2019-07-04

Application of Epidemiology and Biostatistics to Malaria Diagnosis in Returning Travellers

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Application of Epidemiology and Biostatistics to Malaria Diagnosis in Returning Travellers

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

GRADUATE PROGRAM IN MICROBIOLOGY AND INFECTIOUS DISEASES

CALGARY, ALBERTA

JULY, 2019

ABSTRACT

Today, malaria elimination is back on the agenda but for this to be feasible, there must be a coordinated global effort utilizing all available tools. Portable, sensitive diagnostics with a low limit of detection are required to detect the malaria reservoir, and novel antimalarials are required to combat the threat of artemisinin resistance. Returning travellers are a good population in which to investigate malaria physiology and diagnostics because there is a good supply of study participants and an abundance of easily available data.

In Canada, a combination of microscopy and rapid diagnostic tests are used to diagnose malaria, but these lack sensitivity and require repeated testing to rule out the condition. A prospective diagnostic trial of the *illumigene* Malaria, loop-mediated isothermal amplification (LAMP) assay, manufactured by Meridian Bioscience was conducted in symptomatic returning travellers between June 2017 and January 2018. After discrepant resolution with RT-PCR, LAMP had a sensitivity of 100% (95% CI; 95.8-100) and a specificity of 100% (95% CI; 98.7-100). In symptomatic returning travellers, LAMP has the potential to replace traditional malaria diagnostics, allowing for malaria to be ruled out in a timely manner.

It is unclear if uncomplicated malaria causes deranged liver enzymes, which has implications for antimalarial drug development. A retrospective cohort study was evaluated in returning travellers (n=4548) who underwent a malaria test and had liver enzymes measured within 31 days from 2010-2017. After adjusting for gender, age, and use of hepatotoxic medications, returning travellers testing positive for malaria had higher odds of having an abnormal TB [(OR: 12.64, 95% CI: 6.32 – 25.29), $p<0.001$] but not ALP [(OR: 0.32, 95% CI: 0.09 – 1.10), $p=0.072$], ALT

[(OR: 1.01, 95% CI: 0.54 – 1.89), p=0.978] or AST [(OR: 1.26, 95% CI: 0.22 – 7.37), p=0.794], compared to those who tested negative. This is most likely to be due to haemolysis, which normalizes following treatment.

LAMP can be used in the diagnosis of malaria in returning travellers, and it may have a role in malaria elimination. Uncomplicated malaria does not appear to cause raised aminotransferases in returning travellers, and consideration must be given to this in antimalarial drug development.

PREFACE

Chapter 3 of this thesis has been published as Cheaveau J, Nguyen H, Chow B, Marasinghe D, Mohon, AN, Yuan, H, Viana G, van Schalkwyk, Church, D, Chan W, Pillai DR. Clinical Validation of a Commercial LAMP Test for Ruling out Malaria in Returning Travellers: A Prospective Diagnostic Trial. *Open Forum Infect Dis*. 2018;5(11):ofy260. Published 12th Oct 2018.

BC, GV and DRP designed the project. HN, BC, ANM, JC and HY performed the experiments. JC and BC analyzed data. JC and DH performed the statistical analysis. DvS, DC, WC provided feedback on the manuscript. JC and DRP wrote the manuscript. Ethics for this chapter was covered by Ethics Certificate REB17-2220_REN1 issued by the University of Calgary Conjoint Health Ethics Board for the project 'Illumigene Malaria Phase II study: A prospective diagnostic study,' on January 5, 2019

Chapter 4 of this thesis has been published as Cheaveau J, Marasinghe D, Samantha Akakpo S, Deardon R, Naugler, C, Chin A, Pillai DR. The impact of malaria on liver enzymes: A retrospective cohort study (2010-2017). *Open Forum Infect Dis*. 2019;6(6):ofz234. Published 16th May 2019.

JC and DRP designed the project. JC obtained the data and performed the analysis. DH, SA, RD, CN and AC provided feedback on the design and on the manuscript. JC and DRP wrote the

manuscript. Ethics for this chapter was covered by Ethics Certificate REB15-1160_REN4 issued by the University of Calgary Conjoint Health Ethics Board for the project 'Surveillance of Imported Malaria in Calgary,' on October 31, 2018

ACKNOWLEDGMENTS

Many people have helped me throughout my masters. Without their support, none of this would have been possible. I would like to specifically thank the following people:

Dr Dylan Pillai for his support in numerous ways. Dr Pillai gave me the opportunity to attend the University of Calgary, by agreeing to take me on as his Master's student. He gave me the opportunity to move to Canada, something I will be always grateful for and will never forget. He has supported me financially through a stipend and other support for conferences. He has provided guidance for my career, giving valuable input into how to balance the challenges of a clinical and academic medical career. Our conversations have given me valuable insight into the bigger picture, helping me understand how public health, research, regulations and management influence infectious diseases policy.

My supervisory committee members, Dr Daniel Gregson and Dr Rob Deardon for their invaluable input into my publications and thesis. I particularly thank them for their feedback regarding study design and statistics.

Grand Challenges Canada and Medicines for Malaria Venture for providing financial support to Dr Pillai and for my stipend.

Dewdunee Marasinghe, a visiting summer student for her feedback on statistics. We helped each other understand the basics of multivariable statistical analysis.

The Pillai laboratory group past and present members including Abu Naser Mohon, Ranmalee Amarasekara, Maha Alharbi, Ruth Legese, Daniel Castañeda Mogollon and Cody Doolan for their input into my work.

The researchers in Ethiopia with whom the Pillai laboratory collaborate. While my thesis focuses on returning travellers, it was a real privilege to undertake fieldwork in Ethiopia and work with such enthusiastic individuals and help plant the seeds for a large-scale study of LAMP in pregnant mothers.

Chelsea Mitchell, my future wife, for all your support and the great times we have had during the masters. I look forward to having many more. My parents. Bill and Maria for supporting me throughout my studies and Chelsea's parents, Doug and Patti for making me feel so welcome in Canada. My brothers, David and Matthew and their partners for visiting last year and my friends for always being there for me.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACTs	Artemisinin combination therapies
AL	Artemeter-lumefantrine
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
CDC	Centers for Disease Control and Prevention
CHMI	Controlled Human Malaria Infection
CLS	Calgary Laboratory Services
EMM	Effect measure modification
FDA	US Food and Drug Administration
G6PD deficiency	Glucose-6-phosphate dehydrogenase deficiency
GRADE	Grading of Recommendations Assessment, Development and Evaluation
GTS for malaria	Global Technical Strategy for Malaria
HRP2	Histidine-Rich Protein 2
JAMA	Journal of the American Medical Association
LAMP	Loop-mediated isothermal amplification
LMIC	Low and middle income countries
LOD	Limit of Detection
NAT	Nuclei acid testing
NCEs	New Clinical Entities
NPV	Negative Predictive Value
OR	Odds Ratio

PCR	Polymerase Chain Reaction
P.	Plasmodium
pLDH	pan Lactate Dehydrogenase
PPV	Positive Predictive Value
RBM	Roll Back Malaria Initiative
RCTs	Randomized Controlled Trials
RDTs	Rapid Diagnostic Tests
RT-PCR	Real-time Polymerase Chain Reaction
SDGs	Sustainable Development Goals
SOP	Standard Operating Procedure
TB	Total Bilirubin
uRDT	ultra-sensitive Rapid Diagnostic Test
USD	US Dollars
VFR	Visiting Friends and Relatives
WHO	World Health Organisation

CHAPTER 1: MALARIA

Background

In 2017, there were 219 million malaria cases and 435 000 fatalities worldwide (1). 92% of all malaria cases and 93% of malaria deaths occurred in the WHO African Region with mortality concentrated around several high-risk groups, including pregnant women and infants (1). Significant progress has been made in the fight against malaria over the last few decades, but since 2015 progress has stalled with no further reduction in the number of cases. Despite this, since 2016, the public health objective has shifted away from malaria control towards malaria elimination (2, 3).

Symptoms and Signs

Malaria has a wide variety of signs and symptoms, ranging from asymptomatic and a flu-like illness to severe disease and even death. The clinical symptoms of malaria are caused by asexual erythrocytic parasites (4). In uncomplicated malaria, patients typically present with fever, night sweats, headache, nausea and vomiting, myalgia and malaise. There is a continuum from asymptomatic malaria through to uncomplicated malaria and then severe malaria. Several factors affect the disease phenotype including the species of malaria, innate and acquired immunity, the parasitaemia and the speed of treatment (5). Severe malaria typically causes multi-organ failure, cerebral malaria with impaired consciousness and seizures, respiratory distress, seizures, prostration, shock, pulmonary oedema, abnormal bleeding and jaundice (5). Signs include severe anaemia, hypoglycaemia, acidosis, high lactate, acute kidney injury and a high parasitaemia (5).

Severe malaria typically affects non-immune individuals such as children and returning travellers. The most common complication for young children is severe anaemia, while cerebral malaria is more common in older children (6).

Pathophysiology

Malaria is a disease caused by the protozoan parasite of the genus *Plasmodium* with *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae,* and *P. knowlesi* the known causative species. All species of malaria are pathogenic to humans but *P. falciparum* is the most common cause of severe malaria (7). Severe malaria is also possible with *P. vivax* and *P. knowlesi*, but *P. ovale* and *P. malariae* typically present with uncomplicated malaria (8, 9).

Life cycle

To understand how malaria causes pathology, it is important to understand its life cycle. As can be seen in Figure 1, the commonly used diagram produced by the Centres for Disease Control and Prevention; the malaria life cycle involves two hosts, humans and the *Anopheles* mosquito (10).

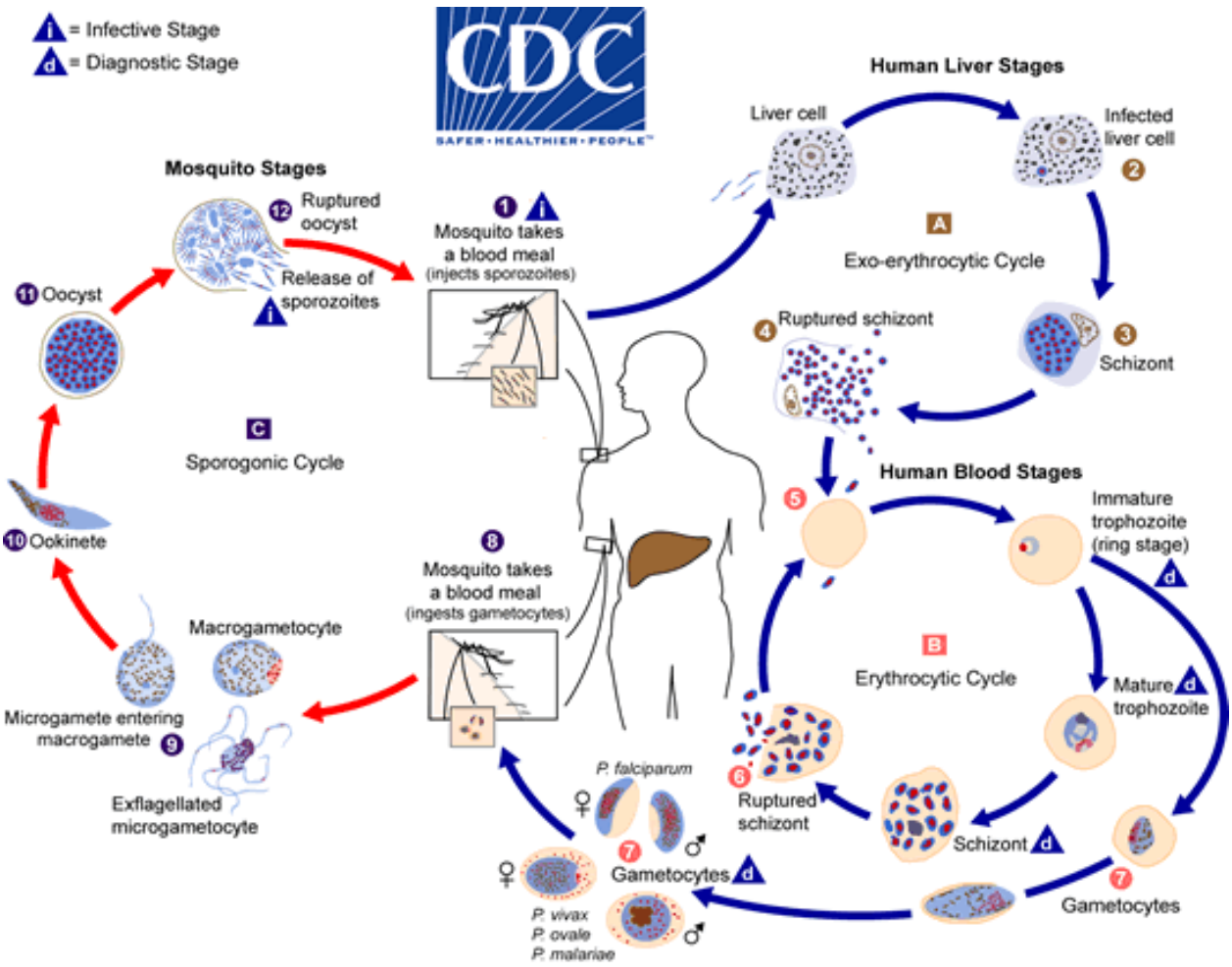


Figure 1: Life Cycle of Malaria

An infected female Anopheles mosquito inoculates malaria sporozoites into a human host. In the initial exo-erythrocytic cycle, these sporozoites infect the liver within 60 minutes and then mature into schizonts, which subsequently rupture and release merozoites (exoerythrocytic schizogony) which then infect red blood cells (erythrocytes). In *P. vivax* and *P. ovale* species, a dormant stage (hypnozoites) can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later. In erythrocytes, the merozoites then develop into ring stage trophozoites, maturing into schizonts which rupture and release merozoites (erythrocytic schizogony); completing the erythrocytic cycle. The species of malaria

differ in the number of merozoites formed and the time that is taken to complete their reproductive cycle.

In addition to the erythrocytic cycle, some blood-stage trophozoites differentiate into gametocytes, which form the start of the parasite's sexual reproduction. Gametocytes are ingested by an *Anopheles* mosquito during a blood meal, which then mature in the mosquito's stomach to form microgametes (male) and macrogametes (female). The microgametes penetrate the macrogametes generating zygotes. The zygotes turn into ookinetes which are motile and elongated. These invade the midgut wall of the mosquito where they develop into oocysts, which then grow, rupture, and release sporozoites. The sporozoites migrate to the mosquito's salivary glands where they are inoculated into the human host during an infective bite to complete the malaria life cycle.

The five species of malaria each have their own geographical distribution. *P. falciparum* is most prevalent in Africa and is the species responsible for the majority of the deaths (5). This is predominantly due to its ability to sequester in the microvasculature, where these occlusions play a role in the development of severe malaria (11). Sequestration is the adherence of infected erythrocytes containing trophozoites and schizonts to the endothelium of capillaries and venules (12). It is thought that the clinical presentation is due to the resulting anoxia and metabolic imbalances (13). Pregnant women, children, and immune-compromised individuals have the highest morbidity and mortality.

P. vivax has a different geographical distribution to *P. falciparum*, mainly due to its dormant liver stage and dependence on the Duffy antigen to invade erythrocytes (14). The hypnozoite

reservoir provides *P. vivax* protection from the immune system during cold seasons free from mosquitoes in areas of seasonal transmission. When higher temperatures return, onwards transmission returns due to re-activation. Although *P. vivax* can occur throughout Africa, the risk of infection with this species is quite low because of the absence in many African populations of the Duffy gene, which produces a protein necessary for it to invade red blood cells (15). *P. vivax* presents with uncomplicated malaria and can also cause severe malaria, but this is less common than in *P. falciarpum* infection.

Of the remaining malaria species, *P. ovale* and *P. malariae* present with the characteristic symptoms of malaria, but the clinical course is typically milder than *P. falciparum* malaria. *P. knowlesi* is predominantly found in SE Asia but is a zoonosis with no known human to human transmission (16). The sylvatic cycle refers to the part of the life cycle that occurs between wild animals and the vector. In *P. knowlesi*, the natural host is long-tailed macaques, which the Plasmodium parasite requires to complete its lifecycle. The reservoir of infection for this species of malaria resides in these animals. However, if inoculated by mosquitoes, humans can become an accidental, or dead-end host. The parasites cannot complete their life cycle, but humans will experience clinical symptoms. *P. knowlesi* replicates every 24 hours, meaning that patients can quickly become severely unwell (16). Until molecular detection methods were developed, it was often mistaken for *P. malariae* which was an issue due to its more severe clinical course (9). RDTs fail to detect *P. knowlesi* but novel diagnostics such as LAMP appear to be able to identify it (17, 18). Due to its short replication cycle, early aggressive treatment is required.

Immunity and Returning Travellers

The presentation of malaria differs in the endemic and non-endemic settings. Many studies have found that the risk of severe malaria is lowest in populations with the highest transmission intensities, as those individuals are exposed frequently so develop naturally acquired immunity (19, 20). Individuals also have increasing exposure with increasing age, so they also develop naturally acquired immunity. This helps to explain why children are more at risk of severe malaria and death (21).

Returning travellers reside in non-endemic areas but visit malarious countries, so are at risk of malaria due to a lack of immunity. The risk can be reduced by using personal protection and antimalarial prophylaxis (22). Individuals who are immigrants visiting friends and relatives (VFRs) typically perceive themselves at reduced risk (23), due to a mis-held belief of immunity and lack of antimalarial prophylaxis. Consequently, returning travellers and particularly those who are VFRs are more likely to be malaria positive than those traveling for alternative reasons (24).

Malaria diagnostics

There are numerous types of malaria diagnostic tests, each with its own standard operating procedure (SOP), limit of detection (LOD) and performance characteristics. Microscopy and rapid diagnostic tests (RDTs) have formed the mainstay of malaria diagnostics for a number of years, alongside polymerase chain reaction (PCR). However, loop-mediated isothermal

amplification (LAMP) and ultrasensitive RDTs (uRDTs) have been developed in recent years and pose a challenge to these traditional diagnostics.

Microscopy

Microscopy blood smears have long been used to diagnose malaria. This involves directly visualizing the *Plasmodium* species in the blood using a microscope. Blood is spread as a thick and thin blood smear, stained with Giemsa and then examined with the microscope. This method is fast, relatively easy to perform, low technology and allows timely identification of the species and parasitaemia which can help guide treatment. The limitations of microscopy are its high LOD and the lack of reliability in returning travellers. Microscopy has a LOD of approximately 50-100 parasites/μL (25-27). Data has shown that microscopy can miss around 50% of *P. falciparum* infections, particularly in areas with low transmission and in those with low-level parasitemias (28). In non-endemic areas that see cases involving returning travellers, laboratories will see only a limited number of cases each year, so technologists may lack expertise and the tests will lack sensitivity (29). Given these limitations, the Public Health Agency of Canada recommends three microscopy smears with at least eight hours intervals to rule out malaria (30).

RDTs

RDTs provide a useful alternative for malaria diagnosis and are designed to address some of the limitations of microscopy. They are lateral flow immuno-chromatographic antigen detection tests, where antibodies labelled with dye targeting specific antigens such as histidine-rich protein 2 (HRP2), lactate dehydrogenase (LDH) and aldolase (31). These antibodies produce a visible band on a strip of nitro-cellulose to allow visual detection (32). Of the most commonly used

antigens, HRP2 is produced by *P. falciparum* only, whereas LDH is produced by all species (32). This has led to the development of *P. falciparum* specific RDTs, pan LDH (all species) RDTs, or a combination of the two. To perform the test, a blood specimen is placed on the RDT sample pad, and then after approximately 15 minutes the presence of a specific band indicates whether the patient is infected with malaria (29). Depending on the type of test and antigen used, RDTs can differentiate *P. falciparum* from the other malaria species.

RDTs are particularly useful in LMICs, due to their ease of use. They are also useful in returning travellers, since expert microscopy might not be available at all hours. They have adequate sensitivity and specificity in symptomatic individuals (33, 34), but like microscopy have a high LOD for *P. falciparum*: 200 parasites/μL before they lose sensitivity (25-27). RDTs are vulnerable to HRP2 gene deletions, leading to false negative results. Since HRP2 gene deletions were first found in Peru in 2010 (35), other studies have found these in other areas including sub-Saharan Africa (36-43). The exact prevalence is unclear, but a national cross-sectional prevalence study from all areas of the Democratic Republic of Congo found a prevalence of 6.4% (42). Should these deletions continue to increase, it will provide a significant challenge for the continued use of RDTs.

