2019-04

Designing and development of multiplex rapid diagnostic test platforms for health-related point-of-care applications in the developing world

Seth, Misago

NM-AIST

DESIGNING AND DEVELOPMENT OF MULTIPLEX RAPID DIAGNOSTIC TEST PLATFORMS FOR HEALTH-RELATED POINT-OF-CARE APPLICATIONS IN THE DEVELOPING WORLD

Misago Seth

A Dissertation Submitted in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Life Sciences and Bioengineering of the Nelson Mandela African Institution of Science and Technology

Arusha, Tanzania

April, 2019

ABSTRACT

Accurate and timely diagnosis is usually an important step towards appropriate disease management. In resource-limited settings, healthcare workers lack proper facilities to perform vital tests, and the diagnosis of disease is often determined by non-specific, physiological symptoms alone. Simple and rapid tests are needed as pre-requisite tools for patient care and must conform to the criteria set out by the World Health Organization (WHO). Such tests should be affordable, sensitive, specific, user-friendly, robust, equipment-free, and deliverable to the needy, and abbreviated as ASSURED diagnostics. To address the challenge of specific diagnosis for diseases that present with similar symptoms, multiplex diagnostic platforms must be designed. The use of paper has been extensively studied for its potential as matrix for microfluidic devices. In fabrication of multiplexed microfluidic devices, patterning is an important step. Various approaches including cutting, photolithography, wax-printing, plotting and etching have been developed and tested on paper. In recent years, the focus has also been directed towards exploring the potential of hydrophilic threads as a convenient and low-cost approach for fabrication of microfluidic channels and as signal substrate. This research was aimed at designing multiplex diagnostic test platforms that could be used at point-of-care especially in resource-limited settings. The first approach was to design multiplex paper-based platforms where paper was patterned using a combination of cutting and wax-printing. In the second approach, ordinary thread was used to create microfluidic channels and paper discs were used for sample loading, reagent storage and results display to develop a colorimetric test platform that was demonstrated using glucose, uric acid and bovine serum albumin. In the third design, thread was used in combination with nitrocellulose membrane to develop an immunochromatographic test platform for infectious diseases diagnosis and was tested using *Helicobacter pylori* (bacteria), Hepatitis B surface antigen (viral antigen), and Immunoglobulin G (antibody). Thread-based designs were evaluated for short-term viability under normal storage conditions. All designs produced rapid results with the lowest limit of detection for antigens being as low as 30 ng/ml for immunochromatographic tests. Treatment of hydrophobic threads improved their wicking, with mercerization being the most efficient with wicking rate up to 42 cm/minute. Reagents, including enzymes and antibodies were found to remain viable in dried form on membranes and paper discs. Developing multiplexed paper- and thread-based diagnostic platforms offers a potential approach in developing ASSURED diagnostics to address health challenges in resource-limited settings. Efforts should be directed towards transforming the proposed designs into viable products that can be used at point-of-care.

Keywords: Point-of-care diagnostics, Multiplex devices, paper-based diagnostic platforms, low-cost diagnostics, ASSURED diagnostics, immunochromatographic, thread-based diagnostics, thread-on-a-tape, thread-based devices, diagnostics, infectious diseases, microfluidic devices, point-of-care, low-cost diagnostics

DECLARATION

I, **Misago Seth**, do hereby declare to the Senate of Nelson Mandela African Institution of Science and Technology that this dissertation is my own original work and that it has neither been submitted nor being concurrently submitted for degree award in any other institution.

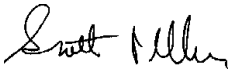
Misago Seth _____ **Date:**_____

Name and signature of candidate

The above declaration is confirmed

Prof. Joram Buza _____ **Date:** _____

Supervisor 1, School of Life Science and Bioengineering, Nelson Mandela African Institution of Science and Technology, Arusha, Tanzania

Prof. Scott Phillips  **Date:** _____

Supervisor 2, Micron School of Materials Science and Engineering, Boise State University, United States

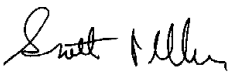
COPYRIGHT

CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance by the Nelson Mandela African Institution of Science and Technology the dissertation titled: **Designing and Development of Multiplex Rapid Diagnostic Platforms for Health-related Point-of-Care Applications in the Developing World** by Misago Seth in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Life Sciences and Bioengineering of the Nelson Mandela African Institution of Science and Technology.

Prof. Joram Buza _____ **Date:** _____

Supervisor, School of Life Science and Bioengineering, NM-AIST, Tanzania

Prof. Scott Phillips  **Date:** _____

Supervisor, Micron School of Materials Science and Engineering, Boise State University, United States

ACKNOWLEDGEMENT

First and foremost, I thank the Almighty God who gives me life and strength to meet with my daily challenges.

The studies which led to the production of this thesis received financial support from the Tanzania Comission for Science and Technology (COSTECH) through the Nelson Mandela African Institution of Science and Technology (NM-AIST) Graduate Scholarship Programme. I am very grateful to COSTECH and NM-AIST for this support which provided me with such a great opportunity to pursue my career dreams.

