Comparing different diagnostic methods and detection platforms for schistosomiasis, giardiasis and malaria in Uganda and Kingdom of Saudi Arabia

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Table of Contents

DEDICATION **11**

DECLARATION **12**

ACKNOWLEDGEMENT **13**

ABBREVIATIONS AND ACRONYMS **14**

LIST OF TABLES **15**

LIST OF FIGURES **17**

ABSTRACT 20

CHAPTER 1: GENERAL OVERVIEW **22**

1. NEGLECTED TROPICAL DISEASES (NTDS) 22

2.1 SCHISTOSOMIASIS 24

2.2 LIFECYCLE 25

2.3 SCHISTOSOMIASIS TRANSMISSION 26

2.3.1 Snail hosts *26*

2.3.2. Human hosts *27*

2.4 PATHOGENESIS AND SYMPTOMATOLOGY 28

2.4.1. Intestinal schistosomiasis *28*

2.4.2 Urogenital schistosomiasis *29*

2.5 DIAGNOSTICS 29

2.5.1 Direct parasitological methods *29*

2.5.2 Antigen detection tests *31*

2.5.3 Antibody detection tests *32*

2.5.4 DNA-based diagnostics for schistosomiasis *32*

2.6 CONTROL AND PREVENTION 33

2.7 SCHISTOSOMIASIS IN UGANDA AND SAUDI ARABIA 36

3.1 GIARDIASIS 37

3.2 LIFECYCLE 39

3.3 EPIDEMIOLOGY 40

3.4 MOLECULAR CHARACTERISATION OF *GIARDIA* 42

3.5 CLINICAL SYMPTOMS AND PATHOLOGY 44

3.6 DIAGNOSTICS 45

3.7 TREATMENT 46

4.1 MALARIA 47

4.2 LIFECYCLE 47

4.3 EPIDEMIOLOGY 48

4.4 CLINICAL SYMPTOMS 51

4.5 DIAGNOSTIC TESTS 52

4.5.1 Microscopy 52

4.5.2 Rapid diagnostic tests 52

4.5.3 Molecular detection of human Plasmodium species 53

4.5.3.1 Molecular diagnosis of malaria by using faecal and blood DNA 55

4.5.3.2 Diagnosis of blood-borne parasites using PCR on faecal DNA 57

4.6 CONTROL AND PREVENTION 57

4.7 TREATMENT 58

5. THE IMPORTANCE OF POLYPARASITISM 60

6. QUANTITATIVE POLYMERASE CHAIN REACTION 60

7. JUSTIFICATION AND RATIONALE OF THE PRESENT STUDY 64

7.1 SCHISTOSOMIASIS 64

7.2 GIARDIASIS 64

7.3 MALARIA 65

8. AIMS OF THE THESIS AND STUDY OBJECTIVES....66

8.1. AIMS....66

8.2. OBJECTIVES....68

9. THESIS LAYOUT....70

CHAPTER 2: SURVEILLANCE OF INTESTINAL SCHISTOSOMIASIS DURING CONTROL: A COMPARISON OF FOUR DIAGNOSTIC TESTS ACROSS FIVE UGANDAN PRIMARY SCHOOLS IN THE LAKE ALBERT REGION....71

2.1 ABSTRACT....73

2.2 INTRODUCTION....74

2.3 METHODS....76

2.3.1 Study area, participants and ethical approval....76

2.3.2 Diagnostics: faecal microscopy with Kato-Katz....77

2.3.3 Diagnostics: schistosome antigens urine-CCA dipsticks....78

2.3.4 Diagnostics: schistosome serology with SEA-ELISA....79

2.3.5 DNA diagnostics: TaqMan® real-time PCR....80

2.3.6 Data management and statistical analysis....81

2.4 RESULTS....82

2.4.1 Prevalence of intestinal schistosomiasis....82

2.4.2 Prevalence of S. mansoni by school distance from Lake Albert....83

2.4.3 Infection intensity by school distance from Lake Albert....84

2.4.4 Urine-CCA as an arbitrary gold standard....84

2.4.5 Empirical and LCA modelling of estimates of diagnostic performance....85

2.5 DISCUSSION....87

2.5.1 Current reference standard, Kato–Katz thick smear....87

2.5.2 Alternative technique for diagnosing schistosomiasis....88

2.5.3 LCA-model for evaluation of diagnostic tests....88

2.5.4 Field applicability-assured urine-CCA diagnostic test *89*

2.6 CONCLUDING REMARKS 90

2.7 APPENDIX 92

Appendix 1: AccuDiag™ Schistosoma IgG ELISA Kit *92*

Appendix 2: Manual data collection sheet used to collate diagnostic information *94*

Appendix 3: Example of a hardcopy of the questionnaire used in the study *95*

CHAPTER 3: AN EXTENSIVE BURDEN OF GIARDIASIS ASSOCIATED WITH INTESTINAL SCHISTOSOMIASIS AND ANAEMIA IN SCHOOL CHILDREN ON THE SHORELINE OF LAKE ALBERT, UGANDA 99

3.1 ABSTRACT 101

3.2 INTRODUCTION 102

3.3 MATERIALS AND METHODS 104

3.3.1 Study location *104*

3.3.2 Examination of children *105*

3.3.3 Examination of stool specimens *106*

3.3.4 Probe-based detection method for Giardia *107*

3.3.5 Statistical analysis *108*

3.3.6 Ethical approval *109*

3.4 RESULTS 110

3.4.1 General characteristics *110*

3.4.2 Prevalence of diseases *111*

3.4.3 Associations with giardiasis *112*

3.4.4 Intestinal schistosomiasis through time *114*

3.5 DISCUSSION 115

3.5.1 Effects of parasitic burdens and environmental factors *115*

3.5.2 Schistosomiasis *116*

3.5.3 Giardiasis *116*

3.5.3 Malaria *117*

3.5.4 Association with Giardia infection *117*

CHAPTER 4: MOLECULAR CHARACTERISATION AND TAXON ASSEMBLAGE TYPING OF GIARDIASIS IN PRIMARY SCHOOL CHILDREN LIVING CLOSE TO THE SHORELINE OF LAKE ALBERT, UGANDA **120**

4.1 ABSTRACT 122

4.2 INTRODUCTION 123

4.3 MATERIALS AND METHODS 125

4.3.1 Faecal material and epidemiological information *125*

4.3.2 Molecular profiling of G. duodenalis assemblages *125*

4.3.3 Statistical analyses *127*

4.4 RESULTS 128

4.4.1 G. duodenalis and assemblages *128*

4.4.2 Epidemiological associations with an assemblage *129*

4.4.3 Sequence analysis of the beta-giardin gene *130*

4.5 DISCUSSION 131

4.5.1 Application of real-time PCR to pathogen detection *131*

4.5.2 Real-Time PCR Assay for Detection and Genotype *131*

4.5.3 Associations between epidemiological information and assemblage *131*

4.5.4 Diversity within assemblages *132*

4.5.5 Conclusion *133*

4.6 APPENDIX 134

Appendix 4: Point mutations detected within assemblage B *134*

CHAPTER 5 NON-INVASIVE SURVEILLANCE OF *PLASMODIUM* INFECTION BY REAL-TIME PCR ANALYSIS OF ETHANOL PRESERVED FAECES FROM UGANDAN SCHOOL CHILDREN WITH INTESTINAL SCHISTOSOMIASIS 135

5.1 ABSTRACT 137

5.2 INTRODUCTION 138

5.3 MATERIALS AND METHODS 141

5.3.1 STUDY AREA 141

5.3.2 Ethical approval 142

5.3.3 Enrolment of participants 142

5.3.4 Clinical examination and demographic information 142

5.3.5 Malaria RDTs detect specific antigens (proteins) 143

5.3.6 DNA extraction from biological samples (EPF and DBS) 143

5.3.7 Malaria detection using malaria species-specific TaqMan™ Assay 144

5.3.8 Sequencing-based species-specific 18s gene for species confirmation 146

5.3.9 Statistical analysis 147

5.4 RESULTS 148

5.4.1 General characteristics 148

5.4.2 Prevalence of malaria infection 149

5.4.3 Geographical distribution of malaria infection 149

5.4.4 Correlation between C_t values and the presence of malaria DNA 150

5.4.5 Evaluating diagnostic accuracy in the face of RDT as 'gold standard' 151

5.4.6 Geographical distribution of Plasmodium species 152

5.5 DISCUSSION 153

5.5.1 Non-invasive surveillance for Plasmodium in non-human primates 153

5.5.2 Validation of a PCR-Based Test for the detection of Plasmodium spp. 153

5.5.3 Altitude-variations in Plasmodium spp. prevalence 154

5.5.4 Analytical specificity and sensitivity of a rtPCR-Based assay *155*

5.5.5 DNA-based species-specific assays *156*

5.6 CONCLUSIONS 157

5.7 APPENDIX 160

Appendix 5: Presence of deletions and mutations in the 18S rRNA-based target sequences ... *160*

CHAPTER 6 EVALUATION AND IMPLEMENTATION OF NEW APPROACHES FOR SURVEILLANCE OF SCHISTOSOMIASIS: APPLICATION OF MOLECULAR DNA METHODS FOR SNAIL SPECIES IDENTIFICATION COMBINED WITH IMMUNO-DIAGNOSTICS USING URINE-CCA STRIP TEST AND FINGER-PRICK BLOOD SEA-ELISA IN SCHOOLCHILDREN IN SAUDI ARABIA 161

6.1 ABSTRACT 163

6.2 INTRODUCTION 164

6.3 METHODOLOGY 168

6.3.1 Study site *168*

6.3.2 Surveys for freshwater snail populations *169*

6.3.3 Selected schools and participants *170*

6.3.4 Field-based evaluation of POC-CCA urine test *171*

6.3.5 Finger-prick blood SEA-ELISA *172*

6.3.6 DNA Methods *173*

6.3.7 Inclusion criteria *174*

6.3.8 Data handling and statistical analysis *174*

6.3.9 Ethical considerations and anthelminthic treatments *174*

6.4 RESULTS 175

6.4.1 Morphometric analysis *175*

6.4.2 Environmental parameters and snail distribution *177*

6.4.3. DNA sequencing *178*

6.4.4 Prevalence of schistosomiasis by an ELISA and CCA *178*

6.4.5 Evaluation of an ELISA for combined with CCA as a standard gold test *179*

6.4.6 Urine filtration assessment with microscopy (Following–up) *180*

6.5 DISCUSSION 181

6.5.1 Schistosomiasis in Saudi Arabia *181*

6.5.2 Spatial distribution of identified Bulinus snails *181*

6.5.3 Environmental effects on snail population density *182*

6.5.4 Employed five morphological parameters for snail shells *182*

6.5.5 Relationship of snail's morphological parameters *184*

6.5.7 Morphological and genetic analysis of Bulinus snails *184*

6.5.8 Immunological detection of schistosomiasis *185*

6.5.9 Following up examination for positive cases *187*

6.5.10 Aspect of future works *187*

6.6 CONCLUSION 188

6.7 APPENDICES 190

Appendix 6: CTAB extraction *190*

Appendix 7: Ethical approval (KSA) *192*

Appendix 8: Analysis and sequence alignment for Bulinus forskalii *193*

CHAPTER 7. GENERAL DISCUSSION 194

7.1. NATIONAL CONTROL PROGRAMME (NCP) 196

7.1.1. Schistosomiasis Control Initiative (SCI) Programme, Uganda *196*

7.1.2 Schistosomiasis Control Programme (SCP), Saudi Arabia *197*

7.1.3 Giardia control programme in Uganda: current and future challenges *198*

7.1.4 Malaria control programme in Uganda *199*

7.2 MOLECULAR DIAGNOSIS IN CLINICAL PARASITOLOGY: CURRENT AND FUTURE ASPECTS 200

7.2.1 Schistosoma spp *201*

7.2.2 Giardia duodenalis *202*

7.2.3 Plasmodium spp *203*

7.3. KEY RECOMMENDATIONS 205

7.4. SUGGESTIONS FOR FUTURE WORK 209

7.5 APPENDIX 213

Appendix 9: A guide to aid the selection of diagnostic tests *213*

8. REFERENCES 214

9. CURRICULUM VITAE AND SELECTED PUBLICATIONS 279

10. PUBLICATIONS 280

Appendix 10:A centenary of Robert T. Leiper's lasting legacy on schistosomiasis and a COUNTDOWN on control of neglected tropical diseases *283*

Appendix 11:Surveillance of intestinal schistosomiasis during control: a comparison of four diagnostic tests across five Ugandan primary schools in the Lake Albert region *284*

Appendix 12:An extensive burden of giardiasis associated with intestinal schistosomiasis and anaemia in school children on the shoreline of Lake Albert, Uganda *285*

Appendix 13:Molecular characterisation and taxon assemblage typing of giardiasis in primary school children living close to the shoreline of Lake Albert, Uganda *286*

Appendix 14:Non-invasive surveillance of Plasmodium infection by real-time PCR analysis of ethanol preserved faeces from Ugandan school children with intestinal schistosomiasis ... *287*

Appendix 15: Molecular detection of equine trypanosomiasis in the Riyadh Province of Saudi Arabia *288*

Appendix 16: Four posters submitted at various scientific meetings *289*

Appendix 17: Certificates of Achievements *293*

Dedication

To my family

Declaration

This thesis is my own work, and the contents have not been presented, nor are currently being presented, either wholly or in part for any other degree or qualification. The data set I analysed in **chapter 2, 3, 4, 5**, and **6** came from the studies conducted by myself in cooperation with teams from LSTM and Kingdom Saudi Arabia. With guidance from my supervisors, I participated in the design, implementation and analysis of the studies and data generated therein. This data set had, up to the time of this analysis, not been analysed and results presented elsewhere.

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Abbreviations and Acronyms

ACT	Artemisinin-based combination therapies
BMGF	Bill & Melinda Gates Foundation
CAA	Circulating anodic antigen
CCA	Circulating cathodic antigen
CO1	Cytochrome oxidase subunit
DALY	Disability-Adjusted Life Year
DFA	Direct fluorescent antibody
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EPG	Eggs per gram
FOBT	Faecal Occult Blood Test
fDNA	Faecal DNA
GDH	Glutamate dehydrogenase
gDNA	Genomic DNA
GPS	Geographical positioning system
HIV	Human Immunodeficiency Virus
IRS	Indoor residual spraying
IVM	Integrated vector management
KK	Kato-Katz faecal smear method
MDA	Mass Drug Administration
MGBP	Minor grove binding probes
MTZ	Metronidazole
NCP	National Control Programme
NPV	Negative predictive value
PPV	Positive predictive value
NTDs	Neglected Tropical Diseases
PC	Preventative chemotherapy
PCR	Polymerase chain reaction
PHV	Phocine Herpes Virus
POC	Point-of-care
PZQ	Praziquantel
RDT	Rapid diagnostic test
rRNA	Ribosomal RNA
SAC	School-aged children
PSAC	Pre- School-aged children
SCI	Schistosomiasis Control Initiative
SEA	Soluble egg antigen
SmSEA	*S. mansoni* soluble egg antigen
STH	Soil-Transmitted Helminth
TPI	Triosephosphate isomerase
VSPs	Variant-specific surface proteins
WASH	Water, sanitation and hygiene
WHO	World Health Organization

List of Tables

Table 1: Summarizing the characteristics, locations and habitats of intermediate hosts for human schistosomiasis 26

Table 2: Microscopic examination of the *Schistosoma* egg morphology..... 30

Table 3: Recommended treatment strategy for schistosomiasis in preventative chemotherapy PZQ . 35

Table 4: Summarizing most commonly snail control strategies used in countries like Japan, Iran, Lebanon, Saudi Arabia..... 35

Table 5: Summary of current epidemiological *G. duodenalis* studies in Uganda which include human infections..... 41

Table 6: Molecular characterisation of *G. duodenalis* isolates and the association of vertebrate host species structure genotype with a transmission cycle 43

Table 7: Tabulated summary of schistosomiasis, giardiasis, and malaria diagnostics..... 67

Table 8: Prevalence (%) of *S.mansoni* by school according to each diagnostic test with 95 % confidence intervals..... 82

Table 9: The prevalence with intensity measured by each diagnostic method for *S. mansoni* across the five primary schools 84

Table 10: Diagnostic comparison of sensitivity (SS), specificity (SP), negative predictive value (NPV) and positive predictive value (PPV) of each method against urine-CCA dipstick as 'gold standard'..... 85

Table 11: Latent class analysis (LCA) estimates of sensitivity and specificity and LCA model of the prevalence of *S. mansoni* by school with 95 % CIs for each diagnostic method 86

Table 12: Prevalence of infection by school across a total of 254 children aged between 5 and 10 years of old 111

Table 13: Adjusted risk factors associated with heavy *Giardia* infection by real-time PCR..... 112

Table 14: Summarizing of assemblage-specific primers and probes..... 126

Table 15: Prevalence (%) of *G. duodenalis* and assemblages across all five schools by real-time PCR; the odds ratio of assemblages A, B or A/B by school compared against the total given (with 95% Confidence Limits) 128

Table 16: Epidemiological associations cross-tabulated against available assemblage information .. 129

Table 17: Polymorphic sites in the *β*-giardin sequences in *Giardia duodenalis* assemblage **A** & **B** isolates..... 130

Table 18: Primers and probes used for screening and identification of *Plasmodium* species 145

Table 19: Prevalence and influence of diagnostic tests for detecting Plasmodium infection in primary school children 149

Table 20: Empirical estimates of sensitivity (SS), specificity (SP), negative predictive value (NPV) and positive predictive value (PPV), Cohen's kappa for each diagnostic test against malaria by RDT as 'gold standard.' 152

Table 21: Distribution of *Plasmodium* species across study sites using TaqMan® probe-based assays 152

Table 22: Morphometric data gathered from *Bulinus* snails at 17 different sites in Saudi Arabia..... 175

Table 23: Showing results of CCA and ELISA from school Huzaifa ibn al-Yaman and Ibn al-Athir school as well as the intensity of infection based on each diagnostic method with 95% confidence interval. 179

Table 24: Diagnostic comparison of sensitivity (SS), specificity (SP), negative predictive value (NPV) and positive predictive value (PPV) of SEA-ELIZA against urine-CCA dipstick as 'gold standard'180

List of Figures

Figure 1: Map is demonstrating the global distribution of different types of *Schistosom*a spp. 24

Figure 2: The lifecycle of the most common human *Schistosoma* spp..... 25

Figure 3: A schematic diagram representing the development of both Th1 and Th2 immune response to parasite antigens..... 28

Figure 4: Microscopic histological images of the urinary tract with *S. haematobium* eggs..... 29

Figure 5: Pictorial representation of the current WHO dose-pole which corresponds to a number of praziquantel tablets 34

Figure 6: An outline map for schistosomiasis transmission in Uganda and Saudi Arabia. 37

Figure 7: Morphology of *Giardia* trophozoites (Schematic and Scanning electron micrograph (SEM) of *Giardia* trophozoite structure). 38

Figure 8: Lifecycle of *G. duodenalis.* 40

Figure 9: Diagram showing essential cycles of transmission for maintaining *Giardia***.** 42

Figure 10: The proposed determinants and mechanisms of *G. duodenalis* pathology within the intestinal 45

Figure 11: The mosquito and human life cycle of the *Plasmodium* parasite 48

Figure 12: The percentage of the population at risk of malaria based on the 2016 World Malaria Report 49

Figure 13: Shows the prevalence of *P. falciparum* malaria in Sub-Saharan Africa in heat map format, in both 2000 and 2015, generated by the Malaria Atlas Project..... 50

Figure 14: A geographical map of Uganda, and a malaria prevalence heat-map (right), generated by the Malaria Atlas Project, highlighting the average prevalence of *P. falciparum* infection in Uganda. 50

Figure 15: The process of a PCR reaction 61

Figure 16: The basic process of amplicons detected by using a labeled probe specific for the target sequence within gDNA 63

Figure 17: A schematic map of the 5 sampled primary schools in the Lake Albert region, with major towns Masindi and Hoima shown 76

Figure 18: Reflecting the enrollment of children in Biiso school for grade 1 to 3, and different steps performed following a school-based survey protocol for NTDs control..... 77

Figure 19: Preparation of a duplicate Kato-Katz thick smear and reading slides at camp in the study location. 78

Figure 20: Detailed photograph of POC-CCA cassettes with control and result bands 79

Figure 21: A-Performing a field-based ELISA test following manufacturer's instruction..... 80

Figure 22: TaqMan® probe -based detection of *S. mansoni* -DNA with categorizing infection intensity based on the cycle threshold (C*t*)..... 81

Figure 23: Prevalence of *S. mansoni* by school distance for each diagnostic method, and by any positive test criterion. 83

Figure 24: Inset, sketch map of Uganda and study area with outline map of the five schools inspected. 104

Figure 25: Participant enrollment and inclusion in study research. 105

Figure 26: Parasitological confirmation of *S. mansoni* and G*iardia* diagnosis**.** 106

Figure 27: Onsite training with LSTM for improving diagnosis of intestinal parasitic infection 107

Figure 28: Pie charts of reported symptoms as reported on the questionnaire..... 110

Figure 29: Box plots of biometric data and haemoglobin levels and heavy infection status with *Giardia.* 113

Figure 30: Malacological survey showing eight sites around five selected schools 115

Figure 31: Showing the results for Bugoigo School children tested for A and B Assemblages, including negative and positive controls 126

Figure 32: Bivariate plot of Ct values for TaqMan® 18S DNA versus Ct value of assemblage-specific TaqMan® TPI probe obtained for each sample with dashed lines showing the 95% prediction interval. 129

Figure 33: Inset: sketch map of the study villages in Buliisa district, Lake Albert, Uganda..... 141

Figure 34: Malaria rapid diagnostic tests showing results from Biiso school-aged children..... 143

Figure 35: rtPCR quantitation as performed by Opticon Monitor 3 software (Biorad), showing the 4 positive samples with malaria infection detected by bDNA in comparison with fDNA using samples collected from school A (Bugoigo). 146

Figure 36: Flow Chart of Study Process, demonstrates various stages being taken for analysing *Plasmodium* spp. assay..... 148

Figure 37: Estimated prevalence of Malaria infection by altitude and school distance from the shoreline 150

Figure 38: Showing the regression between rtPCR-based assays for DBS (C_t) and rtPCR-based assays for EPF (C_t) for the singleplex assay to detect generic *Plasmodium* (18S rRNA gene). 151

Figure 39: An outline map of the KSA with capital Riyadh depicted. 166

Figure 40: A map of administrative boundaries for Asir province with pointing study site, and an outline map of a total number of snails collected at each monitoring site 168

Figure 41: Photograph representing one study site nearby Huzaifa ibn al-Yaman primary school at Al-Majardah District 169

Figure 42: Enrollment and participation in research at the study sites at Al-Majardah District, following school-based survey protocol for NTDs control..... 170

Figure 43: Urine and blood sample examination at Primary Health Care (PHC) public services at Al-Majardah District 171

Figure 44: A field-based ELISA test using the treated SEA to detect specific antibodies to SEA in the sera of eligible children**.** 172

Figure 45: Regression analysis of mean morphometric measurements of 5 snails at each coding site (1-17) are presented with predicating lines for determining the correlation of the mean shell parameter..... 176

Figure 46: Showing the predicted Y (water pH or conductivity (μs)) for the number (X) of freshwater snails collected from 17 sites at Al-Majardah District. 177

Figure 47: Idealized surface plot about showing the number of diagnostic tests performed increases as the rates of infection in the school decline, and hypothetical project areas based on transitions from control to elimination interventions. 198

Figure 48: Schematic figure is highlighting essential differences between the endemic areas and the appropriate control tools..... 205

Figure 49: Modified figure about what expected diagnostic tools should be applied for future schistosomiasis surveillance based on endemicity and control achievements..... 206

Abstract

Introduction: Where access to sanitation is incomplete and environmental water contact high, water-borne diseases such as schistosomiasis can be common in children and occur alongside other parasitic diseases. Parasitological diagnosis based upon microscopy of parasite ova or larvae underestimates true infection prevalence, and as NCPs reduce the intensity of infection(s) with preventive chemotherapy, the diagnostic sensitivity also declines. In Uganda and the Kingdom of Saudi Arabia, for example, school-based disease control has been ongoing over the past decade, and there is a need to develop more sensitive methods of parasite detection and disease surveillance. Against this two-country backdrop, the application of novel diagnostics was investigated to reveal the current status of infections in school children. Parasitological- and serological-based methods were compared against the implementation of real-time PCR methods to detect DNA from faecal or dried blood spot specimens. Diagnostic performance was then compared, and its epidemiological significance discussed for each country setting.

Method: In Uganda, Buliisa District, a total of 271 children were enrolled. Stool, urine and blood were collected and examined for schistosomiasis, giardiasis and malaria using a selection of commercially available RDTs in the field, then compared against real-time PCR methods with TaqMan® probes in the laboratory. In the Kingdom of Saudi Arabia, Al-Majardah District, a total of 163 children were enrolled for this study. As faecal samples could not be collected, blood and urine samples were inspected. Children were interviewed by questionnaire and collection of intermediate snail hosts made. In the following up, all children positive for schistosomiasis where re-examined one year later.

Results: In Uganda, Buliisa District, the overall prevalence of *S. mansoni* was: 44.1% by microscopy of duplicate KK smears from two consecutive stools, 56.9% by urine-CCA dipstick, 67.4% by DNA-TaqMan® and 75.1% by antibodies to SEA- ELISA. Giardiasis was 41.6% by QUIK CHEK™ RDT, and up to 87.0% of children were excreting *Giardia* DNA. The prevalence of heavy infection by real-time PCR assay ($C_t \leq 19$) was 19.5%. Notably, giardiasis was positively associated with egg-patent schistosomiasis and two *Giardia* assemblages, A and B, were found. Co-infections thereof varied by school and by age. A total of 138 (55.8%) cases of malaria were diagnosed for *P. falciparum* (*Pf*HRP-2-detecting RDTs), and 45 samples (18.2%) were positive for pan-*Plasmodium* species (*Pf*HRP-2 and pan-pLDH test lines). Upon analysis of dried blood spots with real-time PCR, a total of 198 (80.1 %) cases for any *Plasmodium* spp. was detected and compared to real-time PCR of faecal samples. In the Kingdom of Saudi Arabia, Al-Majardah District, using urine-CCA strip

assessment and SEA-ELISA, the prevalence of *S. mansoni* was 15.3% and combined *S.mansoni*/*haematobium* was 34.3%, respectively. Molecular DNA analysis using the CO1 gene alongside morphological identification of collected snails demonstrated the presence of *Bulinus forskalii* in this area and the local absence of *Biomphalaria* at the time of the survey.

Conclusion: Using a combination of diagnostic methods, vital contemporary epidemiological information on schistosomiasis, giardiasis and malaria was revealed and then compared across the two countries using the ASSURED criterion. Foremost, whilst real-time PCR methods with TaqMan® probes have the greatest potential in Uganda, in Saudi Arabia however, owing to the difficulties in obtaining a stool, this method is inappropriate. Nonetheless, DNA-based diagnostic platforms will be essential in future public health settings where prevalence and intensity of infections continue to fall, as improved sensitivity is required. Furthermore, the ability to simultaneously co-detect several infectious and parasitic diseases will boost disease surveillance efficiency and capacity.

Chapter 1: General Overview

1. Neglected Tropical Diseases (NTDs)

Neglected Tropical Diseases NTDs have a substantial detrimental health impact on the populace of the developing world, especially in communities trapped within poverty (WHO, 2017a); from actions of prominent international NTD lobbyists, the prominence given to NTDs has increased and NTDs are recognised as a public health scourge of comparable importance to HIV, malaria and tuberculosis (Hotez *et al.*, 2006a). A disease may be deemed 'neglected' when overall funding is inadequate, lower than its need, and insufficient for effective prevention and/or control. Most affected by these debilitating diseases are the poorest of the poor, and most vulnerable within tropical and subtropical populations of the world. More than 1 billion people – a seventh of the world's population, are suffering from one or more NTD (WHO, 2009b, Deribe *et al.*, 2012). The list of NTDs includes helminth, e.g. STH infections (ascariasis, trichuriasis and hookworm infection) and schistosomiasis as well as those of protozoan, bacterial or viral origin (Utzinger *et al.*, 2012). With a lack of innovative diagnostic tools, effective treatment chemotherapies, incomplete sanitation and inadequate knowledge regarding disease transmission, many vulnerable people at risk of significant ill-health. Several NTDs often coexist with poverty because they thrive where access to clean water, putting approximately 500 million people in sub-Saharan Africa susceptible to co-infection (Hotez and Kamath, 2009).

Polyparasitism may also cause long-term effects on child growth, hinders intellectual development and can interfere with iron absorption (anaemia) (Müller *et al.*, 2016). The principles and strategies of control NTDs include improving access to safe, clean drinking water as well as basic sanitation and improved housing. The implementation of these strategies could prevent the spread of the diseases and potentially eradicated (Hotez *et al.*, 2007, Feasey *et al.*, 2009). Prevention strategies available to combat these NTDs may be at the cost of less than US$0.50 per person per year - making the elimination of these NTDs a feasible goal, yet over half a million deaths occur annually (Hotez *et al.*, 2007, Hotez and Pecoul, 2010). A diverse group of disease-specific partnerships represent the different communities involved in NTDs have made a significant contribution to scaling up of programmes. One of those, the BMGF funded the SCI programme which started in 2002. Uganda, one of the six countries developed a control strategy for treating about three-quarters of SAC with preventive chemotherapy, decreasing infection intensity and creating a 'demand' for continuous implementation of the effective large-scale control programme (Fenwick *et al.*, 2009, Fenwick and Jourdan, 2016). With progress and

challenges towards the targeted delivery of treatments, there are genuine prospects to transition from control to elimination of NTDs, or to confirm the presence or absence of further transmission (Molyneux *et al.*, 2018, Rosenberg *et al.*, 2016, French *et al.*, 2018).

Over the last decade, substantial progress in control and elimination has been brought the transmission of the disease under control in different countries due to medicine donations. It has been shown that five of the ten diseases (Guinea worm disease, lymphatic filariasis, leprosy, sleeping sickness and blinding trachoma) would eventually be eliminated and the other five (schistosomiasis, STH, Chagas disease, visceral leishmaniasis and onchocerciasis) have made advanced changes in control over the past 10 years (Hotez *et al.*, 2016). These promising developments encourage researchers to continue to target these long-term debilitating diseases impacting the most vulnerable populations (Hotez *et al.*, 2006b). NTDs are typically addressed through five strategies: preventive chemotherapy, intensified disease management, vector control, veterinary public health measures for neglected zoonotic diseases, and through improved water and sanitation (Molyneux *et al.*, 2017, Molyneux *et al.*, 2018).

In 2013, the World Health Assembly approved Resolution WHA 66.12, which defined strategies for NTDs with clear targets and milestones for 17 NTDs, and endorsed the WHO NTD Roadmap goals linking NTDs to universal health coverage (Molyneux *et al.*, 2017). An integrated control approach, highlighting multiple NTDs is imperative to track the spread of disease transmission (Clements *et al.*, 2010). An initial investment of more than $785 million to support NTD programs, strengthen drug distribution, and increase research and development towards combatting NTDs is needed from developed countries, and commitment from developing countries to improve their uptake and reported compliance in the last mass drug administration (MDA), are the foundations to achieve NTD elimination (Mableson *et al.*, 2014, Molyneux, 2013, Molyneux *et al.*, 2018). Most importantly, NTDs that currently lack appropriate tools for large-scale use. Hence, in general, disease management is presently more complex and costly to manage as there are inherent challenges for their diagnosis, treatment, and follow-up (Rosenberg *et al.*, 2016).

The focus of my thesis is slightly wider than the remit of NTDs alone, but I specifically aim to address a key NTD, schistosomiasis, alongside its occurrence with two other important medically important diseases, giardiasis and malaria. To help position the connections between these three diseases, I will review aspects of the lifecycle and epidemiology, to highlight connects and deficits between actions of each disease individually or collectively.

2.1 Schistosomiasis

Schistosomiasis is a particularly important NTD caused by infection with intravascular digenetic trematodes of the genus *Schistosoma*. Some 700 million people worldwide are exposed to schistosomiasis, with an estimated 200 million people actively infected (Osakunor *et al.*, 2018). Both intestinal (i.e. *S. mansoni*) and urogenital (i.e. *S. haematobium*) schistosomiasis have been widely reported, and the majority of infection occurs in sub-Saharan Africa amongst the most impoverished communities (**Figure 1**) (Colley *et al.*, 2014). Moreover, in Africa, around 300,000 deaths due to schistosomiasis are reported annually (Weerakoon *et al.*, 2018). Due to a lack of hygiene and sanitation facilities, mainly basic healthcare facilities, infection is almost unavoidable.

Infected children are highly likely to become chronic sufferers with attendant fibrosis and an elevated risk for the development of hepatosplenomegaly, hepatic fibrosis, portal hypertension, and colonic polyposis (for the intestinal forms of schistosomiasis caused by *S. mansoni, S. japonicum, S. intercalatum*, and *S. mekongi*) and bladder thickening, ulceration, and polyposis, along with hydronephrosis and renal dysfunction (for urinary schistosomiasis caused by *S. haematobium*) (Osakunor *et al.*, 2018, Gray *et al.*, 2011a). Globally, schistosomiasis, as expressed in DALY, causes an estimated at 3 million DALYs, and costing approximately $57 million annually (Tekle *et al.*, 2012).

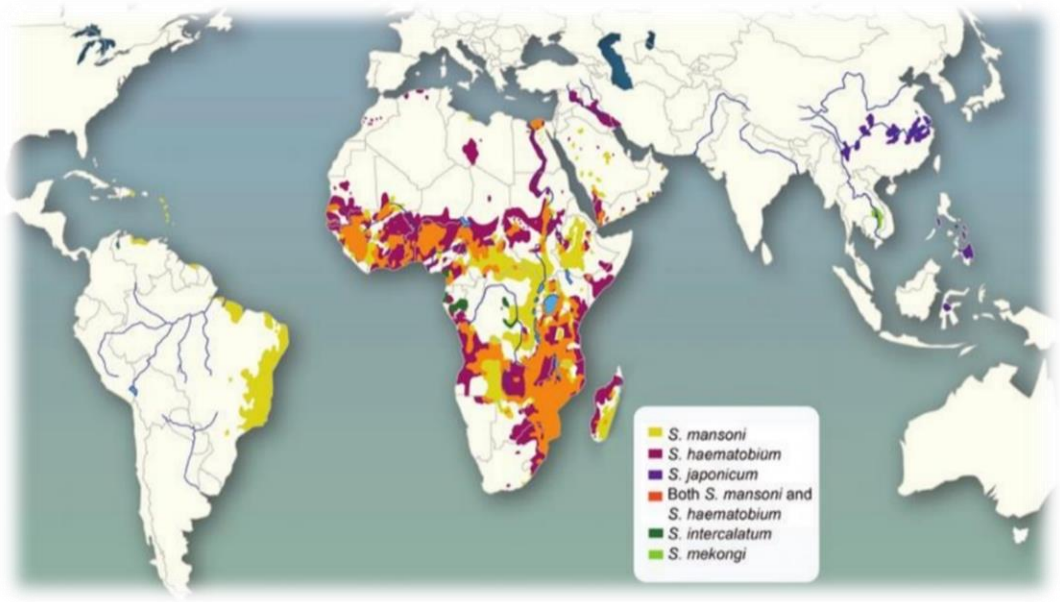


Figure 1: Map is demonstrating the global distribution of different types of *Schistosom*a spp. (The key species are illustrated by different coloured), with sub-Saharan Africa have the highest burden of infection (Weerakoon *et al.*, 2018)

2.2 Lifecycle

Adult schistosome worms (male and female) exist inside visceral blood vessels of their definitive hosts and are commonly known as vascular blood flukes (**Figure 2**). Within the blood vessels, sexual reproduction occurs to fertilise eggs, and then the eggs are laid inside the blood vessel either to be shed into the environment through faeces or urine or are retained in host tissues where they induce an inflammatory response (**Figure 3**) (Gryseels *et al.*, 2006).

The eggs hatch in freshwater sources allowing free-swimming miracidia to be released, which then infect a specific freshwater snail intermediate host. Inside the snail asexually reproduces takes place, creating numerous daughter sporocysts that contain cercariae. After around 4-6 weeks, thousands of cercariae (the larval form infectious for human beings) release into the freshwater (Gryseels *et al.*, 2006). The most widespread types, *S. mansoni* and *S. haematobium*, require a suitable snail intermediate host of the genus; *Biomphalaria* spp. and *Bulinus* spp. species respectively. From this intermediate snail host come the cercariae, which infect their definitive host (humans) by penetrating the skin. Inside the human, the cercariae can then form into an adult capable of producing about 3,000 eggs per day. The process of migration and maturation takes about 4 to 6 weeks post-infection in *S. mansoni* and around 90 days in *S. haematobium* (Colley *et al.*, 2014, Gryseels *et al.*, 2006). Understanding the fundamental schistosome life-stages are essential for developing long-term strategies and tools for the control/elimination of the disease.

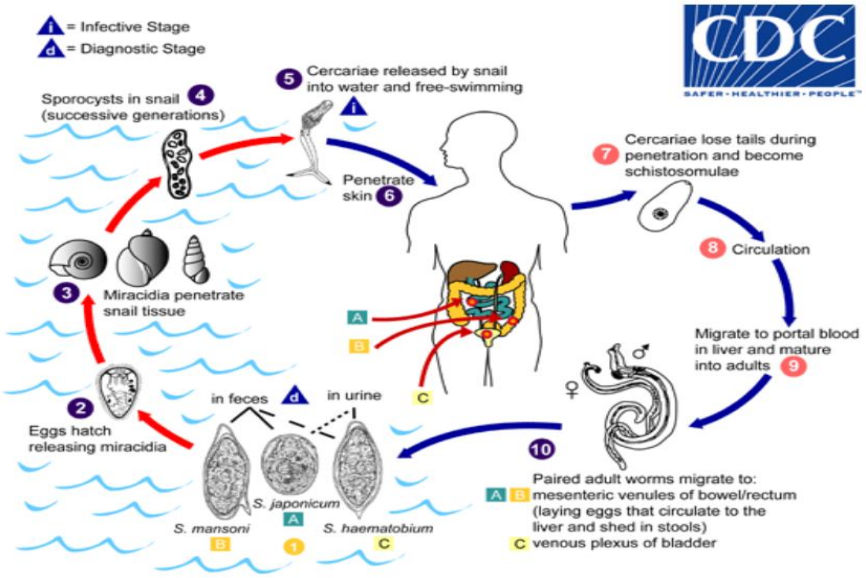


Figure 2: The lifecycle of the most common human *Schistosoma* spp. illustrating that transmission occurs in water by percutaneous transmission (Centre for Disease Control, 2017)

2.3 Schistosomiasis transmission

2.3.1 Snail hosts

In Egypt in 1915, the lifecycles of the African schistosomes were first described by Robert T. Leiper (1881-1969) which demonstrated the vital importance of freshwater snails as an essential intermediate host for this parasitic fluke (Stothard *et al.*, 2017b). Freshwater snails of *Biomphalaria* and *Bulinus* genera (Gastropoda: Planorbidae) are responsible for the transmission of intestinal and urogenital schistosomiasis, respectively (Abe *et al.*, 2018).

Both genera are widely spread in various parts of the Middle East and Africa (Hotez *et al.*, 2012). It has been suggested that miracidia at the first larval stage can recognise specific macromolecular glycoproteins on different snail species, allowing them to partially target and penetrate the suitable snail species and develop into infectious cercariae (Wang *et al.*, 2016). There are more than 400 species of freshwater gastropod detected in the whole of Africa, but only a few of them restricted to the two genera can harbour the parasitic *Schistosoma* worms (Hotez *et al.*, 2012). Their characteristics, locations and habitats are summarised in **Table 1**.

Table 1: Summarizing the characteristics, locations and habitats of intermediate hosts for human schistosomiasis (Madsen, 1992, Rollinson *et al.*, 2001, Abe *et al.*, 2018)

Genera	Morphological traits	Group	Parasite	Location	Habitat and ecology
Biomphalaria 	1. Dextral orientation 2. Biconcave discoid shell 3. Diameter between 7 and 22 millimeters.	*B. pfeifferi*	*S. mansoni*	South Africa, Sahara	Shallow waters close to the shoreline. Common in streams, irrigation channels, reservoirs, swamps, dams and some seasonal waters.
		B. choanopmhala		Areas of the Great lakes	Gravel and sedimentary rocks.
		B. alexandrina		Nile Delta, but spreading to eastern Africa	Swamps and man-made structures such as irrigation systems.
		B. sudanica		Western and eastern Africa	Shallow waters close to the shoreline. Swamps and other habitats associated with lakes and rivers.
Bulinus 	1. Sinistral orientation 2. Conical shell 3. Size between 4 and 20 millimeters.	*B. Africanus*	*S. haematobium*	Northeastern, Central and Southern Africa	Widespread specie with a good tolerance to pollution. Common in habitats varying from streams to small impoundments.
		B. tropicus / truncatus	Only *B. nyassanus* is a natural host for *S. haematobium*	Distributed in most African countries.	Common in small earth dams and residual pools in seasonally flowing streams. Lakes, man-made structures such as irrigation systems and dams.
		B. reticulatus	*S. haematobium*	Eastern and Southern Africa.	Small pools that briefly fill during the rainy season.
		B. forskalii	*S. intercalatum* *S. guineensis* *S. haematobium*	Distributed in most African countries	Lake margins and swamps. Present in both permanent and temporary habitats and in natural and artificial situations.

Environmental conditions such as temperature, pH and conductivity can also affect the development of these snails and therefore effect disease transmission to humans (Liang *et al.*, 2007). It has been documented that conductivity may have an extremely adverse effect on snail density (Rowel *et al.*, 2015). Lower

pH results have also been suggested to an increase of snail density (Levitz *et al.*, 2013). However, there is still a particular discrepancy in findings (Opisa *et al.*, 2011, Rowel *et al.*, 2015).

Although specific for each species, there is evidence for the influence of temperature on survival, growth and fecundity of the freshwater snail. The optimum temperature for aquatic mollusc's development and survival is between 15°C. and. 25°C, but mortality increases after 30°C and over 40°C degrees will cause death (Kazibwe *et al.*, 2006, Opisa *et al.*, 2011). A recurrent concern has been that the host snails' taxonomy not always been entirely distinguished, and the traditional morphology-based character has been inaccurately used for extended times as very similar species can be different from others in their competence (Bakuza *et al.*, 2017). Molecular taxonomic tools for the identification and classification have conclusively been proved in deciphering the phylogenetic relationship of snail populations. A method like targeting the mitochondrial gene CO1 in a PCR and subsequent DNA sequencing has accurately allowed for the identification of very different species from *Bulinus* spp. and *Biomphalaria* spp. populations (Kane *et al.*, 2008, Jørgensen *et al.*, 2007, Stothard and Rollinson, 1997).

2.3.2 Human hosts

Since human hosts become infected by direct penetration of the skin, avoiding contact with infected water is the key to preventing disease spread. Many impoverished communities rely on freshwater that is contaminated by human and animals waste as a primary source for their daily household activities such as drinking and bathing so that decreasing disease transmission based on controlling is quite difficulties (Colley *et al.*, 2014).

WASH control measures require to be implemented to minimise the rate of human infection (Grimes *et al.*, 2015). Sanitation strategies to prevent excreta eggs from contaminating water sources, and eventually providing access to soap and clean water may reduce levels of cercariae and miracidia and therefore the rates of infection (Tchuenté *et al.*, 2017). Targeting population, SAC, children who are living close to freshwater sources, where schistosomiasis is focally transmitted, is vital for control disease, by providing effective medication, such as, PZQ for those most in need (Grimes *et al.*, 2014, Stothard *et al.*, 2013a). The analysis of the local people's living styles, patterns and habits will improve our knowledge required to effectively manage the disease transmission and help in developing control strategies toward elimination (WHO, 2009a).

2.4 Pathogenesis and symptomatology

2.4.1 Intestinal schistosomiasis

Schistosomes localise in the portal and mesenteric veins, with the disease involving the liver, spleen, and gastrointestinal tract, where egg execrating, and eventually release into the environments through faeces (Colley *et al.*, 2014). However, some of these eggs become trapped in the intestinal wall and can lead to multiple-granulomata formation. Subsequently, the muscularis mucosa becomes involved, and the overlying mucosa undergoes hyperplastic changes, the submucosa becomes thickened and dark, swelling (inflammation) of various parts of the digestive tract and yellowish patches may be seen (Pearce and MacDonald, 2002). Diarrhoea, abdominal pain or swelling of the abdomen also can be seen in the chronic stage due to this inflammatory response (**Figure 3**); other gastrointestinal manifestations can be presented such as anaemia, enteropathy and rectal bleeding, and rectal prolapse (Bustinduy *et al.*, 2013, Green *et al.*, 2011).

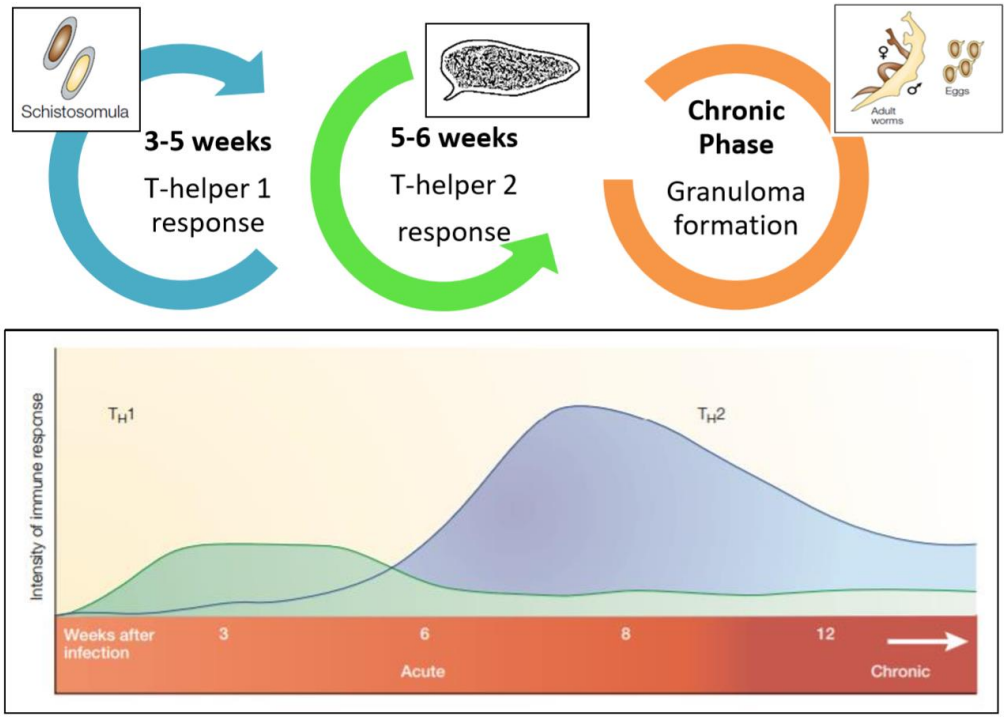


Figure 3: A schematic diagram representing the development of both Th1 and Th2 immune response to parasite antigens (Pearce and MacDonald, 2002)

2.4.2 Urogenital schistosomiasis

Schistosoma haematobium localises in the vesical plexus (venous network around the bladder), where female worms lay eggs into the urine (Colley *et al.*, 2014). Initially, the eggs produced by adult worms in their venous locations are highly immunogenic molecules, known as a soluble egg; they enter into urinary tract tissue, and trigger pro-inflammatory immune responses to schistosome eggs, resulting in granuloma formation (**Figure 3-above**) (Pearce and MacDonald, 2002, Gray *et al.*, 2011b). It has been found that haematuria, dysuria and frequent urination were more frequently associated with urinary tract pathologies of infection (UTPs) which can be observed within three months of infection (**Figure 4**) (De Souza and Olsburgh, 2008). Over extended periods, immune downregulation and reduction of granuloma sizes are exchanged by collagen and fibrotic lesions, eventually leading to strictures, calcifications and urodynamic abnormalities which might persist post-infection and despite treatment (Wilson *et al.*, 2007, Colley and Secor, 2014). Hydronephrosis, kidney failure and death are also long-term sequelae (Smith and Christie, 1986). *S. haematobium* also provides the pre-conditions for squamous cell carcinoma development, a distinguishing feature of this Schistosoma species, believed to cause around 16% of all bladder cancers in Egypt (Zaghloul, 2012).

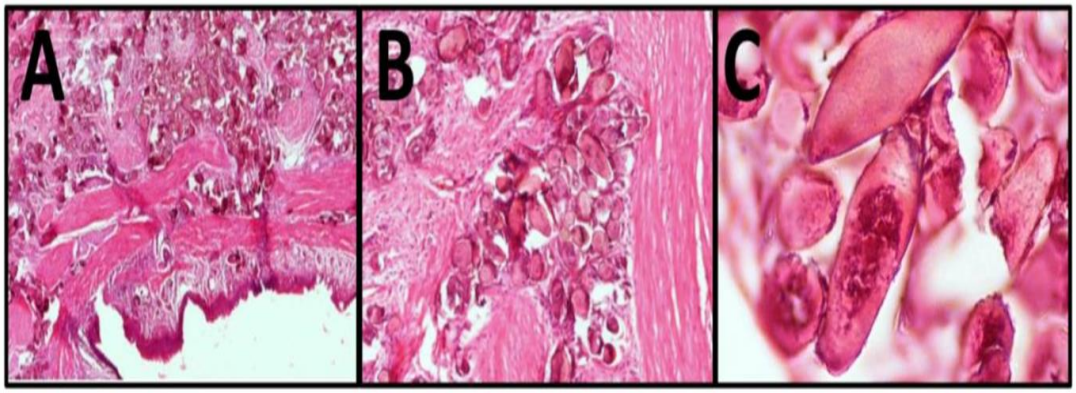


Figure 4: Microscopic histological images of the urinary tract with *S. haematobium* eggs present produced by Fadel, H (Fadel, 2018). (**A**) The presence of *S. haematobium* eggs leads to the transitional epithelium of the ureter, (**B**) *S. haematobium* eggs trap in the muscle layer of the bladder potentially causing urodynamic disorders, (**C**) The terminal spine of *S. haematobium* eggs is presented in the muscle layer of the bladder

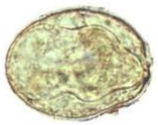
2.5 Diagnostics

2.5.1 Direct parasitological methods

Microscopic examination of excreta (stool, urine) remains the standard gold test for diagnosis of *Schistosoma* spp., but has some well-known limitations

(Gray *et al.*, 2011a). The diagnosis of intestinal schistosomiasis is widely performed by egg detection using the microscopy-based KK (**Figure 4**). According to the WHO recommended thresholds (light: 1-99 EPG, moderate: 100-399 epg, heavy: ≥400 epg)(Hawkins *et al.*, 2016). Despite the low sensitivity of the test in populations who have received previous treatment with PZQ, it is still widely used in limited resource-settings as its simplicity and relatively low-cost, and ability to detect other parasitic diseases such as STH (Colley *et al.*, 2014).

Table 2: Microscopic examination of the *Schistosoma* egg morphology, (**CDC**, 2016)

Schistosoma spp	Type of schistosomiasis	Egg morphology found in stool specimen	Endemic areas
S. mansoni	**Intestinal**		Africa, the Middle East, Central America, South America
S. japonicum			China, Indonesia, the Philippines
S. mekongi			Several districts of Cambodia and the Lao People's Democratic Republic
S. intercalatum			Rain forest areas of central Africa
S. haematobium	**Urogenital**		Africa, the Middle East

The microscopic detection of excreted *S. haematobium* eggs, when 10 ml of urine is syringe-filtered, usually through polycarbonate filters with a pore size of 8–30 µm, is often considered as the gold standard diagnostic (Colley *et al.*, 2014). Based on the WHO guideline, egg counts for *S. haematobium* are given as eggs per 10 mL of urine (ep10mL), and intensities of infections are categorised as light (1–49 ep10mL) and heavy (≥ 50 ep10mL) (Stete *et al.*, 2012). However, the sensitivity of these methods in single stool or urine sample examination is limited by day-to-day variation in egg excretions leading to measurement error in estimating the presence of infection (Tchuenté *et al.*, 2017). This is particularly accentuated in areas with high proportions of light intensity infections (Booth *et al.*, 2003, Weerakoon *et al.*, 2018). The increase of large-scale interventions and repeated mass treatment with PZQ will significantly decrease the prevalence and intensities of schistosome eggs. As a

consequence of the increase of low-intensity schistosome infections, more light infections will be often missed if single samples are examined by standard methods, resulting in a high underestimation of infection. Therefore, there is a need to develop and validate more sensitive diagnostic tools (Tchuenté *et al.*, 2017, Bergquist *et al.*, 2017).

2.5.2 Antigen detection tests

As the burden of schistosomiasis is decreasing, emphasises the importance and essential need for more sensitive methods, apart from the standard diagnostic methods (Bergquist *et al.*, 2017, Stothard *et al.*, 2014, Weerakoon *et al.*, 2018). The detection of circulating adult worm or egg antigens in blood, urine, or sputum is now proven to be highly satisfactory as a diagnostic test (Hawkins *et al.*, 2016, Dawson *et al.*, 2013, Adriko *et al.*, 2014, Knopp *et al.*, 2015, Stothard *et al.*, 2006, Tchuenté *et al.*, 2012). CCA and CAA which are released from the vomitus of worms (Van Dam *et al.*, 1996), are frequently used as target after around 3 weeks post as well as to evaluate the treatment response to the active worm burdens (Stothard, 2009, Van Lieshout *et al.*, 2000, Dawson *et al.*, 2013).

The sensitivity of both CAA in serum and CCA in urine samples are not generally different, enhancing their use in low-high transmission setting (De Jonge *et al.*, 1990, Polman *et al.*, 1995, Stothard, 2009, Danso-Appiah *et al.*, 2016). The CCA test has some drawback in diagnosing *S. haematobium* infection but has better sensitivity for detecting *S. mansoni* infections than single and triplicate KK thick smear (Stothard *et al.*, 2006, Ayele *et al.*, 2008, Knopp *et al.*, 2015, Koukounari *et al.*, 2013).

Although the test is readily applicable in the field and stable at average room temperatures, its use in national control programs is often restricted due to its current price which is around US 1.75 dollars (Stothard, 2009). The advantage of this test is a convenient and efficient method for screening and mapping schistosomiasis in poor resource setting with medium to high transmission (Coulibaly *et al.*, 2013, Colley *et al.*, 2013, Stothard *et al.*, 2011). However, collecting urine samples on different days may be required for more accurate diagnosis because of daily fluctuation of levels of CCA in the urine. Also, it has been shown that the CCA positivity is strongly associated with the intensity of infection (Tchuenté *et al.*, 2012, Stothard, 2009, Stothard *et al.*, 2006, Kittur *et al.*, 2016).

2.5.3 Antibody detection tests

ELISA is a simple, sensitive, and RDTs, which has been applied for detection of the SEA from a small amount of human blood (Smith *et al.*, 2012, Stothard *et al.*, 2009). The SmSEA in the ELISA format has been shown to hold great promise for early diagnosis of schistosomiasis in a higher-risk setting (Hinz *et al.*, 2017, Stothard *et al.*, 2009). This test is commonly used in travellers' medicine clinics where often only light infection can be detected. Currently, a commercially-produced SmSEA-ELISA kit is available which is considered to be suitable for field use (Stothard *et al.*, 2009, Stothard *et al.*, 2014). Indeed, using crude and purified egg antigens of *S. mansoni* in ELISA may result in an overestimation of areas of high transmission since it does not discriminate between active and past infections and because false positives are present due to cross-reactivity with other parasites as hookworms (Correa-Oliveira *et al.*, 1988, Stothard *et al.*, 2009, Stothard *et al.*, 2014).

Using a finger-prick blood sample, the test performed well for the diagnosis of both *S. mansoni* and *S. haematobium* infections in pre-school children (Stothard *et al.*, 2009). The test was at least as sensitive as duplicate KK and a single urine filtration for detection of *S. mansoni* and *S. haematobium*, respectively (Coulibaly *et al.*, 2013). The sensitivity of the test was however decreased in children aged under three years (Dawson *et al.*, 2013). In a comparing study for SmSEA-ELIZA, alkaline phosphatase assay (APIA), circumoral precipitin test (COPT) and KK, suggested that, for field work, the whole targeting population can be diagnosed by SmSEA-ELIZA or APIA. Then, KK and COPT would be suitable tests for confirmation of the positive cases only as detected earlier (Noya *et al.*, 2002).

2.5.4 DNA-based diagnostics for schistosomiasis

To overcome some of the drawbacks of other diagnostic procedures, remarkable progress has been made in upgrading the diagnostic accuracy for schistosomiasis by using the PCR technique (Weerakoon *et al.*, 2018). The conventional PCR methods for the detection of Schistosoma DNA in human samples have been published in different studies (Pontes *et al.*, 2002, Pontes *et al.*, 2003, Sandoval *et al.*, 2006, Weerakoon *et al.*, 2018, Gobert *et al.*, 2005, Gomes *et al.*, 2006). In the beginning, DNA isolation from faecal specimens was hindered by time-consuming procedures and the presence of substances that inhibit the PCR products, but there is nowadays newly, and even faster methods have been developed for DNA isolation which has significantly decrease these difficulties (Verweij *et al.*, 2004). However, real-time chemistries (rtPCR) has advantages over traditional PCR methods: notably, simultaneously amplifies and

visualising the quantities of amplified DNA products, reliably identifying individuals with light-intensity infections, less labour-intensive (Weerakoon *et al.*, 2018, Ten Hove *et al.*, 2008). This technique has been applied for the detection of the *Schistosoma* infections in humans, moreover, in determining the intensity of infection as determined by the cycle threshold (C_t) value (Verweij and Stensvold, 2014, Gomes *et al.*, 2006, Pillay *et al.*, 2014). This rtPCR technique has also been improved for detecting multiple DNA targets in a single reaction mixture, which has been evaluated successfully for case detection, and for discriminating between *S. japonicum*, *S. mansoni*, and *S. haematobium*, and as an critical approaches for detecting and monitoring schistosomiasis control programs (Verweij and Stensvold, 2014, Gobert *et al.*, 2005, Ten Hove *et al.*, 2008). At present, a new, high sensitive PCR approach that allows the genus- and species-specific amplification of the main 5 Schistosoma species (*S. japonicum*, *S. mansoni*, *S. haematobium*, *S. intercalatum*, and *S. bovis*) has been developed based on ribosomal DNA (rDNA) that generated very sensitive and specific amplification (Sandoval *et al.*, 2006). Indeed, PCR identification of *S. mansoni* has documented to be a superior tool in diagnostic accuracy compared to the KK and urine CCA tests in high-endemic setting (Lodh *et al.*, 2013, Melchers *et al.*, 2014, Obeng *et al.*, 2008). Although a more desirable rtPCR assay for detecting both *S. mansoni* and *S. haematobium*, combined with internal control is required for control schistosomiasis on criteria for determining the thresholds between high, and low to moderate transmission settings, this real-time PCR only used for detection *S. mansoni* DNA which will be described in **chapter 2**.

2.6 Control and prevention

Currently, PZQ is the only available PC recommended by the WHO distributed for the treatment of both urogenital and intestinal schistosomiasis (Knopp *et al.*, 2016, French *et al.*, 2018). Insufficient funding in developing countries such as Africa has led to inadequate access to many untreated individuals (Hotez and Pecoul, 2010). In 2002, the SCI highlighted the requirement of an MDA with PZQ (Fenwick *et al.*, 2009, Fenwick and Jourdan, 2016). Fortunately, in 2007 Merck KGaA agreed to supply 200 million tablets of PZQ as part of a 10- year donation via the World Health Organisation (WHO). Later in 2012, the pharmaceutical sector has made committed for a donation of up to 250 million tablets annually, until schistosomiasis is eliminated (Tchuenté *et al.*, 2017). Although the MDA programme is receiving continued financial support, some people who are at high risk of disease in remote regions of Africa are still not being treated (Stothard *et al.*, 2013a). It has been advised to take the tablet with food and water as clinically significant food-drug interactions increase bioavailability (Castro *et al.*, 2000).

The mechanism of action of PZQ involves disrupting calcium ion (Ca2+) channels which are the only moiety so far identified as the molecular target, but the evidence is indirect (Doenhoff *et al.*, 2008). PZQ does not affect immature schistosomes, and the death rate of adult worms can be difficult to measure because of their intravascular sites. Therefore the therapeutic response of PZQ is currently assessed using egg clearance 3 to 4 weeks after treatment (Doenhoff *et al.*, 2008, Sousa-Figueiredo *et al.*, 2012). The main side effects of the drug are stomach discomfort with or without nausea, and headaches and it is recommended to use during pregnancy. These side effects are commonly mild and transient, which are not usually required medical attention (Utzinger *et al.*, 2003). A single right dose of PZQ of 40 mg/kg, is recommended directly by the WHO for schistosomiasis control (Stothard *et al.*, 2013a). SAC target the most vulnerable children during their growth as they are at the risk of intellectual impairment and stunting (Mafe *et al.*, 2005). A dose-pole has been developed to help healthcare professionals when treating children in the target settings (**Figure 5**) (Montresor *et al.*, 2005).

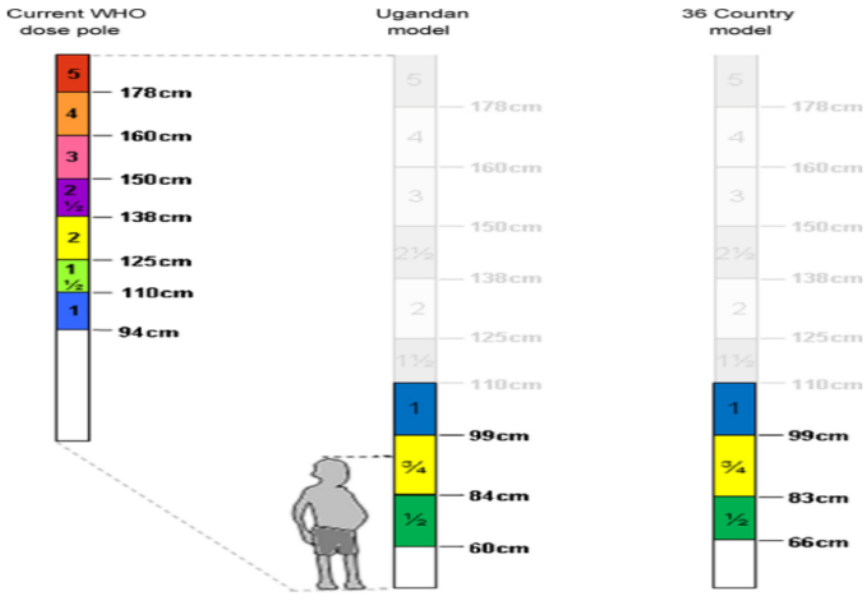


Figure 5: Pictorial representation of the current WHO dose-pole which corresponds to a number of PZQ tablets (600 mg each) (left) and the new dose poles – Ugandan model (center) and a model for the rest of Africa (right) with new height thresholds added to allow for treatment of PSAC (<6-year old). ¾ of a tablet division illustrated, as an example, for child need rather than a single tablet (Sousa-Figueiredo *et al.*, 2010a)

By using school infrastructure, the deworming school-age children can easily be implemented through MDA programmes in which a single oral dose of albendazole and PZQ periodically administered (WHO, 2013b). The demand for PZQ and national distribution of PZQ based on the baseline prevalence of the selected schools (see **Table 3** for recommended values).

Table 3: Recommended treatment strategy for schistosomiasis in preventative chemotherapy PZQ (WHO, 2009b, Stothard *et al.*, 2014)

Category	Baseline prevalence among SAC	Action to be taken[a]	
High-risk community	⩾ 50% by parasitological methods[b] (intestinal and urogenital schistosomiasis) or ⩾ 30% by questionnaire for history of haematuria	Treat all SAC (enrolled and not enrolled) once a year	Also treat adults considered to be at risk (from special groups[c] to entire communities living in endemic areas)
Moderate-risk community	⩾ 10% but <50% by parasitological methods (intestinal and urogenital schistosomiasis) or <30% by questionnaire for history of haematuria	Treat all SAC (enrolled and not enrolled) once every 2 years	Also treat adults considered to be at risk (special groups[c] only)
Low-risk community	<10% by parasitological methods (intestinal and urogenital schistosomiasis)	Treat all SAC (enrolled and not enrolled) twice during their primary schooling age (e.g. once on entry and once on exit)	Praziquantel should be available in dispensaries and clinics for treatment of suspected cases

[a] Equivalent to: high-risk community – all SAC and adults require preventive chemotherapy annually; moderate-risk community – 50% of SAC and 20% of adults require preventive chemotherapy annually; low-risk community – 33% of SAC require preventive chemotherapy annually.
[b] For urogenital schistosomiasis, detection of haematuria by chemical reagent strips gives results equivalent to those determined by urine filtration.
[c] Special groups: pregnant and lactating women, groups with occupations involving contact with infected water such as fishermen, farmers, irrigation workers or women in their domestic tasks, to entire communities living in endemic areas.

Snail-control is a method that has been rarely incorporated into epidemiological studies and control strategies since the comprehensive implementation of MDA. However, the intermediate host of the parasite represents not only fundamental requirement for the development of the infective stage of the parasite but also the site of exponential multiplication of cercaria individuals from one miracidium (Madsen, 1992). This enhances the importance of establishing control with targeting both the parasite and the vector to prevent transmission. The different effective integrated control strategies of the intermediate host of schistosomiasis are summarised in **Table 4**.

Table 4: Summarizing most commonly snail control strategies used in countries like Japan, Iran, Lebanon, Saudi Arabia (Sokolow *et al.*, 2016, Madsen, 1992, Rollinson *et al.*, 2013, Coelho and Caldeira, 2016, Pointier and Jourdane, 2000)

Control strategy	Pro	Against	Future work
Molluscicides	Most commonly used. - Widely used in successful control or eradication in countries like Japan, Iran, Lebanon, Saudi Arabia.	Expensive-Does not prevent recolonization - Toxic for ecosystem	Natural and eco-friendly molluscicides are being developed
Natural enemies	Molluscivorous fishes, crustacean, birds Non-competent snails	Non-target effect. - Self sustainability of species.	
Habitat modification	Reduces snail breeding spaces. - Successfully used in Japan and Saudi Arabia	Expensive	
Genetic modification	Molecular tools for detection of latent schistosomiasis infection in the snail host. - Resistance gene in snail hosts.	Better understanding of the genetic structure or snails is needed	
Others	Repellent molluscicides - Topical DEET - Bait traps	Not really used.	