PCR

Molecular techniques such as PCR are the gold standard in the malaria research setting. PCR can achieve a LOD < 1 parasite/μL, depending on the exact technique used (44, 45); achieving a sensitivity far above traditional methods, particularly in patients with a low parasitaemia. It requires extensive equipment and infrastructure, and may only be available at certain research

centres so it is not feasible to use in routine diagnoses. It a useful adjunct to traditional diagnostics in returning travellers, where it can be used if results are unclear or speciation is challenging.

uRDT

More recently, a uRDT has been developed with a similar workflow to current RDTs, but with improved sensitivity (46, 47). The LOD is thought to be as low as 3.13 parasites/μL (48), far superior to that of traditional diagnostics. However, this study in which this was completed did not reflect the typical field setting of malaria-endemic areas, so the field LOD is likely to be higher. While uRDTs have shown promise, as with regular RDTs, they are vulnerable to HRP2 gene deletions.

LAMP

LAMP is a relatively new technology (49), that has a proven high sensitivity due to its low limit of detection - below 1 parasite/ml (44, 45). It can achieve a diagnosis in about an hour, requires minor equipment which can be battery-powered, requires limited training and the cost is cheaper than for other molecular methods. Additionally, unlike RDTs, LAMP can detect all species of malaria and is not prone to HRP 2/3 deletions which lead to false negative results. LAMP has shown promise in detecting asymptomatic individuals with a low parasitaemia, with a sensitivity far superior to that of traditional diagnostics in recent studies (50).

Malaria treatment

The WHO treatment guidelines for malaria depend on a number of factors including the species of malaria, the severity, pregnancy status and the exact indication for treatment (51). Currently, the first-line treatment for the majority of malaria cases is based on artemisinin, with artemisinin-combination therapies (ACTs) for uncomplicated malaria and IV artesunate for severe malaria (52).

Artemisinin is a potent anti-malarial derived from the *qinghaosu* plant, which was discovered by Tu Youyou, a Chinese scientist. It was an important discovery in the malaria field because artemisinin is more efficacious than previous therapies, and also well tolerated (53, 54). Drug resistance has been found to all commonly used antimalarials including quinine, chloroquine, proquanil, sulfadoxine/pyrimethamine, mefloquine and atorvoquone (55). By combining artemisinin with a second, longer-acting partner drug, the resulting ACT was thought to reduce the probability of developing drug resistance (56). However, in 2009, resistance to artemisinin was discovered at the Thai-Cambodia border, historically a site of emerging antimalarial-drug resistance (57). Should artemisinin resistance spread to Africa, as it has with other antimalarials, this will bring significant mortality and morbidity and disrupt efforts against malaria elimination, with a large financial impact (58).

Due to the threat of artemisinin resistance, there is a need to develop novel antimalarials. Without new medications, significant barriers to control and eradication would remain (59).

NGOs such as Medicines for Malaria Venture (MMV) are attempting to facilitate the development of the next generation of antimalarials (52). However, the cost of drug development is extremely high, and the failure of potential candidates is common (60).

Malaria elimination

Since 2016, public health policy has shifted, in the form of the Sustainable Development Goals (SDGs), WHO Global technical strategy for malaria 2016-2030 (GTS) and the Roll Back Malaria advocacy plan from malaria control to elimination. For the elimination of malaria, there has to be an understanding of the malaria reservoir and how it contributes to the infectious reservoir. As the drive towards malaria elimination continues, an increasing proportion of malarious areas will be of low endemicity. As an area moves towards low endemicity, the proportion of cases with a submicroscopic parasitemia increase (61). It is thought that in low transmission areas, submicroscopic individuals are responsible for 20-50% of all onward transmission. Additionally, in a recent mosquito membrane feeding assay study, asymptomatic carriers contributed to the vast majority of onward transmission to mosquitoes (62). However, these cases with a low parasitemia provide a diagnostic challenge (63). Data has recently shown that traditional diagnostics may underestimate the true burden of malaria, with molecular techniques often required to detect asymptomatic infections (61). However, further studies are required to determine the optimal diagnostic tool for malaria elimination.

CHAPTER 2: EPIDEMIOLOGICAL AND BIOSTATISTICAL METHODS IN CLINICAL RESEARCH

Introduction

The fields of epidemiology and biostatistics are closely intertwined in clinical research. In this chapter, general background information regarding epidemiology and biostatistics relevant to this thesis is discussed.

Epidemiology is the study of the distribution and determinants of disease in populations, and the application of this knowledge to control health problems (64). Distribution refers to the time, place and the subgroups of persons affected in a population, while determinants are the physical, biological, social, cultural, economic and behavioural factors that influence health (65). Descriptive epidemiology focuses on the distribution of disease while analytical epidemiology focuses on the determinants of disease. Biostatistical skills are used in collaboration with epidemiology to ensure a high-quality study and are predominantly required when developing study objectives, hypothesis identification, study design, sample size calculation, and statistical analysis.

The basics of epidemiology

Epidemiology relies on the understanding that diseases and illnesses do not randomly distribute within a population (66). Instead, they distribute depending on a variety of genetic and environmental factors. An epidemiological study using a simple random sample provides us with

data on a population. A simple random sample is arrived at by selecting sample units such that each possible unit has a fixed and known or equal probability of selection (65). This therefore provides a good representation of the population, so it allows us to understand the distribution of disease in that population and the relationship between exposures and outcomes.

Epidemiological parameters

Prevalence and incidence form the basis of many epidemiological parameters used to measure the effects of exposure and disease in a population. Incidence is the number of instances of illness commencing, or of persons falling ill, during a given period in a specified population (65).

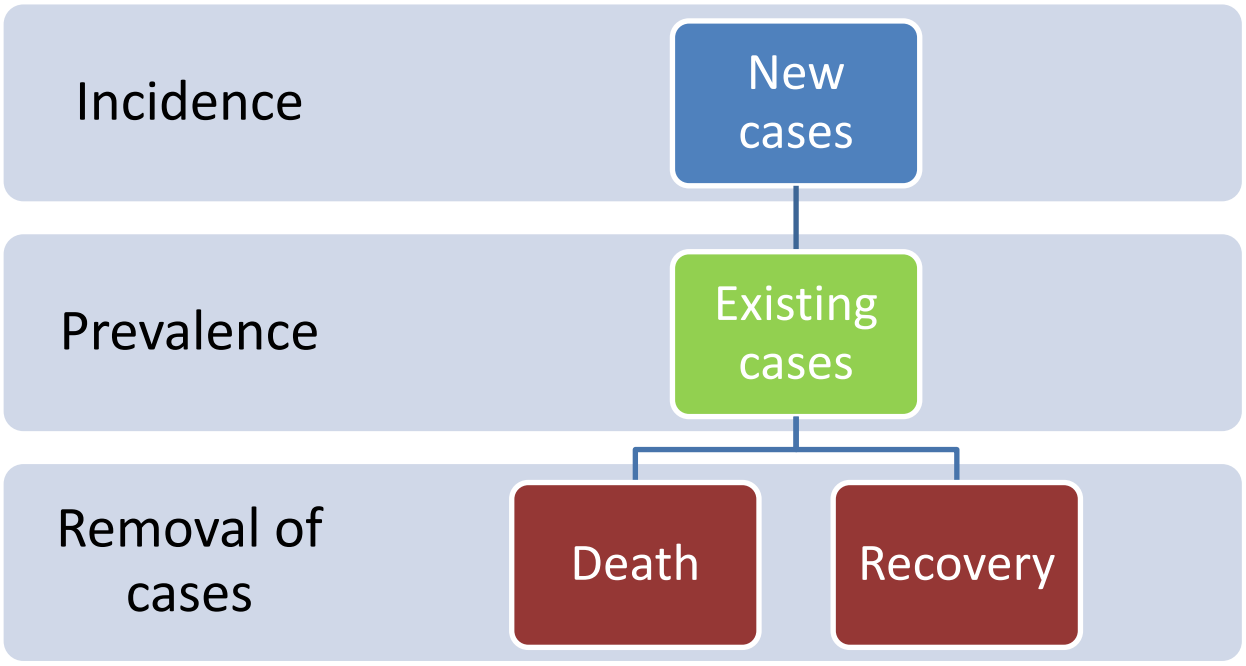


Figure 2: The relationship between incidence and prevalence

It can be reported as a frequency, a rate or a proportion. Prevalence is a measure of disease occurrence: the total number of individuals who have an attribute or disease at a particular time divided by the population at risk at that time (65). Prevalence can be calculated at a specific time (point prevalence), over a specified period (period prevalence), or over a lifetime (lifetime prevalence) (65). This parameter is often used to describe the proportion of individuals in a population who have an outcome of interest (67). Whereas prevalence quantifies how many people have a disease, incidence quantifies the development of new cases of a disease. The relationship between incidence and prevalence is modified by the duration of disease, which depends on the balance between the new cases and either the recovery or death from these cases.

Risks and Odds

The outcome measure reported typically depends on the study design (68). In cross-sectional studies, prevalence is a commonly used outcome and is expressed as a proportion (number of events divided by the total population at risk at a specific point or period in time), but it can also be expressed as odds (number of events divided by the number of non-events). The relationship of prevalence between the two groups is known as the prevalence ratio and the prevalence odds ratio. In cohort studies, risk is the most normal outcome measure. Risk refers to the probability of a new event occurring in a particular time period for those at risk of an event occurrence. This is typically expressed as a risk ratio, where the relative probability of an event occurring among exposed and unexposed individuals is characterized. Finally, if the denominator is not known, such as for a case-control study where the true population prevalence cannot be calculated, an odds ratio is used. Odds can be estimated from proportions using the following equation:

$$Odds = \frac{proportion}{1 - proportion}$$

Use of biostatistics in epidemiological research

Biostatistics are predominantly used in the field of analytical epidemiology. With analytical research, the author is trying to identify the relationship between determinants and disease. For this to be possible, statistical tests must be used to determine if associations are due to chance, or if they represent a true relationship. Consequently, biostatistics are predominantly required at the stages of epidemiological research that include developing objectives, hypothesis identification, study design, sample size calculation, and statistical analysis.

Study objectives

The identification of study objectives occurs at an early stage of the research process, often when devising a research protocol and applying for grants. Identifying potential objectives first requires the researcher to be familiar with the literature related to the field (69). New developments in translational research rarely occur due to a moment of inspiration, and instead, reflect a gradual process of progressing ideas. An excellent understanding of the physiological processes, basic research and epidemiology is required, as this allows the investigator to ask the right questions. The objectives of the research can then be defined, which ideally should be specific in nature and defined prior to any research taking place. One commonly used framework originally adapted from work in business is SMART (Specific, Measurable, Achievable, Realistic and Time-Framed) (70). These specific objectives make it easier to evaluate whether they can and are being achieved.

Hypothesis Identification

The identification of the study objectives allows for the development of a clear research hypothesis. Research should be driven by a clear identification of a null hypothesis (71), to ensure that statistical tests can be applied (72). By assuming that there is no association, statistical tests can estimate the probability that an observed association is due to chance (73), thus allowing for easier identification of type I and II errors. Again, the research hypotheses should be identified well in advance of any data collection, to ensure that bias is minimized.

Study design

The design of both observational and interventional studies is considered at length in this chapter, but specific considerations must be given with respect to biostatistics. The selection of study objective is part of the study design process, and study endpoints should be selected that are able to answer the scientific question being asked (72). The study endpoints should also be set with respect to the null hypothesis to provide evidence to accept or reject them. Commonly, these are either continuous in nature, categorical, ordinal or event-time endpoints. The study design and type of endpoint gathered guides the type of statistical tests that can be performed.

Power and Sample Size

One of the key considerations when developing a study protocol and design is giving thought to the sample size and power. Power is defined as the ability of the study to demonstrate an association if one exits (65). A study needs to be adequately powered to ensure an association is

demonstrated, and one of the key ways in which this can be achieved is by careful selection of the sample size. If the sample size is too small, then the study may not find an anticipated association; while if it is too large, it would be wasteful of resources that are often in short supply.

Components of a sample size calculation

To ensure a study is adequately powered, or to calculate the sample size; a number of different factors need to be considered. These include type I and type II errors, the effect size and the variability of the sample.

A type I error (alpha) is the probability of rejecting the null hypothesis even though it is true. This is most commonly set to a probability of 0.05 (74), and it is the first step in conducting a power calculation. Then the probability of a type II error (beta) is set, which is the probability of accepting the null hypothesis even though it is false. This is most commonly set to 0.1 or 0.2, and given that power is 1-beta; the power would be 0.8 or 0.9 (75).

Additionally, estimates of the effect size should be made, most commonly based on previous literature. This can also take the form of a minimally clinically relevant difference between the two groups (e.g. a 5% difference in malaria positivity or 10mmHg difference in blood pressure). Generally speaking, the smaller the difference between two groups, the larger the sample size required. Finally, the variability of the sample is important and must be estimated or assumed.

Following the calculation or estimation of these parameters, statistical software can be used to calculate a required sample size to achieve a specific power. Alternatively, the power from a specific sample size can be calculated. This process should be done in advance of data collection to ensure an adequate sample size is obtained to answer the study questions and hypotheses previously set out.

Statistical analysis

The statistical analysis will often include significance tests and the estimation of confidence intervals (76), providing information as to whether the rejection of the null hypothesis may be appropriate. The exact method used to calculate this depends on a number of different factors. Briefly, it will depend on the question to be answered, the type of data, the number and type of comparators, and the distribution of the data (77). A full discussion of all statistical methods is beyond the scope of this thesis, but a discussion of the statistics utilized in my manuscripts is found in chapter 5.

Evidence-based medicine

Without the use of evidence-based medicine to guide clinical practice, experts can make incorrect recommendations about the effects of interventions. Experts used to recommend bed rest following a myocardial infarction, but when a review of the evidence was performed, these recommendations were found to be out-dated and based on limited evidence (78). Consequently, evidence-based medicine has developed to provide evidence for clinical practice. However, the

quantity of scientific literature is vast, with significant variations in the quality of study design and statistical analysis.

Critical appraisal

Ever since Gordan Guyatt first formalized the concept of evidence-based medicine in the early 1990s, it has formed the basis of clinical decision making (79). Significant skills in critical appraisal are required to ensure that correct conclusions are drawn from the literature, particularly when making decisions on clinical practice. Part of the critical appraisal process involves identifying sources of error or bias. Systematic errors typically occur due to the study design and favour one study outcome over another (80), whereas random errors occur due to chance. Epidemiologists should be able to critically appraise papers, identify specific types of bias, and understand how they affect conclusions about epidemiological associations or interventions.

The reliability of evidence depends on several aspects, including the study design, risk of bias, appropriate use of statistics and the correct reporting of studies in the literature (81). Various techniques and methodologies exist to attempt to improve the quality of evidence used to make recommendations for clinical guidelines (82). The STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) checklist exists to help improve the methodology of reporting observational studies (83). The Grading of Recommendations Assessment, Development, and Evaluation (GRADE) methodology attempts to rate the quality of evidence used to make recommendations for clinical guidelines (82). Other alternative and commonly used critical appraisal methods include those developed by the Critical Appraisal

Skills Programme (CASP) (84) and the Centre for Evidence-Based Medicine (CEBM) (85). While there are many different methods of critically appraising literature, most follow quite similar principles and are loosely based on the original JAMA 'Users' guides to the medical literature that were published in the early 1990s (86, 87). Many of the key considerations are discussed below, based on the structure described in the book, 'Epidemiology for Canadian Students (64).'

Identify the stated objective

A good study design requires a clearly defined question that needs to be answered. A clearly defined question allows specific study endpoints or hypotheses to be formulated, and statistical tests can be utilized to test these hypotheses. Without a specific question or objective, the study is more vulnerable to type I errors.

Identify the exposure and diseases variables

To understand whether the study can answer its stated objective, the exposure and disease variables should be clearly defined. Ideally, these should be pre-defined in advance of data collection, with a study ideally registered with a clinical trial database (88). This again ensures that the study is less vulnerable to type I errors because exposures and outcomes cannot be easily changed after data collection or analysis has begun.

Classify the study design

Studies can either be observational or interventional in nature. It can often be quite challenging to identify the study design of observational studies, but by identifying the exposure and outcome variables, one can ascertain the study design.

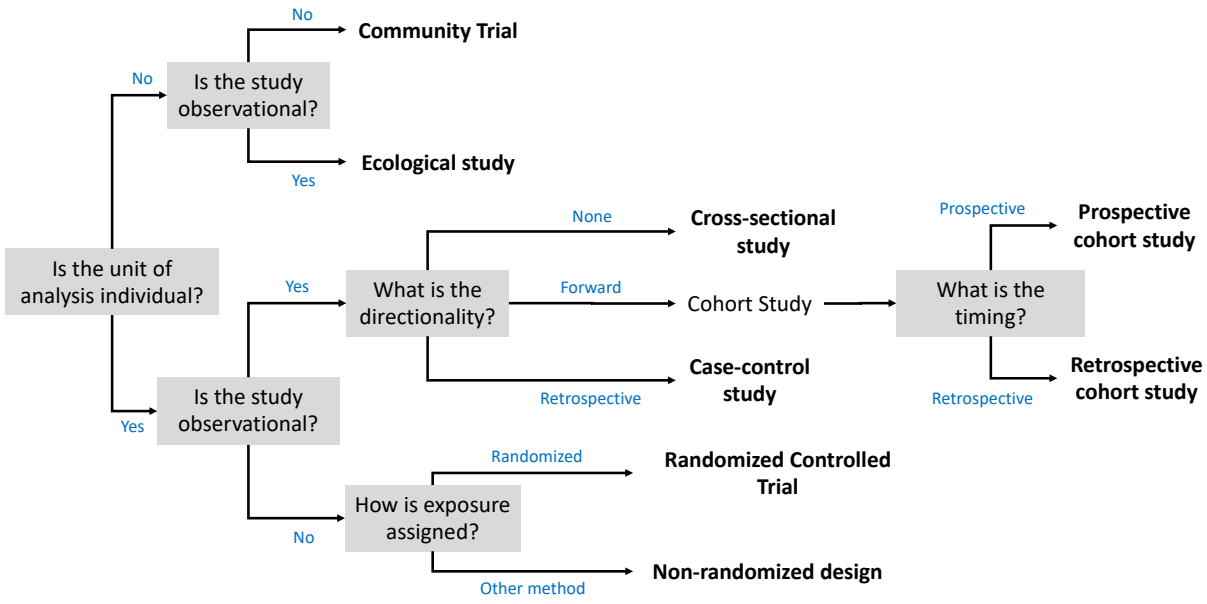


Figure 3: Classification of clinical studies

Figure 3 is adapted from a book by Patten (64), and it demonstrates how to identify study design relatively easily based on four key questions. Study classification starts with the unit of analysis which is either a group or an individual person. It should be classified as individual if there is data on individual participants. Following this, classification depends on whether a study is observational or interventional in nature. For observational studies, the directionality of the relationship between exposure and outcome is key for determining the study type (89):

- Cross-sectional study – the exposure and outcome are measured at the same point in time. The study therefore has no directionality.

- Case-control study – participants are selected retrospectively when the exposure and outcome have already occurred. The outcome is the starting point for the study, with the exposures (or risk factors) then examined, so the study has backwards directionality. Participants with the outcome are the starting point for the study, and individuals without the outcome are then selected. The exposures (or risk factors) in both groups are then examined so the study has backwards directionality.
- Cohort study – the exposure is the starting point for the study, with participants then followed up to see if an outcome occurs. The study has forwards directionality. This process can be done either prospectively (following participants up over many years) or retrospectively (obtaining all the data for a forward directionality study but actually examining this data after the outcome has already occurred). Directionality refers to study design, whereas timing refers to when the study data is obtained.

Randomized controlled trials

Interventional studies are also known as clinical trials, with randomized controlled trials (RCTs) representing the gold standard of interventional study design. They are used to study the effect of interventions, and most commonly used medications. They have a longitudinal design, with participants typically randomized to receive the study drug or a comparator. The comparator can be either placebo or other treatment regimens depending on ethical concerns. This allows investigators to ascertain the effect of a particular intervention.

A crossover study design is longitudinal in nature, and it can be used for interventional trials (90). In a traditional RCT, parallel groups are randomized to a particular treatment or

intervention and are followed up for the study period to investigate an outcome of interest. In a crossover design, groups are initially randomized to a particular intervention or placebo, but then switched to another intervention after a pre-defined time period.

Classification of study design allows for easier identification of bias as part of the critical appraisal process. As will be discussed later, particular types of clinical studies are prone to specific types of bias, which are intrinsic to study design. This has led to a hierarchy of evidence shown in Figure 4 (91). Certain study types provide evidence that is superior in quality to others, with each type having advantages and disadvantages unique to their design (92).

