	1087	3	0	7	1	6	Negative	0	36.56			101	1
IDEA -20°C	1132	3	2	2	0	4	Negative	0	Negative				0
IDEA -20°C	1152	3	0	1	0	5	Negative	0	Negative				0
IDEA -20°C	1181	1	0	2	1	7	Negative	0	Negative				0
Diag 4°C	102	0	0	1	1	6	Negative	0	Negative				0
Diag 4°C	106	0	0	1	0	4	Negative	0	Negative				0
Diag 4°C	108	3	1	0	0	7	49.36	1	31.46			101	1
Diag 4°C	109	1	0	2	1	4	Negative	0	Negative				0
Diag 4°C	110	1	0	2	0	5	Negative	0	Negative				0
Diag 4°C	112	1	0	7	0	7	Negative	0	Negative				0
Diag 4°C	113	1	0	2	0	1	Negative	0	Negative				0
Diag 4°C	118	1	0	0	0	7	Negative	0	Negative				0
Diag 4°C	122	0	0	1	1	7	Negative	0	Negative				0

SOURCE	STUDY NUMBER	LENGTH OF STORAGE CODE	REF STD CODE	TRAVEL CODE	GENDER CODE	AGE CODE	LAMP (TIME IN MINS)	LAMP CODE	qPCR HS (C_t)	1:10 RPT	RE-RUN	GEL BAND (bp)	qPCR CODE
Diag 4°C	124	0	0	7	0	3	Negative	0	Negative	Negative 5.3.16			0
Diag 4°C	123	1	0	2	0	8	Negative	0	Negative	Negative 5.3.16			0
Diag 4°C	126	1	0	1	1	5	Negative	0	Negative				0
Diag 4°C	127	0	0	2	1	6	Negative	0	Negative				0
Diag 4°C	128	0	0	1	1	5	Negative	0	Negative				0
Diag 4°C	131	1	0	1	0	5	Negative	0	Negative				0
Diag 4°C	138	0	2	2	1	4	Negative	0	Negative				0
Diag 4°C	139	0	2	7	0	6	Negative	0	Negative				0
Diag 4°C	144	1	0	2	1	7	Negative	0	Negative				0
Diag 4°C	146	0	0	1	0	2	38.3	1	24.06			101	1
Diag 4°C	155	0	1	0	0	5	Negative	0	Negative	Negative 5.3.16			0
Diag 4°C	160	0	0	4	1	7	Negative	0	Negative				0
Diag 4°C	162	0	0	1	1	5	Negative	0	Negative				0
Diag 4°C	163	1	2	1	1	7	Negative	0	Negative				0
Diag 4°C	170	0	0	0	0	5	Negative	0	Negative				0
Diag 4°C	173	0	2	3	0	3	Negative	0	Negative				0
Diag 4°C	176	1	0	7	1	5	Negative	0	35.99			101	1
Diag 4°C	185	0	0	2	0	6	Negative	0	Negative				0
Diag 4°C	186	0	0	0	0	7	Negative	0	Negative				0
Diag 4°C	187	0	0	7	0	3	Negative	0	Negative				0
Diag 4°C	190	0	0	0	0	5	Negative	0	Negative				0
Diag 4°C	193	0	0	1	0	7	Negative	0	Negative				0
Diag 4°C	194	0	0	1	1	6	Negative	0	Negative				0
Diag 4°C	201	0	0	1	1	7	Negative	0	Negative				0
Diag 4°C	202	0	0	0	1	7	Negative	0	Negative				0
Diag 4°C	204	0	2	2	1	4	Negative	0	Negative				0
Diag 4°C	205	0	0	2	1	7	Negative	0	Negative				0
Diag 4°C	213	0	0	7	0	6	Negative	0	39.62			101	1
Diag 4°C	214	0	0	0	0	7	Negative	0	34.96			101	1
Diag 4°C	218	0	2	3	0	2	Negative	0	32.31			101	1
Diag 4°C	223	0	0	1	1	3	Negative	0	Negative				0
Diag 4°C	224	0	0	1	0	5	Negative	0	Negative				0
Diag 4°C	239	0	3	7	0	5	Negative	0	Negative				0
Diag 4°C	240	1	0	0	0	3	Negative	0	Negative				0
Diag 4°C	243	0	0	1	1	8	Negative	0	Negative				0
Diag 4°C	245	0	2	7	0	7	Negative	0	Negative				0
Diag 4°C	249	0	0	7	1	7	Negative	0	Negative				0
Diag 4°C	251	0	2	2	0	4	Negative	0	Negative				0
Diag 4°C	252	0	0	1	0	7	Negative	0	Negative				0
Diag 4°C	257	0	0	1	0	3	Negative	0	Negative				0
Diag 4°C	258	0	0	1	0	7	Negative	0	Negative				0
Diag 4°C	259	0	0	1	1	6	Negative	0	Negative	Negative 5.3.16			0
Diag 4°C	261	0	0	0	0	3	Negative	0	Negative				0