2.7 Schistosomiasis in Uganda and Saudi Arabia

Schistosomiasis has been of substantial public health importance in many countries of sub-Saharan Africa for long-times, particularly in Uganda. In 2002, an NCP was established for delivering PZQ once every two years to all SAC (aged 5-15 years) (Kabatereine *et al.*, 2007). Although both species of Schistosoma are found in Uganda, *S. mansoni* is the most prevalent, and approximately 20 million people within 73 Ugandan districts are estimated to be at risk of infection, with the shorelines of the Lakes Albert and Victoria and nearby communities being the highest transmission zones (**Figure 6**) (WHO, 2013b). Due to environmental and malacological reasons, disease transmission is thought unlikely in areas of high altitude, mountainous regions, hot and cold places where the conditions are not suitable for snail habitats to develop (Kabatereine *et al.*, 2004, Stanton *et al.*, 2017). These Lakes are a fundamental part of Ugandan life, where water-contact activities are occurred in the regular daily base such as laundry, bathing children play in the freshwater (Loewenberg, 2014, French *et al.*, 2018). Many fishing communities alongside the lakes may also expose to this parasitic infection daily (Fuhrimann *et al.*, 2016, Dunne *et al.*, 2006). Until the clean water is available and basic sanitation is developed, the goal of controlling morbidity to be reached by 2020, along with elimination as a public health problem is an unrealistic goal (Tchuenté *et al.*, 2017, French *et al.*, 2018).

In Saudi Arabia, schistosomiasis is one of the significant health problems, especially in the province of Asir (**Figure 6**) (Al-Madani, 1991, Shati, 2009, Al-Zanbagi, 2013, Lotfy and Alsaqabi, 2010). In 2004, a research study showed that Saudian people accounted for almost 61.2 % of total infected cases reported, and infection is most frequently detected in the age group of 15–39 years, providing evidence about the endogenous source of infection (El-Shahawy *et al.*, 2016). Infection rates by gender were four times higher than females (Barakat *et al.*, 2014). Overall, 75 % of total infection was *S. mansoni*, and the prevalence was more common in most of the southwestern region with a focus in Hail in the North. *Schistosoma haematobium* was restricted to a few foci in the southwestern region in Jazan and Asir (**Figure 6**), and both provinces are bordering Yemen, where the spread of schistosomiasis is still high (Barakat *et al.*, 2014, El-Shahawy *et al.*, 2016, Lotfy and Alsaqabi, 2010). Ministry of Health statistical data in 2008 confirmed that Saudis are more common to be infected than immigrant populations; the percentage of infection was 55.5 % and 45.5 % for Saudis and emigrant populations, respectively (Barakat *et al.*, 2014).

In view of this, it is possible that the actual prevalence of schistosomiasis in the provinces may be underestimated than those shown in the Annual Health Reports, especially in Asir region where schistosomiasis control has apparently little development. It is, therefore, important to address different factors which make the application of schistosomiasis control measures ineffective and to implement new diagnostic tools for better estimation of the prevalence in Asir Province and others (Al-Madani, 1991, Stothard *et al.*, 2014).

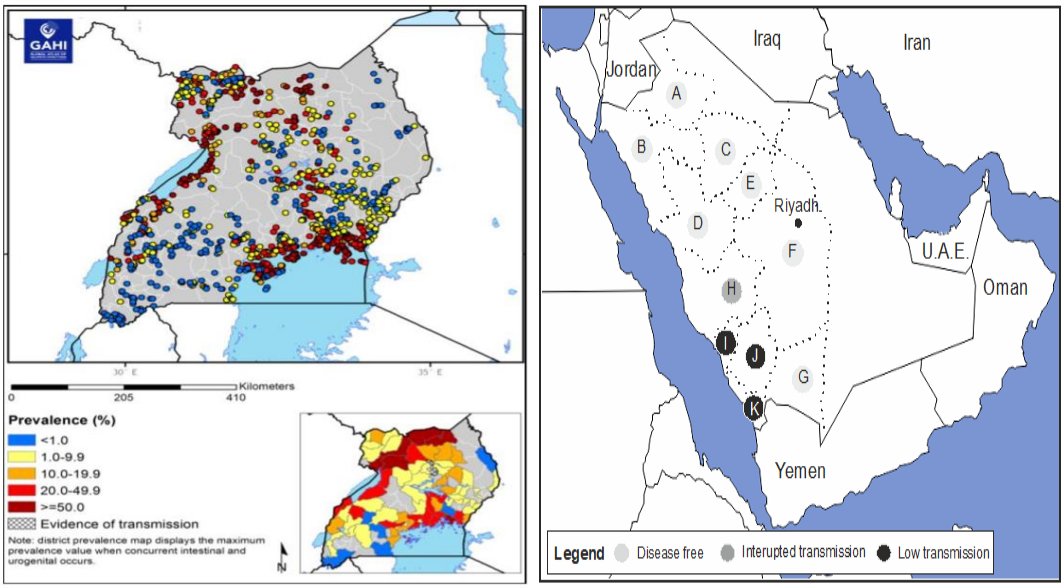


Figure 6: An outline map for schistosomiasis transmission in Uganda and Saudi Arabia. On the left-hand side, the prevalence and location of *S. mansoni* parasitological surveys and average district level prevalence in Uganda as illustrated by Global Atlas (Distribution of schistosomiasis survey data in Uganda, online access (http://www.thiswormyworld.org/maps/distribution-of-schistosomiasis-survey-data-in-uganda). An outline map of the KSA with capital Riyadh depicted (on the right-hand side). Major administrative boundaries are shown, and the associated status of **s**chistosomiasis therein is indicated by shaded circles. (**A**) Al-Jouf; (**B**) Tabuk; (**C**) Hail; (**D**) Al-Madena and Al Monawarah; (**E**) Al Qasim; (**F**) Riyadh; (**G**) Najran; (**H**) Tardif, Makkah, Jeddah, Al Qunfudhah and Alith; (**I**) Bishah and Asir (**J**); Jazan (**K**) (Stothard *et al.*, 2014)

3.1 Giardiasis

Giardiasis, is a common gastrointestinal parasite of mammals, including humans characterised by acute or chronic diarrhoea, caused by protozoan parasites in the genus *Giardia* (Harhay *et al.*, 2010). This protozoan was initially named *Cercomonas intestinalis* by Lambl in 1859 (Kofoid and Christiansen, 1915). *Giardia duodenalis* (syn. *Giardia lamblia*) is a flagellated parasitic micro-organism, is a dominant parasitic species detected worldwide, and the only species commonly causing disease in humans (Thompson, 2000). The morphological features of this parasite exist in two forms: the dormant, infective cysts and the vegetative, disease-causing trophozoites. Cysts are egg-

shaped and approximately 8-12 µm long and 5-9 µm wide. They are enclosed by a 0.3-0.6 µm thick cyst wall, that has a fibrillose structure (**Figure 7**). It is carried in the intestinal tract of human, and many different animals with experience clinical signs shortly after an incubation period of 1 to 14 days (average of 7 days) and usually lasts 1 to 3 weeks of acquiring infection in some individuals, but many others remaining asymptomatic for a remarkably long period (Stark *et al.*, 2007).

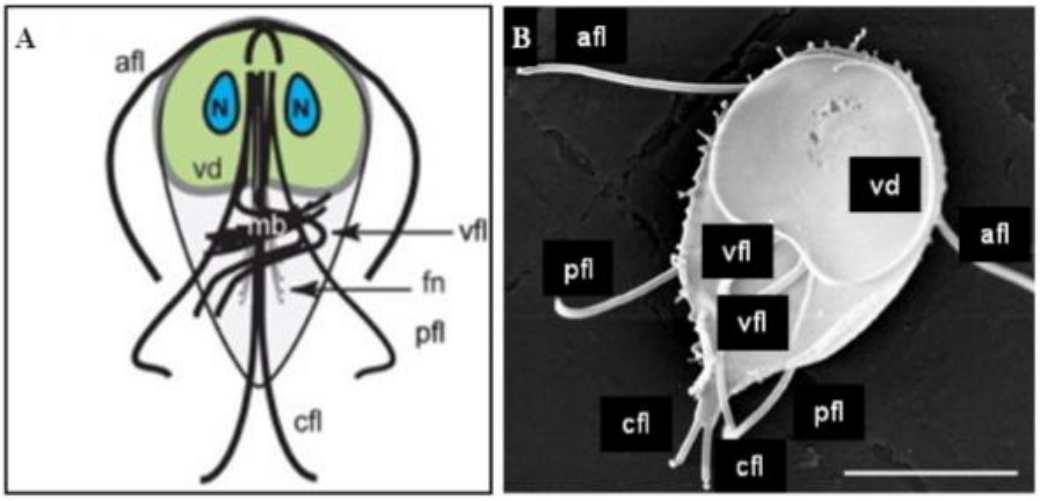


Figure 7: Morphology of *Giardia* trophozoites (**A**) Schematic and (**B**) Scanning electron micrograph (SEM) of *Giardia* trophozoite structure. A ventral view of a *Giardia* trophozoite shows its teardrop shape with nucleus (N), median body (MB), funis (fn), ventral disc (vd) and four flagellar pairs (afl = anterior flagella, pfl = posterior-lateral flagella, vfl = ventral flagella, cfl = caudal flagella). Scale bar = 5 µm (Dawson and House, 2010)

In addition to fatty to watery diarrhoea, which is a hallmark of acute and chronic giardiasis in children, the presence of *G. duodenalis* can result into malabsorption; some studies have shown that some infected children are at higher risk of impaired growth with a decrease in cognitive function in endemic areas, and possibly reduced productivity in adulthood (Fletcher *et al.*, 2012, Escobedo *et al.*, 2014). Occasional outbreaks are documented in people, as the result of mass exposure to contaminated water or food, or direct contact with infected individuals (Robertson *et al.*, 2010). Humans are considered to be the most important reservoir hosts for human giardiasis, but the parasite also infects domestic and wildlife animals (Appelbee *et al.*, 2005, Day, 2007). The genetic characterisation of *G. duodenalis* isolates has revealed the different species and subspecies in humans and domesticated animals (livestock and pets), and infection transmitted from animals to humans is currently thought to be of relatively primary importance in the natural spread of human giardiasis (Esch and Petersen, 2013, Thompson, 2000). Nevertheless, *G. duodenalis* can

be considered as a species complex, and there is evidence that its assemblages represent distinct species-specific isolates (Minetti *et al.*, 2015), and some genetic types of *G. duodenalis* belonging to assemblages A and B isolates should be considered potentially zoonotic (Sprong *et al.*, 2009).

3.2 Lifecycle

Transmission and lifecycle *Giardia* has a two-stage with cysts and trophozoites (**Figure 8**). The infection is acquired through the ingestion of mature cysts, which are excreted in the faeces (Olson *et al.*, 1999, Adam, 2001). Ingestion of as few as 10-25 cysts may be sufficient to occur an infection in some individuals (Smith, 1993), while several species of livestock (sheep, cattle, goat) may get infected by few cysts in surface water sources. However, an infected person might execrate up to 900 million cysts per day and can be transmitted directly between hosts, or on various fomites such as food or specific water sources (e.g., untreated municipal water, backwoods streams, and lakes) contaminated with human or animal faeces. Stable cysts in the environment are evacuated into the faeces, usually can survive for up to 77 days in tap water at 8 ° C, but this decreases with increasing temperature, e.g. 54 days at 21° C and four days at 37 °C (Cole *et al.*, 1989).

Once arrived and differentiated in the lumen of the duodenum, some of the trophozoites begin to reproduce. The parasite generation time is 6-12 hours in vitro (Ankarklev *et al.*, 2010). Both diploid nuclei divide into four diploid nuclei and are multiplies in the intestinal tract by longitudinal binary fission (Yu *et al.*, 2002). Earlier, the two nuclei of one trophozoite were suggested to be equal in length and the quantity of DNA (Yu *et al.*, 2002, Kabnick and Peattie, 1990) being organised on five major chromosomes (Adam, 2001). However, studies highlighted differences in chromosome size and number in both nuclei (Tůmová *et al.*, 2007).

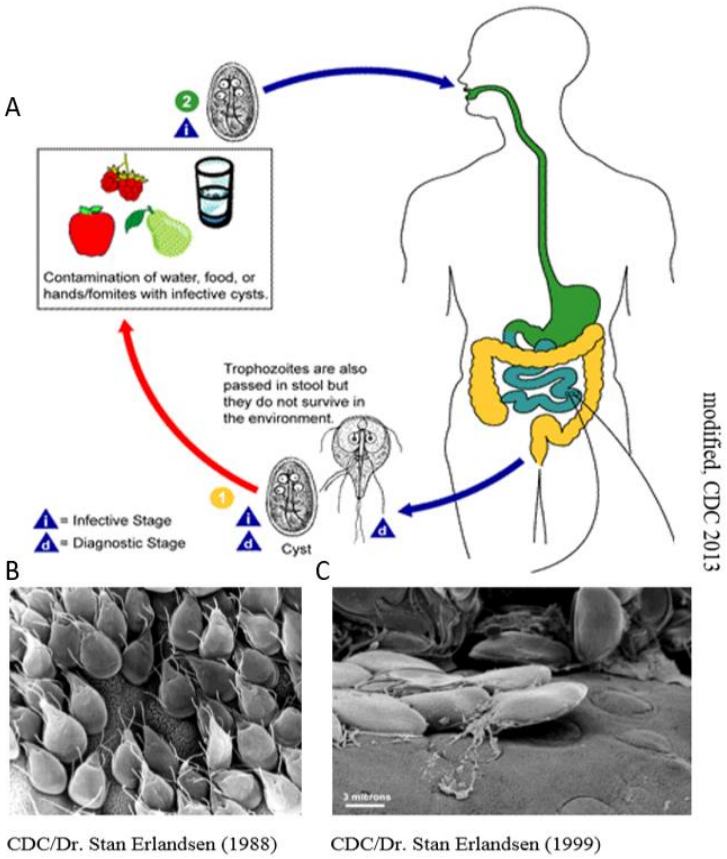


Figure 8: Lifecycle of *G. duodenalis.* (**A**) shows a schematic an overview of the life stages of *G. duodenalis*. (**B**) and (**C**) shows in SEM, colonisation of the host intestinal epithelium by *Giardia* trophozoites after infection. In (C) circular lesions due to securely attachment of the parasite's ventral adhesive disk with the intestinal epithelial surface (Dawson and House, 2010)

3.3 Epidemiology

G. duodenalis occurs worldwide and is particularly common in warm climates (Adam, 2001). The main route of *G. duodenalis* infection is via the faecal-oral route and can occur through direct exposure to and ingestion of infectious cysts. Other means of transmission include direct contact with infected person (anthroponotic transmission) or animals (zoonotic transmission), or by ingestion of contaminated food (foodborne transmission) or water (waterborne transmission) (Cacciò and Ryan, 2008, Yoder *et al.*, 2010). Risk factors for acquiring clinical infection include young age, using un-boiled water, and exposed to pathogens from poorly managed animal faeces (Cacciò and Pozio, 2001, Mohammed Mahdy *et al.*, 2008). *Giardia* infection contributes substantially to the 2.5 million deaths that occur annually as the result of diarrhoeal diseases; the worst affected are those inhabiting developing countries (Bello *et al.*, 2011, Upcroft and Upcroft, 2001).

Giardia is considered the most frequent cause of diarrhoea for animals and humans (Farthing, 1997), making giardiasis one of the most common parasitic human diseases, and approximately 280 million people suffering from symptomatic giardia infection globally (Coelho *et al.*, 2017, Cacciò *et al.*, 2005). The estimated prevalence is 2 to 5% in developed countries and reaching 40% in developing countries (Upcroft, 2001, Nkrumah and Nguah, 2011). In developed countries, infections with Giardia are most common and is regarded as a re-emerging disease (Thompson, 2008, Thompson, 2000). Current statistics probably underestimate the actual incidence of giardiasis because the reporting for a given disease varies between different surveillance systems and is only reportable in certain countries (David *et al.*, 2011). There is currently a paucity of epidemiological data concerning the prevalence, incidence and economic cost of giardiasis in African countries. Although Some small studies (**Table 5**) highlighted the prevalence among different age groups (predominantly children) in individual communities in Uganda, with considering there is no national surveillance system for this parasite in most African countries. Thus, there is a need for epidemiological data on giardiasis in many African countries due to economic costs and the adverse effects on livestock such as wasting and malnutrition and lost productivity.

Table 5: Summary of current epidemiological *G. duodenalis* studies in Uganda which include human infections. Previous epidemiological studies on *Giardia* have indicated cross-species transmission between primates, gorillas, cattle and humans living nearby or using similar water sources (Graczyk *et al.*, 2002, Johnston *et al.*, 2010, Ankarklev *et al.*, 2012, McElligott *et al.*, 2013)

Location	Age group	Prevalence	Assemblage A	Assemblage B	Comments	Reference
Bwindi Impenetrable National Park, southern Uganda	Adults	5% (0.42%-10.42%)	100% (3/3)	0% (0/3)	Cross-sectional survey of humans, gorillas and cattle	Graczyk *et al., 2002*
Kibale National Park, western Uganda	All ages	40.7% (31.9-50.2)	NA	NA	Cross-sectional survey of humans, livestock and primates	Johnston *et al., 2010*
Urban Kampala, southern Uganda	Infants and children	20.1% (16.3%-23.9%)	14.70%	82.30%	Cross-sectional survey of asymptomatic children	Ankarklev *et al., 2012*
Lake Victoria region, southern Uganda	All ages	12% (10–14%)	NA	NA	Cross-sectional survey of Humans.	McElligott *et al., 2013*

Estimating of the economic impact of giardiasis could not be found for Africa. However, the economic impact in the United States of America (USA) has been estimated at $282 million annually (Scharff, 2012, Cork, 2011). In contrast to USA agribusiness, African countries is a largely self-sufficiency farming system for subsistence of individuals and/or groups (Verheye, 2000). In Uganda, as an example, where human-livestock-wildlife interaction is high due to habitat disturbance, the occurrence and spread of giardiasis in the animals and human

population would lead to the cross-species transmission which can make control programs more complex (**Figure 9**). It could also lead to malnutrition in both animals and humans exacerbating the problem of malnutrition in humans further (Johnston *et al.*, 2010, Hunter and Thompson, 2005, Goldberg *et al.*, 2008).

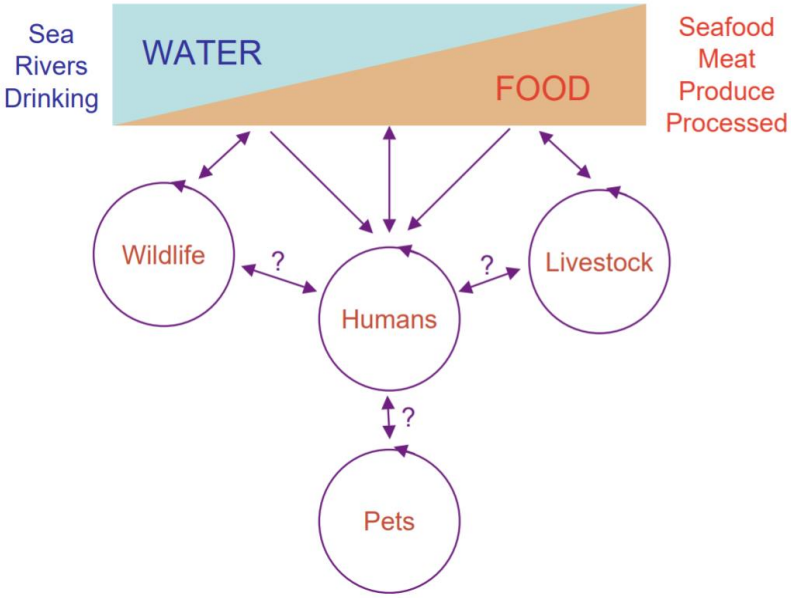


Figure 9: Diagram showing essential cycles of transmission for maintaining *Giardia***.** As well as direct transmission, water and food may also play a critical role in the transmission of the parasites. Question marks demonstrate the unclear frequent interaction between different hosts (Hunter and Thompson, 2005)

3.4 Molecular characterisation of *Giardia*

Isolates of *G. duodenalis* are morphologically indistinguishable but can differ genetically infecting humans and other mammals (Thompson, 2000, Monis *et al.*, 2003). The nucleotide sequence analysis of several housekeeping genes (e.g. *β*-giardin, GDH, TPI and the small subunit rRNA gene for phylogenetic investigation of *Giardia* assemblages, revealed the existence of eight different assemblages, referred to as assemblage A to H (Monis *et al.*, 2003, Cacciò and Ryan, 2008, Lasek-Nesselquist *et al.*, 2010), further divided into sub-assemblages AI, AII, AIII, BIII and BIV(Amar *et al.*, 2002, Cacciò *et al.*, 2002). Each host species can be infected with specific assemblage (**Table 6**), but among these assemblages, only A and B are commonly detected in humans (Vanni *et al.*, 2012). Globally, assemblage B is more frequently found than assemblage A in humans, excluding specific regions of South America and Mexico (Cacciò and Ryan, 2008, Vanni *et al.*, 2012). However, a mixture of co-infecting parasite genotypes within a host have been observed from time to time, e.g. due to ingestion of genetically distinct strains of *Giardia* cysts or re-infection with different *Giardia*

species carrying another assemblage (Sprong *et al.*, 2009, Geurden *et al.*, 2009, Levecke *et al.*, 2009).

An additional interpretation for this phenomenon could be as a result of allelic and genetic heterogeneity and/or genetic recombination that was documented to be a more frequent occurrence in assemblage B then A (Jerlström-Hultqvist *et al.*, 2010a).

Table 6: Molecular characterisation of *G. duodenalis* isolates and the association of vertebrate host species structure genotype with a transmission cycle

Assemblage	Some species commonly infected
AI	humans and animals (cats, dogs, livestock, deer, muskrats, beavers, voles, guinea pigs, ferrets)
AII	humans (more common than A-I)
AIII, AIV	exclusively animals
B	humans and animals (livestock, chinchillas, beavers, marmosets, rodents)
C, D	dogs, coyotes
E	alpacas, cattle, goats, pigs, sheep
F	Cats
G	Rodents
H	Seals

(Modified **CDC, 2013**)

However, the possible source of zoonotic *Giardia* infections and the association between infected animals such as beavers and waterborne outbreaks in people has led to classifying *Giardia* as a zoonosis (WHO, 1979). So far, genetic analysis of three distinct human isolates of *G. duodenalis* have been fully sequenced in detail and are available at http://giardiadb.org/giardiadb/.

In 2007, the 11.7 Mbp WB-assemblage A *G. duodenalis* genome expressed as approximately 4,800 predicted encoding genes was published (Morrison *et al.*, 2007), followed in 2009 with those of GS-assemblage B (Franzen *et al.*, 2009). The sequenced genomes of human assemblage A isolate WB and assemblage B isolate GS showed 77% nucleotide and 78% amino-acid identity in 4,300 orthologous proteins. Due to these genetic differences, both genotypes were considered as two different species (Franzen *et al.*, 2009, Monis *et al.*, 2009), and analysis of P15-assemblage E *G. duodenalis* genome isolated from a pig in 2010 was strengthened this assumption (Jerlström-Hultqvist *et al.*, 2010b).

The molecular analysis of these three isolates identified a highly conserved set of core genes (4,557 genes, 91% of the genome) common to all isolates. Genomic differences were detected in multigene families, for example, VSPs. The

average amino acid identity between the different isolates is 78% (A–B), 81% (E–B), and surprisingly, 90% (A–E) revealing a close relationship between species (Jerlström-Hultqvist *et al.*, 2010b, Franzen *et al.*, 2009). These differences may lead to changing in the parasite survivability and pathogenicity inside the intestinal lumen (Nash, 2002). Therefore, research into the hypothesised differing pathogenicity of assemblages is critical for the further understanding of the variability in symptoms observed for management, intervention and control of giardiasis cases. Nevertheless, the roles of various animals in the transmission of human giardiasis established in different research studies remain subjects of current research.

3.5 Clinical symptoms and pathology

Giardiasis causes a broad spectrum of gastrointestinal symptoms. In its acute phase occurring approximately 1-3 weeks after infection (Gardner and Hill, 2001), an infected person can suffer from greasy or fatty stools, abdominal pain (stomach ache), nausea or vomiting and diarrhoea. Chronic (or recurrent) infections can lead to dehydration, malnutrition, fatigue, weight loss, and impaired bowel function (e.g. inability to absorb vitamin B12) (Olivares *et al.*, 2002). It was reported that 98% of the drug-cured children in a hyperendemic area with *G. duodenalis* became re-infected within six months (Gilman *et al.*, 1988). This is dramatic because giardiasis negatively affects childhood mental and physical development of infected children (Berkman *et al.*, 2002). Furthermore, giardia infections are suggested to be associated with arthritis, allergies, chronic fatigue and irritable bowel syndrome (Gaston and Lillicrap, 2003, Hanevik, 2012, Bartelt and Sartor, 2015). In many cases, the parasite can colonise within the small intestine without any clinical symptoms (asymptomatic passage). The factors influencing the clinical outcome (symptomatic *vs* asymptomatic) are unclear but could include infective dose, differences in immune responses, virulence factors that help to cause disease, and evade immune systems (**Figure 10**)(Farthing, 1997, Buret, 2007, Bartelt and Sartor, 2015).

Thus, the virulence factors probably play a role in pathogenicity, and maybe assemblage and/or sub-assemblage specific, depending on their complement of virulence factors.

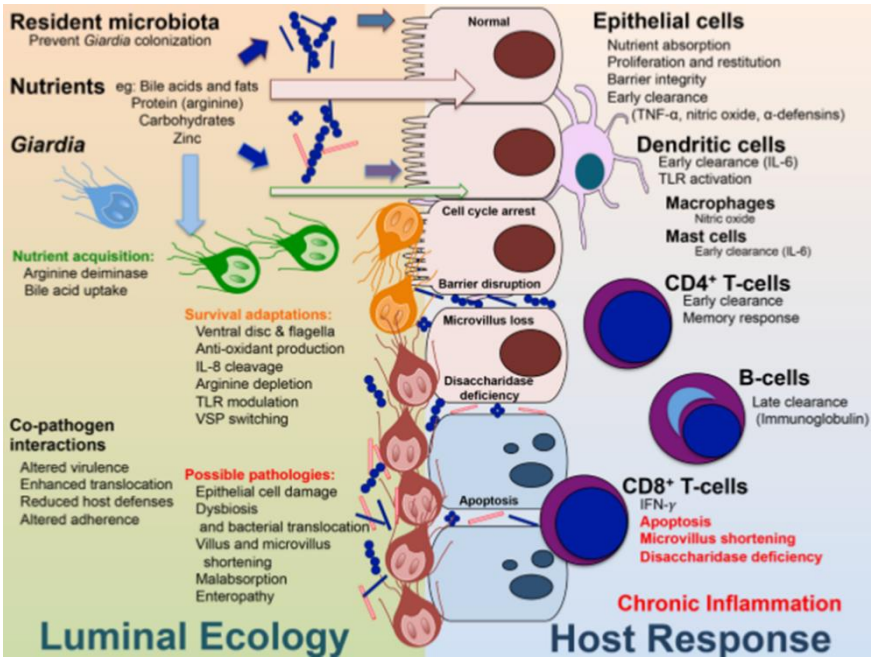


Figure 10: The proposed determinants and mechanisms of *G. duodenalis* pathology within the intestinal, adapted from (Bartelt and Sartor, 2015)

3.6 Diagnostics

G. duodenalis is usually diagnosed by microscopic examination of stool samples for the presence of cysts and/or trophozoites. The excretion of the parasite can be highly variable among individual, and thus an analysis of multiple stool specimens is highly recommended to increase the sensitivity (McHardy *et al.*, 2014). Other suggested techniques for the detection of *G. duodenalis* are concentration techniques using freshly preserved stool samples for detection of vegetative stages (Mank *et al.*, 1995a).

The alternatives for microscopic diagnosis of *G. duodenalis* include detection of *Giardia* antigen by DFA technique, and the ELISA test used to detect specific antigens in stool samples (Maraha and Buiting, 2000, Garcia and Shimizu, 1997, Garcia and Garcia, 2006, Hanson and Cartwright, 2001, Johnston *et al.*, 2003, Mank *et al.*, 1997). Compared to microscopy, immunoassay tests have enhanced the sensitivity of detection particularly for stool samples containing low numbers of cysts with a reduced number of samples required for examination (Mank *et al.*, 1997) but still, multiple stool samples are needed to confirm negative results (Hanson and Cartwright, 2001). The second diagnostic alternative is *G. duodenalis* DNA detection in faeces by a real-time polymerase chain reaction (RT-PCR), which has shown more sensitivity than detection of cysts by microscopy and immunoassay and also can further reduce the required number of stool samples is needed for analysis (Verweij *et al.*, 2003).

DNA-based approaches have also been extensively covered with different studies for classification of subgroups of *G. duodenalis* based upon various target genes. From humans and animals, *G. duodenalis* strains have been isolated and classified as different genotypes, and certain stains can infect humans that are clustered in assemblage A and B (Monis *et al.*, 1996, Minetti *et al.*, 2015), while several studies have suggested the association between assemblages and differences in clinical symptoms. The findings of these studies are difficult to compare and sometimes are even conflicting. These will be further discussed in **chapters 3** and **4**.

3.7 Treatment

The duration of infection and characteristic symptoms are highly variable. Giardiasis is often self-limiting with a mean duration of six weeks (Taylor and Wenman, 1987). Giardiasis can be treated with some antibiotic therapy, including nitroimidazole derivatives (MTZ or Tinidazole), benzimidazole compounds or acridine dyes (Hahn *et al.*, 2013). MTZ or tinidazole are more often used in humans, but other drugs (e.g., furazolidone or paromomycin) may be recommended in some cases. MTZ has efficacy of 80-95% (Gardner and Hill, 2001). Treatment success depends on the crucial information obtained from the medical history, nutritional and immune status of the infected person such as drug-resistant, reinfection or post-Giardia lactose intolerance (Müller *et al.*, 2008, Solaymani-Mohammadi *et al.*, 2010). Therefore, it is sometimes essential to increase the doses, expand the duration of treatment, or to use an alternative drug or a combination of treatments. For instance, the infected person that have a medical intolerance, or patients that are infected with resistant strains, can be treated with quinacrine, alone or in combination with nitroimidazole (Escobedo and Cimerman, 2007).

Currently, recommendations are made for the preferred treatment in different clinical situations for giardiasis include quinacrine hydrochloride or MTZ for a duration of 5 to 10 days, and can be expected to cure over 90% of individuals (Gardner and Hill, 2001, Nash *et al.*, 2001, Escobedo and Cimerman, 2007). In developing countries long-term drug regimens, complex regimens are problematic, as medicines are frequently purchased in large-quantities which represent less than a single day's dose and effective therapies of short duration are preferable in rural communities (Hossain *et al.*, 1982). In an attempt to assess the efficacy of a shorter duration of treatment in giardiasis, two studies were conducted using single-dose tinidazole versus single-dose MTZ and single-dose tinidazole versus a 3-day course of MTZ, concluding that a single oral dose of tinidazole is highly effective for treating giardiasis and is equal in efficacy to a 3-day therapy with MTZ (Speelman, 1985).

4.1 Malaria

Malaria is a life-threatening disease caused by a protozoan of the genus *Plasmodium* spp. parasites, and commonly associated with poverty and leading to a significant economic impact (Gallup and Sachs, 2001, Worrall *et al.*, 2005). There are more than 100 *Plasmodium* species can infect more than one vertebrate host (e.g., reptiles, amphibians, birds, and humans (Millar and Cox-Singh, 2015). In 1897 Ronald Ross (Scottish physician working in India) explored a developing form of a malaria parasite fed on malarial patients, further development of the malaria parasite in the body of the mosquito and described the complete life cycle of malaria (Cox, 2010). However, there are five species of *Plasmodium* (single-celled parasites) can infect humans and cause illness: *P. falciparum*, *P. malariae*, *P. vivax*, *P. ovale*, and *P. knowlesi*. Comparison of *falciparum* and non-*falciparum*, in particular, *P. ovale* and *P. malariae*, have been documented to be in charge of around 25% of imported malaria infection in Europe but is often neglected due to its associated with a less severe clinical course (Ruas *et al.*, 2017).

Although *P. falciparum* is the most prevalent species and responsible for most malaria-related deaths globally, the use of sensitive molecular detection methods for malaria have improved the detection of higher levels of co-infections of *P. ovale* and *P. malariae* with *P. falciparum* than previously thought (Mueller *et al.*, 2007). Thus, morbidity and mortality associated with non-falciparum species have been underestimated. Additionally, current control methods are focused on the reduction of *P. falciparum* infections. However, this may not equally decrease the prevalence of non-falciparum species (Phillips, 2001). Thus, the accurate determination of the current prevalence and distribution of non-falciparum malaria is important so that the response to control approaches can be monitored and improved. This process will aid malaria control efforts globally and will form an essential part of the future goals towards malaria elimination (Phillips, 2001, Mueller *et al.*, 2007, WHO, 2017b).

4.2 Lifecycle

Malaria is caused by a parasite of the *Plasmodium* genus, which is the most common parasitic infection worldwide. The disease is transmitted by the bite of a female *Anopheles* mosquito, though the mosquito's saliva (**Figure 11**), and congenital transmission, although rare, is also a possible mode of transmission (Natama *et al.*, 2017, Martín-Dávila *et al.*, 2018). During a blood meal, an Anopheles female mosquito infected with malaria injects sporozoites into the human host. These sporozoites infect liver cells where they multiply by cell di-

vision for 10-15 days and form schizonts. At maturity, schizonts lysate and release merozoites. For some species (except *P. falciparum*), the liver infection can become latent and allow the parasite to survive for a long time in the body, and become disappeared from the blood (Soulard *et al.*, 2015). This explains the long-term relapses for two of the species that infect humans: *P. vivax* and *P. ovale*. Currently, five species of the *Plasmodium* genus are known to cause malaria in a human host: *P. falciparum, P. vivax, P. ovale* (now split into subspecies *P. ovale curtisi* and *P. ovale wallikeri*), *P. malariae* and *P. knowlesi* (Fuehrer and Noedl, 2014). A sixth *Plasmodium* parasite, *P. cynomolgi,* has been reported in only one naturally-acquired case of human malaria (Ta *et al.*, 2014).

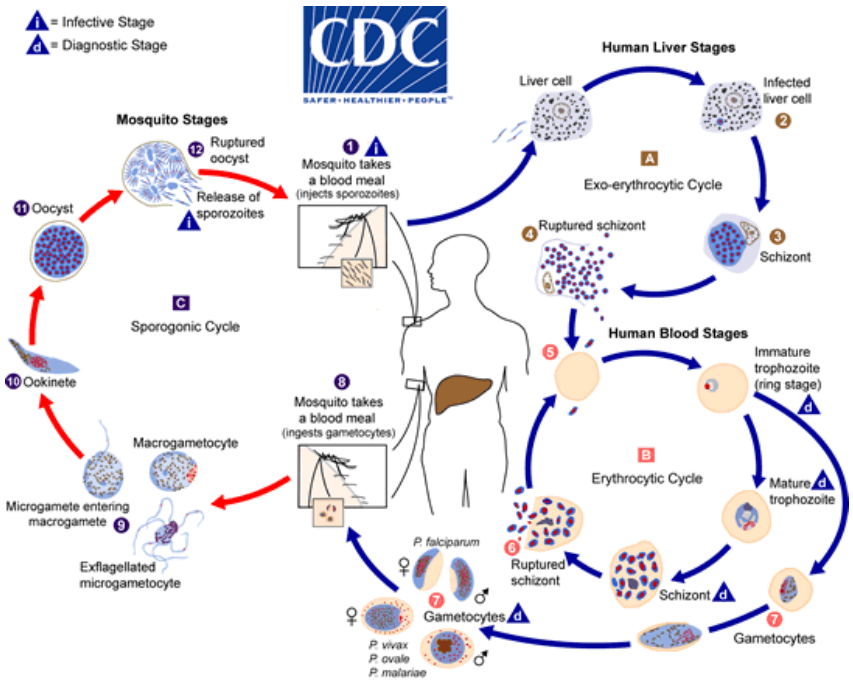


Figure 11: The mosquito and human life cycle of the *Plasmodium* parasite (**CDC. 2011**)

4.3 Epidemiology

In 2016, almost half of the world's population - was at risk of contracting malaria. Most malaria infection and deaths have reported in sub-Saharan Africa. However, the WHO Regions of Southeast Asia, the Americas and the Eastern Mediterranean are also affected (**Figure 12**). In 2016, 91 countries were experiencing continued malaria transmission (WHO, 2016b). Some groups in the population are at much higher risk than others of contracting malaria and being seriously ill: infants, children under 5, pregnant women, people living with HIV or AIDS, non-immune migrants, shifting populations and travellers. According to the latest World Malaria Report, published in November 2017, there were 216 million infections of malaria in 2016, up from 211 million

in 2015. An estimated 445, 000 deaths from malaria in 2016, a figure similar to that of the previous year (446,000) (WHO, 2016b). The WHO Region of Africa bears a disproportionate share of the global burden of malaria. In 2016, 90% of malaria cases and 91% of deaths from this disease occurred in this region. Some 80% of the malaria burden was in some 15 countries - all in sub-Saharan Africa except India (Christiansen-Jucht *et al.*, 2014).

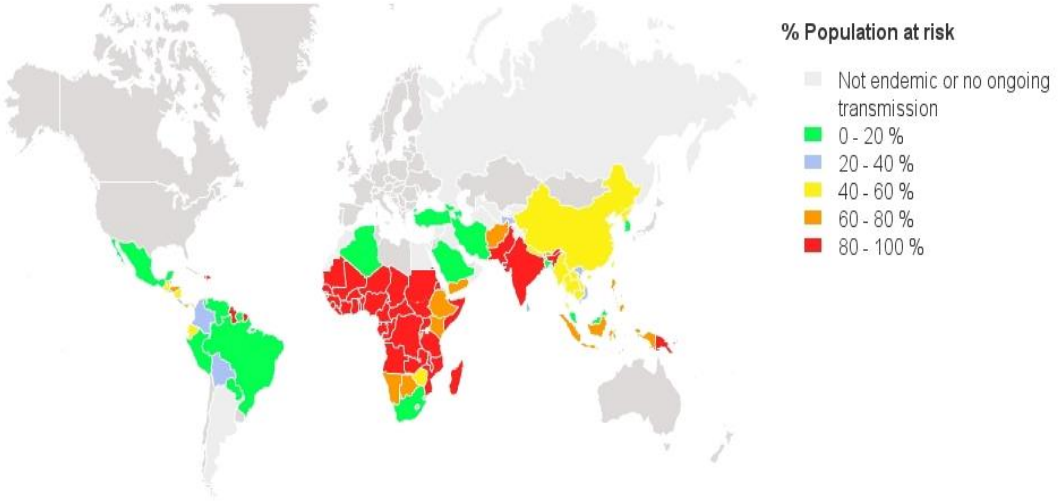


Figure 12: The percentage of the population at risk of malaria based on the 2016 World Malaria Report (WHO, 2016b)

In areas of intense malaria transmission, children under 5 years of age are especially at risk of infection, illness and death; more than two-thirds (70%) of deaths from malaria occur in this age group, while in areas of lower transmission, many cases occur in older children and adults (Nmadu *et al.*, 2015, Roberts and Matthews, 2016, Carneiro *et al.*, 2010). However, malaria remains a major killer of children under five, and a child dies every two minutes (Bhatt *et al.*, 2015). Despite reports that malaria-related hospital admissions have declined in several African regions, Uganda was one of four countries in East Africa that had an increased number of malaria admissions from 2000-2010 (Okiro *et al.*, 2011). It has one of the highest prevalence rates of malaria in the whole of Africa (**Figure 13**). The Ugandan National Malaria Indicator Survey 2014-15 estimated that the malaria prevalence of local children ranges from 6–59 months of age was 29.9% by using RDTs ((UBOS), 2015). By blood examination, the estimated prevalence of *Plasmodium* spp. was 97% *P. falciparum*, 6% *P. malariae*, 1% *P. ovale*, and <1% *P. vivax* ((UBOS), 2015).

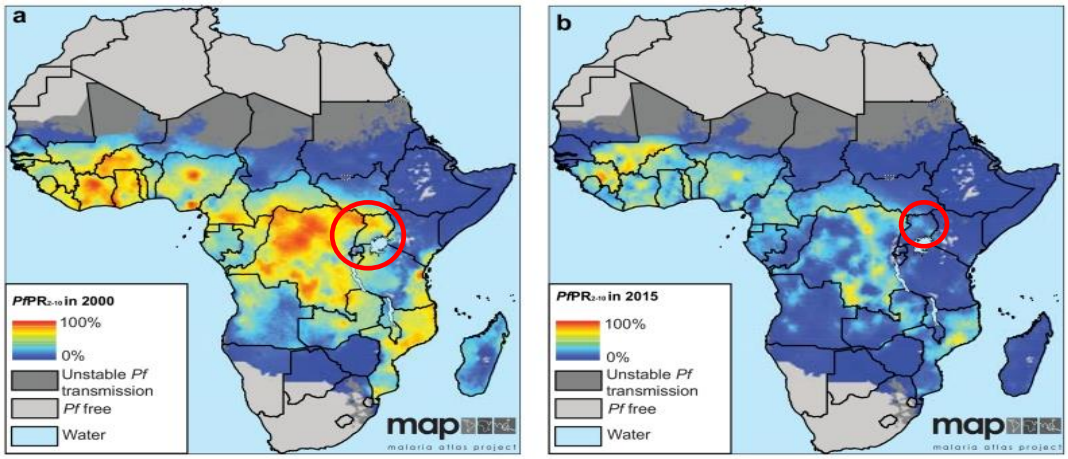


Figure 13: Shows the prevalence of *P. falciparum* malaria in Sub-Saharan Africa in heat map format, in both 2000 (**a**) and 2015 (**b**), generated by the Malaria Atlas Project (Oxford, 2017). It can be seen in Uganda within the red circular; there are still hot spots that have an estimated prevalence of around 50%, even though the substantial reduction of malaria prevalence across countrywide

It has been previously reported that the prevalence of malaria in Uganda is between 48% and 55.8% (Proietti *et al.*, 2011, Oguttu *et al.*, 2017, Maziarz *et al.*, 2017). Previous studies in 2011 have shown that 60.6% of infection with *Plasmodium* species occurred in Lake Albert, on the western border of Uganda (Green *et al.*, 2011). The geographical map of Uganda with prevalence hotspots of up to 50% as shown in **Figure 14**, in Lake Albert, outlined in black, is closed to areas of high malaria prevalence.

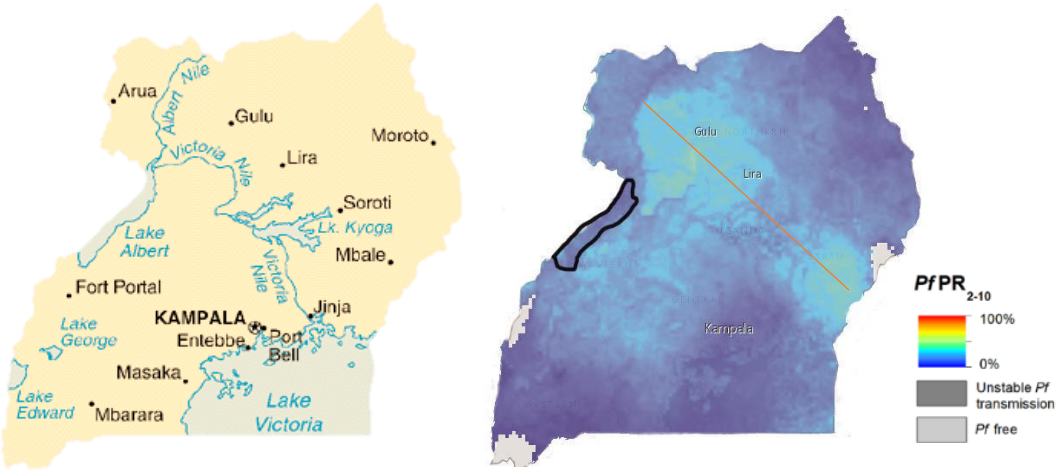


Figure 14: A geographical map of Uganda (left), followed by a malaria prevalence heat-map (right), generated by the Malaria Atlas Project (Oxford, 2017) highlighting the average prevalence of *P. falciparum* infection in Uganda. Overall prevalence was determined at 15.83%. However, the prevalence across the midline of the country (shown by a red line) and Lake Albert (outlined in black) is considerably higher

Although *P. falciparum* is the most common malaria species in Uganda (Yeka *et al.*, 2012), there are only a small number of recent papers on the distribution of non-*falciparum* in Uganda. The majority of research performed in Uganda has focused purely on *P. falciparum* infections, and in some studies, no attempt was made to distinguish between species. There is also a lack of data available on malaria in individuals living at higher altitudes in many regions of Uganda as it is thought that there is generally only a low prevalence of malaria. However, over the last 20 years, there are reports of increasingly frequent epidemics occurring in the highlands of Africa which is attributed to many factors including climate change (Idro *et al.*, 2005). It has also been documented that individuals living in these highland areas are more susceptible to severe disease owing to the lack of acquired immunity (Lindblade *et al.*, 1999, Idro *et al.*, 2005, Wandiga *et al.*, 2010).

4.4 Clinical symptoms

It usually takes between 5 and 30 days, depending on the species of *Plasmodium*, malaria transmission levels and host immunity. Symptoms are mostly caused by parasite activation of a proinflammatory cytokine cascade which plays a central role in the pathogenesis. At first for all species of malaria, some cases develop much milder symptoms that can be misdiagnosed as influenza or other viral infection including fever, chills, malaise, headaches, nausea, abdominal and back pain, respiratory problems (Bartoloni and Zammarchi, 2012). In all types of malaria, the periodic febrile response (fever) is caused by rupture of mature schizonts of the *Plasmodium* parasite and may present with three stages; a cold stage (lasting 30-60 minutes on average); a hot stage (2-6 hours); then a sweating stage (2-3 hours). This occurs daily in *P. falciparum* infection, but every third day in *P. vivax* or *P. ovale* infection (Bartoloni and Zammarchi, 2012). Apart from anaemia, most physical findings in malaria are often non-specific and offer little aid in the diagnosis, although enlargement of some organs may be seen such as hepatomegaly and splenomegaly which are common complications, along with normochromic, normocytic anaemia and thrombocytopenia. Early stage malaria may progress into severe malaria rapidly, with complications such as cerebral malaria (including seizures and coma), respiratory and renal failure can also be developed and progress to death within hours or days if the infected person remains without treatment (Bartoloni and Zammarchi, 2012).

4.5 Diagnostic tests

4.5.1 Microscopy

The current reference is a microscopic examination of the thick film using Giemsa stain (GS), although stains such as Fields and Wrights can also be applied (Tangpukdee *et al.*, 2009). This method is widely used as the best tool for diagnosing malaria in the field due to its low cost, ability to diagnose all species of malaria independently and determine the parasite density (Tangpukdee *et al.*, 2009).

Ideally, this examination should be the first step after conducting a clinical investigation for public signs of predictors. In the GS, the blood elements are concentrated on a much smaller surface than in the thin smear, which speeds up the search for parasites. The destruction of the erythrocytes makes the recognition of the parasites more difficult, but the sensitivity gain over thin smear is approximately 20-fold. If the presence of parasites is detected, the species can be identified more quickly in the thin smear because to the parasite's shape (ring, kitten) is added the appearance of red blood cells (normal or enlarged and deformed, the presence of granulations or not) which provides additional indications. Also, blood should be taken when the patient's temperature is rising, but it is never advisable to postpone the blood test if there is suspicion of malaria. One must be aware that microscopic examination, even in the hands of an expert, is far from having a sensitivity of 100% (Wongsrichanalai *et al.*, 2007). It does not detect very low parasite densities, as they often are in non-immune travellers under chemoprophylaxis (Larréché *et al.*, 2014). This is also the case for all tests currently available. In addition, the cyclic nature of parasite multiplication and sequestration in the target organs further reduces the likelihood of a positive outcome. The NPV is therefore not 100%. Quantitative buffy coat (QBC) fluorescent microscopy can be used to increase the sensitivity of malaria diagnosis, but less readily available due to its expense, and not preferable for species-specific diagnosis (Tangpukdee *et al.*, 2009). This is why it is imperative to repeat the malaria test (s) after 12-24 hours in the absence of a documented alternative diagnosis.

4.5.2 Rapid diagnostic tests

In recent years, new techniques based on immunochromatographic tests using whole blood have been developed for the RDT. These methods are based on the detection of *Plasmodium* antigens, either protein-2 (HRP-2) rich in plasmodial histidine or lactate dehydrogenase (pLDH), the latter being present only in *P.*

falciparum infections. These RDTs are quick, easy to use tests that do not require expensive reagents or electricity, and diagnose malaria with high sensitivity and specificity (Feleke *et al.*, 2017, Doctor *et al.*, 2016, Tseroni *et al.*, 2015, Fancony *et al.*, 2013). There are currently over 200 RDTs available, contributing to 270 million sales in 2015 (WHO, 2016b). Many products are available and can detect all *Plasmodium* species, with different performances. These tests have in common the lack of a clear standard and therefore a potential for a variable quality from one batch to another. In industrialised countries, these tests have been used until recently in addition to microscopy, because of the relatively variable performance obtained in the various studies. HRP-2-based tests were more reliable than those based on pLDH for CP- for the diagnosis of *P. falciparum* (0.08 versus 0.13). Third-generation tests (HRP-2) had CP equivalent to second-generation tests but higher CP + (98.5 versus 34.7).

The results for the detection of *P. vivax* were significantly worse (CP- 0.24 for HRP-2 and 0.13 for pLDH). It should be noted, however, that these tests were all evaluated against a "gold standard". sub-optimal (microscopy)(Mappin *et al.*, 2015). Most RDTs diagnose only *P. falciparum* malaria; however, some can distinguish between species. For example, the Standard Diagnostics (Gyeonggi-do, Republic of Korea) SD Bioline RDT can differentiate between *P. falciparum* and non-*falciparum* malaria (pan). Some RDTs have also been formulated to diagnose *P. vivax,* but these are less common compared to *P. falciparum* tests (Lee *et al.*, 2008). These RTDs, therefore, have the main potential to make the diagnosis of *P. falciparum* faster and more accurate, especially in non-specialized laboratories where inexperienced staff work. Indeed, learning these tests is easy, and a suitable option for use in areas where microscopy is not feasible, with results showing sensitivity and specificity of up to 99.5% and 98% respectively (Tadesse *et al.*, 2016). RDTs are cost effective compared to microscopy, with one of their only downsides being that species-specific diagnosis is not entirely possible. It has also been shown that RDTs may miss low-intensity infections (Golassa *et al.*, 2013). The main limitation is that rapid tests cannot be used to estimate parasite density. It should be noted that HRP-2-based assays can detect circulating antigens even after parasite clearance (2-4 weeks) and therefore cannot be used to evaluate treatment efficacy (Kattenberg *et al.*, 2012).

4.5.3 Molecular detection of human *Plasmodium* species

The rise of molecular biology has made it possible to develop new techniques based on the identification of *Plasmodium* DNA as amplified by the PCR. By targeting short nucleotide sequences which are related to the *Plasmodium*

genome on either side of the portion of interest (primers), and especially to the use of a thermostable enzyme, Taq polymerase (Chien *et al.*, 1976).

DNA is copied/multiplied exponentially to become detectable either by its visualisation under UV light (in the standard PCR) or by detecting fluorescence at each duplication by probes fluorescent or by SYBR Green technology (in the PCR in real- time). This molecular approach can detect very weak parasitic concentrations and distinguish different infectious species even if they are present simultaneously in the same individual (mixed infections)(Heid *et al.*, 1996, Livak *et al.*, 1995). Many other techniques, using different primers and probes (for real-time PCR), have been developed or modified in order to increase the sensitivity and specificity. This technique has been developed and reaching thresholds detection rate of 0.006 parasites /µl (Cnops *et al.*, 2011b), or that developed with combining classical PCR and real-time PCR (nested rtPCR) to reach a threshold of 0.5 parasite /µl, for samples taken from filter papers (Tran *et al.*, 2014).

Most of these PCR techniques have been developed by targeting the coding gene the small subunit of rRNA (18S rRNA). Indeed, this gene found in all eukaryotes contains regions both highly conserved and variable, and it has been the most studied in *Plasmodium*. Five copies of this gene are dispersed on different chromosomes of the parasitic genome (Rooney, 2004, McCutchan *et al.*, 1988). Nevertheless, other genes have also been used, but less frequently, as targets for the molecular identification of *Plasmodium*. For example, an the cytochrome b (cytb) gene, which also has a highly conserved region (Farrugia *et al.*, 2011), the gene encoding the circumsporozoite protein (csp), which was used to identify *P. falciparum* and *P. vivax* (Brown *et al.*, 1992), and dihydrofolate reductase (dhfr) (Barker Jr *et al.*, 1992). These techniques are designed in Monoplex (amplifying a single target) or in Multiplex (amplifying one or more targets). As an illustration, Chew *et al*. (2012) have developed a Hexaplex PCR capable to simultaneously identify six plasmodial species that infect humans (Chew *et al.*, 2012). The precision of these techniques made it possible to discriminate between different species during mixed infections but also to differentiate the two subspecies of *P. ovale* (*P. ovale walikeri* and *P. ovale curtisi*), long misunderstood (Oguike *et al.*, 2011, Calderaro *et al.*, 2012).

Many factors can explain the differences in sensitivities of these different techniques, among others: the type of reagents used, the standards used for quantification, dilutions made, types of thermocyclers, methods of analysis and interpretation of data, (Alemayehu *et al.*, 2013). In addition to the molecular identification of *Plasmodium* generally performed on blood material is liquid, dried on filter paper, or deposited and stained on the blade (Cnops *et al.*, 2010), or

from RDTs (Cnops *et al.*, 2011a), the use of other organic fluids is nevertheless described in the literature. Also, there have been reported in respective work evidence of possible detection by PCR *Plasmodium* in saliva and urine (Mharakurwa *et al.*, 2006, Nwakanma *et al.*, 2009). On the other hand, it has been documented that *P. falciparum* could be detected in the faeces of large African primates (Liu *et al.*, 2010a).

4.5.3.1 Molecular diagnosis of malaria by using faecal and blood DNA

Detection of parasite DNA fragments in fDNA by PCR appears an unconventional approach, as none of the malaria life cycle stages develops in the digestive tract; however, it is known that *Plasmodium* DNA can be detected from blood entering into the bowel lumen. For surveillance of tropical diseases, traditionally since faecal samples are collected and used for diagnosing intestinal parasites such as schistosomiasis, it may be possible to combine, and co-investigate malaria prevalence using faecal samples would increase the amount of epidemiological information collected without substantially increasing costs. However, there is little-published data in this field, with only one past attempt of diagnosing malaria using human fDNA (Jirků *et al.*, 2012), and limited current reports mixed reviews on whether it applies to diagnose malaria using faecal DNA.

Most of the literature carried out over the years presented here is from studies on different species of monkeys and apes, for example, and have inspected macaques (*Macaca* spp.) (Abkallo *et al.*, 2014, Siregar *et al.*, 2015, Kawai *et al.*, 2014), howler monkeys (*Alouatta* spp.) (de Assis *et al.*, 2016), gorillas (*Gorilla* spp.) (Weimin *et al.*, 2010), chimpanzees (*Pan troglodytes*) and bonobos (*Pan paniscus*) (Weimin *et al.*, 2010). One study was conducted experimentally in the laboratory with induced infections in mice (CBA mice) (Abkallo *et al.*, 2014), while another survey carried out on different samples collected from various wild bird species in Washington National Zoological Park (E. S. Martinsen, 2015). In the current literature, only one study has evaluated the possibility of detecting *Plasmodium* spp. using faecal samples collected from mankind (Jirků *et al.*, 2012), with another research conducted on different biological samples from human (saliva and urine) (Putaporntip *et al.*, 2012).

In comparison to faecal DNA, It has been found that 63% of the birds analysed was found to be positive by blood DNA PCR (bPCR), whereas all examined birds were found to be negative via fDNA PCR (fPCR) (E. S. Martinsen, 2015). This may be due to the composition of bird faeces being different to that of mammals, due to previous reports of the possibility of diagnosis in monkey species.

Other reports have shown that it is likely to detect malaria infection using fDNA extracts of various mammal species. For instance, Abkallo *et al.* (2014) showed that the malaria DNA could be detected in faeces consistently after 78 hours of post-infection during erythrocytes from *P.yoelii yoelii* infected mice. Another interesting finding from Abkallo *et al.* (2014) was that malaria parasite could be diagnosed in the pre-erythrocytic stage infection by using fPCR and have detection limit almost twice as high as bPCR. This finding is supported with another research concluded that fPCR could be applied for detecting malaria infection before it is viewed under a microscope, and this is because the parasites not being existed within a blood vessel during the erythrocytic cycle (Kawai *et al.*, 2014).

Interestingly, the study performed by Abkallo *et al.* (2014), on samples collected from a macaque, and has detected two malaria species, one species was found by using blood DNA, and another species being detected in faecal samples. It was suggested that one species was still in the pre-erythrocytic stage of life cycle, therefore was not detectable by microscopy. The estimated prevalence of zoonotic malaria infection in a population of chimpanzees, bonobos and gorillas was found to be between 32% and 48% based on fPCR (Weimin *et al.*, 2010). This was not compared to bPCR since researchers face severe difficulties in obtaining blood samples from wild mammals. However, the estimated sensitivity of fPCR based on repeated examination of samples found to be 57% (Weimin *et al.*, 2010), whereas bPCR was more sensitive for the diagnosis of malaria infection reaching 93% as documented by Da Costa Lima *et al*. (Da Costa Lima *et al.*, 2013). It is undoubtedly that PCR platform will be useful in front-line malaria diagnosis, but a higher sensitivity would be necessary before using this method as a reliable surveillance method within a malaria elimination environment. Siregar *et al*. (2015) highlighted a more promising result, where bPCR has been used for detecting a *Plasmodium* spp*.,* and found that the prevalence of 53.1% among reservoir macaque species, while by using fPCR reaching a sensitivity of 96.7% with no false positives observed. This finding was found to be much more sensitive than the detection of the parasite using prepared thin film under the microscope. It was documented that the comparison between fPCR and bPCR directly could not be performed, probably due to inadequate extraction techniques, however, with high sensitivity, the possibility of diagnosing malaria quickly with a non-invasive technique would have a significant impact for malaria epidemiology in the future. The only available study was performed on human faecal samples, conducted by Jirků *et al.,* (Jirků *et al.*, 2012), showed that the sensitivity of diagnosing *P. falciparum* using fPCR compared to microscopy was 100%. This

is another promising result, however with a sample size of only 16 patients, there is a lack of reliability associated with such conclusions.

4.5.3.2 Diagnosis of blood-borne parasites using PCR on faecal DNA

There is limited information available for the performance of DNA diagnostic tests for blood-borne parasites using faecal DNA. Hornok *et al.* (2015) conducted a study for detecting *Babesia Canis* from stool samples produced by bats of the *Chiroptera* genus. This study showed that in a total of 196 faecal samples assessed, the DNA of *Babesia Canis* was only amplified by PCR from five bat fDNA samples. This was concluded to be one of two things, the bat's infection with *Babesia Canis*, alternatively, the bats have eaten blood-sucking flies (e.g. *Stomoxys* spp.), which is the host-feeding behaviour of the relevant hosts. Hamad *et al.* (2015) looked at detecting *Leishmania major* through non-invasive techniques, using faecal samples from Western Lowland Gorillas (*Gorilla),* with similar methods as the study above, by extensive collections of faecal samples from wild mammals. 13.2% of these faecal samples were considered to be positive using fPCR, of which some were undergoing for sequencing and shown to be the same as those in cases of cutaneous leishmaniasis in humans (Hamad *et al.*, 2015). Although these findings were concluded from different parasites families, the results were based on using similar fPCR methodologies. So, this method can be applied for detecting different blood parasites using non-invasive fPCR. As previously mentioned, PCR has a much higher detection rate than microscopy or RDT, being able to detect 1-5 parasites per microliter (Tangpukdee *et al.*, 2009). PCR is time consuming, often taking over an hour to set up a reaction and around two hours to run. This combined with a high running cost and the need for electricity and freezer storage for reagents makes PCR less accessible in sub-Saharan Africa.

4.6 Control and Prevention

Malaria is a diffculte disease to control mainly due to the highly adaptable natural, requiring a mix of prevention and treatment methods. IVM is a concept introduced by the WHO to promote sustainable, environmentally beneficial and cost-effective strategies for the control livestock diseases, particularly vector-borne diseases, using different options to avoid major impacts on biodiversity (Sutherst, 2004). These strategies are location and vector population dependent. Environmental methods used for mosquito control often include biological control agents, including larvivorous fish and copepods, or bacterial larvicide, which have had a demonstrable role in integrated control when breeding sites are relatively few or are easily identified and treated such

as drinking water sources (Phillips, 2001). These options have limited or no adverse effects on the environment or beneficial organisms.

Other methods include IRS is the process of spraying the inside of dwellings with an insecticide to kill mosquitoes that spread malaria. Prevention can be insecticide-treated nets which are safe for use as personal protection and during pregnancy. As some *Anopheles* mosquitoes feed indoors (endophagic), nets provide barrier protection against biting, while the insecticide hampers indoor-feeding mosquitoes upon landing on the net (WHO, 2017b). Other methods of prevention that are not as widely used include the release of sterile genetically modified mosquitoes into an uninhabited forested area. It is hypothesised that in the future, this will wipe out all strains of infected mosquitoes, reducing the mosquito population and decreasing *Plasmodium* infection rates drastically (Benedict and Robinson, 2003, CDC, 2015).

4.7 Treatment

Malaria is a preventable disease that can be cured with antimalaria medicines. The primary goal of treatment is to achieve a complete cure, that is, rapid and complete elimination of plasmodia in the patient's blood, to prevent uncomplicated malaria from progressing to a severe form potentially fatal or chronic infection causing anaemia. From a public health perspective, the goal of treatment is to reduce the transmission of infection by decreasing the infectious reservoir and to prevent the emergence and spread of antimalarial drug resistance (WHO, 2015a). For all patients suspected of having malaria, parasitological confirmation of the diagnosis should be obtained by microscopic examination or using an RDT before starting treatment. Treatment should be administered on the basis of clinical examination only if it is impossible to carry out diagnostic tests within 2 hours after consultation.

Prompt treatment, within 24 hours of the onset of fever, with a safe and effective antimalarial drug, is essential to allow recovery and prevent life-threatening complications (Trampuz *et al.*, 2003). WHO recommends ACTs to treat uncomplicated malaria caused by *P. falciparum*. Combining 2 active ingredients that have different modes of action, ACTs are the most effective antimalarials available today. Currently, WHO is recommending 5 ACTs for *P. falciparum* malaria. The selection of ACTs should be based on the results of therapeutic efficacy studies against local strains of *P. falciparum* malaria. ACTs are the mainstay of the recommended treatment for *P. falciparum* malaria, and since no other artemisinin derivative should be on the market for several years, their effectiveness must be preserved (Smithuis *et al.*, 2010).

WHO recommends that national malaria control programs regularly monitor the efficacy of existing antimalarials so that selected treatments remain effective (Bhatt *et al.*, 2015). In areas of low transmission, a single dose of primaquine should be added to malaria treatment to reduce transmission of infection (White, 2013). Screening for glucose-6-phosphate dehydrogenase (G6PD) deficiency is not necessary because a low single dose of primaquine is both effective at blocking transmission and unlikely to have toxic effects in subjects deficient in G6PD, regardless of the genotypic variants involved (Eziefula *et al.*, 2014).

Plasmodium vivax infections should be treated with chloroquine in areas where there is no resistance to it. In regions with chloroquine-resistant strains *of P. vivax*, infections should be treated with ACT, preferably a combination in which the drug associated with artemisinin has a long half-life (Price *et al.*, 2009). With the exception of the combination of artesunate + sulfadoxine-pyrimethamine (AS + SP), all ACTs are effective against *P. vivax* infections in the blood stage (Liu *et al.*, 2014, Price *et al.*, 2009). In order to prevent relapses, primaquine should be added to treatment; the dosage and frequency of administration should be adjusted according to the enzymatic activity of the glucose-6-phosphate dehydrogenase (G6PD) of each patient. Severe malaria should be treated with injectable artesunate (intramuscular or intravenous) for at least 24 hours, followed by a full 3-day ACT once the patient can tolerate oral medications. When injectable treatment cannot be administered, children younger than six years of age with severe malaria should receive rectal artesunate treatment before being referred immediately to a centre for complete parenteral treatment (WHO, 2015b). It is imperative that artemisinin-based injectables and artesunate-based suppositories not be used as monotherapy. Initial treatment of severe malaria with these drugs must be completed by a full 3-day ACT. This ensures complete healing and prevents the development of resistance to artemisinin derivatives (WHO, 2015a, WHO, 2015b).

In recent years, ACTs have become significantly more accessible. By the end of 2016, 80 countries had adopted ACTs as first-line treatment. The estimated number of full-treatment ACTs purchased from manufacturers increased from 311 million in 2015 to 409 million in 2016. According to reports, more than 69% of these purchases were made for the public sector. The number of full-treatment ACTs distributed in this sector increased from 192 million in 2013 to 198 million in 2016. Most (99%) were in the WHO African Region (WHO, 2016b).

5. The importance of polyparasitism

Gastrointestinal parasitism with helminths and protozoa of tropical animals and equatorial Africa is frequent, varied and sometimes massive in subjects particularly receptive. Climatic conditions favour and accelerate biological cycles of internal parasites who find a host more easily definitive that the latter is in direct contact and frequent with the environment where the larval life (Hotez *et al.*, 2006a, Hotez *et al.*, 2008). Natural and diversity of polyparasitism vary according to the regional conditions more or less favourable to the ecology of each species involved (temperature, hygrometry, turbulence, nature of soil and flora, presence or absence of intermediate hosts regular, vicarious or waiting, possibly parasite tanks if it is a particularly ubiquitous species) (Viard *et al.*, 1997, Mupfasoni *et al.*, 2009). Synchronised and consecutive transmission of malaria, soil- and water-borne diseases of helminth infection, are among the most prevalent afflictions in areas of poverty in the developing world, particularly in many parts of sub-Saharan Africa (Hotez *et al.*, 2016, Green *et al.*, 2011).

The combined infections highlight that integrated control programs should be flexible and targeting multiple parasitic diseases simultaneously to reduce transmission of several diseases, and improved surveillance of multiple tropical diseases, in the most vulnerable population, SAC (Hotez *et al.*, 2006a, Hotez *et al.*, 2007, WHO, 2004). Data published on joint tropical diseases produce conflicting data. It has been suggested that helminthiasis can have direct and indirect effects on the course of malaria in developing countries (Kwenti *et al.*, 2016). However, various studies have illustrated that co-infection with helminths increase susceptibility to malaria infection (Nacher et al., 2002), and is associated with increased gametocyte production (Nacher *et al.*, 2001), significantly decreased haemoglobin concentrations (Oladele *et al.*, 2014), and a higher risk of severe malaria. On the contrary, other studies have suggested helminth infections may prevent the likelihood of malaria by reducing acute clinical presentations, parasite loads and fatal complications such as kidney failure (Shapiro *et al.*, 2005). These inconsistent results may be due to different research designs, different geographic areas, and regulation of the host immune system by different parasite species (Degarege *et al.*, 2012).