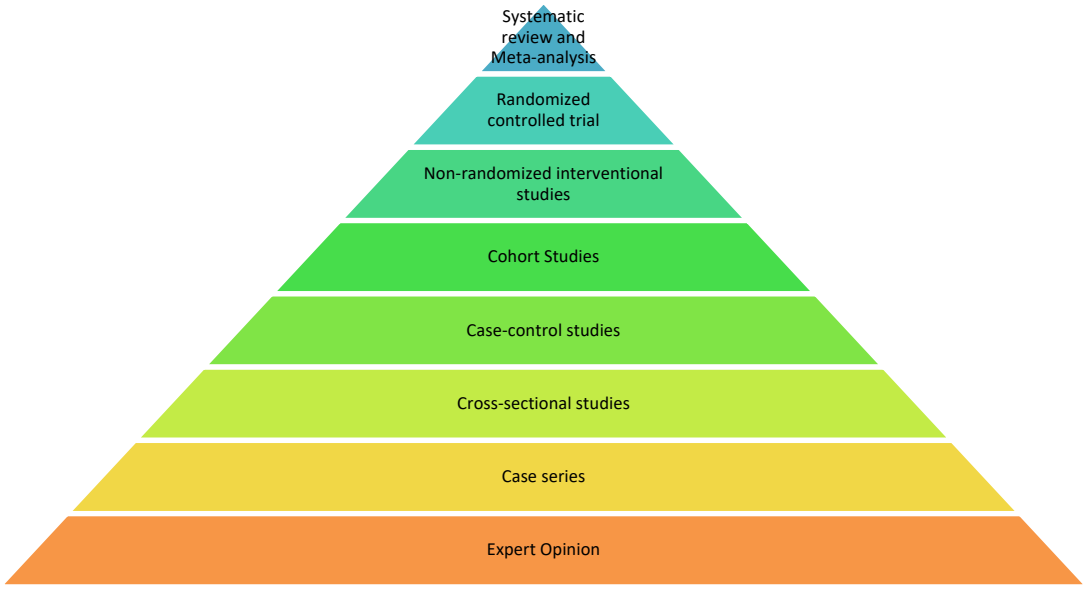


Figure 4: Hierarchy of evidence

Selection bias

Selection bias is a type of systematic bias that occurs from the method used to select study participants (65, 93). For a study to be free of selection bias, careful consideration must be given to the sampling process, so that individuals with a particular exposure of interest have an equal selection probability of participating in the study as those that do not. Other key sources of selection bias are attrition (participants becoming lost to follow-up) and missing data. These are particularly an issue when it comes to longitudinal cohort studies and randomized controlled trials, which can have long follow-up periods.

Since selection bias is a type of systematic bias, a sound study design is required to minimize its impact. If the sampling frame is known, then simple random sampling can be utilized to ensure that there is an equal probability of both exposed and unexposed individuals being enrolled. Other potential sampling methods that could be used include stratified sampling. In case-control studies, it is important to ensure that the participants selected to be in the control group are representative of the population, otherwise selection bias can occur (94).

Misclassification bias

Misclassification bias is another type of systematic error, that occurs due to the incorrect classification of exposure and outcome (95, 96). It can occur for a number of different reasons, including subjective definitions and the use of poor-quality measurements and diagnostics.

Misclassification bias can be differential, where it varies between study groups or non-differential, where the misclassification is consistent across all groups. Non-differential bias

classically biases parameter estimates towards the null, whereas differential bias will bias parameter estimates away from the true population value, therefore over or underestimating any effects.

To understand the direction of differential misclassification bias resulting from inaccurate diagnostics, one must understand the concepts of sensitivity and specificity. A perfect diagnostic test would be able to identify accurately all individuals with, and without, a disease. However, in reality very few diagnostic tests are able to do so, with false positives and false negatives also occurring. Sensitivity is the proportion of people with the condition correctly identified by the test, while specificity is the proportion of people without the condition correctly identified by the test. Therefore, if a test lacks sensitivity, it will tend to mistakenly classify individuals with a disease as not diseased and disease prevalence estimates will be too low. If a test lacks specificity, it will tend to mistakenly classify individuals without a disease as diseased and disease prevalence will be overestimated. This misclassification bias is of particular importance in studies of diagnostic tests.

Confounding

Confounding is a type of systematic error that occurs when the all or part of an association of an exposure and outcome is in fact due to an association of the exposure with other factors that influence the occurrence of the outcome (65). A confounding factor is an extraneous variable not on the causal pathway; this relationship is best demonstrated by a diagram:

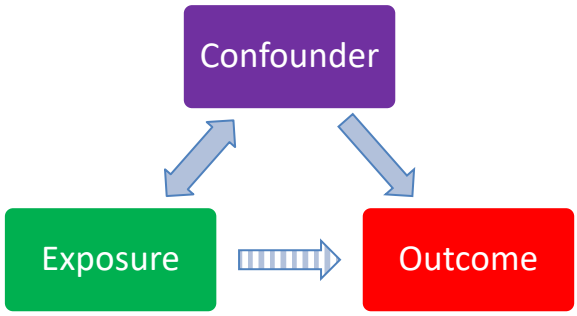


Figure 5: Effect of a confounding factor

For a confounding factor to introduce bias, it must be unequally distributed between the two study groups. Confounding can be eliminated or reduced at the study design stage, such as through randomization which makes two comparison groups similar by equally distributing the confounders between the two groups. Other potential methods that achieve similar aims include restriction and matching. Alternatively, statistical analysis techniques such as stratification and regression can be used to adjust for confounders following data collection (97).

Effect modification

In effect modification, extraneous variables modify the effect of an exposure, and can influence the conclusions drawn by studies. An exposure can have a different effect among different subgroups. In effect modification, the effect in different groups must be reported individually because they represent the real association between exposure and outcome so cannot be adjusted for. If the stratum specific estimates for two groups are similar, the effect is homogenous for the group; if the estimates are different they are termed heterogeneous (95).

Role of chance

Chance is a type of random error that can be assessed by using statistical tests. Critical appraisal focuses on the vulnerability of the study to type I or type II errors, depending on the study findings (64). Type I error refers to rejecting the null hypothesis when it is true, while a type II error refers to a failure to reject a false null hypothesis.

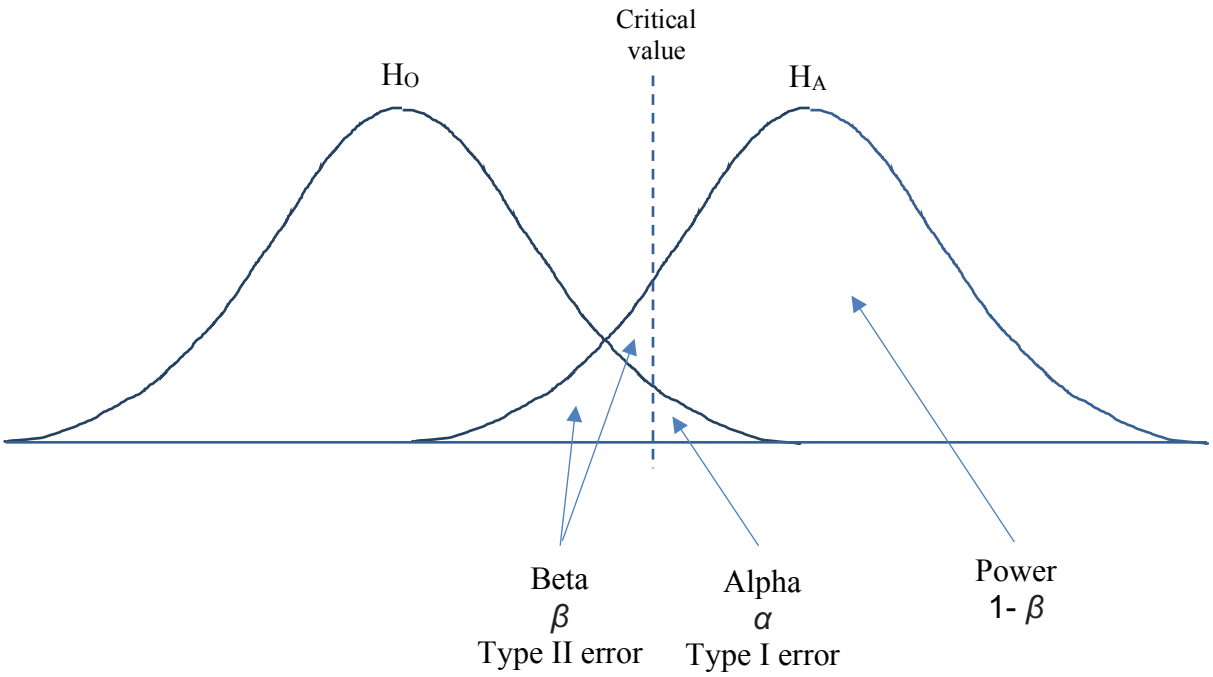


Figure 6: Diagram of hypothesis testing

If statistical tests report a significant result, concern over a type I error arises. With a standard α value of 0.05, then there a 5% probability of rejecting the null hypothesis when it is true. However, this is only the case when there is a single well-defined outcome. The chance of a type I error is fixed for each statistical test (typically 5%), so if multiple tests are performed, the total probability of a type I error increases with each subsequent test because it is 5% for each

statistical test performed. Various techniques can be used for multiple test adjustments including the Bonferroni method (98).

If the study results are not significant, then the chance of a type II error is a concern (failure to reject a null hypothesis when it is false). This raises the question of whether the study is adequately powered. However, the role of chance only applies when there is limited evidence of systematic bias. When this is present, even a large sample size and narrow confidence intervals are unlikely to produce a reliable estimate of the population parameters.

Causation

Previously, the consideration of systematic and random error has been discussed. However, one must also consider the issue of causality when critically appraising clinical studies. Causality refers to whether the evidence obtained from the study proves that the exposure leads to the outcome in the population. This will depend on a number of factors including study design and the data acquired. In 1965, Sir Austin Bradford Hill published nine observations to help determine whether epidemiological relationships are causal (99). Since then, these guidelines have become the gold standard to establish causal inference in epidemiological studies (100). By using the criteria shown in the table below, one can critically appraise whether there is evidence of causality.

Table 1: The Bradford-Hill Criteria

Criteria	**Modern explanation**
Effect size	While a small effect does not preclude causality, larger effects are more likely to imply it.

Reproducibility	If multiple studies find the same effect, causality is more likely
Specificity	A single exposure causes a specific effect
Temporality	The exposure precedes the outcome
Biological gradient	The is evidence of a dose-response relationship with increasing exposure increasing the risk or severity
Plausibility	There is a plausible biological mechanism to explain the results (not essential due to limitations in understanding
Coherence	The association is coherent between epidemiological and laboratory findings
Experiment	The association can be altered or prevented by appropriate interventions
Analogy	Analogous associations between similar factors and diseases

Generalizability

Generalizability is the degree to which results of a study may apply, be relevant, or be generalized to populations or groups that did not participate in the study (65). It may be limited by the genetic or cultural make-up of the participants of the study sample. For example, the results of a study on hypertension conducted in a Caucasian population in North America and Europe may not generalizable to an immigrant population or to Africa, as a black population are at higher risk of developing hypertension (101). Furthermore, the diets in the two populations are likely to be different which further complicates interpretation. Additionally, in regards to healthcare, the funding model and spending on the health system is extremely important, as studies performed in a private health system may not be generalizable to a public system.

Study specific strengths and weaknesses

The different types of studies described are vulnerable to specific types of bias or have particular strengths. While these can vary depending on individual study design, some consistent themes remain throughout.

Ecological studies

In ecological studies, the unit of observation is at the population level. This means that such studies can be performed cheaply and quickly, studying a large number of variables and can be used to generate epidemiological hypotheses. However, since ecological studies do not obtain data on an individual level, they are extremely vulnerable to confounding. The ecological fallacy describes how apparent associations between groups may not accurately represent associations between individuals in the group.

Cross-sectional studies

For cross-sectional studies, data collection occurs at a specific point in time, providing useful information on disease prevalence. The relationship between exposures and outcome can be examined by using prevalence ratios or prevalence odds ratios. Strengths of cross-sectional studies include their efficiency (multiple variables at low cost) and the fact they are not vulnerable to attrition like longitudinal studies. Their main weakness is that they are easily susceptible to confounding and cannot measure incidence. In addition, they cannot demonstrate causality, meaning that follow-up studies are required.

Case-control studies

In case-control studies, the outcome has already occurred at the time the study is being conducted. Data on exposure is collected retrospectively, with a group of controls being chosen following the recruitment of cases. Odds ratios are commonly used as the statistical outcome measure, and this approaches the risk ratio when the disease prevalence is low. Case-control

studies can examine multiple exposures and rare outcomes, and are useful if there is a long latent period for the diseases because they are conducted retrospectively. However, it is difficult to demonstrate a temporal relationship, and therefore causality. Furthermore, they are prone to selection, misclassification and recall bias. As the study sample does not reflect the population, case-control studies cannot be used to estimate prevalence or a probability of an event occurring.

Cohort studies

In cohort studies, the starting point is participants with and without the exposure of choice, with participants followed up and the outcome rates between the two groups compared. The outcome measures are typically risk ratios or rate ratios, but odds ratios can also be used. Cohort studies are particularly good at demonstrating a temporal relationship and are less vulnerable to selection bias. They can also directly measure incidence and are useful for rare exposures. However, cohort studies are time consuming and expensive, with participants often lost to follow up due to the time frames involved. Cohort studies can be carried out retrospectively if adequate records are available. The main limitation is that the retrospective approach limits questions to those where data already exists, and data may not be available for all variables of interest, or may only be available in a biased manner (102).

RCTs

In RCTs, the randomization process helps distribute confounding factors equally between the study arms. Blinding can be implemented to help minimize selection and misclassification bias. RCTs provide the best evidence of causality and are the least vulnerable to bias. However, these trials require extensive resources to plan and carry out. A crossover RCT provides all of the

advantages of traditional parallel RDTs, but each study participant is in all study groups so can act as their own control. This reduces confounding and means that a paired statistical analysis can be carried out. This reduces the study size required to achieve sufficient power (103).

Application of epidemiology and biostatistics to manuscript

In this chapter, the key basic theories relevant to epidemiology and biostatistics in clinical research have been discussed. The application of these skills to my manuscripts is discussed in chapter 5.

CHAPTER 3: CLINICAL VALIDATION OF A COMMERCIAL LAMP TEST FOR RULING OUT MALARIA IN RETURNING TRAVELLERS: A PROSPECTIVE DIAGNOSTIC TRIAL

Background

In 2016, there were approximately 216 million cases of malaria in 91 countries, resulting in 445, 000 deaths (104). The World Health Organization (WHO) African Region bears the largest burden, with around 90% of the malaria cases. The WHO guidelines recommend that all suspected cases of malaria be confirmed with microscopy and/or rapid diagnostic tests. In Canada, there are on average 488 malaria cases per year across the country (105), and approximately 1700 annually in the United States (106). However, in nonendemic settings such as North America, the vast majority of tests performed are negative.

Globally, the diagnosis of malaria is achieved with microscopy and rapid diagnostic tests (RDTs), or a combination of these. The WHO estimates that around 204 million patients were tested by microscopy and 269 million rapid diagnostic tests were sold in 2016 (107). Although the increasing use of diagnostic-based treatment rather than symptom-based treatment undoubtedly has significant benefits, it is not without limitations. Microscopy has been found to have a limit of detection (LOD) of 50–100 parasites/μL under field conditions (25, 26). However, this can only be achieved when laboratory facilities are available, along with a specialized microscopist. Studies have found that the LOD varies depending on the training and experience of the microscopist (108). The method is labor-intensive and requires constant quality

assurance. Due to these limitations, and therefore the possibility of false negatives, the Centers for Disease Control and Prevention (CDC) in the United States and Public Health Canada both recommend that 3 malaria slides be taken (30, 109). This is not just an inconvenience to the patient, who may have to return the emergency room several times; it results in increased health care costs due to re-attendance and increased laboratory time.

Although RDTs have demonstrated a sensitivity and specificity >90% for *Plasmodium falciparum* infections with >200 parasites/ μL, they cannot reliably diagnose cases with parasitemia below this level (27). Additionally, false-negative RDT results can occur from parasites that do not produce histidine-rich protein 2 (HRP2), a growing concern in malaria-endemic regions (35, 43, 110). Molecular testing such as polymerase chain reaction (PCR) can achieve an LOD of 1 parasites/μL with improved sensitivity, but these tests are expensive and simply not feasible without a well-equipped laboratory and expertise (44).

Given the limitations with the current testing methods of microscopy, RDTs, and PCR, there has been interest in validating simpler nucleic acid tests (NATs) such as loop-mediated isothermal amplification (LAMP) (111-115). LAMP is a technology that was developed in 2000 that uses unique DNA polymerases and specially designed primers to amplify nucleic acid without thermocyclers (49). LAMP can differentiate species, and variations can even be used to detect the C580Y mutation in the propeller domain of the kelch 13 gene, the most prevalent marker of artemisinin resistance (116). The LOD varies slightly depending on the exact protocol used, but it can achieve limits of detection of ≤2 parasites/μL, similar to those achieved by PCR and

surpassing those of microscopy and RDTs (117-120). The *illumigene* Malaria LAMP assay (*illumigene* M; Meridian Biosciences, Cincinatti, OH) has shown good sensitivity and specificity vs microscopy, the current gold standard (111). It performs well in terms of technological simplicity and cost. Commercial LAMP technology has been assessed against PCR in a number of studies, with extremely encouraging results (Table 2) (111-114, 117, 120-126). Given the excellent negative predictive value properties of LAMP in malaria, our group has proposed replacing the current testing algorithm, which relies on 3 microscopy tests to rule out infection, with a single LAMP test (111). This would improve testing turnaround time, optimize use of the emergency room, and reduce cost to the health care system.

Table 2: Commercial LAMP performance against PCR

Paper	**Commercial kit**	**Specimen Analysis[b]**	**Location**	**Sensitivity (95% CI)**	**Specificity (95% CI)**
Aydin-Schmidt 2014	Eiken	Retrospective	Zanzibar – pre-elimination	91.5 (84.8 - 95.8)	100 (99.5 - 100)
Hopkins 2013	Eiken	Prospective	Uganda – high endemicity	90.0 (85.0 - 94.0)	85.0 (75.0 - 92.0)
Lucchi 2016	Illumigene	Prospective and retrospective	Senegal – low endemicity	97.2 (92.6 - 99.1)	93.8 (84.2 - 98.0)
Marti 2015	Eiken	Prospective	Switzerland – returning travellers	100 (92.4 - 100)	100 (97.7 - 100)
Polley 2013	Eiken	Prospective	UK – returning travellers	97.0 (89.6 - 99.6)	99.2 (98.1 - 99.7)
Ponce 2017	Illumigene	Prospective	France – returning travellers	100 (95.8 - 100)	98.1 (95.3 - 99.5)
Rypien 2017	Illumigene	Retrospective	Canada – returning travellers	97.3 (90.7 - 99.7)	93.8 (84.8 - 98.3)
Sema 2015[a]	Eiken	Retrospective	Ethiopia – moderate endemicity	96.8 (83.2 - 99.5)	84.3 (71.4 - 92.9)
Tegegne 2017	Eiken	Retrospective	Ethiopia – moderate endemicity	100 (100 - 100)	93.5 (86.5 - 100)

Abbreviations: CI, confidence interval; LAMP, loop-mediated isothermal amplification; PCR, polymerase chain reaction.
[a]Field values taken.
[b]"Prospective" defined as blood specimens tested within 72 hours without preservation; others considered "retrospective".

On a global note, if elimination is to be achieved, a diagnostic tool with a lower limit of detection is required. A recent study modelling eradication showed that reducing the limit of detection of a diagnostic test increased the detection of the asymptomatic infectious reservoir (127). LAMP

meets this criterion, and several studies have described the role it could play in malaria elimination (27, 120, 121, 128, 129).

Although LAMP has been shown in studies to surpass microscopy and RDTs, many studies to date have been retrospective in nature. In addition, studies have often focused on *P. falciparum* rather than all species of malaria. Our study was designed to closely reflect testing scenarios in North America. A single-center evaluation of LAMP performance against microscopy for all species of malaria in a major urban center (1.4 million people) was conducted. The primary objective of the study was to compare the clinical performance of a commercially available LAMP method (*illumigene M*) vs RDT (BinaxNOW) compared with microscopy in symptomatic patients presenting as potential cases of malaria in a nonendemic area. The secondary objective was to determine if certain clinical and epidemiological factors predicted malaria test results using regression analysis.

Methods

Study design, patient enrolment, and ethics.

The study design was pragmatic, containing both prospective and retrospective arms to provide an adequate distribution of all species of malaria. In the prospective arm, adult participants who underwent malaria testing were consecutively enrolled into the study from June 2017 until January 2018. Based on sample size calculation, a goal of 350 specimens was set with at least 10% overall positivity for all species of malaria. To achieve this, testing was performed on a continuous basis with interruptions only to ensure that enough positive samples were tested. Samples arriving at night were tested first thing in the morning by medical laboratory

technologists using the LAMP technology. All prospectively enrolled participants had epidemiological data collected through a case history form that accompanied the specimens. Variables recorded included whether pretravel advice was received, reason for travel, country visited, symptoms, severity, whether the participant took antimalarial prophylaxis, and whether they were on treatment. In addition to the prospective arm of the study, 29 microscopy confirmed malaria specimens were retrospectively collected and included in the study. This design has been used by other studies, and it allows an adequate sample size for rare non–*P. falciparum* species (130). Without this, due to the otherwise prospective nature of the study, only a small number of positive specimens for these species would have been included. Five *Plasmodium vivax* and 5 *Plasmodium ovale* frozen whole-blood specimens (at –80 °C) were obtained from a large teaching hospital in Edmonton, Canada. Thirteen *Plasmodium malariae* specimens were frozen whole-blood specimens obtained from Par., Brazil, and 6 culture-derived *Plasmodium knowlesi* (A1-H.1 clone) specimens of varying parasitemia (range, 0.1–10 000 parasite per μL) were obtained from London, United Kingdom. Retrospective specimens were thawed and underwent the same LAMP testing protocol as the prospective arm. No epidemiological data were available on retrospective specimens. Ethical approval was obtained from the Conjoint Health Research Ethics Board (REB17-2220), and privacy review from Alberta Health Services.