SOURCE	STUDY NUMBER	LENGTH OF STORAGE CODE	REF STD CODE	TRAVEL CODE	GENDER CODE	AGE CODE	LAMP (TIME IN MINS)	LAMP CODE	qPCR HS (C_t)	1:10 RPT	RE-RUN	GEL BAND (bp)	qPCR CODE
Diag 4°C	267	0	0	7	0	6	Negative	0	Negative				0
Diag 4°C	265	0	0	1	0	5	Negative	0	Negative				0
Diag 4°C	268	0	0	7	1	6	Negative	0	Negative				0
Diag 4°C	269	0	1	0	0	5	36.48(21.2.16)	1	26.05			101	1
Diag 4°C	270	1	0	7	1	7	Negative	0	Negative				0
Diag 4°C	272	0	0	0	0	6	Negative	0	Negative	Negative 5.3.16			0
Diag 4°C	273	1	0	0	0	4	Negative	0	Negative				0
Diag 4°C	275	0	1	7	0	6	23.36	1	20.73			101	1
Diag 4°C	276	1	0	5	1	5	Negative	0	Negative				0
Diag 4°C	278	0	2	1	0	4	Negative	0	30.97			101	1
Diag 4°C	283	0	2	7	0	3	Negative	0	Negative				0
Diag 4°C	284	0	0	7	0	6	Negative	0	Negative				0
Diag 4°C	286	0	0	2	0	7	Negative	0	Negative				0
Diag 4°C	287	0	0	2	0	6	Negative	0	Negative				0
Diag 4°C	288	0	1	0	0	8	Negative	0	Negative				0
Diag 4°C	289	0	0	5	0	6	Negative	0	Negative				0
Diag 4°C	291	0	0	7	1	5	Negative	0	Repeat 1 in 10	Negative 25.3.16			0
Diag 4°C	292	0	1	0	0	6	45.36(21.2.16)	1	28.41			101	1
Diag 4°C	294	0	0	0	0	4	Negative	0	40.84			101	1
Diag 4°C	297	0	2	2	0	3	Negative	0	Negative				0
Diag 4°C	298	0	0	1	0	8	Negative	0	Negative				0
Diag 4°C	299	0	0	7	1	7	Negative	0	Negative				0
Diag 4°C	301	0	0	2	1	7	Negative	0	Negative				0
Diag 4°C	304	0	0	1	0	7	Negative	0	Negative				0
Diag 4°C	307	0	0	5	1	4	Negative	0	Negative				0
Diag 4°C	309	0	2	2	0	5	Negative	0	Negative				0
Diag 4°C	310	0	0	0	0	3	Negative	0	Negative				0
Diag 4°C	312	0	0	2	1	6	Negative	0	Negative				0
Diag 4°C	313	0	0	1	1	6	Negative	0	Repeat 1 in 10	Negative 16.4.16			0
Diag 4°C	314	0	0	1	1	3	Negative	0	Negative				0
Diag 4°C	316	0	0	2	1	8	Negative	0	Negative				0
Diag 4°C	318	0	0	0	0	6	Negative	0	41.6			101	1
Diag 4°C	320	0	0	1	0	8	Negative	0	Negative				0
Diag 4°C	323	0	0	0	1	8	Negative	0	Negative				0
Diag 4°C	324	0	0	2	0	5	Negative	0	Negative				0
Diag 4°C	325	0	1	5	1	8	27.24(21.2.16)	1	18.14			101	1
Diag 4°C	326	0	2	5	0	3	Negative	0	Negative				0
Diag 4°C	328	3	1	0	0	7	Negative	0	33.65			101	1
Diag 4°C	336	1	0	1	1	3	Negative	0	Negative				0
Diag 4°C	353	1	0	7	1	8	Negative	0	Negative				0
Diag 4°C	356	0	0	3	1	7	Negative	0	Negative				0
Diag 4°C	357	0	0	7	1	6	Negative	0	Negative				0
Diag 4°C	371	1	0	7	0	6	Negative	0	Negative				0

SOURCE	STUDY NUMBER	LENGTH OF STORAGE CODE	REF STD CODE	TRAVEL CODE	GENDER CODE	AGE CODE	LAMP (TIME IN MINS)	LAMP CODE	qPCR HS (C_t)	1:10 RPT	RE-RUN	GEL BAND (bp)	qPCR CODE
Diag 4°C	384	0	0	2	0	6	Negative	0	Negative				0
Diag 4°C	380	1	0	2	1	7	Negative	0	Negative				0
Diag 4°C	385	0	0	7	1	6	Negative	0	Negative				0
Diag 4°C	391	0	0	0	1	5	Negative	0	Negative				0
Diag 4°C	410	0	0	2	1	4	Negative	0	Negative				0
Diag 4°C	416	0	0	1	1	6	Negative	0	Negative				0
Diag 4°C	420	0	2	0	0	5	Negative	0	Negative				0
Diag 4°C	444	0	0	7	0	5	Negative	0	Negative				0
Diag 4°C	446	1	2	0	0	3	Negative	0	Negative				0
Diag 4°C	449	0	2	2	1	7	Negative	0	Negative				0
Diag 4°C	451	0	0	1	0	6	Negative	0	Negative				0
Diag 4°C	454	0	0	0	1	6	Negative	0	Negative				0
Diag 4°C	455	2	1	0	0	5	Negative	0	32.35			101	1
Diag 4°C	456	0	2	1	0	4	Negative	0	Negative				0
Diag 4°C	457	0	0	1	1	7	Negative	0	Negative	Negative 5.3.16			0
Diag 4°C	458	0	0	0	1	5	Negative	0	Negative	Negative 5.3.16			0
Diag 4°C	460	0	0	0	0	7	Negative	0	Negative				0
Diag 4°C	464	0	0	0	1	8	Negative	0	Negative				0
Diag 4°C	474	1	0	2	0	5	Negative	0	Negative				0
Diag 4°C	476	0	0	1	1	8	Negative	0	Negative				0
Diag 4°C	479	0	0	6	1	5	Negative	0	Negative				0
Diag 4°C	482	0	2	7	1	8	Negative	0	Negative				0
Diag 4°C	486	1	0	7	0	7	Negative	0	Negative				0
Diag 4°C	488	0	0	7	1	8	Negative	0	Negative				0
Diag 4°C	493	1	0	1	1	7	Negative	0	Negative				0
Diag 4°C	496	0	0	7	1	6	Negative	0	Negative				0
Diag 4°C	498	1	0	5	1	7	Negative	0	Negative				0
Diag 4°C	500	0	0	1	1	8	Negative	0	Negative				0
Diag 4°C	502	0	2	7	0	3	Negative	0	Negative				0
Diag 4°C	503	0	0	2	1	7	Negative	0	Negative				0
Diag 4°C	504	0	0	1	0	4	Negative	0	Negative				0
Diag 4°C	506	1	2	2	0	5	Negative	0	Negative				0
Diag 4°C	507	0	0	7	1	8	Negative	0	Negative				0
Diag 4°C	510	0	2	1	1	6	Negative	0	Negative				0
Diag 4°C	511	0	0	2	0	5	Negative	0	Negative				0
Diag 4°C	514	0	0	7	1	6	Negative	0	Negative				0
Diag 4°C	516	0	0	5	0	8	Negative	0	Negative				0
Diag 4°C	523	0	0	7	0	7	Negative	0	Negative				0
Diag 4°C	524	1	0	0	1	6	Negative	0	Negative				0
Diag 4°C	525	1	0	2	1	5	Negative	0	Negative				0
Diag 4°C	526	1	2	1	0	3	Negative	0	Negative				0
Diag 4°C	534	2	1	0	1	6	Negative	0	31.71			101	1
Diag 4°C	540	0	0	2	0	7	Negative	0	Negative				0