6. Quantitative polymerase chain reaction

The technological concept of the PCR, discovered by Kary Mullis in 1983 (Mullis, 1987), and which earned him the Nobel Prize in Chemistry in 1993, has now become an almost universal tool in the field of biology. PCR is a highly sensitive and specific methodology for the detection of nucleic acids. Its use has

extended considerably to many applications, including the quantitative analysis of specific nucleic acids (DNA and cDNA) in a given sample (**Figure 15**). The first approaches to gene quantification by PCR or reverse transcriptase gene transcripts (RT-PCR) were based on a titration analysis using standard nucleic acid (or external standard) and the development of a calibration range obtained by serial dilution of the external standard (Noonan *et al.*, 1990). The result of the analysis gives an estimate of the relative amount of the target matrix relative to the standard. However, the accuracy of the results obtained is very often limited, because of the considerable variability of efficiency from one PCR to another.

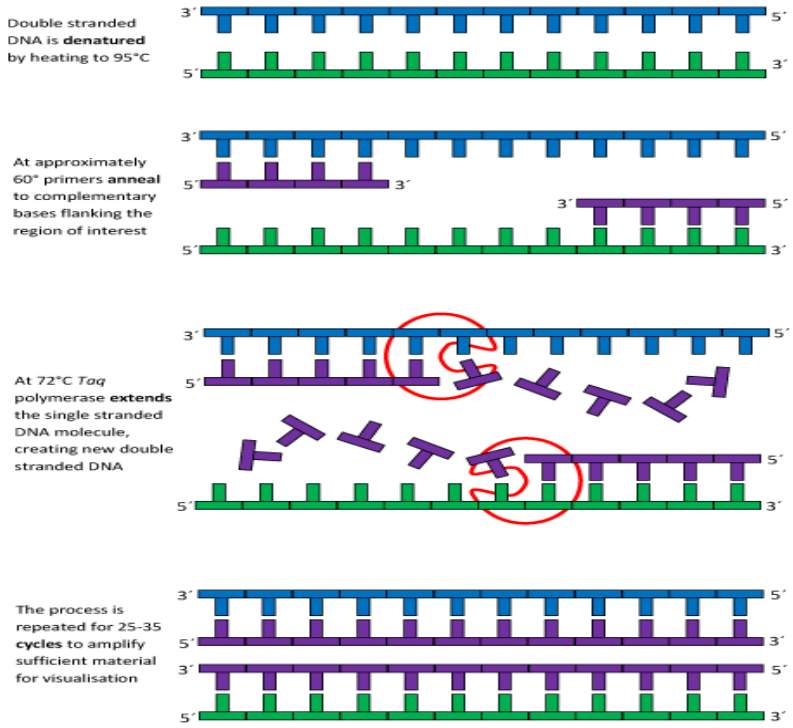


Figure 15: The process of a PCR reaction, showing denaturation at 95^{0}C, followed by annealing at 60^{0}C, then a final extension step at 72^{0}C, making two complementary DNA strands (Leicester, 2017)

The introduction of a co-amplified internal standard during the same PCR reaction partially corrected the tube-to-tube variability and led to the development of two different quantification methodologies: those by differential PCR and those by competitive PCR. In the approaches of quantification by differential PCR (or RT-PCR) (Frye *et al.*, 1989), an endogenous sequence, that is to say belonging to a so-called reference gene present in the tested sample (endogenous sequence of a gene present as a single copy by haploid genome or transcript of a domestic gene whose transcription is constant and independent of the extracellular environment), serves as an internal standard. The standard

endogenous sequence is coamplified with the target sequence during the same PCR reaction using two specific primer pairs. The method makes it possible to evaluate the relative amount of target gene corresponding to the reference gene, the latter making it possible to normalise the quantity and quality of nucleic acid extracted. Nevertheless, quantification using an endogenous gene as an internal standard requires exceptionally standardised application conditions to ensure that the amplification of the two different sequences (target gene and reference gene) is performed with identical efficiencies.

The introduction of a fluorescent reporter molecule into the PCR reaction for the detection of PCR products by a fluorometric method has led to the recent development of quantitative real-time PCR techniques (Higuchi *et al.*, 1993). The latter is no longer based on end-point detection of the PCR products formed but on an analysis of the kinetics of the PCR reaction by means of a system capable of detecting "in a closed tube" the PCR products formed after each amplification cycle. The TaqMan™ probe (**Figure 16**) is an oligonucleotide fragment labelled with two fluorophore groups at it's 5 ', and 3' ends (Livak *et al.*, 1995). The 5 'end carries the donor fluorophore which is a derivative of fluorescein (FAM, TET, JOE, HEX or VICTM). At the 3 'end is the quencher fluorophore which is usually a rhodamine derivative (TAMRA). Due to the proximity of the two fluorophore groups, linked to the small size of the probe (25 to 30 nucleotides, i.e. less than 55 angstroms between the two fluorophores), the energy absorbed by the excited fluorophore donor is transferred by FRET to the fluorophore acceptor. Since the excitation spectrum of the TAMRA does not overlap the emission spectrum of the donor fluorophore, the quencher absorbs the energy transmitted to it but emits no fluorescence (**Figure 16**)(Navarro, 2015).

The peculiarity of the TaqMan™ system is to exploit the 5'-3 'nuclease activity of the DNA polymerase which makes it possible to hydrolyse the probe hybridised to its specific target during the stage of elongation of the primers (Holland *et al.*, 1991). Cleavage of the probe during this step has the effect of removing the two fluorophores, to release the donor fluorophore quenching effect exerted by TAMRA and thus restore its fluorescence emission. The intensity of the fluorescence emitted by the donor fluorophore, commonly referred to as reporter fluorophore in a TaqMan™ probe, is measured at the end of each amplification cycle.

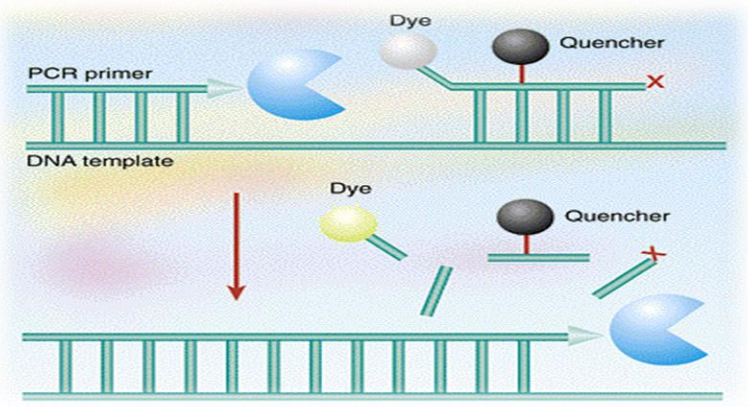


Figure 16: The basic process of amplicons detected by using a labeled probe specific for the target sequence within gDNA. During annealing, the probe binds to its intramolecular complement in the amplicon. During extension, the probe cleaved as a result of the 5′ nuclease activity of the DNA polymerase enzyme, detaching the quencher from the reporter and produces an emission of fluorescence. This is then quantified by rtPCR apparatus (Navarro, 2015)

By its mode of operation, the TaqMan™ system uses particular amplification conditions in two stages, a denaturation step at 95 ° C. and a second step combining hybridisation and elongation carried out at an identical temperature (around 60 ° C.). Since the probe can only be cleaved when hybridised to its complementary strand, the extension temperature of the primers must be compatible with the hybridisation temperature of the probe. The Tm of a TaqMan™ probe must, therefore, be high and close to 70 ° C, which imposes a rigorous design of the primers but facilitates the application of a standard thermocycling profile. In addition, the TaqMan™ system requires the use of a DNA polymerase that must have a 5'-3 'exonuclease activity whose efficiency will determine the quality and intensity of the signal emitted (Kreuzer *et al.*, 2000). The cleavage of the probe being irreversible, the TaqMan™ system does not allow the establishment of a post-PCR fusion curve.

The development of a new type of TaqMan™ probes, the TaqMan™ - MGB probes (Kutyavin *et al.*, 2000), made it possible to overcome certain limitations of the TaqMan™ system. The introduction at the 3 'end of a molecule with an affinity for the MGB or nucleus increases the Tm of the probe and more strongly stabilises its interaction with the DNA matrix, which makes it possible to draw probes of smaller size (13 to 20 nucleotides) and able to hybridise with a matrix rich in dinucleotides GC or AT. Moreover, in a TaqMan™-MGB probe, the reporter fluorophore is associated with a non-fluorescent quencher; which makes it possible to detect two different targets (or multiplex) during the same PCR reaction. These chemical modifications made to the TaqMan™ probes also made it possible to extend the applications of this chemistry to genotyping by allelic discrimination.

7. Justification and rationale of the present study

7.1 Schistosomiasis

Improved coverage of PC with PZQ across sub-Saharan Africa has been the aims of the WHO for some time. In 2014, 49 million SAC received treatment, equating to a coverage level of 34.6% within this age groups (WHO, 2016a). However, many children living in several parts of sub-Saharan Africa, particular in Uganda are not targeted in MDA programmes (Stothard *et al.*, 2013a). Therefore further parasitological surveys are essential to determine the disease prevalence in these remote regions to enable PZQ to be administered.

Resources available for the control are often limited, making it essential to know the distribution of schistosomiasis at different settings in Uganda for effective treatment regimens with PZQ. The impact of *S. mansoni* on communities living on the shoreline of Lake Albert has been studied in depth (Kabatereine *et al.*, 2004, Seto *et al.*, 2012, Dunne *et al.*, 2006, Levitz *et al.*, 2013), yet the prevalence of intestinal schistosomiasis is still a substantial burden within these communities. SCI, an international charity, is led by Fenwick that has been in charge of treating SAC in Uganda since 2003 (Loewenberg, 2014). Typically, traditional parasitological methods of diagnosis that visualise parasite ova underestimate true prevalence. Using the Kingdom of Saudi Arabia as a contemporary example, the primary care health system plays a critical role in schistosomiasis control activities since 1977 (Lotfy and Alsaqabi, 2010). So, the implementation of new diagnostic methods within PCH is important for moving forward from control to elimination (Stothard *et al.*, 2014). Our study aims to reassess parasitological- and serological-based methods alongside real-time PCR across a landscape where disease control is ongoing in Buliisa District, Lake Albert, and Asir region, Saudi Arabia and focus upon examination of children of school-age, using several different techniques alongside TaqMan® assay in attempt to produce the current picture of *Schistosoma* spp. prevalence in the selected study sites.

7.2 Giardiasis

Giardia duodenalis occurs worldwide and causes an estimated 2·5 million cases of diarrhoea in developing countries annually (Mahdy *et al.*, 2009). *Giardia* affects mainly children and has a wide range of clinical manifestations (Thompson and Monis, 2004). Although seven main genotypes of *G. duodenalis* have been identified (A–G), only genotypes A and B are commonly responsible for human infection (Thompson, 2004). The reasons behind the variability observed in giardia infection in humans are not fully understood,

and there is no clear evidence between the infecting genotype (A or B) and any clinical manifestation (Cacciò *et al.*, 2005). In Uganda, epidemiological studies on *G. duodenalis* have found cross-species transmission between primates, gorillas, cattle and humans living nearby or using similar water sources (Graczyk *et al.*, 2002, Johnston *et al.*, 2010). The majority of studies from Uganda focus on the zoonotic potential of giardiasis and not the pathological effects on the human population. This is the first epidemiological study to determine the prevalence and associated risk factors of *G. duodenalis* assemblages undertaken in five primary schools around Lake Albert, Uganda.

7.3 Malaria

As part of the WHO Global Technical Strategy for Malaria 2016-30, it has been highlighted that diagnostic testing has fallen behind in the fight against malaria (WHO, 2015a). Malaria surveillance data is often unreliable, making programme planning challenging. In 2015 it was estimated that only 19% of malaria cases were detected by surveillance systems (WHO, 2016b). Efforts in reducing burden in the high burden African countries have intensified recently through a combination of intensified malaria control and tools, notably long-lasting insecticide-treated nets (ITNs), IRS of insecticides, ACTs, and intermittent preventive therapy (IPT) for high-risk groups (Yeka *et al.*, 2012). Without adequate surveillance data, the actual prevalence of malaria cannot be determined, making control and prevention efforts complex, especially with the aims of malaria elimination in many countries within the next 10-15 years (WHO, 2017b, WHO, 2015a). Drug resistance is a growing problem, particularly to artemisinin compounds, the mainstay of modern malaria treatment (Singh *et al.*, 2018), and another problem that cannot be monitored effectively without adequate surveillance methods. As previously illustrated, little research has been undertaken in the field of diagnosing malaria using fPCR. Only one study has been found to use this method on human samples, and with a small sample size of 16, this is not adequate to gain a good estimate of the reliability of this diagnostic method (Jirků *et al.*, 2012). This is the first study that will assess the potential of TaqMan™ Assay targeting mitochondrial genome 18S as previously described by Shokoples *et al*. (Shokoples *et al.*, 2009) to diagnose malaria and determine species-specific *Plasmodium* in humans from either blood spot or faecal samples. Evaluating the effectiveness of a new species-specific surveillance approach fits in with the objectives of the WHO global technical strategy for monitoring and assessment of disease prevention and control programmes of malaria (WHO, 2015a).

8. Aims of the thesis and study objectives

8.1 Aims

The overall aim of the thesis is to explore different diagnostic platforms for providing insights into the epidemiology and surveillance of important parasitic diseases, with attention on schistosomiasis, giardiasis, and malaria (*Plasmodium* spp.) in Uganda, a country where the disease burdens are still high despite large-scale control (see **Table 7- below**). These diagnostics can be used as a surveillance tool, in lower endemic countries, e.g. Kingdon of Saudi Arabia, not only to determine that infection levels of these parasitic infections are also sustained below target thresholds (WHO 2015a). By making a comparison between country settings, I hope to develop the dicussion on how to monitor, evaluate, and assess the impact of different control and intervention measures being implemented against these parasitic diseases.

Table 7: Tabulated summary of schistosomiasis, giardiasis, and malaria diagnostics

Health-related	*Schistosomiasis spp*	*Giardia spp.*	*Plasmodium spp*
Clinical	*Schistosomiasis has three clinical states. -The first stage is characterized by dermatitis and is caused by the cercariae. -The second stage is marked by fever and constitutional complaints (Katayama fever) -The third stage results in chronic fibro-obstructive disease which is caused by the eggs. *Acute Clinical Presentation -Dermatitis at the site of penetration -Fever results in *S. mansoni* and *S. japonicum* lasting 4-8 weeks -Acute onset: fever, headache, and cough -Heptosplenomegaly and Lymphadenopathy -Symptoms resolve in weeks but death may occur. -Eosinophilia *Chronic Clinical Presentation -Often asymptomatic -Fatigue, colicky abdominal pain -Intermittent diarrhea -Effects on the liver and intestines or bladder in *S. haematobium* -Liver pre-sinusoidal blockage and increased portal pressure. Earliest sign Hepatomegaly -Intestinal granuloma, CNS (3%), Pulmonary (with patent portosystemic collateral circulation)	*Gastrointestinal Symptoms: -Sudden onset of explosive, watery diarrhea, abdominal cramps, foul flatus, vomiting, fever, and malaise -The majority of patients experience a less severe onset of symptoms, but rather suffer from more subtle maladies which are recurrent or resistant. -Stools become malodorous, mushy, and greasy. Watery diarrhea may alternate with soft stools or even constipation. Stools do not contain blood or pus. -Upper GI symptoms, including cramping, nausea, lack of appetite, bloating, substernal burning, and acid indigestion. *Constitutional symptoms: -Anorexia, fatigue, malaise, and weight loss are common. -Weight loss occurs in more than 50% of patients and averages 10 pounds per person. -Chronic illness may occur with adults presenting with long-standing malabsorption syndrome and children with failure to thrive. *Lactose intolerance *Miscellaneous: Unusual presentations include allergic manifestations such as urticaria, erythema multiforme, bronchospasm, reactive arthritis, and biliary tract disease. *Physical: -Physical examination not revealing. -Abdominal examination may reveal nonspecific tenderness . -Rectal examination should reveal heme-negative stools. -In severe cases, evidence of dehydration or wasting may be present.	*Uncomplicated malaria: symptomatic malaria without signs of severity or evidence of vital organ dysfunction. The manifestations of uncomplicated disease are the following: (Fever, Chills, Headache, Dizziness, Back pain, Myalgia, joint and bone pains, Cough, chest pain, Weakness, prostration, Gastrointestinal disturbances (nausea, vomiting, diarrhea) The typical but infrequently observed malaria attack consists of three stages: 1-Cold stage (characterized by feeling of cold and shivering followed by whole body shaking that lasts 15- 60 minutes, cold, dry and pale skin) 2-Hot stage (high fever up to 40- 41 oC that lasts 2-6 hours, severe headache, palpitations, tachypnoea, flushed and dry skin) 3-Sweating stage (profuse sweating for 2- 4 hours, feeling of exhaustion) *Severe (complicated) malaria: "the presence, in a patient with falciparum malaria, of clinical manifestations such as: (Prostration,Hyperpyrexia,Dehydration,Hypotension,Circulatory collapse, Electrolyte imbalances, Hypoglycaemia, Severe anaemia, Spontaneous bleeding, Disseminated Intravascular, Coagulation, Haemoblobinuria, Jaundice,Hepatosplenomegaly,Generalized convulsions, Impaired consciousness, Coma, Acidosis, Acute abdominal pain, Acute Respiratory Distress Syndrome, Pulmonary edema, Renal failure)
Parasitological	1-Urine filtration for *S.haematobium* infection where the eggs are quantified by means of filtering 10 ml of a vigorously shaken mid-day urine sample through a polycarbonate filter or by centrifugation 2- Kato-Katz thick smears (Kato-Katzs) from a single stool are currently recommended for diagnosing *S.mansoni* infections to map areas for intervention 3-RDT for hematuria (urogenital schistosomiasis),fecal occult blood (FOB), and calprotectin detection (entero-schistosomiasis) are also point-of-care approaches	*Giardia cysts can be excreted intermittently, multiple stool collections (i.e., three stool specimens collected on separate days) increase test sensitivity. The use of concentration methods and trichrome staining might not be sufficient to identify Giardia because variability in the concentration of organisms in the stool can make this infection difficult to diagnose.	*Microscopy of peripheral blood thin and thick films remains the reference for malaria diagnosis, with Giemsa staining is most commonly used in routine Lab
Immunological	1-POC-CCA,a commercial point-of-care circulating cathodic antigen test for assessing *S. mansoni* infection prevalence in areas at risk. 2-SEA -ELISA , a good serological screening test for schistosomiasis, but gives no indication of the infecting species of schistosome	* Evaluation of Rapid Antigen Point-of-Care Tests (Quik Chek) for Detection of *Giardia* and *Cryptosporidium* Species in Human Fecal Specimens (Novel)	*SD BIOLINE Malaria Ag P.f/Pan test is a rapid, qualitative and differential test for the detection of histidine-rich protein II (HRP-II) antigen of Plasmodium falciparum and common Plasmodium lactate dehydrogenase (pLDH) of Plasmodium species in human whole blood
Molecular	*Real-Time PCR using an internal transcribed spacer (ITS)-based real-time polymerase chain reaction (PCR) for the detection of Schistosoma DNA in feces with targeting high-low endemic setting in Uganda (Novel)	*Implementation of a quantitative PCR (Real-Time PCR) for Giardia and analysis of the prevalence, cyst shedding and genotypes of Giardia present in selected schools (Novel)	*Real-Time PCR for Detection and Identification of *Plasmodium spp* from either dry blood spots or faecal preserved ethanol (Novel)

8.2 Objectives

Specific study objectives of the thesis are:

- **(i)** To explore the geographical distribution of the parasitic infection among school children by assessing the infection prevalence of the target species and conducting spatial analysis;
- **(ii)** To describe the extent and geographic distribution of schistosomiasis infections among school children in Uganda and Saudi Arabia using advanced diagnostic tools for addressing the impact of nation-wide preventive chemotherapy via MDA campaigns;
- **(iii)** To understand the impact of school location and household conditions, as well as behavioural components, on schistosomiasis transmission and infection intensity among schoolchildren, to be served as evidence-based information to guide future schistosomiasis infection control strategies;
- **(iv)** To document the distribution of intermediate snail hosts in the different settings in Uganda and Saudi Arabia, and exploring the impact of physico-chemical parameters of the water body including pH, conductivity on an abundance of intermediate snails
- **(v)** To perform moleclare idenfitfcation of collected intermiadiate snail gost using a DNA-based molecular technique as an advanced-tool for species-specific identification.
- **(vi)** To determine the current prevalence and to identify risk factors associated with Giardia infection in selected school children in five primary schools on the shoreline of Lake Albert, Uganda, by using a rapid antigen test and TaqMan® probe-based assays from faecal DNA;
- **(vii)** To shed light on putative interactions between giardiasis, intestinal schistosomiasis and anaemia in theses school children;
- **(viii)** To identify the taxonomic assemblages of *Giardia* within school children, by using assemblage-specific TaqMan®TPI probes with confirming their presence by sequence analysis of β-giardin gene, to identify potential coinfections, risk factors and pathologies associated with differing assemblages;
- **(ix)** To conduct an epidemiological assessment of malaria infections in Low-high altitude in the Buliisa district of Uganda and application of molecular techniques as diagnostic quality control for species-specific TaqMan® method to provide a more sensitive tool for species identification;
- **(x)** To explore the possibility of the implementation of the TaqMan® assay as a platform to monitor, evaluate and assess the impact of different

public health interventions to control and eliminate critical communicable diseases.

This thesis examines the extent to which these objectives can be achieved when the assessment is carried out not as a stand-alone initiative, but in conjunction with NTDs teams in Saudi Arabia and Uganda, utilising locally available techniques and resources. This assessment is carried out to provide evidence to decision-makers: **1**) when and where most of the disease burdens have been lightened, and interventions at national levels can be scaled up or down, or **2**) when the distribution and extent of the disease burden are not previously well documented, but the information is needed to develop effective control strategies or to raise awareness on the necessities of developing the strategies. Especially for the giardiasis, schistosomiasis and malaria infection control, components of the interventions can be tailored based on the risk factors identified, where there are relatively limited resources, and the complementary interventions can be rolled-outed for small-scale implementation on top of preventive chemotherapy. Although parasitological tools provide a cost-effective platform to minimise the financial expenditure of general disease assessments, this was not investigated formally, and focus was given to epidemiological surveillance using alternative approaches alongside molecular methods for accurate estimation of NTDs (**Table 7**).

8.3 Thesis layout

The thesis chapters for this Ph.D. will be laid out per the following structure:

Chapter 1 Background and literaturere review: Describes the current knowledge of NTD with focusing on schistosomiasis, giardiasis, and malaria, as well as different diagnostic approaches for the epidemiology of these communicable with details about the current prevalence and national control activities within in the study locations;

Chapter 2 Surveillance of intestinal schistosomiasis during control: a comparison of four diagnostic tests across five Ugandan primary schools in the Lake Albert region;

Chapter 3 An extensive burden of giardiasis associated with intestinal schistosomiasis and anaemia in school children on the shoreline of Lake Albert, Uganda;

Chapter 4 Molecular characterisation and taxon assemblage typing of giardiasis in primary school children living close to the shore of Lake Albert, Uganda;

Chapter 5 Non-invasive surveillance of *Plasmodium* infection by real-time PCR analysis of ethanol preserved faeces from Ugandan school children with intestinal schistosomiasis

Chapter 6 Evaluation and implementation of new approaches for surveillance of schistosomiasis: application of molecular DNA methods for snail species identification combined with immuno-diagnostics using urine-CCA strip test and finger-prick blood SEA-ELISA in schoolchildren in Saudi Arabia;

Chapter 7 General discussion provides implications of strategies using alternative diagnostic tools and advanced molecular techniques in the studies presented, related to control or elimination of the critical communicable diseases mentioned, and outlines suggestions for future work.

Chapter 2: Surveillance of intestinal schistosomiasis during control: a comparison of four diagnostic tests across five Ugandan primary schools in the Lake Albert region

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Cover sheet for each research paper included in a research thesis

1.For a research paper already published

1.1. Where was the work published**? Cambridge.org/core/journals/parasitology**

1.2. When was the work published? **21 March 2018 (Appendix 11)**

2. If the work was published prior to registration for your research degree, give a brief of the rationale for its inclusion **N/A**

2.1. Was the work subject to academic peer review? **N/A**

2.3. Have you retained the copyright for the work? **N/A**

3. For a research paper prepared for publication but not yet published

3.1. Where is the work intended to be published? **N/A**

3.2. List the authors of the paper in the intended authorship order **N/A**

3.3. Stage of publication-Not yet submitted/submitted/Undergoing revision

from peer reviewer's comments/In press **N/A**

4. For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper.

HAl-S contributed to the design of the studies; participated in data collection; participated in data entry, participated in data interpretation, conducted an analysis of data, prepared the manuscript and approved the final version.

Candidate's signature

Mr Hajri Alshehri

Supervisor or senior author's signature to confirm role as stated in ()

Professor Russell Stothard Primary Supervisor

2.1 Abstract

Introduction: Programmatic surveillance of intestinal schistosomiasis during control can typically use four diagnostic tests, either singularly or in combination, but these have yet to be cross-compared directly. Our study assembled a complete diagnostic dataset, inclusive of infection intensities, from 258 children from five Ugandan primary schools. The schools were purposely selected as typical of the endemic landscape near Lake Albert and reflective of high- and low-transmission settings.

Methods: In Buliisa District, a total of 258 (5-10 years old) were enrolled. Children were also interviewed with a standardised questionnaire to ascertain recent PZQ treatment history. A field-based, duplicate Kato-Katz thick smear slides were prepared from provided two stool samples each stool. Urine-CCA dipstick and SEA-ELISA from a single finger-prick blood sample were used to test for schistosome antigen/antibody, respectively, following manufacturer's instructions. DNA-TaqMan® assays were performed in each aliquot of stool spiked with Phocine Herpes Virus (PhHV-1) to act as an internal control for each DNA extraction and later real-time PCR assay for inhibition.

Results: Overall prevalence was: 44.1% by microscopy of duplicate KK smears from two consecutive stools, 56.9% by urine-CCA dipstick, 67.4% by DNA-TaqMan® and 75.1% by SEA-ELISA. A cross-comparison of diagnostic sensitivities, specificities, positive and NPVs were undertaken, inclusive of latent class analysis (LCA) with an LCA-model estimate of prevalence by each school. The latter ranged from 9.6% to 100.0%, and prevalence by the school for each diagnostic test followed a static ascending order or monotonic series of KK, urine-CCA dipstick, DNA-TaqMan® and SEA-ELISA.

Conclusion: We confirm that KK remains a satisfactory diagnostic standalone in high-transmission settings, but in low transmission, settings should be augmented or replaced by urine-CCA dipsticks. DNA TaqMan® appears suitable in both endemic environments though is only implementable if resources permit. In low-transmission settings, SEA-ELISA remains the method of choice to evidence an absence of infection. We discuss the pros and cons of each technique concluding that future surveillance of intestinal schistosomiasis would benefit from a flexible, context-specific approach both in choice and application of each diagnostic method, rather than a single one-size fits all approach.

Keywords: *Schistosoma mansoni*, KK, urine-CCA, SEA-ELISA, DNA-TaqMan®, latent class analysis

2.2 Introduction

Developing appropriate diagnostics tools, methods and protocols to track parasitic diseases before, during and after control is an essential component within the multi-disciplinarily of parasitology. It has been previously highlighted (Stothard and Adams, 2014) and with regard to schistosomiasis, intestinal schistosomiasis poses a considerable public health burden in Uganda (Loewenberg, 2014). Since 2003 there has been an active NCP against it (Kabatereine *et al.*, 2006, Kabatereine *et al.*, 2007, Fenwick *et al.*, 2009, Stanton *et al.*, 2017), as primarily based on preventive chemotherapy campaigns (Montresor *et al.*, 2012, Stothard *et al.*, 2013b). Despite much progress in the delivery of PZQ treatments to SAC, infections with *S. mansoni* continue to be pervasive, particularly along the immediate shoreline of Lake Albert (Seto *et al.*, 2012, Al-Shehri *et al.*, 2016). Moving some 10–20 km inland, however, the prevalence of infection by school can decline dramatically, at least if measured by faecal egg-patency for if more sensitive diagnostic tools were used, such as urine-antigen dipsticks, such declines are less precipitous (Stothard *et al.*, 2006, Stothard *et al.*, 2017a).

The incongruence between 'estimated' and 'true' prevalence is a well-known diagnostic dilemma in surveillance of intestinal schistosomiasis primarily due to an operational compromise between imperfect detection tools and insufficient specimen sampling (Bergquist *et al.*, 2009, Stothard *et al.*, 2014, Utzinger *et al.*, 2015). Nonetheless, if control programmes are to be monitored effectively and also permit evidence-based adaptation or revision of control tactics (Tchuenté *et al.*, 2017), infection dynamics at an individual level need to be captured alongside any broader changes in the epidemiological landscape amenable to measurement (Hawkins *et al.*, 2016, Stothard *et al.*, 2017a). As the strive towards elimination grows (Hawkins *et al.*, 2016, Colley *et al.*, 2017), previous diagnostic shortcomings are revealed highlighting new diagnostic needs that guide future target product profiles (Utzinger *et al.*, 2015, Hawkins *et al.*, 2016, Savioli *et al.*, 2017, Tchuenté *et al.*, 2017, Weerakoon *et al.*, 2018).

At an individual level, often the school-aged child, the diagnostic repertoire for surveillance of intestinal schistosomiasis within NCPs has remained surprisingly meagre; for many years, it has been exclusively founded on parasitological methods alone (Stothard *et al.*, 2014), with only sporadic application of serological methods (Chernet *et al.*, 2017, Hinz *et al.*, 2017). With the growing need for modernisation and interest in the adoption of more sensitive disease diagnostics in general (Mabey *et al.*, 2004, Solomon *et al.*, 2012, Stothard and Adams, 2014). In recent years, there have been two critical developments that centre upon scale-up in the use of urine-CCA dipsticks

(Colley *et al.*, 2013, Sousa-Figueiredo *et al.*, 2013, Foo *et al.*, 2015, Danso-Appiah *et al.*, 2016, Greter *et al.*, 2016, Kittur *et al.*, 2016) and development of DNA-detection platforms with real-time PCR with parasite-specific TaqMan® hydrolysis probes (Ten Hove *et al.*, 2008, Mejia *et al.*, 2013, Easton *et al.*, 2016, Weerakoon *et al.*, 2018). Furthermore, recent application of more sophisticated statistical modelling such as latent class analysis (LCA) (Hadgu *et al.*, 2005), has advanced diagnostic tool performance comparisons beyond the direct need of a fixed reference 'gold' standard which, for schistosomiasis, is something we currently do not have (Shane *et al.*, 2011, Ibironke *et al.*, 2012, Koukounari *et al.*, 2013, Beltrame *et al.*, 2017).

In this study, we attempt to make a diagnostic comparison for surveillance of intestinal schistosomiasis in schoolchildren across five primary schools using four methods namely: microscopy of duplicate KK smears from two consecutive stools, urine-CCA dipsticks, real-time PCR of stool with a Schistosoma-specific TaqMan® probe (**Table 7**) and serological analysis of finger-prick blood for antibodies against schistosome SEA. Diagnostic congruence was first assessed by empirical cross-tabulations, assuming a 'gold standard', then later by LCA with disease prevalence by the school also estimated with an LCA model.

2.3 Methods

2.3.1 Study area, participants and ethical approval

Field sampling and examinations of children took place in May 2015 in five primary schools in Buliisa District located within the Lake Albert region. Three of which have been visited previously as sentinel surveillance sites of the NCP (Kabatereine *et al.*, 2007) and the global positioning system locations (GPS) known (**Figure 17**). The schools Walakuba (GPS 01°50.323N, 031°22.740E), Bugoigo (GPS 01°54.004N, 031°24.750E) and Runga (GPS 01°43.828N, 031°18.603E) were located on the immediate shoreline, while Biiso (GPS 01°45.516N, 031°25.236E) and Busingiro (GPS 01°44.090N, 031° 26.855E) were over 10 km away inland which aimed to represent the current control landscape across high- and low-endemic settings, respectively.

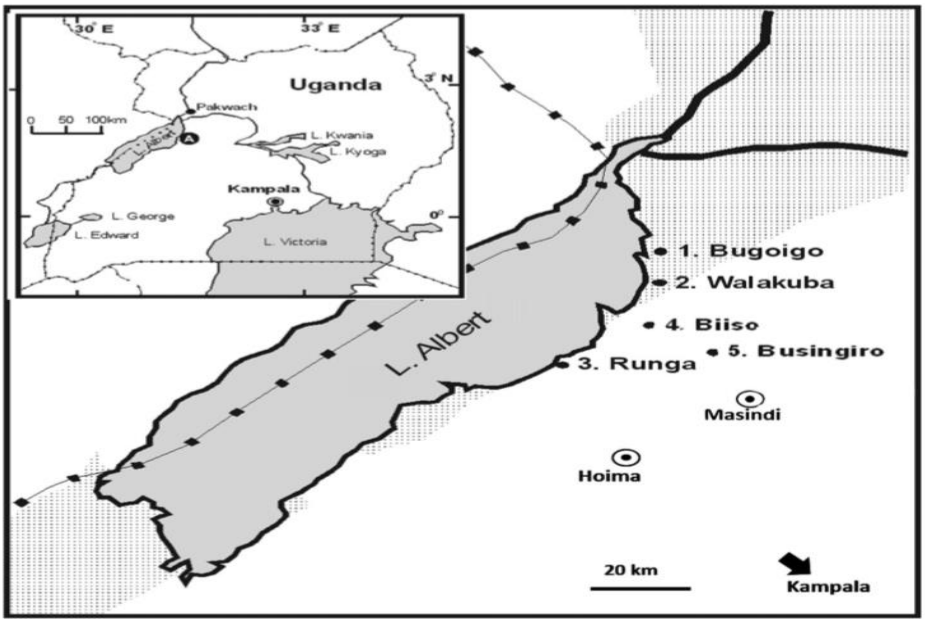

Figure 17: A schematic map of the 5 sampled primary schools in the Lake Albert region, with major towns Masindi and Hoima shown

After obtaining written informed consent and verbal assent (**Figure 18**), a pre-target of 60 children of equal gender aged between 5 and 10 years of age were enrolled and requested to provide two stool samples on consecutive days, a single urine sample and single finger-prick blood sample. Children were also interviewed with a standardised questionnaire to ascertain recent PZQ treatment history (**Appendix 3**). All participants were provided with a single PZQ (40mg/kg) treatment by the attending nurse following WHO guidelines (Montresor *et al.*, 1998). The Ugandan Council for Science and Technology and the Liverpool School of Tropical Medicine granted approval for this study.

Figure 18: Reflecting the enrollment of children in Biiso school for grade 1 to 3, and different steps performed following school-based survey protocol for NTDs control for each school (Montresor *et al.*, 1998). During the survey, **A**-Each participant 5-10-year-olds are interviewed together with parents or caregivers to determine symptoms and potential risk factors for parasitic infection using a standardised interview questionnaire. **B**-A participant information sheet and an informed consent form were provided to all eligible children, who enrolled into the study only after at least one parent provides consent as well as received health education sheet about schistosomiasis and STHs, transmission, ill effects and prevention. **C**- Participants provided faecal, urine and blood samples with numerical coding for each sample to be diagnosed for schistosomiasis, STHs and malaria under field conditions. **D**-All study participants in the following day, who are positive for parasitic infections offered anti-malaria, a single dose of PZQ (40mg/Kg), albendazole(600mg) tablet calculated according to the weight and height of each child (See **page** 34) (left) and the new dose poles – Ugandan model (center) and a model for the rest of Africa (right) with new height thresholds added to allow for treatment of PSAC (<6-year old). ¾ of a tablet division illustrated, as an example, for child need rather than a single tablet (Sousa-Figueiredo et *al.,* 2010a), and crushed into flavoured fruit syrup before administration

2.3.2 Diagnostics: faecal microscopy with Kato-Katz

Duplicate KK thick smear slides (41.7 mg templates) were prepared from each stool received after first sieving through a 212 µM metal mesh (Montresor *et al.*, 1998). Schistosome- eggs were viewed by microscopy (×100 magnification) (**Figure 19**), quantified and expressed as EPG of faeces with the intensity of infection classified as: light (1–99 EPG), medium (100–399 EPG) and heavy (⩾400 EPG) following the WHO guidelines (Montresor *et al.*, 1998). For quality control and validation, 10% of the slides were randomly selected and re-examined by a second senior technician. For later DNA analysis, a 0.8 g aliquot of

sieved stool was each prepared and stored in 95% ethanol before transportation to the UK for processing.

Figure 19: Preparation of a duplicate KK thick smear and reading slides at camp in the study location. All collected samples were transferred to field laboratory located in the camp in Bugoigo and processed as follows. First, **A**-A duplicate KK thick smear was prepared by VCD teams from each stool sample on microscope slides using 41.7 mg punched plastic templates. After a clearing time of 30-45 min, the slides were examined under a light microscope by experienced laboratory technicians. The numbers of schistosome-eggs and STHs (i.e. *A. lumbricoides* and *T. trichiura*) were counted and recorded separately (**Appendix 2**). For quality control, **B**-10% of the slides were re-examined by the senior technician from VCD and Hajri AL-shehri with assisting by Professor Russell. A result was assumed to be false-positive or false-negative if the result from the initial reading did not agree with the quality control as well as the third reading. Relationship between egg- and *S. mansoni* infection intensity. Egg-based infection categories are based on microscopy with standard cut-offs (Stothard *et al.*, 2017a): infections with 1–99 epg were classified as light-intensity, those with 100–399 epg as moderate, and those with ≥400 epg as heavy-intensity infections

2.3.3 Diagnostics: schistosome antigens urine-CCA dipsticks

The commercially available urine-CCA dipstick was used to test for schistosome antigens in each urine sample received following manufacturer's instructions (Rapid Medical Diagnostics, Pretoria, South Africa). The CCA test can detect active *S. mansoni* infection. After adding one drop of urine and one drop of the buffer to the cassette, the CCA antigen attaches to the monoclonal antibody on the membrane of the sample. An antigen-antibody complex forms as the liquid moves along with the cassette and fixes to another monoclonal antibody. If positive, a pink line appears on the strip. A further line is produced indicating the validity of the test (**Figure 20 A**). The test should be read after 20 minutes of adding the buffer. The strength of the pink line correlates to the intensity of schistosome infection. So, the test result was classified by visual

inspection against a colour chart as used previously (Sousa-Figueiredo *et al.*, 2013), by two individuals as negative, trace (±), light positive (+), medium positive (++) and heavy positive (+++) (**Figure 20 B**). In this setting, all trace reactions were later considered to be positive as justified previously upon biological causality and by prior epidemiological analyses (Standley *et al.*, 2010, Sousa-Figueiredo *et al.*, 2013, Adriko *et al.*, 2014).

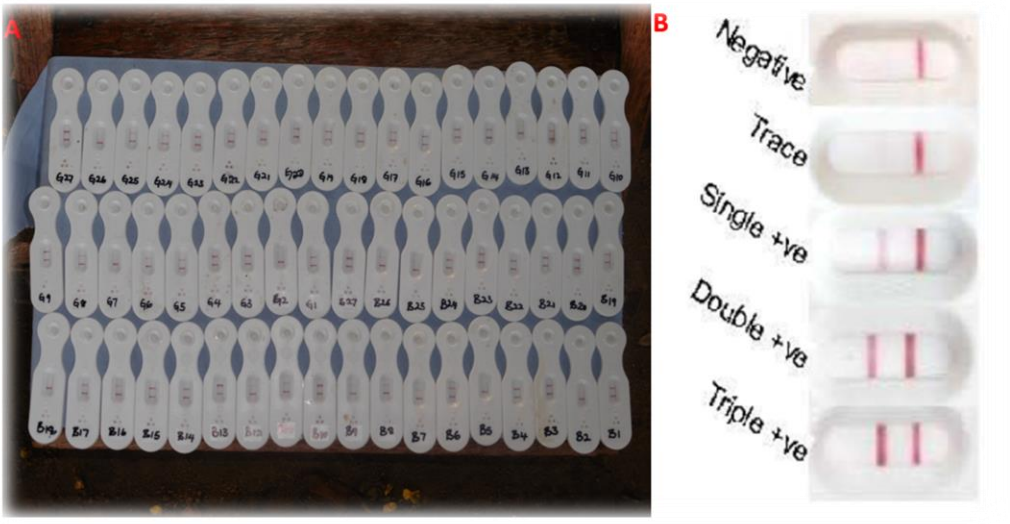

Figure 20: Detailed photograph of POC-CCA cassettes with control and result bands. To the left (**A**), POC-CCA shows endemic area for schistosomiasis infected SAC living in Runga village. To the right (**B**) POC-CCA shows classified visually into four infection status groups

2.3.4 Diagnostics: schistosome serology with SEA-ELISA

The SEA-ELISA is referred to as an IEDM (indirect egg detection method for diagnosis Schistosomiasis. A commercially available ELISA kit (IVD Inc.; Carlsbad, USA) was used to test for host antibodies (IgG/M) to soluble egg antigens (SEA) using a field-based ELISA test following manufacturer's instruction (**Appendix 1:** AccuDiag™ Schistosoma IgG ELISA Kit). A lancet was used to withdraw approximately 100 µl of a finger-prick blood sample from each child and stored in a 1.5ml Eppendorf tube at room temperature. After 2 hours, the samples were centrifuged for 3 minutes at 9,000 rpm, and serum from each child was harvested. 2 µl of harvested serum was then collected, and diluted in 2:80 with specimen dilution buffer before loading a total of 100 µl into each ELISA micro-well (**Figure 21 A**). Upon completion, the micro-titre plate was placed on a white card to view the visual colour of each reaction as graded into pale yellow (light positive), yellow (medium positive) and dark yellow (heavy positive), **Figure 21 B** and **C**, upon visual comparison with the control sera as recorded previously (Stothard *et al.*, 2009). Two laboratory technicians analysed the colour change to improve reliability.

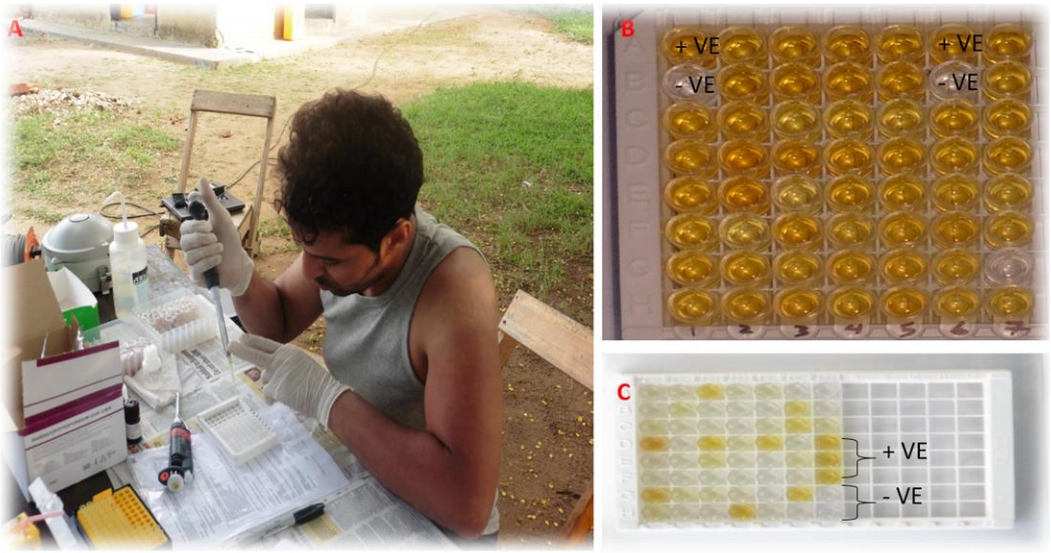

Figure 21: A-Performing a field-based ELISA test following manufacturer's instruction by Hajri Al-Shehri under supervision Professor.Russell. **B**- Demonstrating colour change (clear to yellow), reflecting schistosome antibody detection, and highlighting the high prevalence of *S.monsonia* detected by ELISA in high transmission setting (Runga) compared to **C**- low transmission setting (Busingiro)

2.3.5 DNA diagnostics: TaqMan® real-time PCR

After transfer to the UK, each aliquot of stool was spiked with PHV (PhHV-1) to act as an internal control for each DNA extraction and later real-time PCR assay for inhibition following protocols of Meurs *et al.* (Meurs *et al.*, 2015, Ten Hove *et al.*, 2008), which targetted a 77 base pair segment within the ribosomal internal transcribed spacer (ITS-2) region which can be identified using *S.mansoni* (GenBank: AF503487) as reference sequence (Meurs *et al.*, 2015, Obeng *et al.*, 2008). Schistosome DNA was detected with the Schistosoma-specific primers of Ssp48F (*5'GGT CTA GAT GAC TTG ATY GAG ATG CT'3*) and Ssp124R (*5'TCC CGA GCG YGT ATA ATG TCA TTA'3*) and TaqMan® probe Ssp78T (ROX -*TGG GTT GTG CTC GAG TCG TGGC*– Black Hole Quencher-3) as developed by (Meurs *et al.*, 2015, Obeng *et al.*, 2008). DNA-TaqMan® assays were performed in a Chromo-4 with Opticon monitor Version 3.1. (Biorad, Hemel Hempstead, UK) with Biorad iQ™ Supermix and thermal cycling conditions of 15 minutes at 95°C, followed by 50 cycles, each of 15 s at 95°C, 20 s at 60°C, and 25 s at 72°C. The infection intensity was classified according to C_t values: negative ($C_t > 45$), light positive ($35 > C_t \leqslant 45$), medium positive ($25 > C_t \leqslant 35$), and heavy positive ($C_t \leqslant 25$) (see **Figure 22**).

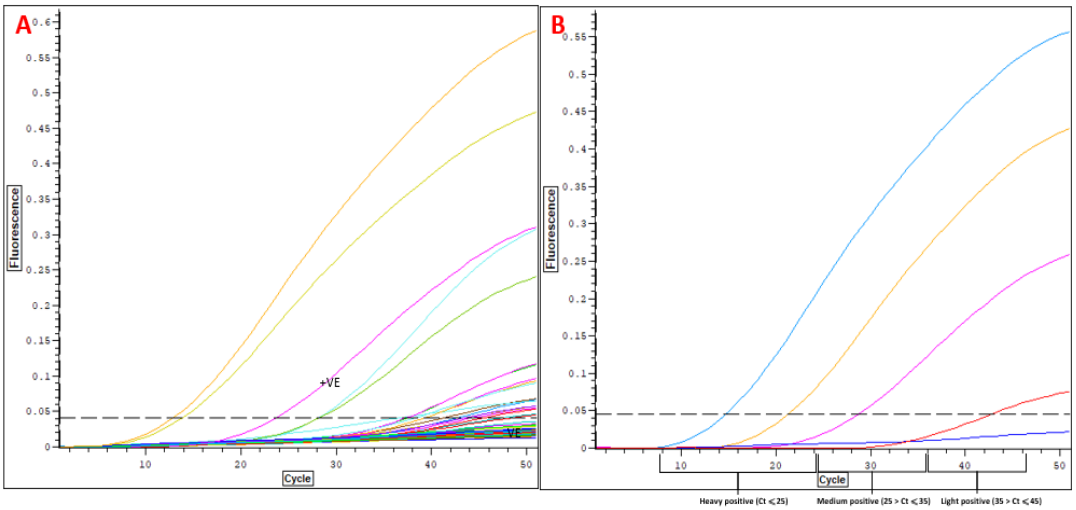

Figure 22: TaqMan® probe-based detection of *S. mansoni* -DNA with categorising infection intensity based on the cycle threshold (C_t) as described in the above text. In Graph **A**, qRT-PCR were performed on stool samples from Busingiro children, 4.0% (2/50) of children with heavy infection, 4.0% (2/50) with moderate, and 16.0% (8/50) with light infection based on the cycle threshold (C_t) value of the Schisto-PCR plus positive control (+VE). In Graph **B**, RT-PCR-based infection categories are based on known egg counts samples by microscopy with standard cut-offs: sample with 72 epg was classified as light-intensity (C_t-43), a sample with 200 epg as moderate (C_t-29), and sample with 600 epg as heavy-infection (C_t-23). The solid line indicates the C_t-value for each sample individually with standard deviation +/-5 being adjusted using cut-off value C_t-20 heavy, 30 moderate, and 40 light infections for pairwise comparisons with multiple testing sample

2.3.6 Data management and statistical analysis

All data collected in the field and processed in the laboratory were recorded on proforma data sheets (**Appendix 2** and **Appendix 3**). These were then double entered in Microsoft Excel prior to the generation of summary tables for prevalence and intensity of infection (**Table 8** and **Table 9**). Empirical estimates of sensitivity, specificity, NPV and PPV was calculated in R statistical package v 2·10·1 (The R Foundation for Statistical Computing, Vienna, Austria) and SPSS software (v 24.0, SPSS Inc., IBM, USA) assuming the urine-CCA as the 'gold' standard against the remaining three diagnostic tests (**Table 10**). For percentage values, 95% confidence intervals (95% CI) were estimated using the exact method (Armitage *et al.*, 1994). We have decided to assume the urine-CCA as the gold standard for our descriptive analyses (i.e. empirical estimates of diagnostic performance), since there have been extensive evaluations of urine-CCA dipsticks (Colley *et al.*, 2013) and WHO recommendation of its use in surveillance mapping (Danso-Appiah *et al.*, 2016).

Subsequently, to tackle the inherent problems with diagnostic measurement error, we employed an LCA and full information maximum likelihood estimation (**Table 11**). LCA allows grouping of categorical data (in the current study

not infected and infected from the diagnostic tests under examination) into latent classes indicating *S. mansoni* infection via a probability model. Given the well-known epidemiological landscape of Lake Albert region, such a model was designed to allow LCA estimated prevalence of *S. mansoni* to vary by school (**Table 11**). Through this approach, model-based estimates of sensitivity and specificity across diagnostic tests without assuming a gold standard were also obtained. The classification certainty of this model was evaluated through entropy; values of entropy near one indicate high certainty in classification while values near zero indicate low certainty (Celeux and Soromenho, 1996). LCA assumes the relationships between the observed variables (i.e. diagnostic tests in the current study) are accounted for by their class membership and thus conditioning on class membership (i.e. the disease status in the current study) such that if the model estimated disease status is misclassified by one test, the probability that it will be misclassified by another test would not be affected. We assessed this assumption by speculating the standardised residuals for each response pattern from the diagnostic tests as estimated from the LCA model. Further technical details of these models in the context of schistosomiasis have been described elsewhere, and thus they are not repeated here (Ibironke *et al.*, 2012). The LCA model was fitted using MPlus version 7.3 (Muthén and Muthén, 2015).

2.4 Results

2.4.1 Prevalence of intestinal schistosomiasis

A total data set was assembled from 258 children with a prevalence of intestinal schistosomiasis by each diagnostic test presented, see **Table 8**. Overall prevalence of intestinal schistosomiasis was: 44.1% (95% CI; 38.0–50.2) by microscopy of duplicate KK smears from two consecutive stools, 56.9% (95% CI; 50.8–63.0) by urine-CCA dipstick, 75.1% (95% CI; 69.8–80.4) by SEA-ELISA, and 67.4% (95% CI; 61.6–73.1) by DNA-TaqMan® assay.

Table 8: Prevalence (%) of *S.mansoni* by school according to each diagnostic test with 95 % confidence intervals

Diagnostic method	School name					
	Walakuba (n=50) % [95% CI]	Runga (n=50) % [95% CI]	Bugoigo (n=58) % [95% CI]	Biiso (n=50) % [95% CI]	Busingiro (n=50) % [95% CI]	Total (n=258) % [95% CI]
Kato-Katz	70.0 [56.8-83.1]	86.0 [76.0-95.9]	37.9 [25.0-50.8]	20.0 [8.5-31.4]	16.0 [0.2-15.7]	44.1 [38.0-50.2]
Urine-CCA dipstick	78.0 [66.1-89.8]	100 [NA]	55.1 [41.9-68.3]	38.0 [24.0-51.9]	14.0 [4.0-23.9]	56.7 [50.8-63.0]
SEA-ELISA	94.0 [87.1-100.8]	96.0 [90.3-101.6]	81.0 [70.6-91.4]	60.0 [45.9-74.0]	44.0 [29.7-58.2]	75.1 [69.8-80.4]
DNA-TaqMan	84.0 [73.4-94.5]	92.0 [84.2-99.7]	82.7 [72.7-92.7]	52.0 [37.6-66.3]	24.0 [10.1-33.8]	67.4 [61.6-73.1]
Positive by any test	98.0 [93.9-102.0]	100 [NA]	94.8 [88.9-100.0]	76.0 [63.7-88.2]	54.0 [39.6-68.3]	84.8 [80.4-89.2]

As was expected the prevalence of infection at Runga and Walakuba was observed to be highest locally, exceeding 50% in all methods, whereas the prevalence of infection at Busingiro was lowest falling well short of 50% by any methods. However, pooling infection status by being positive by any test revealed that just under half of the children attending this school could be considered to be 'free' from infection. For the total data set only just over a quarter of children (n=69) could be considered to have no evidence of intestinal schistosomiasis demonstrating the pervasiveness of intestinal schistosomiasis in the Lake Albert region.

2.4.2 Prevalence of *S. mansoni* by school distance from Lake Albert

The geographical proximity of each of the five schools to Lake Albert shoreline is depicted in schematic in **Figure 23 A**; on-the-ground shortest distance to the lake shoreline can be ranked in the following order of Walakuba (0.2 km), Runga (0.4 km), Bugoigo (0.9 km), Biiso (9.4 km) and Busingiro (13.2 km)(**Figure 23 B**). Notably, both Runga and Bugoigo schools are located for safety and convenience on slightly higher ground behind each village so as not to flood, which during wetter periods has detrimentally affected Walakuba in the past (**J.R.S**., personal observation).

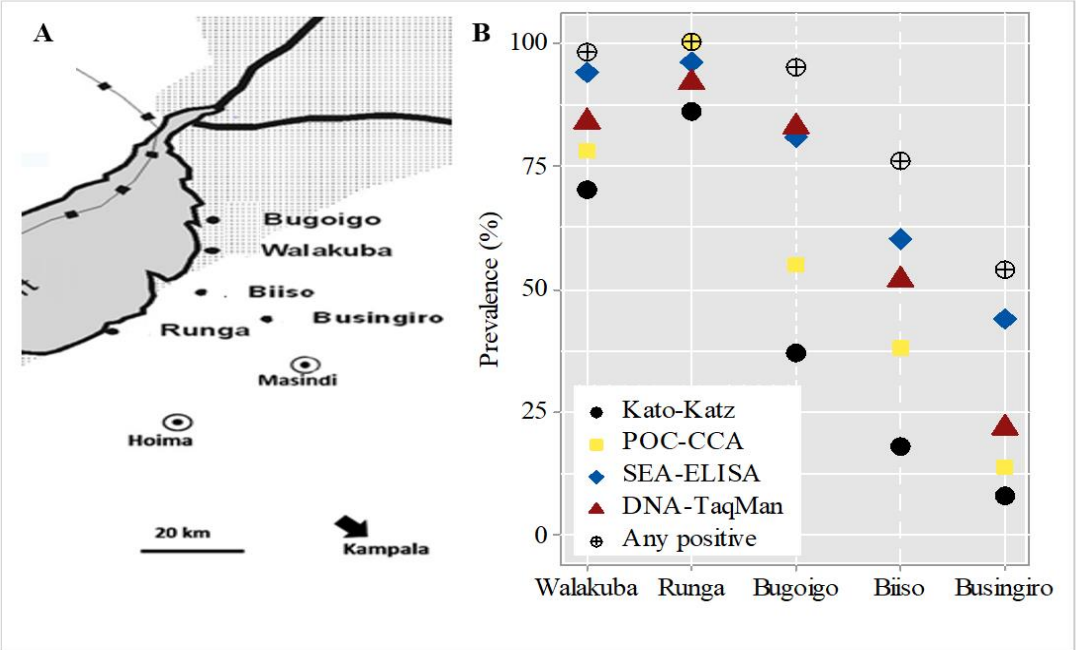

Figure 23: Prevalence of *S. mansoni* by school distance for each diagnostic method, and by any positive test criterion. **A,** schematic map of the 5 sampled primary schools in the Lake Albert region, with major towns Masindi and Hoima shown. The grey area is Lake Albert while the grey hatched area is the lowland plain where inflowing and outflowing Victoria and Albert Nile rivers, respectively make their course. **B.** Prevalence of *S. mansoni* by school for each diagnostic method, typically reveals a wide range of prevalence by a method for each location

2.4.3 Infection intensity by school distance from Lake Albert

While diagnostic comparisons are made on the basis of binary associated with endemic setting data, it is worth noting that infection intensity also varied by school setting, in that 'heavy intensity' infections or 'strong positive' by any test were particularly common at Runga, but were rare at Busingiro, **Table 9**. As shown in **Figure 23 B**, the changing prevalence by school for each method is apparently visible in that the prevalence of inferred from each diagnostic test typically followed a static ascending order or monotonic series of KK, urine-CCA dipstick, SEA-ELISA, and DNA-TaqMan® Assay although the relative position of the estimated prevalence by urine-CCA at Runga slightly exceeds SEA-ELISA and DNA-TaqMan® Assay.

Table 9: The prevalence with intensity measured by each diagnostic method for *S. mansoni* across the five primary schools

Method and measure of intensity	School name					
	Wala-kuba (N=50)	Runga (N=50)	Bugoigo (N=58)	Biiso (N=50)	Busingiro (N=50)	
	n (%)	n (%)	n (%)	n (%)	n (%)	In total (%)
Kato-Katz[a]						
negative	15 (10.4)	7 (4.8)	36 (25.0)	40 (27.7)	46 (31.9)	144 (55.8)
light (< 100 epg)	5 (15.6)	7 (21.8)	16 (50.0)	3 (9.3)	1 (3.1)	32 (12.4)
medium (100 – 400 epg)	10 (40.0)	5 (20.0)	3 (12.0)	5 (20.0)	2 (8.0)	25 (9.7)
heavy (> 400 epg)	20 (35.0)	31 (54.3)	3 (5.2)	2 (3.5)	1 (1.7)	57 (22.1)
Urine-CCA						
negative	11 (9.9)	0 (NA)	26 (23.4)	31 (27.9)	43 (38.7)	111 (43.0)
light (+, incl. trace)	5 (11.1)	10 (22.2)	19 (42.2)	7 (15.5)	4 (8.8)	45 (17.4)
medium (++)	6 (20.6)	9 (31.0)	7 (24.1)	6 (20.6)	1 (3.4)	29 (11.2)
heavy (+++)	28 (38.3)	31 (42.4)	6 (8.2)	6 (8.2)	2 (2.7)	73 (28.3)
SEA-ELISA)						
negative	3 (4.6)	2 (3.1)	11 (17.1)	20 (31.2)	28 (43.7)	64 (24.8)
light (+, incl. trace)	4 (8.0)	3 (6.0)	14 (28.0)	16 (32.0)	13 (26.0)	50 (19.3)
medium (++)	33 (33.6)	19 (19.3)	27 (27.5)	11 (11.2)	8 (8.1)	98 (37.9)
heavy (+++)	10 (21.7)	26 (56.5)	6 (13.0)	3 (6.5)	1 (2.1)	46 (17.8)
DNA TaqMan®						
negative (Ct > 45)	8 (9.5)	4 (4.7)	10 (11.9)	24 (28.5)	38 (45.2)	84 (32.5)
light (35 > Ct ≤ 45)	4 (7.6)	2 (3.8)	20 (38.4)	18 (34.6)	8 (15.3)	52 (20.1)
medium (25 > Ct ≤ 35)	19 (35.1)	9 (16.6)	19 (35.1)	5 (9.2)	2 (3.7)	54 (20.9)
heavy intensity (Ct ≤ 25)	19 (27.9)	35 (51.4)	9 (13.2)	3 (4.4)	2 (2.9)	68 (26.3)

2.4.4 Urine-CCA as an arbitrary gold standard

Assuming the urine-CCA as an arbitrary gold standard, the diagnostic performance for the three remaining tests is shown along with diagnostic accuracy and Cohen's kappa statistic, **Table 10**. The sensitivity of SEA-ELISA is the highest (96.6%) but also has the lowest specificity (53.2%), with the highest NPV of all methods. By contrast, the sensitivity of KK is the lowest (76.9%) but also has the highest specificity (99.1%), with the highest PPV of all methods.

Table 10: Diagnostic comparison of sensitivity (SS), specificity (SP), negative predictive value (NPV) and positive predictive value (PPV) of each method against urine-CCA dipstick as 'gold standard'

	Urine-CCA as reference 'gold standard'						
Evaluating Diagnostic test	**Negative (%)**	**Positive (%)**		**Measurement estimate % [95% CIs]**		**Diagnostic accuracy % [95% CIs]**	**Cohen's kappa [95% CIs]**
DNA-TaqMan®			Total (%)				
negative	62 (24.0)	22 (8.5)	84 (32.6)	Sensitivity	85.0 [78.4-89.9]	72.5 [66.7-77.6]	0.4 [0.3-0.5]
positive	49 (18.9)	125 (48.5)	174 (67.4)	Specificity	55.9 [46.6-64.7]		
total (%)	111 (43.0)	147 (57.0)	258 (100.0)	PPV	71.8 [64.7-77.9]		
				NPV	73.8 [63.5-82.0]		
SEA-ELISA							
negative	59 (22.9)	5 (1.9)	64 (24.8)	Sensitivity	96.6 [92.3-98.5]	77.9 [72.5-82.5]	0.5 [0.4-0.6]
positive	52(20.2)	142 (55.0)	194 (75.2)	Specificity	53.2 [43.9-62.2]		
total (%)	111 (43.0)	147 (57.0)	258 (100.0)	PPV	73.2 [66.6-78.9]		
				NPV	92.2 [82.9-96.6]		
Kato-Katz							
negative	110 (42.6)	34 (13.2)	144 (55.8)	Sensitivity	76.9 [69.2-82.9]	86.4 [81.7-90.1]	0.7 [0.6-0.9]
positive	1 (0.4)	113 (43.8)	144 (44.2)	Specificity	99.1 [95.1-99.8]		
total (%)	111 (43.0)	147 (56.9)	258 (100.0)	PPV	99.1 [95.2-99.8]		
				NPV	76.4 [68.8-82.6]		

2.4.5 Empirical and LCA modelling of estimates of diagnostic performance

On the basis of LCA analysis, the sensitivity and specificity of each method can be estimated on the basis of their latent class assignment which highlights the trade-off between diagnostic specificity (i.e. false positive) and sensitivity (i.e. false negative). In this analysis, sensitivity and specificity of SEA-ELISA and urine-CCA are broadly equivalent with DNA-TaqMan® appearing to have slightly lower sensitivity and specificity. Estimating the prevalence of infection by school with LCA, **Table 11**, reveals a lower prevalence than that on the basis of positivity by any test but follows the same static ascending order or monotonic series (**Figure 23 B**). It is evident that at Runga intestinal schistosomiasis is universal whereas at Busingiro around 9.6% of children are suspected of harbouring infections.

Table 11: Latent class analysis (LCA) estimates of sensitivity and specificity and LCA model of the prevalence of *S. mansoni* by school with 95 % CIs for each diagnostic method

Diagnostic method	Sensitivity	Specificity
Kato-Katz	84.4 % [76.0–92.9]	100 % [NA]
Urine-CCA	99.1 % [97.3 –100]	89.3 % [80.9 – 97.6]
SEA-ELISA	97.7 % [95.1– 100]	49.5 % [39.4 –59.6]
DNA-TaqMan®	90.2 % [84.2 - 96.2]	57.5 % [48.6 – 66.5]
Prevalence by school by LCA model		
Walakuba	75.7 % [62.9 – 88.5]	
Runga	100.0 % [NA]	
Bugoigo	49.7 % [35.0 – 64.5]	
Biiso	27.0 % [12.2– 41.9]	
Busingiro	9.6 % [9.0 – 18.4]	

The LCA model generated similar sensitivity for SEA-ELISA and urine-CCA but was slightly lower for DNA-TaqMan®, Table 4. KK was again shown through LCA to have the highest specificity among all the four tests. The specificity of 89.3% (95% CI; 80.9–97.6) for the urine-CCA test was acceptable but for the SEA-ELISA and the DNA-TaqMan, specificities were less so and estimated to be 49.5% (95% CI; 39.4–59.6) and 57.5% (95% CI; 48.6–66.5), trending as with empirical calculations, see **Table 11**. Furthermore, LCA estimated infection prevalence of *S. mansoni* by the school to be lower than that on the basis of positivity by any test (for the latter see **Figure 23 B**). Nevertheless, both of these approaches suggested that intestinal schistosomiasis was universal at Runga, however, at Busingiro LCA estimated a prevalence of *S. mansoni* infection to be 9.6% (95% CI; 9.0–18.4), much lower than that revealed by positivity upon any test.

Finally, the entropy of the LCA model was estimated to be 0.921. This indicated a clear delineation of classes in the fitted model standardised residuals for each response pattern from the four diagnostic tests from this model were between −2 and 2, evidencing that local independence of the four diagnostic tests is not obviously violated.

2.5 Discussion

2.5.1 Current reference standard, Kato–Katz thick smear

Owing to the complicated developmental and population biology of the schistosome within the mammalian host, it is well known that accurate detection of intestinal schistosomiasis by any biomarker can be problematic and has been the topic of at-length discussions previously (Bergquist *et al.*, 2009, Stothard *et al.*, 2014, Utzinger *et al.*, 2015). Foremost, the insensitivity of the KK, especially in the detection of light egg-patent infections or in patients with a recent history of PZQ treatment, is perhaps the most obvious obstacle to overcome (Kongs *et al.*, 2001, Koukounari *et al.*, 2013, Leuenberger *et al.*, 2016).

Indeed, how we debate and assess the significance of egg-negative infections is changing alongside measuring morbidity associated with intestinal schistosomiasis which goes beyond what KK assessments can offer (King, 2015). Nevertheless, KK can still be promoted as a field-applicable standalone and appropriate in high-endemic settings, as seen here in both Runga and Walakuba, where prevalence and intensity of infection were high. Nonetheless, KK has several deficits when applied to lower transmission settings, as exemplified by the other schools sampled here and is more misleading perhaps than informative. To compensate, De Vlas *et al*. (1993) developed a useful corrective prevalence chart which took into account infection intensity; however, its uptake was not as good as anticipated (De Vlas *et al.*, 1993). It is also outside the scope of the present paper to discuss economic cost-benefit of faecal microscopy (Meurs *et al.*, 2015) other than that mobile microscopy with handheld devices offers some attractive cost-saving solutions for surveillance of intestinal schistosomiasis in high-endemic areas (Stothard *et al.*, 2005, Bogoch *et al.*, 2014). However, as control programmes move forward towards elimination, the KK methodology will be inappropriate and will be unable to provide sufficient quality epidemiological information for precision mapping of disease foci (Tchuenté *et al.*, 2017, French *et al.*, 2018, Molyneux *et al.*, 2018, Weerakoon *et al.*, 2018).

The latter is pivotal in the local intensification of delivery of treatments and surveillance interventions to confirm interruption of transmission (Rollinson *et al.*, 2013, Stothard *et al.*, 2017a, Bergquist *et al.*, 2017). Indeed, from the information reported here, we would suggest that control efforts in locations such as Busingiro should be intensified rather than reduced.