Routine laboratory testing for malaria

Venous whole-blood samples were collected in EDTA vacutainers from medical centers in Calgary and the surrounding area and transported to Calgary Laboratory Services (CLS). CLS is a large centralized public microbiology laboratory service covering 1.4 million people. Samples from malaria-positive patients who were initially symptomatic, treated, and then retested were

included in the study. Each specimen was tested according to standard operating procedure: Giemsa stained thick and thin peripheral blood smears and RDTs (BinaxNOW Malaria, Alere, Waltham, MA) on study participants. For the purposesof analysis, microscopy repeated 3 times from the same individual to confirm negativity was classified once only. Microscopy, RDTs, and LAMP testing were conducted in real time by the same technologist who was trained in all diagnostic tests due to personnel availability.

Malaria testing with illumigene M LAMP

illumigene Malaria (Meridian Biosciences, Cincinatti, OH) is a commercially available, loop-mediated amplification test that detects all species of human malaria. The assay provides a positive or negative result only and does not speciate. Medical laboratory technologists received training for the LAMP assay. Testing using LAMP occurred simultaneously with current standard operating procedure (SOP) for the duration of the study. Specimens received at night were tested by LAMP in the day shift due to technologist availability. LAMP was performed according to the manufacturer's recommendations.Complete standard operating procedures for *illumigene* are available online (http://www.meridianbioscience.eu/media/pdf/Package%20Insert/280925_281125_MULTI_REV1215.pdf). Samples were frozen once at –80°C for subsequent real-time PCR (RT-PCR) analysis, which was performed off-site in a blinded manner.

Discrepancy resolution

Discrepant results occurred when the results of microscopy and the LAMP assay results were different. All microscopy-positive and discrepant specimens underwent RT-PCR testing at an off-site location at the Provincial Reference Laboratory in Edmonton, Canada, in a blinded

manner. The RT-PCR method was clinically validated and used routinely at the reference laboratory (131). Concordant negative specimens were also tested by RT-PCR in Calgary, Canada.

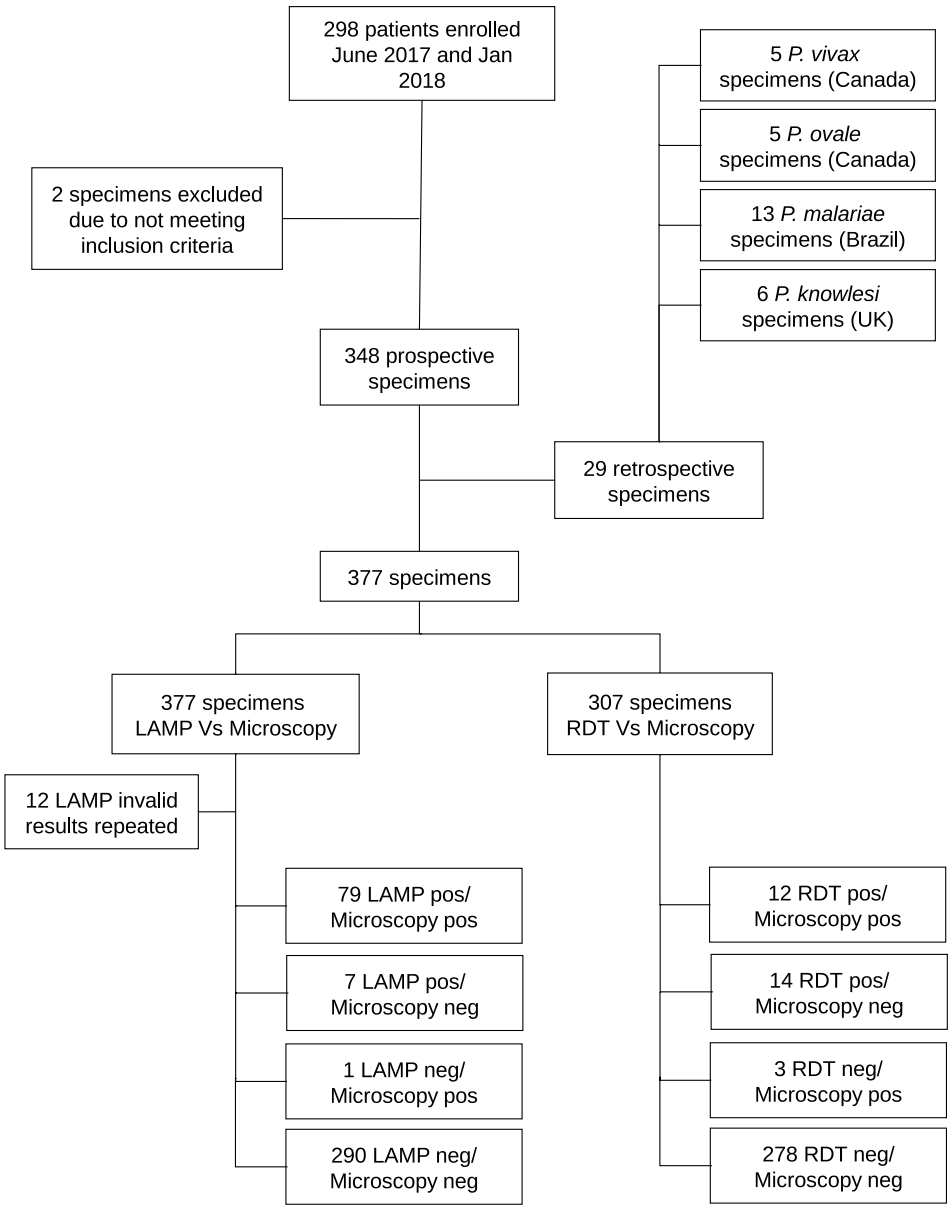
Statistical Analysis

Data were tabulated in Microsoft Excel, version 16.11.1, and were analyzed using Stata, version 13.0. Crude statistical analysis of epidemiological dichotomous variables was determined using chi-square or Fisher exact statistical tests; t tests were used for comparison of means. Variables found to be statistically significant with initial analysis were then characterized through multivariate analysis with logistic regression. Retrospectively and prospectively collected specimens were pooled and analyzed together. Sensitivity, specificity, positive predictive rate, and negative predictive rate for LAMP were calculated against microscopy both before and after discrepant resolution with PCR. After being assessed for significance, multivariate logistic regression was utilized to calculate odds ratios. Both important variables and statistically significant ones were incorporated into the analysis. Admission status was excluded because it was a dependent variable, which was influenced by the malaria test result. Due to the small sample size of malaria-positive patients, variables were dichotomized to allow for reliable analysis. Continent visited was split into Africa and non-Africa, and reason for travel was split into those visiting friends and relatives (VFR) and non-VFR. The sample size for sub-analyses was insufficient without this breakdown. As observed in the correlation matrix (Appendix A-i), there was reason to believe that there was a correlation between pretravel advice and prophylaxis taken, so only a single variable, "prophylaxis taken," was utilized in the logistic analysis.

Results

In the prospective arm, 350 specimens were collected from a total of 298 consecutive patients enrolled (Figure 7). Two specimens were excluded due to having insufficient samples for specimen resolution, leaving 348 specimens.

Figure 7: Flow chart of study design and initial results of malaria testing by each method



Fifty specimens were repeats from some of the participants who initially tested positive, as it is routine practice to monitor parasitemia during treatment. In the retrospective arm, 29 additional frozen specimens were used to supplement the analysis. The prospective and retrospective specimens were combined, so there were 377 specimens in total.

Microscopy and LAMP were available on all specimens, but RDT testing was only performed on participants in the prospective arm if deemed appropriate (n = 307). Frozen specimens had prior microscopy data at the time of diagnosis. There was an initial "invalid" test rate with LAMP *illumigene* M of 3.18%; however, all of these resolved with a single repeat test.

Table 3: Comparison of clinical and epidemiological characteristics of study participants according to their malaria test outcome (n=298)

	Total	**Malaria positive**	**Malaria negative**	**P value[a]**
Number of patients	298	25	273	
Age (mean, 95% CI)	32.5 (29.9 - 35.0)	25.2 (18.3 – 32.1)	33.1 (30.5 - 35.8)	0.089[b]
Male n (%)	151 (50.7)	13 (52.0)	138 (50.5)	0.890[e]
Parasitemia (median, IQR)		0.2 (1.275)		
Pre-travel advice n (%) n=208[c]	69 (33.2)	2 (11.8)	67 (35.1)	0.061[f]
Malaria prophylaxis taken n (%) n=234[d]	40 (17.1)	2 (9.1)	38 (17.9)	0.385[f]
Reason for travel n (%)[d]				
Business	15 (5.0)	1 (4.0)	14 (5.1)	1.000[f]
Not recorded	26 (8.7)	2 (8.0)	24 (8.8)	1.000[f]
Visiting friends/relatives	129 (43.3)	10 (40.0)	119 (43.6)	0.692[e]
Volunteer	1 (0.3)	1 (4.0)	0	0.085[f]
New Immigrant	32 (10.7)	6 (24.0)	26 (9.5)	0.038[f]
Tourism	89 (29.9)	4 (16.0)	85 (31.1)	0.111[f]
Visitor to Canada	6 (2.0)	1 (4.0)	5 (1.8)	0.414[f]
Continent visited n (%) n=287[c]				
Non-Africa	186 (64.8)	4 (16.0)	182 (69.5)	
Africa	101 (35.2)	21 (84.0)	80 (30.5)	<0.001[f]
Symptoms n (%)[d]				
Fever	265 (88.9)	23 (92.0)	242 (88.7)	0.704[f]
Night sweats	96 (32.2)	8 (32.0)	88 (32.2)	0.862[e]
Myalgia	92 (30.9)	16 (64.0)	76 (27.8)	<0.001[e]
Headache	124 (41.6)	13 (52.0)	111 (37.2)	0.361[e]
Cough	102 (34.3)	4 (16.0)	98 (35.9)	0.047[f]
Sore throat	66 (22.1)	2 (8.0)	64 (23.4)	0.080[f]
Diarrhoea	66 (22.1)	5 (20.0)	61 (22.3)	0.808[f]
Other	8 (2.7)	2 (8.0)	6 (2.2)	0.148[f]
Pre-employment	4 (1.3)	0	4 (1.5)	1.000[f]

Not recorded	12 (4.0)	0	12 (4.4)	0.608[f]
New immigrant screen	4 (1.3)	0	4 (1.5)	1.000[f]
Admitted n (%) n = 295[c]	47 (15.9)	12 (50.0)	35 (12.9)	<0.001[e]

[a]p value compares malaria positive to malaria negative group, missing values excluded
[b]T test of means
[c]n less than grand total due to missing data, percentages calculated excluding missing data and statistical tests excluding missing data
[d]Total does not add to 100 as participants reported more than one symptom
[e]Chi square test
[f]Fisher exact test

In the prospective arm, clinical and epidemiological data were analyzed based on the participant's final malaria result after discrepant resolution. Twenty-five participants out of 298 tested positive for malaria after discrepant resolution. Twenty-two out of the 25 participants who tested positive were positive by microscopy; the remaining 3 were confirmed by PCR. The median parasitemia (interquartile range) was 0.2 (1.275). The mean age in the cohort was 32.5 (95% confidence interval [CI], 29.9–35.0) years, and 50.7% of participants were male (Fifty specimens were repeats from some of the participants who initially tested positive, as it is routine practice to monitor parasitemia during treatment. In the retrospective arm, 29 additional frozen specimens were used to supplement the analysis. The prospective and retrospective specimens were combined, so there were 377 specimens in total.

Microscopy and LAMP were available on all specimens, but RDT testing was only performed on participants in the prospective arm if deemed appropriate (n = 307). Frozen specimens had prior microscopy data at the time of diagnosis. There was an initial "invalid" test rate with LAMP *illumigene* M of 3.18%; however, all of these resolved with a single repeat test.

Table 3). Fever was by far the most common symptom (265/298; 88.9%), followed by headache (124/298; 41.6%), cough (102/298; 34.3%), night sweats (96/298; 32.2%), and myalgia (92/298; 30.9%). Myalgia was statistically more common in the malaria-positive group (16/25; 64.0%)

compared with the malaria-negative group (76/273; 27.8%; P < .001) (Figure 8). Cough was statistically less common in the malaria- positive group (4/25; 16.0%) compared with the malaria-negative group (98/273; 35.9%; P = .047). Malaria-positive patients were more likely to be admitted than those who tested negative (P < .001). For every year increase in age, the odds of testing positive for malaria decreased by 6% (odds ratio [OR], 0.94), which was statistically significant (95% CI, 0.91–0.98). The odds of testing positive for males was slightly lower than females (OR, 0.60; 95% CI, 0.18–1.98), but this was not statistically significant. Participants traveling to Africa were significantly more likely to test positive for malaria (OR, 18.90; 95% CI, 4.81–74.48) compared with those visiting another location.

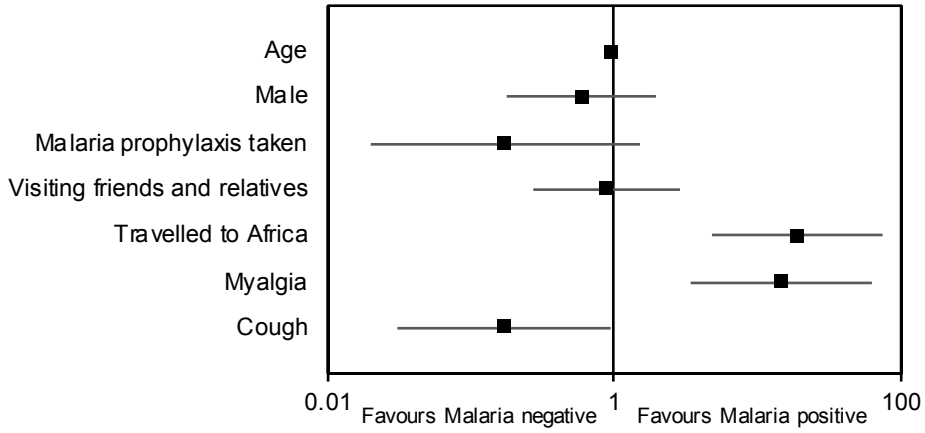


Figure 8: Odds Ratio (OR) and 95% CI for clinical and epidemiological characteristics among study participants for malaria positive versus malaria negative patients. ORs and 95% CIs enumerated on the x axis were calculated using logistic regression

There was no difference in the odds for those visiting friends and relatives compared with those traveling for other reasons (OR, 0.88; 95% CI, 0.27–2.89). Antimalarial prophylaxis was protective, with the odds ratio for those taking it being 0.17 (95% CI, 0.02–1.48), but this was

not statistically significant. Evidence of effect measure modification (EMM) was looked for between those taking prophylaxis and also visiting Africa, but none was observed. In addition, EMM was assessed for those taking prophylaxis and also in the VFR group, but the sample size was insufficient to perform the analysis.

Data were analyzed both including and excluding retrospective specimens. For the prospective arm, when repeat testing on the same participant was included, 57 specimens tested positive by LAMP, whereas 51 tested positive by microscopy (Table 4). For microscopy-positive specimens, 38 tested positive for *P. falciparum*, 7 for *P. vivax*, 6 for *P. ovale*, and none for *P. malariae* or *P. knowlesi*; the remainder tested negative. Due to repeat testing of malaria-positive patients, a test positivity rate of 14.7% was obtained in this study. Surveillance data in Calgary from 2013–2018 and excluding repeat testing within 2 months of an index revealed a test positivity rate of 4.4% (unpublished data) used to calculate the PPV and NPV.

Table 4: Performance of illumigene M assay versus microscopy on returning travellers in the prospective arm before and after discrepant resolution by RTPCR (n=348)

Before discrepant resolution		**Microscopy**			After discrepant resolution		**PCR**		
		Positive	**Negative**	**Total**			**Positive**	**Negative**	**Total**
LAMP	**Positive**	50	7	57	LAMP	**Positive**	57	0	57
	Negative	1	290	291		**Negative**	0	291	291
	Total	51	297	348		**Total**	57	291	348
		Percentage	**95% CI**				**Percentage**	**95% CI**	
Sensitivity		98.1	90.0 – 100		**Sensitivity**		100	93.7 – 100	
Specificity		97.6	95.2 – 99.1		**Specificity**		100	98.7 – 100	
Positive predictive value[a]		65.3			**Positive predictive value[a]**		100		
Negative predictive value[a]		99.9			**Negative predictive value[a]**		100		
[a]Based on a prevalence of 4.4%									

In the prospective arm, LAMP had a sensitivity of 98.1% (95% CI, 90.0%–100%) and a specificity of 97.6% (95% CI, 95.2%–99.1%) vs microscopy before discrepant resolution for all samples. Based on a prevalence of 4.4%, the positive predictive value (PPV) was 65.3%, and the negative predictive value (NPV) was 99.9%. Seven specimens were negative with microscopy but tested positive with LAMP. One specimen was positive with microscopy but negative with LAMP. All specimens, both discordant and concordant, were tested by RT-PCR for confirmation. Discrepant resolution confirmed that the 7 false-positive specimens by LAMP tested positive by RT-PCR (Table 5).

Table 5: Results of discrepant resolution by sample number (n=8)

Sample number	**Microscopy**	***illumigene M***	**Alternate PCR**	**Final**
018	Neg	Pos	Pos	True Positive - *P. falciparum*
035	Neg	Pos	Pos	True Positive - *P. falciparum*
068	Neg	Pos	Pos	True Positive - *P. falciparum*
095	Neg	Pos	Pos	True Positive - *P. vivax*
112	Neg	Pos	Pos	True Positive - *P. falciparum*
122	Po gametocytes	Neg	Neg	True Negative
253	Neg	Pos	Pos	True Positive - *P. falciparum*
265	Neg	Pos	Pos	True Positive - *P. falciparum*

One false-negative specimen by LAMP tested negative by RT-PCR. After discrepant resolution, LAMP had a sensitivity of 100% (95% CI, 93.9%–100%) and a specificity of 100% (95% CI, 98.7%–100%) vs microscopy. The PPV and NPV were both 100%. For 1 negative specimen by LAMP and RT-PCR, only *P. ovale* gametocytes were found. When including retrospective specimens, an additional 5 *P. vivax*, 5 P. *ovale*, 13 *P. malariae*, and 6 *P. knowlesi* specimens were included, so that in total by microscopy there were 38 *P. falciparum* specimens, *12 P. vivax*, 11 *P. ovale*, 13 *P. malariae*, and 6 *P. knowlesi* specimens, with the remainder being negative (Table 6).

Table 6: Performance of illumigene M assay versus microscopy on returning travellers in the prospective and retrospective arms combined (n=377) before and after discrepant resolution

Before discrepant resolution		**Microscopy**			After discrepant resolution		**PCR**		
		Positive	**Negative**	**Total**			**Positive**	**Negative**	**Total**
LAMP	**Positive**	79	7	86	LAMP	**Positive**	86	0	86
	Negative	1	290	291		**Negative**	0	291	291
	Total	80	297	377		**Total**	86	291	377
		Percentage	**95% CI**				**Percentage**	**95% CI**	
Sensitivity		98.8	93.2 – 100		**Sensitivity**		100	95.8 – 100	
Specificity		97.6	95.2 – 99.1		**Specificity**		100	98.7 – 100	
Positive predictive value[a]		65.5			**Positive predictive value**[a]		100		
Negative predictive value[a]		100			**Negative predictive value**[a]		100		
[a]Based on a prevalence of 4.4%									

With retrospective specimens included, LAMP had a sensitivity of 98.8% (95% CI, 93.2%–100%) and a specificity of 97.6% (95% CI, 95.2%–99.1%) vs microscopy. After discrepant resolution, LAMP had a sensitivity of 100% (95% CI, 95.8%–100%) and a specificity of 100% (95% CI, 98.7%–100%). There were no additional discrepancies following inclusion of retrospective specimens. The performance of RDT compared with microscopy was evaluated (n = 307) (Appendix A(ii)). After discrepant resolution using RT-PCR, the sensitivity was 83.3% (95% CI, 58.6%–96.4%) and specificity was 96.2% (95% CI, 93.2%– 98.1%). These results correlated with a PPV of 50.2% and an NPV of 99.2%. The species-specific performance of LAMP after discrepant resolution is shown in (Appendix A(ii)).