SOURCE	STUDY NUMBER	LENGTH OF STORAGE CODE	REF STD CODE	TRAVEL CODE	GENDER CODE	AGE CODE	LAMP (TIME IN MINS)	LAMP CODE	qPCR HS (C_t)	1:10 RPT	RE-RUN	GEL BAND (bp)	qPCR CODE
Diag 4°C	548	0	0	2	0	5	Negative	0	Negative				0
Diag 4°C	550	0	0	7	1	5	Negative	0	Negative				0
Diag 4°C	555	0	0	5	0	6	Negative	0	Negative				0
Diag 4°C	556	0	0	2	0	8	Negative	0	Negative				0
Diag 4°C	570	0	0	0	1	8	Negative	0	Negative				0
Diag 4°C	579	1	0	2	0	3	Negative	0	Negative				0
Diag 4°C	581	1	0	2	1	8	Negative	0	Negative				0
Diag 4°C	582	1	0	4	1	7	Negative	0	Negative				0
Diag 4°C	612	0	0	0	0	5	Negative	0	Negative				0
Diag 4°C	614	1	0	5	1	6	Negative	0	Negative				0
Diag 4°C	624	0	0	7	0	7	Negative	0	Negative				0
Diag 4°C	635	0	1	3	0	4	24.54(21.2.16)	1	17.07			101	1
Diag 4°C	641	1	0	1	1	7	Negative	0	Negative				0
Diag 4°C	643	0	0	0	0	4	Negative	0	Negative				0
Diag 4°C	650	0	0	1	1	4	Negative	0	Negative				0
Diag 4°C	653	0	0	0	0	5	Negative	0	Negative				0
Diag 4°C	656	0	0	1	0	8	Negative	0	Negative				0
Diag 4°C	660	2	0	2	0	7	Negative	0	Negative				0
Diag 4°C	668	0	0	0	1	7	Negative	0	Negative				0
Diag 4°C	677	0	0	7	1	7	Negative	0	38.91			?	2
Diag 4°C	691	0	0	7	1	5	Negative	0	Negative				0
Diag 4°C	696	1	0	5	1	5	Negative	0	Negative				0
Diag 4°C	701	0	0	5	0	6	Negative	0	Negative				0
Diag 4°C	708	1	0	2	1	3	Negative	0	Negative				0
Diag 4°C	719	0	0	4	1	6	Negative	0	Negative				0
Diag 4°C	723	0	0	0	1	5	Negative	0	Repeat 1 in 10	Negative 25.3.16			0
Diag 4°C	725	0	0	7	1	5	Negative	0	Negative				0
Diag 4°C	727	0	2	2	0	1	Negative	0	Repeat 1 in 10	Negative 26.12.15			0
Diag 4°C	739	1	0	7	1	4	Negative	0	Negative				0
Diag 4°C	741	0	2	7	1	8	Negative	0	Negative				0
Diag 4°C	743	1	0	0	1	5	Negative	0	Negative				0
Diag 4°C	746	0	0	4	0	5	Negative	0	Negative				0
Diag 4°C	748	0	0	2	1	8	Negative	0	34.43			101	1
Diag 4°C	757	0	0	3	0	6	Negative	0	Negative				0
Diag 4°C	767	0	0	1	1	5	Negative	0	Negative				0
Diag 4°C	778	0	0	0	1	4	Negative	0	Negative				0
Diag 4°C	780	0	0	2	1	7	Negative	0	Negative				0
Diag 4°C	781	0	0	0	1	7	Negative	0	Negative				0
Diag 4°C	786	1	0	7	1	7	Negative	0	35.4			101	1
Diag 4°C	788	0	0	2	0	7	Negative	0	Negative				0
Diag 4°C	797	3	1	0	0	7	Negative	0	30.84			101	1
Diag 4°C	799	0	0	7	0	7	Negative	0	Negative				0
Diag 4°C	800	0	0	5	1	8	42.18(1in10)	1	26.76	30.36		101	1