It is my pleasure to thank my supervisors Professors Joram Buza and Scott Phillips for their excellent supervision. The journey was long and tortuous, but with their dedicated moral and material support, this work has been completed. Prof. Buza is sincerely thanked for his continuous support and alternative plans at critical times, whereas Prof. Phillips is thanked for hosting part of my initial laboratory work and training within his research group "The Phillips Group" at Penn State University.

The laboratory work was carried out at Penn State, NM-AIST and national Institute for Medical Research (NIMR) Tanga Centre. I therefore, thank the Phillips Group at Penn State, especially Prof. Scott Phillips and Greg Lewis for their support while learning the art of paper-based microfluidic devices fabrication. I would like to thank my colleagues in the Life Science laboratory (NM-AIST) and Amani Biomedical Research Laboratory (NIMR-Tanga) for their cooperation and assistance during my lab work on development and evaluation of thread-based diagnostic platforms.

I am deeply indebted to the National Institute for Medical Research (NIMR), my employer for granting me a fully-supported study leave and permission to pursue my studies. On that behalf, let me sincerely thank Dr. Martha M. Lemnge, Dr. Deus Ishengoma and Prof. John Lusingu who at different times during the course of my training were Centre Directors at NIMR Tanga; Dr. Mwelecele Malecela (the former) and Prof. Yunus Mgaya, the incumbent NIMR Director General.

Finally, my sincere gratitude goes to my family: My wife Pendo, our daughter Brightness and our son Brian for their patience as they had to endure long periods of my absence during my studies. Thank you so much Pendo, Brightness and Brian for your understanding and coping. I will forever appreciate your immense contribution towards my achievements. I also

appreciate the moral, material and psychological support from my other relatives. May God bless you very much.

Many people played individual or collective role of one kind or other, knowingly or unknowingly and it is impossible to mention all of them. I therefore, extend my hand of appreciation to all of you and say: “Thank you very much and may God continue to bless you abundantly”.

DEDICATION

This dissertation is dedicated to my beloved family.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

ARIs	Acute Respiratory Infections
ASSURED	Affordable, Sensitive, Specific, User-friendly, Robust, Equipment-free, Deliverable (to end users)
BSA	Bovine Serum Albumin
COSTECH	Commission for Science and Technology (Tanzania)
CT	Computerized Tomography
HBsAg	Hepatitis B Surface Antigen
HP	*Helicobacter Pylori*
HRP	Horseradish peroxidase
ICAT	Immunochromatographic Assay on Thread
ICH	International Conference on Harmonisation
IgG	Immunoglobulin G
ILFT	Immunochromatographic Lateral Flow Test
KCMC	Kilimanjaro Christian Medical Centre
MoHCDGEC	Ministry of Health, Community Development, Gender, Elderly, and Children
mRDTs	malaria Rapid Diagnostic Tests
MRI	Magnetic Resonance Imaging
MSD	Medical Stores Department
NAI	Naturally-Acquired Immunity
NC	Nitrocellulose
ng/ml	nanograms per millilitre
NIMR	National Institute for Medical Research
NM-AIST	Nelson Mandela-African Institution of Science and Technology
NMFIs	Non-Malarial Febrile Illnesses
OD	Optical density
PBS	Phosphate-Buffered Saline
PCD	Passive Case Detection
POC	Point-of-Care
RDT	Rapid Diagnostic Test
STIs	Sexually-transmitted Infections
USA	United States of America

UTI	Urinary Tract Infections
WHO	World Health Organisation
µPADs	*microfluidic* Paper-based Analytical Devices
µTAD	*microfluidic* Thread-based Analytical Device
µTPAD	*microfluidic* Thread-and Paper-based Analytical Device

CHAPTER ONE

1.0 General Introduction

1.1 Background Information

1.1.1 Preamble

Accurate diagnosis is pre-requisite for appropriate management of any problem, disease or condition since the problem must be known before it can be solved. Health facilities in developing countries face acute shortages of diagnostic options. Significant efforts are therefore directed towards development of affordable technologies in order to enable low-resource communities to improve healthcare, animal care, food quality, and environmental safety (Acharya *et al.,* 2004; Aluwong and Bello, 2010). Simple and low-cost diagnostic technologies are very important for improving health-care, and for making health-care more accessible and affordable in developing nations and resource-limited settings. However, making these diagnostics affordable and accessible to the poor, remains a major challenge (Whitesides, 2009). This work therefore, attempts to address this challenge through designing and testing low-cost multiplex diagnostic platforms that can be used at point-of-care for diagnostic purposes in developing world settings.

1.1.2 Diagnostic approaches to infectious diseases

Diagnosis of infectious diseases can be achieved through a variety of ways ranging from history and clinical presentation (signs and symptoms), simple rapid tests, molecular tests and even advanced technology imaging approaches. Apart from history and clinical presentation, various tests ranging from simple microscopy, histopathology, immunohistochemistry, immunochromatography, and various forms of imaging techniques such as x-rays, ultrasound, computerised tomography (CT) scanning and magnetic resonance imaging (MRI) may be applied depending on initial evaluation. Others may include urinalysis, microbial culture, flow cytometry, and many more.

In hospitals equipped with modern facilities, the diagnostic process is often thorough, with high probability for establishment of accurate/definitive diagnosis since most of the above diagnostic options are available. This influences treatment choice and outcome. However, the situation is different in resource-limited setting where even basic diagnostic capacity is lacking. In such settings, clinicians rely on patient history and clinical presentation to make diagnosis in most cases. As a result, inaccurate diagnosis often leads to improper or uninformed

treatment, which jeopardises chances of complete and timely recovery.