Discussion

The commonest reason for travel was recent immigrants traveling to visit friends and relatives abroad. These individuals have been found in other studies to stay in endemic areas for longer, are more likely to stay in local houses, so they are more exposed to mosquitos, and are less likely

to seek pretravel advice and antimalarial prophylaxis (132). The patient cohort here represents a typical population presenting with the symptoms of malaria. As a result, this study is generalizable to urban centers with a large immigrant population across North America and Europe. Although numerous studies have examined the role of LAMP for the diagnosis of malaria in both the nonendemic and endemic settings, this is the first evaluation of LAMP in a "realtime" prospective trial in returning travelers to North America. A recent meta-analysis found that LAMP appears to have excellent sensitivity against PCR (133). Due to its limit of detection, LAMP is thought to be particularly useful in diagnosing patients with a low parasitemia below the LOD of microscopy (121). It has several advantages over other molecular tests: simple to use, no major capital equipment needs, and limited training needed (49, 112, 134). Laboratory technologists were trained in *illumigene* M, and they were able to perform it without difficulty in under an hour once they received the specimen.

Currently, the CDC recommendation for malaria testing is to release an RDT result as a preliminary result and then repeat thick and thin films every 6 to 8 hours on average 3 times to ensure that no parasites are present (109). This is accompanied by a significant labour cost in the laboratory and repeated emergency room use. A previous study from our group proposed a new testing algorithm for malaria with LAMP as the first line diagnostic test (111). In the nonendemic setting, the proportion of malaria tests that are positive is relatively small. Repeated testing of patients without malaria based on existing algorithms results in additional costs to the laboratory and hospital. We estimated from this study that a single LAMP test with a high NPV would lead to a laboratory cost savings of up to USD$13 per malaria test in a nonendemic setting. Further savings for the hospital may be possible resulting from the earlier discharge of

the patient from the emergency room or doctor's office. Multiple studies have found that LAMP had an excellent NPV compared with PCR. However, they are often retrospective, do not run continuously, and/or commonly have a small number of non–*P. falciparum* species included.

A strength of the study is that all species of malaria were tested and correctly identified, which is essential if this test is to be used routinely. The study was prospective in nature and ran continuously in real time for 8 months to reflect current practice, albeitsupplemented with retrospective specimens. Technologists were easily trained and were able to complete testing with *illumigene* M without supervision. This has potential advantages afterhours as technologists may not be experienced with reading the thick films required to ultimately rule out infection. Based on the LAMP sensitivity and NPV (100%) found in this study, patients presenting after-hours who test negative can safely be discharged home within 1 hour of specimen receipt at the laboratory (Figure 9).

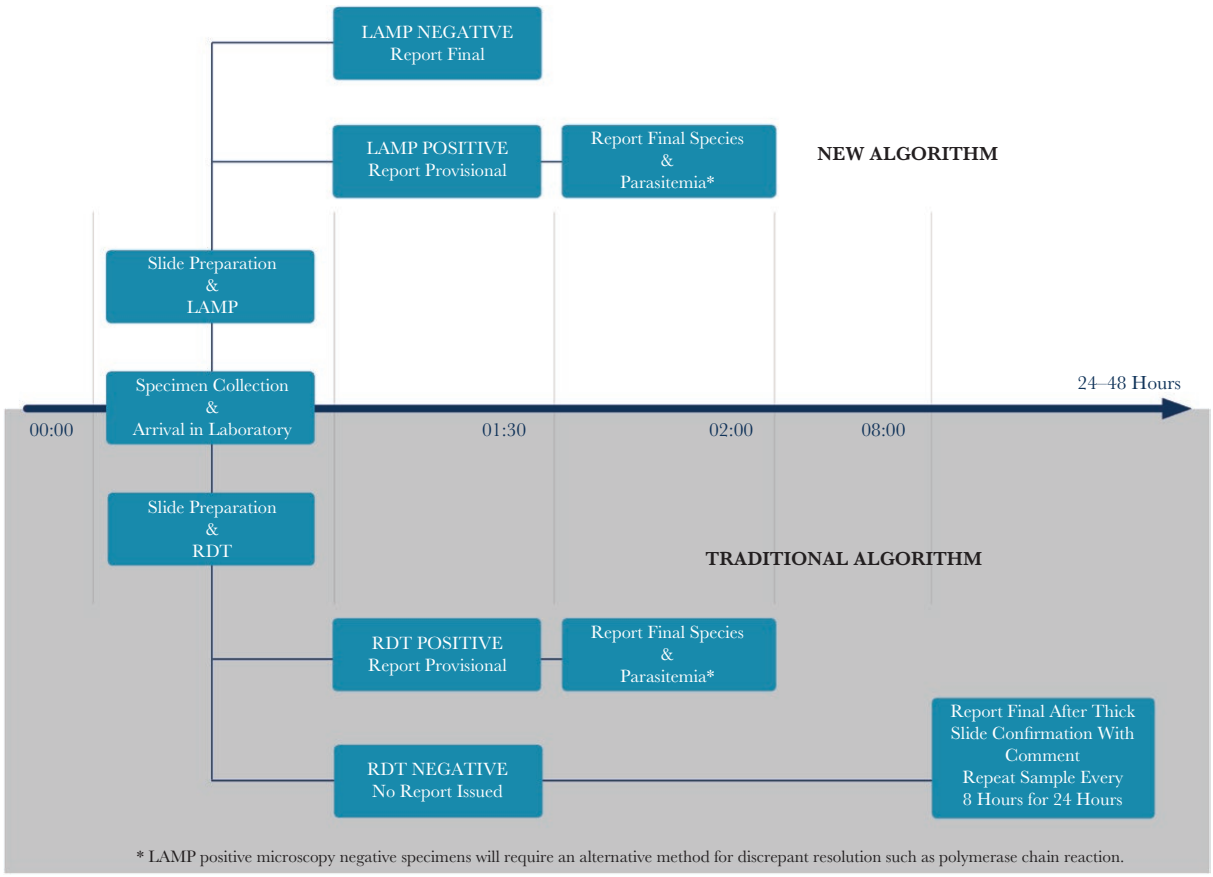


Figure 9: Comparison of a traditional malaria testing algorithm with the novel algorithm proposed in this study

The lower sensitivity of RDT (83.3%) in this study does not permit this confidence, especially for non–*P. falciparum* species, for which it fails more routinely to detect infection. Previously, the time to final reading of the thick film (where sensitivity is achieved) was 9 to 24 hours, depending on specialist technologist availability, because of the lack of confidence in the RDT result. The ability to discharge patients leads to a cost savings for the health facility because patients do not have to re-attend for repeat microscopy smears.

A limitation of this study is that the same technologist often performed LAMP, microscopy, and RDT testing for each specimen. It was not feasible to have multiple technologists available who could perform testing, so consequently operators were not blinded to other results. This may have introduced some misclassification bias. For discordant results, PCR testing occurred off-site and operators were blinded, thereby reducing misclassification bias for the most important results. Also, although *P. knowlesi* samples were contrived from a lab strain, LAMP has been able to detect patient samples on dried blood spots by our group (our unpublished observations) and patient whole-blood samples by others (Martin et al., unpublished data, 2018).

A limitation of the *illumigene* M platform is that it does not identify the species of malaria. This is not essential as a rapid screening test. Positive LAMP tests can be followed up with microscopy to determine species and parasitemia. An "invalid" rate of 3.18% was observed. The reasons for this are unclear (possibly due to high parasitemia, DNA amplification inhibitor, delay in the extraction process), but this study did have a smaller proportion of invalid results compared with other relevant studies (111, 125). In this study, invalid results resolved with single repeated testing; however, they caused a delay in diagnosis. Specimens that did not resolve with repeated testing would require an alternative method to be used.

In a nonendemic setting where the prevalence of malaria in returning travelers is relatively low, LAMP is able to rule out malaria with a faster turnaround time without the need for repeat testing. A novel, highly sensitive testing algorithm for malaria screening with associated cost savings in the nonendemic setting is proposed.

CHAPTER 4: THE IMPACT OF MALARIA ON LIVER ENZYMES: A RETROSPECTIVE COHORT STUDY (2010-2017)

Background

With 92% of malaria cases and 93% of malaria deaths occurring in sub-Saharan Africa in 2017, the threat of artemisinin resistance spreading to the region is of great public health concern (1). For malaria control and eradication to be feasible by the target of 2030 (2, 135), there is a necessity to develop novel therapeutic compounds; but no new clinical entities (NCEs) treating blood-stage infections have been developed since artemisinin (136). The likelihood of artemisinin resistance spreading means that without new medications, significant barriers to control and eradication would remain (59).

As with any drug development, attrition of antimalarial drugs in development pipelines is common and also extremely costly (60). Characterizing liver enzymes in uncomplicated malaria is becoming increasingly important, as clinical trials of novel antimalarial NCEs have reported abnormalities in liver enzymes, specifically alanine aminotransferase (ALT) and/or total bilirubin (TB) (137-143). Regulatory bodies such as the US Food and Drug Administration (FDA) typically use Hy's law for evidence of liver toxicity in clinical trials, as severe drug induced liver injury (DILI) tends to occur at a frequency of at least 10% of this rate (144). Hy's law sets a danger threshold of 3x upper limit of normal (xULN) fold elevations of aminotransferases compared with a control arm, with an elevation of serum total bilirubin to >2x ULN (144, 145).

The World Health Organization (WHO) guidelines reports that severe malaria can cause multi-organ failure and liver damage, with jaundice relatively common (5). In non-severe malaria, guidelines state that hyperbilirubinemia is relatively common, and aminotransferase liver enzymes may be elevated up to tenfold but signs of liver failure are extremely rare. The extent of abnormalities in aminotransferases are not further characterized by the WHO and in general, data on non-severe malaria more limited. Studies are often heterogeneous, lack control groups, are cross-sectional in nature or do not control for confounders such as disease severity making it difficult to draw inference. Jaundice can occur (possibly due to haemolysis) as well as raised aminotransferases, but the exact frequency of these results varies depending on the study (146-149).

Furthermore, some approved anti-malarial medications can cause mildly deranged liver enzymes (150), leading to further difficulties in interpreting trial results. Liver dysfunction can occur due to many currently used anti-malarial therapies, either through malaria prophylaxis or treatment. Chloroquine has not been found to cause liver toxicity, and appears to be safe in mild liver disease (151). Sulfadoxine/pyrimethamine is well known to cause hepatic toxicity, including liver granulomas, mixed cholestatic-hepatocellular hepatitis, acute hepatic necrosis and chronic hepatitis (152-154). Malarone has been associated with deranged liver enzymes (150) and mefloquine can cause a transient rise in liver enzymes, but is not associated with significant hepatotoxicity (155, 156). Hepatotoxicity with the use of quinine and doxycycline is rare (157, 158). Artemisinin combination therapies (ACTs) have a good safety profile, and generally have replaced other antimalarials due to their efficacy and tolerability. They are known to cause

delayed haemolysis and therefore hyperbilirubinemia (159), but some studies have also found that self-limiting, mild rises in aminotransferases can also occur (157, 160).

Historically, the impact of malaria and antimalarials on liver enzymes is unclear. Drug development trials have shown conflicting results(138, 139, 142, 161, 162), but these often enroll small numbers, lack a control group and are not specifically designed to determine the cause of the liver enzyme abnormalities. This makes it challenging to differentiate whether any liver enzyme changes are due to malaria the itself or the NCEs being tested. However, more recently a number of studies have been conducted that have specifically attempted to address issue(137, 163-165). A sub-study of the West African Network for Clinical Trials of Antimalarial Drugs (WANECAM) phase 3b/4 trials was specifically designed to investigate the prevalence of liver abnormalities among individuals ≥ 6 months in age with uncomplicated malaria, those taking pyronaridine-artesunate (PA) were compared with artemether-lumefantrine (AL) (137). This study found an ALT rise in 28/1015 (3%) and 7/671 (1%) of PA treated and AL treated individuals respectively, with a TB rise in both groups of <1%, suggesting the liver abnormalities are rare in malaria even when taking medications. Silva-Pinto *et al* characterized liver enzymes in returning travellers admitted with uncomplicated malaria, finding individuals taking AL had a higher proportion of abnormal aminotransferases than those taking quinine and doxycycline (163). A Woodford *et al* (164) retrospective cohort study of in-patient returning travellers described a peak TB on the day of admission the early period, with a raised ALT in the early and late period, but this study lacks a control group and and was prone to selection bias, as individuals without serial liver enzyme measurements were excluded. Reuling *et al* (165) studied liver enzymes in in-patient returning travellers with uncomplicated malaria and also in

Controlled Human Malaria Infection (CHMI) studies. Among returning travellers, they reported a peak TB at time of diagnosis and a prevalence of aminotransferase abnormalities of 69%, far above those found in other studies. In the CHMI group who were not returning travellers, similar rises were seen for the aminotransferases, but not for TB. This was felt to be due to the lower parasitaemia in the CHMI group, leading to less haemolysis. Overall, ALT/AST peaked 2-6 days following initiation of therapy whereas TB peaked prior to antimalarial administration. However, there are differences between naturally acquired malaria and CHMI (166), and the time course of rises makes it difficult to differentiate between the effects of malaria and treatments.

To characterize liver enzymes in malaria, a retrospective cohort study was designed to obtain longitudinal data on liver enzymes in returning travellers. The primary objective was to ascertain the effect of malaria on aminotransferases and TB, with the secondary objective to ascertain the time frame when any changes in liver enzymes occur from the initial malaria test.

Methods

Study design and Ethics

Symptomatic returning travellers were eligible for enrolment if they presented for malaria testing due to in the period 2010-2017 at Calgary Laboratory Services (CLS) in Calgary and had at least one liver enzyme tested within 31 days of the index malaria test. All eligible individuals were enrolled for the liver enzyme analyses, but due to the large number of non-malaria exposed participants, individuals were randomly selected to be enrolled for the epidemiological analyses. Individuals had epidemiological data recorded on a patient information sheet by the clinician doing the malaria testing. A chart review was undertaken which obtained liver enzymes,

epidemiological data, disease severity and out-patient medication data. Malaria severity was defined in line with WHO guidelines (5). Out-patient prescriptions for the six months prior to malaria testing was obtained and reviewed for evidence of hepatotoxic medications, as defined by the National Institutes of Health LiverTox database (157). The liver enzymes recorded were Alkaline Phosphatase (ALP), Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and Total Bilirubin (TB). Ethical approval was obtained from University of Calgary Ethics Board (Ethics ID: REB15-1160).

Outcomes

The primary outcome was to determine whether malaria leads to increased ALT and TB within 31 days of malaria testing. These liver enzymes were chosen because they were felt to be most commonly used and relevant markers of liver injury. AST is not regularly tested at CLS and ALP is more typically raised in obstructive causes of deranged liver enzymes. The secondary objective was to determine the time period when any abnormalities in ALT and TB occur, starting from the initial blood draw. Time periods from index malaria test were split in line with previous literature into an early period: 0-3 days, intermediate period: 4-11 days and a late period: 12-31 days(164).

Malaria and liver enzyme testing

Each individual eligible for enrolment was tested according to standard operating procedure at the time: three Giemsa-stained thick and thin peripheral blood smears at least 6 to 8 hours apart and rapid diagnostic tests (RDTs) (BinaxNOW Malaria, Alere, Waltham, MA). Malaria species were identified by microscopy. Liver enzyme testing occurred as part of routine clinical testing

on whole blood specimens, with upper limits of normal used in clinical practice (167). All liver enzyme measurements were converted into multiples of the age and gender specific upper limit of normal, termed xULN. This accounted for the differing upper limits of normal for different genders and age groups. The results for the xULN values were highly positively skewed, so they were dichotomized into either normal or abnormal values.

Statistical Analysis

Firstly, the impact of epidemiological risk factors on malaria test results were analyzed. Univariable statistical analysis of epidemiological dichotomous variables was determined using chi-square or Fisher exact statistical tests with t tests used for continuous variables, then by multivariable logistic regression. Results for the four liver enzymes were analyzed separately. To allow for the generation of results for the univariable analysis, if a participant had multiple values taken within the same time period, the mean value was taken. The prevalence of Hy's law was calculated according to these values. For the multivariable analysis, results were obtained over a 31-day period, so were treated as longitudinal data. Due to evidence of clustering (Appendix B(ix): Graph showing the distribution of mean liver enzyme results for each participant within each time period and Appendix B(x): Correlation matrix of liver enzymes and time periods), logistic regression of binomial longitudinal panel data was performed with robust standard errors and adjustments for serial correlation. Time period was included with early, intermediate and late values to ascertain their influence on liver enzyme results. Analysis was performed on liver enzymes individually, with missing data excluded from the model. Further details of the methods can be found in the appendix material.

Results

Prevalence of epidemiological characteristics with malaria

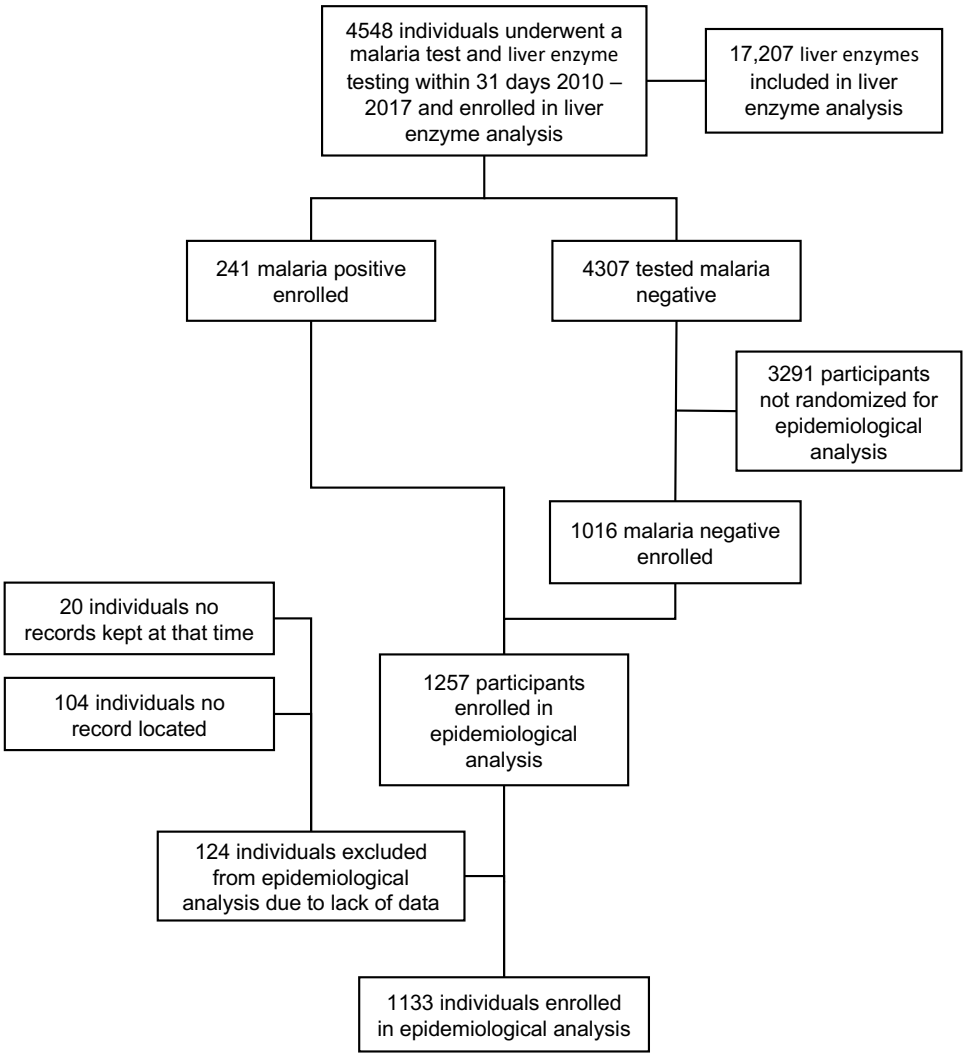


Figure 10: Flow diagram of study

As shown in Figure 10, initial analysis revealed a total 17,207 liver enzymes results from 4,548 individuals receiving a malaria test, with 241/4548 (5.3%) malaria positivity rate. The univariable analysis shown in Table 7 revealed that malaria positive and negative individuals were of a similar mean age [malaria positive: 33.30, (95% CI: 31.11 – 35.49), malaria negative:

35.84 (95% CI 34.62 – 37.06)]; but there were statistically more males (p= 0.001) in the malaria positive group (156/241, 64.7%) compared to the malaria negative group (538/1016, 53.0%).