SOURCE	STUDY NUMBER	LENGTH OF STORAGE CODE	REF STD CODE	TRAVEL CODE	GENDER CODE	AGE CODE	LAMP (TIME IN MINS)	LAMP CODE	qPCR HS (C_t)	1:10 RPT	RE-RUN	GEL BAND (bp)	qPCR CODE
Diag 4°C	815	0	2	0	0	5	Negative	0	Negative				0
Diag 4°C	816	0	0	2	0	6	Negative	0	Negative				0
Diag 4°C	821	0	0	4	1	7	Negative	0	Negative				0
Diag 4°C	823	0	0	1	0	5	Negative	0	Negative				0
Diag 4°C	827	0	0	7	0	6	Negative	0	Negative				0
Diag 4°C	852	0	0	2	0	8	Negative	0	Negative				0
Diag 4°C	859	0	0	0	0	5	Negative	0	Negative				0
Diag 4°C	862	0	1	1	1	6	Negative	0	35.71				1
Diag 4°C	868	0	0	4	0	8	Negative	0	Negative				0
Diag 4°C	873	0	0	7	0	8	Negative	0	Negative				0
Diag 4°C	886	1	3	1	1	7	Negative	0	Negative				0
Diag 4°C	889	0	0	4	1	4	Negative	0	Negative				0
Diag 4°C	893	0	0	7	1	5	Negative	0	Negative				0
Diag 4°C	903	0	2	0		6	Negative	0	35.92			101	1
Diag 4°C	908	0	0	6	1	6	Negative	0	Negative				0
Diag 4°C	919	0	0	1	0	8	Negative	0	Negative				0
Diag 4°C	925	0	0	5	1	7	Negative	0	Negative				0
Diag 4°C	929	1	0	2	0	6	Negative	0	Negative				0
Diag 4°C	935	0	2	7	1	4	Negative	0	Negative				0
Diag 4°C	943	0	0	2	0	8	Negative	0	Negative				0
Diag 4°C	946	0	0	0	0	4	Negative	0	Negative	Negative 5.3.16			0
Diag 4°C	961	1	0	5	1	2	Negative	0	Negative				0
Diag 4°C	965	3	1	0	0	7	Negative	0	34.61			101	1
Diag 4°C	984	0	0	0	1	8	Negative	0	Negative				0
Diag 4°C	992	0	2	2	0	2	Negative	0	Negative				0
Diag 4°C	994	0	0	5	1	6	Negative	0	35.84			?	2
Diag 4°C	1002	0	0	2	0	5	Negative	0	37.53			101	1
Diag 4°C	1006	0	2	1	1	7	Negative	0	35.27			101	1
Diag 4°C	1016	0	0	1	0	3	Negative	0	Repeat 1 in 10	Negative 25.3.16			0
Diag 4°C	1025	0	0	7	1	5	Negative	0	Negative				0
Diag 4°C	1027	0	0	7	1	7	Negative	0	Negative				0
Diag 4°C	1035	1	0	0	0	5	Negative	0	Negative				0
Diag 4°C	1056	1	0	0	0	7	Negative	0	Negative				0
Diag 4°C	1061	0	0	0	1	7	Negative	0	Negative				0
Diag 4°C	1064	0	0	7	1	8	Negative	0	Negative				0
Diag 4°C	1067	0	2	1	0	5	Negative	0	Negative				0
Diag 4°C	1075	0	0	0	1	3	Negative	0	Negative				0
Diag 4°C	1084	0	0	0	0	7	Negative	0	Negative				0
Diag 4°C	1088	1	0	7	0	3	Negative	0	Negative				0
Diag 4°C	1090	1	0	0	1	3	Negative	0	Negative				0
Diag 4°C	1091	1	0	7	1	7	Negative	0	Negative				0
Diag 4°C	1092	0	0	1	1	8	Negative	0	Negative				0
Diag 4°C	1094	0	0	2	0	6	Negative	0	Negative				0

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Diag 4°C	1101	0	0	7	1	4	Negative	0	Negative				0
Diag 4°C	1108	0	0	1	0	7	Negative	0	Negative				0
Diag 4°C	1112	0	0	7	0	7	Negative	0	Negative				0
Diag 4°C	1136	0	0	0	1	6	Negative	0	Negative				0
Diag 4°C	1145	1	0	7	1	6	Negative	0	Negative				0
Diag 4°C	1151	0	2	2	0	6	Negative	0	Negative				0
Diag 4°C	1154	0	0	0	0	6	Negative	0	Negative				0
Diag 4°C	1163	1	1	2	1	5	Negative	0	Negative				0
Diag 4°C	1164	0	0	1	1	8	Negative	0	Negative				0
Diag 4°C	1168	0	0	1	0	6	Negative	0	Negative				0
Diag 4°C	1169	0	0	4	1	2	Negative	0	35.57			101	2
Diag 4°C	1179	0	0	7	1	4	Negative	0	Negative				0
Diag 4°C	1180	1	0	5	1	5	Negative	0	Negative				0
Diag 4°C	1183	1	0	2	0	6	Negative	0	Negative				0
Diag 4°C	1183	0	0	2	1	6	Negative	0	Negative				0
Diag 4°C	1187	0	0	7	1	2	Negative	0	Negative				0
Diag 4°C	1189	0	0	2	1	7	Negative	0	Negative				0
Diag 4°C	1194	0	0	2	1	4	Negative	0	Negative				0
Diag 4°C	1196	0	0	1	1	7	Negative	0	40.7			?	2
Diag -20°C	103	0	2	7	0	7	Negative	0	Negative				0
Diag -20°C	104	0	0	0	1	5	Negative	0	Negative				0
Diag -20°C	120	0	0	1	0	5	Negative	0	35.3			101	1
Diag -20°C	130	1	0	1	1	3	Negative	0	Negative				0
Diag -20°C	133	0	0	0	0	5	Negative	0	Negative				0
Diag -20°C	137	1	0	2	1	7	Negative	0	Negative				0
Diag -20°C	146	0	2	0	1	6	38.3	1	24.06			101	1
Diag -20°C	149	0	0	2	0	6	Negative	0	Negative				0
Diag -20°C	152	0	0	1	1	3	Negative	0	Negative				0
Diag -20°C	167	0	2	1	0	4	Negative	0	Negative				0
Diag -20°C	169	1	0	2	0	8	Negative	0	Negative				0
Diag -20°C	171	0	0	1	0	3	Negative	0	35.13	31.5(25.3.16)	0	0	0
Diag -20°C	175	2	1	2	1	3	Negative	0	Negative				0
Diag -20°C	177	0	0	7	1	6	Negative	0	Negative				0
Diag -20°C	178	0	0	3	1	7	Negative	0	Negative				0
Diag -20°C	179	0	0	1	0	7	Negative	0	Negative				0
Diag -20°C	183	0	0	7	0	7	Negative	0	Negative				0
Diag -20°C	184	0	1	3	0	4	29.48(21.2.16)	1	20.93			101	1
Diag -20°C	186	0	0	2	1	6	Negative	0	Negative				0
Diag -20°C	191	1	0	2	0	6	Negative	0	Negative				0
Diag -20°C	192	0	0	4	0	5	Negative	0	Negative				0
Diag -20°C	197	1	0	1	0	5	Negative	0	Negative				0
Diag -20°C	198	0	2	2	1	4	Negative	0	40.05			101	1
Diag -20°C	199	0	0	2	1	8	Negative	0	Negative				0