2.5.2 Alternative technique for diagnosing schistosomiasis

Of the remaining diagnostic methods, the diagnostic pros and cons of each method have been discussed elsewhere often using the ASSURED framework (Bergquist *et al.*, 2009, Stothard *et al.*, 2014, Utzinger *et al.*, 2015). DNA-TaqMan® methods are, however, increasingly gaining favour and offer a multiplex DNA-platform for co-detection of several NTDs as well as many other infectious agents (Verweij and Stensvold, 2014, Llewellyn *et al.*, 2016, Maksimov *et al.*, 2017, Verweij *et al.*, 2007, Won *et al.*, 2016); much more so than other any other current biomarker method can provide (Ten Hove *et al.*, 2008, Solomon *et al.*, 2012, Mejia *et al.*, 2013, Easton *et al.*, 2016). There also is the suggestion that DNA-TaqMan® could become an acceptable 'gold' standard (Meurs *et al.*, 2015, Weerakoon *et al.*, 2018), and whilst we ultimately share some enthusiasm in this there are some impediments to discuss. Foremost, DNA-TaqMan® requires specialist equipment and is not currently amenable to point-of-contact settings although there is growing interest in the use of more field-friendly methods (Minetti *et al.*, 2016, Weerakoon *et al.*, 2018), such as loop-mediated isothermal amplification (LAMP) (He *et al.*, 2016) and recombinase polymerase amplification (RPA) (Rosser *et al.*, 2015). Nonetheless, from our results here the DNA-TaqMan® has been somewhat outperformed upon consideration of **Table 8**. In our opinion, perhaps the most significant advantage of DNA-based platform is that DNA-TaqMan® assays can cross-over into environmental monitoring through detection of environmental(e) DNA and therefore broaden the vision of schistosomiasis control in general potentially uniting clinical and environmental surveillance (Rollinson *et al.*, 2013, Stothard *et al.*, 2017a, Stothard *et al.*, 2017b).

2.5.3 LCA-model for evaluation of diagnostic tests

In the absence of a 'gold' standard diagnostic test and complexity of the changing epidemiological landscape in which tests are being applied in Uganda (Standley *et al.*, 2010, Adriko *et al.*, 2014, Al-Shehri *et al.*, 2016), our analysis presented in **Table 10** postulated that urine-CCA dipsticks could be an 'error-free' standard which, in **Table 11**, was further explored by LCA. Here the probabilistic statistical model applied does not assume any 'gold' standard and therefore points towards the urine-CCA as having near-optimal diagnostic scores of sensitivity (99.1%) and specificity of (89.3%). Moreover, these scores are significantly better than those reported previously by empirical comparisons (Stothard *et al.*, 2006) and illustrate how advances in statistical modelling developed elsewhere on urine-CCA dipsticks (Knopp *et al.*, 2015, Koukounari *et al.*, 2013) can provide a deeper insight into diagnostic score evaluations over

and above simple empirical calculations (Colley *et al.*, 2013, Danso-Appiah *et al.*, 2016).

Nonetheless, a theoretical issue of adopting LCA-models exclusively is an assumption of independence of tests which, given the biological biomarkers employed here could be somewhat confounded; KK detects eggs directly, DNA-TaqMan® measures Schistosoma-DNA in the stool (presumably from excreted eggs), and SEA-ELISA detects antibodies to secreted egg-antigens. Thus, these three methods are somewhat interrelated to similar biomarkers of the egg itself although will have each have differing physical, biochemical and physiological components **Table 9**. However, the urine-CCA dipstick is less directly connected to egg biomarkers for it utilises carbohydrate-antigens released from feeding worms of either sex, and hence offers an alternative biomarker appraisal. Since violations of the conditional independence assumption can lead to biased LCA estimates of accuracy and prevalence, performing and reporting checks of whether assumptions are met is essential which was why we compared LCA estimates of diagnostic performance with empirical ones, concluding each of the diagnostic tests used. In addition, speculation of standardised residuals from the LCA model indicated that the assumption of local independence of the four diagnostic tests under examination was not obviously violated.

2.5.4 Field applicability-assured urine-CCA diagnostic test

Over and above the routine diagnostic scores of sensitivity, specificity, negative and PPVs with or without LCA models, however, it is also necessary to further consider each diagnostic tool against the ASSURED criteria (Kosack *et al.*, 2017). This seeks to understand whether a diagnostic test can be used at scale and is ultimately useful in several clinical and epidemiological surveillance settings (Mabey *et al.*, 2004, Peeling *et al.*, 2006, Stothard and Adams, 2014). The roll-out of the urine-CCA test has been discussed previously (Stothard, 2009), and it is pleasing to see it become further endorsed at the policy level (Danso-Appiah *et al.*, 2016). The most desirable features of this test are its affordability, stable commercial production, the use of urine-sampling, the speed of analysis and a short time to obtain results which has a very pragmatic consideration for the end-user in this emphasis. All of the above potentially make the dissemination of epidemiological results back to the local community obtained by the urine-CCA dipstick much quicker, which is vital to increase local ownership of preventive chemotherapy campaigns in future (Tchuenté *et al.*, 2017).

2.6 Concluding Remarks

The study has shown that intestinal schistosomiasis continues to be a public health challenge on the shoreline of Lake Albert which now presents as a heterogenous epidemiological landscape of high- and low-transmission settings. A total of four diagnostic tests were each assessed regarding contemporary surveillance for intestinal schistosomiasis finding that KK sampling is a satisfactory diagnostic standalone in high-transmission settings but in low-transmission settings should be augmented or replaced by urine-CCA dipsticks. DNA-TaqMan® appears suitable in both endemic settings though is only implementable if resources permit. In low-transmission settings, SEA-ELISA remains the method of choice to evidence an absence of infection. In the dearth of a diagnostic 'gold' standard for intestinal schistosomiasis, LCA offered useful computations of diagnostic performance between tests.

Acknowledgement

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Authors' contributions: HAl-S, JRS, NBK conceived the study with further participation in cross-sectional surveys by JElaC, AA, MAr, AW and MAd. Real-time PCR assays were performed by Hal-S with assistance from JElaC and JRS. Data were tabulated by HAl-S and analysed with the support of MCS and JRS. The models for latent class analysis were developed by AK upon discussion with Hal-S. All authors contributed to the drafting and revision of the manuscript with JRS as guarantor.

Competing interests: None declared.

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Ethical approval: The study was approved by the Liverpool School of Tropical Medicine and Uganda National Council for Science and Technology.

2.7 Appendix

Appendix 1: AccuDiag™ Schistosoma IgG ELISA Kit adapted 11-11 2015, omline access (http://www.rapidtest.com/pdf/Schistosoma%20IgG%20_8209-35(11-11-2015).pdf)

CE

Diagnostic Automation / Cortez Diagnostics, Inc.
IMMUNO DIAGNOSTICS

AccuDiag™ Schistosoma IgG ELISA Kit

REF 8209-35

IVD See external Label 96 Tests

Test	Schistosoma IgG ELISA
Method	Enzyme Linked Immunosorbent Assay
Principle	Sandwich Complex
Detection Range	Qualitative : Positive, Negative
Sample	5 µL serum
Total Time	~ 25 min.
Shelf Life	12 Months from the manufacturing date
Specificity	85%
Sensitivity	100%

INTENDED USE

The Schistosoma ELISA Kit is For the qualitative determination of serum antibodies in humans, primarily IgG, to Schistosoma spp. using the ELISA technique.

SUMMARY AND EXPLANATION

Schistosomiasis is a disease caused by parasitic worms of the genus Schistosoma. People are at risk for Schistosomiasis infection when they are exposed to the parasites, normally by bathing or swimming in contaminated water. Infection is transmitted when larvae from the parasite (usually freshwater snails) penetrates the skin and travels through the bloodstream. Acute and chronic symptoms result when the egg migration of the worms affects vital tissue and organs. Some affected people may be asymptomatic, but generally symptoms range skin irritation, to fever, intestinal and urinary tract infections, to possibly life-threatening complications. The disease has affected more than 200 million people worldwide, and has been classified as the second most common tropical disease following Malaria.

TEST PRINCIPLE

During the first incubation, the antibodies in the patients' serum bind to the antigens in the test well. The next incubation allows the enzyme complex to bind to the antigen-antibody complex. After a few washings to remove unbound enzymes, a substrate is added that develops a blue color in the presence of the enzyme complex and peroxide. The stop solution ends the reaction, turning the blue assay color to yellow.

SPECIMEN COLLECTION AND PREPARATION

Coagulate blood and remove serum. Freeze sample at -20 °C or lower if not used immediately.

Do not heat inactivate serum.
Avoid repeated freezing and thawing of samples.

MATERIALS AND COMPONENTS

Materials provided with the test kits

1. **Plate:** Microwells containing *Schistosoma* SEA antigens - 96 test wells in a test strip holder.
2. **Enzyme Conjugate**: One (1) bottle containing 11 ml of Protein A Peroxidase (HRP) in a stabilizing buffer with Thimerosal.
3. **Positive Control**: One (1) vial containing 1 ml of diluted rabbit *Schistosoma*-positive sera in buffer with Thimerosal.
4. **Negative Control:** One (1) vial containing 1 ml of diluted *Schistosoma*-negative human sera in buffer with Thimerosal.
5. **TMB Substrate Solution:** One (1) bottle containing 11 ml of the TMB tetramethylbenzidine (TMB).
6. **Wash Concentrate 20X**: One (1) bottle containing 25 ml of concentrated buffer and surfactant with Thimerosal.
7. **Dilution Buffer:** Two (2) bottles containing 30 ml of buffered protein solution with Thimerosal
8. **Stop Solution**: One (1) bottle containing 11 ml of 1 M phosphoric acid.

Materials required but not provided

1. Pipettes
2. Squeeze bottle for washing strips
3. DI water
4. Tubes for serum dilutions
5. ELISA plate reader with a 450 nm and a 620-650 nm filter (optional if results are read visually).

Preparation

Wash Buffer - Remove cap and add contents of bottle to 475 ml DI water. Place diluted wash buffer into a squeeze bottle.
Note: Washings consist of filling to the top of each well, shaking out the contents and refilling.
Avoid generating bubbles in the wells during the washing steps.

Test samples: Make a 1:40 dilution of patients' sera using the dilution buffer.

ASSAY PROCEDURE

1. Break off number of wells needed (two for controls plus number of samples) and place in strip holder.
2. Add 100 µl of negative control to well #1, 100 µl of positive control to well #2, and 100 µl of the diluted (1:40) test samples to the remaining wells.
   Note: Negative and positive controls are supplied as prediluted. Do not dilute.
3. Incubate at room temperature (15 °C to 25 °C) for 10 minutes.
4. Shake out contents and wash 3 times with diluted wash buffer.*
5. Add 2 drops of enzyme conjugate to each well.
6. Incubate at room temperature for 10 minutes.
7. Shake out contents and wash 3 times with wash buffer.
8. Add 2 drops of Chromogen to every well.
9. Incubate at room temperature for 5 minutes.
10. Add 2 drops of stop solution.
11. Zero ELISA reader on air, read wells at 450 nm with a reference filter at 620-650 nm or read results visually.

* Washings consist of using the diluted wash buffer to fill to the top of each well, shaking out the contents and refilling the wells for a total of 3 times.

Avoid generating bubbles in the wells during the washing steps.

Controls must be included each time the kit is run.

Diagnostic Automation/ Cortez Diagnostics, Inc.
21250 Califa St, Suite 102 and 116, Woodland Hills, CA 91367 USA Phone: 818-591-3030, Fax : 818-591-8383
Email: onestep@rapidtest.com Website: www.rapidtest.com

***CAUTION! WHEN USING AN AUTOMATED OR SEMI-AUTOMATED WASHING SYSTEM THE FOLLOWING MUST BE FOLLOWED. FAILURE TO DO SO WILL RESULT IN INADEQUATE WASHING OF THE WELLS AND MAY LEAD TO FALSE POSITIVE RESULTS!**

Washing Procedure for Auto and Semi-Automated Washers

- **Perform five (5) washes per step instead of three**
- **Set machine to "soak" for one minute between each step**
- **After each set of washings, slap wells against an absorbent towel.**

RESULTS

Spectrophotometer:
Zero ELISA reader on air. Read all wells using a bichromatic reading with filters at 450 nm and 620-650 nm.
Positive - Absorbance reading greater or equal to 0.2 OD units.

Negative - Absorbance reading less than 0.2 OD units.

Interpretation of Results -Visual
A sample should be interpreted as positive if the degree of color development is obvious and significant.

Troubleshooting

Problem: Negative control has substantial color development.
***Correction*:** Inadequate washings. Rerun test with more vigorous washings.

QUALITY CONTROL

The use of a positive and negative control allows easy validation of kit stability. For a valid test, the positive control must be over 0.5 OD units and the negative control must be under 0.2 units. Should the values fall outside these ranges, the kit should not be used.

PERFORMANCE CHARACTERISTICS

		Reference Method *	
		+	-
Diagnostic Automation,Inc.	+	12	6
	-	0	34

Positive Agreement: 100% (12/12)
Negative Agreement: 85% (34/40)

*Reference Method refers to a commercially available ELISA.

LIMITATIONS OF PROCEDURE

Serological results should be used as an aid in diagnosis and should not be interpreted as diagnostic by themselves.

PRECAUTIONS

1. Do not use solutions if they precipitate or become cloudy.
2. Wash concentrate may show crystallization upon storage at 4 °C. Crystallization will disappear after diluting to working strength.
3. Do not use serum that may have supported microbial growth, or is cloudy due to high lipid content. Samples high in lipids should be clarified before use.
4. Do not add azides to the samples or any of the reagents.
5. Controls and some reagents contain Thimerosal as a preservative.
6. Treat all sera as if capable of being infectious.
7. The negative control has been tested and found negative for Hepatitis B surface antigen and for the antibody to HIV by required test methods. Since no test can offer complete assurance that infectious agents are not present, this product should be used under appropriate safety conditions that would be used for any potentially infectious agent.

STORAGE

1. Reagents, strips and bottled components should be stored at 2-8 °C
2. Squeeze bottle containing diluted wash buffer may be stored at room temperature.

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ISO 13485
ISO 9001

Diagnostic Automation/Cortez Diagnostics, Inc.
21250 Califa Street, Suite 102 and 116, Woodland Hills, California 91367 USA

Date Adopted	**2016-10-05**
REF **8209-35**	**AccuDiag™- Schistosoma IgG ELISA**
EC REP	**CEpartner4U, Esdoornlaan 13, 3951DB Maarn. The Netherlands.** www.cepartner4u.eu

Revision Date: 2015-02

Appendix 2: Manual data collection sheet used to collate diagnostic information

ID	RDTs		FOB	CCA	SEA-ELISA	Kato Katz								Direct Smear	
	Giardia	Crypto				SCH		T.t.		A.l.		Hkm		Others	
						A	B	A	B	A	B	A	B	A	B
AM1															
AM2															
AM3															
AM4															
AM5															
AM6															
AM7															
AM8															
AM9															
AM10															
AM11															
AM12															
AM13															
AM14															
AM15															
AM16															
AM17															
AM18															
AM19															
AM20															

Appendix 3: Example of a hardcopy of the questionnaire used in the study

Registration

1. Survey date

   .....

2. School ID

   - ☐ A
   - ☐ B
   - ☐ C
   - ☐ D
   - ☐ E

3. Name

   .....

4. Age

   .....

5. ☐ Male
   ☐ Female

6. Student ID

   .....

Student information

7. Height (cm)

   .....

8. Weight (kg)

   .....

9. Under arm temperature (°C)

   .....

Previous treatment information

10. Have you ever had *Bilharzia*?

    ☐ Yes ☐ No

11. If yes, what treatment were you given?

    - ☐ PZQ
    - ☐ ALB
    - ☐ Other
    - ☐ Don't know
    - ☐ Not applicable

12. Do you have blood in stool?

    ☐ Yes ☐ No

13. Do you have blood in urine?

    ☐ Yes ☐ No

14. Please specify which other treatments have been received .....

Location

15. How far do you live from school (in minutes)? .....

16. Have you ever lived anywhere else?
    - ☐ Yes
    - ☐ No

17. Where have you previously lived? .....

18. When did you move here? .....

Visited other areas

19. Have you left the area recently?
    - ☐ Yes
    - ☐ No

20. If yes, where did you visit? .....
21. If yes, when did you visit these areas? .....

Water source information

22. Do you have access to tap/pumped water?
    - ☐ Yes
    - ☐ No

23. Do you use other water sources e.g. for drinking, bathing, swimming?
    - ☐ Lake
    - ☐ Pond
    - ☐ River
    - ☐ Rain
    - ☐ Other

Lake use information

24. Which part of the lake? .....
25. Lake water uses:
    - ☐ Drinking
    - ☐ Washing/bathing
    - ☐ Swimming
    - ☐ Preparing food
    - ☐ Washing clothes/dishes

26. How frequently do you use the lake?

☐ Daily ☐ Weekly ☐ Less than once a week

Pond use information

27. Where is the pond? .....
28. Pond water uses:
    - ☐ Drinking
    - ☐ Washing/bathing
    - ☐ Swimming
    - ☐ Preparing food
    - ☐ Washing clothes/dishes
29. How frequently do you use the pond?

☐ Daily ☐ Weekly ☐ Less than once a week

River use information

30. Which part of the river?
31. River water uses:
    - ☐ Drinking
    - ☐ Washing/bathing
    - ☐ Swimming
    - ☐ Preparing food
    - ☐ Washing clothes/dishes

32. How frequently do you use the river?

☐ Daily ☐ Weekly ☐ Less than once a week

Rain water information

33. Rain water uses:
    - ☐ Drinking
    - ☐ Washing/bathing
    - ☐ Swimming
    - ☐ Preparing food
    - ☐ Washing clothes/dishes

34. How frequently do you use rain water?

☐ Daily ☐ Weekly ☐ Less than once a week

Other water source use information

35. What other water sources do you use? .....
36. Water uses for this water source:
    - ☐ Drinking
    - ☐ Washing/bathing
    - ☐ Swimming
    - ☐ Preparing food

☐ Washing clothes/dishes

37. How frequently do you use this water source?

☐ Daily ☐ Weekly ☐ Less than once a week

Other risk factors

38. Do you wear shoes regularly?

☐ Yes

☐ No

39. Do you play in soil/clay regularly?

☐ Yes

☐ No

40. Do you openly defaecate or urinate?

☐ Yes

☐ No

Symptoms

41. Have you experienced any of the following in the last week?

Fever

☐ Yes ☐ No ☐ Unknown

Headaches

☐ Yes ☐ No ☐ Unknown

Aching muscles

☐ Yes ☐ No ☐ Unknown

Stomach ache

☐ Yes ☐ No ☐ Unknown

Vomiting

☐ Yes ☐ No ☐ Unknown

Diarrhoea

☐ Yes ☐ No ☐ Unknown

Cough

☐ Yes ☐ No ☐ Unknown

Tired

☐ Yes ☐ No ☐ Unknown

Hungry

☐ Yes ☐ No ☐ Unknown

Chapter 3: An extensive burden of giardiasis associated with intestinal schistosomiasis and anaemia in school children on the shoreline of Lake Albert, Uganda

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Cover sheet for each research paper included in a research thesis

1.For a research paper already published

1.1. Where was the work published**? Transactions of The Royal Society of Tropical Medicine and Hygiene**

1.2. When was the work published? **09 December 2016 (Appendix 12)**

2. If the work was published prior to registration for your research degree, give a brief of the rationale for its inclusion **N/A**

2.1. Was the work subject to academic peer review? **N/A**

2.3. Have you retained the copyright for the work? **N/A**

3. For a research paper prepared for publication but not yet published

3.1. Where is the work intended to be published? **N/A**

3.2. List the authors of the paper in the intended authorship order **N/A**

3.3. Stage of publication-Not yet submitted/submitted/Undergoing revision

from peer reviewer's comments/In press **N/A**

4. For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper.

HAl-S contributed to the design of the studies; participated in data collection; participated in data entry, participated in data interpretation, conducted an analysis of data, prepared the manuscript and approved the final version.

Candidate's signature

Mr Hajri Alshehri

Supervisor or senior author's signature to confirm role as stated in ()

Professor Russell Stothard Primary Supervisor

3.1 Abstract

Introduction: Water-borne parasitic diseases associated with poverty still blight the lives of African school children. In Uganda, intestinal schistosomiasis is still common along the shoreline of Lake Albert, despite ongoing control, and co-infection with giardiasis and malaria is poorly described. To shed light on putative interactions between diseases, a prospective cross-sectional parasitological survey was undertaken across five primary schools on the shoreline of Lake Albert.

Methods: Stool samples from a total of 254 school children, aged 5-10 years, were examined by microscopy and RDTs, with additional quantitative real-time PCR assay for detection of *Giardia* DNA. Each child was interviewed, anthropometric measurements taken, and a finger-prick blood sample was also collected from each child and tested for malaria with haemoglobin levels measured. Associations between disease and health status were assessed.

Results: Intestinal schistosomiasis (46.5%), giardiasis (41.6%) and malaria (56.1%) were prevalent, and a quarter of children were anaemic (<115 g/L). Up to 87.0% of children were excreting *Giardia* DNA, and the prevalence of heavy infection by quantitative real-time PCR assay ($C_t \leq 19$) was 19.5%, being positively associated with light, moderate and heavy egg-patent schistosomiasis, as well as with anaemia and being under-weight.

Conclusion: In this setting, an extensive burden of giardiasis was revealed with heavy intensity infections associated with egg-patent intestinal schistosomiasis and anaemia. To improve child health, greater attention on giardiasis is needed, along with exploring joined-up actions across diseases that promote better water hygiene and sanitation measures that better co-ordinate interventions on the ground.

Keywords: *Schistosoma*, *Giardia*, *Plasmodium*, epidemiology, co-infection, control

3.2 Introduction

In impoverished regions of sub-Saharan Africa and in addition to malaria, water-borne parasitic diseases pose a significant threat to the well-being of children. In parts of Uganda, for example, intestinal schistosomiasis is hyper-endemic along the shorelines of the Great Lakes and is particularly rife around Lake Albert (Kabatereine *et al.*, 2004, Dunne *et al.*, 2006, Loewenberg, 2014). Local aetiological factors include favourable freshwater snail habitats, poor local sanitation and hygiene, as well as extensive levels of daily water contact by shoreline communities (Stothard *et al.*, 2004, De Moira *et al.*, 2007, Rowel *et al.*, 2015). While an extensive burden of malaria is known (Booth *et al.*, 2004, Sousa-Figueiredo *et al.*, 2010a, Wilson *et al.*, 2011, Brooker *et al.*, 2012), the occurrence and extent of another waterborne, poverty-related disease, giardiasis, has yet to be investigated. In 2003, a NCP against schistosomiasis and intestinal worms was launched at Pakwach (see **Figure 24**) (Fenwick *et al.*, 2009). Since then there have been substantive actions to deliver PZQ and albendazole by MDA to at-risk children attending primary schools and adults in endemic areas (Seto *et al.*, 2012). Owing to high levels of re-infection, an extensive burden of intestinal schistosomiasis remains, and children with the hepatosplenic and gastrointestinal disease can still be found (Bustinduy *et al.*, 2014).

Giardiasis is caused by *Giardia* spp., a binucleate flagellated protozoan, and is associated with ingestion of infectious cysts on foods or in drinking water, as contaminated by faecal waste from humans and (or) animals carrying patent or cryptic infections (Painter *et al.*, 2015, Halliez and Buret, 2013). Although infections with *Giardia* spp. may be acute and self-limiting, chronic disease, such as persistent diarrhoea, leads to malabsorption and weight loss (Savioli *et al.*, 2006, Escobedo *et al.*, 2014, Katz and Taylor, 2001, Huang and White, 2006). Many people with *Giardia* infection remain asymptomatic, and therefore it took many years until the parasite was classified as a pathogen (Mank *et al.*, 1995b, Thompson and Monis, 2004, Halliez and Buret, 2013, Escobedo *et al.*, 2014).

In low and middle-income countries, the prevalence of human giardiasis can vary widely, typically from 0-30%, and depends on which diagnostic is applied. It can be almost universal in specific populations (Halliez and Buret, 2013, DuPont, 2013). Diagnosis of *G. lamblia* is usually performed by microscopic examination of stool samples for the presence of cysts and/or trophozoites (**Table 7**). The excretion of the parasite can be highly variable, and therefore analyses of multiple stool samples and concentration techniques are recommended to increase sensitivity (Danciger and Lopez, 1975, Hanson and Cartwright, 2001, Staat *et al.*, 2011, El-Nahas *et al.*, 2013).

Other suggested procedures for the detection of *G. lamblia* are concentration techniques and examination of freshly preserved stool samples for the *detection* of *cysts* and trophozoites of *G.lamblia* (Mank *et al.*, 1997, El-Nahas *et al.*, 2013). The alternatives for microscopic diagnosis of *G. lamblia* include immunoassays for DFA and *G. lamblia* antigen detection (Garcia *et al.*, 2000, Garcia and Garcia, 2006, Baig *et al.*, 2012). Compared to microscopy, immunoassay tests have shown increased sensitivity with reduced number of samples required for examination (Garcia and Shimizu, 1997, Johnston *et al.*, 2003, Mank *et al.*, 1997, Mank *et al.*, 1995a), but still multiple stool sample analysis is needed for optimal sensitivity (Hanson and Cartwright, 2001). The second diagnostic alternative is *G. lamblia* DNA detection in stool by quantitative real-time PCR assay, which showed a higher sensitivity than microscopy and immunoassay analyses and can further reduce the required number of stool samples for analysis (Guy *et al.*, 2004, Verweij *et al.*, 2004, Schuurman *et al.*, 2007, ten Hove *et al.*, 2007, David *et al.*, 2011, Verkerke *et al.*, 2014).

Co-infection with other poverty-related diseases, such as soil-transmitted helminthiasis occurs, and with ascariasis, immunomodulation of the gut mucosal surface potentially favours chronic giardiasis (Teixeira *et al.*, 2007, Redpath *et al.*, 2014). Within Uganda, regular reporting and surveillance of giardiasis are very scant (Ankarklev *et al.*, 2012, McElligott *et al.*, 2013, Fuhrimann *et al.*, 2016). While the disease is zoonotic in livestock and an essential anthropozoonosis of the mountain gorilla (Hogan *et al.*, 2014, Sak *et al.*, 2014), the burden of giardiasis in school children is mostly unknown, as are any associations with intestinal schistosomiasis.

This present investigation aimed to conduct a prospective cross-sectional parasitological survey to ascertain current levels of intestinal schistosomiasis, giardiasis and malaria among schoolchildren (5-10 years of age) on the shoreline of Lake Albert, Uganda. Associations between diseases were assessed and as three of the five schools first surveyed for intestinal schistosomiasis in 2002-2003, a comparison through time was made.

3.3 Materials and methods

3.3.1 Study location

In June 2015 in Buliisa District, Western Uganda, a prospective cross-sectional parasitological survey, using parasitological methods augmented with RDTs, was undertaken within five primary schools. Three schools were selected on the basis that a baseline assessment had been undertaken in 2002-2003, and historical data were available from: Biiso (GPS co-ordinates: 01° 45.516 N, 031° 25.236 E), Walakuba (GPS 01° 50.323 N 031° 22.740 E) and Runga (GPS 01° 43.828 N 031° 18.603 E) (Balen *et al.*, 2006, Kabatereine *et al.*, 2007). Two additional schools were selected Bugoigo (01° 54.004 N 031° 24.750 E) and Busingiro (GPS 01° 44.090 N 031° 26.855 E) on the basis that the former school was sited very close to the shoreline while the latter school was furthest away from the lake (~13 kilometres). Busingiro was accessible on a daily basis from Bugoigo, where the field team was encamped, and intestinal schistosomiasis was presumed to be minimal there allowing associations between diseases in the putative absence of *Schistosoma mansoni* to be assessed (see **Figure 24**).

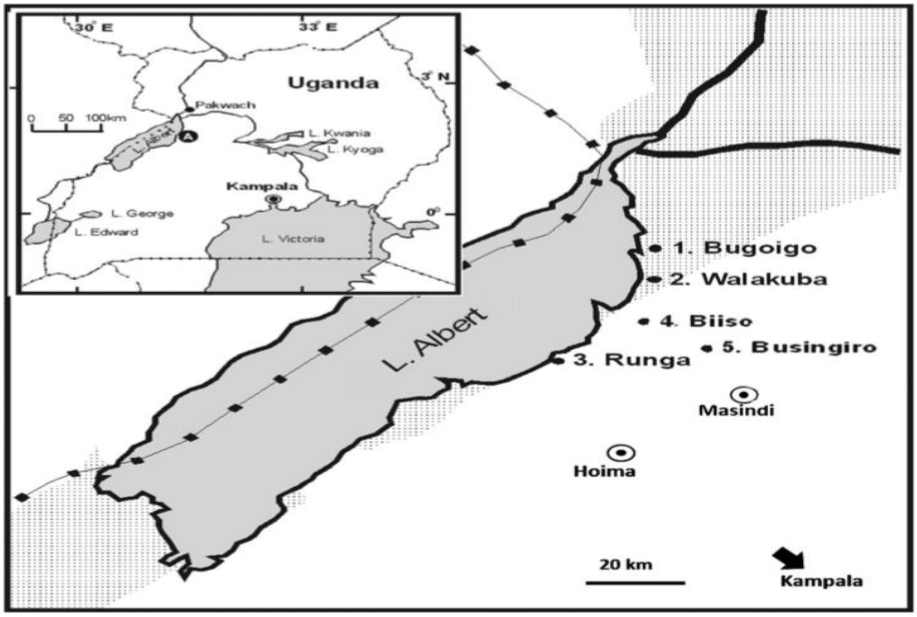

Figure 24: Inset, a sketch map of Uganda and study area (**A**). Outline map of the five schools inspected. The primary schools of Biiso and Busingiro are located on top of the escarpment whereas Bugoigo, Walakuba and Runga on the lakeshore plain. Once part of Masindi District, the schools are now located within Buliisa District after administrative areas were revised. The northern village of Pakwach was where the NCP against Bilharzia and intestinal worms was launched in 2003

3.3.2 Examination of children

In liaison with the headmaster and class teachers, a pre-target enrolment of 60 children per school, of equal gender, randomly recruited from classes primary 1 to 3, was performed. Each child was asked a suite of questions using standardised questionnaire recording general demographics and epidemiological data pertinent to intestinal schistosomiasis, giardiasis and malaria (**Appendix 3 -Page 95**). Finger-prick blood was collected from each child and tested for malaria by RDT (SD BIOLINE Malaria Ag P.f/Pan test, SD Diagnostics, Korea) and haemoglobin levels by HemoCue®portable haemoglobin photometer (HemoCue, CA 92630, USA). Children were considered anaemic if haemoglobin levels were below 115 g/L (WHO, 2011). Height was measured using a portable stadiometer (model 214; SECA, Hanover, MD), weight was recorded using a digital weighing scale (model 803; SECA, Hanover; MD) (see **Figure 25**). Both metrics were used for following growth indicators: a) height-for-age Z-score (HAZ) to assess stunting; b) weight-for-age Z-score (WAZ) to assess underweight; c) Body mass index (BMI)-for-age z-scores variations reflect the changes in nutritional status (Onyango *et al.*, 2008). Children were defined as stunted if their height-for-age Z score was -3 ≤ SD, as underweight if their weight-for-age Z score was -2 ≤ SD, and children with Z-scores between 1 and -2 SD were considered mildly malnourished.

Figure 25: Participant enrollment and inclusion in study research. In each selected primary school, **A**-School administrators were requested to provide students' enrolment list. **B**-Each selected subject was registered in a separate registration format and invited to participate in the study and was given a urine container and a plastic sheet with an applicator stick to bring approximately 4 g stool sample. **C**- Interview based questionnaire prepared in English and then translated into the local language and translated back to English was used to collect socio-demographic and associated risk factor data for parasitic infection (**Appendix 3 Page-95**). Also,

body weight and height measurement were taken for each child in a standing and relaxed position while breathing normally. **D**-Finger-prick blood was collected from each child that was willing to give blood. This was done by a certified nurse in the research team

3.3.3 Examination of stool specimens

Each child was requested to provide a stool sample on two consecutive days. All stool samples received on the first day were tested for FOBT (Mission Test, Acon Laboratories, SanDiego, CA). In Bugoigo and Runga schools, all children were tested with Quik-Chek tests for *Giardia* and *Cryptosporidium* (*Giardia/Cryptosporidium* Quik-Chek, Alere, UK) (see **Figure 26 B**). The Quik-Chek RDT is typically used to measure clinically relevant giardiasis with a level of detection of 6000 cysts/ml of faeces. Owing to a limited supply of 100 Quik-Check tests, only children from these two schools were tested while a more general detection of giardiasis was undertaken by recourse to real-time PCR assays. Duplicate faecal smears were prepared from each stool sample from consecutive days using the KK thick smear technique (41.7 mg) (see **Figure 26 A**), and examined by compound light microscopy, and helminth egg tallies were recorded according to EGP of stool (light 1-99, medium 100-399, heavy 400+) (Sousa-Figueiredo *et al.*, 2012). A later 10% re-read of slides was performed as quality control. After filtration through a metal mesh (212µm pore size), a small pellet-sized faecal sample from each child was preserved in absolute ethanol for later DNA extraction and real-time PCR analysis.

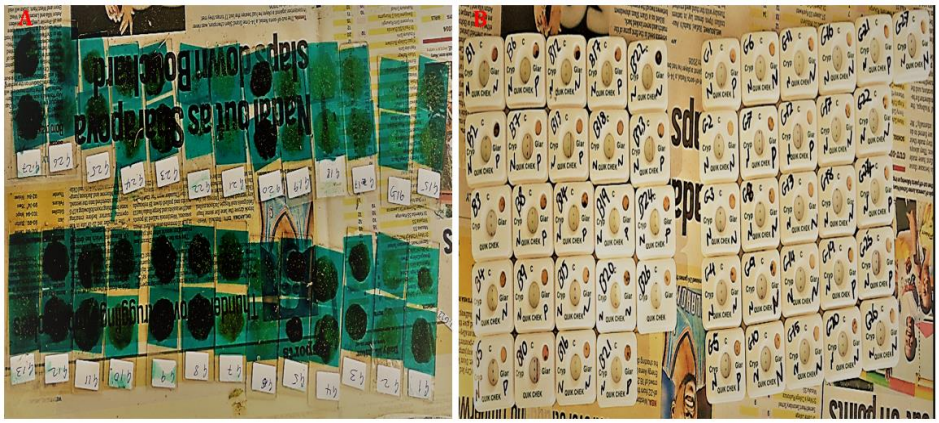

Figure 26: Parasitological confirmation of *S. mansoni* and *giardia* diagnosis**. A**-Preparing KK slides and searching for Schistosoma- eggs. **B**-Reading *GIARDIA/CRYPTOSPORIDIUM* QUIK CHEK test (RDTs) used for qualitative detection and differentiation of *Giardia* cyst antigen and *Cryptosporidium* oocyst antigen in a single test device (**Table 7**)

3.3.4 Probe-based detection method for *Giardia*

Upon arrival, gDNA was extracted, and detection of *Giardia* DNA was performed using the TaqMan® assay following primer, probes and protocols of Verweij *et al.* (Verweij *et al.*, 2004) (see **Figure 27**). In brief, approximately 0.2g of faecal sample preserved in ethanol was vigorously shaken, centrifuged and the pellet was washed twice with 1000 µl of phosphate-buffered saline (abbreviated PBS). The sample then was suspended in 250 µl of 2% polyvinylpolypyrrolidone (PVPP; Cat-77627-25G, Sigma) before frozen overnight at - 20°C in a MagNA Lyser Green Beads (Product No. 03358941001, Roche). After tissue lysis treatment with proteinase K (Supplier No, BIO-37037, Bioline) for two hours at 55°C, DNA was then extracted from the pellet using the commercial QIAamp® DNA Mini Kit spin columns (Qiagen, Hilden, Germany). In each sample, 10^3 plaque-forming units (PFU)/ml phocine herpesvirus-1 (PhHV-1) was added to the isolation lysis buffer, to serve as an internal control for the isolation.

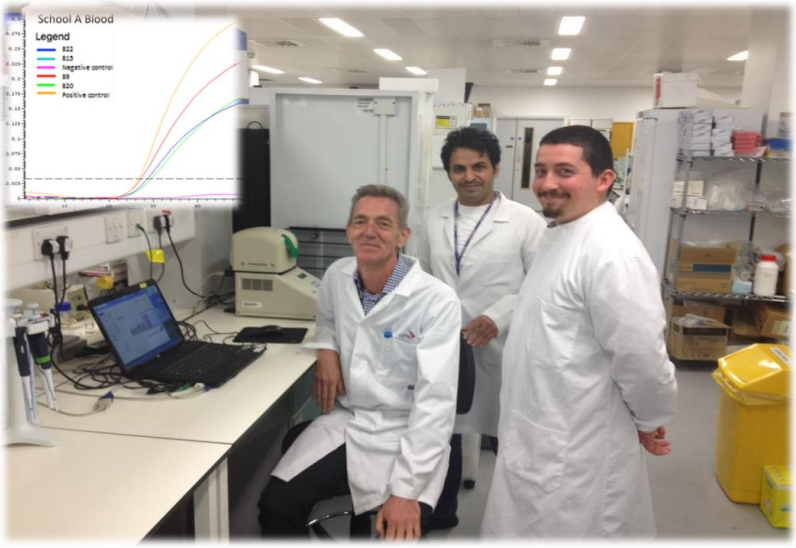

Figure 27: Onsite training with LSTM for improving diagnosis of intestinal parasitic infection by using a bead-beating procedure on stool samples preserved in ethanol prior to DNA isolation and the performance of Multiplex TaqMan® Real-Time PCR assay with Dr Jaco J. Verweij and Dr Lucas Cunningham for detection and differentiation of giardiasis and other intestinal parasites by dual TaqMan® assays

The amplification reaction was performed as a duplex TaqMan® Real-Time PCR assay in a volume of 25 µl, containing 12.5 µl ™ iQ Supermix (ProductNo.170-8860-MSDS, Bio-Rad), 1 µl of forward primer (400nM)-*Giardia* 18S-99 (5'*GACGGCTCAGGACAACGGTT'3*), and 1 µl of reverse primer (400nM)-*Giardia*18S-125 (5'*TTGCCAGCGGTGTCCG3'3*), 0.5 µl of *G. lamblia*-specific double (200nM)-labelled probe (JOE-*CCCGCGGCGGTCCCTGCTAG*-

BHQ1),1µl of PhHV-1-specific primer (400nM)-PhHV-267s (5'*GGGCGAATCACAGATTGAATC'3*) and 1µl of PhHV (400nM)-337as (5'*GCGGTTCCAAACGTACCAA'3*), 0.5 µl of PhHV-1-specific double(200nM)-labelled probe (Cy5-*TTTTTATGTGTCCGCCACCATCTGGATC*-BHQ2), supplied by Eurofins scientific (Eurofins Genomics Anzinger Str. 7a 85560 Ebersberg, Germany), 2.5 µl of nuclease-free H_2O, and 5 µl DNA sample. Amplification consisted of 3 min at 95°C followed by 50 cycles of 15s at 95°C, 20s at 60°C and 30s at 72°C. Negative controls (Eluted buffer), and positive controls (stools samples with confirmation cysts seen provided by diagnosis Lab in LSTM) was included in all reaction sets. High cycle threshold values (C_t-values) obtained by duplex TaqMan® Real-Time PCR assay are considered to be less reproducible due to deficient copy numbers of the specific target. Assays were performed in a Chromo-4 with Opticon monitor™ Version 3.1. (Bio-Rad, UK) and levels of infection categorised according to C_t-values: no infection ($C_t \geq 40$), light ($30 \leq C_t \leq 39$), moderate ($20 \leq C_t \leq 29$), and heavy ($C_t \leq 19$) intensity infection. Samples were excluded if the amplification remained inhibited (Verweij *et al.*, 2004).

3.3.5 Statistical analysis

All data collected in the field were recorded on pro-forma data sheets (**Appendix 3**), then tabulated in Microsoft Excel with double entry cross-check. The analysis was performed with R statistical package v 2·10·1 (The R Foundation for Statistical Computing, Vienna, Austria) and SPSS software (v 24.0, SPSS Inc., IBM, USA). For percentage values, 95% confidence intervals (CI_{95}) were estimated using the exact method (Armitage *et al.*, 1994). With an extensive burden of giardiasis revealed by real-time PCR, greater focus was placed on exploring the association of heavy infections with *Giardia* inclusive with: gender, age, the intensity of other parasitic diseases and clinical signs and symptoms in the examined children. A univariate logistic regression analysis was performed to study the association between infection status and the risk factors under consideration. The level of significance of $p < 0.05$ was set for multivariable analysis; the model was interpreted using adjusted ORs and 95% CIs. The prevalence of intestinal schistosomiasis at Runga, Walakuba and Biiso between 2002 and 2015 was compared by a 1-tailed Z-test (Armitage *et al.*, 1994).

3.3.6 Ethical approval

The Ugandan Council for Science and Technology and the Liverpool School of Tropical Medicine granted approval for this study. Each child was examined by the project nurse, and PZQ (40 mg/Kg, Merck, Germany) and albendazole (400 mg, GSK, UK) treatment were administered to all participants irrespective of their infection status. Children with a positive malaria RDT were each offered a take-home, 3-day course of Coartem (Lonart®, Cipla, India) with the first and third administrations directly overseen by the project nurse.

3.4 Results

3.4.1 General characteristics

A total of 274 children were initially recruited, however, owing to instances of failure to produce stool, a final dataset was composed of 254 children, mean age of 6.8 years (95% CI=6.6, 6.9) with a balanced male (49.4%) to female (50.5%) ratio. From the questionnaire, 45% of children reported to have received a deworming medication in the past year. Levels of itinerancy were low across the schools, with 9% reported to have moved into their current school location within in the past three years. Access to electricity at home was limited (11.2 %) as was access to tap water (17.8%), which varied by location: Bugoigo (0.0 %), Runga (0.0 %), Walakuba (11.3 %), Biiso (50.7 %) and Busingiro (46.4%). Typically, between a half to three-quarters of children reported fever, headache, tummy cramps and diarrhoea within the past week (i.e., recently) and previous month (i.e., previously), **Figure 28**.

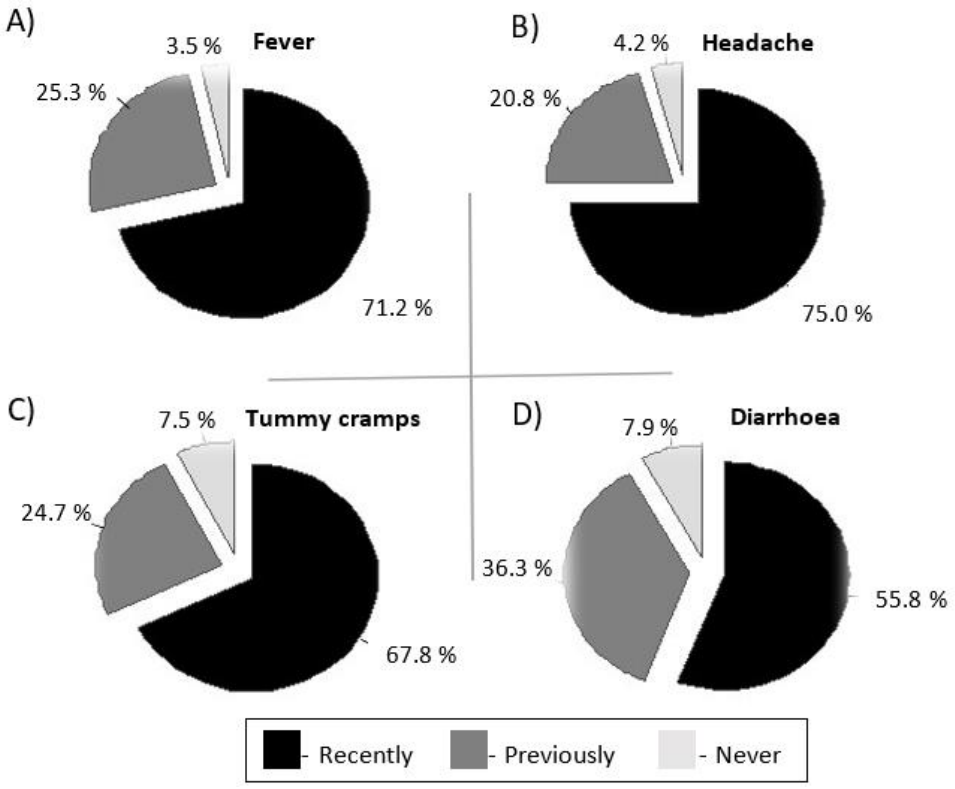

Figure 28: Pie charts of reported symptoms as reported on the questionnaire illustrate the majority of children have common complaints (recently – within the past week; previously – within the past month). **A**) fever, **B**) a headache, **C**) tummy cramps, **D**) diarrhoea

3.4.2 Prevalence of diseases

The prevalence of infection across the five schools as shown in **Table 12**, egg-patent intestinal schistosomiasis of 46.5%, giardiasis and malaria by RDT of 41.6% and 56.1%, respectively. The prevalence of egg-patent intestinal schistosomiasis at Walakuba and Runga was 82.0% and 86.8%, with the majority of children having heavy-intensity infections. Overall, the prevalence of *Giardia* by RDT was 41.6% but by real-time PCR increased to 87.0 % which was graded into light (27.1%), medium (40.5%) and heavy (19.2%) categories upon comparison with C_t values. Just over half of the children were positive for malaria by RDT, with triple co-infection in just under a fifth of the population. Over a quarter of children were judged anaemic (30.7 %), and FOB was found in 11.0% of stool samples.

Table 12: Prevalence of infection by school across a total of 254 children aged between 5 and 10 years of old

School locations Prevalence	Bugoigo (n=60) %	Walukuba (n=56) %	Runga (n=54) %	Biso (n=51) %	Busingiro (n=50) %	Total (n=271) %
Schistosoma mansoni (Kato-Katz)						
Negative	63.3 (38/60)	18.0 (9/50)	13.2 (7/53)	80.3 (41/51)	92.0 (46/50)	53.4 (141/264)
Light	26.6 (16/60)	10.0 (5/50)	13.2 (7/53)	5.8 (3/51)	2.0 (1/50)	12.1 (32/264)
Moderate	5.0 (3/60)	26.0 (13/50)	11.3 (6/53)	9.8 (5/51)	4.0 (2/50)	10.9 (29/264)
Heavy	5.0 (3/60)	46.0 (23/50)	62.2 (33/53)	3.9 (2/51)	2.0 (1/50)	23.4 (62/264)
Giardia (QUIK CHEK[a])						
Negative	63.0 (29/46)	ND	54.0 (27/50)	ND	ND	58.3 (56/96)
Positive	36.9 (17/46)	ND	46.0 (23/50)	ND	ND	41.6 (40/96)
Giardia (TaqMan)						
Negative	5.4 (3/55)	12.0 (6/50)	5.8 (3/51)	16.0 (8/50)	27.0 (13/48)	12.9 (33/254)
Light	47.2 (26/55)	12.0 (6/50)	1.9 (1/51)	28.0 (14/50)	45.8 (22/48)	27.1 (69/254)
Moderate	36.3 (20/55)	54.0 (27/50)	50.9 (26/51)	38.0 (19/50)	22.9 (11/48)	40.5 (103/254)
Heavy	10.9 (6/55)	22.0 (11/50)	41.1 (21/51)	18.0 (9/50)	4.1 (2/48)	19.2 (49/254)
Malaria (RDTs)						
Negative	48.2 (28/58)	64.8 (35/54)	38.8 (21/54)	31.3 (16/51)	34.0 (17/50)	43.8 (117/267)
Positive	51.7 (30/58)	35.1 (19/54)	61.1 (33/54)	68.6 (35/51)	66.0 (33/50)	56.1 (150/267)
Co-infection[b]						
Negative	85.0 (51/60)	80.0 (45/56)	51.8 (28/54)	86.2 (44/51)	98.0 (49/50)	80.0 (217/271)
Positive	15.0 (9/60)	19.6 (11/56)	48.1 (26/54)	13.7 (7/51)	2.0 (1/50)	19.9 (54/271)
Anaemia (<115Hb)	36.6 (22/60)	33.9 (19/56)	44.4 (24/54)	5.8 (3/51)	ND	30.7 (68/221)

ND: not determined; RDT: rapid diagnostic test.
[a] QUIK CHEK *GIARDIA/CRYPTOSPORIDIUM* test detects *Giardia* cyst antigens and *Cryptosporidium* oocyst antigens.
[b] Co-infection of all three parasitic infections (GIA-giardiasis, SCH-schistosomiasis and MAL-malaria).

3.4.3 Associations with giardiasis

Factors potentially associated with the presence of heavy *Giardia* infection as ascertained by real-time PCR were analysed, including all available socio-economic data, are shown in **Table 13**. The prevalence of heavy *Giardia* infection was strongly associated with Runga school [OR, 5.7 (95% CI, 2.18–17.0)] while both age and gender had no noticeable impact as shown in **Table 13**. The positive association [OR, 2.3 (95%CI, 0.89-5.1)] with FOBT was marginal, but all egg-patent intestinal schistosomiasis infection intensities were positively associated some 3 to 4 times greater than those without schistosome eggs in stool. By contrast, there was no significant association with heavy giardiasis infection and access to tap water [OR, 0.76 (95%CI, 0.29-1.7)]. There was a significant association between anaemic children and heavy giardiasis [OR, 2.2 (95%CI, 1.41-4.3)]**.**

Table 13: Adjusted risk factors associated with heavy *Giardia* infection by real-time PCR

Heavy *Giardia* infection[a]	Positive (n=49) %	Negative (n=205) %	OR	95% CI
School				
Bugoigo	10.9	89.1	–	–
Runga	41.2	58.8	5.7	2.2–17.0
Walukuba	22.0	78.0	2.3	0.8–7.2
Biiso	18.0	82.0	1.8	0.6–5.7
Busingiro	4.2	95.8	0.4	0.1–1.6
Age group				
5–6	19.3	80.7	–	–
7–8	20.8	79.2	1.1	0.56–2.1
9–10	15.4	84.6	0.7	0.26–1.9
Gender				
Female	15.9	84.0	–	–
Male	23.0	77.0	1.5	0.84–2.9
Faecal occult blood				
Negative	17.7	82.3	–	–
Positive	32.1	67.8	2.2	0.89–5.1
Kato-Katz intensity				
Negative	11.2	88.8	–	–
Light	33.3	66.6	3.9	1.54–10.0
Moderate	26.9	73.0	2.9	1.01–7.9
Heavy	28.1	71.9	3.1	1.41–6.8
Anaemia (<115Hb g/L)				
Negative	16.0	84.0	–	–
Positive	30.0	70.0	2.2	1.14–4.3

[a] Heavy *Giardia* infection categorised by real-time PCR (Ct value ≤19) vs negative, light and moderate infection; – reference comparator.

The box-plots (as seen in **Figure 29**) children identified with heavy *Giardia* infection against those that were not. The Weight-for-Age Z-score threshold of -2 SD **≤**, 24.5% of children with heavy infection showed evidence of wasting [OR,

0.66 (95% CI, 0.46-0.93)], compared to 18.0% who had either moderate, light or negative infection status **Figure 29A**. Height-for-Age (HAZ), heavy *Giardia* infection was not significantly associated with stunting [OR, 0.81 (95% CI, 0.62-1.05)], **Figure 29B**. Body Mass Index (BMI) was not associated with heavy infection [OR, 0.86 (95%CI, 0.61-1.21)], **Figure 29C**. Although there was an association with anaemia when considering a cut off of 115 mg/L, general levels of haemoglobin were not associated [OR, 0.98 (95%CI, 0.61-1.21)], **Figure 29D**.

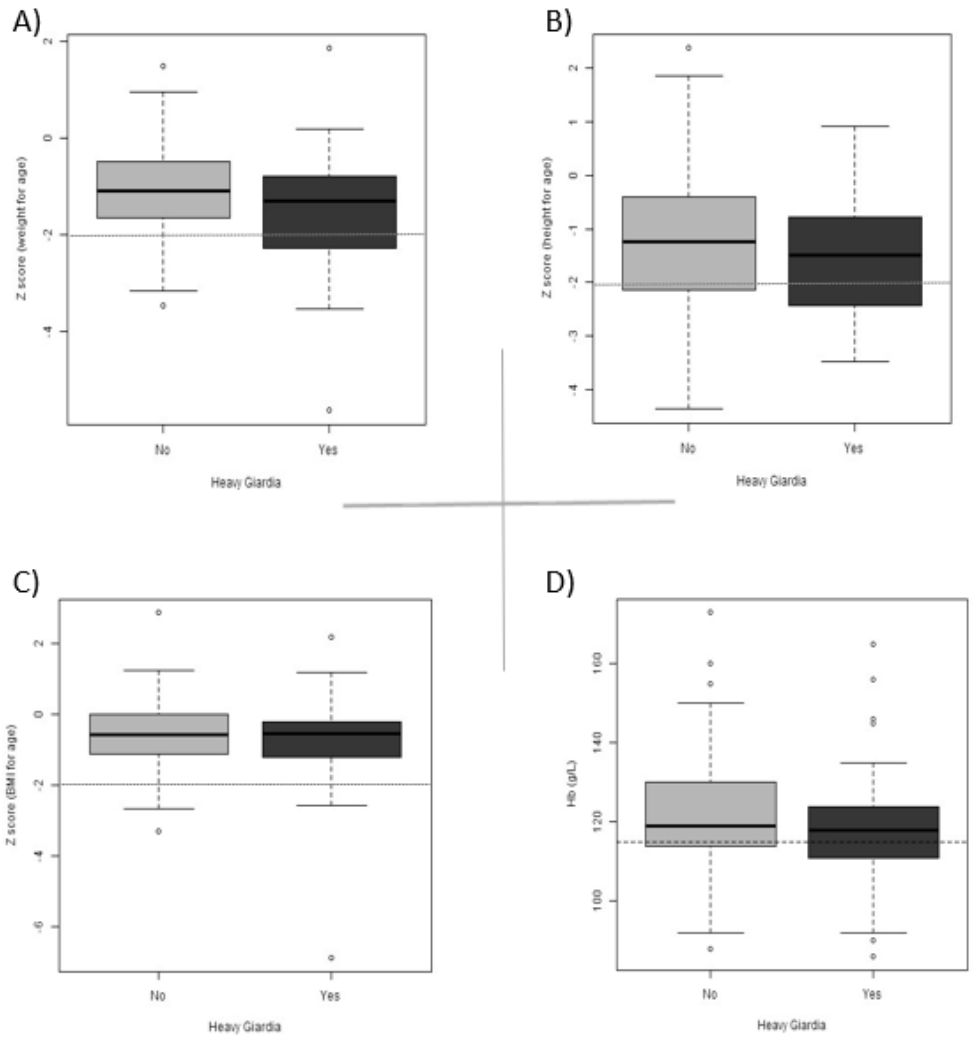

Figure 29: Box plots of biometric data and haemoglobin levels and heavy infection status with *Giardia.* **A**) Weight-for-age, **B**) Height-for-age, **C**) Body mass index, **D**) Haemoglobin levels

3.4.4 Intestinal schistosomiasis through time

As resources available for control are often limited, it is essential to know the distribution of schistosomiasis to devise and target optimal intervention strategies. Previous approaches in describing schistosomiasis in Africa have typically been made at the national level, using data from the few studies available within a country and then extrapolating these to the country as a whole. While such an approach may be effective for advocacy, it is of limited practical relevance to the targeting of control efforts. Between 1998 and 2002, the Vector Control Division of the Ministry of Health undertook epidemiological surveys across the country, among both school and community populations. Schools were selected based on ecological differences in rainfall, altitude and temperature. Ecological zones were identified using a combination of expert opinion and ecological zone mapping (Kabatereine *et al.*, 2004). In 2002, the prevalence and the arithmetic mean intensity of children with intestinal schistosomiasis at Runga, Walakuba and Biiso in 2002 versus 2015 was 95.2% (631 epg) *v* 86.7% (1723 epg), 90.4% (1026 epg) *v* 80.3% (814 epg) and 17.2% (52 epg) *v* 19.6% (198 epg) respectively. The declines (approximately 10%) in prevalence at Runga and Walakuba were statistically significant ($p<0.05$) while the small increase at Biiso was not statistically significant ($p>0.3$) (Stothard *et al.*, 2017b).

3.5 Discussion

3.5.1 Effects of parasitic burdens and environmental factors

In this cross-sectional survey in 5 primary schools, an extensive burden of parasitic diseases, inclusive of co-infections, has been revealed, see **Table 12**. Triple infections of intestinal schistosomiasis, giardiasis and malaria affected just under a fifth of all sampled children. Although all were sufficiently fit to attend school, more general markers of ill-health, such as a half to three-quarters of children reported fever, headache, tummy cramps, diarrhoea (**Figure 28**), anaemia (30.7%) and FOBT (11.0%), revealed an underlying morbidity, inclusive of chronic wasting (**Figure 29**), previously seen in this area (Bustinduy *et al.*, 2013, Green *et al.*, 2011). Along the lakeshore, access to tap water is still very poor which no doubt helps both intestinal schistosomiasis and giardiasis flourish (DuPont, 2013, Grimes *et al.*, 2015, Grimes *et al.*, 2014, Mohammed Mahdy *et al.*, 2008). Moreover, a concurrent malacological survey of the shoreline was undertaken and found numerous snails shedding schistosome cercariae, (**Figure 30** below) (Stothard *et al.*, 2017b), as upon several occasions before (Rowel *et al.*, 2015, Levitz *et al.*, 2013). Clearly, there is a pressing need to establish and sustain safe water sources for both drinking and washing on the immediate shoreline as a foundation step towards promoting well-being (Bartram and Cairncross, 2010).

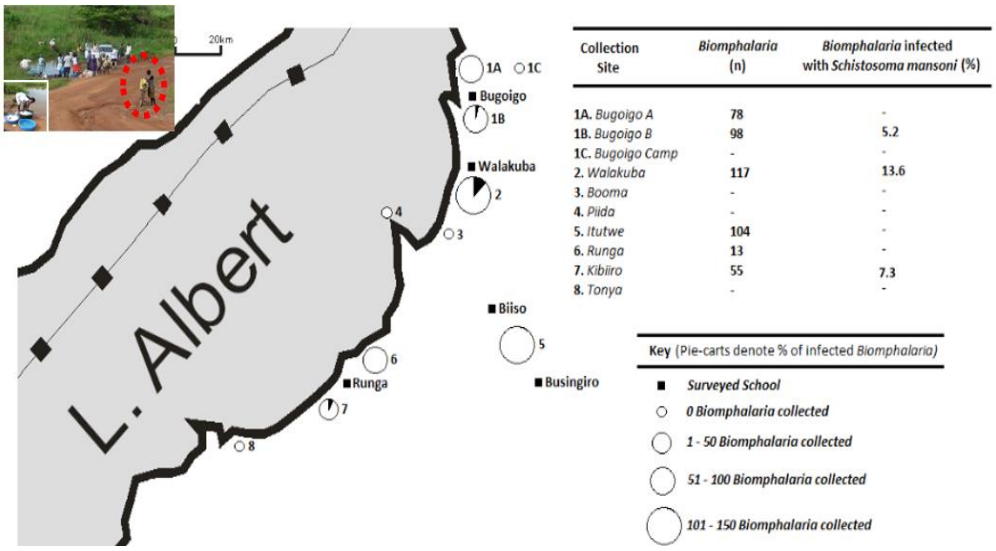

Figure 30: Malacological survey involving eight sites around five selected schools was also carried out to identify intermediate snail hosts and larval infection rate in the snail. The snails collected were checked for trematode infection by shedding to assess the establishment of transmission. The observation was also made on water contact habits of the study population (Stothard *et al.*, 2017b)

3.5.2 Schistosomiasis

The burden of intestinal schistosomiasis appears greatest at Runga and Walakuba schools, which has been noted previously upon comparison with other locations along this lake (Balen *et al.*, 2006, Kabatereine *et al.*, 2007). Despite best efforts of control with school-based delivery by MDA of PZQ, the resultant declines in egg-patent prevalence (~10%) were small although statistically significant. Whilst this is movement in the right direction, rather worryingly, there were large numbers of children with heavy intensity infections, **Table 12**. Looking to the future, a more aggressive treatment strategy against intestinal schistosomiasis is needed to gain and sustain control such as exploring biannual treatment cycles in both children and adults as well as snail control for prevention of disease transmission (see **Figure 30**). The reported annual treatment coverage was low (45.0%) and is in definite need of being raised. To do this, a more significant effort in time and resourcing for future MDA is needed, for example by expanding the period allocated for MDA at each school, to ensure that as many children receive treatment as possible. Similarly, in these high transmission settings biannual treatment cycles in both children and adults should be explored, as well as tackling the burden of infection in PSAC (Stothard *et al.*, 2017b, Stothard *et al.*, 2013a).

3.5.3 Giardiasis

Underlying the striking levels of intestinal schistosomiasis and malaria, with the pervasive nature of giardiasis is of concern and warrants greater attention on this waterborne disease in future, **Table 12**. Use of the Quik-Chek RDT revealed that, whilst no cryptosporidiosis was encountered (**Table 7**), a considerable number of children at Runga and Bugoigo were patently infected with clinically relevant *Giardia* (**Figure 28**). This was confirmed by real-time PCR and, by having a higher diagnostic sensitivity (Verweij *et al.*, 2004, Easton *et al.*, 2016, Schuurman *et al.*, 2007), found a larger proportion of children with some evidence of infection (**Table 12**). This points towards very high levels of exposure likely by ingestion of cysts from lake water. Notably, livestock regularly enters into the lake and contaminate, and each day such water is directly drawn in plastic jerry-cans for drinking and domestic use (**Figure 30**), exacerbating exposure and fostering zoonotic potential (Thompson, 2004). When only heavy infections by real-time PCR were considered there was clear focalisation of chronic giardiasis at Runga, a positive association with egg-patent intestinal schistosomiasis as well as associations with FOBT and anaemia, **Table 13**.

3.5.3 Malaria

Analysis of the blood by malaria RDTs has provided a much deeper insight into the complex dynamics and significance of *Plasmodium* infections in children living on the shorelines of Lake Albert in Uganda. Infection prevalence varied by village and was more common in children living in Biiso and Busingiro, **Table 12**. Indeed, some of the most widespread parasitic infections in tropical and subtropical countries and many people suffer from multiple infestations are plasmodia and helminth species, and their extensive geographical overlap in the tropics means that the coinfection is commonly found (Brooker *et al.*, 2007, Brooker *et al.*, 2006). Thus, in the presence of helminth infections, the immune response evoked by helminth infections and probably modify immune responses to *Plasmodium* and consequently alter infection and disease risks (Sangweme *et al.*, 2010). Although all of the studied schools were located in regions of very high schistosomiasis and malaria endemicity (Booth *et al.*, 2004, Yeka *et al.*, 2012), there are climatic factors that may influence local anopheline biology (Ochieng'Opondo *et al.*, 2016), with the potential favourable location for transmission of different *Plasmodium* species. The prevalence of *Plasmodium* species detected in these communities will be discussed in details in **chapter 5**.

3.5.4 Association with *Giardia* infection

The strong association of heavy *Giardia* infection and egg-patent intestinal schistosomiasis remains to be fully explained (**Table 13**). While each disease is associated with water contaminated by faecal material, there are different major modes of exposure, namely oral ingestion of cysts and per-cutaneous transmission by cercariae, respectively. It is also reasonable to speculate that intestinal schistosomiasis alters the environment of the bowel to become more conducive for colonisation of ingested cysts (Bartelt and Sartor, 2015). For example, the numerous schistosome egg-induced lesions that perforate and immunomodulate the mucosal surface (Bustinduy *et al.*, 2013), perhaps promote chronic giardiasis. In terms of anaemia, while it is difficult to disentangle precisely the relative roles intestinal schistosomiasis, giardiasis and malaria are playing, there was a clear association with heavy giardiasis [OR, 2.2 (95%CI, 1.41-4.39)]. Amongst other causal factors, this could be mediated by more general gastro-intestinal inflammation and malabsorption, alongside daily blood loss as evidenced by FOBT (Wilson *et al.*, 2011, Green *et al.*, 2011). If anaemia is to be tackled effectively here, from a public health perspective, it is important to consider and tackle as far as possible all three diseases simultaneously.

It is outside the immediate scope of this chapter to discuss in full most appropriate interventions in the short and long-term, an immediate suggestion would be to expand preventive chemotherapy approaches to include other anti-parasitic drugs. The use of intermittent preventive treatment for malaria along with intensified vector management would appear appropriate here (Nankabirwa *et al.*, 2014, Njau *et al.*, 2013). Expanded access to treatment and combination therapies against *Giardia* is also needed, even though these have much longer administration regimes (Harhay *et al.*, 2010). Moreover, the efficacy of any drug should also be assessed and be cognisant of the underlying disease states resultant from other parasites. For example, the efficacy of drugs against *Giardia* may be confounded if schistosome egg-induced lesions fail to regress after PZQ treatment. Like-wise, incomplete management of giardiasis diminishes attempts to regain gut function, thus developing a strategy for multi-parasitic disease management is needed here.

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Chapter 4: Molecular characterisation and taxon assemblage typing of giardiasis in primary school children living close to the shoreline of Lake Albert, Uganda

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Supervisor or senior author's signature to confirm role as stated in ()

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4.1 Abstract

Introduction: *Giardia duodenalis* is an important zoonotic parasite which can parasitise in the intestines of humans and various animals. However, the information about the prevalence and genetic diversity of *G. duodenalis* in Uganda is limited.

Methods: As part of an epidemiological survey for gastrointestinal parasites in school children across five primary schools on the shoreline of Lake Albert, the prevalence of giardiasis was 87.0% (n=254) as determined by real-time PCR analysis of faecal samples with a genus-specific *Giardia* 18S rDNA probe. Faecal samples were further characterised with taxon assemblage-specific TPI TaqMan® probes and by sequence characterisation of the *β*-giardin gene.

Results: While less sensitive than the 18S rDNA assay, general prevalence by TPI probes was 52.4%, with prevalence by taxon assemblage of 8.3% (assemblage A), 35.8% (assemblage B) and 8.3% co-infection (A & B assemblages). While assemblage B was dominant across the sample, proportions of assemblages A and B, and co-infections thereof, varied by school and by the age of child; mixed infections were particularly common at Runga school (OR = 6.9 [95% CI; 2.5, 19.3]) and in children aged 6 and under (OR = 2.7 [95% CI; 1.0, 7.3]). Infection with assemblage B was associated with underweight children (OR = 2.0 [95% CI; 1.0, 3.9]). The presence of each assemblage was also confirmed by sequence analysis of the *β*-giardin gene finding sub-assemblage AII and further genetic diversity within assemblage B.

Conclusion: To our knowledge, this is the first report of *G. duodenalis* prevalence and genotypes in the shoreline of the Lake Albert in Uganda, which extended the host range of *G. duodenalis* and provided basic data for controlling *G. duodenalis* infection in theses settings. To better explore the local epidemiology of giardiasis and its impact on child health, additional sampling of school children with assemblage typing would be worthwhile.