Table 7: Demographics of study participants by malaria test result

	Total	**Malaria positive**	**Malaria negative**	**p value[1]**
Number of patients	1257	241	1016	
Age (mean, 95% CI)	35.35 (34.28 – 36.42)	33.30 (31.11 – 35.49)	35.84 (34.62 – 37.06)	0.067[2]
Male n (%)	694 (55.21)	156 (64.73)	538 (52.95)	0.001[3]
Parasitaemia (median, IQR)		0.2 (1.1)		
Malaria species n (%)				
P. falciparum		151 (62.66)		
P. falciparum/P. ovale		5 (2.07)		
P. malariae		1 (0.41)		
P. ovale		16 (6.64)		
P. vivax		56 (23.24)		
P. vivax/P. ovale		1 (0.41)		
Not documented		11 (4.56)		
Malaria severity n (%) n=192		44 (22.92)		
Treatment n (%)				
Artesunate		38 (15.77)		
Chloroquine		29 (12.03)		
Malarone		91 (37.76)		
Qunine/Doxycycline		22 (9.13)		
Other		6 (2.49)		
Not documented		55 (22.82)		
Pre-travel advice n (%) n=867[4]	297/867 (34.26)	39/161 (24.22)	258/706 (36.54)	0.003[3]
Malaria prophylaxis taken n (%) n=495[4]	119/495 (24.0)	18/104 (17.31)	101/391 (25.83)	0.071[3]
Reason for test n (%) n=1133[4]				
Business	96/1133 (8.47)	13/199 (6.53)	83/934 (8.89)	0.279[3]
Visiting friends/relatives	485/1133 (42.81)	104/199 (52.26)	381/934 (40.79)	0.003[3]
New Immigrant	132/1133 (11.65)	40/199 (20.10)	92/934 (9.85)	<0.001[3]
Tourism	295/1133 (26.04)	17/199 (8.54)	278/934 (29.76)	<0.001[3]
Visitor to Canada	16/1133 (1.41)	10/199 (5.03)	6/934 (0.64)	<0.001[3]
Not recorded	109/1133 (9.62)	15/199 (7.54)	94/934 (10.06)	0.272[3]
Continent visited n (%) n=1073[4]				
Non-Africa	631/1073 (58.81)	36/194 (18.56)	595/879 (67.69)	
Africa	442/1073 (41.19)	158/194 (81.44)	284/879 (32.31)	<0.001[3]
Symptoms n (%)[5] n=1054[4]				
Fever	889/1054 (84.35)	162/181 (89.50)	727/873 (83.28)	0.036[3]
Night sweats	391/1054 (37.10)	73/181 (40.33)	318/872 (36.43)	0.322[3]
Headache	561/1054 (53.23)	116/181 (64.09)	445/873 (50.97)	0.001[3]
Cough	317/1054 (30.08)	45/181 (24.86)	272/873 (31.16)	0.093[3]
Sore throat	243/1054 (23.06)	21/185 (11.60)	222/873 (25.43)	<0.001[3]
Myalgia	390/1054 (37.00)	78/181 (43.09)	312/873 (35.74)	0.062[3]
Other	298/1054 (28.27)	48/181 (26.52)	250/873 (28.64)	0.565[3]
Hepatotoxic medication n (%)	588 (46.78)	105 (43.57)	483 (47.54)	0.267[3]

[1]p value compares malaria positive to malaria negative group, missing values excluded
[2]t test of means
[3]chi-square test
[4]n less than grand total due to missing data, percentages calculated excluding missing data and statistical tests excluding missing data
[5]Total does not add to 100 as participants reported more than one symptom

Among the malaria positive group, the most common species responsible was *P. falciparum* (156/241, 64.7%), with 22.9% of the total malaria positive individuals suffering from severe malaria. For the multivariable logistic regression, there was evidence of correlation (Appendix B(v): Correlation matrix of variables included in epidemiology multivariate regression) between pre-travel advice and use of anti-malarial prophylaxis so only anti-malarial prophylaxis was included alongside the other variables.

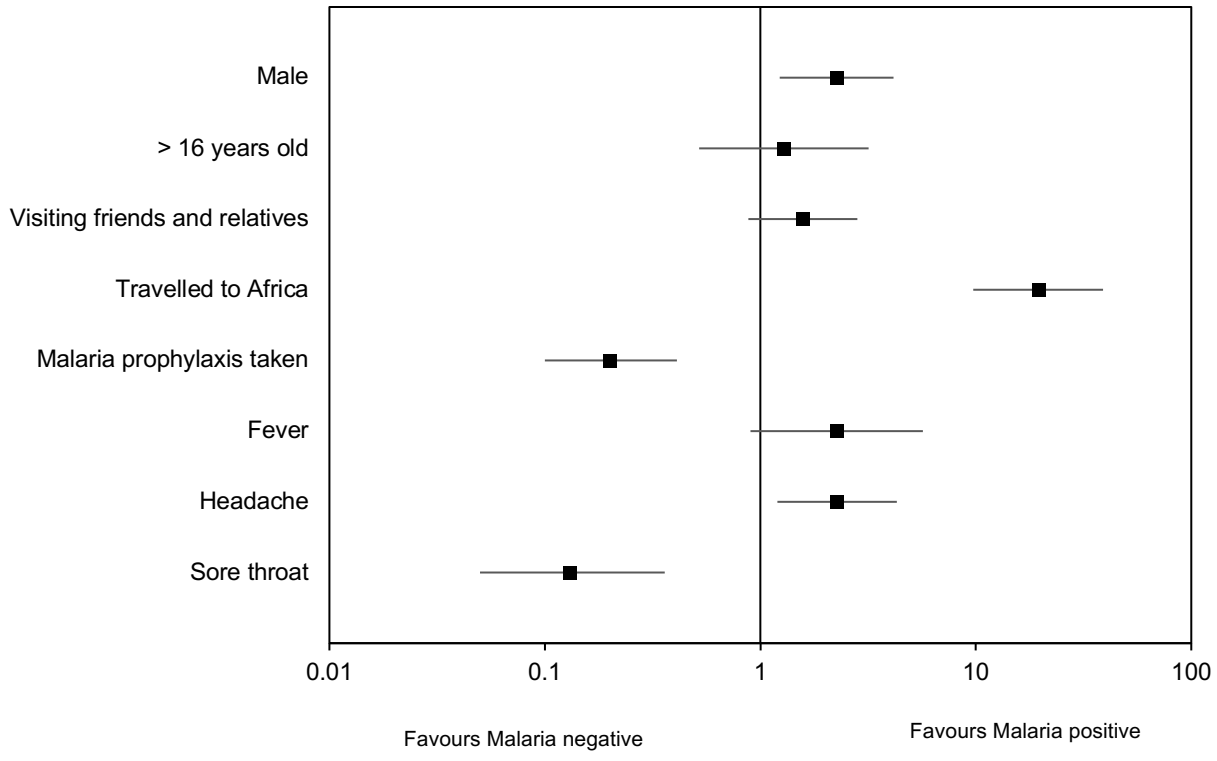


Figure 11: Logistic regression analysis of epidemiological risk factors

As seen in Figure 11, returning travellers who were male (OR: 2.26, 95% CI: 1.23 – 4.15) and those who had visited Africa (OR: 19.47, 95% CI: 9.73 – 38.95) had statistically higher odds of

testing positive for malaria, while those taking anti-malarial prophylaxis had lower odds (OR: 0.20, 95% CI: 0.10 – 0.41). Individuals with a headache (OR: 2.27, 95% CI: 1.20 – 4.30) had statistically higher odds of being malaria positive, while those presenting with a sore throat (OR: 0.13, 95% CI: 0.05 – 0.36) had lower odds.

Impact of malaria on liver enzymes

A total of 17,207 individual liver enzyme measurements were performed in the 31 days following the first malaria test. In the early period, 221/241 (91.7%) of malaria positive individuals had an ALT measured, compared to 3816/4037 (94.5%) of malaria negative individuals. However, the percentage of malaria positive individuals with ALT measurements in the intermediate and late periods was 11.6% and 11.2% respectively, with for 11.8% and 15.2% for negative individuals. This demonstrates how there is an increased testing rate in the early period compared to the intermediate and late periods. For TB, the respective values for malaria positive individuals are 72.6%, 11.6% and 10.0% respectively, with 38.7%, 8.2% and 9.9% for negative individuals, meaning a greater proportion of malaria positive individuals had a TB measured. For ALP and AST, a similar pattern of testing was noted, with the vast proportion of tests performed in the early period. The median values in each time period for all liver enzymes are shown in Appendix B(viii): Median fold-upper limit of normal (xULN) and interquartile range (IQR) values for liver enzymes for all species of malaria. This analysis suggested that the median TB of malaria exposed individuals may be higher than for the unexposed, but no difference was found for ALP, ALT and AST. The proportion of individuals with abnormal liver enzymes are shown in Table 8.

Table 8: Proportion of abnormal liver enzymes by malaria result

	ALP n (%)			**ALT n (%)**			**AST n (%)**			**TB n (%)**		
Time period	Early n = 3098	Interm edi-ate n = 431	Late n = 555	Early n = 4037	Interm edi-ate n = 538	Late n = 682	Early n = 640	Interm edi-ate n = 118	Late n = 156	Early n = 1840	Interm ed-iate n = 381	Late n = 450
Malaria result												
Malaria negative	197/2888 (6.82)	114/409 (27.87)	105/532 (19.74)	624/3816 (16.35)	241/510 (47.25)	168/655 (25.65)	166/602 (27.57)	65/111 (58.56)	57/153 (37.25)	129/1665 (7.75)	63/353 (17.85)	57/426 (13.38)
Malaria positive	7/210 (3.33)	2/22 (9.09)	1/23 (4.35)	42/221 (19.00)	12/28 (42.86)	5/27 (18.52)	12/38 (31.58)	4/7 (57.14)	2/3 (66.67)	48/175 (27.43)	2/28 (7.14)	4/24 (16.67)
p value	0.049	0.080	0.099	0.302	0.650	0.404	0.593	1.000	0.557	<0.001	0.195	0.551

If multiple tests done on participants within same time frame, mean of results used. All by chi-square or Fisher exact; statistically significant differences shown in bold.
Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferases; AST, aspartate aminotransferases; TB, total bilirubin.

The proportion of individuals with abnormal ALT was not statistically different in any period, with the proportion abnormal in the early period 42/221 (19.0%) in the malaria exposed group and 624/3816 (16.4%) in the unexposed group. For TB in the early period, the proportional with an abnormal result was 48/175 (27.4%) in the exposed group, compared to 129/1665 (7.8%) in the unexposed group, with no difference in the intermediate and late periods. Malaria negative individuals were more likely to have an abnormal ALP in the early period than malaria individuals (p=0.049), but no other statistically difference was found in other timeframes. For AST, no statistically significant difference was found in any time period. Univariable analyses of severity of malaria, species and use of hepatotoxic medications, seen in 'Appendix B(xi): Proportion of abnormal liver enzymes for severity of malaria, use of hepatotoxic medication and species of malaria,' showed that individuals with severe malaria were statistically more likely to have an abnormal TB than those with non-severe malaria in the early period. Use of hepatotoxic medication appeared to influence ALP in the early period (p=0.001) and ALT in the intermediate period (p=0.039), but no other associations had a statistically significant difference and results were not consistent across all time periods.

Table 9: Proportion of participants that meet the criteria for Hy's law depending on malaria test result for the early, intermediate and late time periods

		Early period		**Intermediate period**		**Late period**	
		Malaria result		Malaria result		Malaria result	
		Negative (n=1581)	Positive (n=166)	Negative (n=336)	Positive (n=18)	Negative (n=409)	Positive (n=18)
Hy's law[1]	No	1549 (97.98)	162 (97.59)	316 (94.05)	17 (94.44)	398 (97.31)	17 (94.44)
	Yes	32 (2.02)	4 (2.41)	20 (5.95)	1 (5.56)	11 (2.69)	1 (5.56)
	p value[2]	0.771		1.000		0.408	

[1]Criteria for Hy's law based on mean of individual participants' results within any given time period
[2]Fisher exact test

In terms of Hy's law (Table 9), there was no difference in the proportion of malaria positive individuals versus malaria negative individuals meeting the criteria in the early (2.4%, 2.0%, p=0.771), intermediate (5.6%, 6.0%, p=1.000) or late (5.6%, 2.7%, p=0.408) periods. Of the four malaria positive individuals meeting Hy's law, one individual had severe *P. falciparum* and subsequently succumbed to the illness, one non-severe *P. vivax* and had taken multiple illicit drugs, one had non-severe *P. ovale* with G6PD deficiency and the final individual had severe *P. falciparum* with an acute hepatitis A co-infection.

For the multivariable analysis, ALP, ALT, AST and TB were analyzed as the dependent variables separately, to assess the result for individual liver enzymes. To allow for longitudinal logistic regression to be performed, only the first liver enzyme result performed on an individual on any given day was taken, leading to the exclusion of 5 ALP, 6 ALT, 0 AST and 5 TB results. This left 5412 ALP, 6843 ALT, 1075 AST and 3861 TB measurements respectively included in the analysis. For ALT, 6843 observations were performed on 1271 individuals, with a mean number of observations per individual of 1.5. For TB, 3861 observations were performed on 2186 individuals, leading to mean of 1.8 observations per individual. Gender (p=0.451), age (p=0.337) and hepatotoxic medication (p=0.186) appeared to have no effect on the odds of

having an abnormal ALT result (Table 10). In addition, malaria positivity also appeared to have no effect [(OR: 1.01, 95% CI: 0.54 – 1.89), p=0.978]. Using the early period as a reference, ALT results from the intermediate period had significantly higher odds of being abnormal [(OR: 2.57, 95% CI: 1.68 – 3.92), p<0.001], but the late period did not [(OR: 0.67, 95% CI: 0.40 – 1.14), p=0.139]. For TB, males had higher odds of an abnormal result [(OR: 5.72, 95% CI: 2.85 – 11.49), p<0.001], adults had lower odds than children [(OR: 0.43, 95% CI: 0.21 – 0.91), p=0.026] but hepatotoxic medication (p=0.751) did not. Individuals testing positive for malaria had significantly higher odds of having an abnormal TB result [(OR: 12.64, 95% CI: 6.32 – 25.29), p<0.001]. Compared to the early period, TB measured in the intermediate [(OR: 0.58, 95% CI: 0.33 – 1.04), p=0.070] and late period [(OR: 0.72, 95% CI: 0.37 – 1.39), p=0.324] had lower of being abnormal, but no these were not significant. Malaria result had no effect on the odds of having an abnormal ALP [(OR: 0.32, 95% CI: 0.09 – 1.10), p=0.072] or AST [(OR: 1.26, 95% CI: 0.22 – 7.37), p=0.794]. For the time periods, after adjusting for the other variables; the results for ALP and AST followed a similar pattern to ALT, with values measured in the intermediate period having higher odds of being abnormal than in the early period. Examining malaria positive results only (Appendix B(xii): Odds ratios and their corresponding 95% CIs and p-values for selected determinants of having abnormal liver enzymes if malaria positive), disease severity was borderline statistically significant for abnormal TB [(OR 2.56, 95% CI: 0.99 – 6.62), p=0.052] but *P. falciparum* species (OR 0.70, 95% CI: (0.24 – 2.05), p=0.511] did not appear to significantly increase the odds of having an abnormal TB. No statistically significant difference was found for ALT.

Table 10: Odds ratios and their corresponding 95% Cis and p-values for selected determinants of having abnormal liver enzymes

Liver assay	**ALP**		**ALT**		**AST**		**Bilirubin**	
Number of observations	5412		6843		1075		3861	
Number of Individuals	3443		4417		806		2186	
Mean obs per individual	1.6		1.5		1.3		1.8	
	Odds ratio	p value	Odds ratio	p value	Odds ratio	p value	Odds ratio	p value
Gender (male)	0.75 (0.47 – 1.21)	0.242	0.89 (0.66 – 1.21)	0.451	0.60 (0.26 – 1.36)	0.220	5.72 (2.85 – 11.49)	<0.001
Age (adult)	23.30 (7.30 – 74.40)	<0.001	1.22 (0.81 – 1.83)	0.337	2.82 (1.06 – 7.46)	0.037	0.43 (0.21 – 0.91)	0.026
Hepatotoxic medication (taken)	1.42 (0.89 – 2.28)	0.140	1.23 (0.90 – 1.68)	0.186	1.45 (0.65 – 3.28)	0.367	0.91 (0.50 – 1.63)	0.751
Malaria result (positive)	0.32 (0.09 – 1.10)	0.072	1.01 (0.54 – 1.89)	0.978	1.26 (0.22 – 7.37)	0.794	12.64 (6.32 – 25.29)	<0.001
Early time period (reference)	1.00		1.00		1.00		1.00	
Intermediate time period	4.23 (2.66 – 6.72)	<0.001	2.57 (1.68 – 3.92)	<0.001	3.56 (1.26 – 10.04)	0.016	0.58 (0.33 – 1.04)	0.070
Late time period	2.28 (1.25 – 4.16)	0.007	0.67 (0.40 – 1.14)	0.139	0.61 (0.18 – 2.03)	0.419	0.72 (0.37 – 1.39)	0.324

Discussion

The primary objective of this study was to investigate whether malaria could lead to abnormal liver enzymes in returning travellers and, if so, to ascertain the time course of any changes. In the multivariable analysis, males had increased odds of having a higher TB but not ALT, when compared to females. As described in a number of different studies, males appear to have a higher baseline TB compared to females (168, 169). Our laboratory uses an identical upper limit of normal for both sexes, so this most likely introduced some misclassification bias for abnormal TB and therefore an overestimation of the odds ratio. Interestingly, those taking hepatotoxic medication were not at higher risk of having abnormal ALT or TB. However, these medications were being taken in the 6 months prior to the index malaria test, so individuals are likely to be relatively stable on their medications. Malaria-positive individuals were more likely to have an

abnormal TB, with rises in this group most likely to occur in the early period. This is in line with previous literature in returning travellers (164, 165), and appears to fit with a raised TB occurring with malaria, which then begins to return to normal as the individual is treated. As reviewed by Anand *et al*, hyperbilirubinemia can be conjugated or unconjugated, and the causes are multifactorial including intravascular haemolysis, disseminated intravascular coagulation, drugs, hepatitis and G6PD deficiency (149). The results from this study, along with the biologically plausible explanation appears confirm results from other studies that malaria causes raised TB. In contrast to the TB, no increased odds of abnormal ALT results with malaria positivity were found. Other studies have described a raised ALT in returning travellers (164, 165), but these studies lack a control group may be prone to selection bias. Anand *et al* reports histological changes in the liver including centrizonal necrosis, Kupffer cell hyperplasia, hemozoin deposition, lymphocytic infiltration and steatosis (149). However, evidence for this comes from studies with small sample sizes and specimens from fatal cases. After adjusting for a multivariable analysis, given a strong association was seen with TB, one would have expected to find an association should one exist. In contrast to Woodford *et al* (164), even when examining ALT results for malaria positive individuals only, there was no statistically significant difference seen across the three time periods, but the sample size is small in the intermediate and late time periods.

A key strength of this study is the use of a multivariable analysis, which has not been done by other studies. Other studies of returning travellers typically exclude those without serial liver enzyme measurements or do not adjust for clustering, leaving them vulnerable to selection bias and therefore overestimating the prevalence of liver enzyme abnormalities. Symptomatic

returning travellers are obviously a different group to those enrolled CHMI trials, with returning traveller more likely to have been exposed to many novel pathogens, taken new medications or been exposed to blood borne viruses. The use of a control group allows us to make an accurate assessment on the impact of malaria in returning travellers, because these confounding factors are similar across the two groups. Furthermore, compared to other studies the sample size is quite large, so it should be powered to detect any difference in ALT.

One potential criticism of this study is that the malaria group is not compared to a completely 'normal' group, but instead one that is symptomatic and often presenting to hospital. This was deliberate, as we attempted to control for confounding factors in returning travellers and reduce sources of bias. The non-malaria group will have been exposed to an array of different diagnoses, so we must be cautious when interpreting these results. This limits the generalizability of this study in respect to antimalarial drug development, as this control group does not reflect a population in which drug development occurs, making comparisons challenging. Furthermore, as these are real-life patients and clinical specimens, the liver enzymes available are those from routine clinical care. Consequently, only total bilirubin, and not unconjugated bilirubin was available for analysis, making it challenging to differentiate the effect of malaria, haemolysis and genetic conditions such as Gilbert's syndrome on bilirubin results. Our statistical analysis of this may also be criticized. While this is a retrospective cohort study, we have chosen to use logistic regression as our statistical test of choice. This may be considered controversial because risk ratio is traditionally used as an outcome measure for cohort studies.

This is the first study to utilize multivariable regression analysis in a longitudinal study specifically examining liver enzymes in malaria. In this comparison of malaria exposed and non-exposed returning travellers, the odds of having an abnormal TB are significantly higher in the malaria exposed group in the early period compared to the non-exposed, but no difference is seen for ALT, AST, or ALP. Further prospective studies are required to clarify liver enzymes in the setting of drug development.

CHAPTER 5: APPLICATION OF EPIDEMIOLOGY AND STATISTICS TO MANUSCRIPTS

In this chapter, the application of epidemiological and statistical theory to both of my manuscripts is considered.