1.1.3 Diagnostic resource limitations and the need for ASSURED diagnostics for developing countries

Diagnosis based on history and clinical signs alone, is most of the times inaccurate due to non-specificity of clinical signs and symptoms (Sonkar *et al.,* 2016; Sonkar *et al.,* 2017), as well as inability of the patients or parents to recall and clearly explain the symptoms. Due to this drawback, more specific diagnostic procedures must always be instituted to complement clinical diagnosis. In resource-limited parts of the world, choice of diagnostic algorithm is also limited. In such settings, the use of advanced technology diagnostic procedures requiring highly trained personnel, expensive laboratory equipment and reliable power supply is highly unrealistic. This is because in these settings, it is currently almost impossible to fulfil the conditions required for high technology, expertise-demanding diagnosis. Simple diagnostics are therefore required to overcome this problem. To ensure that these tests are useful to those living in remote, low-resource areas in developing countries, such tests must conform to the requirements set by the World Health Organization (WHO). The tests must fulfil the WHO's ASSURED criteria for ideal diagnostic tests for developing countries. The test must be *Affordable* by the target group, *Sensitive* (few false-negative results or with lowest limit of detection), *Specific* (few false-positive results), *User-friendly* (simple to perform by persons with little training), Rapid and robust, Equipment-free and Deliverable to end users (must not require special storage and transportation conditions such as refrigeration) (Urdea *et al.,*2006; Peeling *et al.,* 2006). Current approaches to addressing these challenges focus on lateral flow and microfluidic paper-based devices (Koczula and Gallota, 2016; Yang *et al.,* 2017). These devices are fabricated using different forms of paper as medium for diagnosis. Such paper forms include chromatography paper, glass fiber, and nitrocellulose membrane. These papers are modified by various patterning methods to allow flow of small volumes of reagents in desirable directions towards reaction zones where test results are read. Such patterning methods include photolithography (Martinez *et al.,* 2008a), plotting (Bruzewicz *et al.,* 2008), ink etching (Abe *et al.,* 2008), plasma etching (Li *et al.,* 2008), cutting (Fenton *et al.,* 2009) and wax printing (Carrilho *et al.,* 2009; Lu *et al.,* 2009). In some cases cutting is used in combination with other methods such as wax printing (Seth and Buza, 2017) but these methods are sometimes used independent of each other (Lewis *et al*., 2014). Lateral flow assays have been in the market for a long time now. Although most of the microfluidic approaches have not yet resulted in commercial products, effort is being directed towards achieving this goal.

Apart from paper, recent research is also focusing on the use of thread as wicking and reaction medium for simple, low-cost diagnostics or sensors (Li *et al.*, 2009; Nilghaz *et al*., 2013). Several studies have demonstrated the potential for thread-based devices for blood grouping (Ballerini *et al.,* 2011a), infectious diseases pathogen detection and clinical chemistry (Li *et al.,* 2009). This approach obviates the need for patterning described above for paper microfluidic devices hence may reduce cost and time required for assembly of these devices (Li *et al.,* 2009). Microfluidic thread-based analytical devices (μTADs) may also be combined with paper platforms to make microfluidic thread-and-paper-based analytical devices (μTPADs) for increased versatility (Gonzalez *et al.,* 2016).

1.1.4 Success stories using ASSURED diagnostics

Simple multiplex platforms for low-cost diagnostics are finding application in diagnosis of different diseases or conditions. They have been used in areas of infectious diseases diagnosis for medical and veterinary fields (Nielsen *et al*., 2008; Rohrman *et al.,* 2012; Kamphee *et al.*, 2015), urinalysis for non-infectious health biomarkers and other chemicals e.g. detection of glucose, proteins, ketones, drug abuse, nitrite, bilirubin, leucocytes (Martinez *et al*., 2007; Reches *et al*., 2010; Li *et al*., 2010a, b), bio-threats such as detection of anthrax, plague, tularemia, nerve agents, ricin and botulism (Shyu *et al.,* 2002; Gessler *et al.,* 2007; Zasada *et al.,* 2015), food safety such as detection of mycotoxins, drug residues, food allergens, food-borne pathogens (Toldra and Reig, 2006; Shankar *et al.,* 2010; Law *et al*., 2014; Weng *et al*., 2016), environmental safety monitoring such as monitoring chemical or microbial contaminations in soil, water and air (Hossain *et al*., 2012; Sicard *et al.,* 2015), fertility testing such as pregnancy diagnosis and sperm quality evaluation (Matsuura *et al.,* 2014), and many more applications.

1.2 Research problem and justification of study

Diagnosis is the first step towards treatment of a disease or correction of any unfavourable condition, but is a major challenge in developing countries. In public health arena, it is important that health facilities are well equipped to properly discharge their roles. Failure to accurately diagnose the cause of problem always results in wrong treatment or management. Health facilities in most developing countries or resource-limited settings are poorly equipped as they have poor diagnostic facilities, less trained personnel and unreliable or no power supply to run available equipment or help in cold storage of diagnostic reagents that require refrigeration or freezing. Most people in such settings therefore, face acute shortage

in diagnostic services and healthcare providers have fewer options during provision of health services. Due to this fact, many conditions afflicting people are poorly diagnosed, and this results into poor disease management and outcomes.