Keywords: *Giardia duodenalis*; real-time PCR; assemblage B; *β*-giardin; wasting

4.2 Introduction

The binucleate flagellated protozoan *Giardia duodenalis* (syn. *G. lamblia, G. intestinalis*) is a common gastrointestinal parasite able to infect a variety of mammals(Adam, 2001, Helmy *et al.*, 2014). Where sanitation and hygiene are poor, these parasites can cause acute and/or chronic giardiasis across all ages (Wegayehu *et al.*, 2016, Rogawski *et al.*, 2017, Tellevik *et al.*, 2015, Muhsen and Levine, 2012). While levels of endemicity of giardiasis may vary across the world, it can be common in children living within low and middle-income countries (Laishram *et al.*, 2012, Muhsen and Levine, 2012); for example, in Uganda giardiasis can be particularly rife (Al-Shehri *et al.*, 2018, Fuhrimann *et al.*, 2016), but its effect on child health is not fully appreciated but in Rwanda nearby, the very high prevalence of *G. duodenalis* in children aged 5 and under, was associated with being underweight (Ignatius *et al.*, 2012).

There are eight distinct groups or taxonomic assemblages (A to H) within *Giardia* currently recognised (Sprong *et al.*, 2009, Almeida *et al.*, 2010, Takumi *et al.*, 2012). Assemblages A and B are typically held most responsible for human infections, with the latter assemblage associated with zoonotic transmission (Almeida *et al.*, 2010, Feng and Xiao, 2011, Vanni *et al.*, 2012, Asher *et al.*, 2014, Thompson and Ash, 2016); each assemblage can be further divided into sub-assemblages, e.g. A: AI, AII & AIII and B: BIII and BIV on the basis of sequence variation within molecular markers e.g.GDH, *β*-giardin, small subunit ribosomal DNA (18S rDNA), and triose phosphate isomerase (TPI) (Durigan *et al.*, 2014, Karim *et al.*, 2015, Minetti *et al.*, 2015). Despite efforts to investigate specific assemblages with disease symptoms and severity, there is no absolute association to date (Sprong *et al.*, 2009, Thompson and Ash, 2016).

In Uganda, general investigations on the epidemiology of giardiasis are increasing (Nizeyi *et al.*, 1999, Graczyk *et al.*, 2002, Nizeyi *et al.*, 2002, Johnston *et al.*, 2010), although only a single study has employed molecular methods of characterisation (Ankarklev *et al.*, 2012). Ankarlev *et al.* (2012) investigated associations between taxon assemblages and *Heliobacter pylori* infection in apparently healthy children aged 0–12 living in Kampala, the capital. Assemblage B was found dominant and a risk factor for *H. pylori* infection (Ankarklev *et al.*, 2012) and like in other parts of the world, assemblage B was more associated with symptomatic infections (Pelayo *et al.*, 2008, Puebla *et al.*, 2014).

To shed light on the taxonomic assemblages of *Giardia* within school children living on the shoreline of Lake Albert, we undertook a molecular characterisation of previously characterised stool samples as reported by Al-Shehri *et al.* (Al-Shehri *et al.*, 2016). Faecal samples were further characterised with assemblage-specific TaqMan® triose phosphate isomerase (TPI) probes and the presence of each taxon assemblage confirmed by sequence analysis of the *β*-giardin gene (**Table 7**). Associations between taxon assemblage and collected epidemiological data were explored.

4.3 Materials and Methods

4.3.1 Faecal material and epidemiological information

Faecal samples were available for further molecular analysis (see below) that were initially collected within the epidemiological survey of 254 school children from five primary schools (**Figure 24**, **Page-104**) as reported by Al-Shehri *et al.* (Al-Shehri *et al.*, 2016). Each sampled child underwent an epidemiological questionnaire and clinical examination; data on socio-*demographical aspects* and standard biometry were recorded (height with a clinical stadiometer, model 214; SECA, Hanover, MD and weight by weighing scales with a model 803; SECA, Hanover; MD). Heights and weights were used to assess stunting, height-for-age Z-score (HAZ), and wasting, weight-for-age Z-score (WAZ). Children were defined as stunted if their height-for-age Z score was -2 ≤ SD and underweight if their weight-for-age Z score was -2 ≤ SD (WHO, 2013a). Finger-prick blood was collected from each child and tested for haemoglobin levels by HemoCue® portable haemoglobin photometer (HemoCue, CA 92630, USA). Children were considered anaemic if haemoglobin levels were below 115 g/L (WHO, 2011).

During the surveys, all sampled stools were tested for FOBT (Mission Test, Acon Laboratories, San Diego, CA, USA), but owing to a limited supply of RDTs, only stools collected from Bugiogo and Runga were tested in-field with Quik-Chek RDTs (*GIADIA/CRYPTOSPORIDIUM* Quik-Chek, Alere, Galway, Ireland). Stools were then stored in absolute ethanol for later DNA analysis.

4.3.2 Molecular profiling of *G. duodenalis* assemblages

After transfer to the UK and each faecal sample was spiked with PHV to act as an internal control for extraction and performance of later real-time PCR assays. gDNA was extracted, and detection of *Giardia* small-subunit ribosomal DNA was performed using TaqMan® assay following primers, probes and protocols of Verweij *et al.* (Verweij *et al.*, 2004), as explained in **chapter 3**. These extractions were again retested with a duplex real-time PCR assay with assemblage-specific A and B probes using the triose phosphate isomerase (TPI) locus **Table 14,** published by Elwin *et al.*, (Elwin *et al.*, 2014).

Table 14: Summarizing of assemblage-specific primers and probes

Target	Primer/ Probe	Nucleotide position	Sequence (5'→3')
G. duodenalis assemblage A	GDAF	647–662	CATTGCCCCTTCCGCC
	GDAR	702–722	CTGCGCTGCTATCCTCAACTG
	GDAT	679–693	VIC-CCATTGCGGCAAACA-MGB-NFQ
G. duodenalis assemblage B	GDBF	939–958	GATGAACGCAAGGCCAATAA
	GDBR	996–1020	TCTTTGATTCTCCAATCTCCTTCTT
	GDBT	959–976	FAM-AATATTGCTCAGCTCGAG-MGB-NFQ

The real-time PCR analysis of faecal extractions from each school was completed in separate PCR plates that each contained negative and positive controls; a negative control (without gDNA template) of extraction *elution buffer* (10 mM Tris-HCl [pH 8], 1 mM EDTA) and a positive control (with reference genomic *Giardia* DNA template) from a heavily infected individual excreting approximately 1000 cysts per gram of faeces as estimated by microscopy. As a further quality control, reamplification of 10% of samples was undertaken to assess assemblage assay reliability. Assays were performed in a Chromo-4 with Opticon monitor™ Version 3.1. (Bio-Rad, UK). The infection was determined according to C_t values; for the 18S rDNA TaqMan® assay no-infection was $C_t \geq 40$ and positive infection $C_t \leq 39$ while for assemblages-specific probes was $C_t \leq 45$, see **Figure 31**.

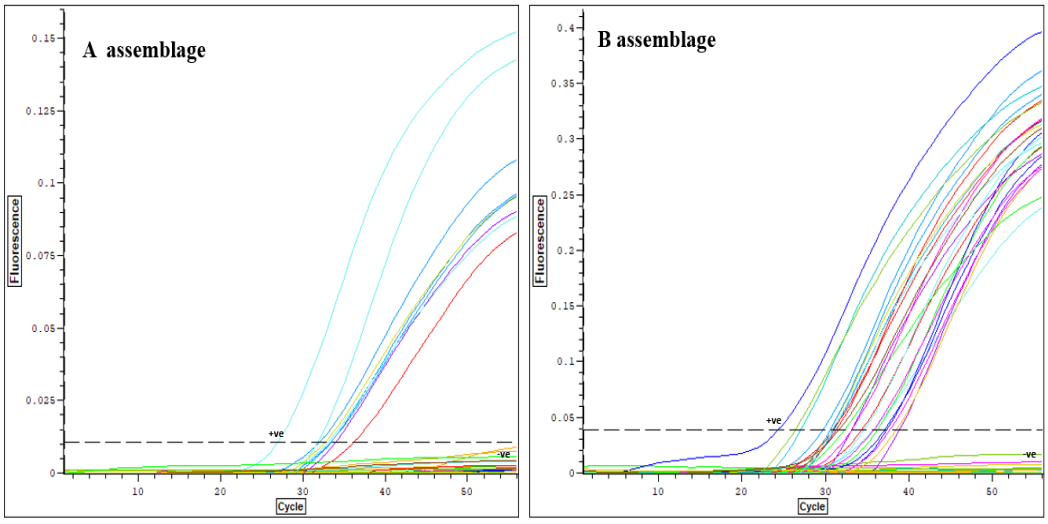

Figure 31: Showing the results for Bugoigo School children tested for **A** and **B** Assemblages, including negative and positive controls

To further confirm assemblage A and B, the *β*-giardin gene was amplified from samples from six children using nested PCR following protocols of Minetti *et al.* (Minetti *et al.*, 2015). PCR products were purified using the QIAquick® PCR Purification kit (QIAGEN Ltd.) and were sequenced in both directions by Sanger sequencing. Nucleotide sequences and chromatograms were analysed and edited using Geneious software (Vejlsøvej55, 8600 Silkeborg, Denmark). Sequences from this study were aligned with each other and reference sequences downloaded from GenBank (listed below). The assemblages and sub-assemblages at each locus were identified by BLAST searches against the following reference sequences: *β*-giardin (accession nos. X14185.1–**AI**, AY072723.1– **AII**, DQ650649.1–**AIII**, AY072726.1–**BIII**, AY072725.1– **BIV**).

4.3.3 Statistical analyses

Statistical analysis was performed using Minitab Ltd® (Brandon Court, Unit E1-E2 Coventry CV3 2TE UK). Binary logistic regression tests were performed to compare data from each school and as well as risk variables as an independent indicator to assess any associations with specific assemblages.

4.4 Results

4.4.1 *G. duodenalis* and assemblages

Out of the 254 samples examined, 221 tested positive (87.0%) by targeting *Giardia* 18S rDNA assay while 133 (52.3%) tested positive with TPI assemblage-specific probes. Across Bugoigo and Runga schools, the prevalence of giardiasis by Quik-Chek RDT was 41.6% (**Table 12-Page-111**). Of the 133-tested positive by TPI probes, 21 samples were positive for assemblage A (15.8%) only, 91 positives for assemblage B (68.4%) only and 21 positives for both assemblages A and B (15.8%), mixed assemblage infections **Table 15**.

Table 15: Prevalence (%) of *G. duodenalis* and assemblages across all five schools by real-time PCR; the odds ratio of assemblages A, B or A/B by school compared against the total given (with 95% Confidence Limits)

	Giardia TaqMan® 18S rDNA probe		Assemblage (A & B) TaqMan® TPI probe				
School	Number of positives % (x/y)	95% CL	Number of positives % (x/y)	95% CL	A % (x/y) OR [95% CI]	B % (x/y) OR [95% CI]	AB % (x/y) OR [95% CI]
Bugoigo	94.5% (52/55)	[85.8-98.6]	56.3% (31/55)	[43.1-69.0]	5.4% (3/55)	43.6% (24/55)	7.2% (4/55)
					0.6 [0.2, 2.5]	**1.4 [0.8, 2.8]**	**0.9 [0.3, 3.1]**
Runga	94.1% (48/51)	[84.8-98.5]	72.5% (37/51)	[59.2-83.4]	15.6% (8/51)	37.2% (19/51)	19.6% (10/51)
					4.7 [1.7, 13.3]	**2.0 [1.0, 4.3]**	**6.9 [2.5,19.3]**
Walukuba	88.0% (44/50)	[76.7-95.0]	40.0% (20/50)	[27.2-54.0]	2.0% (1/50)	32.0% (16/50)	6.0% (3/50)
					0.1 [0.0, 1.2]	**0.6 [0.3, 1.8]**	**0.5 [0.1, 1.8]**
Biiso	84.0% (42/50)	[71.9-92.3]	54.0% (27/50)	[40.2-67.4]	14.0% (7/50)	36.0% (18/50)	4.0% (2/50)
					2.1 [0.8, 5.9]	**1.0 [0.5, 2.1]**	**0.4 [0.1, 2.1]**

Across these samples' assemblage, A was less common than assemblage B, an approximate ratio of 1: 2.7, with assemblage B dominant. To ascertain if there was any amplification bias in assemblage detection, **Figure 32** below (Graph **A**) shows a bivariate plot of C_t values for *Giardia* 18S rDNA TaqMan® probe and the corresponding Ct value of assemblage A TPI TaqMan® probe (18S rDNA = 0.203 + 0.6991 TPI, with R-squared 34.91% (P <0.005), and positive correlation (r=0.60); **Figure 32** (Graph **B**) shows bivariate plot for assemblage B (18S rDNA = 0.228 + 0.6947 TPI, with R-squared 28.39 % (P <0.005), and positive correlation (r=0.54). The performance of each TaqMan® assay appeared equivalent. Of note, however, is that mixed assemblage infections appear more common at Runga school where the local prevalence of assemblage A was also much higher.

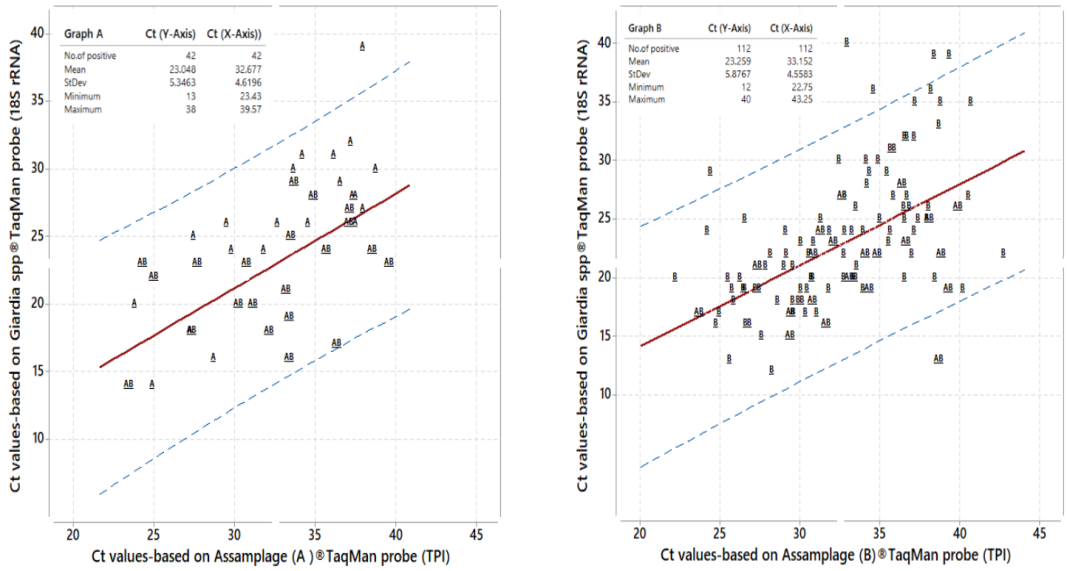

Figure 32: Bivariate plot of Ct values for TaqMan® 18S DNA versus Ct value of assemblage-specific TaqMan® TPI probe obtained for each sample with dashed lines showing the 95% prediction interval. **Graph A**, using assemblage, **A** probe; **Graph B,** using assemblage **B** probe

4.4.2 Epidemiological associations with an assemblage

The most notable in **Table 16** is the association of mixed assemblages in younger children (OR = 2.7 [95% CI; 1.0, 7.3]), and assemblage B was associated with the presence of FOBT (OR = 2.2 [95% CI; 1.0, 5.2]). It appeared that there was also a significant association of infection with assemblage B and children of lower weight-for-age, i.e. wasting (OR = 2.0 [95% CI; 1.0, 3.9])

Table 16: Epidemiological associations cross-tabulated against available assemblage information

Epidemiological factors	Assemblage (A, B & AB)®TaqMan probe (TPI)					
	Infected with A	OR [95 CL]	Infected with B	OR [95 CL]	Infected with AB	OR [95 CL]
Gender						
Male	10	1.0 [0.41, 2.66]	44	1.0 [0.63, 1.87]	12	1.5 [0.60, 3.94]
Female	11	0.9 [0.37, 2.39]	47	0.9 [0.53, 1.58]	9	0.6 [0.25, 1.64]
Age group						
5 to 6	11	1.5 [0.59, 3.82]	38	0.9 [0.56, 1.70]	14	2.7 [1.03, 7.28]
7 to 8	9	0.9 [0.39, 2.53]	34	0.7 [0.45, 1.38]	6	0.5 [0.19, 1.46]
9 to 10	1	0.2 [0.03, 2.26]	19	1.5 [0.74, 3.07]	1	0.2 [0.03, 2.26]
Faecal occult blood (FOB)						
Negative	18	0.6 [0.15, 2.36]	74	0.4 [0.19, 0.98]	17	0.4 [0.12, 1.48]
Positive	3	1.6 [0.42, 6.56]	17	2.2 [1.01, 5.18]	4	2.3 [0.67, 8.23]
Height-for-age Z score, mean						
-2 > SD height-for-age Z score	17	1.3 [0.41, 4.30]	63	0.7 [0.38, 1.30]	14	0.6 [0.23, 1.71]
-2 ≤ SD height-for-age Z score	4	0.7 [0.23, 2.39]	28	1.4 [0.76, 2.59]	7	1.5 [0.58, 4.30]
Weight-for-age Z score, mean						
-2 > SD weight-for-age Z score	20	3.9 [0.50,31.22]	65	0.4 [0.25, 0.95]	16	0.6 [0.20, 1.92]
-2 ≤ SD weight-for-age Z score	1	0.2 [0.03, 1.99]	26	2.0 [1.04, 3.91]	5	1.5 [0.51, 4.80]
Anaemia (<115 Hbg/L)						
Negative	9	0.4 [0.13, 1.36]	46	0.5 [0.27, 1.18]	9	0.3 [0.10, 0.95]
Positive	6	2.3 [0.73, 7.54]	23	1.7 [0.84, 3.68]	8	3.1 [1.05, 9.36]
Undeterminate	6	———	22	———	4	———

4.4.3 Sequence analysis of the beta-giardin gene

Table 17 details the point mutations with the six representative samples for the *β*-giardin gene, finding an exact match with sub-assemblage AII and no sequence within the three sample inspected. By contrast, each of the three samples for assemblage B was different and did not match either BIII and BIV precisely. The sequence from Sample 102 is particularly notable as there appeared to be allelic variation within the TPI gene as evidenced by split-peak chromatograms of A/G or T/C at three locations present within this region (see **Appendix 4** supplemental figure).

Table 17: Polymorphic sites in the *β*-giardin sequences in *Giardia duodenalis* assemblage **A** & **B** isolates

		Nucleotide position								
Assemblage	Isolate/Genbank number									
A isolates	Beta-giardin (bg)	**284**	**383**	**407**	**473**	**491**	**563**	**593**	**596**	**611**
AI	X14185.1	C	T	T	T	A	G	T	C	A
AII	AY072723.1	C	T	T	T	A	G	T	T	A
AIII	DQ650649.1	T	C	C	C	G	A	C	C	G
	Sample 9	C	T	T	T	A	G	T	T	A
	Sample 22	C	T	T	T	A	G	T	T	A
	Sample 103	C	T	T	T	A	G	T	T	A
B isolates		**170**	**176**	**188**	**233**	**287**	**314**	**317**	**398**	
BIII	AY072726.1	C	A	A	G	C	C	C	C	
BIV	AY072725.1	T	A	A	A	T	T	T	T	
	Sample 24	C	A	A	A	C	T	T	C	
	Sample 104	C	A	A	A	C	C	T	C	
	Sample 102	C	A/G	A/G	A	C	T/C	T	C	

4.5 Discussion

4.5.1 Application of real-time PCR to pathogen detection

The high prevalence of giardiasis reported here by real-time PCR with the 18S rDNA probe analysis (87.0%) demonstrates that children living on the shoreline of Lake Albert are at very high risk of both acute and more likely, chronic infections. The high burden of giardiasis was also corroborated in the field by the Quik-Check RDTs at Runga and Bugoigo schools confirming that some 41.6% of children were patently shedding copious amounts of *Giardia* cysts within their stools (**see chapter 3, Table 12**). It is unsurprising perhaps that the levels are so high since this lakeshore environment has very poor local sanitation and water hygiene, as well as being hyperendemic for intestinal schistosomiasis, another waterborne disease (Al-Shehri *et al.*, 2016). Nonetheless, the prevalence of giardiasis here is much elevated in comparison to other parts of the world (Thompson and Smith, 2011), although in Rwanda over 60% of rural children have been shown to be infected with *Giardia* by molecular typing methods (Ignatius *et al.*, 2014). More broadly, the diagnostic sensitivity of real-time PCR methods is known to be superior to alternative diagnostic methods, often revealing giardiasis to be more pervasive (Gotfred-Rasmussen *et al.*, 2016), and also creates opportunities for investigations of (sub) assemblage transmission dynamics (Thompson and Ash, 2016).

4.5.2 Real-Time PCR Assay for Detection and Genotype

Given the multi-copy nature of the 18S rDNA against the lower copy number of TPI, the diagnostic sensitivity of TPI probes is lower, such that just under a half of the infected cases detected by 18S rDNA were missed (**Table 15**). It has been stated previously that the detection limit of *Giardia* 18S rDNA probe assay is approaching 10 pg DNA/μL (Jaros *et al.*, 2011). Presumably, that of TPI assay is much higher (Elwin *et al.*, 2014) such that assemblage typing of 'light' intensity infections is not always possible. A similar level of diagnostic discordance has been observed elsewhere (Ignatius *et al.*, 2014) which hopefully does not lead to a systematic bias in general reporting of each assemblage, as evidenced by C_t values in **Figure 32**, but rather than typing parasites with assemblage-specific primers is not possible when shedding cysts are too few in number.

4.5.3 Associations between epidemiological information and assemblage

Nonetheless, in this sample assemblage B dominates upon comparison to assemblage A. Notably this 1:2.7 ratio varied by school with Runga having a

greater proportion of assemblage A, as well as co-infection with assemblage B thereof, see **Table 15**, and more broadly, there appeared to be some interesting epidemiological associations by assemblage, see **Table 16**. Although there was no association with gender, younger children appeared to harbour a greater proportion of mixed assemblage infections than older counterparts (OR = 2.7 [95% CI; 1.0, 7.3]). There was also an indication that FOBT was associated with assemblage B (OR = 2.2 [95% CI; 1.0, 5.2]) and in children being underweight (OR = 2.0 [95% CI; 1.0, 3.9]). These findings add to the general debate on the health consequences of giardiasis with particular emphasis on assemblage B, which also appears more genetically heterogeneous that assemblage A here (Thompson and Ash, 2016).

4.5.4 Diversity within assemblages

It is an interesting observation that of the six samples subjected to sequence analysis of *β*-giardin, the three samples selected from assemblage A were identical and could be further unequivocally assigned to sub-lineage AII, which has been reported in other studies (Cacciò and Ryan, 2008, Plutzer *et al.*, 2010, Cacciò and Sprong, 2010, Ryan and Cacciò, 2013, Beck *et al.*, 2012, Zhang *et al.*, 2012). By contrast, of the three samples selected from assemblage B, there were each different, see **Table 17**, and none matched exactly either BIII or BIV sub-assemblages. Most notable are the point mutations at positions 176, 188 and 314, where split-peak chromatograms were observed (see **Appendix 4**). This is indicative of mixed amplicon templates inferring putative allelic variation within the TPI locus. The genomic complexity of *Giardia* is complex, being binucleate and sometimes aneuploid (Aguiar *et al.*, 2016) which might infer sample 102 was either a mixed co-infection of two independent B lineages or contains a single infection lineage with an unusual genomic TPI variant. Nonetheless, there is greater diversity within assemblage B and with further genetic profiling would reveal additional variants which might point towards currently unknown heterogeneities in local transmission cycles. For example, there is numerous livestock, e.g. cattle and goats, that regularly enter into the lake and while drinking openly defecate into the water which may add to raised zoonotic potential in such domestic water directly drawn from the lake.

4.5.5 Conclusion

To conclude, additional sampling of school children would be worthwhile if putative associations between assemblage B and detrimental health outcomes reported here are to be fully verified statistically. Furthermore, to better monitor local transmission cycles of *Giardia*, we encourage future studies that track each assemblage within local livestock and undertake environmental sampling of lake water where domestic water is drawn.

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Authors' contributions: HAl-S, JRS, JElaC conceived the study with further participation in cross-sectional surveys by JElaC, AA, MAr, AW and MAd. Real-time PCR assays were performed by Hal-S with assistance from JElaC, OKl-H and JRS. Data were tabulated by HAl-S and analysed with the support of OKl-H and JRS. All authors contributed to the drafting and revision of the manuscript with JRS as guarantor.

Competing interests: None declared.

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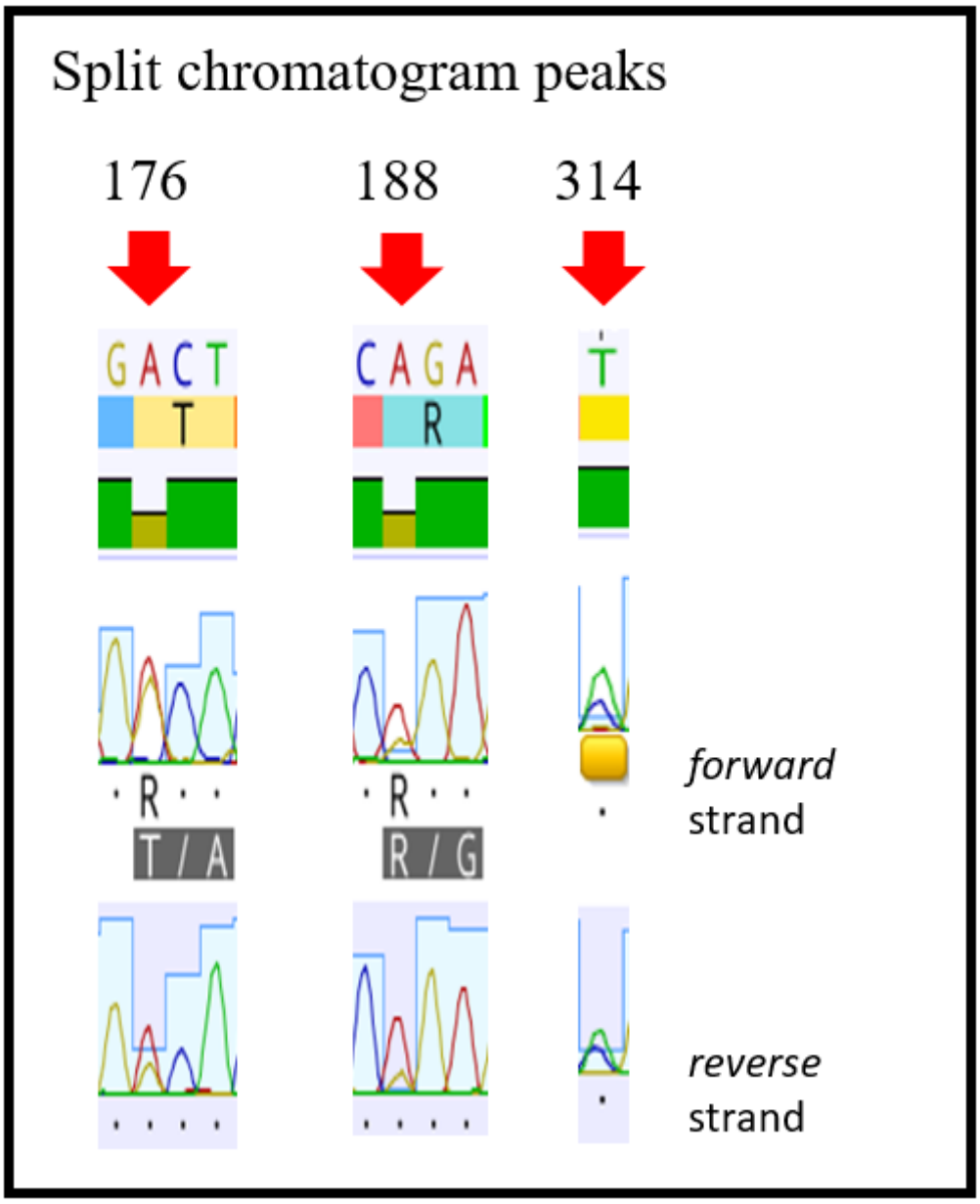
Ethical approval: The study was approved by the Liverpool School of Tropical Medicine and Uganda National Council for Science and Technology.

4.6 Appendix

Appendix 4: Point mutations detected within assemblage B



Chapter 5 Non-invasive surveillance of *Plasmodium* infection by real-time PCR analysis of ethanol preserved faeces from Ugandan school children with intestinal schistosomiasis

This chapter was published in modified form in Malaria Journal, 2019, 18(**1**):109.

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Cover sheet for each research paper included in a research thesis

1.For a research paper already published

1.1. Where was the work published**?** **Malaria Journal**

1.2. When was the work published? **1 April 2019 (Appendix 14)**

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HAl-S contributed to the design of the studies; participated in data collection; participated in data entry, participated in data interpretation, conducted an analysis of data, prepared the manuscript and approved the final version.

Candidate's signature

Mr Hajri Alshehri

Supervisor or senior author's signature to confirm role as stated in ()

Professor Russell Stothard Primary Supervisor

5.1 Abstract

Introduction: In 2016, an estimated 216 million cases of malaria were reported, resulting in approximately 445,000 deaths. With increasing incidences of resistance to standard anti-malarial therapies and the unprecedented failure of RDTs in Sub-Saharan Africa, there is an onus on the scientific community to identify new mechanisms by which parasites of the *Plasmodium* genus can be detected and targeted for eradication. This study aimed to establish whether quantitative PCR (rtPCR) analysis of either dried blood spots (DBS) from finger-prick samples or ethanol preserved faces (EPF) samples collected as a part of surveillance programmes for schistosomiasis, could be used effectively as a highly sensitive and specific surveillance method for *Plasmodium* spp.

Methods: 247 dried blood spots (DBS) and ethanol preserved faecal (EPF) samples were analysed using generic, genus-specific *Plasmodium* TaqMan™ assay methods, using a single primer/probe set for detecting *Plasmodium* spp., followed by species-specific probes targeting a conserved region of the small subunit 18S rRNA gene for species-specific *Plasmodium* detection. 7 of positive samples by species-specific TaqMan™ assay underwent sequencing for species-specific confirmation. Prevalence, sensitivity, specificity, positive and NPVs were calculated to assess the efficacy of DNA from either blood or faecal samples using rtPCR as a diagnostic test for *Plasmodium* infection, compared to RDTs as the gold standard.

Results: In Lake Albert, Uganda, of the 247 SAC included in this study who were asymptomatic, 138 (55.8%; 95% CI: 49.6– 61.9) cases of malaria were diagnosed for *P. falciparum* (*Pf*HRP-2-detecting RDTs), and 45 samples (18.2%; 95% CI: 13.9–23.5) were positive for *Plasmodium* species (*Pf*HRP-2 and pan-pLDH test lines). While rtPCR has detected 198 (80.1 %, 95% CI: 74.7–84.6) for any species of *Plasmodium* spp., whereas rtPCR-based assays for EPF were 158 (63.9%; 95% CI: 57.8–69.7).

Conclusions: rtPCR analysis of fDNA samples produced mainly similar prevalence estimates to RDT data when surveilling of genus-specific *Plasmodium* infections. With the primer set used by this study, species-specific diagnosis of *Plasmodium* spp. was not possible. rtPCR using blood samples currently offers a superior surveillance method for species-specific *Plasmodium* detection.

Keywords: *Plasmodium*; real-time PCR; surveillance; RDT; faecal samples;

5.2 Introduction

With an estimated 216 million cases of malaria, resulting in 445,000 deaths, malaria remains a considerable cause of mortality and morbidity in 91 countries worldwide (WHO, 2016b). Approximately 90% of this malaria infection and deaths occurred in the WHO African Region, where ecological conditions are conducive to the transmission of *Plasmodium* spp. by their Anopheline insect host (Christiansen-Jucht *et al.*, 2014). Certain demographics are more vulnerable than others to malarial illness, such as children under 5 years of age in highly endemic areas, while in areas of lower transmission, many cases occur in older children and adults (Nmadu *et al.*, 2015, Roberts and Matthews, 2016, Carneiro *et al.*, 2010).

Malaria is caused by a parasite of the *Plasmodium* genus, which in most cases is transmitted by the bite of a female *Anopheles* mosquito, though blood-borne transmission (through accidental nosocomial transmission via blood transfusion or transplantation) and congenital transmission are also known to occur (Natama *et al.*, 2017, Martín-Dávila *et al.*, 2018). Upon inoculation of the human host with a motile *Plasmodium* sporozoite, the parasite spreads to the circulatory system via the liver, initiating a cytokine cascade which results in flu-like symptoms such as fever, malaise, myalgia, nausea and vomiting. Symptoms may progress quickly as parasites sequestrate in cerebral blood vessels, causing seizures, coma and eventually death (Bartoloni and Zammarchi, 2012). At present, five species of the *Plasmodium* genus are known to cause malaria in a human host: *P. falciparum, P. vivax, P. ovale* (now split into subspecies *P. ovale curtisi* and *P. ovale wallikeri*)*, P. malariae* and *P. knowlesi* (Fuehrer and Noedl, 2014). A sixth *Plasmodium* parasite, *P. cynomolgi,* has been reported in only one naturally-acquired case of human malaria (Ta *et al.*, 2014).

Poverty in sub-Saharan African countries, such as Uganda, create a favourable environment for mosquito breeding, with ample mosquito breeding grounds in stagnant water sources created by villagers living in poor conditions ((UBOS), 2015, Kibret *et al.*, 2018). Malaria prevalence has been previously reported here as between 55% and 96% (Betson *et al.*, 2014, Al-Shehri *et al.*, 2016). Communities living near Lake Albert in Uganda have also been found to have an extensive burden of other parasitic diseases such as schistosomiasis and giardiasis (Al-Shehri *et al.*, 2016), requiring treatment via MDA to school children in the area (Fenwick *et al.*, 2009).

Proper management lies mainly in the speed and accuracy of diagnosis, but also in an appropriate prescription (adapted antimalarial, dosage and appropriate duration of treatment) (Shah *et al.*, 2016, Kabaghe *et al.*, 2017). In Uganda, a

2009 health facility survey for monitoring quality of severe malaria case management showed that, of all patients assessed, only 27 % were correctly diagnosed with severe malaria and 30 % did not receive correct initial parenteral antimalarial medicine prescribed at the appropriate dose and frequency (Achan *et al.*, 2011). The World Health Organisation (WHO) has emphasised diagnosis and surveillance as critical aspects of controlling and eventually eliminating malaria (WHO, 2012). Malaria is currently diagnosed either by microscopy, RDT or PCR of blood spots (Tangpukdee *et al.*, 2009, Feleke *et al.*, 2017, Doctor *et al.*, 2016, Fancony *et al.*, 2013, Golassa *et al.*, 2013).

Microscopy remains to this day the most commonly applied test for the diagnosis of malaria (**Table 7**). Not requiring sophisticated equipment or a permanent source of electricity, the use of both 'thick' blood smears (also referred to as 'thick blood films') and 'thin' blood smears using Giemsa-stained blood are the diagnostics best suited for low-middle income countries such as Uganda. A 'thick' blood film consists of several layers of red blood cells and can confirm the presence of a *Plasmodium* parasite, while the 'thin' blood film (a single layer of red blood cells) makes it easier to identify species-specific morphological features (Bejon *et al.*, 2006). Correct diagnosis of a *Plasmodium* infection using microscopy is dependent upon the experience of the microscopist, with many *Plasmodium* spp. appearing indistinguishable under light microscopy, even to the trained eye (Lee *et al.*, 2009). Light microscopy can only successfully detect a *Plasmodium* spp. infection (in approximately 0.025-0.0625 µl of blood) at a density of 50-100 parasites/µl, rendering this technique less effective for lower parasite densities, such as those occurring in patients following initial antimalarial treatment (Koepfli *et al.*, 2016).

In the past two decades, new applications have become available for malaria diagnosis, such as the so-called "rapid" immunochromatographic tests (RDTs) (**Table 7**). These RDTs detect parasite-specific antigens, such as *Plasmodium* lactate dehydrogenase (pLDH) and *P. falciparum* histidine-rich protein II (*Pf*HRP- II), with the former antigen being genus-specific, and the latter being specific only for the detection of a *P. falciparum* infection (Wongsrichanalai *et al.*, 2007). In addition to their reliability in high endemic settings, RDTs are easy to use and interpret. However, some RDTs have a low sensitivity (ranging from 50 to 1000 parasites /µl (Huong *et al.*, 2002, Makler *et al.*, 1998) and a relatively high cost, thus preventing their routine use in low-resourced field settings of sub-Saharan Africa (Murray *et al.*, 2003).

It is currently recognised that PCR diagnosis of a *Plasmodium* infection is a more sensitive approach than the aforementioned techniques of light micros-

copy and RDTs, but remains reserved for specialised laboratories given its material and financial requirements. Nested PCR (nPCR) can detect up to 1 parasite/µl (Snounou *et al.*, 1993) and real-time PCR (also known as quantitative PCR or rtPCR), up to 0.2 parasite/µl or even 0.02µl parasite/µl (Mangold *et al.*, 2005, Payne, 1988, Cnops *et al.*, 2010).

Diagnostics play a critical role in guiding both the deployment of existing NTDs program resources and the implementation and evaluation of intervention strategies. Currently, faecal samples are widely used for surveillance of diseases such as schistosomiasis and STHs, but limited studies have been carried out in the detection of *Plasmodium* gDNA from human faecal samples (Jirků *et al.*, 2012). In non-human primates, however, detection of *Plasmodium* spp. infections is routinely performed using DNA extracted from faecal samples, even providing evidence for the African origin of *P. vivax* (Liu *et al.*, 2014, Martinsen *et al.*, 2014, Plenderleith *et al.*, 2018, Abkallo *et al.*, 2014, Siregar *et al.*, 2015, de Assis *et al.*, 2016, Weimin *et al.*, 2010, Kawai *et al.*, 2014). As such, faecal samples from human patients could also be used to detect malaria infections in field surveys alongside a molecular diagnosis of schistosomiasis and STHs in order to monitor a program's impact on transmission of these NTDs with minimal risk of recrudescence. The uses of such screening would range from mapping *Plasmodium*-endemic locations, monitoring and evaluation of malaria transmission, assessment of whether MDA could be ceased in favour of a targeted approach, and finally, as a means of post-MDA surveillance.

We report here the development and implementation of quantitative PCR (rtPCR) for the diagnosis of *Plasmodium* species from faecal samples in Lake Albert, Uganda (**Table 7**). This study aimed to detect gDNA of *Plasmodium* species from either ethanol preserved faeces (EPF) or dried blood spot (DBS) samples previously collected by Hajri Al-Shehri *et al.,* (Al-Shehri *et al.*, 2016), and comparing the results with malaria RDTs as the gold standard. The aim of this study was to examine whether rtPCR of DNA from faecal samples could be used for the surveillance of species-specific *Plasmodium* infections in human populations, especially in countries such as Uganda where school-based control of these infections (Schistosomiasis, STHs, and Malaria) with MDA has taken place.

5.3 Materials and methods

5.3.1 Study area

In this cross-sectional survey, field sampling and examinations of children took place during May 2015 in five primary schools in Buliisa district located within the Lake Albert region. Three of these five schools have been visited previously as sentinel surveillance sites of the NCP (Kabatereine *et al.*, 2007) and the global positioning system locations (GPS) for each school was recorded (**Figure 33**). The schools Walakuba (GPS 01°50.323N, 031°22.740E), Bugoigo (GPS 01°54.004N, 031°24.750E) and Runga (GPS 01°43.828N, 031°18.603E) were located on the immediate shoreline, while Biiso (GPS 01° 45.516N, 031°25.236E) and Busingiro (GPS 01°44.090N, 031° 26.855E) were located over 10 km away inland with an altitude of 1,295 metres on average above sea level which aimed to represent the current control landscape across high- and low-endemic settings, respectively.

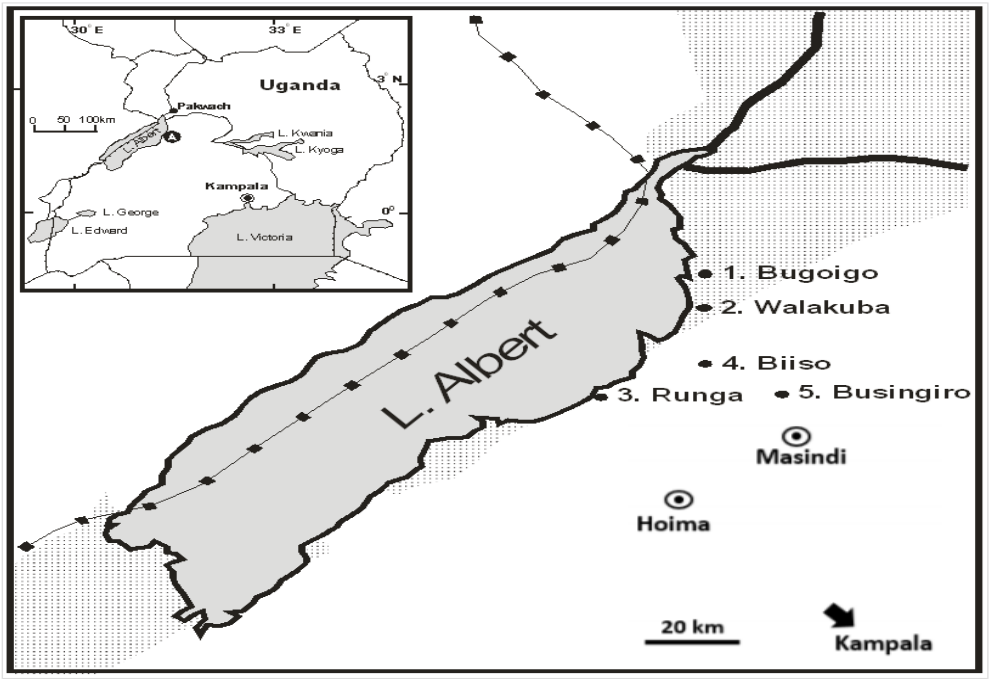

Figure 33: Inset: sketch map of the study villages in Buliisa district, Lake Albert, Uganda. The grey area is Lake Albert while the grey hatched area is the lowland plain where inflowing and outflowing Victoria and Albert Nile rivers, respectively make their course. The northern village of Pakwach was where the NCP against bilharzia and intestinal worms was launched in 2003, and study area denoted (**A**). **B.** Schematic map of the five sampled primary schools inspected in the Lake Albert region. The primary schools of Bugoigo (**1**), Walakuba (**2**) and Runga (**3**) on the lakeshore plain (Low-Altitude), whereas Biiso (**4**) and Busingiro (**5**) are located on top of the escarpment (High-Altitude). Once part of Masindi District, the schools are now located within Buliisa District after administrative areas were revised.

5.3.2 Ethical approval

The Ugandan Council for Science and Technology and the Liverpool School of Tropical Medicine granted approval for this study. Each child was examined by the project nurse and PZQ (40mg/Kg, Merck KGaA, Darmstadt, Germany) and Albendazole (400mg, GSK, Brentford, UK) treatment was administered by the attending nurse following WHO guidelines (Montresor *et al.*, 1998) to all participants irrespective of their infection status. Children with a positive malaria RDT were each offered a take-home, 3-day course of Artemether-lumefantrine (Coartem® Lonart; Cipla, Mumbai, India) with the first and third administrations directly overseen by the project nurse.

5.3.3 Enrolment of participants

After obtaining written informed consent and verbal assent, a pre-target enrolment of 60 children per school, of equal gender, randomly recruited from classes primary 1 to 3, were assessed for study eligibility and requested to provide two stool samples on consecutive days, a single urine sample and single finger-prick blood sample(**Figure 18 Page-77**). The inclusion criteria were*:* children aged between five- and ten-years-old presenting at selected school during the survey with a history of water contact activities in the previous months, and fever with measured axillary body temperature >37.5 °C at presentation. However, the absence of fever in a child did not exclude from malaria diagnosis, and treatment. Exclusion criteria included severe and chronic illnesses, trauma/injury, and intake of anti-malarial medication within the previous weeks.

5.3.4 Clinical examination and demographic information

In liaison with the headmaster and class teachers, medical history and clinical examination were performed on each child by the study clinician and information was entered into a standardised questionnaire form (**Appendix 3 Page -95**). This included demographic information, clinical and treatment history, vital signs, body weight, and epidemiological data pertinent to intestinal schistosomiasis, and malaria. Schistosome-eggs were detected by microscopy (×100 magnification) after duplicate KK thick smear slides (41.7 mg templates) were prepared from each stool. The eggs were then quantified and expressed as EPG of faeces with the intensity of infection classified as: light (1–99 EPG), medium (100–399 EPG) and heavy (⩾ 400 EPG) following the WHO guidelines (Montresor *et al.*, 1998). Clinical diagnosis of malaria was made according to national guidelines. A maximum of 3 μL Finger-Prick blood was drawn from every child for RDTs, as well as one drop of blood, was spotted onto filter paper

(Whatman 3 MM, Whatman International, Maidstone, England). Spots were then dried at ambient temperature before storage in individual plastic bags with silica gel to preserve DNA integrity, and then transported to LSTM. Here, they were stored at −20 °C for molecular analysis with Malaria species-specific TaqMan™ Assay.

5.3.5 Malaria RDTs detect specific antigens (proteins)

At the study site, Finger-Prick blood was collected from each child, and all whole blood specimens were tested in singlet using the SD Bioline Malaria Ag P.f/Pan test (RDTs) (SD Diagnostics, Korea) (**Figure 34**). The tests were performed and interpreted following the manufacturer's instructions. Briefly, the RDT were stored and used at room temperature, and approx. 2 µL of whole blood was transferred by provided plastic pipette to the round specimen well, followed by the addition of two drops of diluent into the square well. The test results were read at 15 minutes. The results were interpreted as invalid (no control line), positive (control line and test line present), mixed infection (control line and two test lines), or negative (control line present, no test line). The malaria RDT test results were provided directly to the on-site nurse and assisted the clinician on the clinical decision for treatment.

Figure 34: Malaria RDTs showing results from Biiso SAC

5.3.6 DNA extraction from biological samples (EPF and DBS)

Upon arrival, gDNA extraction, from ethanol preserved faeces (EPF) was performed by Al-Shehri *et al.* (Al-Shehri *et al.*, 2016). In brief, approximately 0.2 g of faecal sample preserved in ethanol was vigorously shaken, centrifuged and the pellet was washed twice with 1000 µl of Phosphate-buffered saline (abbreviated PBS). The sample then was suspended in 250 µl of 2% polyvinylpolypyrrolidone (PVPP; Cat-77627-25G, Sigma) before frozen

overnight at – 20 °C in a MagNA Lyser Green Beads (Product No. 03358941001, Roche). After tissue lysis treatment with proteinase K (Supplier No, BIO-37037, Bioline) for two hours at 55°C, DNA was then extracted from the pellet using the commercial QIAamp® DNA Mini Kit spin columns (QI gen, Hilden, Germany) following to the manufacturer's instructions. In each sample, 10^3 plaque-forming units (PFU)/ml phocine herpesvirus-1 (PhHV-1) was added to the isolation lysis buffer, to serve as an internal control for the isolation using the method of *Verweij et al.*(Verweij *et al.*, 2004).

The blood gDNA was extracted from Dried Blood Spots (DBS) collected with a finger stick (FS-DBS) using Chelex Resin (Chelex® 100 sodium form 50-100 mesh dry, No 11139-85-8, Sigma) (Strøm *et al.*, 2014). A standard hole punch was used to cut hole 2-mm diameter circular from stored blood samples onto filter paper (Whatman 3 MM, England), and placed into a labelled 1.5 mL Eppendorf tube (2.0 ml Crystal Clear Microcentrifuge Tube, No. E1420-2000, Starlab). The filter paper punches were then manually pushed towards the bottom of the tube several times with a pipette tip before one mL distilled water being added to each sample, and then incubating for 30 minutes at 55°C. All supernatant was removed, before adding another 1 mL of distilled water and incubating in the same conditions for 15 minutes. After removing all supernatant, 200 µL of adjusted 10% Chelex-100 concentration with distilled water, 100 µL TE buffer (Tris-EDTA (TE) buffer solution, pH 8.0, 93283-100ML, Sigma), and 1 µL sodium dodecyl proteinase K (Proteinase K, recombinant, PCR Grade, Sigma) was added, vortexed and centrifuged for 15 seconds at 14500 RPM, before incubating in the same conditions for one hour. A further vortex and centrifuge took place as above, before incubating in a VWR incubator (VWR® Ovens and Incubators) at 300 RPM at 93°C for 30 minutes. Samples were agitated and centrifuged at 10000 RPM for one minute. 100 µL of supernatant was removed, with taking care not to disturb the Chelex particles and added to another labelled 1.5 mL Eppendorf tube, before being stored at -20°C.

5.3.7 Malaria detection using malaria species-specific TaqMan™ Assay

Extracted DNA was analysed using TaqMan™ Assay targeting mitochondrial genome 18S gene as previously described by Shokoples *et al*. (Shokoples *et al.*, 2009). A singleplex real-time RT-PCR assay was initially performed to detect a region of the *Plasmodium* 18S gene that is conserved across all five species *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* (Rougemont *et al.*, 2004). Generic *Plasmodium* DNA was detected from DBS and EPF with the malaria-specific primers of Plasmo-1 F primer (5'*GTT AAG GGA GTG AAG ACG ATC AGA*'3) and Plasmo-2 R primer (5'*AAC CCA AAG ACT TTG ATT TCT CAT AA*'3), and Plasprobe

(FAM-*ACC GTC GTA ATC TTA ACC ATA AAC TAT GCC GAC TAG*-BHQ-1), supplied by Eurofins scientific (Eurofins Genomics Anzinger Str. 7a 85560 Ebersberg, Germany). The reaction was performed with a final volume of 25 µL containing 12.5 µL of iQ Supermix ((iQ™ Supermix, 100 x 50 µl reactions, 2x real-time PCR mix, contains dNTPs, 6 mM MgCl2, 50 U/ml hot-start iTaq™ DNA polymerase, Bio-Rad Laboratories Ltd.uk), 5 µL DNA, 0.4 µL (10 pmol/ul) - of each Plasmo-1 F primer / Plasmo-2 R primer, 0.2 µL (10 pmol/ul)-of Plasprobe, and 6.5µL of nuclease-free H20 (Ambion® RT-PCR Grade Water).

Samples were confirmed *Plasmodium* spp. positive with genus-specific primers, and the 6-carboxyfluorescein (FAM)-Labelled "Plasprobe" were subjected to species differentiation using TaqMan™ Assays. *Plasmodium* species were determined with species-specific forward primers, Plasmo-2 R primer and species-specific probes using multiplex rtPCR of the primers and probes listed in **Table 18** with the respective concentrations for each reaction, as published by *S*hokoples *et al.* (Shokoples *et al.*, 2009).

Table 18: Primers and probes used for screening and identification of *Plasmodium* species[a]

Species	Primer or probe	Concn (nM)	Sequence (5 –3)
P. falciparum	Fal-F primer	200	CCG ACT AGG TGT TGG ATG AAA GTG TTA A
P. falciparum	Falcprobe	200	JOE-AGC AAT CTA AAA GTC ACC TCG AAA GAT GAC T-BHQ-1
P. vivax	Viv-F primer	200	CCG ACT AGG CTT TGG ATG AAA GAT TTT A
P. vivax	Vivprobe	80	ROX-AGC AAT CTA AGA ATA AAC TCC GAA GAG AAA ATT CT-BHQ-2
P. ovale	Ova-F primer	200	CCG ACT AGG TTT TGG ATG AAA GAT TTT T
P. ovale	Ovaprobe[b]	80	FAM-CGA AAG GAA TTT TCT TAT T-MGBNFQ
P. malariae	Mal-F primer	200	CCG ACT AGG TGT TGG ATG ATA GAG TAA A
P. malariae	Malaprobe[b]	80	Cy5-CTA TCT AAA AGA AAC ACT CAT-MGBNFQ

a Primer and probe sequences are as previously published (Shokoples et al., 2009)

b,Ovaprobe and Malaprobe were synthesised by Biosearch Technologies

In total of 25 µL in each reaction, consisting of 12.5 µL IQ supermix, 5 µL of DNA template, species-specific forward primers, Plasmo-2 R primer, species-specific probes, and the reaction was completed with nuclease-free H20 (Sterile, RNase and DNase Free, 3098-2ML, UK) until a final volume of 25 µl was reached. Extracted DNA-DBS and DNA-EPF for each school were run on different plates, included a number of controls: extraction of TE (10 mM Tris-HCl [pH 8], 1 mM EDTA) as a negative control, and four positive controls, using DNA extracted from heavily infected samples diagnosed under microscope, with either *P. falciparum, P. vivax, P. ovale* or *P. malariae,* provided by diagnostic LAB at LSTM, as well as 10% experimental results repeated to ensure reliability. DNA-TaqMan® assays were performed in a Chromo-4 with Opticon

monitor Version 3.1. (Biorad, Hemel Hempstead, UK) with Biorad iQ™ Supermix and thermal cycling conditions of an initial denaturation of 3 minutes at 95°C, followed by 50 cycles of 15 seconds at 95°C, 20 seconds at 60°C and 15 seconds at 72°C. Infected children were identified according to C_t values: either positive ($10 > C_t \leqslant 45$) or negative ($C_t > 45$), see **Figure 35**

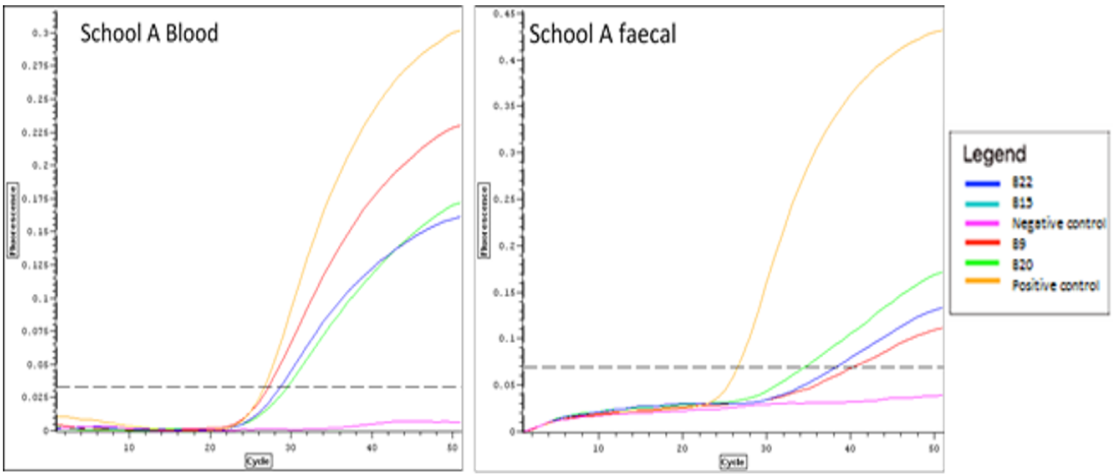

Figure 35: rtPCR quantitation as performed by Opticon Monitor 3 software (Biorad), showing the 4 positive samples with malaria infection detected by bDNA in comparison with fDNA using samples collected from school **A** (Bugoigo). It can be observed that, bDNA often gives lower C_t values and higher fluorescence in opposition to fDNA, for example, school A, sample code- B20, diagnosed by blood has a C_t value of ~27 and fluorescence of ~0.225 nm, whereas diagnosed by fDNA has a C_t value of ~40 and fluorescence of ~0.1 nm

5.3.8 Sequencing-based species-specific 18s gene for species confirmation

In order to confirm the species present and assess the genetic variability of the *Plasmodium* 18S gene, the positive products of multiplex rtPCR for each species (*P. falciparum, P. ovale* and *P. malariae)*, and one mixed infection (*P. falciparum, and P. ovale)* were performed by PCR-sequencing-based on marker 18s gene. Seven samples of PCR products from positive DNA-DBS samples (and a minimum of 500 ng total DNA) were purified with the QIAquick® PCR Purification kit (QIAGEN Ltd.) according to the manufacturer's instructions, and 5 µL of purified PCR product was sequenced externally in forwarding direction by Source Bioscience (Nottingham, UK) with their specific primers (1 ng/µL per 100 bp). Sequence reads were either 54 bp or 76 bp long, aligned using Geneious v9.1.5 software (Biomatters Limited) package, then compared with gene sequences of *Plasmodium* available from the NCBI server, using the basic local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the malaria species. Only sequences which mapped uniquely within the *Plasmodium* spp. reference genome and map to the human reference genome were

included in our analyses. *Plasmodium* 18S gene reference sequences used for *Plasmodium* species were *P. falciparum*: **M19172.1**, *P. ovale*: **L48987.1**, *P. malariae*: **AF488000.1**, and *P.vivax*: **XR_003001206.1**.

5.3.9 Statistical analysis

Statistical analysis was performed using Minitab Ltd® (Brandon Court, Unit E1-E2 Coventry CV3 2TE UK), calculating prevalence, sensitivity, specificity, PPV and NPV of each of the diagnostic methods. Logistic regression tests were performed using risk variables as an independent indicator to assess any associations with diseases based on diagnostic tools. For sequencing analysis, it was performed using the essential local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and using the Geneious v9.1.5 software (Biomatters Limited) package to find matching gene fragments, followed by checking the specificity of pre-existing primers and probes.

5.4 Results

5.4.1 General characteristics

A total of 271 children participating in this cross-sectional survey from May to June 2015 were screened; 247/271 (91.1%) met the inclusion criteria and were enrolled, mean age of 6.8 years (95% CI 6.6–6.9) with males accounting for 124/247 (50.2 %), and females 123/247 (49.7 %). Samples from 24/271 (8.8 %) children were not available for data analysis; 4/271 (1.4 %) children did not provide sufficient blood samples, 6 /271 (2.2 %) children refused to be tested on site with RDTs, 7 /271 (2.5 %) children had inhibitors in their faeces, 2/271 (0.7 %) had low yield DNA extracted from dried blood spots (DBS) on filter paper, and 5/271 (1.8%) of children demographic information was missing. The complete flow of the performed diagnostic tests and results are outlined in **Figure 29**.

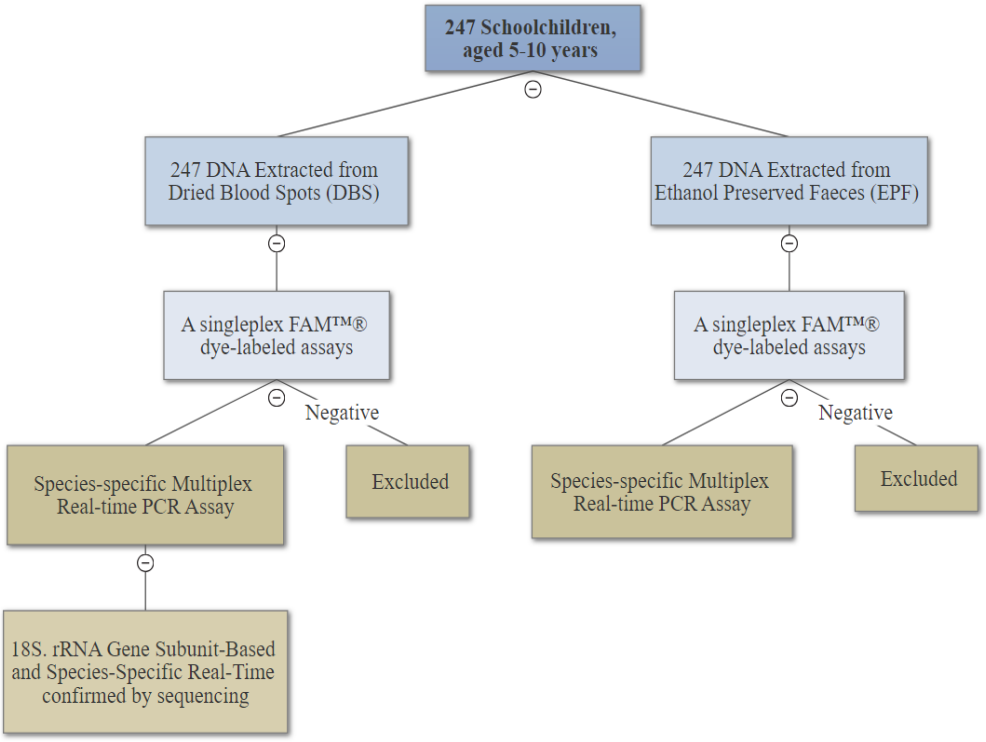

Figure 36: Flow Chart of Study Process, demonstrates various stages being taken for analysing *Plasmodium* spp. assay

5.4.2 Prevalence of malaria infection

Of 247 SAC were tested for malaria infection using malaria Ag P.f/Pan test (RDTs), 138 (55.8%; 95% CI: 49.6– 61.9) were positive for *P. falciparum* (*Pf*HRP-2-detecting RDTs). Forty-five samples (18.2%; 95% CI: 13.9–23.5) were positive for *Plasmodium* species (*Pf*HRP-II and pan-pLDH test lines). A singleplex rtPCR-based assay for DBS on filter paper was prepared and performed, of which 198 (80.1 %, 95% CI: 74.7–84.6) were positive for any species of *Plasmodium* spp., whereas a singleplex rtPCR-based assay for EPF were 158 (63.9%; 95% CI: 57.8–69.7). Co-infections among schoolchildren tested determined by the singleplex Real-Time TaqMan™ assays using eDNA-DBS and eggs for *S. mansoni* detected by two KK thick smears from each a single stool sample of two consecutive days were 88 (44.5%; 95% CI: 37.6–51.4) as shown in **Table 19**

Table 19: Prevalence and influence of diagnostic tests for detecting Plasmodium infection in primary school children

School name	Walukuba (n = 43) n (%)	Runga (n = 50) n (%)	Bugoigo (n = 56) n (%)	Biiso (n = 49) n (%)	Busingiro (n = 49) n (%)	Total (n = 247) n (%)
Prevalence						
Rapid diagnostic test[a]						
Negative	29 (67.4)	21 (42.0)	26 (46.4)	16 (32.6)	17 (34.6)	109 (44.1)
Positive	14 (32.5)	29 (58.0)	30 (53.5)	33 (67.3)	32 (65.3)	138 (55.8)
95% CI	[20.4–47.4]	[44.2–70.6]	[40.7–65.9]	[53.3–78.7]	[51.3–77.0]	[49.6–61.9]
rtPCR-based assays for DBS[b]						
Negative	10 (23.2)	8 (16.0)	24 (42.8)	5 (10.2)	2 (4.0)	49 (19.8)
Positive	33 (76.7)	42 (84.0)	32 (57.1)	44 (89.8)	47 (95.9)	198 (80.1)
95% CI	[62.2–86.8]	[71.4–91.6]	[44.1–69.2]	[78.2–95.5]	[86.2–98.8]	[74.7–84.6]
rtPCR-based assays for EPF[c]						
Negative	28 (65.1)	31 (62.0)	13 (23.2)	4 (8.1)	13 (26.5)	89 (36.0)
Positive	15 (34.8)	19 (38.0)	43 (76.7)	45 (91.8)	36 (73.4)	158 (63.9)
95% CI	[22.4–49.8]	[25.8–51.8]	[64.2–85.9]	[80.8–96.7]	[59.7–83.7]	[57.8–69.7]
No. of infected	**(n = 33)**	**(n = 42)**	**(n = 32)**	**(n = 44)**	**(n = 47)**	**Total (n = 198)**
Co-infection (SCH, MAL[d])						
Negative	4 (12.1)	6 (14.2)	21 (65.6)	36 (81.8)	43 (91.4)	110 (55.5)
Positive	29 (87.8)	36 (85.7)	11 (34.3)	8 (18.1)	4 (8.5)	88 (44.5)
95% CI	[72.6–95.1]	[72.1–93.2]	[20.4–51.6]	[9.5–31.9]	[3.3–19.9]	[37.6–51.4]

[a] Malaria by RDT (SD Bioline Malaria Ag P.f/Pan test, SD Diagnostics, Yongin-si, Gyeonggi-do, Korea)

[b] A TaqMan-based real-time PCR assay that collectively detected all 4 *Plasmodium* species from DBS

[c] A TaqMan-based real-time PCR assay that collectively detected all 4 *Plasmodium* species from EPF

[d] Co-infections among schoolchildren with *Plasmodium* species detected by rtPCR-based assay for DBS and *Schistosoma mansoni* by Kato-Katz faecal microscopy

5.4.3 Geographical distribution of malaria infection

The trends of malaria prevalence by "Low-High Altitude" and "distance from Lake Albert" were mostly similar between the three diagnostic methods, and the rate of being diagnosed positive increased significantly by variation in distance from the Lake within the study sites ($p < 0.001$ for all three diagnostic tools), Figure 5.4.3. The highest rates of malaria infection detection by using

Real-Time TaqMan™ assays for eDNA-DBS targeting generic *Plasmodium* (18S gene) was 47 (95.9%; 95% CI: 86.2–98.8) at Businginiro SAC, and for eDNA-EPF was 45 (91.8%; 95% CI: 80.8 – 96.7) at Biiso school children. In Low Altitude, closest schools to the shoreline, Walukba and Runga , the rate of prevalence was low by A RDT 14 (32.5%; 95% CI: 20.4-47.4), 29 (58.0%; 95% CI: 44.2-70.6), respectively, and by Real-Time TaqMan™ Assays for eDNA-EPF 15 (34.8%; 95% CI: 22.4-49.8), 19 (38.0%; 95% CI: 25.8-51.8), compared to Real-Time TaqMan™ Assays for eDNA-DBS 33 (76.7%; 95% CI: 62.2-86.8), 42 (84.0%; 95% CI: 71.4-91.6). In Bugoigo school, the prevalence of malaria by Real-Time TaqMan™ Assays for eDNA-EPF was increased 43 (76.7%; 95% CI: 64.2-85.9), compared to the other two diagnostic tools, see **Figure 37**.