Application of Epidemiological and Biostatistical Methods in Chapter 3: Clinical Validation of a Commercial LAMP test for ruling out malaria in returning travellers: A prospective Diagnostic Trial

This manuscript was a diagnostic trial of LAMP, so the primary objective was not to identify an epidemiological relationship between an exposure and outcome in the traditional way. Nevertheless, the first section focused on the relationship between epidemiological risk factors and testing positive for malaria, with the second part of the manuscript focusing on the diagnostic capabilities of LAMP.

To investigate the epidemiological risk factors, a univariable analysis was performed to produce some initial descriptive statistics. Age was a normally distributed variable, so as illustrated in Fifty specimens were repeats from some of the participants who initially tested positive, as it is routine practice to monitor parasitemia during treatment. In the retrospective arm, 29 additional frozen specimens were used to supplement the analysis. The prospective and retrospective specimens were combined, so there were 377 specimens in total.

Microscopy and LAMP were available on all specimens, but RDT testing was only performed on participants in the prospective arm if deemed appropriate (n = 307). Frozen specimens had prior microscopy data at the time of diagnosis. There was an initial "invalid" test rate with LAMP *illumigene* M of 3.18%; however, all of these resolved with a single repeat test.

Table 3, a *t* test comparison of the means compared the age of malaria positive and negative participants. A *t* test is typically used to compare two means, and there must be one independent, categorical variable with two groups and one continuous dependent variable (170, 171). The following formulas are used to calculate it, with $\overline{D} = difference\ between\ the\ means$, $se(\overline{D}) = standard\ error$, and $s_p = pooled\ standard\ deviation$:

$$t_D = \frac{\overline{D} - \Delta_0}{se(\overline{D})}$$

$$se(\overline{D}) = s_p \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}$$

$$s_p = \sqrt{\frac{(n_1 - 1){s_1}^2 + (n_2 - 1){s_2}^2}{n_1 + n_2 - 2}}$$

To ensure that a *t* test is appropriate for use, the following assumptions must be met. The sample must be a simple random sample, it should be normally distributed, a large sample size must be used and the variance in the two groups must be equal in the population (170, 171).

Table 11: Example of 2x2 table

Exposure	**Malaria test positive**	**Malaria test negative**	Total

Fever present	**a**	**b**	a+b
Fever not present	**c**	**d**	c+d
Total	a+c	b+d	a+b+c+d

The other exposure variables found in Table 3 were expressed as categorical variables e.g. female or male, fever or no fever. Exposures and outcome (malaria positivity) were expressed as a 2x2 table similar to the one shown below to allow for the calculation of p values. For this process, a chi-square test was performed where there were 5 or more frequencies in each quadrant of the 2x2 table, or fisher exact if there were fewer than 5. This approach was used because it displayed the proportions for each exposure to the reader while also giving an idea of significance with p values.

The chi-square test is a non-parametric tool designed to analyze group differences when the dependent variable is measured at a nominal level (172). The formula for a chi-square test is shown below:

$$\chi^2 = \sum \frac{(Observed_{cell} - Expected_{cell})^2}{Expected_{cell}}$$

To perform a chi-square test, the study groups must be independent and the categories mutually exclusive (172). The sample size must be large enough so that the value of the contingency cell is 5 or more in at least 80% of the cells. In addition, no cell should have a value of less than one (172, 173).

Following this, a multivariable analysis of the exposures when testing malaria positive was carried out. Odds ratios were chosen as the outcome measure since the starting point for this study was the malaria test result, so it has backwards directionality. Consequently, logistic

regression was chosen as the statistical method to analyze this data. Appendix A(i): The matrix of variables included in multi-variate analysis shows evidence of correlation between pre-travel advice and antimalarial prophylaxis with a value of 0.565. Correlation demonstrates how closely two variables co-vary, varying from -1 (perfect negative correlation) through to 0 (no correlation) to +1 (perfect positive correlation) (174). A value of 0.565 is a moderately positive correlation. Theoretically, this makes sense as participants would require a visit to a health provider for pre-travel advice to obtain antimalarial prophylaxis. As antimalarial prophylaxis has a direct effect on the risk of getting malaria, pre-travel advice is excluded from the regression model. The remaining variables were then included in a regression model with the results shown in Figure 8.

However, contrasting results are noted for the epidemiological variables Chapter 3 and Chapter 4 of this thesis. In Chapter 3, myalgia appears to favour malaria positivity, but no statistically significant difference is seen in Chapter 4, which has a far superior sample size. This raises the possibility that in Chapter 3, the statistically significant result is, in fact, a type I error. This shows the importance of defining clear hypotheses that can be easily defined and tested for. Minimizing the number of statistical tests performed helps reduce type I errors since the error rate is set at 5% per test so they become more likely to occur with repeated statistical tests. Data dredging is thought to be an increasing issue in science, where incentives to publish statically significant results lead to publication bias (175).

The second section of the manuscript focused on the diagnostic performance of LAMP against the gold standard of PCR. For this, sensitivity and specificity were used as a statistical measure

of performance, with positive predictive value and negative predictive value also calculated. The methods used in the calculation of these measures are shown below. The interpretation of sensitivity and specificity is discussed under misclassification bias in chapter 2.

Table 12: Calculation of diagnostic performance

		Gold standard		
		Positive	**Negative**	
Test result	**Positive**	**TP**	**FP**	**PPV** TP/(TP+FP)
	Negative	**FN**	**TN**	**NPV** TN/(FN+TN)
		Sensitivity TP/(TP+FN)	**Specificity** TN/(FP+TN)	
TP = True Positive FP = False Positive FN = False Negative TN = True Negative				

One important thing to note is that PPV and NPV will depend on the prevalence of the disease in the population. In this study, the prevalence of malaria does not reflect the prevalence in the population. Retrospective specimens positive for malaria were added to the sample and repeated tests were performed on malaria positive individuals to ensure parasite clearance. In the study, the true malaria prevalence rate in returning travellers of 4.4% (unpublished data) was used to calculate the PPV and NPV, rather than the study prevalence rate of 14.7%. If the study prevalence was used rather than the baseline population prevalence, PPV for the prospective arm would be 87.7% rather than 65.3%. This indicates how the prevalence of a disease in the population can influence PPV and NPV.

The key outcome in the study is sensitivity because a sensitivity less than 100% reflects that false negative results may occur. This is extremely important in clinical medicine where individuals falsely testing negative for malaria would be discharged from care and come to serious harm. Introducing retrospective specimens to a prospective study design was a slightly unusual but necessary step of this study. Since there are five malaria species, an attempt was made to include all of them in the study. With a prospective study design and a low prevalence of malaria species such as *P. ovale, P. malariae and P. knowlesi* the study would have been insufficiently powered to test the diagnostic performance of LAMP against PCR. Given that the prevalence of malaria in returning travellers is low, adding the retrospective positive specimens creates narrower confidence intervals for the species-specific estimates of sensitivity.

Further consideration must be given to specimen 122 shown in Table 5. *P. ovale* gametocytes were seen by microscopy but *illumigene* tested negative was negative for malaria, with PCR confirming a negative result. The specimen was classified as a false negative for microscopy. However, as seen in Figure 1, gametocytes are not part of the asexual erythrocytic cycle so do not cause disease. In addition, there are different targets for PCR for the asexual erythrocytic stage and gametocytes (176, 177), meaning that PCR used in this context does not target gametocytes. Therefore, while this a classified as a microbiological false negative, gametocytes cannot cause disease so it is not a true clinical false negative. In addition, there is a microbiological explanation as to why these results have occurred.

Critically appraising this section of the paper, the study is prospective in nature, so does provide better evidence of causality than retrospective studies of LAMP. Furthermore, blinding was used to conceal the results of *illumigene*, microscopy, and RDTs from the investigators performing the

PCR analysis. This ensures that they are not influenced by previous results leading to bias. However, a superior study design would include an element of blinding to specimen processing. In the study, the same technologist performed microscopy smears, RDTs and LAMP, and they were not blinded to other results. This was primarily due to practical issues, such as not having enough technologists available to perform each test independently. This potentially led to an element of misclassification bias, with technicians potentially overestimating LAMP performance, particularly if traditional diagnostics were positive. However, since LAMP reports a binary negative or positive, this minimized the chance of misclassification of results.

Application of Epidemiological and Biostatistical Methods in Chapter 4: The impact of malaria on liver enzymes: A retrospective cohort study (2010-2017)

This manuscript focuses on the relationship between malaria infection in returning travellers and liver enzymes. The first part of the manuscript examined epidemiological risk factors and malaria positivity, utilizing similar statistical methods as those in chapter 3. However, there were some subtle differences in the two studies that need to be discussed. In this study the malaria positivity rate was 5.3%, reflecting the prevalence of malaria in symptomatic returning travellers presenting to healthcare professionals over the study period. Additionally, the sample size was larger, with a significant number of malaria negative participants (see Figure 10: Flow diagram of study). In the data collection phase, epidemiological characteristics were collected from malaria history forms, which was extremely time-consuming as the data was collected manually. Therefore, to reduce the time required for data collection, a simple random sample of history forms was taken to achieve a minimum of 4 malaria negative for every malaria positive. This

was chosen because this ratio achieves the greatest power for a case-control study (178), which was felt to be justifiable for this sub-analysis within the overall study design.

Analyzing the relationship between the malaria test result and liver enzymes is more complex. In this case, the starting point of the study was the malaria test, with participants followed up for 31 days and their liver enzymes measured (the outcome).

There were a number of factors that were challenging about the statistical analysis of this study. First, liver enzymes are actually made up of 4 different tests including ALP, ALT, AST, and TB. In clinical medicine, these have different interpretations. They were also measured inconsistently, with AST measured less frequently than the others. ALP is commonly increased by extrahepatic obstructive causes of liver injury, such as choledocholithiasis (179), while total bilirubin can also be increased by haemolysis (179), which is also known to be caused by malaria (146-149). The aminotransferases including ALT and AST are probably the simplest measure of hepatotoxicity. Overall, there is no simple measure of hepatotoxicity than can be used. Other studies have used the term malarial hepatopathy, with TB level > 2 x ULN with associated aminotransferase elevation > 3 × ULN, but the significance of this has never been demonstrated (164). The variability of liver enzymes and the lack of clearly defined end-point in the literature makes hypothesis identification extremely challenging. Consequently, the study attempted to identify the associations of the malaria test result and each of the individual liver enzymes.

Second, raw liver enzyme values are not continuous data and have age and sex-specific upper limits of normal. Also, the vast majority of individuals had normal liver enzymes, with a small

minority having highly abnormal values so the data was not normally distributed. Simple parametric tests such as *t* tests could not be used. Transforming data to a log scale did not create a normal distribution. Therefore, data was dichotomized to be either normal or abnormal. Consideration was given to using multiples of age and sex-specific upper limits of normal with ordinal regression, but it did not appear to change the overall results, so categorical data with logistic regression was used instead.

Third, individuals were followed up for 31 days, but there was no formal end-point on day 31. The liver enzyme measurements are from real-life clinical data, so the vast majority of individuals were discharged before this time and had no further liver enzyme measurements. Liver enzymes were measured on different days for different participants, meaning that a comparison of an outcome at a specific point in time was challenging. Other studies have excluded individuals without serial liver enzyme measurements (164), but this leads to selection bias. Therefore, the data was treated as longitudinal data, also known as panel data, so that all liver enzyme measurements in the study were included in the analysis.

Fourth, the number of liver enzymes for participants was not consistent. Participants who had abnormal liver enzymes were more likely to have them measured than those with normal values, and these subsequent results were more likely to be abnormal. This is serial clustering, where individuals with abnormal liver enzymes are oversampled and without adjusting for this, the result would have been biased (180).

In the final manuscript, a number of different methods were used in the analysis to adjust for the longitudinal nature of the data and the clustering. Initially, the data was split into early (day 0 -3), intermediate (day 4 – 11) and late (day 12 – 31) and a univariate outcome formed by taking the mean value for each participant within each time period. This removed the correlation issue and provided results that are easy to interpret by clinicians. The results of this analysis can be found in Table 8.

One of the main objectives of this study was to adjust for confounding factors by utilizing generalized linear models. To allow for the longitudinal nature of the data and clustering, a mixed model was used. Robust standard errors allowing for intragroup correlation were used because observations were independent across clusters, but not within groups. Missing data was not missing at random, as individuals who were well were discharged from medical care. These individuals were less likely to have their liver enzymes measured so to avoid biasing results, imputation was not used to substitute missing data. Therefore, missing data was simply excluded from the analysis, which is shown in Table 10. Odds ratios were chosen as the outcome measure of choice, despite the fact the study was classified as a retrospective cohort study. Consideration was given to displaying risk ratios, but it was not possible to obtain this while taking the aforementioned considerations into account. Unfortunately, in this study the rare disease assumption does not apply, so the outcome measure has to be interpreted appropriately.

Summary

Malaria diagnostics is a rapidly developing field and there are potential benefits to be accrued from implementing novel diagnostics with a high sensitivity that can obtain results within a short

time period. Currently, the mainstay of malaria diagnosis relies on RDTs and microscopy, with both methods lacking analytical sensitivity. This leads to repeat testing to rule out malaria. In the first manuscript of this thesis, a prospective diagnostic trial of the Meridian *illumigene* Malaria assay (LAMP) was conducted comparing it to reference microscopy and RDT (BinaxNOW Malaria) in returning travellers between June 2017 and January 2018. Returning travellers with signs and symptoms of fever were enrolled in the study. RDT, microscopy and LAMP assays were performed simultaneously. A total of n=298 patients (50.7% male, mean age 32.5) were enrolled, most visiting friends and relatives (43.3%), presenting with fever (88.9%), not taking prophylaxis (82.9%), and treated as outpatients (84.1%). In the prospective arm (n=348), LAMP had a sensitivity of 98.1% (95% CI; 90.0-100) and a specificity of 97.6% (95%; CI 95.2-99.1) versus microscopy. After discrepant resolution with RTPCR, LAMP had a sensitivity of 100% (95% CI; 93.7-100) and a specificity of 100% (95%; CI 98.7-100) versus microscopy. When including retrospective specimens (n=377), LAMP had a sensitivity of 98.8% (95% CI; 93.2-100) and a specificity of 97.6% (95% CI; 95.2-99.1) versus microscopy, and after discrepant resolution of this set, LAMP had a sensitivity of 100% (95% CI; 95.8-100) and a specificity of 100% (95% CI; 98.7-100). After discrepant resolution, RDT had a sensitivity of 83.3% (95% CI; 58.6-96.4) and a specificity of 96.2% (95% CI; 93.2-98.1) versus microscopy. A cost-benefit analysis of reagents and labour suggests up to USD 13 savings per specimen using a novel algorithm with LAMP screening.

In the second manuscript of this thesis, I focused on the relationship between malaria in returning travellers and liver enzymes. It is unclear if malaria causes deranged liver enzymes. This has implications in both clinical practice and also in research, particularly for antimalarial drug

development. A retrospective cohort study of returning travellers (n=4548) who underwent a malaria test and had enzymes measured within 31 days in Calgary, Canada from 2010-2017 was performed. Odds ratios of having an abnormal alkaline phosphatase (ALP), alanine aminotransferases (ALT), aspartate aminotransferases (AST) and total bilirubin (TB) were calculated using multivariable longitudinal analysis with binomial response. After adjusting for gender, age, and use of hepatotoxic medications, returning travellers testing positive for malaria had higher odds of having an abnormal TB [(OR: 12.64, 95% CI: 6.32 – 25.29), p<0.001] but not ALP [(OR: 0.32, 95% CI: 0.09 – 1.10), p=0.072], ALT [(OR: 1.01, 95% CI: 0.54 – 1.89), p=0.978] or AST [(OR: 1.26, 95% CI: 0.22 – 7.37), p=0.794], compared to those who tested negative. TB was most likely to be abnormal in the 'early' period (day 0 – day 3) but then normalized in subsequent intervals. Returning travellers with severe malaria [(OR 2.56, 95% CI: 0.99 – 6.62), p=0.052] had borderline increased odds of having an abnormal TB, but malaria species [(OR 0.70, 95% CI: (0.24 – 2.05), p=0.511] did not. In malaria exposed returning travellers, the TB is abnormal, especially in the early period, but no abnormalities are seen for ALT, AST, or ALP.

Implications of the Use of LAMP in Returning Travellers and Future Recommendations

Currently, to rule out malaria in returning travellers, the Public Health Agency of Canada recommends three microscopy smears, at least eight hours apart (30). As Figure 9 shows, this leads to a long time frame to rule out malaria. This delay is inconvenient for the patient and is associated with increased costs in the laboratory (181). The low LOD for LAMP and high sensitivity means that repeated testing is not required, so patients can be discharged after a single test. Unquantified cost savings can be achieved in the emergency department and to hospitals in

general, in terms of a reduced length of stay or fewer attendances for repeated thick and thin smears. Since this research, LAMP has been implemented as the first line malaria diagnostic across Western Canada. As the body of literature continues to develop, other countries should begin to use LAMP in their malaria testing pathways. Further research is required post-implementation to quantify the direct and indirect savings to the healthcare system.

LAMP appears to be a useful tool in diagnosing malaria in returning travellers, but further research is required in LMICs. Currently, there are two commercially available LAMP tests, *illumigene M* test made by Meridian Bioscience and Malaria-LAMP (also known as LoopAmp Malaria) made by Eiken Chemical Co, LTD and distributed by Human Diagnostics. *Illumigene* has the advantage of a quick and simple extraction method, whereas Eiken allows a greater number of tests to be run at any time, with results read either by turbidity or fluorescence. Both technologies have been tested in returning travellers and in the field in LMICs, and have shown excellent sensitivity (111-114, 117, 120-126). However, barriers remain in terms of cost and practicalities. First, commercial LAMP assays currently require a continuous and stable power supply, something that is a problem in the field. Including a battery or uninterruptible power source would improve the usability in LMICs. Second, a simple extraction method, similar to that used by *illumigene* is required, negating the need for heating or centrifugation. Third, adaptability for high-throughput specimen processing is essential, particularly when considering the use in areas targeting malaria elimination. While high-throughput specimen processing is possible in the field, to date no trials have shown a high sensitivity when using this method in elimination studies (128). Finally, the price point per test is too high for use in resource-limited settings, at approximately 10-25 USD per test. Without evidence to show that outcomes are

improved and therefore justify this increased cost, health systems will probably continue to favour traditional diagnostics. Outcome-based longitudinal studies are required to investigate whether the improved sensitivity alongside portability leads to improved clinical outcomes. If this evidence is obtained, then the extra cost is justifiable. The WHO would be pressured to recommend LAMP as a first-line diagnostic and this creates extra leverage for negotiations with private donors for funding. Private-public partnerships similar to those used to finance Cepheid GeneXpert® cartridges for tuberculosis (TB) diagnosis and rifampicin resistance testing in rural settings may be a way to obtain funding. Under this initiative, key actors and NGOs including the United States President's Emergency Plan for AIDS Relief (PEPFAR), the United States Agency for International Development (USAID), UNITAID and the Gates Foundation reduced the cost of the WHO recommended diagnostic by 40%, allowing its distribution in LMICs (182).

Implications of the Impact of Malaria on Liver Enzymes and Future Recommendations

Artemisinin resistance provides a considerable threat globally, both in terms of increased mortality and morbidity in Africa and also in the fight against malaria elimination (183). Without new antimalarials, malaria elimination will be extremely challenging (59). As previously discussed, there have been difficulties in the development of new antimalarials due to abnormal liver enzymes (137-139, 141-143, 162). From this thesis, it appears that in returning travellers, there is an association between malaria and an increased TB, but not ALP or the aminotransferases. This means that the increases in aminotransferases may be due to NCEs in antimalarial drug development and not malaria itself, as was previously thought. This study has a control group and is the first multivariable analysis of the relationship between malaria and liver enzymes, so it provides higher quality evidence than other studies. This has negative

consequences for drug development, as development of potential drug targets would have to cease if they are hepatotoxic.

In this study, there has been criticism of the comparison group of returning travellers, mainly due to the lack of generalizability to the drug development field. Consequently, further studies are required to fully investigate liver enzymes in this population. Ideally, a prospective randomized controlled trial of malaria infection should be performed, possibly using CHMI but with steps taken to make it reflect natural infection as much as possible. Ideally, the control arm should be administered antimalarials as well because these are known to cause abnormal liver enzymes. However, gaining ethical approval to perform this study will be challenging.

Conclusion

Overall, this research provides useful information regarding diagnostics and liver function in returning travellers. Data presented here supports the conclusion that uncomplicated malaria does not appear to cause abnormal aminotransferase against a control group of symptomatic returning travellers. Further evidence is provided that LAMP assays can be used to screen for malaria in returning travellers, with potential cost savings for the healthcare system.