SOURCE	STUDY NUMBER	LENGTH OF STORAGE CODE	REF STD CODE	TRAVEL CODE	GENDER CODE	AGE CODE	LAMP (TIME IN MINS)	LAMP CODE	qPCR HS (C_t)	1:10 RPT	RE-RUN	GEL BAND (bp)	qPCR CODE
Diag -20°C	200	0	0	0	0	3	Negative	0	36.9			101	1
Diag -20°C	207	0	0	1	0	5	Negative	0	35.29			500	2
Diag -20°C	208	0	2	2	0	3	Negative	0	Negative				0
Diag -20°C	209	0	0	6	1	5	Negative	0	35.4			101	1
Diag -20°C	215	0	0	2	1	8	Negative	0	Negative				0
Diag -20°C	220	0	0	7	0	7	Negative	0	Negative				0
Diag -20°C	226	0	0	2	1	6	Negative	0	Negative				0
Diag -20°C	227	0	2	3	0	2	47.42	1	26.21			101	1
Diag -20°C	231	0	0	5	1	6	Negative	0	39.5			?	2
Diag -20°C	232	0	0	1	0	4	Negative	0	Negative				0
Diag -20°C	233	0	0	7	1	5	Negative	0	Negative				0
Diag -20°C	234	0	0	0	0	6	Negative	0	Negative				0
Diag -20°C	235	0	0	7	1	5	Negative	0	Negative				0
Diag -20°C	237	0	0	1	1	3	Negative	0	Negative				0
Diag -20°C	237	0	0	5	0	6	Negative	0	Negative				0
Diag -20°C	238	0	2	2	0	6	Negative	0	Negative				0
Diag -20°C	241	0	0	2	0	5	Negative	0	Negative				0
Diag -20°C	242	1	0	5	1	5	Negative	0	Negative				0
Diag -20°C	247	0	0	7	1	2	Negative	0	Negative				0
Diag -20°C	248	0	0	1	1	6	Negative	0	Negative	Negative 5.3.16			0
Diag -20°C	250	0	0	0	0	5	Negative	0	Negative				0
Diag -20°C	256	0	0	1	0	3	Negative	0	Negative	Negative 5.3.16			0
Diag -20°C	264	0	0	5	1	8	40.12(57.12 1:10)	1	27.15	30.48		101	1
Diag -20°C	271	0	0	7	0	6	Negative	0	Negative				0
Diag -20°C	277	0	0	2	0	6	Negative	0	Negative				0
Diag -20°C	279	0	0	2	1	7	Negative	0	Negative				0
Diag -20°C	281	0	2	7	0	6	Negative	0	Negative				0
Diag -20°C	290	0	0	0	0	5	Negative	0	Negative				0
Diag -20°C	296	0	0	5	0	6	Negative	0	38.14			101	1
Diag -20°C	305	0	2	3	0	3	Negative	0	Negative				0
Diag -20°C	308	2	1	0	0	5	52.24 (Negative 1:10)	1	27.49			101	1
Diag -20°C	330	0	0	1	1	7	Negative	0	Negative				0
Diag -20°C	331	0	0	7	0	8	Negative	0	35.7			101	1
Diag -20°C	332	0	0	7	1	6	Negative	0	Negative				0
Diag -20°C	335	0	0	1	0	7	Negative	0	Negative				0
Diag -20°C	337	0	1	7	0	6	29.54	1	14.98			101	1
Diag -20°C	340	0	0	7	0	6	Negative	0	Negative				0
Diag -20°C	342	0	0	1	1	8	Negative	0	Negative				0
Diag -20°C	344	0	0	0	0	7	Negative	0	Negative				0
Diag -20°C	345	0	0	7	0	3	Negative	0	Negative	Negative			0
Diag -20°C	346	0	0	7	1	7	Negative	0	Negative				0
Diag -20°C	348	0	0	4	1	7	Negative	0	Negative				0
Diag -20°C	349	0	0	4	1	4	Negative	0	Negative				0