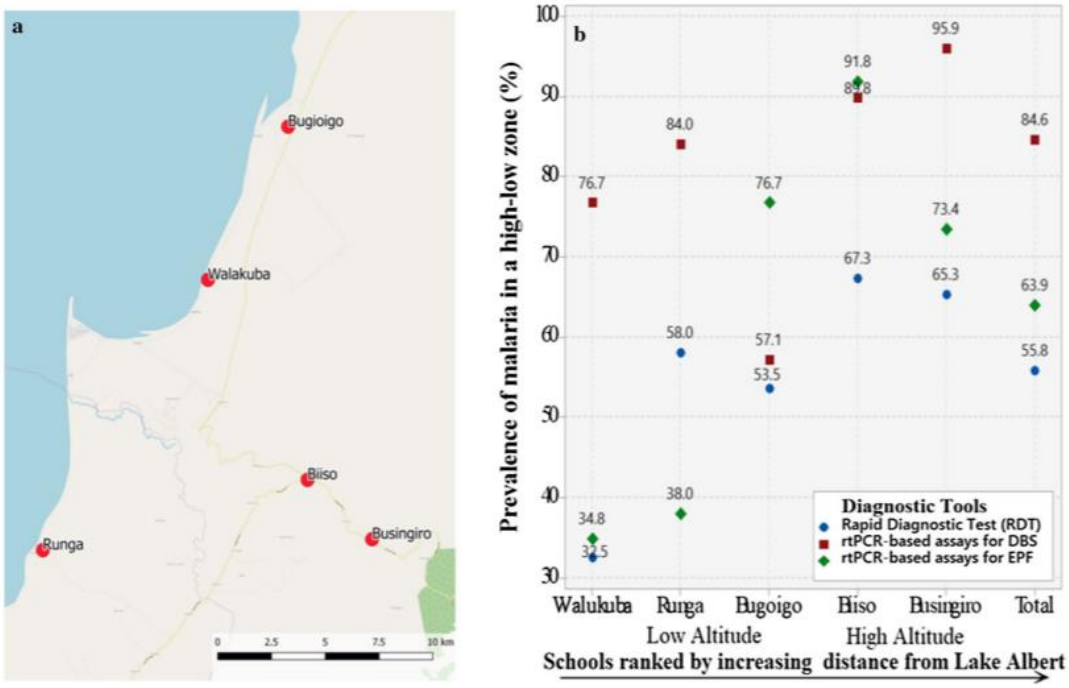

Figure 37: Estimated prevalence of Malaria infection by altitude and school distance from the shoreline. **(A)** Schematic map of the five sampled primary schools in the Lake Albert region, the blue area indicates Lake Albert. **(B)** Estimated prevalence of Malaria infection by altitude and school distance from the shoreline for each examined diagnostic test; prevalence by any positive test criterion is also illustrated.

5.4.4 Correlation between C_t values and the presence of malaria DNA

The relationship between C_t value of rtPCR-based assays for DBS and C_t value of rtPCR-based assays for EPF in diagnosing generic *Plasmodium* (18S rRNA gene), is DBS (C_t) = 26.93 + 0.1763 EPF (C_t) and is statistically significant (P = 0.01), with R-squared 4.14 % (P < 0.005), and positive correlation as shown is r = 0.2 (**Figure 38**), indicating that when EPF (C_t) increase, the DBS (C_t) also tends to increase (*p-value* < 0.05).

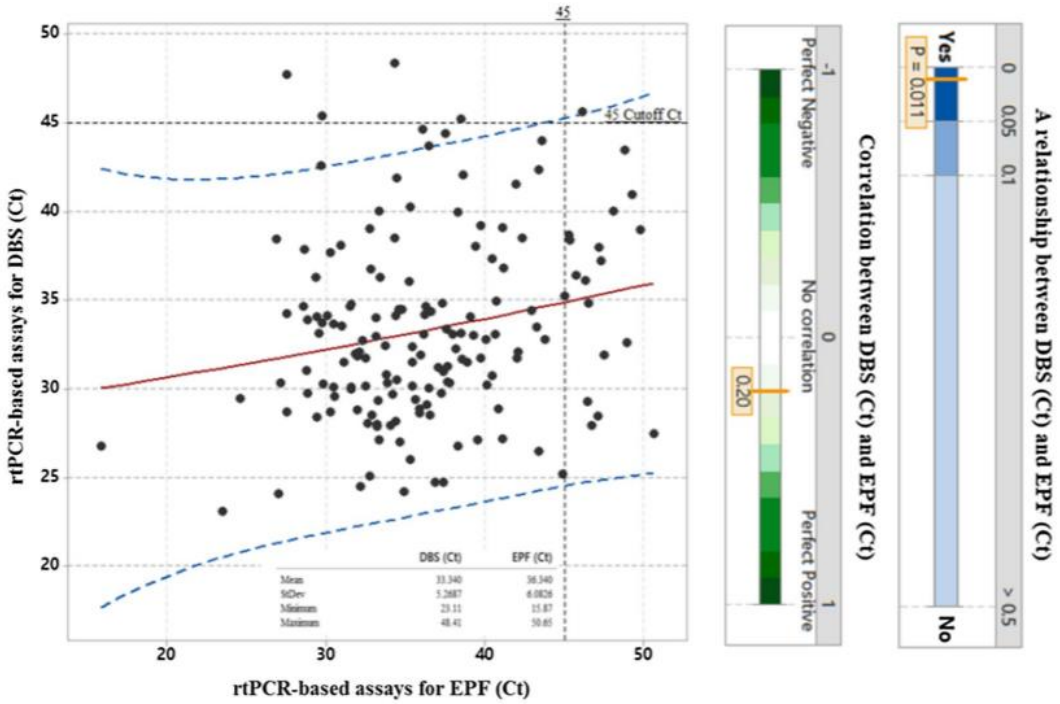

Figure 38: Showing the regression between rtPCR-based assays for DBS (C_t) and rtPCR-based assays for EPF (C_t) for the singleplex assay to detect generic *Plasmodium* (18S rRNA gene). The red fitted line shows the predicted value and cross line is the cut off 45 C_t. The blue dashed lines show the 95% prediction interval. The mean C_t value of EPF (C_t) is 36.3, and DBS (C_t) is 33.3, and the actual difference between DBS (C_t) and EPD (C_t) is 2.3 (95 Cl; 1.1-3.5).

5.4.5 Evaluating diagnostic accuracy in the face of RDT as 'gold standard'

Using RDT as 'gold standard' **Table 20**, the estimated sensitivity of rtPCR-based assays for DBS targeting generic *plasmodium* is 94.2% (95% CI: 88.9-97.0), with diagnostic odds 9.7 (95% CI; 4.3 - 22.0), while rtPCR-based assays for EPF is 73.1% (95% CI: 65.2-79.8), and diagnostic odds 2.4 (95% CI: 1.4 - 4.2). The specificity for DBS was 37.6% (95% CI: 29.0-46.9), and the PPV of 65.6% (95% CI: 58.8-71.9) and NPV of 83.6 % (95% CI: 70.9-91.4), with agreement (Cohen's kappa) was 0.3 (95% CI: 0.2- 0.4). rtPCR-based assays for EPF had specificity of 47.7 % (95% CI: 38.5-57.0), PPV of 63.9 % (95% CI: 56.1-71.0) and NPV of 58.4 % (95% CI: 48.0-68.1), with agreement (Cohen's kappa) was 0.2 (95% CI: 0.1-0.3).

Table 20: Empirical estimates of sensitivity (SS), specificity (SP), negative predictive value (NPV) and positive predictive value (PPV), Cohen's kappa for each diagnostic test against malaria by RDT as 'gold standard.'

Assay	Negative (%)	Positive (%)	Total (%)	Estimate % (95% CIs)		Diagnostic accuracy (95% CIs)	Diagnostic odds (95% CIs)	Cohen's kappa
Rapid diagnostic tests (RDTs)								
rtPCR of DBS[a]								
Negative	41 (83.6)	8 (16.3)	49 (19.8)	Sensitivity	94.2% [88.9–97.0]	69.2% [63.2–74.6]	9.7 [4.3–22.0]	0.3 [0.2–0.4]
Positive	68 (34.3)	130 (65.6)	198 (80.1)	Specificity	37.6% [29.0–46.9]			
Total (%)	109 (44.1)	138 (55.8)	247 (100.0)	PPV	65.6% [58.8–71.9]			
				NPV	83.6% [70.9–91.4]			
rtPCR of EPF[a]								
Negative	52 (58.4)	37 (41.5)	89 (36.0)	Sensitivity	73.1% [65.2–79.8]	61.9% [55.7–67.7]	2.4 [1.4–4.2]	0.2 [0.1–0.3]
Positive	57 (36.0)	101 (63.9)	158 (63.9)	Specificity	47.7% [38.5–57.0]			
Total (%)	109 (44.1)	138 (55.8)	247 (100.0)	PPV	63.9% [56.1–71.0]			
				NPV	58.4% [48.0–68.1]			

[a] rtPCR-based assays based on detecting a region of the *Plasmodium* 18S DNA gene that is conserved across all five species *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*

5.4.6 Geographical distribution of *Plasmodium* species

Multiplex PCR that detects the four most common human *Plasmodium* species (*P. falciparum, P. vivax*, *P. ovale,* and *P. malariae*) using four different specific probes for Real-Time TaqMan™ Assay (**Table 21**).

Table 21: Distribution of *Plasmodium* species across study sites using TaqMan® probe-based assays

School Locations	*Walukuba (n=43)* n (%)	*Runga (n= 50)* n (%)	*Bugoigo (n=56)* n (%)	*Biiso (n=49)* n (%)	*Busingiro (n=49)* n (%)	*Total (n=247)* n (%)
Prevalence						
Species-specific Multiplex rtPCR for DBS						
P. falciparum	8 (18.6)	21 (42.0)	9 (16.0)	38 (77.5)	35 (71.4)	111 (44.9)
P. vivax	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
P. ovale	0 (0.0)	2 (4.0)	1 (1.7)	7 (14.2)	6 (12.2)	16 (6.4)
P. malariae	0 (0.0)	0 (0.0)	0 (0.0)	5 (10.2)	4 (8.1)	9 (3.6)
Species-specific Multiplex rtPCR for EPF						
P. falciparum	1 (2.3)	2 (4.0)	0 (0.0)	2 (4.0)	13 (26.5)	18 (7.2)
P. vivax	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
P. ovale	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
P. malariae	0 (0.0)	0 (0.0)	0 (0.0)	5 (10.2)	4 (8.1)	9 (3.6)

a. species-specific multiplex PCR that detects the four most common human *Plasmodium* species (*P. falciparum, P. ovale, P. vivax* and *P. malariae*) using four different specific TaqMan probes targeting specific region from extracted DNA from either Dry Blood spot (DBS) or Ethanol Preserved Faeces (EPF)

5.5 Discussion

5.5.1 Non-invasive surveillance for *Plasmodium* in non-human primates

Owing to the difficulty of obtaining blood samples from great apes and other non-human primates, mapping the diversity of *Plasmodium* parasite infections from this group has relied almost solely on PCR-based detection of DNA extracted from faeces (Kaiser *et al.*, 2010, Liu *et al.*, 2010a, Liu *et al.*, 2014, Boundenga *et al.*, 2015, Plenderleith *et al.*, 2018). Extraction of *Plasmodium* DNA from whole blood was only necessitated when whole genome sequencing (WGS) was to be carried out, and must be done so under sedation and with ethical approval (Otto *et al.*, 2014, Otto *et al.*, 2018, Sundararaman *et al.*, 2016, Pasini *et al.*, 2017, Rutledge *et al.*, 2017).

In general, monitoring *Plasmodium* spp. infections in non-human primates from faecal samples is carried out using single genome amplification (SGA), followed by direct sequencing of SGA amplicons; a procedure developed to eliminate *Taq* polymerase template-switching which can result in recombinants that do not exist naturally (Liu *et al.*, 2010b). Initial bulk PCR of fDNA samples first identifies samples with amplifiable *Plasmodium* spp. mitochondrial, apicoplast, and nuclear sequences. Bulk PCR-positive faecal samples are then selected for SGA analyses, with the original faecal sample diluted beforehand in nuclease-free water, again to avoid *Taq* polymerase artefacts and the possibility of skewing PCR results in favour of a predominant *Plasmodium* species infection (in cases where multiple species may be present)(Liu *et al.*, 2010b). Using this SGA approach, in combination with primers specific for *Plasmodium* mitochondrial cytochrome B (*cytB*) sequences, nuclear gene sequences (such as erythrocyte-binding antigen 165 (*eba165*) and gametocyte surface protein p47 (*p47*)), or apicoplast gene sequences (such as Clp chaperone M (*clpM*)), analyses of nearly 3,500 SGA-derived sequences from both faecal and blood samples have been used to confirm the existence of six *Laverania* spp. (a *Plasmodium* subgenus) among non-human primates (Liu *et al.*, 2016, Loy *et al.*, 2017).

5.5.2 Validation of a PCR-Based Test for the detection of *Plasmodium* spp

In the present study, DNA samples derived from both faecal (EPF) and dried blood spot (DBS) samples were subject to real-time PCR as described in Shokoples *et al.*, 2009 (a non-SGA approach) (Shokoples *et al.*, 2009). Initially, genus-specific *Plasmodium* 18S rRNA sequence primers were used to detect the presence of a *Plasmodium* spp. infection, with *Plasmodium* spp.-positive sam-

ples subject to further species-specific rtPCR analyses to determine the presence of either *P. falciparum, P. vivax, P. malariae*, *P. ovale* (both subspecies) or a mixed infection (Rougemont *et al.*, 2004) (**Table 18**).

Using these criteria, 198 of 247 children (80.1%; 95% CI: 74.7-84.6) tested positive for a *Plasmodium* spp. infection from DBS samples, with 158 of 247 children (63.9%; 95% CI: 57.8-69.7) testing positive for a *Plasmodium* spp. infection using EPF-derived DNA (**Error! Reference source not found.**). Taking these data alone, both DBS- and EPF-derived DNA, followed by rtPCR diagnosis, identified more cases of *Plasmodium* spp. infection compared to the Ag P.f/Pan RDT, which tested positive for a *P. falciparum* infection (taking *Pf*HRP-II as a proxy) in only 138 children (55.8%; 95% CI: 49.6-61.9), with a pan *Plasmodium* spp. lactate dehydrogenase (pLDH) detected in only 45 cases (18.2%; 95% CI: 13.9-23.5). Assuming rtPCR data to be correct (no false positive results), discrepancies between these numbers may be accounted for by one of two reasons: (i) the increased sensitivity of PCR diagnosis compared to even the most sensitive of RDTs (Britton *et al.*, 2016); or (ii) the failure of the Ag P.f/Pan RDT (detecting *Pf*HRP-2/pan HRP-2) to detect the target antigen due to variation in, or deletion of, the histidine-rich repeat region of the *hrp2* gene, an issue that has been documented in *P. falciparum* (Koita *et al.*, 2012, Deme *et al.*, 2014, Okoth *et al.*, 2015). Diagnosis of a *Plasmodium* spp. infection by a pLDH-positive result was an even less reliable method of detecting malaria, despite the observed low genetic diversity of the *P. falciparum ldh* gene (Simpalipan *et al.*, 2018).

5.5.3 Altitude-variations in *Plasmodium* spp. prevalence

Taking altitude and distance from Lake Albert into account, the highest prevalence of *Plasmodium* spp. infection by any method was at Busingiro school, where 47 out of 49 pupils (95.9%; 95% CI: 86.2-98.8) tested positive by rtPCR-based assay of DBS samples (**Figure 37**). In contrast, only 73.4% (95% CI: 65.2-79.8) of the same pupils tested positive for a *Plasmodium* spp. infection by rtPCR-based assay of EPF samples. The highest result for rtPCR-based detection of *Plasmodium* spp. DNA from EPF samples was at Biiso school, where 91.8% of pupils tested positive for a *Plasmodium* spp. infection, with a similar number (89.8% of pupils) testing positive using rtPCR-based assay of DBS samples. Strikingly, in all but one case (at the Runga school) both rtPCR-based assays using either DBS or EPF samples proved more sensitive in the diagnosis of a malaria parasite infection than an Ag P.f/Pan RDT. These findings confirm those previously seen by Jirků *et al.*(Jirků *et al.*, 2012), in that this study showed that *Plasmodium* spp.-infected humans shed a detectable amount of *Plasmodium* spp. DNA in their faeces for PCR-based amplification and diagnosis.

Unlike in previous studies where *P. falciparum* infection and parasite density was negatively associated with rising altitude (Drakeley *et al.*, 2005, Chandler *et al.*, 2006, Pothin *et al.*, 2016, Omondi *et al.*, 2017), our study found that the highest percentages of *Plasmodium* spp. detection by Real-Time TaqMan™ -based assay of DBS samples and RDTs were at Busingiro (95.9% of pupils) and Biiso (91.8% of pupils) respectively, both regions being at higher altitudes and at a greater distance from Lake Albert compared to the three remaining sites (**Figure 37B**). In both cases, Real-Time TaqMan™ -based detection of *Plasmodium* spp. from EPF samples detected a higher percentage of infection than RDTs; at Busingiro, 73.4% diagnosed by Real-Time TaqMan™ -based assay from EPF compared to 65.3% by RDTs, and at Biiso; 91.8% diagnosis by Real-Time TaqMan™ -based assay from EPF samples, compared to 67.3% diagnosis by RDTs.

At lower altitudes, and adjacent to Lake Albert, rtPCR-based assay of DBS samples demonstrated a greater detection of *Plasmodium* spp. infection when compared to RDT detection at all sites (**Figure 37B**)**.** However, rtPCR -based detection of *Plasmodium* spp. DNA from EPF samples were unpredictable. At Bugoigo, DNA extracted from EPF samples detected the greatest percentage of *Plamodium* spp. infection (76.7% of pupils) compared to both rtPCR-based assay from DBS samples or RDTs (57.1% and 53.5% respectively). At Runga, rtPCR-based assay of EPF samples detected the lowest percentage of *Plasmodium* spp. infection (38.0%) compared to RDTs (58.0%) or rtPCR-based assay of DBS samples (84.0%). At the lowest altitude, and in closest proximity to the water's edge at Lake Albert, Walukuba school pupils tested positive for a *Plasmodium* spp. infection in 32.5% of cases using RDTs, with rtPCR analysis of EPF-extracted DNA faring better with 34.8% detection. At Walukuba, rtPCR-based assay of DBS samples demonstrated the greatest percentage detection, at 76.7%. At all five sites, rtPCR-based detection of *Plasmodium* spp. DNA from DBS samples diagnosed more pupils than RDT-based diagnosis, with the rtPCR-based assay of EPF samples detecting a greater percentage of *Plasmodium* infections at 4 out of 5 sites.

5.5.4 Analytical specificity and sensitivity of a rtPCR-Based assay

When determining the sensitivity and specificity of rtPCR-based assays; an initial evaluation of cycle threshold (C_t) values showed that there was a positive correlation between DBS- and EPF-derived samples (r =0.2), with a mean EPF Ct value of 36.3, and a mean DBS Ct value of 33.3 (**Figure 38**)**.** When correlating these results to RDT diagnosis as our 'gold standard', rtPCR-based assay of DBS samples showed a greater sensitivity than EPF samples (94.2% versus 73.1%), with rtPCR of DBS samples having a specificity of 37.6% and a PPV of 65.6%. In

contrast, the specificity of the rtPCR-based assay of EPF samples was 47.7%, with a PPV of 63.9% (**Table 20**)**.** Overall, the diagnostic accuracy of a rtPCR-based assay of DBS samples was 69.2% (95% CI: 63.2-74.6) and for EPF samples was 61.9% (95% CI: 55.7-67.7). Therefore, EPF samples in this study proved less diagnostically accurate and had lower sensitivity when compared to DBS samples when diagnosing infection of a parasite of the *Plasmodium* genus. However, one must take into consideration that PCR analysis and microscopy are generally considered more accurate in the diagnosis of a malaria infection in all cases besides a *P. knowlesi* infection, and so, without microscopy results in addition to RDT results, it is unknown in our case whether both rtPCR-based assays in fact detected a greater number of cases accurately (**Figure 37**) in incidences where RDTs failed to detect a *Plasmodium* antigen(Mahende *et al.*, 2016, Han *et al.*, 2017, Mogeni *et al.*, 2017).

5.5.5 DNA-based species-specific assays

In the next phase of our examination, i.e. when attempting to determine *Plasmodium* species using species-specific primers (Rougemont *et al.*, 2004) for Real-Time TaqMan™ -based analyses of both DBS- and EPF-derived DNA, three species of *Plasmodium* (*P. falciparum, P. malariae,* and *P. ovale* (either subspecies)) were identified from DBS samples (**Table 21**)**.** The presence of *P. vivax* was not detected in this study, in keeping with findings from previous studies in the same region (Betson *et al.*, 2018). When examining EPF samples, and using the same probe-based assays, the rtPCR analysis failed to detect the presence of either *P. vivax* or *P. ovale* subspecies (though a total of 16 pupils tested positive for *P. ovale* DNA from DBSs). In the case of *P. malariae*, rtPCR-based assays from both DBS and EPF samples detected 9 cases (3.6% of the total number of pupils). However, when testing for *P. falciparum* DNA, rtPCR of EPF samples identified only 18 cases, though 111 cases were identified using DBS samples (**Table 21**). When using the same primers, Shokoples *et al.* (Shokoples *et al.*, 2009) demonstrated a sensitivity and specificity of 100% in 91 single infections, with 13/16 mixed infections successfully identified. Assuming 100% sensitivity and specificity of these Real-Time TaqMan™ probe-based assays, species-specific multiplex PCR using DNA extracted from EPF samples was not sufficient to accurately identify species-specific *Plasmodium* spp. infections. Analysis of the target sequence in the 18S rRNA gene using reference gene for each species revealed flaws in species-specific TaqMan™ Assay as for three species (*P. falciparum*, *P. malariae*, and *P. ovale*) were correctly identified by using DNA extracted from DBS samples. However, the infections with these species (*P. malariae*, and *P. ovale*) may be missed by the 18S rRNA-based PCR diagnostic method in the presence of deletions and mutations in the 18S rRNA-based

target sequences (**Appendix 5 Page-160**). Thus, it can be concluded that in addition to molecular biological methods for the detection and characterisation of *Plasmodium* spp, careful microscopic examination of stained thin blood films is still essential in high-prevalence malaria regions, including sub-Saharan Africa for identifying different malaria species (Kawamoto *et al.*, 1996, Zhou *et al.*, 1998).

Despite these results, the sensitivity and specificity of a rtPCR-based assay using EPF samples, with *Plasmodium* spp. genus-specific primers were similar to those seen when using DBS samples (using the P.f/Pan RDT as a 'gold standard') (**Table 20**). In addition, *Plasmodium* spp. identification from faeces could be carried out in tandem with tests for schistosomiasis and STHs, and is a less invasive (and possibly more accurate (**Figure 37B**) method of determining malaria status when compared to DBS samples and RDTs, especially in small children who are most at risk when infected with a *Plasmodium* parasite(Jirků *et al.*, 2012)

In addition, in both this study and that of Jirků *et al.,* 2012 carried out, PCR-based analysis of *Plasmodium* spp. DNA using an initial 'bulk' PCR approach, in contrast to the limiting dilution and SGA procedure that has become a mainstay in the identification of *Plasmodium* spp. DNA from the faeces of non-human primates (Liu *et al.*, 2010b, Liu *et al.*, 2016, Loy *et al.*, 2017). Future examinations using this protocol, alone or in combination with more sensitive rtPCR probes (Dormond *et al.*, 2011, Gavina *et al.*, 2017), may prove more sensitive in both genus-specific and species-specific *Plasmodium* spp. detection and identification from human faecal samples. Advances in handheld rtPCR equipment may also provide an avenue by which these diagnostics can become more portable and affordable in field conditions (Ahrberg *et al.*, 2016, Li *et al.*, 2016). What is evident from our results is that, in accordance with the findings of Jirků *et al.,* human faecal samples, even those transported as EPF samples for diagnosis of schistosome or STH infections, contain sufficient *Plasmodium* spp. DNA to enable Real-Time TaqMan™ -based identification of malaria parasite infection.

5.6 Conclusions

According to the studies, the sensitivity thresholds of the different PCRs targeting the *Plasmodium* genes vary from 0.001 to 30 parasites / µl, whereas microscopy when correctly performed at a detection threshold of 50-100 parasites / µl, and RDTs ranging from 50 to 1000 parasites / µl. In addition, microscopic diagnostic performance is closely related to the microbiologist 's experience, reading time, and intensity of parasitaemia so that microscopic

examination may be negative in pauciparasitic forms. Hence the introduction of molecular biology techniques should be explored which are more sensitive and more specific. It should be noted that internal control of inhibition was used for all the samples of our study to detect the false negative results by the rtPCR. This discordant results would probably be related to an error of identification of *Plasmodium* spp by RDTs, compared to DBS or EPF that the level of parasitaemia was low and under the threshold of detection. Multiplex PCR has demonstrated the existence of a mixed *P. falciparum*, *P.ovale* and *P. malariae* infection among SAC, the RDTs of which only allowed the diagnosis of *P. falciparum* and mixed infection. In this sense, different studies have shown that PCR is more sensitive and more specific than RDTs examination for the detection of mixed infections (Siwal *et al.*, 2018). The multiplexed PCR used saves time and reagents by using multiple primer pairs; allowing the detection of the four malaria species in a single stage. In addition, this technique is suitable for a large number of blood samples, which would allow it to be applied to malaria screening in subjects from endemic areas. Nevertheless, although the introduction of PCR in malaria screening is fascinating, the cost of this molecular technique remains high compared to the microscopic examination which represents the reference technique for malaria, schistosomiasis and STHs. Also, PCR requires equipment that is only available in undoubtedly specialised laboratories.

Authors' contributions

Authors contributions were: HAS, AC, JA, EJLaC, BJP, ADA & JRS performed laboratory DNA investigations while fieldwork in Uganda was undertaken by HAS, JA, MAd, MAr, AA, NBK and JRS. All authors contributed to the initial draft of the manuscript and assented on the final version submitted for consent for publication. All authors read and approved the final manuscript.

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Competing interests

The authors declare they have no competing interests.

Consent for publication

Not applicable.

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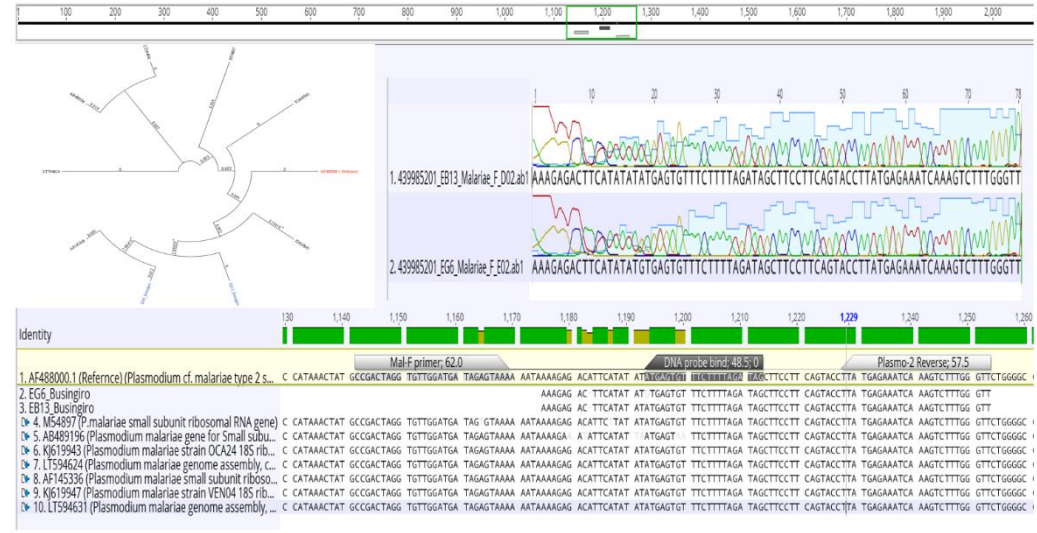
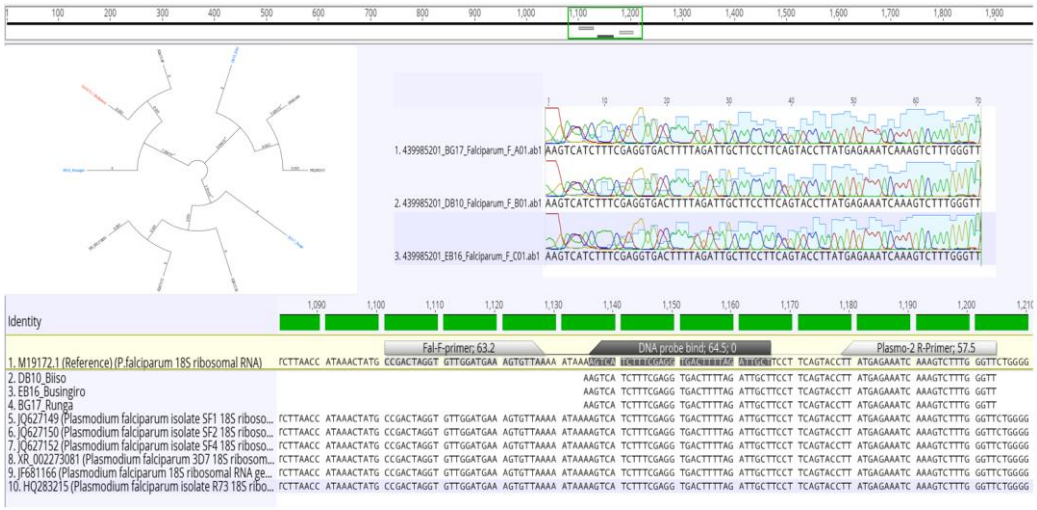
5.7 Appendix

Appendix 5: Presence of deletions and mutations in the 18S rRNA-based target sequences





Chapter 6 Evaluation and implementation of new approaches for surveillance of schistosomiasis: application of molecular DNA methods for snail species identification combined with immuno-diagnostics using urine-CCA strip test and finger-prick blood SEA-ELISA in schoolchildren in Saudi Arabia

This chapter is being prepared for publication.

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Professor Russell Stothard Primary Supervisor

6.1 Abstract

Background: Schistosomiasis, a well-known disease in the Kingdom of Saudi Arabia, is a major public health challenge in the Middle East regions, especially in Asir Province, the southwestern part of Saudi Arabia. Within the WHO 2012-2020 Roadmap on NTDs has set control and elimination of schistosomiasis by the scale-up of MDA with PZQ, snail control using chemical molluscicides, and early detection and treatment of infected cases, which has led to change the epidemiological landscape across the Kingdom of Saudi Arabia.

Methods: To develop better monitoring protocols, two commercially available diagnostic tests –Urine- CCA strip and SEA-ELISA – were evaluated for detection of *Schistosoma* infections in 163 schoolchildren from two primary schools (Huzaifa Ibn al-Yaman and Ibn al-Athir schools) representative of disease endemicity at Al-Majardah District in Saudi Arabia. DNA analysis using the COX1 gene combined with the morphological identification of snail species was also implemented in this study. In an attempt to characterise infection status further, urine filtration assessment with microscopy and CCA-testing was also used in a follow-up survey for children being positive in the baseline.

Results: DNA analysis using the COX1 gene combined with the morphological identification of snail species demonstrated the presence of *Bulinus forskalii* in the selected study area and absence of *Biomphalaria* during malacological surveys. Using urine-CCA strip assessment and SEA-ELISA, the prevalence of *S. mansoni* was 15.3% and combined *S.mansoni*/*haematobium* 34.3%, respectively. Using the CCA-urine as a reference test, the diagnostic scores of the SEA-ELISA using sera from fingerprick blood were not satisfactory; a sensitivity of 60.0%, a specificity of 70.2 %, a PPV of 26.7 % and a NPV of 90.6 %. At the unit of the school, statistical tests were performed, which showed no apparent relationship between age and inferred from CCA or ELISA tests ($P > 0.05$). In the following up, one year later, standard urine filtration assessment with microscopy and CCA-testing did not reveal active infections, pointing toward the importance of past infection and prior treatment status.

Conclusions: The SEA-ELISA test holds promise as a complementary field-based test for monitoring the effectiveness of preventive and infection control programs in schoolchildren over and above standard examined methods especially when current and previous infection status is considered negative.

Keywords: schistosomiasis, elimination, a freshwater snail, point-of-contact, urine-CCA strip, SEA-ELISA, Kingdom of Saudi Arabia

6.2 Introduction

Schistosomiasis is a parasitic disease, caused by platyhelminth worms, schistosomes, o more commonly known as 'blood flukes' (Hotez *et al.*, 2008, Colley *et al.*, 2014). Over 66.5 million people were treated for the disease in 2015 (WHO, 2016a). It is the second most common parasitic disease globally after malaria, and is listed in the WHO portfolio of NTDs (WHO, 2015c, WHO, 2016a). Worldwide, the number of people thought to be infected with *S. haematobium* is approximately 112 million and for *S. mansoni* 54 million (Koukounari *et al.*, 2007). It can cause various symptoms, depending on the chronicity of infection, the level of infection and the individual. Chronic infections can lead to anaemia, learning difficulties and in severe cases even death (Helmy *et al.*, 2017, Colley *et al.*, 2014). Urogenital and gastrointestinal pathology can also occur, the symptoms of which may include haematuria, genital lesions, abdominal pain and diarrhoea (Gray *et al.*, 2011a). Bladder cancer has also been reported in the late stages of infection (Mostafa *et al.*, 1999, Marbjerg *et al.*, 2015).

Schistosoma mansoni and *S. haematobium* are commonly found in Africa as well as the Middle East, and both species being detected in the Kingdom of Saudi Arabia (KSA) (Lotfy and Alsaqabi, 2010, El-Shahawy *et al.*, 2016, Barakat *et al.*, 2014). Schistosomiasis has been reported in Saudi Arabia since the first *S. haematobium* case was confirmed in 1887, but it is believed that the disease in Saudi Arabia has been endemic since the 10th century (Barakat *et al.*, 2014, WHO, 1993). When the first national survey was carried out for comprehensively tracking schistosomiasis and its intermediate host distribution (s) across countrywide in Saudi Arabia, estimated overall prevalence was 17%. However, it was thought by other surveyors that this statistic was an over-estimate owing to geographical complexity of the disease due to the relative risks associated with a unsafe water source in urban centres and the Eastern region, and the focal nature of the disease in rural areas, which should be better delineated for better strategic control and prevention at local levels (Lotfy and Alsaqabi, 2010).

There are different specific types of freshwater snails serving as intermediate hosts for *Schistosoma* spp. in Saudi Arabia: (1) *Biomphalaria pfeifferi*– presented in perennial and intermittent streams, rivers, lakes, reservoirs, and ponds in Northern, Northwestern, Southwestern, Mid-northern, and Mid-southern areas; (2) *Bulinus beccarii* – found in shallow slow running perennial and intermittent streams, and created bodies of water, including ponds in Western and Southern provinces; (3) *Bulinus truncatus* – is abundant on stones and masonry surroundings small pools practically lacking water plants, and more common species in the Mid-western and Southern areas; (4) *Bulinus*

wrighti – present in most common on clean rock surfaces in the shade far from human settlement, and according to Brown and Gallagher (1985), this species of snail appears less ecologically specialised and restricted to pristine habitats, and therefore, unlikely important in human disease transmission in general, although it is a permissive intermediate host for *S. haematobium* and widely used in the experimental laboratory (Lotfy and Alsaqabi, 2010, Brown *et al.*, 1985, Arfaa *et al.*, 1989, Amr and Alshammari, 2012, Mostafa *et al.*, 2012, Bin, 2009); (5) *Bulinus forskalii* is one of *Bulinus* species that has complex genetic variations, and is typically responsible for the transmission of non-human *Schistosoma* in Africa and adjacent endemic areas (Jones *et al.*, 2001), and its distribution remains unclear due to insufficient data of many parts of the Arabian Peninsula, particularly in KSA (Jones *et al.*, 2001, Neubert, 1998, Amr and Alshammari, 2012).

The national programme to control schistosomiasis (bilharzia) in KSA is focused on the detection and PZQ treatment of infected cases, snail control, and health education which led to a considerable decrease in the prevalence of the disease in most regions of Saudi Arabia (Ashi *et al.*, 1989, WHO, 1993, Ageel and Amin, 1997, Fenwick *et al.*, 2006, Hotez *et al.*, 2012). Earlier in environmental control, copper sulphate was the first substance used to control snails. Also, the available drugs being used to treat infected people included antimonials, niridazole and then later metrifonate; however, when PZQ was introduced in 1982 (Ashi *et al.*, 1989), it replaced all these drugs, and from that point onward being used in mass control, the prevalence of schistosomiasis in Saudi Arabia declined significantly (Hotez *et al.*, 2012). The prevalence in 1983 was 9.8% which reduced to 0.6% in 2004, because of regular treatment of all risk groups with PZQ alongside treating identifiable sites of frequent human contact with snail-infested waters (Lotfy and Alsaqabi, 2010). In 2005, the elimination control program for schistosomiasis was launched in KSA, targeting 12 remaining focal transmission sites (Lotfy and Alsaqabi, 2010).

The strategy for elimination included: active case detection and treatment of 80–100% of the endemic population; biannual-directed treatment of 80-100% children of school-age; availability of adequate water supply and sanitation as well as good hygiene to endemic communities; snails control with chemical molluscicides; and finally, health promotion and ill-health prevention by health education (Kloos, 1995, McCullough, 1986, Fenwick, 1987, WHO, 1993). In 2008, the WHO Department of Control of NTDs convened up to 30 global experts and country representatives where schistosomiasis has been under control for a long time which could contribute to the future expansion of strategies for monitoring schistosomiasis in very low-transmission region as well as criteria for examining and evaluating disease elimination (WHO,

2009a). As of 2010, the estimated country prevalence (%) of schistosomiasis in 2010 dropped quickly to <0.01% in Saudi Arabia (Rollinson *et al.*, 2013). Areas within Saudi Arabia today are classed as **a**) disease free; **b**) transmission interrupted; **c**) low transmission (**Figure 39**) (Stothard *et al.*, 2014).

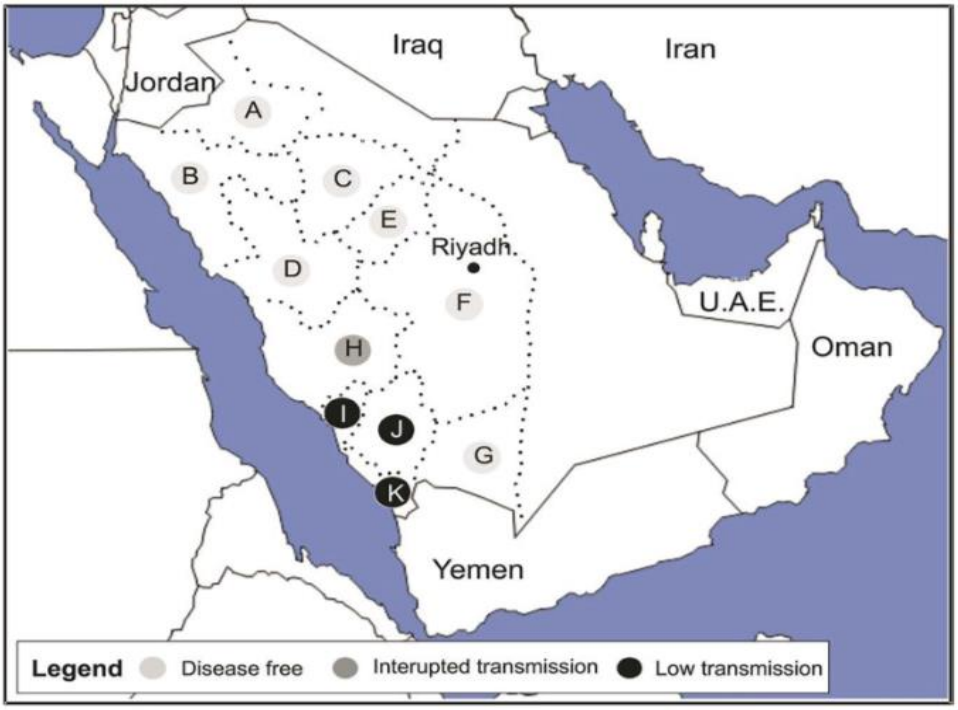

Figure 39: An outline map of the KSA with capital Riyadh depicted. Major administrative boundaries are shown, and the associated status of Schistosomiasis therein is indicated by shaded circles. (**A**) Al-Jouf; (**B**) Tabuk; (**C**) Hail; (**D**) Al-Madena and Al Monawarah; (**E**) Al Qasim; (**F**) Riyadh; (**G**) Najran; (**H**) Tardif, Makkah, Jeddah, Al Qunfudhah and Alith; (**I**) Bishah and Asir (**J**); Jazan (**K**) (Stothard *et al.*, 2014)

Asir Province (see **J** in **Figure 39**) is in the southwestern part of KSA, bordering Yemen in a very limited area. Topographical classification of this Region has led to difficulties in controlling schistosomiasis as it is comprising of vast and steep rocky mountains, Asir Plateau which is very suitable for flourishing of snail breeding sites, and the coastal plain is known as Tihama (**Figure 40A**). This topography leads to disseminating the snails from the high -lower areas (Al-Madani, 1991). In this scenario, there is a requirement for more sensitive and field-based methods for the detection of *Schistosoma* infection in people as well as in snails. Unfortunately, parasitological diagnosis, which lacks sensitivity, within a community is still one of the mainstays in planning, implementation and sustainability of MDA, and guidelines for evaluating the schistosomiasis control in subject areas (Bergquist *et al.*, 2009, Cavalcanti *et al.*, 2013, Knopp *et al.*, 2013). Importantly, there is no one generic diagnostic tool for monitoring and evaluating the current status of infection prevalence that can be entirely

accurate or reliable to detect any infected person for achieving elimination stages (Bergquist *et al.*, 2009).

As KSA has started moving from schistosomiasis control towards elimination and eradication, careful consideration and planning are essential (Rollinson *et al.*, 2013, Barakat *et al.*, 2014). As an alternative to replace time-consuming parasitological examinations is presently available, the point-of-contact (POC)-tests, urine-CCA and SEA-ELISA seem to be appropriate tests for implementation in low-transmission settings (Stothard, 2009, Stothard *et al.*, 2009, Grenfell *et al.*, 2014).

Without such a reliable test, future monitoring and evaluation of the performance of control programmes involves assessing the different transmission settings, characterised by 'light' egg-patent infections have become ever more required as several hundreds of children are needed to be screened quickly and easily in this manner to draw a conclusion about whether or not intensive or more preventive control actions are needed (Stothard *et al.*, 2014). In the present context of control, there is also a need for better documentation of snail species in such a country like KSA, where there is a lack of information about contemporary snail's distribution. This work, therefore, sought to employ morphological and molecular characterisation of snails targeting the mitochondrial COX1 gene for identification of encountered snails and their distribution alongside the main river at Al-Majardah district in South-Western Saudi Arabia. Also, contemporary disease surveillance data to assess putative transmission of schistosomiasis was explored by assessing the prevalence of schistosomiasis in two selected school's locations, and comparing the results of different diagnostic tests: CCA-urine test and SEA- ELISA, using the CCA-urine as a reference test.

6.3 Methodology

6.3.1 Study site

Saudi Arabia is in the south-west of the Asian continent and is the largest country in the Middle East. It occupies four-fifths of the Arabian Peninsula covering an area of 2.25 million km2. According to the system of regions issued, this country has been divided into 13 administrative regions. The schistosomiasis control programme is operating across all primary schools in 11 regions (**Figure 40**), including Asir region (**J**). For this study, two primary schools at Al-Majardah district (Huzaifa ibn al-Yaman Primary School, and Ibn al-Athir Primary School) were purposively selected based on previous *S. haematobium* prevalence data, and their geographical locations to the known disease-transmission zone. Huzaifa ibn al-Yaman represented a low/non-transmission zone; Ibn al-Athir represented a suspected- transmission zone (**Figure 40 B**). The study was carried out in early December 2016, approximately one month after the last round of vector control teams with annual diagnostic and monitoring in both schools.

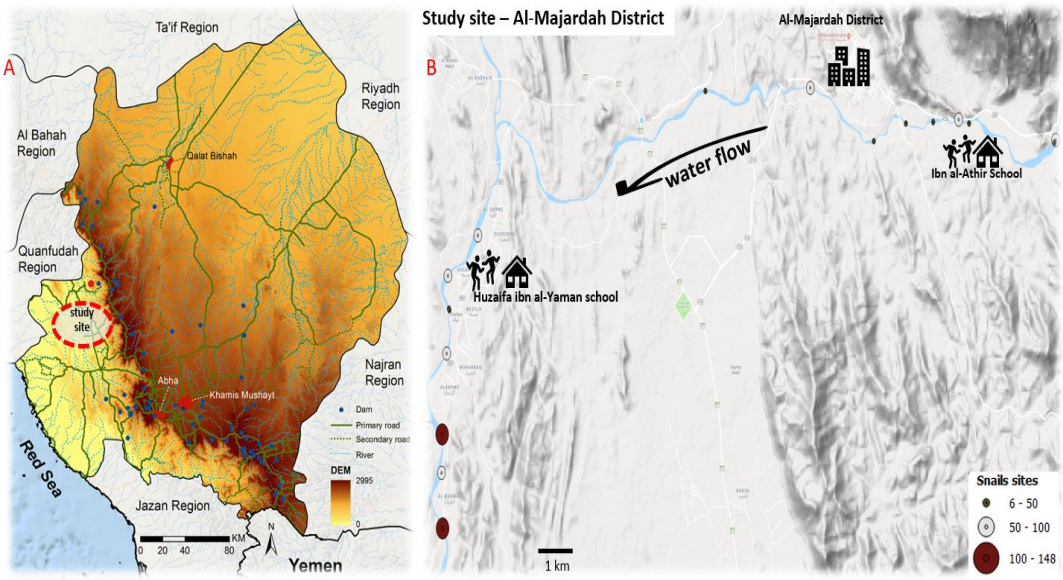

Figure 40: A map of administrative boundaries for Asir province with pointing study sites**. A**, a map of administrative boundaries for Asir province with red dots indicating three capital cities (Abha, Khamis Mushayt, and Bishah) (Alshahrani *et al.*, 2016), and study area (Al-Majardah district) highlighted with red circular (GPS co-ordinates: 19°06'18.2"N, 41°55'46.2"E). **B**: An outline map of a total number of snails collected at each monitoring site, and a map of the two schools inspected with an arrow showing water flow from mountains down to the coastal plain known as Tihama. The primary schools of Huzaifa ibn al-Yaman are located around 17 Km away from Al-Majardah town (GPS co-ordinates: 19°02'57.8"N, 41°46'38.6"E), and Ibn al-Athir is around 4 Km (GPS co-ordinates:19°05'22.1"N, 41°57'25.9"E). The distance between two schools approximately 20 Km, and both schools located close to the main river at Al-Majardah district.

6.3.2 Surveys for freshwater snail populations

It is during the warm and wet season of the year (a period of local people frequently contact with rivers and other natural water resources which are abundant) that shoreline of the main river at Al-Majardah district is considered a space of at-risk status. The malacological surveys were conducted in the middle of the season in December 2016 on a diverse set of sampled locations within this river to identify the potential transmission sites. 17 sites were prospected, each of these sites being likely to be used or frequented by the local human populace. As such, each site can be a favourable site for the transmission of schistosomiasis as intermediate hosts (i.e. *Bulinus*) were present (

Figure 41). A standard snail scoop was used at each location, water pH and conductivity *(µs*) were measured as well in each site using a hand-held water meter (Hanna pH environmental combo-meter). Potential aquatic and difficult access sites were sampled by hand. All molluscs present were systematically collected, identified and kept for morphological analysis. All snails stored later in pure ethanol according to collection location within a labelled Conical Centrifuge Tubes (Thermo Scientific Nunc 339653 Conical Centrifuge Tubes 300/CS, RACKED 50 mL) for later species identification (**Figure 41 D**).

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Figure 41: A - Photographed representing one study site nearby Huzaifa ibn al-Yaman primary school (GPS co-ordinates: 19°01'08.5"N 41°46'12.8"E), around 4Km away from school. **B**: Measuring surface-water chemistry and collecting snails around this site (500 square meters). **C**: Snails found on the stones which are common in these areas (148-Freshwater gastropods of the genus *Bulinus* collected from this site). **D**: Alive snails kept for morphological analysis to identify snail species and cercariae by using the dissecting microscope in the Vector Control Laboratory at Al-Majardah District

6.3.3 Selected schools and participants

After explaining the objectives of this study with the vector control division at Asir region, two schools were chosen based on their location, and prior information on schistosomiasis infection status. First, written informed consent was obtained from the headteacher of selected schools (**Figure 42**). Informed oral consent was obtained from the children attending grade 1-6 (typically aged between 7 and 12 years) before randomly screening all eligible children from each school (112 boys from Huzaifa Ibn al-Yaman primary school, and 51 boys from Ibn al-Athir primary School), following guidelines released by the WHO (Montresor *et al.*, 1998). Each child then gave a mid-morning urine specimen in a 250-ml plastic container (Thermo Scientific™ Urine Specimen Container) (**Figure 42C**), and a finger-prick blood sample was taken using a disposable safety lancet (Haemolance Plus® Safety Lancets (PACK OF 50)) and Gilson pipette (Gilson® P10, STARLAB) (**Figure 42D**). Finally, each child was asked four questions; full name (i) data of birth, (ii) location of living, (iii) duration of attendance at this school, and (iv) previous anthelminthic treatment (s).

Figure 42: Enrollment and participation in research at the study sites. **A**: participating children in the survey, and discussing any concerns with oral health staff, and the headteacher at Huzaifa Ibn al-Yaman school, following school-based survey protocol for NTDs control (Montresor *et al.*, 1998). **B**: setting up the equipment for data collection and sampling. **C**: participants provided a mid-morning urine specimen in a 250-ml plastic container. **D**: is taking a finger-prick blood sample using a disposable safety lancet

6.3.4 Field-based evaluation of POC-CCA urine test

The urine-CCA strip tests were used according to the protocol supplied by manufacturer's Rapid Medical Diagnostics, Pretoria, South Africa. In brief, a total of 163 individual urine samples were each tested by mixing with two drops of provided hydration buffer then dropped onto the plastic cassette containing labelled antibodies, and then the strip was incubated at room temperature. After an incubation period of 20min, the test line for each urine-CCA strip was compared against that of the five quality control standards (**Figure 6.3.4E**); a negative test criterion was based upon a test line either not visible or weaker than standard, while the internal control line was visible (**Figure 6.3.4 C, D**). An antigen-antibody complex forms as the liquid moves along the cassette and fixes to another monoclonal antibody. If positive, a pink line appears on the strip. The strength of the pink line correlates to the intensity of schistosome infection. So, The test result was classified by visual inspection against a colour chart as used previously (Sousa-Figueiredo *et al.*, 2013), by two individuals as negative, trace (±), light positive (+), medium positive (++) and heavy positive (+++).

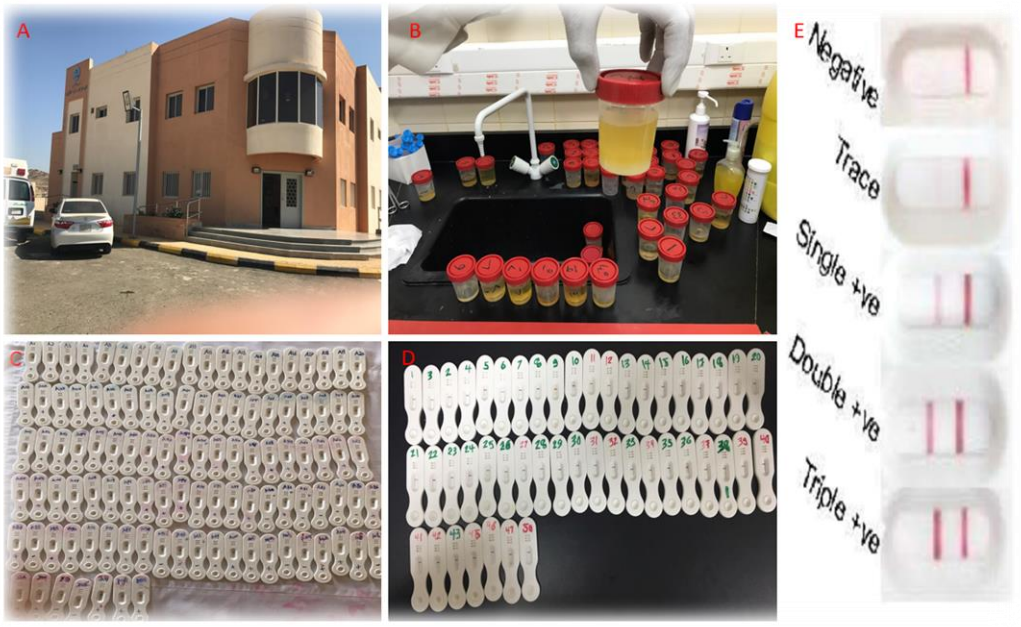

Figure 43: **A**- Urine and blood sample examination at Primary Health Care (PHC) public services in Tharban (GPS co-ordinates:19°03'20.9"N 41°47'09.6"E), around 3,708.64 ft away from Huzaifa Ibn al-Yaman primary school, where urine sample examination was performed. **B**: checking all labelled and the turbidity of the collected urine samples from participants. Detailed photograph of POC-CCA cassettes with control and result bands. To the left (**C**), POC-CCA shows the results for some infected SAC with schistosomiasis at Huzaifa Ibn al-Yaman school. To the right (**D**), POC-CCA shows the results for SAC at Ibn al-Athir school. **E**: POC-CCA shows classified visually into four infection status groups.

6.3.5 Finger-prick blood SEA-ELISA

A commercially available ELISA kit (IVD Inc.; Carlsbad, USA) was evaluated to detect host antibodies (IgG/M) againts soluble egg antigens (SEA) using a field-based ELISA test following manufacturer's instructions (Check **chapter 2** for assay protocol, (**Appendix 1 Page-92**). A disposable lancet was used to withdraw approximately 100 µl of a finger-prick blood sample from each child and stored in a 1.5ml Eppendorf tube at room temperature (**Figure 44 A**). After 2 hours, the samples were centrifuged for 3 minutes at 9,000 rpm, and serum from each child was harvested. 2 µl of harvested serum was then collected and diluted in 2:80 with specimen dilution buffer before loading a total of 100 µl into each ELISA micro-well. Upon completion,the micro-titre plate was placed on a white card to view the visual colour of each reaction as graded into pale yellow (light positive), yellow (medium positive) and dark yellow (heavy positive) (**Figure 44 C**), upon visual comparison with the control sera as recorded previously (Stothard *et al.*, 2009). Two laboratory technicians analysed the colour change to improve reliability.

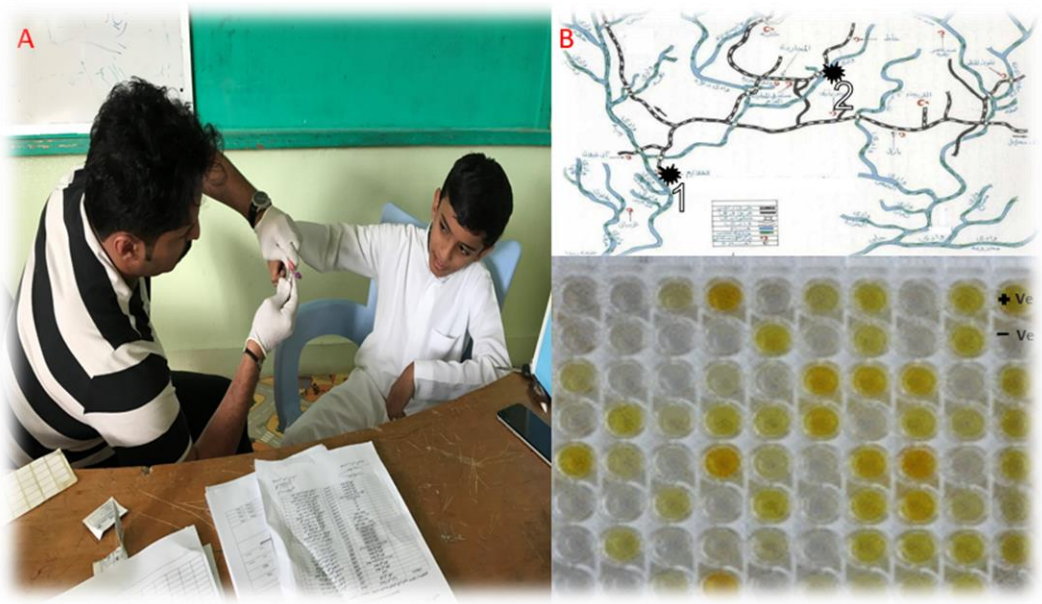

Figure 44: A field-based ELISA test using the treated SEA to detect specific antibodies to SEA in the sera of eligible children**. A**: Withdraw approximately 100 µl of a finger-prick blood sample from each child and stored in a 1.5 ml Eppendorf tube for performing a field-based ELISA test following the manufacturer's instructions by Hajri Al-Shehri. **B**: shows the schools sites alongside the bank river (**1**; Huzaifa ibn al-Yaman, and **2**-Ibn al-Athir schools). **C**: ELISA plate is demonstrating colour change (apparent to yellow), indicating schistosome antibody detection in SAC study at Huzaifa ibn al-Yaman, including two positives and two negatives control

6.3.6 DNA Methods

gDNA was extracted from 21 snails (one snail from each site including identifying snails for control) using the CTAB method as described by Stothard & Rollinson *et al.* (Stothard and Rollinson, 1997) (see **Appendix 6 Page-190**). Extracted DNA was re-dissolved in 0.1TE buffer (TE Buffer contains 1M Tris-HCl (pH approximately 8.0), containing 0.1M EDTA, Cat. 12090015, Thermo Fisher Scientific), and stored at 4°C until being used. Fragment of the gene CO1 was amplified from nucleic acid extraction by PCR. A *co1* region was amplified using forward primer CO1-LC1490 (5'*GGT CAA CAA ATC ATA AAG ATA TTG G* 3'), and reverse primer CO2-HCO2198 (5'*TAA ACT TCA GGG TGA CCA AAA AAT CA* 3')(Kane *et al.*, 2008). The PCR conditions were: initial denaturing step at 95 ° C (3 min) followed by 50 cycles of denaturing, annealing and extension of 95 ° C (15 s), 60 ° C (20 s), 72 ° C (25 s), with a final extension step at 72 ° C (2 min).

A master mix was made up with 1.6 µL of each a 10 µM primer working solution, and 21.4 µL of Nuclease-Free water (Sterile, RNase and DNase Free, 3098-2ML, UK) dissolving in PuReTaq Ready-To-Go PCR Beads (GE Healthcare, 27955702, solid, Sigma), and 2 µL of DNA was finally being added to each PCR tubes. The PCR was run on one plate, included a number of controls: extraction of TE (10 mM Tris-HCl [pH 8], 1 mM EDTA) as a negative control, and tow positive controls, using DNA extracted from characterised *Biomphalaria pfeifferi* (N= 2) and *Bulinus truncatus* (N= 2) collected by Professor Stothard in 2014 from Abha city, around 200Km in the distance from the study site. The tubes were briefly centrifuged, and placed in a thermocycler using Bio-Rad Thermal Cyclers (T100™ Thermal Cycler / a 96-well block format). The DNA was then purified using the QIA quick® PCR Purification Kit (Qiagen, for direct purification of double-single stranded PCR products >100 bp), following the manufacturer's Protocol. Purified PCR products were labelled with the sample name and coded, were sent for sequencing at Source Bioscience. Additional tubes of Forward and Reverse primers were sent at concentrations of 10ng/ul for PCR samples.

6.3.7 Inclusion criteria

To be included in the study participants had to meet following general criteria: 1) should not have received anti-helminthic treatment prior to the study (assessed by asked each participant with his headteacher individually, and their parents if they are around, 2) be healthy as assessed by a clinical examination conducted by the local nurse working with NTDs control teams at Al-Majardah District. Children were excluded from the cross-checking analysis for the following up in December 2017 if their results of either CCA-ve or SEA-ELISA-ve. A total of 56 children met these criteria and were included in the following up study.

6.3.8 Data handling and statistical analysis

All data collected in the field and processed in the laboratory were recorded on proforma data sheets. These were then double entered in Microsoft Excel prior to the generation of summary tables for prevalence and intensity of infection. Empirical estimates of prevalence were calculated in the Minitab statistical package.18 (Minitab Ltd for Statistical Computing, Coventry CV3 2TE, UK), assuming the urine-CCA as the diagnostic 'gold standard' against the SEA-ELISA, as alternate urinary schistosomiasis diagnosis diagnostic tests. For percentage values, 95% confidence intervals (95% CI) were estimated using the exact method (Armitage *et al.*, 1994). We have decided to assume the urine-CCA as the gold standard for our descriptive analyses (i.e. empirical estimates of diagnostic performance), since there have been extensive evaluations of urine-CCA dipsticks (Colley *et al.*, 2013) and WHO recommendation of its use in surveillance mapping (Danso-Appiah *et al.*, 2016). We calculated sensitivity (SS), specificity (SP), PPV and NPV for the SEA-ELISA diagnostic approach.

6.3.9 Ethical considerations and anthelminthic treatments

The Research Council of the Saudi Arabia Ministry of Health and the Liverpool School of Tropical Medicine granted approval for this study by Professor Luis Cuevas, Chair of Review panel at LSTM in April 2015, and ethical approval granted by Dr Mohammed H Alzahrani, Supervisor of Disease Vector Units & Director of Malaria Program at the Ministry of Health, Saudi Arabia in October 2016 (**Appendix 7 Page-192**). The local NTDs control teams were given the full results for enrolling, children, and any infected children or even being suspected to have Schistosomiasis infection, and then being referred to local primary health care for treatment. Case-reported were also taking place according to vector control and NTDs control at Asir region.

6.4 Results

6.4.1 Morphometric analysis

An overview of freshwater snail distribution and abundance, as well as water pH reading and conductivity levels (µs), was taken at all sample sites (**Table 22**). *Biomphalaria* was not encountered at all visited sites, while a species of the genus *Bulinus* was often found in this river in large numbers. Out of 851 snails collected from 17 locations (**Figure 40B**), five snails from each sample site (85 snails in total) were selected randomly with taking into account for a different size (from the biggest snail shell to smallest). All these chosen snails then underwent morphological measurement of the mean ($\bar{x}$) and the standard deviation ($\sigma_{\bar{x}}$) for Shell Width (**SW**), Shell Height (**SH**), Aperture Height (**AH**), Aperture Width (**AW**), Shell Length (**SL**) (see **Figure 45A**). Also, water-quality (water **pH** and conductivity (μs)) was measured at least twice at each sampling site (**Table 22**).

Table 22: Morphometric data gathered from *Bulinus* snails at 17 different sites in Saudi Arabia. The standard deviation of the mean values ($\sigma_{\bar{x}}$) for the shell width (SW), shell height (SH), aperture height (AH), aperture width (AW), and shell length (SL) of each 5 snails collected from each site are shown. All measurements are in mm. Also, water pH and conductivity (μs) values measured at all sample sites, and a total number of snail specimens collected with GPS Co-ordinates on Google Maps for highlighting the risk sites based on the distance from selected schools.

GPS Co-ordinates	Site ID	Number at site	Mean SW	Mean SH	Mean AH	Mean AW	Mean SL	pH	Conductivity (µS)
19°02'47.2"N 41°46'22.9"E	1	34	2.96 ±011	6.60±0.20	3.20±0.18	1.50±0.15	3.10±0.09	6.1	621.2
19°03'14.4"N 41°46'20.5"E	2	53	3.00±0.31	7.50±0.94	3.80±0.27	1.90±0.19	3.60±0.55	8.62	651.2
19°03'46.6"N 41°47'02.5"E	3	51	3.50±0.34	8.60±067	4.05±0.13	2.60±0.44	4.00±0.48	8.22	612.9
18°59'53.9"N 41°46'13.3"E	4	122	3.30±0.25	8.20±0.79	3.90±0.36	2.00±0.34	4.10±0.27	8.81	695.4
19°02'48.4"N 41°46'22.1"E	5	17	2.80±0.48	5.30±0.74	2.70±0.30	1.50±0.18	2.50±0.51	7.2	618.3
19°02'12.6"N 41°46'19.2"E	6	56	3.00±0.19	7.50±0.13	3.00±0.33	1.90±0.23	4.00±0.20	7.13	661.8
19°01'08.5"N 41°46'12.8"E	7	148	3.00±0.54	7.00±091	3.70±0.58	2.00±0.18	3.50±0.79	8.92	603.2
19°00'38.1"N 41°46'11.6"E	8	61	3.04±0.38	7.16±0.78	3.28±0.70	1.86±0.11	3.46±0.56	8.3	531.8
19°05'15.4"N 41°58'23.3"E	9	40	2.58±0.32	5.50±0.72	2.86±0.30	1.70±0.24	2.48±0.48	8.33	567.1
19°05'02.0"N 42°00'23.8"E	10	62	3.40±0.34	8.02±0.92	3.48±0.45	2.26±0.25	4.22±0.49	8.52	602.5
19°05'15.4"N 41°56'56.1"E	11	24	2.94±0.23	5.01±1.31	3.26±0.54	1.86±0.09	3.32±0.82	7.30	647.3
19°05'16.4"N 41°57'33.9"E	12	23	3.48±0.45	8.70±1.80	3.72±0.60	2.34±0.53	4.74±1.32	7.45	658.5
19°05'18.0"N 41°58'09.1"E	13	56	3.52±0.56	7.56±0.90	3.42±0.29	1.98±0.13	3.88±0.57	8.51	689.8
19°05'40.8"N 41°52'17.9"E	14	21	5.96±1.14	9.88±3.81	6.64±2.19	4.02±0.60	2.42±0.73	7.14	648.2
19°04'60.0"N 42°00'22.2"E	15	12	7.18±1.98	11.08±3.81	7.53±3.14	4.98±1.42	3.20±1.47	7.01	678.9
19°05'43.3"N 41°54'43.1"E	16	65	2.60±0.35	5.05±0.97	2.53±0.80	1.45±0.26	2.33±0.31	8.24	618.5
19°05'01.1"N 41°56'11.6"E	17	6	2.64±0.36	4.74±1.09	2.70±0.48	1.30±0.52	1.94±0.68	7.08	667.2
	Sites	Nearby Huzaifa ibn al-Yaman (Average distance 0.1 -6 km)							
	Sites	Nearby Ibn al-Athir (Average distance 0.1 -10 km)							

During morphological measurements, the five snails were placed under a light microscope next to a known scale at LSTM (**Figure 45 A**) and were photographed using Motic Images Plus 2.0 software (www.motic.com). The five morphometric parameters were plotted based on the mean ($\bar{x}$) of each 5 snails at each site individually. A scatter plot with regression line predicting the varia-

tion of the mean morphometric parameters ($\bar{x}$) was generated for *Bulinus*: Aperture Height (AH) against Aperture Width (AW), Shell Height (SH) against Shell Width (SW), and Shell Length (SL) against Shell Height (SH). See **Figure 45 B, C, D**.