To address this problem and save many lives in resource-limited settings, low-cost diagnostic tests need to be developed. Apart from being sensitive and specific, these tests must be affordable to poor people and user-friendly, so they can be used with minimal training. Paper- and thread-based diagnostics have shown to be promising in addressing this problem. Using paper and threads, simple, microfluidic diagnostic tests can be designed and developed. Such simple technologies do not require expensive and highly specialised manufacturing equipment therefore can be applied in areas where resources are limited.

Despite the progress made in identification and testing of paper and threads as new substrates for low cost POC diagnostics, gaps remain in the knowledge and its application towards development of such devices. One of the issues that need to be addressed are the contemporary challenges in diagnosis of febrile illnesses in the current era, following decline in malaria especially in areas where malaria has always been the predominant cause of febrile illness. Decline of malaria comes with challenges associated with detection of sub-microscopic malaria infections, diagnosis of malaria using mRDTs, diminishing naturally acquired immunity due to reduction in low-dose infective mosquito bites, and the attitude and practice of the community and health workers towards malaria decline.

Reports have recommended multiplexing paper-based diagnostics as one of the best ways to develop affordable and robust diagnostic platforms that are highly needed in resource-limited settings. Despite this fact, there is limited evidence of devices that have been made available for use. Therefore, more effort is required to explore all potential avenues in order to realize this goal.

Apart from paper, threads have also emerged as promising alternatives in fabrication of POC diagnostics. Most thread-based device platforms explored have employed the thread as both the distribution channel for sample and reagents as well as detection or display zone. A few have utilised paper disc as results display zones for colorimetric devices, while none have attempted to combine thread and nitrocellulose or nylon membranes for immunochromatographic devices. This approach is potential for optimal exploitation of thread strength as well as binding and contrast advantage of the nitrocellulose or nylon membranes. If well optimized, it will generate devices with high contrast, hence easy to read even by those

with limited expertise. Combining thread and paper discs is meant to achieve the same goal, taking advantage of the great contrast afforded by paper discs when used in colorimetric tests. Since threads are relatively new as substrates or matrices for fabrication of rapid diagnostics, it is necessary to explore the durability and viability of the devices they produce in order to assess their potential for producing ASSURED diagnostics.

This work describes simple technologies for designing and manufacturing of low-cost diagnostics using thread and paper. The developed technologies are suitable for use in most remote parts of the world to improve health conditions of people through improved disease diagnosis and hence appropriate and timely management.

1.3 Objectives

1.3.1 General objective

The main objective of this study was to design, develop and evaluate low-cost multiplex test platforms for diagnostic applications in the developing world

1.3.2 Specific objectives

i. To review the challenges in diagnosis of febrile illnesses in Tanzania in the era of declining malaria
ii. To design multiplex double-antibody sandwich immunochromatographic lateral flow platforms for point-of-care (POC) diagnostics
iii. To design a multiplex *thread-on-a-tape* colorimetric device for diagnosis of important health biomarkers
iv. To design a multiplex *thread-on-a-tape* immunochromatographic test for point-of-care detection of infectious diseases
v. To assess short-term stability/viability of thread-on-tape devices under ambient tropical conditions

1.4 Significance of the research

This study highlights promising future in the field of development of point-of-care diagnostics in Tanzania. ASSURED diagnostics developed will have direct application in areas like differential diagnosis of febrile illnesses, neonatal and maternal septicaemia, ante-natal sexually-transmitted diseases screening, acute bacterial and viral diarrheal syndromes. Platforms designed will undergo further development and improvement stages and be used for developing target-specific tests with potential for clinical applications. Controlled testing will

be carried out to assess performance in terms of sensitivity and specificity. Prototypes that pass set criteria may be deployed for use in areas where diagnostic infrastructure or capacity is limited.

The test principles and the capacity developed during this study will form a baseline on which more applications aimed at development of diagnostic tests for infectious and non-infectious conditions will be built. The scope includes but is not limited to disease conditions characterised by immunological responses, detection of microbial, anti-microbial and chemical contamination of animal-derived foods such as milk, meat and eggs, and monitoring environmental contamination by pathogens, pesticides and heavy metals. Most of the designs described here utilize simple materials that are cheap, readily available and used in daily household applications like threads, needles, adhesive tapes, glue, and paper. Moreover, this work is the first of its kind in Tanzania and methods used require only moderate training, making them easy to adopt and adapt in developing countries such as Tanzania, where both infrastructural and trained personnel resources are limited. This will therefore stimulate more diagnostic development since the simple technology applied can easily be transferred to local scientists with minimal training, to facilitate local development and manufacture of necessary diagnostics. Therefore, this project serves as a foundation for building capacity in research, development and production of affordable diagnostics in Tanzania.