SOURCE	STUDY NUMBER	LENGTH OF STORAGE CODE	REF STD CODE	TRAVEL CODE	GENDER CODE	AGE CODE	LAMP (TIME IN MINS)	LAMP CODE	qPCR HS (C_t)	1:10 RPT	RE-RUN	GEL BAND (bp)	qPCR CODE
Diag -20°C	350	0	0	2	0	5	Negative	0	Negative				0
Diag -20°C	351	1	2	2	0	5	Negative	0	Negative				0
Diag -20°C	354	0	2	2	1	7	Negative	0	Negative				0
Diag -20°C	358	0	0	1	0	2	Negative	0	32.29			101	1
Diag -20°C	360	1	0	2	0	1	Negative	0	Negative				0
Diag -20°C	365	0	2	2	0	5	Negative	0	Negative				0
Diag -20°C	370	0	0	4	1	7	Negative	0	Negative				0
Diag -20°C	372	0	0	1	1	8	Negative	0	Negative				0
Diag -20°C	374	0	0	7	1	6	Negative	0	Negative				0
Diag -20°C	375	0	1	5	1	8	32.54	1	17.9			101	1
Diag -20°C	376	0	0	5	1	7	Negative	0	Negative				0
Diag -20°C	377	1	0	1	1	5	Negative	0	Negative				0
Diag -20°C	378	0	0	2	1	6	Negative	0	Negative	Negative 30.1.16			0
Diag -20°C	379	0	0	4	0	8	Negative	0	Negative				0
Diag -20°C	381	1	2	1		7	Negative	0	Negative				0
Diag -20°C	383	0	0	0	1	7	Negative	0	Negative				0
Diag -20°C	388	0	0	4	1	2	Negative	0	Negative				0
Diag -20°C	389	0	0	7	0	7	Negative	0	Negative				0
Diag -20°C	390	0	2	7	0	3	27.24(21.2.16)	1	24.2			101	1
Diag -20°C	392	0	0	0	0	4	Negative	0	Negative				0
Diag -20°C	394	0	2	1	1	6	Negative	0	Negative				0
Diag -20°C	396	0	0	0	1	4	Negative	0	Negative				0
Diag -20°C	401	0	0	1	0	8	Negative	0	Negative				0
Diag -20°C	403	0	0	2	0	8	Negative	0	Negative				0
Diag -20°C	404	0	0	7	1	7	Negative	0	Negative				0
Diag -20°C	406	0	0	1	1	6	Negative	0	Negative				0
Diag -20°C	407	1	0	0	0	7	Negative	0	Negative				0
Diag -20°C	412	0	0	0	0	5	Negative	0	Negative				0
Diag -20°C	414	0	0	2	1	7	Negative	0	Negative				0
Diag -20°C	415	1	0	7	1	7	Negative	0	Negative				0
Diag -20°C	416	0	0	1	1	6	Negative	0	Negative				0
Diag -20°C	417	0	0	2	0	6	Negative	0	Negative				0
Diag -20°C	418	0	0	0	1	5	Negative	0	Negative				0
Diag -20°C	421	0	0	1	1	6	Negative	0	Negative				0
Diag -20°C	425	0	2	0	0	5	Negative	0	Negative				0
Diag -20°C	426	0	0	1	0	6	Negative	0	Negative				0
Diag -20°C	427	0	2	7	1	8	Negative	0	Negative				0
Diag -20°C	428	0	2	1	0	4	Negative	0	Negative				0
Diag -20°C	430	0	0	7	1	5	Negative	0	Negative				0
Diag -20°C	431	1	2	0	0	3	Negative	0	Negative				0
Diag -20°C	432	0	2	0	0	5	Negative	0	32.27			101	1
Diag -20°C	433	0	0	1	0	6	Negative	0	Negative				0
Diag -20°C	434	0	0	7	0	5	Negative	0	Negative				0

SOURCE	STUDY NUMBER	LENGTH OF STORAGE CODE	REF STD CODE	TRAVEL CODE	GENDER CODE	AGE CODE	LAMP (TIME IN MINS)	LAMP CODE	qPCR HS (C_t)	1:10 RPT	RE-RUN	GEL BAND (bp)	qPCR CODE
Diag -20°C	435	0	0	0	1	5	Negative	0	Negative				0
Diag -20°C	437	1	0	7	1	7	Negative	0	Negative				0
Diag -20°C	438	0	0	1	1	5	Negative	0	Negative				0
Diag -20°C	439	1	0	2	1	4	Negative	0	Negative				0
Diag -20°C	441	0	0	0	1	6	Negative	0	Negative				0
Diag -20°C	443	0	0	0	1	8	Negative	0	Negative	Negative 5.3.16			0
Diag -20°C	445	1	0	7	1	8	Negative	0	Negative				0
Diag -20°C	447	1	0	0	0	7	Negative	0	Negative				0
Diag -20°C	448	1	0	7	0	6	Negative	0	Negative				0
Diag -20°C	450	0	0	0	0	7	Negative	0	Negative				0
Diag -20°C	452	1	0	0	0	4	Negative	0	Negative				0
Diag -20°C	453	0	0	0	0	4	Negative	0	Negative				0
Diag -20°C	459	1	0	7	0	7	Negative	0	Negative				0
Diag -20°C	462		1	2	0	7	Negative	0	Negative				0
Diag -20°C	463	0	0	2	1	8	Negative	0	Negative				0
Diag -20°C	465	0	0	6	1	6	Negative	0	Negative				0
Diag -20°C	480	2	1	0	0	4	Negative	0	29.35			101	1
Diag -20°C	480.2	2	1	0	0	4	42	1	27.6				1
Diag -20°C	483	0	0	1	0	8	Negative	0	Negative				0
Diag -20°C	495	0	0	0	0	7	Negative	0	Negative				0
Diag -20°C	501	1	0	0	0	5	Negative	0	Negative				0
Diag -20°C	505	2	1	0	1	6	33.36	1	24.43			101	1
Diag -20°C	508	0	0	5	0	6	Negative	0	Negative				0
Diag -20°C	521	0	0	1	0	7	Negative	0	Negative				0
Diag -20°C	529	1	0	0	1	3	Negative	0	Negative				0
Diag -20°C	530	0	0	1	1	8	Negative	0	Negative				0
Diag -20°C	542	0	0	1	1	7	Negative	0	Negative				0
Diag -20°C	552	0	0	7	1	5	Negative	0	Negative				0
Diag -20°C	558	0	0	2	0	7	Negative	0	Negative				0
Diag -20°C	580	0	0	0	0	7	Negative	0	Negative				0
Diag -20°C	584	0	0	1	1	5	Negative	0	Negative				0
Diag -20°C	588	0	0	2	0	8	Negative	0	Negative				0
Diag -20°C	595	0	0	1	1	4	Negative	0	Negative				0
Diag -20°C	595	0	0	0	1	8	Negative	0	Negative				0
Diag -20°C	598	0	0	4	1	6	Negative	0	Negative				0
Diag -20°C	599	0	1	0	0	7	Negative	0	31.84			101	1
Diag -20°C	613	0	0	2	1	4	Negative	0	Negative	Negative 5.3.16			0
Diag -20°C	622	0	1	0	0	7	Negative	0	31.29			101	1
Diag -20°C	632	1	0	0	0	3	Negative	0	Negative				0
Diag -20°C	632.2	1	0	7	0	7	Negative	0	Negative				0
Diag -20°C	636	1	2	1	0	3	Negative	0	Negative				0
Diag -20°C	645	0	0	2	1	7	Negative	0	Negative				0
Diag -20°C	646	1	0	4	1	7	Negative	0	Negative				0