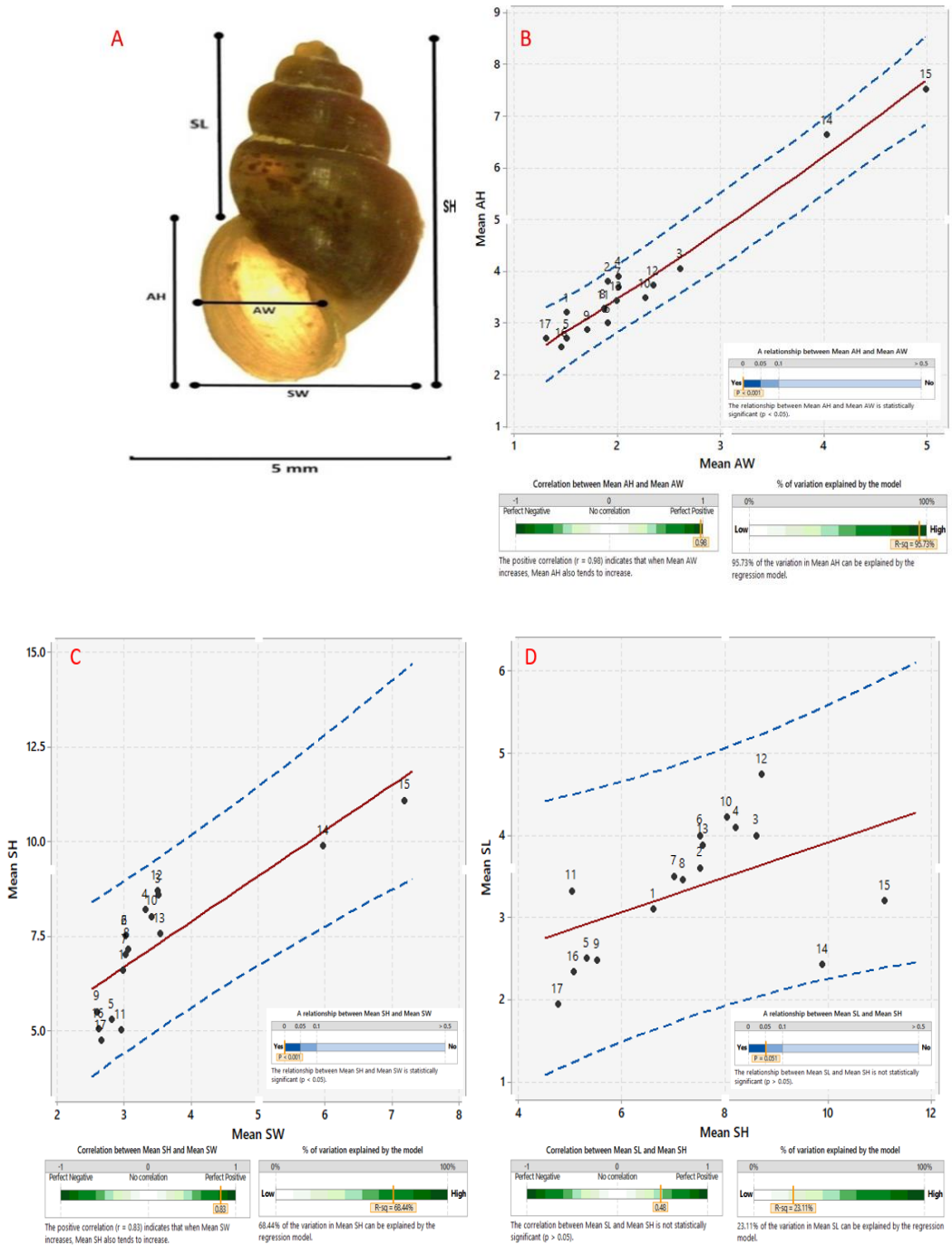

Figure 45: Regression analysis of Mean morphometric measurements of 5 snails at each coding site (1-17) are presented with predicating lines for determining the correlation of the mean shell parameter. The tree red fitted lines show the predicted Y for any X value. The blue dashed lines show the 95% prediction interval. **A**: photo taken at LSTM lab shows dimensions of the

shell illustrated for a specimen of *Bulinus* spp. Aperture Height (AH), Aperture Width (AW), Shell Hight (SH), Shell Width (SW), and Shell length (SL) were measured in scale bar= 5mm. AW was taken at the point equidistant between the top and bottom of the aperture. Following this morphometric analysis, the mean morphometric measurements of each 5 *Bulinus* snails at each site was calculated and plotted using a linear regression model, so the variation between the measurements can be visualised. Measuring snails were to give the experience of morphological variation. Tree lines were added to show how intimately connected (or unconnected) of the snails based on mean morphometrics. In the right-hand graphs. **B**: A statistically significant relationship between Mean AH and Mean AW (*P Value* < 0.01, R=0.83, R-sq 95.73%). C: The relationship between Mean SH and Mean SW is statistically significant (*P Value* < 0.05, R=0.98, R-sq 68.44%). D: The regression variant in the mean measurement of Mean SL against Mean SH is not statistically similar (*P Value*= 0.051, R=0.4, R-sq 23.11%)

6.4.2 Environmental parameters and snail distribution

A significant positive relationship was found between a number of freshwater snails collected at each site and water pH (R = 0.72, *P* = 0.001) (**Figure 46**). In addition, a significant negative relationship was found between the number of freshwater snails collected and water conductivity (μs) (R = -0.12, *P* = 0.65)

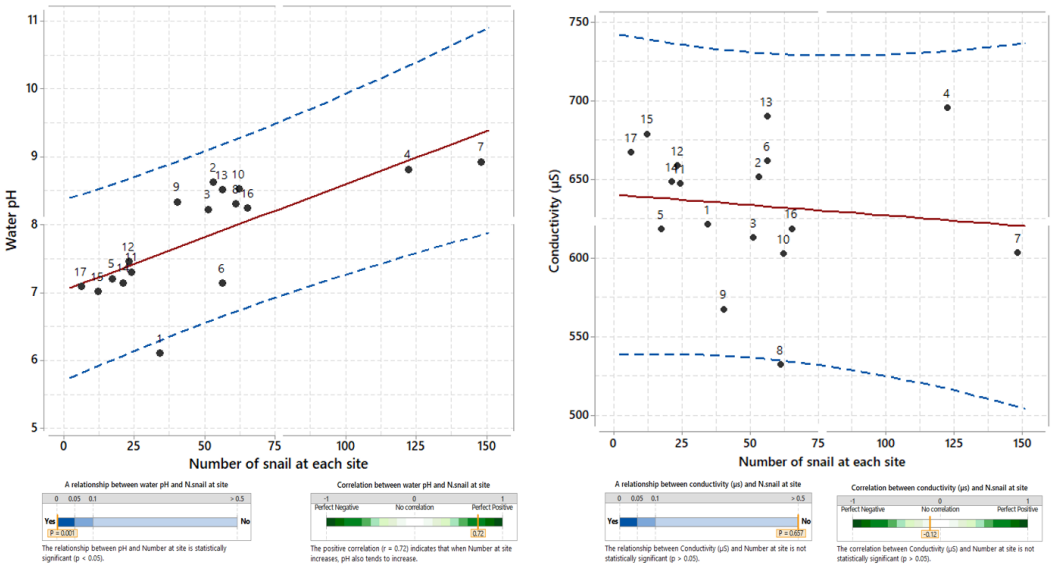

Figure 46: Showing the predicted Y (water pH or conductivity (μs)) for the number (X) of freshwater snails collected from 17 sites at Al-Majardah District. The blue dashed lines show the 95% prediction interval. The fitted equation for the linear model that describes the relationship between water pH and the number of freshwater snails is: pH = 7.032 + 0.01566 number of snails, with R-sq=52.33%, while the relationship between water conductivity (μs) and the number of freshwater snails is: μs= 640.4 - 0.1335 number of snails, with R-sq=1.33%

6.4.3 DNA sequencing

Random 17 snails identified using morphological characteristics of the shells, geographic distribution **Table 22**, was subjected to sequencing analysis for COI. From 1-85 individuals of each snail from each of the 17 sites were analysed, including known snails as controls. The sequences from all selected snails loaded onto BLASTn confirmed that the species found is *Bulinus forskalii*. The sequence showed a 99% identity score with *B. forskalii* collected in the study sites (Appendix 8: Analysis and sequence alignment for *Bulinus forskalii*).
6.4.4 Prevalence of schistosomiasis by an ELISA and CCA

A total data set was assembled from 163 children with a prevalence of schistosomiasis by each diagnostic test presented, see **Table 23**. All male children, between the ages of 7 and 12, were tested for schistosomiasis using CCA-Urine dipstick and SEA-ELISA tests. The overall percentage of the prevalence from both tests were different (*P Value* < 0.05), by Urine-CCA, was 25/163 [15.3%; 95_{CI} %, 10.6-21.6] and SEA-ELISA 56/163 [34.3%; 95_{CI} %, 27.5-41.9], with children at Huzaifa Ibn al-Yaman school, has the higher infected children (*P Value* < 0.05). Statistical tests were performed, which showed no apparent relationship between age and CCA or ELISA result (*P Value* < 0.05). The prevalence of schistosomiasis was: 18/112 [16.0%; 95_{CI} %, 10.4-23.9] by urine-CCA dipstick at Huzaifa Ibn al-Yaman school and 7/51 [11.7 %; 95_{CI} %, 5.5-23.3] at Ibn al-Athir school. By SEA-ELISA, the rate of infection was quite higher at Huzaifa Ibn al-Yaman school, 40/112 [35.7%; 95_{CI} %, 27.4-44.9], while lower at Ibn al-Athir school 16/51 [31.3%; 95_{CI} %, 20.3-45.0]. The intensity of infection was only detected at Huzaifa Ibn al-Yaman school, with only 2/112 [1.7%; 95_{CI} %,0.4-6.2], being heavy infected with schistosomiasis determined by SEA-ELISA.

Table 23: Showing results of CCA and ELISA from school Huzaifa ibn al-Yaman and Ibn al-Athir school as well as the intensity of infection based on each diagnostic method with 95% confidence interval. The majority of schoolchildren from both schools had a negative result for schistosomiasis with both CCA and ELISA tests (*P Value* < 0.05). Huzaifa ibn al-Yaman School had more children with an ELISA result of either moderate or light infection compared with school Ibn al-Athir. However, two children from school Huzaifa ibn al-Yaman have a heavy infection by the SEA- ELISA, while school Ibn al-Athir, where no heavy infected children detected by both methods.

	Selecting sites for school-aged children at Asir region	
Prevalence of schistosomiasis by each diagnostic methods	**Huzaifa ibn al-Yaman Primary School (n=112)**	**Ibn al-Athir Primary School (n=51)**
	No.Postive (%)	*No.Postive (%)*
Schistosome antigens in urine (CCA incl. trace)		
Negative	94 (83.9)	44 (86.2)
Light	14 (12.5)	6 (11.7)
Medium	4 (3.5)	1 (1.9)
Heavy	0 (0.0)	0 (0.0)
95% confidence intervals for the prevalence (%)	18 [16.0%; 95 %, 10.4-23.9]	7 [11.7 %; 95 %, 5.5-23.3]
Antibodies to soluble egg antigens (SEA-ELISA)		
Negative	72 (64.2)	35 (68.6)
Light	28 (25.0)	10 (19.6)
Medium	10 (8.9)	6 (11.7)
Heavy	2 (1.7)	0 (0.0)
95% confidence intervals for the prevalence (%))	40 [35.7%; 95 %, 27.4-44.9]	16 [31.3%; 95 %, 20.3-45.0]

6.4.5 Evaluation of an ELISA for combined with CCA as a standard gold test

Assuming the urine-CCA as an arbitrary diagnostic 'gold' standard, the diagnostic performance for SEA-ELISA tests is shown along with diagnostic accuracy and Cohen's kappa statistic, **Table 24**. The sensitivity of SEA-ELISA is (60.0 %), also the specificity (70.2 %), with the PPV (26.7%), and a higher percentage for NPV (90.6%).

Table 24: Diagnostic comparison of sensitivity (SS), specificity (SP), negative predictive value (NPV) and positive predictive value (PPV) of SEA-ELIZA against urine-CCA dipstick as 'gold standard'

	CCA-urine point-of-care (POC) as gold standard							
Evaluating Diagnostic test	Negative (%)	Positive (%)		Measurement Estimate		Diagnostic Accuracy	Diagnostic Odds	Cohen's kappa
				% (95% CIs)		(95% CIs)	% (95% CIs)	
SEA-ELISA			Total (%)					
Negative	97 (90.6%)	10 (9.3%)	107 (65.6%)	Sensitivity	60.0 [40.7-76.6]	68.7 [61.2-75.3]	3.5 [1.4 - 8.5]	0.2 [0.0-0.3]
Positive	41 (73.2%)	15 (26.7%)	56 (34.3%)	Specificity	70.2 [62.2-77.2]			
Total (%)	138 (84.6%)	25 (15.3%)	163 (100%)	PPV	26.7 [16.9-39.5]			
				NPV	90.6 [83.6-94.8]			

6.4.6 Urine filtration assessment with microscopy (Following–up)

A total of 56 children with results of either CCA+ ve or SEA-ELISA +ve were re-tested by using the urine-CCA strip as well as urine filtration in the following up study. All specimens were processed and filtered in the field. The urine sample was taken between the hours of 10:00 and 14:00 for optimum egg passage. The urine of egg-positive specimens was used as the template. All tested children were negative with urine-CCA and no Schistosoma-eggs being seen with urine filtration method.

6.5 Discussion

6.5.1 Schistosomiasis in Saudi Arabia

Schistosomiasis remains a life-threatening public health problem in many developing countries particularly in rural communities (Rollinson *et al.*, 2013, Stothard *et al.*, 2017a, Stothard *et al.*, 2014). The transmission of this parasitic infection relies on the three disease levels (human, parasite and intermediate host) and surface waters (Ponce-Terashima *et al.*, 2014). Both *S. haematobium* and *S. mansoni* has been documented in Saudi Arabia, and their distribution in KSA is focal because of the scattered nature of the population (Lotfy and Alsaqabi, 2010). Saudi Arabia has implemented schistosomiasis control and elimination programs resulting in a reduced prevalence of the disease throughout the country to very low levels. Since schistosomes are transmitted by freshwater snails; each schistosome species has its characteristic intermediate-host snails then combined snail investigations are important (Ashi *et al.*, 1989, Ageel and Amin, 1997, WHO, 1993). The intermediate hosts of *S. mansoni* and *S. haematobium,* while belonging to the same pulmonate family: Planorbidae differ by snail genus. *Schistosoma mansoni* is transmitted by the genus *Biomphalaria*, while *S. haematobium* is transmitted by the genus *Bulinus* (Brant and Loker, 2013). Thus, *Bulinus* serves as an intermediate-host for *S. haematobium* in Saudi Arabia, but not all species are thought to be permissive hosts (Al-Zanbagi, 2013).

To capture the environmental heterogeneities and biological discontinuities of the epidemiological landscape of schistosomiasis in the KSA, this field survey was conducted to be reflective of an area of low endemicity within Al-Majardah District, in Asir region. A total of 851 genus *Bulinus* snails were collected from the 17 sites along the shoreline of the main river at Al-Majardah District in south-western Saudi Arabia through this species was identified by morphology **(Figure 45**) and molecular methods as *B. forskalii* (**Appendix 8**)*,* typically considered a refractory host for *S. haematobium* (Abe *et al.*, 2018, Labbo *et al.*, 2007). *Biomphalaria* was not encountered in any of the selected sites indicative of the absence of transmission potential for *S. mansoni*.

6.5.2 Spatial distribution of identified *Bulinus* snails

Out of the 17 sites sampled (**Figure 40**), the spatial distribution of *Bulinus* snails was higher in the different sites (from 1 to 8 sites), which is around Huzaifa Ibn al-Yaman (*n*= 542 in total) with average distance from school 0.1 -6 Km in comparison to other sites (from 9 to 17 sites) that are around Ibn al-Athir (*n*= 117 in total) with an average distance of 0.1 -10 km (*P Value* < 0.05) (**Table 22**). The highest number of *Bulinus* snails (*n*= 148) were collected from site-7 (GPS co-ordinates:19°05'02.0"N 42°00'23.8"E), approx. 3.56 km in the distance from Huzaifa Ibn al-Yaman, while 62 *Bulinus* snails were the highest sampled site-10 (GPS co-ordinates: 19°05'02.0"N

42°00'23.8"E), around 5.18 km in the distance from Ibn al-Athir school. Indeed, water-level fluctuations and summer draught negatively affect the population dynamics of the freshwater snails as well as the transmission of schistosomiasis in KSA (Al-Akel, 2012). From the outset, some features emerge as a tropical climate or temperate, the presence of surface waters, a modest socio-economic level and a specific population density (Barbosa *et al.*, 2013, Mari *et al.*, 2017).

6.5.3 Environmental effects on snail population density

In considering environmental factors and ecological drivers, however, it needs to be remembered that the variability of environmental conditions has many effects on snail populations through time and can limit overall freshwater snail abundance (Monde *et al.*, 2016). Intriguingly, despite apparently favourable water pH and conductivity readings, not all sites were found to be inhabited by many freshwater snails (see **Table 22**), however; likely due to unsuitable environmental conditions, indicating perhaps that negatively charged ions in the water known to disrupt snail development, such as chloride, nitrate, sulfate, and phosphate anions (ions that carry a negative charge), whereas positively charged inorganic dissolved solids required for snail growth, such as calcium, magnesium, potassium, and sodium (Utzinger *et al.*, 1997). Also, few snails (*n*=7) was collected from Site-17 (GPS co-ordinates: 19°05'01.1"N 41°56'11.6"E), which had a water conductivity reading of 667.2 *µs*, suggesting that other environmental conditions, such as water flow rate or temperature, may be preventing snail colonisation *(*McCreesh and Booth*, 2014)*. For example, site- 1 had much fewer freshwater snails (*n*=34), possibly due to such low water pH readings (Spyra, 2017).

6.5.4 Employed five morphological parameters for snail shells

Mollusca is the most extensive and diverse phylum of invertebrate animals, which are found in a wide range of freshwater habitats and have various life-history strategies (Strong *et al.*, 2007, Benkendorff, 2010). Thus, characterisation of snail species in the concerned area is of value not only to malacologists but also parasitologists because of the role they perform as intermediate hosts for a variety of parasitic infections (Aguiar-Silva *et al.*, 2014, Oso and Odaibo, 2018). The most characteristic feature of

molluscs is their shell, the shape of which varies among taxa, partly reflecting their phylogenetic history and relationship between species where they are living (Bogan, 2008, Perez and Minton, 2008, Falade and Otarigho, 2015). Therefore, an analysis of collecting 85 *Bulinus* snail's morphological parameters with the standard division is presented in **Table 22**. The snails collected around Ibn al-Athir school, as an example, (Site-15, GPS co-ordinates: 19°04'60.0"N 42°00'22.2"E) had the highest Mean SW (7.18±1.98), Mean SH (11.08±3.81), Mean AH (7.53±3.14), Mean AW (4.98±1.92), with comparable Mean SL (3.20±0.31) with other sites, and site-9 (GPS co-ordinates: 19°05'15.4"N 41°58'23.3"E) had the lowest Mean SW (2.58±0.32), Mean SH (5.50±0.72), and comparable Mean AH (2.86±0.30), Mean AW (1.70±0.24), Mean SL (2.48±0.48) with other sites. But, those nearby Huzaifa Ibn al-Yaman, few snails collected had low-morphological parameters, with site-5 (GPS co-ordinates: 19°02'48.4"N 41°46'22.1"E) had the lowest Mean SW (2.80±0.48), Mean SH (5.30±0.74), Mean AH (2.70±0.30), Mean AW (1.50±0.18), and Mean SL (2.50±0.51). Indeed, a variation of shell morphology have seen can be explained on the basis of genetic factors and/or environmental influences, and the interaction between them (Hill, 2004). In Low-transmission zone, particularly in rural communities in Saudia Arabia, human-made habitats, such as irrigation canals, serving as aquaria for the intermediate snail hosts have been frequently attributed to the disease transmission (Al-Madani, 1991, Kloos, 1995).

Although no report exists of human schistosomiasis around Al-Majardah District recently, the general presence of *Bulinus* and other potential intermediate hosts in the main river may pose the risk of the disease being introduced to the surrounding communities in the future. This is because agricultural activities are being carried out daily, which are allowing human water contact. Thus, the epidemiological study of molluscan-borne diseases through, e.g., shell morphology represents a significant advance tool for identifying different snail species of medical importance, and can be critical steps for making public health-control decisions in order to take a step forward to control these snail vectors by molluscicide (Ibikounlé *et al.*, 2008, Falade and Otarigho, 2015).

6.5.5 Relationship of snail's morphological parameters

In an analysis of five mean morphological parameters of 85 *Bulinus* snails (5 snails from each site), the relationship of snail's morphological parameters showed linearity for Mean AH against Mean AW, and Mean SH against Mean SW (**Figure 45**). Also, the highest and lowest coefficient of the determinant (R-square) was observed, respectively (**Figure 45 B, C, D**). A linear relationship for Mean AH when compared to Mean AW (*P Value* < 0.01, R=0.83, R-sq 95.73%), and the relationship of Mean SH when compared to Mean SW (*P Value* < 0.05, R=0.98, R-sq 68.44%) was observed, while the linear relationship of Mean SL against Mean SH is not statistically significant (*P Value*= 0.051, R=0.4, R-sq 23.11%). This pattern of association has been observed in many studies, among freshwater (Ismail and Elkarmi, 2006, Falade and Otarigho, 2015). Morphological differences may also be due to several factors of particular populations (e.g., by geographic distance); shell morphology may also be shaped mainly by the environment (Vergara *et al.*, 2017).

6.5.7 Morphological and genetic analysis of *Bulinus* snails

Besides, morphological shell characterisation of snail populations and species within the genus from different geographical locations, the molecular evolutionary genetics analysis can significantly contribute to the taxonomy and systematics of species groups by correlating identified morphological parameters with the one-step molecular analyses of the mitochondria 1gene cytochrome oxidase I (COI) (Jones *et al.*, 1999). Consequently, sequencing and analysis of the complete I gene (COI) from 17 collecting snails undertaken here, indicated the presence of *Bulinus forskalii* (Appendix 8). This study has proven that this species is abundant at all sites (17- Sites), where their role as intermediate hosts in the transmission of *S. haematobium* are well known (Abe *et al.*, 2018, Labbo *et al.*, 2007). Nevertheless, the implementation of unambiguous and reliable DNA-based approaches for species identification seems to be an essential aim for schistosomiasis control in Saudi Arabia. Particular analyses of Random Amplified Polymorphic DNA (RAPDs) and the I (COX) has been recommended for effectively differentiating *B. forskalii* group (Jones *et al.*, 1999), and more generally have been documented as a useful tool in an evaluation of the stability of the derived

phylogenetic relationships within the genus *Bulinus,* either by a direct comparison of genetic variation within and between species of *Bulinus* profiles (Langand *et al.*, 1993, Kane *et al.*, 2008, Rollinson *et al.*, 1998), or by cluster analysis of genetically derived distance estimates (Stothard *et al.*, 1996).

6.5.8 Immunological detection of schistosomiasis

Various studies have shown that the presence of an intermediate snail host may determine the prevalence and explain the transmission of infection in the subject area (Abe *et al.*, 2018). So, developing better diagnostic protocols with increased sensitivity and specificity is central for the monitoring and surveillance programme of schistosomiasis in KSA Since traditional parasitological approaches become more unreliable as both infection prevalence and intensity diminish through control (Stothard *et al.*, 2014). Recently, urine-CCA strip test (POC-CCA®) and finger-prick blood SEA-ELISA tests have become commercially available and evaluating their diagnostic accuracy in high-low prevalence settings for schistosomiasis was performed recently (Al-Shehri *et al.*, 2018, Knopp *et al.*, 2015). These tests can potentially indicate the occurrence of disease transmission locally and also providing cost-effective strategies to ensue from chemotherapy-based control in comparison with traditional methods (Stothard *et al.*, 2014, Weerakoon *et al.*, 2018, Al-Shehri *et al.*, 2018).

As was expected (see **Table 23**), the prevalence of infection at Huzaifa ibn al-Yaman school was observed to be highest locally 18/112 [16.0%;95$_{CI}$ %, 10.4-23.9] by urine-CCA dipstick, and 40/112 [35.7%;95$_{CI}$ %, 27.4-44.9] by SEA-ELISA, exceeding 16.0 % in all methods, whereas the prevalence of infection at Ibn al-Athir school was lowest 7/51 [11.7 %;95$_{CI}$ %, 5.5-23.3] by urine-CCA dipstick and 16/51 [31.3%;95$_{CI}$ %, 20.3-45.0] by SEA-ELISA. However, pooling infection status by being positive by any test revealed that just above half of the children attending both schools could be considered 'free' from infection. For the total data set only just a 65.6 % of children, 107/163 [95$_{CI}$ %, 58.0-72.5] could be considered to have no evidence of schistosomiasis infection demonstrating the low-transmission of schistosomiasis at Al-Majardah District in the Asir region. Presently, the CCA assay has been further developed and became established as part of WHO guidelines as a point of care (POC) urine-dipstick (van Dam

et al., 2004, Utzinger *et al.*, 2015). Unexpectedly, in field evaluation studies, the test has showed some limitations in detecting *S. haematobium* infections (Stothard *et al.*, 2006, Stothard *et al.*, 2009, Ayele *et al.*, 2008), but performed well for *S. mansoni* infection (Legesse and Erko, 2007, Stothard *et al.*, 2006). Also, the correlation between the intensity of infection, as measured by egg counts against CCA concentration can be applicable and reliable in field-based research (Stothard *et al.*, 2006) . Another hurdle limiting further use of the CCA test in such poor resource settings, even within an area mono-endemic for schistosomiasis is the cost of the dipsticks, currently more than US$2 per test (Stothard *et al.*, 2009). On another hand, the ELISA technique, using SEA as the target, is the most widely used technique for its high sensitivity and stability (McLaren *et al.*, 1978, Mott and Dixon, 1982, Mott *et al.*, 1987, Doenhoff *et al.*, 2004).

However, this serodiagnosis of schistosomiasis has few drawbacks which are common to antibody detection techniques for parasite infection (Wu and Halim, 2000, Doenhoff *et al.*, 2004). For example, antibody-detection tests are often criticised for lacking its specificity, including the difficulty in accurately differentiating between active from past infection, with parasite-specific antibodies remaining a long time after cure (Doenhoff *et al.*, 2004, Hinz *et al.*, 2017). Antibody false-positive result is of course also possible due to overlapping in heterologous immunity between infectious agents (Smith *et al.*, 2012). Despite its instability in schistosome detection, immunodiagnostic techniques remain the best available methods for diagnosis in areas of low intensity of infection where the sensitivity and specificity of these methods appear to be satisfactory (Goncalves *et al.*, 2006, McCarthy *et al.*, 2012, Bergquist *et al.*, 2009). Comparing urine-CCA strip to SEA-ELISA gives a more accurate estimate of the reliability of using SEA-ELISA as a diagnostic method (**Table 24**). The sensitivity of SEA-ELISA compared to urine-CCA strip was shown as 60.0 % (95_{CI} %; 40.7%-76.6%). Understandably, compared to the sensitivity established using the urine-CCA strip as a reference test, this is lower due to the higher prevalence obtained using SEA-ELISA test. The specificity was calculated to be 70.2 % (95_{CI} %; 62.2%-77.2%), again lower when compared to urine-CCA as the gold standard. The PPV was determined to be 26.7 % (95_{CI} %;16.9%-39.5%). The NPV was estimated at 90.6 % (95_{CI} %; 83.6%-94.8%), and Cohen's kappa 0.2% (95_{CI} %;0.0%-0.3%). As for many other helminth infections, the implementation of parasitological diagnosis as the 'gold standard' leads to other

diagnostic test results (incorrectly) appear as inaccurate and unreliable tests for the monitoring and surveillance programme of parasitic infections (Doenhoff *et al.*, 2004).

6.5.9 Following up examination for positive cases with urine filteration included

In the following up in December 2017 for children with results of either CCA+ Ve or SEA-ELISA+Ve. A total of 56 children met these criteria and were tested by using the urine-CCA strip as well as urine filtration. Upon follow-up of children who were considered to have an infection, a re-examination of cases appeared negative with the urine-CCA strip, and no eggs were seen by using urine filtration under the microscope in this study. This is probably due to PZQ treatment in between, so a follow-up in a year or two might reveal new infections. It has been suggested to succeed in dealing with the known limitation of this parasitological detection method in low- endemic settings, replicate specimens or the number of preparing slides from a single sample needs to be increased. However, this increases costs may hamper the survey for the need of repeated samples and would complicate control strategies based on screen and treat (Wu and Halim, 2000). In China, where the target of some control programmes is the elimination of *S. japonicum*, a two-step diagnostic approach has been used, entailing initial serodiagnostic screening, followed by stool examination using the KK technique in seropositive individuals (Zhu, 2005). So, further evaluations are required in a region where schistosomiasis is non-endemic to determine whether there is cross-reactivity with antibodies against other infections.

6.5.10 Aspect of future works

In Asir Region, however, the infection with schistosomiasis has been documented to be endemic within this area and age group as the age group of 15-35 showed the highest infection rate, as well as the incidence in males, was higher than females, providing evidence about the endogenous source of infection (Shati, 2009). So, evaluations these test's diagnostic accuracy should now be expanded with using larger sample sizes, including more diverse age groups and in this areas and others with lower endemicity

for *S. haematobium*. A range of approaches to improving the accuracy of immunodiagnostic assays has been described. More sensitive assays (such as antibody-detection techniques) could be employed in future studies to confirm this to be true. More accurate reference standard tests, such as an increased number of parasitological examinations and the use of DNA screening of urine and stool with the Dra 1 PCR technique (Ibironke *et al.*, 2011) or using a TaqMan™ real-time PCR assay using specific primer-probe as s described in Uganda study for *S. mansoni* (Al-Shehri *et al.*, 2018), should also be employed for more realistic estimates of both sensitivity and specificity. Also, a sensitive diagnostic tool that can also distinguish between past and present infections will be most useful for monitoring and evaluate control programmes with the ultimate goal of elimination (Bergquist *et al.*, 2009).

6.6 Conclusion

Both intestinal and urinary schistosomiasis have been previously documented in this province, *S. mansoni* is mainly prevalent in the Asir Range, while *S. haematobium* occurs on the Asir Plateau and in the Tihama area (Lotfy and Alsaqabi, 2010). The intermediate hosts of the latter species are *B. truncatus* and *B. beccarii*. A total of 851 snails found in the 17 sites along the shoreline of the main river at Al-Majardah District, only *Bulinus* species found during this survey and confirmed the present of *B. forskalii* by morphological and molecular tools. However, this species is not typically associated with *S. haematobium* infection (Labbo *et al.*, 2007). There were not cercariae shed by *B. forskalii* were observed from 17 sites, and none of them was not of medical importance. That could be due to the opportunistic sampling that might have missed the transmission spots. DNA COX1 of collecting snails confirmed its identity 99% (497/501) with the closest sample on GenBank of HQ121585.1. No *Biomphalaria pfeifferi* (intermediate host snail of *S. mansoni*) were encountered despite repeated and intensive searching, which is suggestive of limited local transmission potential of *S. mansoni* during this time or have been a couple of years before. From screening children, there was evidence of past infection by SEA-ELISA and active *S mansoni* infection by CCA test. However, upon parasitological-serological follow-up a year later no further infection was seen presumably due to PZQ treatment in intervening at time point (Tchuenté *et al.*, 2013). Further surveillance of *S mansoni* needed and broadened snail habitat

sampling to see if *Biomphalaria* can be encountered in future as well as increasing number of samples with including different age groups and gender alongside molecular tools such as rtPCR could help in highlighting the actual prevalence of schistosomiasis in rural communities sitting in KSA.

Acknowledgements: We extend sincere thanks to the school children, teachers, districts officials and the population around the main river Majeridia District, Schistosomiasis and Vector control teams for their participation and assistance in conducting the field-work. We are grateful to Professor Russell Stothard for sharing his knowledge and support.

Authors' contributions: HAl-S, JRS conceived the study with further participation in cross-sectional surveys by Mal-Z, M Al-H. The diagnostic tests (CCA-Urine tests & SEA-ELIZA) were performed by Hal-S with assistance from PHCC technician at selected site. Data were tabulated by HAl-S and analysed with the support of JRS. All authors contributed to the drafting and revision of this chapter with JRS as guarantor.

Competing interests: None declared.

Funding: HAl-S is in receipt of a PhD Scholarship from the Ministry of Health, Kingdom of Saudi Arabia.

Ethical approval: The study was approved by the Liverpool School of Tropical Medicine and and ethical approval granted by Dr Mohammed H Alzahrani, Supervisor of Disease Vector Units & Director of Malaria Program at the Ministry of Health, Saudi Arabia in October 2016

6.7 Appendices

Appendix 6: CTAB extraction

REAGENTS

- CTAB solution
  - For 200ml
    - 2.42g Tris
    - 16.4g NaCl
    - 1.2g EDTA
    - 4g CTAB (Hexadecyltrimethylammonium bromide)
    - 0.4ml 2-Mercapto Ethanol
    - Mess up to 200ml with sterilized water
- Protease K
- Chloroform + Isoamyl alcohol (Chloroform: isoamyl alcohol = 24: 1 (v/v), CIA)
- Absolute Ethanol
- 70% Ethanol
- 0.1 x TE
- Control samples

PROCEDURES

1. Prepare CTAB + ProK solution.
   i) Heat up CTAB solution at 65ºC in water bath (or incubator).
   ii) Add 10µl of Protease K into each 600 µl of CTAB solution for each sample and mix.
2. Put each filter sample into a NEW 2ml tube with screw cap (use a tube with sealed cap to prevent leaking).
3. Add 600µl of CTAB + ProK solution MIX into each tube.
4. Add virus solution in all tubes to confirm success of DNA extraction. Add 10µl of PHPV solution for all prepared samples.
5. Keep at 65ºC for 3 h with mixing using a rotary.

6. Add 600μl of CIA to each tube, and centrifuge at 14000~15000rpm (8000g) for 3min.
7. Pick up 450μl of supernatant and transfer it into a new microtube. Do not pick up white debris at liquid border.
8. Add 1ml of absolute Ethanol into each tube and mix by inverting tubes. After mixing well, centrifuge at 14000~15000rpm (8000g) for 20min at 4ºC. If much DNA is collected, you can see fibrous thing in the liquid when you add ethanol.

* This is usual ethanol precipitation procedure. So if you want, keep tubes at -20ºC for a while before centrifuge.

9. Decant liquid carefully.
10. Add 1ml of 70% ethanol to wash out salt. After mixing by inverting tubes, centrifuge for 5min at 14000~15000rpm (8000g), 4ºC.
11. Decant ethanol and remove remained liquid using a micropipette.
12. Dry with a heat block at 80ºC for few minutes (keep open the top of tube).

* Be careful not to dry too much and not to leave liquid.

13. Add 25~50μl of 0.1 x TE and rotate for 15min at 35ºC using a rotary.
14. Centrifuge for 3min at 14000~15000rpm (8000g).
15. Check by electrophoresis and PCR

Appendix 7: Ethical approval (KSA)

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المرفقات 01-10-2016

المملكة العربية السعودية
وزارة الصحة

وزارة الصحة
Ministry of Health

TO WHOM IT MAY CONCERN

RE: samples collection for research at Liverpool School of Tropical Medicine in collaboration with Vector Borne Disease Control Department, Ministry of Health, Saudi Arabia.

Reference is made to the above subject. Vector Borne Disease Department of the Ministry of Health has collaboration agreement with the Liverpool School of Tropical Medicine, Liverpool, the UK. The agreement is in the area of research on control of vector borne disease including Schistosomiasis, Malaria, Dengue and Leishmaniasis. The bearer of this letter, **Mr. Hajri Rajeh Alshehri** is PhD student from Liverpool School of Tropical Medicine. He will be allowed to collect human blood samples from International workers for research purposes.

I believe that these projects will be a credit to the Ministry of Health and we will do our best to support the student in this matter.

With best regards

M Alzahrani

Mohammed Alzahrani

وزارة الصحة
إدارة الأمراض المتنقلة
وناقل المرض

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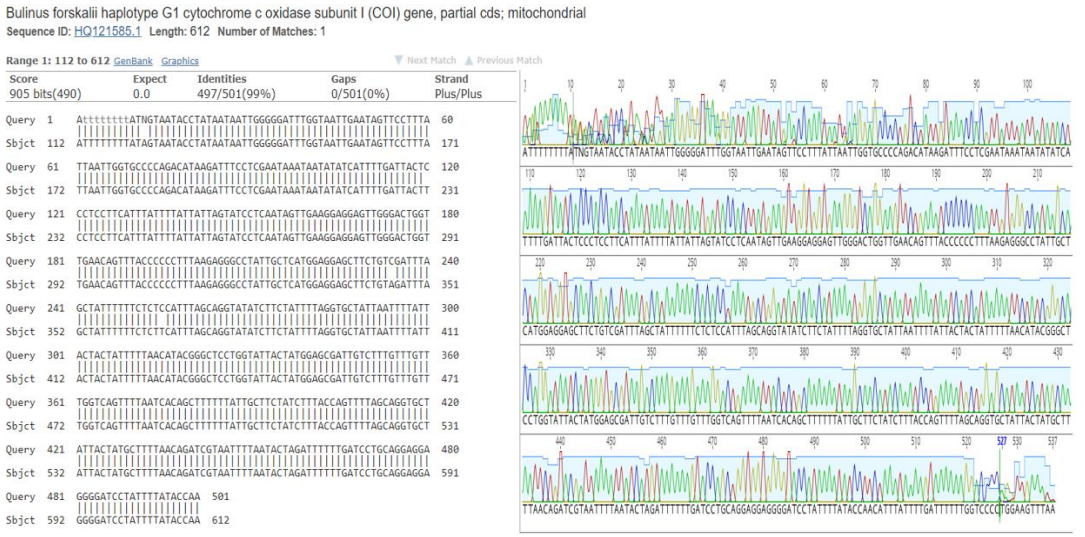
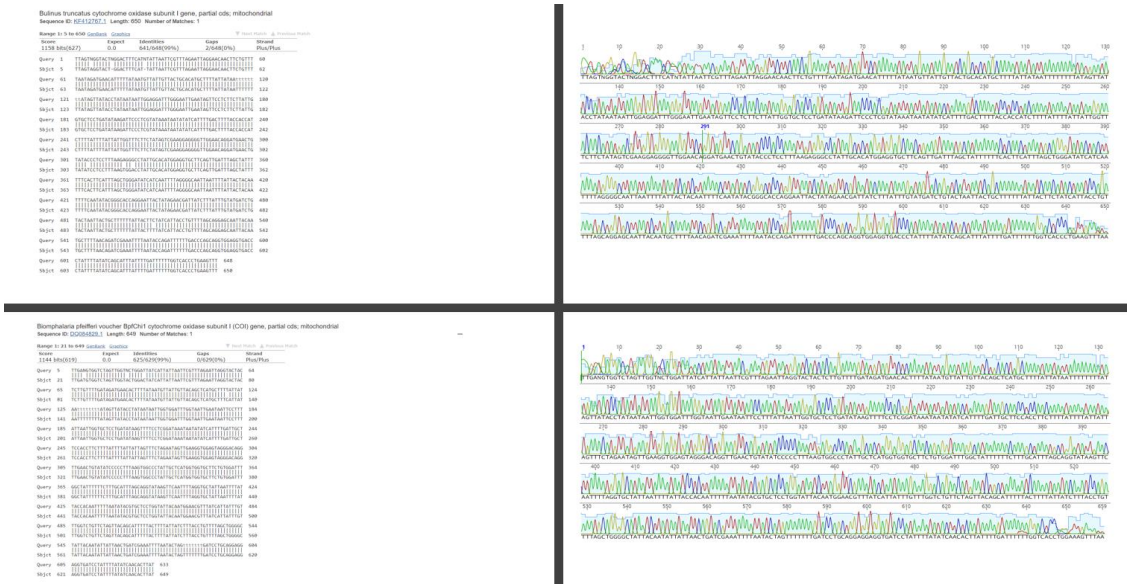
Appendix 8: Analysis and sequence alignment for Bulinus forskalii



Controls



Chapter 7. General discussion

The present thesis has contributed to obtaining better appraisal and knowledge about the current epidemiology of key parasitic and communicable diseases in Uganda and Saudi Arabia. It has sought to provide evidence-based information for the health authorities to guide the respective national programmes to move forward in achieving their programmatic goals. To do so, we have carried out cross-sectional studies and assessed the prevalence of schistosomiasis, giardiasis and malaria using a combination of novel and traditional diagnostic tools. This has linked standard microscopic examination of collected samples with rapid antigen diagnostic tests. Furthermore, DNA based molecular techniques, for detecting *Schistosoma* spp. (with the characterisation of intermediate snail hosts), giardiasis and genotyping assemblages of *Giardia*, and generic 18S DNA *Plasmodium* detection following *Plasmodium* species-specific real-time PCR for differing between species. On top of the epidemiological profiles of these parasites, we also identified factors associated with their infections and negative impacts upon on child health. Also, the thesis has provided actual examples of how the national programmes could evidence-based programmatic decisions based on the information obtained from these surveys, in order to help meet their control or elimination goals.

The thesis consists of diagnostic and detection studies that were based on parasitological- and serological-based methods alongside real-time PCR assays (**Table 7**), and focused upon examination of children of school-age as access opportunities for monitoring and evaluation of the national control or elimination programmes. In particular, the thesis has

a) Explored the geographical distribution of the most common parasitic infections among SAC by assessing the infection prevalence of the target species and conducting spatial analysis;
b) Assessed the extent and geographical distribution of schistosomiasis infections among school children in Uganda and Saudi Arabia using advanced diagnostic tools for documenting the effectiveness and cost-effectiveness of nation-wide

preventive chemotherapy via MDA campaigns with rising the impact of target different diseases; in specific,

- Assessed the impact of the MDA in Uganda, in order to provide evidence to intensify drug administration decisions along the lake shoreline or as a critical part of post-MDA surveillance activities away from the lake;
- Explored the prevalence levels of schistosomiasis in Saudi Arabia, as well as the accuracy of immunologic tools being evaluated among SAC, where the NCP was taking place for elimination of transmission;
- Expolred the distribution of intermediate snail hosts in the different settings in Uganda and Saudi Arabia, and documented the impact of physico-chemical parameters of the water body including pH, conductivity on an abundance of intermediate snails;
- Performed molecular characterisation of collected intermediate snail hosts using a DNA-based molecular technique as an advanced-tool for species-specific identification;
- Explored the impact of school location and household conditions, as well as behavioural components, on schistosomiasis transmission and infection intensity among schoolchildren, to be served as evidence-based information to guide future schistosomiasis infection control strategies;
- Investigated factors associated with schistosomiasis infections in critical districts in Uganda and Saudi Arabia, not only limited to sanitation, and hygiene, but also environmental and socioeconomic characteristics, and assessed the distribution pattern of infection at the school-level using spatial analysis; and

c) Determined the prevalence of giardiasis infection using rapid antigen detection and TaqMan@ assay to determine the diagnostic accuracy as well as the distribution patterns of assemblages of *Giardia* as evaluated by using assemblage-specific TaqMan®TPI probes with confirming their presence by sequence analysis of *β*-giardin gene, to identify potential coinfections, risk factors and pathologies associated with differing assemblages;
d) Highlighted the interactions between giardiasis, intestinal schistosomiasis and anaemia in theses school children;

e) Recorded the prevalence of malaria infections at at the district-level in Uganda, to evaluate the impact of the malaria control programme and assess future options for DNA-based surveillance to detect *Plasmodium* spp. making use of samples typically collected by schistosomiasis control programme.
f) Explored the possibility of the implementation of the TaqMan® assay as a platform for monitoring and evaluation of of different public health interventions and assess the impact to control and eliminate critical communicable diseases.

7.1. National control programme (NCP)

7.1.1 Schistosomiasis Control Initiative (SCI) Programme, Uganda

In recent years, several countries in sub-Saharan Africa have made significant progress in treating and reducing the spread of NTDs (WHO, 2017a). Uganda, for example, was one of the six countries initially selected by SCI to instigate a nationwide preventive chemotherapy intervention as implemented by the Vector Control Division, Ministry of Health (Fenwick *et al.*, 2009). In 2002, Uganda was the first country in sub-Saharan Africa to start schistosomiasis MDA at scale. In April 2003, over 400,000 children were treated with deworming medication, with SCI aiming to target three-quarters of SAC (SAC: 5–14 years old) with preventive chemotherapy and reducing infection intensity and promoting the national and regional prevention programmes. The programme expanded to cover increasing numbers of schoolchildren and communities in only 23 of the 56 districts in Uganda over the six-year period (Fenwick *et al.*, 2009), consequently highlighting the need for further surveys in the remaining districts, and include communities that are poorly served by local health services (Tchuenté *et al.*, 2017). In 2018, 46 districts will be received PZQ MDA through the NCP (Adriko *et al.*, 2018). Although the higher rate of schistosomiasis infection among children, PSAC, i.e., those aged ≤5 years were considered to be at a low risk of infection for a long time (Adriko *et al.*, 2018, Fenwick and Jourdan, 2016), and also this age group was not originally included within the remit of the SCI (Fenwick *et al.*, 2009). However, several studies have documented the burden of disease in this neglected population; a study carried out on the shoreline of Lakes Albert, and Victoria in Uganda

estimated the prevalence of *S. mansoni* at 62.3% in PSAC, further evidence these children need to be treated (Sousa-Figueiredo *et al.*, 2010b). In the absence of significant socio-economic development and environmental change, progress towards elimination remains elusive or at least a distant goal in most countries (French *et al.*, 2018, Fenwick and Jourdan, 2016).

7.1.2 Schistosomiasis Control Programme (SCP), Saudi Arabia

Primary health care centres have played a critical role in the comprehensive implementation of control activities targeting schistosomiasis which is launched by the MOH in 1971 (McDowell and Rafati, 2014). In 2003, the prevalence of this disease had significantly declined to 0.007%, and this is due to multiple activities implemented and regular overseeing the effectiveness of intervention strategies (Barakat *et al.*, 2014, Stothard *et al.*, 2014, Lotfy and Alsaqabi, 2010). In addition to mass chemotherapy using PZQ, public health education focusing on behaviour changes towards risk factors, improving sanitation, provision of clean water supply, and snail control were applied (WHO, 2007). According to the annual MOH report, the outcome of the program was very optimistic; the rates of infection were 2.2, 2.9, and 2.78/100,000 in 2000, 2004, and 2008 respectively. Also, from an examination of 34,305 water bodies, there were only 778 sites found to harbour intermediate snail hosts (McDowell and Rafati, 2014).

Thereafter, a comprehensive review of the status of this disease and elimination actions were taken; accordingly, KSA can be categories into 3: (a) disease free, (b) transmission interrupted; and (c) low transmission (general prevalence <1%) (Stothard *et al.*, 2014). However, the overall effects of the control programme in South-East regions were not satisfactory when compare it to patterns reported from other regions (Al-Madani, 1991, McDowell and Rafati, 2014, Shati, 2009, Stothard *et al.*, 2014, Lotfy and Alsaqabi, 2010). In this context, appropriate diagnostics becomes a central role in providing accurate information for guiding the implementation strategies. It is clear that in high-endemic settings the number of diagnostic tests used to identify whether or not treatment is required is much less critical than that needed in elimination set-

tings (**Figure 47**) (Stothard *et al.*, 2014). Also, diagnostic methods used in near-elimination settings, e.g. serology, involve the use of more invasive methods such as capillary blood rather than stool or urine specimens.

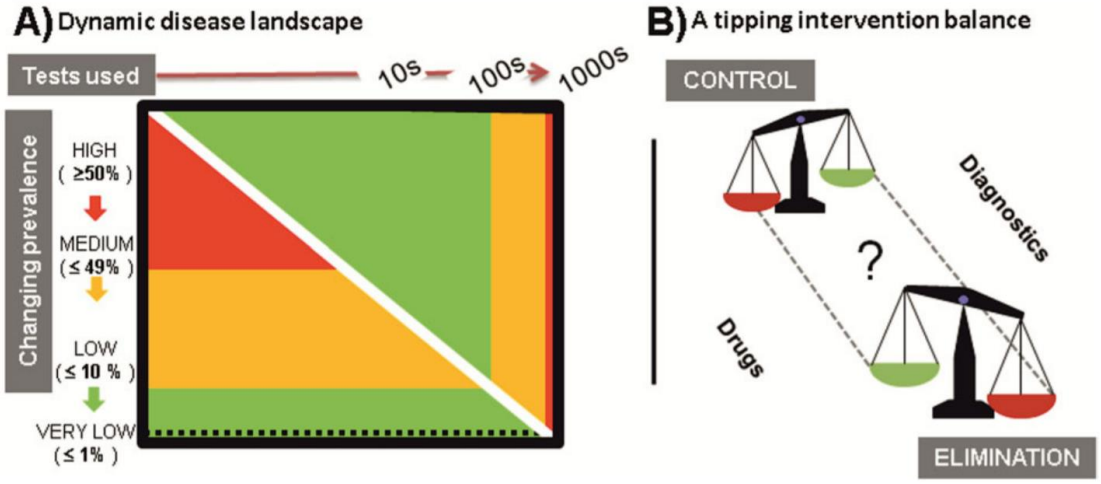


Figure 47: (**A**) Idealized surface plot about showing the number of diagnostic tests performed increases as the rates of infection in the school decline. (**B**) Treatment and diagnosis areas are divided into hypothetical project areas based on transitions from control to elimination interventions. Also, there will be a question mark when the costs of the diagnostic tests vary in different settings; this may outweigh the costs of MDA treatment(s) (Stothard *et al.*, 2014)

7.1.3 *Giardia* control programme in Uganda: current and future challenges

Relatively little is known about the epidemiology of giardiasis in African countries as this disease does not benefit from a disease-specific programme (Squire and Ryan, 2017), although a recent review of giardiasis in Uganda focussed on the epidemiology and zoonotic transmission dynamics between primates, gorillas, cattle and humans living in close proximity or using similar water sources (Graczyk *et al.*, 2002, Johnston *et al.*, 2010). In Uganda, current prevalence estimates for humans varies from study to study primarily because of the age groups studied. The prevalence in children and infants is usually higher than adults (Graczyk *et al.*, 2002, Johnston *et al.*, 2010, Ankarklev *et al.*, 2012, McElligott *et al.*, 2013). However, the majority of studies from

Uganda focus on the zoonotic potential of giardiasis and not the pathological effects on the human population.

Our efforts in characterising the molecular epidemiology of giardiasis and the roles of various animals in the transmission of human giardiasis are hindered by the lack of case-control and longitudinal cohort studies. This sampling bias restricts the range of testing of humans and animals living in the same community. Nevertheless the frequent detection of infections with different genotypes and subtypes, and the link between heterozygosity and apparent mutation at some genetic loci for some *G. duodenalis* genotypes (Feng and Xiao, 2011). Therefore, the purpose of this study was to determine the prevalence and molecular epidemiology of *Giardia* assemblages around Lake Albert in Uganda, with a focus on current and future challenges and to develop recommendations for better control of this important parasite. At the very least, a high burden of infection and disease has been clearly presented which has drawn better attention to the importance of this disease locally.

7.1.4 Malaria control programme in Uganda

Despite a significant decline in the global malaria burden have been reported, declines were slower in the 15 highest -burden countries, the majority of which are located in sub-Saharan Africa (WHO, 2016b). Among the high-burden countries, Uganda has reported some of the highest levels of transmission intensity in the world and ranks fourth among these high malaria burden countries (Katureebe *et al.*, 2016). Since 2006, Roll Back Malaria (RBM) has involved in malaria prevention and control in the country and periodically funding the malaria indicator surveys (MIS) (Ssempiira *et al.*, 2017). The MIS is standardised nationally-representative surveys for determining the prevalence of parasitaemia and its risk in children ≤ 5 years old and evaluating malaria control interventions coverage. To date, two studies have been carried out by malaria indicator surveys (MIS) in Uganda; MIS 2009 and MIS 2014–2015 (Ssempiira *et al.*, 2017). The first study showed a high burden of malaria in different districts, with three regions had the prevalence between 50–75% and six were 10–50%, and only one community < 10% ((UBOS), 2010). In the second survey of MIS,

there was a significant reduction of parasitaemia risk of 50%, and this is due to improvement in the coverage of Insecticide Treated Nets (ITNs) and ACTs intervention at all level of care. Additionally, parasitaemia prevalence has declined in the majority of districts to mesoendemic, 11%–50% ((UBOS), 2015, Ssempiira *et al.*, 2017). The actual effects of each intervention on parasitaemia reduction are not clear at national and subnational levels, but a new update national malaria control and elimination plans as a framework has been developed by the Ministry of Health (MoH) to accelerate malaria control efforts (Ssempiira *et al.*, 2017).

In this framework known as Uganda Malaria Reduction Strategic Plan (UMRSP) 2014–2020, clear aims have been establishing to reduce malaria mortality rates from very high levels to close to zero, morbidity to 30 cases per 1,000 persons per year, and the prevalence of parasitic infections to less than 7% ((NMC), 2016). To achieve these targets and ensure from the more efficient use of scarce resources and an active surveillance programme, it is important to know the impact of each intervention on the prevalence. So, identifying diagnostic platforms seem to be important for accurate estimation of malaria prevalence in such this setting. The information presented in this thesis has shown that there remains a substantial burden of infection in school children in Buliisa District which would benefit from intensification of control interventions.

7.2 Molecular diagnosis in clinical parasitology: current and future aspects

Within the fields of molecular diagnostics, DNA-based detection has been established itself as a sensitive and specific qualitative and quantitative technique that has become extensively used as diagnostic tools (Kralik and Ricchi, 2017, Chew *et al.*, 2012, Craw and Balachandran, 2012, Schuurman *et al.*, 2007, Melchers *et al.*, 2014). In the field of parasitic diagnosis, several real-time PCR assays for the detection of intestinal parasites have been developed and shown to have excellent specificity and sensitivity; they have been accepted as objective tools for case confirmation and as gold standard in the development of alternative new diagnostic tools as they have been registered to ISO standards (Verweij *et al.*, 2004). Recent developments in improved and automated DNA extraction methodologies and a more comprehensive application of real-

time PCR in molecular diagnostic laboratories for viral and bacterial diseases will allow DNA-based methods to be used as an alternative tool for the detection and identification for a range of different pathogens including parasitic infections (Verweij and Stensvold, 2014, Siwal *et al.*, 2018, Ndao, 2009, Won *et al.*, 2016). This thesis has described different approaches on the implementation of real-time PCR for the detection and quantification of schistosomiasis, giardiasis and malaria using different biological samples to validate and optimise its use in future epidemiological surveys (**Table 7**).

7.2.1 *Schistosoma* spp.

In chapter 2, a real-time PCR approach targeting the internal transcribed spacer 2 (ITS-2) has been developed and showed higher sensitivity for detecting schistosomiasis (Obeng *et al.*, 2008). The ITS-2 PCR improved *Schistosoma* spp. detection considerably, in particular for the detection of *S. mansoni* (**Table 8**)(Weerakoon *et al.*, 2018). A real-time PCR targeting the gene CO1of *S. mansoni* detected as similar as percentages of *S. mansoni*-positives with standard microscopy in a Senegalese population (Ten Hove *et al.*, 2008). Unlike the cox1 gene, which can exhibit sequence variation between populations, the real-time PCR assay on ITS-2 does not discriminate between DNA of *S. mansoni* and DNA of *S. haematobium* and is less sensitive to reaction failures due to intra-specific variation (Ibironke *et al.*, 2012, Meurs *et al.*, 2015). In this thesis, the ITS-2 PCR was further evaluated using urine samples collected in different studies and compared with Urine- CCA strips and microscopy (Obeng *et al.*, 2008, Melchers *et al.*, 2014), which showed higher sensitive than CCA test. Also, ITS2-based schistosomiasis PCR on stool samples for the detection of *S. mansoni* and found that it outperforms standard microscopy on parasitological faecal smears, which makes it particularly useful in low–endemic setting areas, and consequently, in post-control settings (Meurs *et al.*, 2015). Therefore, the outcome of results indicates that real-time PCR assay is a valuable tool for diagnosis, and could potentially serve as a gold standard to estimate the prevalence and intensity of *Schistosoma* infections in epidemiological surveys (French *et al.*, 2018). However, serology remains the best method to exclude infection and have the highest NPV.

7.2.2 *Giardia duodenalis*

Real-time PCR for this parasite has been already evaluated and shown 100% specificity and a higher sensitivity as compared to conventional microscopy and antigen-based detection (Verweij *et al.*, 2004, Verweij *et al.*, 2003, Stark *et al.*, 2011, Laude *et al.*, 2016). **In chapter 3**, **Table 12** gives the results of the diagnostic tests for *G. duodenalis* in Lake Albert, Uganda where the schistosomiasis control is taking place since 2002. The results show that the real-time PCR assay has the highest sensitivity compared to the antigen-based test for the detection of *G. duodenalis*. In this thesis, *G. duodenalis* real-time PCR was evaluated as part of an epidemiological study for intestinal schistosomiasis in LSTM research laboratory in Liverpool. In previous studies, *G. duodenalis* are very scant in Uganda (Ankarklev *et al.*, 2012, McElligott *et al.*, 2013, Fuhrimann *et al.*, 2016). While the disease is zoonotic in livestock and an essential anthropozoonosis of the mountain gorilla (Hogan *et al.*, 2014, Sak *et al.*, 2014), the burden of giardiasis in school children is mostly unknown across all country. A comparable prevalence with antigen-based detection is reported in **chapter 3** after screening eligible children with *GIARDIA/CRYPTOSPORIDIUM* QUIK CHEK™ test under field condition. When real-time PCR was used on the same samples, the prevalence increased to 87.0 % which was graded into light (27.1%), medium (40.5%) and heavy (19.2%) categories upon comparison with C_t values. So, real-time PCR assay seems to be much more sensitive and beneficial for accurate detection of *G. duodenalis* in human stools and this finding is supported with those from other studies in different setting worldwide (Beyhan and CENGİZ, 2017, Verweij *et al.*, 2004). **In chapter 3**, all detected cases of *G. duodenalis* originated from SAC without previous data about the prevalence of this parasite in those communities, but these settings are well-known about endemicity of *S. mansoni*. The finding of the association between tow diseases, (see **Table 13**), brought the importance for implementing effective controls targeting both or more of this water-borne diseases in this communities using real-time multiplex PCR assay as described in several studies (Verweij *et al.*, 2004, Verweij *et al.*, 2007, Ten Hove *et al.*, 2008), and also creates opportunities for investigations of (sub)assemblage transmission dynamics (Thompson and Ash, 2016).

In chapter 4, the *G. duodenalis* -positive samples were further characterised in an attempt to determine the geographic distribution of assemblages and identifying its risk factors. The data showed that assemblage B was dominant across the sample, which is supported by another study in Rwandan children (Ignatius *et al.*, 2012), and assemblages A and B is varied by school sites and by the age of the child. Mixed infections were so prevalent at Runga school in concordance with the highest rate of intestinal schistosomiasis infections, translated as a co-morbidity and transmission-score (**Table 15**). Also, assemblage B revealed an association with underweight children in concordance with another study carried out in Rwandan children (Ignatius *et al.*, 2012). Previous studies have suggested the epidemiological role of *G. duodenalis* assemblages in children as a factor associated with clinical manifestation (Ignatius *et al.*, 2012, Jerez Puebla *et al.*, 2015, Kohli *et al.*, 2008, Mahdy *et al.*, 2009, Puebla *et al.*, 2017, Puebla *et al.*, 2014).

Like most research subjects in **chapters 3** and **4**, and despite extensive attempts to associate symptoms with genotype in human infections, conflicting results have been obtained, and there is to date no clear correlation between assemblage and symptoms. However, it has been documented that genotype AI is more frequently detected in animals, whereas genotype AII is mainly found in humans (Sprong *et al.*, 2009, Cacciò and Ryan, 2008). It has been suggested that the presence of clinical presentation of infection depends on different factors such as the degree of host adaptation, nutritional and immunological status (Bartelt and Sartor, 2015). The results of studies on the association of *G. duodenalis* assemblages with epidemiological factors are summarised in **Table 16**. Future molecular studies on transmission patterns in these communities in combinations with a detailed description of underlying conditions might provide more insight into the source and impact of *G. duodenalis* assemblages on those children.

7.2.3 *Plasmodium* spp.

Measures of accuracy include sensitivity and specificity for malaria detection always rely on using the blood samples (Chotivanich *et al.*, 2007), making several attempts to develop diagnostic tools using other biological samples from human to detect this

parasite unnecessary or impractical. Furthermore, It could not be expected that a blood parasite could shed detectable DNA amounts in faeces (Prugnolle *et al.*, 2011, Jirků *et al.*, 2012). However, there is limited published data in this field, with only one past attempt of diagnosing malaria using human fDNA (Jirků *et al.*, 2012), with another looking at diagnosis using human saliva and urine samples (Putaporntip *et al.*, 2012). In the current attempt, **chapter 5**, quantitative PCR (rtPCR) assays have been optimised to detect and quantify malaria parasite from school-aged–children using generic primer-probe published by Shokoples *et al.* (Shokoples *et al.*, 2009) for *Plasmodium*-DNA detection from either faeces and blood spots. The findings were promising to enhance assay used in malaria control sitting , which fits in with the objectives of the WHO global technical strategy for malaria (WHO, 2015a).

Despite a century of research, current knowledge of African great ape malaria and identifies gaps is limited, and future research for zoonotic transmission is currently unclear (Nys *et al.*, 2017). However, several molecular studies have shown surprisingly high genetic diversity and population of these species typically inspecting fDNA material (Duval *et al.*, 2009, Krief *et al.*, 2010, Liu *et al.*, 2010a, Liu *et al.*, 2010b, Liu *et al.*, 2014, Liu *et al.*, 2016, Rayner *et al.*, 2011, Boundenga *et al.*, 2015, Otto *et al.*, 2014). Available evidence has been published for supporting *P. falciparum* from western gorillas can be transmitted to humans (Duval *et al.*, 2009, Prugnolle *et al.*, 2011). All data for identification of *Plasmodium* spp. were based on the molecular analysis using PCR-based amplification of faeces (Jirků *et al.*, 2012). Because it is difficult to obtain their blood samples, which are used for detection of *Plasmodium* spp., the estimation of the prevalence of *Plasmodium* spp. is problematic (Jirků *et al.*, 2012). However, faecal-based diagnostics open new platform tools for addressing the questions regarding the prevalence, epidemiology, and clinical role of zoonotic malaria in such these setting. In addition, it could be used for other parasitic infection, which is detectable in faeces or blood from either humans and wildlife, and if so, this will have a high impact on malaria controls in recent future.

7.3. Key recommendations

Control efforts for schistosomiasis and other NTDs, if followed, can reduce endemicities to levels that are no longer considered as public health problems. With the general success achieved by the schistosomiasis control and elimination programmes in Africa and Saudi Arabia (Bergquist *et al.*, 2017, Stothard *et al.*, 2014, French *et al.*, 2018, Fenwick and Jourdan, 2016), there are few recommendations for improving the schistosomiasis control at national levels but at local levels where transmission can remain high, more specific revision of control tactics is needed (**Figure 48**) (Bergquist *et al.*, 2017).

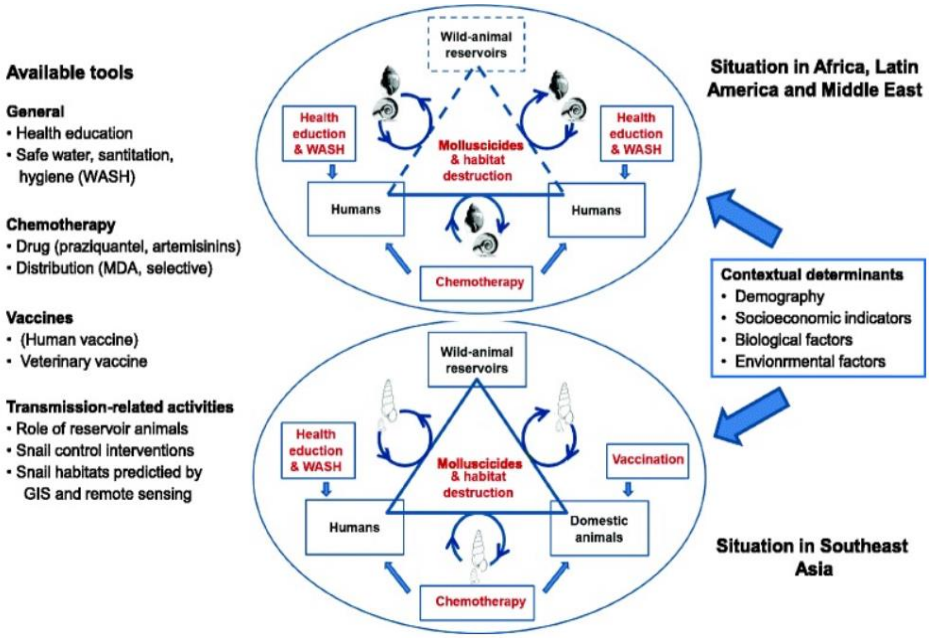


Figure 48: Schematic figure is highlighting essential differences between the endemic areas and the appropriate control tools (Bergquist *et al.*, 2017)

Above all, using more sensitive diagnostics are now urgently needed since current methods (which are suitable for gaining control) are not sensitive enough in the longer term. When the goal is the elimination of the disease, active case detection can be a problem. The clinical and parasitological conditions commonly used are not

sufficiently sensitive. Approaches will need to be modified to incorporate more sensitive techniques. Currently available tests for antibodies and antigens may be used for the diagnosis of schistosomiasis in areas of low transmission (e.g. in Saudia Arabia), but new field-based antigenic or molecular tests will be needed (**Figure 49**).

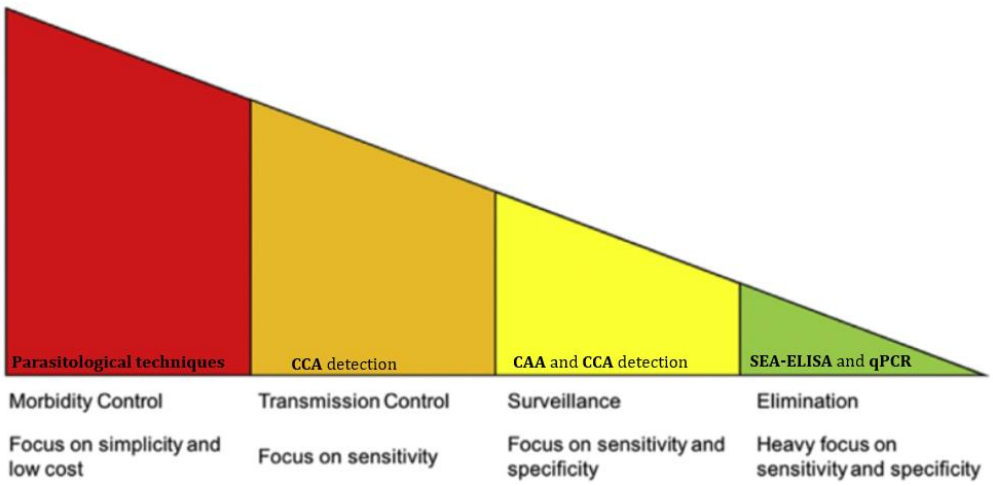


Figure 49: Modified figure about what expected diagnostic tools should be applied for future schistosomiasis surveillance based on endemicity and control achievements (Le and Hsieh, 2017)

Whatever technology is available, it is essential that programme managers maintain staff interest in case finding and treatment. As the endemicity decreases, the sustainable control of transmission must become the major consideration; it should be based on improved hygiene and sanitation and the fight against molluscs (**Figure 48**). These measures will help prevent a resurgence of schistosomiasis on the one hand, and on the other hand, consolidate the results already achieved in terms of improving health.

In areas of low transmission, resources may be more effectively used if central authorities delegate the implementation of schistosomiasis control to regional authorities. This will require greater reliance on health resources and local education; training and skills transfer from the central to the peripheral level will be essential.