CHAPTER TWO

Challenges in Diagnosis of Febrile illnesses in Tanzania in the Era of Declining Malaria[1]

Abstract

Malaria and other febrile illnesses are very common especially in children in developing countries. Due to reliance on clinical algorithms for diagnosis in resource-poor settings, most febrile episodes have always been attributed to malaria. However, continuous malaria monitoring and recent improvements in malaria diagnosis have revealed a progressive decline in malaria and significant involvement of non-malarial etiologies in most febrile cases. This paper highlights the situation of malarial and non-malarial fevers, challenges facing the health sector, and possible approaches to addressing these challenges for better diagnosis of non- malarial febrile illnesses in Tanzania.

Key words: Non-malarial fevers, febrile illness, malaria, diagnostic challenges, Tanzania

[1]*American Journal of Research Communication*, 2015, **3**(5): 88-110} www.usa-journals.com, ISSN: 2325-4076.

2.1 Introduction

Malaria is ranked among the leading causes of morbidity and mortality in Africa and other malarial endemic regions with fever being the predominant clinical manifestation (Luxemburger *et al.,* 1998; Muhe *et al.,* 1999; Chandramohan *et al.,* 2002; Snow *et al.,* 2005; WHO, 2009). Diagnosis of febrile episodes using fever as the sole criteria is somewhat cumbersome as many illnesses are accompanied by elevated axillary body temperatures. Studies conducted over 5-10 years ago have estimated the number of annual fever episodes among African children to be as high as 870 million (Snow *et al.,* 2003; Snow *et al.,* 2005; Gething *et al.,* 2010a; Gething *et al.,* 2011), a large proportion of which has been associated with malaria. However, these data have been found to overestimate malaria burden due to the lack of specificity of a purely clinical diagnosis. Studies have revealed a diminishing contribution of malaria to the febrile illnesses whose prevalence remains high (D'Acremont *et al.,* 2010; Bisoffi and Buonfrate, 2013; Crump *et al.,* 2013; D'Acremont *et al.,* 2014a). With the current decline in malaria transmission in many of the malaria endemic regions, the preponderance of febrile episodes could therefore be attributed to non-malarial febrile illnesses (NMFIs) (Okiro and Snow, 2010; Acestor *et al.,* 2012; Bisoffi and Buonfrate, 2013). Since most fevers without any other obvious cause were in the past almost considered malarial, identification of alternative causes of fever has always been perceived as unnecessary and causes of NMFIs have remained mostly unknown. Today, the world has made a significant step in terms of malaria diagnosis. With deployment of malaria rapid diagnostic tests (mRDTs), over-diagnosis, and consequently over-treatment of malaria has been reduced and the contribution of non-malarial etiologies to febrile illnesses has now been recognized (D'Acremont *et al.,* 2010). Some studies reported a proportion of non-malarial fevers ranging between 57% to above 90% (D'Acremont *et al.,* 2010; Gething *et al.,* 2010b). With malaria having been ruled out and without sufficient means to specifically reach etiologic diagnosis of the febrile conditions (Petti *et al.,* 2006), clinicians often find themselves in a dilemma under such circumstances. Consequently, clinical management is often driven by syndrome-based guidelines employing empiric treatment (WHO, 2011; Baltzell *et al.,* 2013). In the next sections, we discuss the situation of malarial and non-malarial fevers, challenges facing the health sector, and possible approaches to addressing these challenges for better diagnosis of NMFIs in Tanzania.

2.2 Febrile illnesses among children and adults in Tanzania

Febrile illnesses among Tanzanian children and adults are due to a wide range of etiologies including malaria and other non-malarial causes. A recent study on malaria passive case detection (PCD) in communities of Korogwe District in Tanga, north-eastern Tanzania, has revealed that despite a significant decline in malaria prevalence in these communities, fever prevalence remains high (Rutta *et al*., 2012). In this study, the malaria positivity rate in 15 729 febrile cases was 20% in 2006, but progressively declined to below 10% in 2009 while fever cases remained unchanged (over 40% for children under five, and over 20% for children older than five (Rutta *et al*., 2012). This study did not identify the causes of non-malarial fevers, but its findings underscore the fact that even though malaria has declined, the prevalence of fever cases remains high. For example, a 2013 hospital-based study in the same district investigating causes of fever in children aged 2 to 59 months showed multiple etiology of febrile illnesses with respiratory infections occurring in more than 60% of cases (Mahende *et al*., 2014). Other syndromes include gastroenteritis (>21%), bacteremia and urinary tract infections (>21%) and malaria (8%). More than 26% of patients were found to be co-infected with more than one pathogen in this study (Mahende *et al*., 2014).

A separate study in rural and urban Tanzania investigated causes of fevers in children up to 10 years of age. As in other studies, acute respiratory infections were by far the most common aetiology (62%) followed by malaria (11%), gastroenteritis (10%) and urinary tract infection (6%). Others included typhoid fever, mucosal infections, meningitis, and some with no known causative organism (D'Acremont *et al.,* 2014b). Furthermore, hospital-based studies investigating all cases admitted as severe malaria cases at Bombo (Tanga), Magunga (Korogwe), and KCMC (Kilimanjaro) hospitals revealed that a considerable proportion of admissions were malaria negative on microscopy (Reyburn *et al.,* 2004; Msangeni *et al.,* 2011). Specific tests on bacteremia patient samples revealed infection with *Salmonella typhi*, *Streptococcus s*pp, *Staphylococcus spp*, non-typhi Salmonella, brucellosis, rickettsiosis, leptospirosis and others (Biggs *et al.,* 2011, 2013, 2014; Msangeni *et al.,* 2011; Prabhu *et al.*, 2011; Crump, 2012; Crump *et al.,* 2013; D'Acremont *et al.,* 2014a; D'Acremont *et al.*, 2014b; Mahende *et al.*, 2014). It should also be noted that not all cases were positive on blood culture, indicating that most of the patients were infected with other groups of pathogens such as viruses, protozoans, fungi as well as other bacteria that do not grow well on blood cultures and whose detection methods were beyond the scope(s) or objective(s) of these studies (Msangeni *et al.,* 2011). It is apparent that febrile illnesses are still highly prevalent in Tanzania despite