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APPENDICES

Appendix A – Supplementary material for chapter 3: Clinical Validation of a Commercial LAMP test for ruling out malaria in returning travellers: A prospective diagnostic trial

Appendix A(i): The matrix of variables included in multi-variate analysis

	Pre-travel advice	Africa	VFR	Sex coded	Age	Prophylaxis taken
Pre-travel advice	1					
Africa	0.101	1				
VFR	-0.069	-0.011	1			
Sex coded	-0.024	0.005	0.096	1		
Age	0.068	-0.01	-0.177	0.031	1	
Prophylaxis taken	0.565	0.178	0.073	-0.034	0.02	1

Appendix A(ii): Performance of BinaxNOW RDT versus microscopy on returning travellers in the prospective arm before and discrepant resolution by alternate PCR (n=307)

Before discrepant resolution		**Microscopy**			After discrepant resolution		**PCR**		
		Positive	**Negative**	**Total**			**Positive**	**Negative**	**Total**
LAMP	**Positive**	15	11	26	LAMP	**Positive**	23	3	26
	Negative	3	278	281		**Negative**	3	278	281
	Total	18	289	307[1]		**Total**	26	281	307
		Percentage	**95% CI**				**Percentage**	**95% CI**	
Sensitivity		98.1	90.0 – 100		**Sensitivity**		88.5	69.9 – 97.6	
Specificity		97.6	95.2 – 99.1		**Specificity**		98.9	96.9 – 99.8	
Positive predictive value[2]		65.3			**Positive predictive value[2]**		79.2		
Negative predictive value[2]		99.9			**Negative predictive value[2]**		99.5		

[1]BinaxNOW RDT not performed on all specimens in the prospective arm
[2]Based on a prevalence of 4.4%

Appendix A(iii): Species specific LAMP performance versus PCR for all specimens after discrepant resolution by alternate PCR (n=377)

	Species of malaria				
	P. falciparum	***P. vivax***	***P. ovale***	***P. malariae***	***P. knowlesi***
LAMP true positive	44	13	10	13	6
LAMP false positive	0	0	0	0	0
LAMP false negative	0	0	0	0	0
LAMP true negative	291	291	291	291	291
Other species excluded	42	73	76	73	80
Sensitivity	100 (91.7 – 100)	100 (75.3 – 100)	100 (69.2 – 100)	100 (75.3 – 100)	100 (54.1 – 100)
Specificity	100 (98.7 – 100)	100 (98.7 – 100)	100 (98.7 – 100)	100 (98.7 – 100)	100 (98.4 – 100)
Positive predictive value[1]	100	100	100	100	100
Negative predictive value[1]	100	100	100	100	100
[1]Based on a prevalence on 4.4%					

Appendix B – Supplementary material for Chapter 4: The Impact of Malaria on Liver Enzymes: A Retrospective Cohort Study (2010-2017)

Appendix B(i): Discussion of findings in drug development trials

A meta-analysis of studies investigating the efficacy and safety of ACTs for the treatment of non-severe malaria reported no liver abnormalities among the serious adverse effects events reported (161), but didn't report less severe adverse events. In contrast, in a study of 82 individuals with non-severe malaria receiving a new antimalarial, some individuals suffered from a raised aminotransferase and TB. However, these were mild, transient and rare among the participants (138) and the effect was not dose-dependent, hinting at a direct effect from malaria itself. Solyok et al found only a raised TB but not aminotransferases in a group of 7 in a phase 1 trial receiving a new antimalarial (162), but it is impossible to separate the effect of malaria and the drug in this study. In a sub-study of the West African Network for Clinical Trials of Antimalarial Drugs (WANECAM) phase 3b/4 trials specifically designed to investigate the prevalence of liver abnormalities among individuals ≥ 6 months with uncomplicated malaria, those taking pyronaridine-artesunate (PA) were compared with artemether-lumefantrine (AL) (137). This study found an ALT rise in 28/1015 (3%) and 7/671 (1%) of PA treated and AL treated individuals respectively, with a TB rise in both groups of <1%, suggesting the liver abnormalities are rare in malaria even when taking medications. McCarty *et al* found in a phase II clinical trial of a novel antimalarial ferroquine raised aminotransferases in 3/8 subjects but normal TB, with one having raised aminotransferases administration prior to ferroquine administration but the following two having a temporal association following drug administration (142). Finally, in the setting of a Controlled Human Malaria Infection (CHMI)

study, mild aminotransferases rises in those receiving AL (1/6 individuals) and Malarone (1/7 individuals), but no TB rises (139).

Appendix B(ii): Study design

Individuals undergoing repeated malaria tests within 31 days of the index test were only included once. CLS is a large centralized public microbiology laboratory service covering 1.4 million people. Epidemiological variables including reason for travel, country visited, symptoms and whether the individual sought travel advice or malaria prophylaxis were recorded. Due to the enrolment criteria, testing predominantly occurred in hospital emergency departments but out-patient liver enzymes were also recorded.

Appendix B(iii): Malaria and liver enzyme testing

For malaria testing, venous whole blood samples were collected in EDTA vacutainers from medical centres in Calgary and the surrounding area and transported to Calgary Laboratory Services (CLS). Results were obtained on one of following instruments depending on blood draw location: Cobas 8000 c701 analyzer, Roche Diagnostics, USA; Cobas 8000 c702 analyzer, Roche Diagnostics, USA; Cobas 6000 c501 analyzer, Roche Diagnostics, USA and Vitros 350 analyzer, Ortho Clinical Diagnostic, USA. All machines were calibrated with identical upper limits of normal.

Appendix B(iv): Statistical Analysis

Firstly, the impact of epidemiological risk factors on malaria test results were analyzed. Univariable statistical analysis of epidemiological dichotomous variables was determined using

chi-square or Fisher exact statistical tests with t tests used for continuous variables, then by multivariable logistic regression. Continent visited was split into Africa and non-Africa, and reason for travel was split into those visiting friends and relatives (VFR) and non-VFR. As observed in the correlation matrix (Appendix B(v): Correlation matrix of variables included in epidemiology multivariate regression), there was evidence of correlation between pre-travel advice and prophylaxis taken, so only prophylaxis was utilized in the analysis. Confounding factors such as disease severity and medications taken were included in the model. Time period was included with early, intermediate and late values to ascertain their influence on liver enzyme results. Analysis was performed on liver enzymes individually, with missing data excluded from the model.

For the liver analyses, raw liver results were transformed into age and sex specific multiples of the upper limit of abnormal (xULN). This accounted for the differing upper limits of normal for different genders and age groups. The results for the xULN values were highly positively skewed, so they were dichotomized into either normal or abnormal values. Results for the four liver enzymes were analyzed separately. Results were first split into the early, intermediate and late periods to allow for the generation of descriptive data and identification of time frames. To allow for the generation of results for the univariable analysis, if a participant had multiple values taken within the same time period, the mean value was taken. The prevalence of Hy's law was calculated according to these values. For the multivariable analysis, results were obtained over a 31-day period, so were treated as longitudinal data. Due to evidence of clustering (Appendix B(ix): Graph showing the distribution of mean liver enzyme results for each participant within each time period and Appendix B(x): Correlation matrix of liver enzymes and time periods), logistic regression of binomial longitudinal panel data was performed with robust

standard errors and adjustments for serial correlation. Logistic regression was performed as opposed to ordinal regression because it was felt to be easier to interpret and did not change the study conclusions. Confounding factors such as disease severity and medications taken were included in the model. Time period was included with early, intermediate and late values to ascertain their influence on liver enzyme results. Analysis was performed on liver enzymes individually, with missing data excluded from the model. Ethical approval was obtained from University of Calgary Ethics Board (Ethics ID: REB15-1160).

Appendix B(v): Correlation matrix of variables included in epidemiology multivariate regression

	Gender	Age	Malaria test	VFR	Africa	Pre-travel advice	Prophy-laxis	Fever	Night sweats	Headache	Sore throat	Cough	Myalgia
Gender	1.0000												
Age	0.0086	1.0000											
Malaria test	0.0932	-0.0027	1.0000										
VFR	0.0262	-0.1841	0.0882	1.0000									
Africa	0.0357	0.1026	0.3842	-0.0400	1.0000								
Pre-travel advice	-0.0505	0.0791	-0.1010	-0.1465	0.1532	1.0000							
Prophylaxis	-0.0577	0.1513	-0.0813	-0.1687	0.3163	**0.6300**	1.0000						
Fever	0.0078	-0.1399	0.0646	0.0814	-0.0669	-0.0142	-0.0959	1.0000					
Night sweats	0.0023	0.2745	0.0305	-0.0267	0.0361	0.0388	0.0569	0.1795	1.0000				
Headache	-0.1209	0.1853	0.0991	-0.0591	0.1036	0.0402	0.0769	0.1561	0.2515	1.0000			
Sore throat	-0.0510	0.0539	-0.1238	0.0193	-0.0414	0.0853	-0.0110	0.0932	0.1392	0.1926	1.0000		
Cough	0.0506	-0.1046	-0.0518	0.0698	-0.0767	-0.0425	-0.0858	0.1402	0.0874	0.0758	0.3385	1.0000	
Myalgia	-0.0666	0.2390	0.0575	-0.0725	0.0770	0.0675	0.0539	0.1193	0.1518	0.2655	0.0937	-0.0355	1.0000

Appendix B(vi): Odds ratios and their corresponding 95% Cis for determinants of malaria positivity

Variable	AOR (95% CI)	p values
Male	2.26 (1.23 – 4.15)	0.009
Age > 16 years old	1.28 (0.52 – 3.18)	0.595
Visiting friends and relatives	1.58 (0.88 – 2.82)	0.123
Travelled to Africa	19.47 (9.73 – 38.95)	<0.001
Malaria prophylaxis taken	0.20 (0.10 – 0.41)	<0.001
Fever	2.26 (0.90 – 5.68)	0.082
Night sweats	0.96 (0.51 – 1.83)	0.912
Headache	2.27 (1.20 – 4.30)	0.012
Sore throat	0.13 (0.05 – 0.36)	<0.001
Cough	0.94 (0.47 – 1.90)	0.868
Myalgia	0.95 (0.51 – 1.74)	0.858
Other	1.09 (0.58 – 2.06)	0.779

Appendix B(vii): Correlation matrix of variables included in ALT and TB multivariate regression

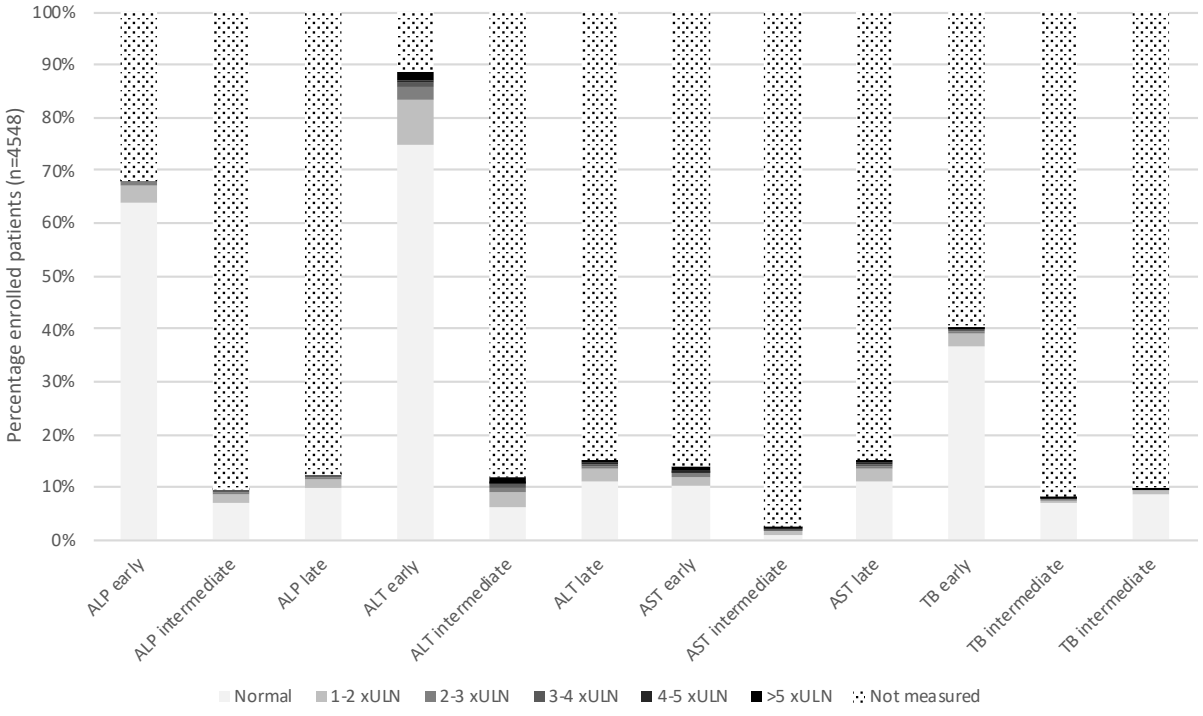
	Gender	**Adults**	**Malaria test**	**Hepatotoxic medication**	**Severity**	***P. falciparum***	**Time period**
Gender	1.0000						
Adults	0.0075	1.0000					
Malaria test	0.0523	-0.0016	1.0000				
Hepatotoxic medication	-0.0782	0.0651	-0.0025	1.0000			
Severity	0.0605	-0.0410	N/A	0.0052	1.0000		
P. falciparum	0.0703	0.0436	N/A	0.1594	0.2298	1.0000	
Time period	-0.0192	-0.0801	-0.0363	-0.1913	-0.0099	-0.1327	1.0000

Appendix B(viii): Median fold-upper limit of normal (xULN) and interquartile range (IQR) values for liver enzymes for all species of malaria

	Time period	Malaria positive n, median (IQR)	Malaria negative n, median (IQR)	p value[1]
ALP	Early	n = 210, 0.52 (0.24)	n = 2888, 0.53 (0.23)	0.170
	Intermediate	n = 22, 0.62 (0.26)	n = 409, 0.66 (0.59)	0.307
	Late	n = 23, 0.56 (0.20)	n = 532, 0.63 (0.38)	0.161
ALT	Early	n = 221, 0.48 (0.48)	n = 3816, 0.49 (0.41)	0.896
	Intermediate	n = 28, 0.76 (0.85)	n = 510, 0.92 (1.52)	0.559
	Late	n = 27, 0.48 (0.57)	n = 655, 0.57 (0.65)	0.152
AST	Early	n = 38, 0.75 (0.69)	n = 602, 0.70 (0.54)	0.957
	Intermediate	n = 7, 1.19 (3.95)	n = 111, 1.25 (2.76)	0.946
	Late	n = 3, 1.06 (4.26)	n = 153, 0.83 (0.96)	0.439
TB	Early	n = 175, 0.67 (0.58)	n = 1665, 0.33 (0.29)	<0.001
	Intermediate	n = 28, 0.48 (0.30)	n = 353, 0.35 (0.46)	0.050
	Late	n = 24, 0.51 (0.42)	n = 426, 0.33 (0.37)	0.006

[1]Mann–Whitney U test

Appendix B(ix): Graph showing the distribution of mean liver enzyme results for each participant within each time period



Appendix B(x): Correlation matrix of liver enzymes and time periods

	ALP early	ALP inter-mediate	ALP late	ALT early	ALT inter-mediate	ALT late	AST early	AST inter-mediate	AST late	TB early	TB inter-mediate	TB late
ALP early	1.0000											
ALP intermediate	**0.8954**	1.0000										
ALP late	0.5527	0.5046	1.0000									
ALT early	0.0493	-0.2202	-0.2101	1.0000								
ALT intermediate	0.0658	-0.1836	-0.2583	**0.9934**	1.0000							
ALT late	0.0354	-0.2340	0.5531	0.1358	0.0418	1.0000						
AST early	0.0551	-0.1722	-0.1849	**0.9849**	**0.9872**	0.1116	1.0000					
AST intermediate	0.3864	0.0313	-0.1472	**0.8173**	**0.8179**	0.0601	**0.7384**	1.0000				
AST late	0.0225	-0.1419	0.6811	-0.1981	-0.2695	**0.8923**	-0.1875	-0.2498	1.0000			
TB early	0.3594	-0.0579	0.3682	0.5091	0.4481	0.8009	0.4574	**0.6090**	0.5794	1.0000		
TB intermediate	-0.1622	-0.1742	0.2863	**0.7587**	**0.6848**	**0.6915**	**0.7142**	**0.6340**	0.3509	**0.8258**	1.0000	
TB late	0.1794	-0.1837	0.5146	0.3625	0.2690	**0.9193**	0.2931	0.3997	**0.7160**	**0.9083**	**0.8497**	1.0000

Appendix B(xi): Proportion of abnormal liver enzymes for severity of malaria, use of hepatotoxic medication and species of malaria

	ALP n (%)			**ALT n (%)**			**AST n (%)**			**TB n (%)**		
Time period	Early	Intermed-iate	Late	Early	Intermed-iate	Late	Early	Intermed-iate	Late	Early	Intermed-iate	Late
Severity of malaria												
Non-severe	4/136 (2.94)	2/17 (11.76)	1/16 (6.25)	28/139 (20.14)	10/21 (47.62)	5/20 (25.00)	8/26 (30.77)	4/7 (57.14)	2/2 (100.00)	26/108 (24.07)	2/21 (9.52)	3/18 (16.67)
Severe	1/37 (2.70)	0/2 (0.00)	0/4 (0.00)	8/39 (20.51)	2/3 (66.67)	0/4 (0.00)	2/7 (28.57)	0/0 (0.00)	0/1 (0.00)	18/39 (46.15)	0/4 (0.00)	1/4 (25.00)
p value	0.939	1.000	1.000	0.960	1.000	0.544	1.000	N/A	0.333	0.010	1.000	1.000
Hepatotoxic medication												
No medication	86/1647 (5.22)	61/254 (24.02)	61/331 (18.43)	330/2133 (15.47)	144/331 (43.50)	107/409 (26.16)	96/382 (25.13)	42/70 (60.00)	38/93 (40.86)	100/989 (10.11)	41/222 (18.47)	42/277 (15.16)
On medication	118/1451 (8.13)	55/177 (31.07)	45/224 (20.09)	336/1904 (17.65)	109/207 (52.66)	66/273 (24.18)	82/258 (31.78)	27/48 (56.25)	21/63 (33.33)	77/851 (9.05)	24/159 (15.09)	19/173 (10.98)
p value	0.001	0.104	0.625	0.063	0.039	0.559	0.065	0.685	0.341	0.441	0.388	0.208
Species of malaria												
Non-P. falciparum	2/62 (3.23)	1/9 (11.11)	0/9 (0.00)	15/67 (22.39)	4/13 (30.77)	2/11 (18.18)	3/16 (18.75)	2/4 (50.00)	0/0 (0.00)	11/47 (23.40)	1/16 (6.25)	3/8 (37.50)
P. falciparum	5/142 (3.52)	1/13 (7.69)	1/14 (7.14)	26/145 (17.93)	8/14 (57.14)	3/15 (20.00)	8/20 (40.00)	2/3 (66.67)	1/2 (50.00)	37/122 (30.33)	1/12 (8.33)	1/16 (6.25)
p value	1.000	1.000	1.000	0.445	0.252	1.000	0.277	1.000	N/A	0.371	1.000	0.091

If multiple tests done on participants within same time frame, mean of results used
All by chi-square or Fisher exact, statistically significant differences shown in bold

Appendix B(xii): Odds ratios and their corresponding 95% CIs and p-values for selected determinants of having abnormal liver enzymes if malaria positive

Liver assay	**ALP**		**ALT**		**AST**		**Bilirubin**	
Number of observations	359		392		48		322	
Number of Individuals	177		185		38		156	
Mean obs per individual	2.0		2.1		1.3		2.1	
	Odds ratio	p value	Odds ratio	p value	Odds ratio	p value	Odds ratio	p value
Gender (male)	0.22 (0.02 – 2.16)	0.193	0.41 (0.10 – 1.70)	0.219	Insufficient data		3.28 (1.18 – 9.17)	0.023
Age (adult)	3.70 (0.10 – 141.60)	0.481	0.21 (0.03 – 1.67)	0.140			0.57 (0.16 – 2.03)	0.390
Hepatotoxic medication (taken)	1.24 (0.17 – 8.88)	0.828	2.13 (0.46 – 9.93)	0.337			0.69 (0.27 – 1.81)	0.455
Disease severity (severe)	0.29 (0.02 – 3.98)	0.351	0.83 (0.13 – 5.31)	0.848			2.56 (0.99 – 6.62	0.052
Species (f*alciparum*)	0.40 (0.04 – 3.73)	0.421	2.87 (0.53 – 15.56)	0.222			0.70 (0.24 – 2.05)	0.511
Early time period (reference)	1.00		1.00				1.00	
Intermediate time period	0.56 (0.03 – 9.89)	0.690	2.10 (0.28 – 15.94)	0.472			0.12 (0.03 – 0.50)	0.004
Late time period	0.66 (0.03 – 13.51)	0.787	0.20 (0.02 – 1.71)	0.142			0.31 (0.07 – 1.27)	0.102

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