The integration of control activities into the health and hygiene-related services will further benefit health as a whole. In addition, the integration of schistosomiasis control and activities to control other diseases. Furthermore, the integration of control activities into existing health and school facilities on the one hand, and the development of intervention with other existing programs, such as lymphatic filariasis, malaria, nutritional programs, Immunization and other public health interventions, on the other hand, should be sought and encouraged, especially if new and more sensitive diagnostic tests are available. Staff motivation and retraining should also be strengthened by organising "cross-training" of laboratory staffs.

Because asymptomatic cases are common and there are animal reservoirs for some species of these parasitic infections, the definition of elimination criteria would be particularly tricky to achieve. The risk of reintroduction of the disease in an area from which it had been eliminated, for example as a result of water resources development projects or population movements, further complicates the picture. By establishing a reliable surveillance system, any new locally acquired infections can be detected within a suitable period of time, and this is essential. Similarly, the degree of the reliability of the surveillance system - the sensitivity of the diagnostic method used and the efficiency of the reporting system should be updated based on the endemicity of the diseases. When the endemicity of the disease has been reduced, thresholds and treatment strategies must be modified. When treatment has been administered repeatedly for many years, active surveillance is essential to detect any emergence of drug resistance.

The results in this thesis reflect the role played by human activities in the transmission of schistosomiasis. The risk of infection with schistosomiasis is related to the presence or absence of the parasite, but also all the conditions necessary for the completion of the transmission cycle. So, the contact more or less frequent and sustainable with water, contaminated by stool and urine of infected persons constitute the main risk factor for infection with schistosomiasis (Stothard *et al.*, 2017a). Thus, schistosomiasis is sensitive to the relationship between the human community and the aquatic environment. Typically streams, ponds and lakes remain significant sources of

infection in Uganda and Saudi Arabia, so investment in sustainable water-related infrastructure will be important for reducing schistosomiasis transmission through prevention of new habitats.

Discussions on the improvement of the molecular diagnostic tools and collecting samples for schistosomiasis, giardiasis and malaria control programme in Uganda and the findings of this thesis will help inform decisions on how best to improve the programme outputs for these several diseases. Moreover, there is a need to focus on better inter-sectoral approaches for the effective delivery of treatments. With levels of infection evidence in the total number of SAC in the study areas in Lake Albert inspected, several thousand children are likely infected *Giardia* spp. and are living with an unmet treatment need (Al-Shehri *et al.*, 2016). Whilst not all the infected children would develop symptoms; it is likely that in these settings with these infections (giardiasis and schistosomiasis), children have been in regular contacting with infested water which is known to be a source of the disease (Adam, 2001, Colley *et al.*, 2014). Thus, we recommend establishing the overall public health importance for these infections in Uganda and what would be a core set for improving the case detection at the school level as a part of the integrated approach, reflecting this finding.

Considering that the *Giardia* infection prevalence levels were even higher in several hotspots, attention should also be paid to waster, sanitation and hygiene (WASH), the sensitivity of diagnostic tests available, and zoonotic transmission as a significant risk for human infections. Enhanced surveillance efforts should be considered in these communities against *Giardia* infections. A close collaboration with the health promotion programme under the environmental health programme for health education and access to improved water and sanitation would be useful as part of 'inter-sectoral approaches', which will shorten the duration of deworming required to interrupt parasitic infection transmission. WASH programmes should be implemented in these communities and actively advocated against water-borne diseases, by establishing this programme in a long run and the primary preventive measures such as personal hygiene (washing hands after defecation and defecating in the toilet) and sanitation

should be reinforced. Malaria infection screening should be considered in the schistosomiasis and STH infection surveillance activities in Uganda, employing molecular tools whenever possible.

In Saudi Arabia, the contribution of genetic diversity of schistosomiasis and snails in Asir needs to be investigated. Several studies in KSA identified genetic variability within the same species collected from different regions at different periods (Mostafa *et al.*, 2012, Shalaby *et al.*, 2011). Schistosomiasis was detected in Hamadryas baboons, which live near local human settlements in the Saudi-Yemeni border areas (Yamane *et al.*, 2003), so the role of animal reservoir hosts for schistosomiasis in KSA should also be considered. Other factors that might affect the successful implementation of such control programs are illegal immigration from the highly endemic countries and the creation of multiple water bodies, which can be suitable for breeding of snails (Lotfy and Alsaqabi, 2010, McDowell and Rafati, 2014).

7.4. Suggestions for future work

Real-time PCR, hereafter abbreviated RT PCR, is becoming a common tool for diagnosis of several pathogens including intestinal parasites. Nevertheless, there is ongoing research for improving diagnostic applicability in different resource settings. It is evident that real-time PCR platforms will improve substantially, including faster thermocycling times, flexibility, multiplexed amplification of multiple targets, speeding up of the assay, processing and generating multi well-plates for a larger number of samples. Indeed, a real-time PCR technique has already been implemented for the detection of several diseases, such as *Schistosoma* spp. (Melchers *et al.*, 2014, Ten Hove *et al.*, 2008), *Giardia* spp. (David *et al.*, 2011, Verweij *et al.*, 2004), *Plasmodium* spp. (Shokoples *et al.*, 2009, Chew *et al.*, 2012, Gavina *et al.*, 2017, Siwal *et al.*, 2018), viral pathogens (Mirza *et al.*, 2018). So, it is scale-up of implementation, therefore, that will allow early disease detection and control methods. Moreover, diagnostic technologies, such as DNA microarray and biosensor technologies are rapidly advancing (Peña-Bahamonde *et al.*, 2018). Also, recombinase polymerase amplification (RPA) has shown great potential, as evidenced by different studied (Daher *et al.*, 2016, Poulton and Webster, 2018, Kosack *et al.*, 2017).

The present thesis highlights that real-time PCR provides a feasible and cost-effective alternative diagnostic method for the detection of different parasitic infections in the presented epidemiological studies. The initial implementation of real-time PCR in a laboratory requires the substantial monetary investment required for "real-time" PCR instrumentation as well as investing in rather costly reagents are major stumbling blocks for its routine use in most epidemiological settings. On the other hand, real-time PCR could be cost-effectiveness when its diagnostic applications are expanded for detecting multiple targets such as bacteria, viruses, protozoa, fungi, and parasites. Nevertheless, it needs to be considered that despite cost efficiency regarding cost component for most molecular genetic assays, real-time PCR-based detection can still favour when using multiple primers directed at multiple templates within a single reaction. This is where the most significant advantage lies. For future work there are several components that need to be considered outlined as follows:

- To adapt real-time PCR assays into more field-applicable platforms that allow closer-to-patient diagnosis and low-cost community disease surveillance.
- To explore the performance of these real-time PCR assays on other non-invasive sampling specimens e.g. urine and saliva, for co-detection of parasites.
- To embed these clinical diagnostics into wider environmental surveillance of parasites screening of water or intermediate hosts in high-through-put formats.

Altogether these advances have the potential to provide a mechanism for the broader application of more accurate and convenient DNA detection methods that will be invaluable in future NTDs control and elimination efforts. A significant challenge that remains to be addressed is the implementation of highly sensitive and specific molecular diagnostic tools in several resource-poor settings. Although the real-time PCR method has brought forward new interesting prospects for molecular diagnosis of most common intestinal parasites in different endemic settings, it has little application for epidemiological surveys in most of these settings. However, real-time PCR can be

currently used as a gold standard for the evaluation of new rapid and inexpensive tests for field diagnostics. Ultimately, field- PCR platform (such as RPA) has recently become promising tools with equipment and processing times being further miniaturised with integrated processing of DNA extraction, which could apply to resource-poor settings (Crannell *et al.*, 2015, Daher *et al.*, 2016, Weerakoon *et al.*, 2018). Such advanced diagnostic tools would have a significant impact on controls of these NTDs in resource-poor settings, particularly in early case detection and monitoring treatment efficacies.

As a general rule, sensitivity is an essential factor in diagnosis. It is important for a clinician to know the specifications of the PCR methods used by the laboratory conducting the analysis and/or to have a result accompanied by a specific comment in order to interpret the results correctly. The most important factors influencing the sensitivity of PCR methods are the choice of primers (to the genetic locus selected), the quality and the quantity of the extracted sample for analysis. The delays and the conditions of transport influence the reliability of the results. The commercialised techniques allow, when they exist, a level of standardisation of these results and an easy comparison from one laboratory to another, but there remain difficulties in coordination. In all other cases, the developed techniques must be validated with maximum sensitivity. Adequate controls for contamination and inhibition should be included. Participation in external quality controls is also essential. A positive PCR result ensures the presence of the desired pathogen, while in some cases a negative result may be related to sensitivity problems. During a strong clinical suspicion, a repetition of the exam a few days is often useful. In the context of certain chronic infections, a positive PCR is not synonymous with disease, the development and the standardisation of the quantitative PCR will make it possible to define thresholds.

In view of the continuous development of new diagnostic methods, it becomes all the more essential that the clinician or researchers are able to choose the right test, based on its characteristics and clinical applicability in a given clinical situation or public health setting. For this purpose, as part of a series of recommendations for critical reading published and recently updated in “A guide to aid the selection of diagnostic tests” (Kosack *et al.*, 2017), the ASSURED (Affordable, Sensitive, Specific, User-friendly,

Rapid and robust, Equipment-free and Deliverable to end-users) criteria can be considered as a benchmark for deciding which diagnostic tests could be used in different resource-constrained settings (**Appendix 9**) (Kosack *et al.*, 2017). However, these criteria are generic and are required to be adapted to the needs of each of the diagnostic tests and the manuscripts reported in this thesis hope to direct and stimulate this debate.

7.5 Appendix

Appendix 9: A guide to aid the selection of diagnostic tests

Step 1: Define the test's purpose – why, what, where, who?

- Decide whether an acute or chronic infection is to be diagnosed
- Decide whether the test is to be used for diagnosis, disease monitoring or verifying a cure
- Decide whether the test should be quantitative or qualitative
- Decide whether test results will be analysed at the point of care or in a central laboratory
- Define the test's end-users: trained laboratory technicians or primary health-care workers?
- What is the required performance of the test?

Step 2: Review the market

- Identify the products of interest available
- Obtain details of the tests available, including: (i) the manufacturer's name; (ii) the product's name; (iii) the product's catalogue number; (iv) package size; (v) storage requirements; (vi) shelf life; (vii) sample type (e.g. serum, plasma, whole blood or urine) and volume required; (viii) control reagents available; (ix) instruction languages; (x) how long the test takes and the number of steps required; (xi) additional equipment required; and (xii) cost
- Determine whether analysers are used and, if so, what the manufacturer's requirements are for training, installation and maintenance

Step 3: Review regulatory approval by international and national bodies

- Determine whether the test has the European CE mark
- Determine whether the test has been approved by the FDA
- Determine whether the test's manufacturing site meets the ISO 13485 standard
- Determine whether the test is prequalified or endorsed by WHO
- If not prequalified or endorsed by WHO, determine whether the test has been approved by the Expert Review Panel of the Global Fund to Fight AIDS, Tuberculosis and Malaria
- Determine whether the test has been approved by national authorities

Step 4: Determine the test's optimal diagnostic accuracy

- Review publications on the test's performance under ideal conditions (i.e. at reference laboratories)

Step 5: Determine the test's diagnostic accuracy in practice

- Review publications on the test's performance under real-life conditions (i.e. at the end-user level)

Step 6: Monitor the test in routine use

- Carry out quality control
- Carry out proficiency testing
- Supervise and train end-users

CE: Conformité Européenne; FDA: Food and Drug Administration; ISO: International Organization for Standardization; WHO: World Health Organization.

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9. Curriculum vitae and selected publications

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EDUCATION

2014-2018	Ph.D. Candidate, Liverpool School of Tropical Medicine, Liverpool, UK
2009-2011	Master of Bimolecular & Biomedical Sciences, Glasgow Caledonian University, Scotland, UK Thesis: Investigation of the mechanisms underlying the potential EVI-1 target gene involving in transforms Rat1 fibroblasts
2001-2005	Bachelor of Medical Sciences. Specialising: Medical Microbiology Qassim University - College of Medical Science

WORK EXPERIENCE

2007-Current	General Hospitals of Ministry of Health, as (Laboratory's Specialist - Department of Medical Microbiology).
2006-2007	Malaria Elimination Programme, Asir Health Affairs, Abha, Kingdom of Saudi Arabia
2005-2007	One year (Internship) at King Fahad Hospital – Jeddah

10. PUBLICATIONS

1) A centenary of Robert T. Leiper's lasting legacy on schistosomiasis and a COUNTDOWN on control of neglected tropical diseases

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- **Parasitology** https://doi.org/10.1017/S0031182016000998

2) Surveillance of intestinal schistosomiasis during control: a comparison of four diagnostic tests across five Ugandan primary schools in the Lake Albert region

Hajri Al-Shehri[1,2], Artemis Koukounari[3], Michelle C. Stanton[1,4], Moses Adriko[5], Moses Arinaitwe[5], Aaron Atuhaire[5], Narcis B. Kabatereine[5] and J. Russell Stothard[1]

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- **Parasitology** https://doi.org/10.1017/S003118201800029X

3) An extensive burden of giardiasis associated with intestinal schistosomiasis and anaemia in school children on the shoreline of Lake Albert, Uganda

Hajri Al-Shehri[a,b], Michelle C. Stanton[a], James E. LaCourse[a], Aaron Atuhaire[c], Moses Arinaitwe[c], Aida Wamboko[c], Moses Adriko[c], Narcis B. Kabatereine[c,d] and J. Russell Stothard[a,*]

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4) Molecular characterisation and taxon assemblage typing of giardiasis in primary school children living close to the shoreline of Lake Albert, Uganda

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5) Non-invasive surveillance of *Plasmodium* infection by real-time PCR analysis of ethanol preserved faeces from Ugandan school children with intestinal schistosomiasis

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Malaria Journal doi: https://doi.org/10.1186/s12936-019-2748-4

6) Molecular detection of equine trypanosomiasis in the Riyadh Province of Saudi Arabia

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11 Appendices

Appendix 10:A centenary of Robert T. Leiper's lasting legacy on schistosomiasis and a COUNT-DOWN on control of neglected tropical diseases

A centenary of Robert T. Leiper's lasting legacy on schistosomiasis and a COUNTDOWN on control of neglected tropical diseases

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SUMMARY

Part of Robert T. Leiper's (1881–1969) lasting legacy in medical helminthology is grounded on his pioneering work on schistosomiasis (Bilharzia). Having undertaken many expeditions to the tropics, his fascination with parasite life cycles typically allowed him to devise simple preventive measures that curtailed transmission. Building on his formative work with others in Africa and Asia, and again in Egypt in 1915, he elucidated the life cycles of African schistosomes. His mandate, then commissioned by the British War Office, was to prevent and break transmission of this disease in British troops. This he did by raising standing orders based on simple water hygiene measures. Whilst feasible in military camp settings, today their routine implementation is sadly out of reach for millions of Africans living in poverty. Whilst we celebrate the centenary of Leiper's research we draw attention to some of his lesser known colleagues, then focus on schistosomiasis in Uganda discussing why expanded access to treatment with praziquantel is needed now. Looking to WHO 2020 targets for neglected tropical diseases, we introduce COUNTDOWN, an implementation research consortium funded by DFID, UK, which fosters the scale-up of interventions and confirm the current relevance of Leiper's original research.

Key words: *Schistosoma*, life cycle, schistosomiasis, R. T. Leiper, Uganda, praziquantel, NTD scorecard.

PATRICK MANSON'S FIRST HELMINTHOLOGIST

There is a long list of eminent parasitologists of Scottish decent active in the 1870–1920 period, who were at the forefront of the great advances made in the battle against parasitic diseases (Barrett *et al*. 2015; Cox, in press). This period has often been described as 'the golden age of parasitology' and a most prominent figure throughout was undoubtedly Sir Patrick Manson (1844–1922). Manson is widely acknowledged as "The Father of Tropical Medicine' and was largely responsible for the creation of the London School of Tropical Medicine that opened in 1899 as part of the Seamen's Hospital Society's Branch Hospital at the Albert Dock (Cook, 2007). Under his influence many made significant international contributions, for example Ronald Ross (1878–1937) in protozoology, and in the nascent field of medical helminthology, his first appointee, Robert Thomson Leiper (1881–1969), was to be particularly productive (Willmott, 1981). Indeed, Leiper was to have an influential role in all aspects of pure and applied helminthology not only within the London School but also much further afield (Grove, 1990).

LEIPER AND THE LONDON SCHOOL

At the age of 24, Leiper joined the London School in 1905 after graduating from the University of Glasgow in medicine and very quickly gained international prominence whilst on an expedition to Accra, Ghana as Carnegie Research Scholar. While in Ghana, he elucidated the life cycle of the Guinea Worm (*Dracunculus medinensis*) and devised simple measures for its control, still used today (Grove, 1990). First introduced to schistosomiasis in Egypt by Arthur Looss (1861–1923) between 1906 and 1907, he also took part in the Egyptian Government's helminthological survey of Uganda. It was not until 8 years later and again in Egypt between 1915 and 1916, that he resolved the controversy behind Bilharzia. Second time around, he clearly described the essentials of life cycles of *Schistosoma mansoni* and

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Appendix 11:Surveillance of intestinal schistosomiasis during control: a comparison of four diagnostic tests across five Ugandan primary schools in the Lake Albert region

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Surveillance of intestinal schistosomiasis during control: a comparison of four diagnostic tests across five Ugandan primary schools in the Lake Albert region

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Abstract

Programmatic surveillance of intestinal schistosomiasis during control can typically use four diagnostic tests, either singularly or in combination, but these have yet to be cross-compared directly. Our study assembled a complete diagnostic dataset, inclusive of infection intensities, from 258 children from five Ugandan primary schools. The schools were purposely selected as typical of the endemic landscape near Lake Albert and reflective of high- and low-transmission settings. Overall prevalence was: 44.1% (95% CI 38.0–50.2) by microscopy of duplicate Kato-Katz smears from two consecutive stools, 56.9% (95% CI 50.8–63.0) by urine-circulating cathodic antigen (CCA) dipstick, 67.4% (95% CI 61.6–73.1) by DNA-TaqMan® and 75.1% (95% CI 69.8–80.4) by soluble egg antigen enzyme-linked immunosorbent assay (SEA-ELISA). A cross-comparison of diagnostic sensitivities, specificities, positive and negative predictive values was undertaken, inclusive of a latent class analysis (LCA) with a LCA-model estimate of prevalence by each school. The latter ranged from 9.6% to 100.0%, and prevalence by school for each diagnostic test followed a static ascending order or monotonic series of Kato-Katz, urine-CCA dipstick, DNA-TaqMan® and SEA-ELISA. We confirm that Kato-Katz remains a satisfactory diagnostic standalone in high-transmission settings but in low-transmission settings should be augmented or replaced by urine-CCA dipsticks. DNA-TaqMan® appears suitable in both endemic settings though is only implementable if resources permit. In low-transmission settings, SEA-ELISA remains the method of choice to evidence an absence infection. We discuss the pros and cons of each method concluding that future surveillance of intestinal schistosomiasis would benefit from a flexible, context-specific approach both in choice and application of each diagnostic method, rather than a single one-size fits all approach.

Introduction

Developing appropriate diagnostics tools, methods and protocols to track parasitic diseases before, during and after control is an important component within the multi-disciplinarity of parasitology. It has been previously highlighted (Stothard and Adams, 2014) and with regard to schistosomiasis, intestinal schistosomiasis poses a considerable public health burden in Uganda (Loewenberg, 2014). Since 2003 there has been an active national control programme against it (Kabatereine *et al.* 2006, 2007; Fenwick *et al.* 2009; Stanton *et al.* 2017), as primarily based on preventive chemotherapy campaigns (Montresor *et al.* 2012; Stothard *et al.* 2013). Despite much progress in the delivery of praziquantel (PZQ) treatments to school-aged children, infections with *Schistosoma mansoni* continue to be pervasive, particularly along the immediate shoreline of Lake Albert (Al-Shehri *et al.* 2016). Moving some 10–20 km inland, however, infection prevalence by school can decline dramatically, at least if measured by faecal egg-patency for if more sensitive diagnostic tools were used, such as urine-antigen dipsticks, such declines are less precipitous (Stothard *et al.* 2006, 2017*b*).

The incongruence between 'estimated' and 'true' prevalence is a well-known diagnostic dilemma in surveillance of intestinal schistosomiasis largely due to an operational compromise between imperfect detection tools and insufficient specimen sampling (Bergquist *et al.* 2009, 2015; Stothard *et al.* 2014; Utzinger *et al.* 2015; Weerakoon *et al.* 2015). Nonetheless, if control programmes are to be monitored effectively and also permit evidence-based adaptation or revision of control tactics (Tchuenté *et al.* 2017), infection dynamics at an individual level need to be captured alongside any broader changes in the epidemiological landscape amenable to measurement (Hawkins *et al.* 2016; Stothard *et al.* 2017*a*). As the strive towards elimination grows (Hawkins *et al.* 2016; Colley *et al.* 2017), previous diagnostic shortcomings are revealed

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Appendix 12:An extensive burden of giardiasis associated with intestinal schistosomiasis and anaemia in school children on the shoreline of Lake Albert, Uganda

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ORIGINAL ARTICLE

An extensive burden of giardiasis associated with intestinal schistosomiasis and anaemia in school children on the shoreline of Lake Albert, Uganda

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Background: Water-borne parasitic diseases associated with poverty still blight the lives of African school children. In Uganda, intestinal schistosomiasis is still common along the shoreline of Lake Albert, despite ongoing control, and co-infection with giardiasis and malaria is poorly described. To shed light on putative interactions between diseases, a prospective cross-sectional parasitological survey was undertaken in five primary schools.

Methods: Stool samples from 254 school children, aged 5–10 years, were examined by microscopy and rapid diagnostic tests (RDTs), with additional real-time PCR assays for detection of *Giardia* DNA. A finger-prick blood sample was also taken from each child and tested for malaria, and haemoblobin levels measured. Associations between diseases and anaemia were assessed.

Results: Intestinal schistosomiasis (46.5%), giardiasis (41.6%) and malaria (56.2%) were common, and a quarter of children were anaemic (<115 g/L). Up to 87.0% of children were excreting *Giardia* DNA and the prevalence of heavy infection by real-time PCR (Ct≤19) was 19.5%, being positively associated with light, moderate and heavy egg-patent schistosomiasis, as well as with anaemia.

Conclusions: In this setting, an extensive burden of giardiasis was revealed with heavy intensity infections associated with egg-patent intestinal schistosomiasis and anaemia. To improve child health, greater attention on giardiasis is needed along with exploring joined-up actions across diseases that promote better water hygiene and sanitation measures.

Keywords: Co-infection, Control, Epidemiology, *Giardia*, *Plasmodium*, *Schistosoma*

Introduction

In impoverished regions of sub-Saharan Africa and in addition to malaria, water-borne parasitic diseases pose a significant threat to the well-being of children. In parts of Uganda, for example, intestinal schistosomiasis is hyper-endemic along the shorelines of the Great Lakes and is particularly rife around Lake Albert.[1–3] Local aetiological factors include favourable freshwater snail habitats, poor local sanitation and hygiene, as well as extensive levels of daily water contact by shoreline communities.[4–6] While an extensive burden of malaria is known,[7–10] the occurrence and extent of another waterborne, poverty-related disease, giardiasis, has yet to be investigated. In 2003, a national control programme against schistosomiasis and intestinal worms was launched at Pakwach (see Figure 1).[11] Since then there have been substantive actions to deliver praziquantel and albendazole by mass drug administration to at-risk children attending primary schools and adults in endemic areas.[2] Owing to high levels of re-infection,[12] an extensive burden of intestinal schistosomiasis remains and children with hepatosplenic and gastrointestinal disease can still be found.[13]

Giardiasis is caused by *Giardia* spp., a binucleate flagellated protozoan, and is associated with ingestion of infectious cysts on foods or in drinking water, as contaminated by faecal waste from humans or animals carrying patent or cryptic infections.[14,15] In low and middle income countries, prevalence of human giardiasis can vary widely, typically from 0–30%, and

Appendix 13:Molecular characterisation and taxon assemblage typing of giardiasis in pri-mary school children living close to the shoreline of Lake Albert, Uganda

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Molecular characterisation and taxon assemblage typing of giardiasis in primary school children living close to the shoreline of Lake Albert, Uganda

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ABSTRACT

As part of an epidemiological survey for gastrointestinal parasites in school children across five primary schools on the shoreline of Lake Albert, the prevalence of giardiasis was 87.0% (n = 254) as determined by real-time PCR analysis of faecal samples with a genus-specific *Giardia* 18S rDNA probe. Faecal samples were further characterised with taxon assemblage-specific triose phosphate isomerase (TPI) Taqman® probes and by sequence characterisation of the β-giardin gene. While less sensitive than the 18S rDNA assay, general prevalence by TPI probes was 52.4%, with prevalence by taxon assemblage of 8.3% (assemblage A), 35.8% (assemblage B) and 8.3% co-infection (A & B assemblages). While assemblage B was dominant across the sample, proportions of assemblages A and B, and co-infections thereof, varied by school and by age of child; mixed infections were particularly common at Runga school (OR = 6.9 [95% CI; 2.5, 19.3]) and in children aged 6 and under (OR = 2.7 [95% CI; 1.0, 7.3]). Infection with assemblage B was associated with underweight children (OR = 2.0 [95% CI; 1.0, 3.9]). The presence of each assemblage was also confirmed by sequence analysis of the β-giardin gene finding sub-assemblage AII and further genetic diversity within assemblage B. To better explore the local epidemiology of giardiasis and its impact on child health, additional sampling of school children with assemblage typing would be worthwhile.

1. Introduction

The binucleate flagellated protozoan *Giardia duodenalis* (syn. *G. lamblia, G. intestinalis*) is a common gastrointestinal parasite able to infect a variety of mammals (Adam, 2001; Helmy et al., 2014). Where sanitation and hygiene are poor, these parasites can cause acute and/or chronic giardiasis across all ages (Wegayehu et al., 2016; Muhsen and Levine, 2012; Rogawski et al., 2017; Tellevik et al., 2015). While levels of endemicity of giardiasis may vary across the world, it can be common in children living within low and middle income countries (Laishram et al., 2012; Muhsen and Levine, 2012); for example, in Uganda giardiasis can be particularly rife (Al-Shehri et al., 2016; Fuhrimann et al., 2016), but its effect on child health is not fully appreciated but in Rwanda nearby, the very high prevalence of *G. duodenalis* in children aged 5 and under, was associated with being underweight (Ignatius et al., 2012).

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Appendix 14:Non-invasive surveillance of Plasmodium infection by real-time PCR analysis of ethanol preserved faeces from Ugandan school children with intestinal schistosomiasis

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Malaria Journal

RESEARCH Open Access

Non-invasive surveillance of *Plasmodium* infection by real-time PCR analysis of ethanol preserved faeces from Ugandan school children with intestinal schistosomiasis

Hajri Al-Shehri[1,2], B. Joanne Power[3], John Archer[1], Alice Cousins[1], Aaron Atuhaire[4], Moses Adriko[4], Moses Arinaitwe[4], Abdullah D. Alanazi[5], E. James LaCourse[1], Narcis B. Kabatereine[4] and J. Russell Stothard[1*]

Abstract

Background: As part of ongoing co-surveillance of intestinal schistosomiasis and malaria in Ugandan school children, a non-invasive detection method for amplification of *Plasmodium* DNA using real-time (rt)PCR analysis of ethanol preserved faeces (EPF) was assessed. For diagnostic tabulations, results were compared to rtPCR analysis of dried blood spots (DBS) and field-based point-of-care (POC) rapid diagnostic tests (RDTs).

Methods: A total of 247 school children from 5 primary schools along the shoreline of Lake Albert were examined with matched EPF and DBS obtained. Mean prevalence and prevalence by school was calculated by detection of *Plasmodium* DNA by rtPCR using a 18S rDNA Taqman® probe. Diagnostic sensitivity, specificity, positive and negative predictive values were tabulated and compared against RDTs.

Results: By rtPCR of EPF and DBS, 158 (63.9%; 95% CI 57.8–69.7) and 198 (80.1%, 95% CI 74.7–84.6) children were positive for *Plasmodium* spp. By RDT, 138 (55.8%; 95% CI 49.6–61.9) and 45 (18.2%; 95% CI 13.9–23.5) children were positive for *Plasmodium falciparum*, and with non-*P. falciparum* co-infections, respectively. Using RDT results as a convenient field-based reference, the sensitivity of rtPCR of EPF and DBS was 73.1% (95% CI 65.2–79.8) and 94.2% (95% CI 88.9–97.0) while specificity was 47.7% (95% CI 38.5–57.0) and 37.6% (95% CI 29.0–46.9), respectively. With one exception, school prevalence estimated by analysis of EPF was higher than that by RDT. Positive and negative predictive values were compared and discussed.

Conclusions: In this high transmission setting, EPF sampling with rtPCR analysis has satisfactory diagnostic performance in estimation of mean prevalence and prevalence by school upon direct comparison with POC-RDTs. Although analysis of EPF was judged inferior to that of DBS, it permits an alternative non-invasive sampling regime that could be implemented alongside general monitoring and surveillance for other faecal parasites. EPF analysis may also have future value in passive surveillance of low transmission settings.

Keywords: *Plasmodium*, *Schistosoma mansoni*, Real-time PCR, Surveillance, RDT, Faecal sampling

Background

Malaria continues to be a public health problem in 90 countries worldwide [1], with the impact of global control flat-lining over the past 2 years; the greatest disease burden remains in sub-Saharan Africa (SSA) where over 90% of deaths occur [2]. Here, weak health systems with restricted diagnostic repertoires and inadequate access to prompt treatment preside, alongside favorable conditions for *Plasmodium* transmission [2, 3]. Collectively, this places certain demographical groups, e.g.

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Appendix 15: Molecular detection of equine trypanosomiasis in the Riyadh Province of Saudi Arabia

Brief Communication

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Molecular detection of equine trypanosomiasis in the Riyadh Province of Saudi Arabia

Abdullah D. Alanazi,[1] Robert Puschendorf, Bashir Salim, Mohamed S. Alyousif, Ibrahim O. Alanazi, Hajri R. Al-shehri

Abstract. We conducted a cross-sectional study to detect trypanosome infections of horses and donkeys in the Riyadh Province of Saudi Arabia. DNA was extracted from blood samples collected from 368 horses and 142 donkeys, and subjected to universal first ribosomal internal transcribed spacer region (ITS1)-PCR followed by *Trypanosoma evansi* species–specific RoTat1.2-PCR. The universal ITS1-PCR revealed *T. evansi* infection in horses ($n = 12$; 3.3%) and donkeys ($n = 4$; 2.8%). There was no significant effect of sex or age on the prevalence of trypanosomiasis in horses or donkeys. Application of the RoTat1.2-PCR revealed that the RoTat1.2 *VSG* gene was absent from the positive ITS1-PCR samples of 3 horses and 1 donkey. This discrepancy could be explained by the circulation of *T. evansi* type B in Saudi Arabia; however, this suspicion requires confirmation.

Key words: Equine; ITS1; PCR; RoTat1.2; Saudi Arabia; trypanosomiasis.

Trypanosomiasis, or surra, caused by *Trypanosoma evansi*, is a serious disease that affects camels and horses in tropical and subtropical countries and often leads to reduced productivity and economic losses.[6] The parasite is found in both intra- and extravascular fluids of multiple hosts and is transmitted mechanically by several genera of hematophagous flies such as *Tabanus, Chrysops, Atylotus, Haematopota*, and *Stomoxys*.[11] In camels and horses, trypanosomiasis is found in acute and chronic forms, and clinical signs include intermittent fever, lacrimation, conjunctival petechiae, anemia, edema, enlarged lymph nodes, abortion, decreased fertility, and loss of body weight, which can result in death.[11]

In Saudi Arabia, the horse population is estimated to be >33,000 (http://www.fao.org/faostat/en/#data/QA, using filters: Saudi Arabia, Stocks, Horses, 2016), and more than 500 horses are imported annually from different countries such as the United Arab Emirates, the United States, and Europe. Horses are used for various purposes, including husbandry activities, transportation, racing, showing, and breeding. Diagnosis of equine trypanosomiasis relies on conventional parasitologic examinations and serologic assays.[2,3] DNA-based technologies including PCR have been widely used in the diagnosis of trypanosomiasis infection in camels, horses, cattle, and pets, given its sensitivity and specificity in detecting all stages of parasitic infection.[4,12,14,15,17] There is little information available on the prevalence or epidemiology of equine trypanosomiasis in Saudi Arabia. Thus, we aimed to estimate the prevalence of *Trypanosoma* spp. in horses and donkeys in the Riyadh Province of Saudi Arabia.

Our study was reviewed and approved by the Ethics Committee of the Department of Biological Science, Shaqra University (Riyadh, Saudi Arabia). This cross-sectional investigation was conducted from May 2015 to August 2017 in Riyadh Province, which is in the central part of Saudi Arabia between 24°38°N and 46°43° E (Fig. 1). Riyadh Province has very hot summers, when the temperature approaches 50°C. The average high temperature in July is 45°C. Winters are cold but seldom dropping below 0°C, with windy nights. The overall climate is arid, receiving very little annual rainfall (21.4 mm), with a relative humidity of 10–47% throughout the year. Riyadh Province is also known to have many dust storms (http://sdwebx.worldbank.org/climateportal/index.cfm?page=country_historical_climate&ThisCCode=SAU#).

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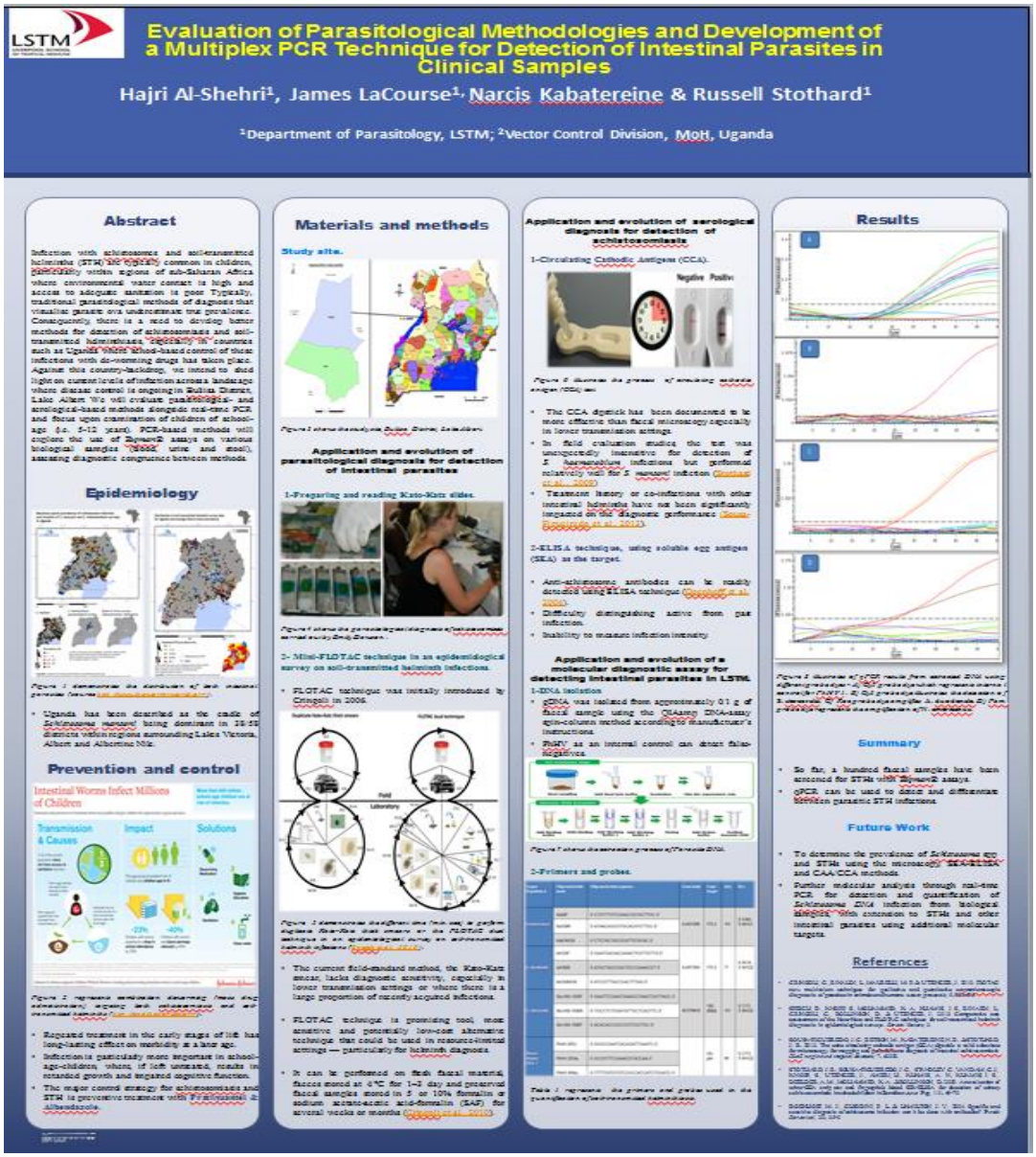
Appendix 16: Four posters submitted at various scientific meetings



Giardiasis in Ugandan schoolchildren: A survey with rapid diagnostic tests (RDTs) and TaqMan real-time PCR assays

Hajri Al-Shehri[1], James LaCourse[1], Narcis Kabatereine[2] & Russell Stothard[1]

[1]Department of Parasitology, LSTM, UK; [2]Vector Control Division, MoH, Uganda

Summary: An epidemiological survey with advanced diagnostic methods revealed an extensive burden of giardiasis in a total of 94 school children (prevalence by RDTs= 41% and by qPCR= 88%) examined from two shoreline villages on Lake Albert, Uganda.

Acknowledgements: To KSA MoH, the VCD field team, Martha Betson & Jaco Verweij

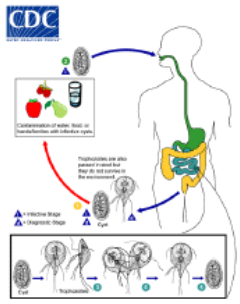
Background

Giardiasis is a common gastrointestinal illness in both high- and low-income countries, caused by the flagellated protozoan *Giardia lamblia*. The prevalence of the disease is greatest where there is insufficient or no access to safe water, hence faecal-oral transmission is extensive.

Giardiasis is typically diagnosed using standard faecal microscopy, however, DNA-based assays are the 'gold' standard. Rapid diagnostic tests based on copro-antigens are now available allowing point-of-care screening in impoverished settings. In Uganda, for example, whilst the prevalence of other parasitic diseases is known well, little has been documented on giardiasis.

Known risk factors for infection

- *Giardia* cysts are often picked up from contaminated surfaces (such as eating utensils or bathroom handles etc.) that contain trace amounts of faeces from an infected person or animal.
- Drinking water or using ice made from water sources where *Giardia* may live (for example, untreated or improperly treated water from lakes, streams, or wells).
- Ingesting environmental water while swimming or playing in water where *Giardia* may live, especially in lakes, rivers and ponds.
- Eating uncooked food that contains *Giardia* cysts.



Zoonotic transmission

- *Giardia* is also able to colonise a variety of other mammals and establish transmission cycles independently from man.
- In certain areas there is extensive spill-over from animal infections into human transmission cycle and the epidemiology can be complex.

Major cycles of transmission of *Giardia* demonstrates some assemblages/species are host specific and cycle between their respective hosts (blue), whereas others have low host specificity and are capable of infecting humans and other animals (red) (Monis et al., 2009).

Aims

1. To conduct an epidemiological survey in school-aged children living on the shoreline of Lake Albert, Uganda.
2. To assess the prevalence of giardiasis and helminthiasis.
3. To compare the diagnostic performance of RDT and TaqMan® assays.

Material and Methodology

A- Study sites

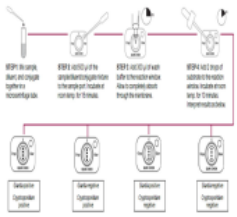
Two shoreline villages – Bugoigo and Runga were visited and 100 primary school children enrolled in the study.

B- Faecal specimens

- A total of 94 stool samples were obtained to evaluate the Quik-Chek© versus real-time PCR for *Giardia*.
- All fresh faecal samples were also subjected to Kato-Katz technique before being preserved in 96% ethanol for DNA extraction (Stothard et al., 2013).

C- The RDT for *Giardia* and *Cryptosporidium*

- Commercially available at £5 per test, used according to kit instructions, and detects *Giardia* cyst antigen and *Cryptosporidium* oocyst antigen.



D- Quantitative PCR (qPCR)

- Samples (N=94) were analysed by TaqMan® assays.
- Amplification and detection of *G. lamblia* DNA and PhHV-1 internal control were performed on all samples.
- qPCR reactions contained the following primer/probes:

Forward PhHV-267s	(GGGCGAATCACAGATTGAATC)
Reverse PhHV-337as	(GCGGTTCCAAACGTACCAA)
Probe PhHV	(Cy5 – TTTTTATGTGTCCGCCACCATCTGGATC)
Giardia185-99F	(GACGGCTCAGGACAACGGTT)
Giardia185-125R	(TTGCCAGCGGTGTCCG),
Giardia probe	(JOE-CCCGCGGCGGTCCCTGCTAG)

(Verweij et al., 2004).

Results

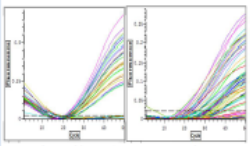
A- RDT for detection of *Giardia/Cryptosporidium*

- Needed fresh faecal specimens but was less labour intensive than traditional methods with results available in 30 minutes.
- Although cost per test was high it was judged the best in-field test and detected numerous children infected. Only 3 tests were invalid and were repeated.

Fig 1. RDTs used in Runga school showing many children with giardiasis, no *Cryptosporidium* seen.

B- TaqMan quantitative PCR (qPCR) for *Giardia*

- Species-specific DNA assays with PhHV assay acting as an internal control to determine efficiency of the PCR and detect inhibition in the sample.



Internal control (PhHV-1) *Giardia*

Fig 2. Chromatogram traces from real-time PCR reactions.

C- Prevalence of giardiasis by school & assay

- The RDT revealed that prevalence by location was high, 36% and 48% at Bugoigo and Runga, respectively.
- A gender related biasis in the prevalence of infections was also observed but this changed by location.
- The prevalence by qPCR (88%) was approximately twice that revealed by RDT.

Table 1. Results from Bugoigo school

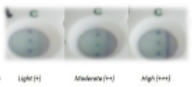
RDTs	Tested	M	F	qPCR	Tested	M	F
Positive	17	5	12	Positive	38	17	21
Negative	29	17	12	Negative	8	5	3
Prevalence %	37	23	50	Prevalence %	83	77	87

Table 2. Results from Runga school

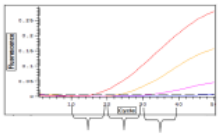
RDTs	Tested	M	F	qPCR	Tested	M	F
Positive	22	15	7	Positive	45	22	23
Negative	26	9	17	Negative	3	1	2
Prevalence %	48	63	41	Prevalence %	94	96	92

D- Intensity of infections by RDT and real-time PCR

- RDTs could be classified into 3 levels according to blue-line colour.



- Real-time PCRs could be into 3 levels according to the Ct value.



Intensity High (+++) Moderate (++) Light (+)

- Infection intensities were recorded by school and by assay.

Bugoigo school

RDTs (N=46)	M	F	qPCR (N=46)	M	F
High	1	6	High	0	6
Moderate	3	4	Moderate	4	9
Light	1	2	Light	13	6
Negative	17	12	Negative	5	3
Total	22	24	Total	22	24

Runga school

RDTs (N=48)	M	F	qPCR (N=48)	M	F
High	6	5	High	12	7
Moderate	5	0	Moderate	9	16
Light	4	2	Light	1	0
Negative	9	17	Negative	2	1
Total	24	24	Total	24	24

Table 3. Shows the performance of the *QUIK CHEK* compared to qPCR based on intensity of infection.

Conclusions

- The RDTs were excellent as an initial in-field screen, however, they underestimate the prevalence of infection as 'light' infections (as defined by qPCR) were missed.
- Whilst RDTs are useful for 'outbreak' settings or in the detection of 'heavy' infections, their use in general surveys should be viewed with some caution for its sensitivity is much lower than qPCR.
- The high prevalence of giardiasis revealed in this survey highlights a cryptic burden of disease in these lake shoreline communities.
- Given the observed close water contact of communities and livestock e.g. cattle, it is likely that there are combined epidemiological cycles of transmission occurring.

Future work

- Refine multiplex-qPCR assays for simultaneous detection of *Entamoeba histolytica*, *Giardia lamblia* and *Cryptosporidium parvum*.
- Use PCR-RFLP to reveal the putative zoonotic genotypes of *Giardia* by analysis of *bg* and *gdh* genes.

References : Monis et al., 2009, *Trends in parasitology*, 25, 93-100 Stothard et al., 2013, *Trends in parasitology*, 29, 197-205, Verweij et al., 2004, *Journal of clinical microbiology*, 42, 1220-1223.

1275 Comparing Diagnostics of Intestinal Schistosomiasis in Uganda Across High and Low Endemic Settings

Hajri Al-Shehri[1,*], Michelle C. Stanton[1], James E. LaCourse[2], Aaron Atuhaire[3], Moses Arinaitwe[3], Aida Wamboke[3], Moses Adriko[3], Narcis B. Kabatereine[3,4] & J. Russell Stothard[2,*]

[1] Department of Parasitology, Liverpool School of Tropical Medicine, Liverpool, L3 5QA UK; [2] Ministry of Health, Aziz District, Kingdom of Saudi Arabia; [3] Vector Control Division, Ministry of Health, Kampala, Uganda; [4] Schistosomiasis Control Initiative, School of Public Health, Imperial College London, London W2 1PG, UK.

ABSTRACT

Infection with intestinal schistosomiasis is typically common in school-aged children, particularly within regions of sub-Saharan Africa. Here environmental water contact is typically high and access to adequate sanitation is often poor. Traditional parasitological methods of diagnosis that visualise parasite ova underestimate true prevalence and as control programmes progress, infection egg-tallies may also decline and become an insensitive surveillance tool. Consequently, there is a need to develop better methods for detection of intestinal schistosomiasis, especially in countries such as Uganda where ongoing school-based control has taken place for over a decade. Against this country-backdrop, we investigate the application of novel diagnostics using parasitological, serological and molecular DNA methods to shed light on current levels of infection across 5 primary schools within Bullisa District, Lake Albert. While Kato-Katz sampling is sufficient in high endemic settings, even against the others used here, it is not in low endemic settings. In the latter, we propose that if resources permit, DNA TaqMan is favourable but serological methods remain the 'gold-standard' to evidence an absence of infection. However, urine-CCA dipsticks are perhaps the best point-of-care test available today.

AIMS

1. To conduct an epidemiological survey in school-aged children living on the shoreline of Lake Albert, Uganda.
2. To assess the prevalence of intestinal schistosomiasis
3. To compare the parasitological and serological diagnostic performance against TaqMan® assays.

Material and Methodology

A- Study sites

Five shoreline villages were visited and 271 primary school children enrolled in the study.

B-Application of diagnostic tools for detection of intestinal parasites

1-Preparing and reading Kato-Katz slides.

- A modified thick-smear technique.
- Helminth eggs examined quantitatively.
- Not sensitive compared to serological tests

2-Circulating Cathodic Antigens (CCA).

- The CCA dipstick has been documented to be more effective than faecal microscopy especially in lower transmission settings.

3-ELISA technique, using soluble egg antigen (SEA) as the target.

- Anti-schistosome antibodies can be readily detected using ELISA technique (Doenhoff et al., 2004).
- Difficulty distinguishing active from past infection.
- Ability to measure infection intensity

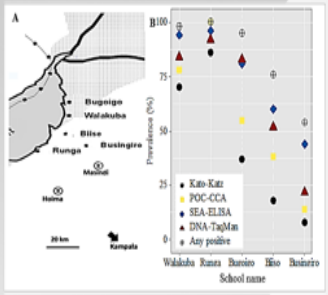
4-Quantitative PCR (qPCR)

- Samples (N=258) were analysed by TaqMan® assays.
- Amplification and detection of *Schistosomiasis* DNA and PhHV-1 internal control were performed on all samples
- qPCR reactions contained the following primer/probes:

PhHV-267s	GGGCGAATCACAGATTGAATC
PhHV-337as	GCGGTTCCAAACGTACCAA
PhHV-305tq	Cy5-TTTTTATGTGTCCGCCACCATCTGGATC-BHQ3
Sxp_ITS_48F	GGT CTA GAT GAC TTG ATY GAG ATG CT
Sxp_ITS_124R	TCC CGA GCG YGT ATA ATG TCA TTA
Sxp78T	ROX-TGG GTT GTG CTC GAG TCG TGG C-BHQ2

RESULTS

Figure 1A. The geographical location of the 5 sampled schools, (B) prevalence of *Schistosoma mansoni* across schools by each diagnostic method and also 'pooled' prevalence based on criterion of any test positive.



- KK – 44.1% [95%CI 38.0-50.2]
- Urine-CCA – 56.9% [95%CI 50.8-63.0]
- SEA-ELISA – 75.1% [95%CI 69.8-80.4]
- DNA TaqMan – 67.4% [95%CI 61.6-73.1]
- Any positive test – 84.8% [95%CI 80.4-89.2]

Overall prevalence by diagnostic technique was: The CCA dipstick has been documented to be more effective than faecal microscopy especially in lower transmission settings.

Table 1. Evaluation of parasitological- and serological-based methods alongside real-time PCR against screening Tests (CCA) as gold standard *diagnosis*: Validity and Reliability

	CCA-urine point-of-care (POC) as gold standard			
Evaluating Diagnostic test	Measurement Estimate % [95% CIs]		Diagnostic Accuracy [95% CIs]	Cohen's kappa [95% CIs]
TaqMan®				
Negative	Sensitivity	85.03 [78.38- 89.9]	72.48% [66.73-77.57]	0.4215 [0.3024-0.5405]
Positive	Specificity	55.86 [46.58-64.7]		
Total (%)	PPV	71.84 [64.74-77.9]		
	NPV	73.81 [63.51-82.0]		
SEA-ELIZA				
Negative	Sensitivity	96.6 [92.29-98.54]	77.91% [72.46-82.54]	0.5247 [0.4125-0.637]
Positive	Specificity	53.13[43.92-62.17]		
Total (%)	PPV	73.2 [66.56-78.95]		
	NPV	92.18[82.98-96.62]		
Kato-Katz				
Negative	Sensitivity	76.67 [69.42-82.95]	86.43% [81.72-90.18]	0.733 [0.6149-0.8513]
Positive	Specificity	99.1 [95.07-99.84]		
Total (%)	PPV	99.12 [95.2-99.84]		
	NPV	76.39 [68.82-82.58]		

- Each method was cross-tabulated by empirical calculation to assess sensitivity, specificity, negative predictive values and positivetive predictive value.
- Sensitivity of SEA-ELISA is the highest (96.6%) but also has the lowest specificity (53.2%), with the highest negative predictive value of all methods
- Sensitivity of Kato-Katz is the lowest (76.9%) but also has the highest specificity (99.1%), with the highest positive predictive value of all methods.
- Diagnostic scores for DNA-TaqMan assay appear intermediate between these two methods

Table2. LCA estimates of sensitivity and specificity and *Schistosoma mansoni* prevalence with 95 % CIs for four diagnostic tests

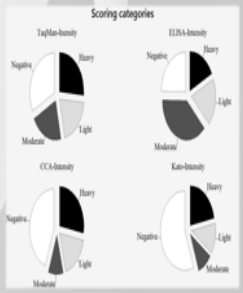
Diagnostic indicator	Sensitivity	Specificity
Kato-Katz	84.4 % [76.0-92.9]	100 % [NA]
Urine-CCA dipsticks	95.1 % [87.3 – 100]	89.3 % [80.9 – 97.6]
SEA-ELISA	97.7 % [95.1 – 100]	49.5 % [39.4 – 59.6]
DNA-TaqMan	90.2 % [84.2 - 96.2]	57.5 % [48.6 – 66.5]
LCA Model based S. mansoni prevalence in Walakuba	75.7 % [62.9 – 88.5]	
LCA Model based S. mansoni prevalence in Runga	100.0 % [NA]	
LCA Model based S. mansoni prevalence in Bugoigo	49.7 % [35.0 – 64.5]	
LCA Model based S. mansoni prevalence in Biiso	27.0 % [12.2 – 41.9]	
LCA Model based S. mansoni prevalence in Busingiro	9.6 % [3.0 – 18.4]	

CONCLUSIONS

- There is still a very high prevalence of active intestinal schistosomiasis on the immediate shoreline of Lake Albert, despite control.
- In high-endemic settings, Kato-Katz sampling is a satisfactory diagnostic standalone but in low-transmission settings should be augmented or replaced by urine-CCA dipsticks.
- DNA-TaqMan assay appears suitable in both endemic settings though is only implementable if resources permit and cannot be not used at point-of-care settings.
- SEA-ELISA remains the method of choice to evidence an absence infection and urine-CCA dipsticks appear the best point-of-contact method across all endemic settings.

FUTURE WORK

- Further analysis of diagnostic scores by individual against hepato-splenic disease for tracking morbidity.
- Faecal occult blood as point-of-care test for morbidity associated with infection



References : Mwanje et al., 2002. Trends in parasitology, 22. 93-100 Doenhoff et al., 2010. Trends in parasitology, 26. 197-203. Ishaleh et al., 2004. Journal of clinical microbiology, 42. 1220-1223.

NP-0457

Assessing the significance of giardiasis in Ugandan school children and strain assemblages of *Giardia duodenalis* on the shoreline of Lake Albert

Hajri Al-Shehri[1,2], James E. LaCourse[1], Otto Klimach[1], Narcis B. Kabatereine[3] & J. Russell Stothard[1,*]

[1]Department of Parasitology, Liverpool School of Tropical Medicine, Liverpool, L3 5QA UK; [2] Ministry of Health, Asir District, Kingdom of Saudi Arabia; [3] Vector Control Division, Ministry of Health, Kampala, Uganda.

ABSTRACT

Worldwide, giardiasis is considered the most prevalent gastro-intestinal parasitic disease and in sub-Saharan Africa is often under-reported even though its chronic clinical impact on child health is well known. As part of a general epidemiological survey for intestinal schistosomiasis in school children in Western Uganda, 254 children [aged 5–10] were also examined for giardiasis by RDTs (Alere: GIARDIA/CRYPTOSPORIDIUM QUIK CHEK™). Infection prevalence by RDT was 41.6% (40/96), and a later analysis of archived stool in ethanol by real-time PCR revealed prevalence of 87.0% (221/254). As there is known strain diversity within *Giardia duodenalis*, faecal samples were also typed using specific strain-assemblage probes, finding both A and B strains individuals as well as A/B co-infections, especially in children aged 6 and under (OR = 2.7 [95 CI: 1.03, 7.28]). More broadly, underweight children (cut-off, -2 ≤ SD weight-for-age Z score) appeared positively associated assemblage B (OR = 2.0 [95 CI; 1.04,3.91]). Overall, infection with *G. duodenalis* was not associated with gender although further expanded sampling and longitudinal studies at the sub-assemblage level are needed to better monitor infection dynamics and associations with other parasitic diseases.

AIMS

1. To conduct an epidemiological survey in school-aged children living on the shoreline of Lake Albert, Uganda
2. To assess the prevalence of *G. duodenalis* with molecular methods
3. To perform a molecular characterisation of *G. duodenalis* using TaqMan® strain-specific assays

Material and Methodology

A - Study sites

Five shoreline villages were visited and 254 primary school children enrolled in the study

Fig 1. shows the study site, Buliisa District, Lake Albert

B - Application of diagnostic tools for detection and a molecular characterisation of *G. duodenalis*

1- Infection detected by RDT

(Alere: GIARDIA/CRYPTOSPORIDIUM QUIK CHEK™).

Fig 2. shows sample collection and QUIK CHEK protocol and processing

2-Quantitative PCR (qPCR)

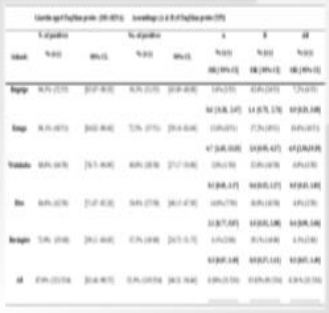
- Samples (N=258) were analysed by **TaqMan®** assays.
- Amplification and detection of *G. duodenalis* DNA (18S-RNA) and PhHV-1 Internal control were performed on all samples
- Duplex qPCR for specific A and B assemblages targeting (TPI) , *qPCR* reactions contained the following primer/probes (Table 1)

Table 1: Primers & probes used for the MGBP genotyping assay (Elwin *et al* 2014)

Target	Primer/ Probe	Nucleotide position	Sequence (5' - 3')
G. duodenalis assemblage A	GDAF	647-662	CATTGCCCTTCCCGCC
	GDAR	703-722	CTGCGCTGCTATCCTGACTG
	GDAT	679-693	VIC-CCATTGCGCGACAAGB-MGB-NFQ
G. duodenalis assemblage B	GDBF	559-580	GATGAACGCAAGGCCAATAA
	GDBR	606-1629	TCTTTGATTCTCCAATCTCTTCTT
	GDBT	580-675	FAM-AATATTCTCAGCTCGAGAMGB-NFQ

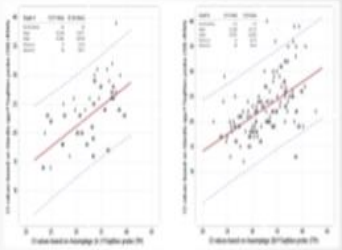
RESULTS

Table 2: shows the prevalence of *G. duodenalis* and its assemblages across all five schools with odd ratio and 95% Confidence Limit of association between A, B and mixed AB assemblages with study sites



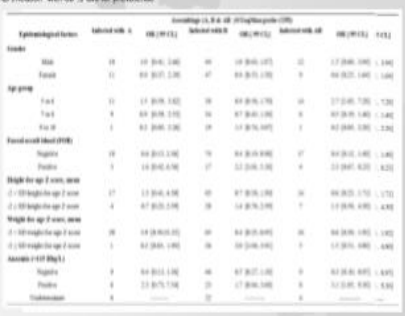
- Out of the 254 samples,221 tested positive (87.0%) by targeting 18S rRNA and 133 (52.3%) by specific assemblages TPI gene.
- Out of the 133 positive results, 21 of the samples were identified to contain both assemblage A and B

Fig 3. linear regression model indicating the relationship between Ct value of ***Giardia*** spp ®TaqMan probe (18S rRNA) and Ct value of Assemblage (A) or (B) ®TaqMan probe (TPI) with blue dashed lines showing the 95% prediction interval



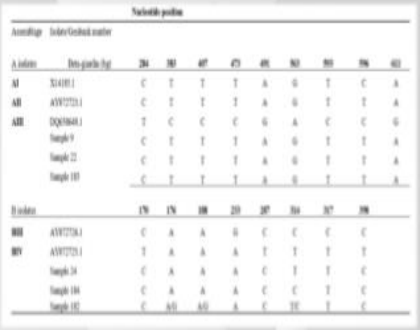
- Linear model (Graph A) describes the relationship between Ct value of ***Giardia*** spp ®TaqMan probe (18S rRNA) and Ct value of Assemblage A is 18S rRNA = 0.203 + 0.6891 TPI, with R-squared 34.91% (P <0.005),and positive correlation(r=0.60). Out of the 133 positive results, 21 of the samples were identified to contain both assemblage A and B
- The fitted equation for the linear model of assemblage B (Graph B) and ***Giardia*** spp is 18S rRNA = 0.228 + 0.6947 TPI, with R-squared 28.39 % (P <0.005), and positive correlation(r=0.54).

Table 3: Binary logistic regression analysis of potential risk factors associated with assemblages A, B or AB infection with 95 % CIs for prevalence



- Association of mixed assemblages in younger children (OR = 2.7 [95% CI; 1.0, 7.3])
- Assemblage B was associated with the presence of faecal occult blood (OR = 2.2 [95% CI; 1.0, 5.2])
- A significant association of infection with assemblage B and children of lower weight-for-age, i.e. wasting (OR = 2.0 [95% CI; 1.0, 3.9]).

Table 4: Details the point mutations with the six representative samples for the Z-giardin gene, finding an exact match with sub-assemblage AII and some variation between BIII and BIV.



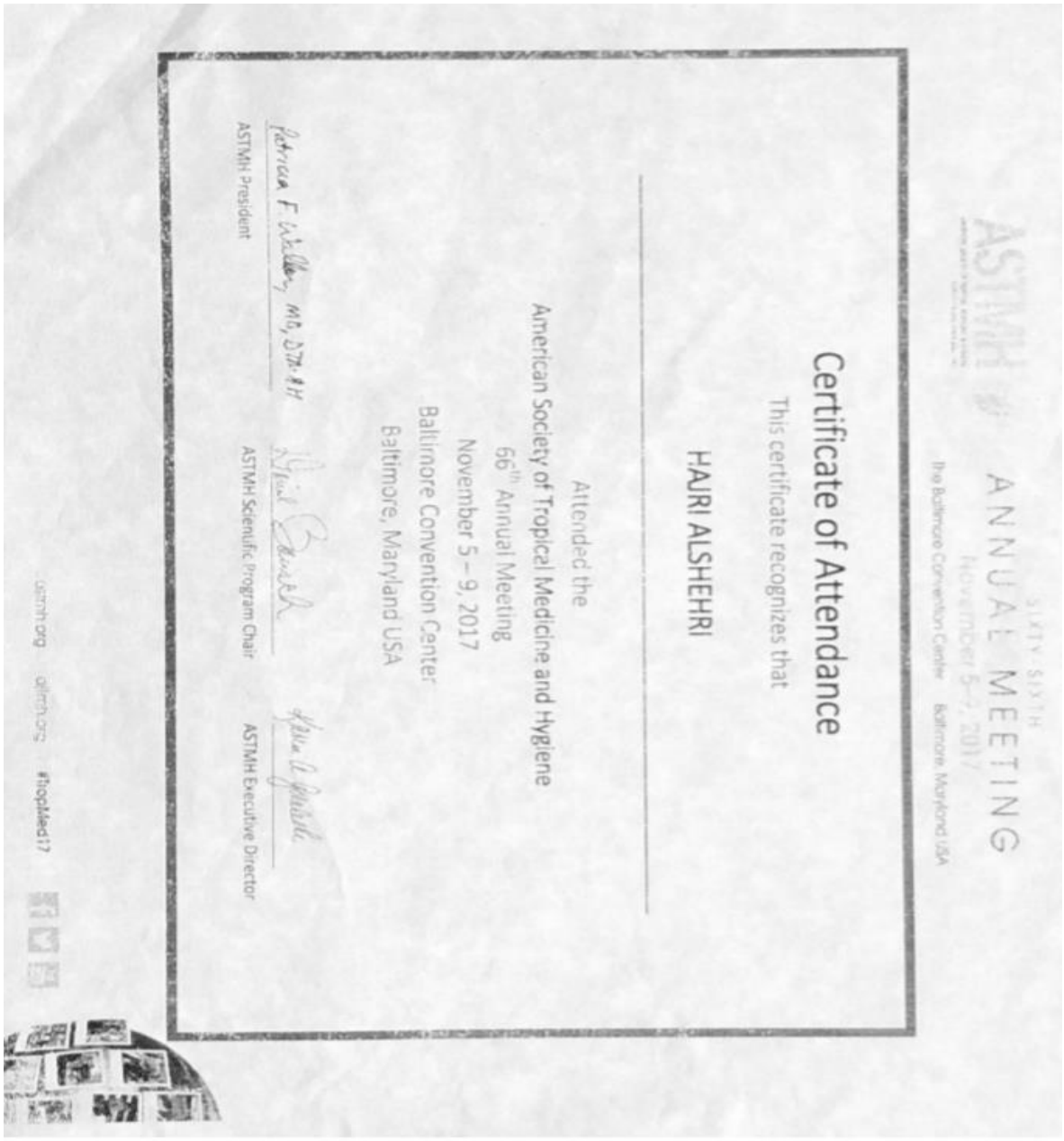
CONCLUSIONS

- There is a very high prevalence of *G. duodenalis* with 87.0% of 254 children younger than ten years with active infections
- Molecular characterization has revealed the existence of two genetically distinct species (A and B) in children
- In high-endemic settings, DNA-TaqMan assay appears a satisfactory diagnostic standalone for detection and assemblages of *G. duodenalis*

References:

AL-SHEHRI H., STOTHARD J. R. 2016. An extensive burden of giardiasis associated with intestinal schistosomiasis and anaemia in school children on the shoreline of Lake Albert, Uganda. Transactions of the Royal Society of Tropical Medicine and Hygiene, 110, 597-603.

Appendix 17: Certificates of Achievements



Certificate of Attendance

This certificate recognizes that

HAJRI ALSHEHRI

Attended the

American Society of Tropical Medicine and Hygiene

66th Annual Meeting

November 5 – 9, 2017

Baltimore Convention Center

Baltimore, Maryland USA

ASTMH President

ASTMH Scientific Program Chair

ASTMH Executive Director

SIXTY SIXTH ANNUAL MEETING

November 5–9, 2017

The Baltimore Convention Center Baltimore, Maryland USA

astmh.org #ASTMH17

Certificate of Participation

Awarded to

Hajri Rajh Alshehri

acknowledging the valuable participation in the 9th Saudi Students' Conference

The ICC, Birmingham, 13 - 14 Feb 2016

Faisal M. Almohanna Abaalkhail

Saudi Arabian Cultural Attaché in the UK

9th SSC

Organisers

Sponsor University

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Publisher

Springer

This is to certify that

Mr. Hajri Alshehri

has completed the IBMS credited activity

The 2016 Parasitic Infection

at The O2, London, UK code MD026N16

on 28th -30th June 2016 credits18

signature of organiser

87 Gladstone Street, Bedford MK41 7RS

Tel: +44 (0) 1234 211015

Fax: +44 (0) 1234 481015

Email: info@bsp.uk.net

Certificate of Attendance

British Society for Parasitology

BSP Spring Meeting 2016

From Science to Solutions:

Optimising control of parasitic diseases

Mr Hajri Alshehri

Was a participant in the:

BSP Spring Meeting

11th April -13th April 2016, held at Imperial College London

Julian Fuller

BSP Secretariat

INSTITUTO DE HIGIENE E MEDICINA TROPICAL
Universidade Nova de Lisboa

Certificate of Participation

35th European Course in Tropical Epidemiology

Hajri Alshehri attended the 35th European Course in Tropical Epidemiology, held from the 16th of August to the 2nd of September 2016, at the Institute of Hygiene and Tropical Medicine, Lisbon, Portugal.

Instituto de Higiene e Medicina Tropical, on September 2, 2016

Director

Professor Paulo Ferrinho, PhD

LSTM
LIVERPOOL SCHOOL
OF TROPICAL MEDICINE

This is to certify that

Hajri Alshehri

having satisfactorily completed the approved course of study and having passed the prescribed examinations is awarded the

Professional Certificate in Supporting Learning

October 2016

Sue Assinder
Director of Education

Marita Grimwood
Director of Studies

This is to certify that

Hajri Alshehri

has successfully completed

Professional Certificate in Supporting Learning
at
Liverpool School of Tropical Medicine

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Yaz El Hakim
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Date: March 2018

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