the reported decline in malaria. However, unlike the situation in the past, frequent causes of current febrile illnesses are non-malarial.

2.3 Diagnostic dilemma of non-malarial fevers

Accurate diagnosis is a prerequisite for appropriate management of any disease or condition. Therefore, diagnostic facilities should be strengthened in order to make sure that appropriate treatment is instituted for the right disease. Failure to meet the above standards usually leads to wrong treatments, poor outcomes, and an increased risk for development of drug resistance. While the above is true, the Tanzanian health sector is still facing a challenge of poor quality diagnostic facilities, which are contributing to difficulties in disease management (Ishengoma *et al*., 2009; Ishengoma *et al*., 2010). Some of the ongoing challenges facing health systems in Tanzania with respect to diagnosis of malarial and non-malarial fevers are discussed below.

2.3.1 Sub-microscopic malaria infection

Microscopic examination of Giemsa-stained blood slides remains the gold standard and the most widely-used laboratory tool for diagnosis of malaria. Although mRDTs provide a more convenient option at point-of-care, and nucleic acid-based detection and identification techniques offer more sensitivity and specificity, it is microscopy that has continued to be the mainstay for malaria diagnosis in settings where resources are available. This is because, while mRDTs are rapid and user-friendly and require no specialized training, equipment, or power to read the results, they are relatively new in application and some of the original versions lacked the ability to distinguish different species of plasmodium. Most mRDTs also are qualitative, and products from different suppliers vary in sensitivity and specificity (Forney *et al.,* 2003; Wongsrichanalai *et al.,* 2003a; Wongsrichanalai *et al.,* 2007). Nucleic acid-based detection methods are more sensitive and specific than microscopy, but require expensive and highly specialized equipment, highly trained personnel and continuous power supplies (Coleman *et al.,* 2006; Nicastri *et al.,* 2009; Santana-Morales *et al.,* 2012). Most of these essential conditions are limited or missing in most low-resource (developing) countries where malaria is endemic.

Despite the usefulness of microscopy as a tool for malaria diagnosis, its relatively high limit of detection is a concern. The established theoretical limits of detection of malaria parasites by microscopy lie between 5-20 parasites per microliter of blood (Dowling and Shute, 1966; Bruce-Chwatt, 1984; Payne, 1988; Babiker and Schneider, 2008). However, due to a number of factors including sample collection, processing, slide reading, equipment, and malaria incidence and prevalence, the practical limit of detection is over 100 parasites per microliter of

blood (WHO, 1988). This detection limit implies that microscopy may miss lower density infections, especially in endemic areas and in areas where malaria is declining. For example, it has been found that about 88% of all infections are missed in areas where malaria prevalence measured by PCR is less than 10% (Okell *et al.*, 2009). This proportion of patients with parasite levels below the limit of detection by microscopy are referred to as patients with submicroscopic malaria infection (parasitemia).

Studies have demonstrated that submicroscopic parasites contribute substantially to malaria transmission (Babiker *et al.*, 2008; Karl *et al.*, 2011). Although the proportion of febrile infections as a result of submicroscopic parasite density has not been well characterized, the possibility has been highlighted (Snounou, 1993; Cohee *et al.*, 2014). The contribution of submicroscopic parasites to transmission intensity as well as febrile episodes is likely to significantly increase in areas of declining malaria burden as a result of diminishing naturally acquired immunity to malaria (Doolan *et al.*, 2009). It has also been shown that, apart from acting as reservoirs of transmission, submicroscopic infections could have an impact on hemoglobin levels (Mockenhaupt *et al.*, 2000; Cohee *et al.*, 2014). Febrile cases due to submicroscopic parasites are expected to be few, but whenever they occur, they are likely to be missed by conventional microscopic diagnosis. Such cases will be considered to be non-malarial and patients will receive inappropriate treatment, hence minimizing their chances of timely and complete recovery, and thus further propagating not only the malaria transmission cycle, but also promoting antimicrobial resistance.