SOURCE	STUDY NUMBER	LENGTH OF STORAGE CODE	REF STD CODE	TRAVEL CODE	GENDER CODE	AGE CODE	LAMP (TIME IN MINS)	LAMP CODE	qPCR HS (C_t)	1:10 RPT	RE-RUN	GEL BAND (bp)	qPCR CODE
Diag -20°C	647	0	1	1	1	6	53 Positive didn't reach threshold	1	26.78			101	1
Diag -20°C	651	0	0	0	1	8	Negative	0	Negative				0
Diag -20°C	657	0	0	1	1	5	Negative	0	Negative				0
Diag -20°C	658	0	0	1	0	7	Negative	0	Negative				0
Diag -20°C	671	0	0	5	1	4	Negative	0	Negative				0
Diag -20°C	672	1	1	2	1	5	Negative	0	33.89			101	1
Diag -20°C	673	0	0	0	1	3	Negative	0	Negative				0
Diag -20°C	680	1	0	0	1	5	Negative	0	Negative				0
Diag -20°C	688	1	0	7	1	7	Negative	0	Negative				0
Diag -20°C	693	0	0	1	1	6	Negative	0	Negative				0
Diag -20°C	698	1	0	2	0	5	Negative	0	Negative				0
Diag -20°C	703	0	1	0	0	8	Negative	0	Negative				0
Diag -20°C	709	1	0	2	0	3	Negative	0	Negative				0
Diag -20°C	725	0	0	7	0	6	Negative	0	41.67			?150	0
Diag -20°C	732	1	0	7	1	6	Negative	0	Negative				0
Diag -20°C	744	0	0	1	1	8	Negative	0	Negative				0
Diag -20°C	751	1	0	2	1	3	Negative	0	Negative				0
Diag -20°C	753	0	3	7	0	5	Negative	0	Negative				0
Diag -20°C	755	0	0	1	1	8	Negative	0	Negative				0
Diag -20°C	761	0	1	2	1	3	Negative	0	Negative				0
Diag -20°C	764	0	0	1	0	5	Negative	0	Negative				0
Diag -20°C	770	0	0	7	1	7	Negative	0	38.15			?	2
Diag -20°C	779	1	0	2	0	5	Negative	0	Negative				0
Diag -20°C	785	1	0	5	1	6	Negative	0	Negative				0
Diag -20°C	791	0	0	2	1	7	Negative	0	Negative	Negative 5.3.16			0
Diag -20°C	793	0	2	1	1	5	Negative	0	Negative				0
Diag -20°C	805	0	0	5	0	8	Negative	0	Negative				0
Diag -20°C	833	0	0	7	1	5	Negative	0	Negative				0
Diag -20°C	835	0	0	7	0	3	Negative	0	Negative				0
Diag -20°C	838	0	0	7	1	8	Negative	0	35.2			101	1
Diag -20°C	847	0	0	0	0	6	Negative	0	Negative				0
Diag -20°C	851	0	0	0	1	6	Negative	0	Negative				0
Diag -20°C	854	1	0	2	1	8	Negative	0	Negative				0
Diag -20°C	865	0	0	2	0	7	Negative	0	Negative				0
Diag -20°C	869	0	1	0	0	7	Negative	0	35.56				1
Diag -20°C	872	0	0	7	1	4	Negative	0	Negative				0
Diag -20°C	874	0	0	0	1	7	Negative	0	Negative				0
Diag -20°C	890	0	0	2	1	7	Negative	0	Negative				0
Diag -20°C	895	1	0	2	1	5	Negative	0	Negative				0
Diag -20°C	897	0	0	1	0	7	Negative	0	Negative				0
Diag -20°C	905	0	0	7	1	4	Negative	0	Negative				0
Diag -20°C	913	0	0	1	1	8	Negative	0	Negative				0
Diag -20°C	920	0	1	0	0	6	27.06(21.2.16)	1	20.81			101	1