2.3.2 The use of malaria rapid diagnostic tests (mRDTs)

Along with microscopic examination of Giemsa-stained thick and thin blood smears, mRDTs are currently the best available and affordable alternative means of diagnosing malaria, especially in resource-poor settings (Ochola *et al.*, 2006; Ishengoma *et al.*, 2011; Mubi *et al.*, 2011; Minja *et al.*, 2012; Santana-Morales *et al.*, 2012). The use of mRDTs significantly facilitates targeted malaria treatment and reduces over-treatment with anti-malarials (Ishengoma *et al.*, 2011; Mubi *et al.*, 2011; Rutta *et al.*, 2012). These tests are useful because they do not require sophisticated equipment or complicated procedures or training, and can be stored at room temperature, which enables their use in areas where refrigeration is lacking. However, most mRDTs are not ideal. Their sensitivity and specificity under different manufacturing, storage, and operating conditions vary (Iqbal *et al.*, 2003; Ishengoma *et al.*, 2011). Varying performance implies that under certain circumstances, mRDTs may fail to accurately diagnose malaria, thus compromising the choice of treatment. Low sensitivity

results in failure to detect malaria parasites in an infected patient (false negative), while low specificity results into over-diagnosis, hence overtreatment of malaria. It is paramount that mRDTs are sufficiently accurate for proper diagnosis, which is crucial in effecting optimal management of fever patients. Fortunately, in most cases their sensitivity and specificity are sufficiently high for *Plasmodium falciparum* (Iqbal *et al.,* 2003; Pattanasin *et al.,* 2003).

Some brands of mRDTs are designed to detect only one Plasmodium species and may be the best choice in areas where that species is the predominant cause of malaria. For example, most cases in Sub-Saharan Africa are caused by *Plasmodium falciparum* (Snow *et al.,* 2005; Hay *et al.,* 2009; Gething *et al.,* 2011). In other parts of the world like Asia, North and South America, the Middle East, North Africa, and the South Pacific, *P. vivax* is the predominant malaria parasite (Li *et al.,* 2001; Oliveira-Ferreira *et al.,* 2010). However, between these extremes, other minor species such as *P. ovale,* and *P. malariae* also are present and cannot be ignored. For this reason, some of the mRDTs being developed today are capable of detecting non- falciparum malaria (Ashton *et al.,* 2010). This diversity in malaria etiology poses a challenge to health care workers in developing countries, and sometimes leads to improper diagnosis. In Tanzania for instance, mRDTs are usually provided by the Ministry of Health, Community Development, Gender, Elderly, and Children (MoHCDGEC) through the Medical Stores Department (MSD) (Chipwaza *et al.,* 2014a). If the stock delivered to health facilities is capable of detecting *P. falciparum* only, then any non-falciparum malaria will be missed and may be treated symptomatically as a non-malarial febrile case. Therefore, an ideal mRDT would be highly sensitive, specific, and capable of detecting all common causes of malaria in a particular epidemiological and clinical setting (Mouatcho and Goldring, 2013). Despite favourable mRDT performance parameters, malaria diagnosis still poses a substantial challenge resulting from potential concomitant infection with other causes of fever (Chipwaza *et al.,* 2014a). When other pathogens are involved, mere demonstration of malaria parasites may not be conclusive as to what treatment should be applied to the patient (Wongsrichanalai *et al.,* 2003b; Brouqui *et al.,* 2005; Keong and Sulaiman, 2006; Uneke 2008; Agwu *et al.,* 2009). In malaria endemic countries, infection with *Plasmodium spp* does not necessarily result in malaria, since individuals may harbor malaria parasites without becoming ill. Therefore, for every febrile case, a thorough check-up should be performed to confirm malaria or rule out non-malaria causes of fever. Unfortunately, in low-resource countries like Tanzania, healthcare facilities are ill-equipped to make this distinction.

2.3.3 Diminishing naturally acquired immunity to malaria and its potential role in fever of malarial origin

Naturally acquired immunity (NAI) to malaria has been associated with non-sterile protection against malaria disease in individuals who have been infected by malaria parasites (Doolan *et al.,* 2009; Richards *et al.,* 2010; Douglas *et al.,* 2011; Pinkevych *et al.,* 2012). This immunity may last for years but can be overcome. For example, exposure to high infectious doses of the parasites, changes in the genetics of the parasite resulting in more virulent forms and compromised immune systems all may overcome and thus result in diminished naturally acquired immunity.

In these situations, endemic stability is likely to be disrupted. In recent years, the world has observed a substantial drop in malaria incidence and prevalence across most of the malaria-endemic regions (Mmbando *et al.,* 2010; O'Meara *et al.,* 2010; Ishengoma *et al.,*2013; Mboera *et al.,* 2013), with the exception of malaria hotspots where no decline has been observed or reported. In certain areas, this decline has been occurring for more than 15 years (Ishengoma *et al.,* 2013). If withdrawal of continuous exposure to low-dose infective bites is sustained over a long period, then NAI subsequently diminishes, which will render adults susceptible to malaria (Doolan *et al.,* 2009). Unpublished data from Korogwe, Tanzania show a time lag in acquisition of natural immunity to malaria in children from the age of 5 to the age of 10 years and above.

Apart from predisposing the population to rebound epidemics (Doolan *et al.,* 2009; Ursing *et al.,* 2014), diminished immunity will shift the endemic stability balance. The level of parasite dose to which the population is tolerant will drop in accordance with diminishing natural immunity. As a consequence, certain levels of parasites that previously resulted in asymptomatic infection could eventually cause clinical cases of fever (Doolan *et al.,* 2009). Some fever cases may even be caused by parasite levels below detection limits of current rapid diagnostics and microscopy. Although greatly confounded by the non-malarial scenario, this is a possibility that needs to be investigated, and which should be addressed by developing more sensitive tests for both malarial and non-malarial febrile illnesses.