SOURCE	STUDY NUMBER	LENGTH OF STORAGE CODE	REF STD CODE	TRAVEL CODE	GENDER CODE	AGE CODE	LAMP (TIME IN MINS)	LAMP CODE	qPCR HS (C_t)	1:10 RPT	RE-RUN	GEL BAND (bp)	qPCR CODE
Diag -20°C	924	0	0	2	0	7	Negative	0	Negative				0
Diag -20°C	930	0	0	7	1	8	Negative	0	Negative				0
Diag -20°C	941	0	1	0	0	5	34.12	1	19.15			101	1
Diag -20°C	949	1	0	5	1	2	Negative	0	Negative				0
Diag -20°C	962	1	3	1	1	7	Negative	0	Negative	Negative 5.3.16			0
Diag -20°C	963	0	0	7	0	7	Negative	0	Negative				0
Diag -20°C	966	0	2	1	0	5	Negative	0	Negative				0
Diag -20°C	983	0	0	0	0	4	Negative	0	Negative				0
Diag -20°C	986	0	2	2	1	4	Negative	0	Negative				0
Diag -20°C	988	0	0	2	1	4	Negative	0	Negative				0
Diag -20°C	989	1	0	0	1	6	Negative	0	Negative				0
Diag -20°C	995	0	0	1	1	7	Negative	0	Negative				0
Diag -20°C	998	0	0	2	0	6	Negative	0	Negative				0
Diag -20°C	999	0	0	0	0	6	Negative	0	Negative				0
Diag -20°C	1001	0	2	2	0	4	Negative	0	Negative				0
Diag -20°C	1003	1	0	7	0	3	Negative	0	Negative				0
Diag -20°C	1010	0	0	1	0	8	Negative	0	Negative				0
Diag -20°C	1023	1	0	5	1	7	Negative	0	Negative				0
Diag -20°C	1033	0	0	0	1	8	Negative	0	Negative				0
Diag -20°C	1041	0	0	2	1	4	Negative	0	Negative				0
Diag -20°C	1043	0	2	7	1	8	Negative	0	Negative	Negative 5.3.16			0
Diag -20°C	1055	0	2	2	0	1	Negative	0	Negative				0
Diag -20°C	1058	0	0	1	1	6	Negative	0	Negative				0
Diag -20°C	1059	2	1	0	0	5	Negative	0	Negative				0
Diag -20°C	1063	0	2	2	0	2	Negative	0	Negative				0
Diag -20°C	1069	1	0	1	1	7	Negative	0	Negative				0
Diag -20°C	1073	0	0	0	1	7	Negative	0	Negative				0
Diag -20°C	1080	0	0	0	0	5	Negative	0	Negative				0
Diag -20°C	1089	0	2	7	1	4	Negative	0	31.29			101	1
Diag -20°C	1095	3	1	0	0	7	Negative	0	33.57			101	1
Diag -20°C	1099	0	0	3	0	6	Negative	0	Negative				0
Diag -20°C	1107	0	2	1	1	7	Negative	0	Negative				0
Diag -20°C	1120	1	0	7	1	5	Negative	0	Negative				0
Diag -20°C	1125	0	0	7	0	6	Negative	0	Negative			0	0
Diag -20°C	1126	2	1	1	1	6	Negative	0	Negative				0
Diag -20°C	1127	0	0	1	1	7	Negative	0	42.74			?	2
Diag -20°C	1141	0	0	7	1	8	Negative	0	Negative				0
Diag -20°C	1142	0	0	1	0	8	Negative	0	Negative				0
Diag -20°C	1157	0	0	7	1	7	Negative	0	Negative				0
Diag -20°C	1158	0	0	0	0	3	Negative	0	Negative				0
Diag -20°C	1165	1	0	1	1	7	Negative	0	Negative				0
Diag -20°C	1171	1	0	5	1	5	Negative	0	Negative				0
Diag -20°C	1174	0	0	0	1	7	Negative	0	Negative				0

SOURCE	STUDY NUMBER	LENGTH OF STORAGE CODE	REF STD CODE	TRAVEL CODE	GENDER CODE	AGE CODE	LAMP (TIME IN MINS)	LAMP CODE	qPCR HS (C_t)	1:10 RPT	RE-RUN	GEL BAND (bp)	qPCR CODE
Diag -20°C	1176	1	0	2	0	6	Negative	0	Negative				0
Diag -20°C	1193	0	0	2	0	5	Negative	0	Negative				0
Diag -20°C	1200	0	0	1	0	4	Negative	0	Negative				0
Diag -20°C	212/489	0	0	3	1	5	Negative	0	Negative				0
Diag -20°C	519a	0	0	2	0	5	Negative	0	Negative				0
Diag -20°C	520a	0	0	2	0	8	Negative	0	Negative				0
Diag -20°C	522a	0	0	7	1	6	Negative	0	Negative				0
Diag -20°C	527a	0	0	7	1	5	Negative	0	Negative				0
Diag -20°C	531a	0	0	2	0	6	Negative	0	Negative				0
Diag -20°C	533a	0	2	5	0	3	Negative	0	Negative				0
Diag -20°C	537a	0	0	0	1	5	Negative	0	Repeat 1in 10	Negative 25.3.16			0
Diag -20°C	538a	0	2	7	0	3	Negative	0	Negative				0

KEY TO CODES FOR APPENDIX 6:

LENGTH OF STORAGE	2011=4, 2012=3, 2013=2, 2014=1, 2015/ 2016=0
REF STD (COMPOSITE REFERENCE STANDARD)	CRS negative= 0, Microscopy/ culture positive only = 1, Serology positive only = 2, CRS positive = 3
TRAVEL	Unknown=0, Africa=1, Asia=2, Caribbean=3, Europe=4, Latin America=5, Middle East=6, Worldwide=7
GENDER	Male=0, Female=1
AGE (YEARS)	1920-1929=1, 1930-1939=2, 1940-1949=3, 1950-1959=4, 1960-1969=5, 1970-1979=6, 1980-1989=7, 1990-1999=8
LAMP AND qPCR ASSAYS	Negative= 0, Positive= 1

APPENDIX 7: GANTT CHART FOR PROGRESS TO A PROFESSIONAL DOCTORATE DEGREE