2.3.4 Knowledge, attitudes and practices of the community and health workers regarding non-malaria fevers

Although the prime clinical manifestation of malaria is fever, malaria is not the only disease

that presents with fever (Punjabi *et al.,* 2012; Acestor *et al.,* 2012; Baltzell *et al.,* 2013; Bisoffi and Buonfrate, 2013; Ishengoma *et al.,* 2013; Mayxay *et al.,* 2013; D'Acremont *et al.,* 2014a; D'Acremont *et al.,* 2014b). It is therefore important to distinguish all cases of fever and correctly diagnose them to their respective etiologies or groups in order to institute appropriate treatment. However, the current state of affairs attributes most febrile conditions as malaria. A significant proportion of community members in Tanzania and other countries do not have appropriate knowledge about febrile conditions and most believe that fever and malaria are synonymous (Baltzell *et al.,* 2013; Chipwaza *et al.,* 2014a, b). Only a small proportion was able to associate fever with true causes such as respiratory and urinary tract infections (Chipwaza *et al.,* 2014a). Due to this lack of knowledge, most febrile patients or parents and guardians of febrile children expect a positive malaria test. When they are given negative results, sometimes they do not accept them. They would rather have their children given antimalarial drugs even if not diagnosed with malaria. This situation also leads to self-medication with antimalarial drugs, with or without consulting healthcare professionals (McCombie, 2002; Kunda *et al.,* 2007; Chipwaza *et al.,* 2014b). In studies involving community members, most admitted that they did not know other causes of fever apart from malaria (Crump, 2012; D'Acremont *et al.,* 2014a).

Healthcare workers face a dilemma in diagnosis and management of febrile illnesses. Clinical diagnosis of fever based on symptoms and signs is not sufficient to render appropriate treatment. The use of mRDTs helps in definitive identification of positive malaria cases but has no use for negative cases. Non-malarial febrile illnesses due to bacteria, protozoa, fungi and viruses, for example typhoid fever, urinary tract infections (UTI), acute respiratory tract infections (ARIs), rotavirus infection, brucellosis, tick-borne relapsing fever, leptospirosis, dengue fever, and Chikungunya virus infection have been reported in Tanzania (Schoonman and Swai, 2009; Prabhu *et al.,* 2011; Crump *et al.,* 2013; D'Acremont *et al.,* 2014b; Mahende *et al.,* 2014), however there is limited diagnostic capacity for non-malarial fever etiologies. Thus, healthcare workers always resort to clinical diagnosis or symptomatic treatment of febrile cases (Chipwaza *et al.,* 2014a).

2.4 What should be the way forward?

2.4.1 Create community awareness on the existence of non-malarial febrile aetiologies

It is important for patients and the general community to understand the existence of febrile etiologies other than malaria. Healthcare providers should play a leading role in this education.

Patients and the general community also should be made aware that a negative malaria test of a febrile person does not necessarily mean that the method, device, or personnel doing the testing is incompetent. It also does not mean that the patient 'is not ill'. Instead, a negative test creates the need for further testing to establish the real cause of the febrile illness in the patient. Such awareness can be accomplished at different levels. First, healthcare providers could educate patients during consultation sessions after receiving test results regardless of the status of the test. Second, the health system should offer alternative diagnosis for non-malarial fever cases. It is certainly not assuring when, having all indications of febrile illness, a patient is told to go home without being treated simply because *only one* test was found to be negative. To meet this requirement, researchers and scientists must continue to explore all possible causes of febrile disease in different epidemiological settings. Third, mass education through local and regional media should be employed to strengthen awareness and knowledge about the existence of non-malarial febrile illnesses, and the approaches for diagnosing and treating them.

2.4.2 Strengthen diagnostic systems for malaria and create options for diagnosis of other causes of fever

Most of what appears to be the non-malarial fever dilemma in Tanzania and other low-resource countries has gradually resulted from over-dependence on clinical algorithm for malaria diagnosis (Font *et al.,* 2001; Reyburn *et al.,* 2004; Petti *et al.,* 2006; Craig *et al.,* 2010). Lack of diagnostic infrastructure or resources, trained personnel, instruments such as microscopes, and accompanying reagents has always made it impossible to correctly diagnose malaria in the developing world. Slight improvements in malaria diagnostics coupled with recent declines in malaria infections revealed that not all febrile cases are due to malaria (Snounou *et al.*, 1993; Wongsrichanalai and Miller, 2002; Murray *et al.,* 2003; Wongsrichanalai *et al.,* 2007; Van Den Ende *et al.,* 2010; Reyburn 2010; Yukich *et al.,* 2010; Mouatcho and Goldring, 2013). It is therefore proposed that improved diagnostics be extended to remote areas where such capacity is not available. Building malaria diagnostic capacity in health facilities will help discriminate true malarial and non-malarial fevers, thereby enabling accurate, effective and optimal malaria case management. However, improved malaria diagnostics will be only one step towards success. Capacity needs to be built or strengthened to enable diagnosis of alternative causes of fever in malaria-negative febrile patients (Pang and Peeling, 2007; Chappuis *et al.,* 2013). To ensure that these tests are useful to those living in remote, low-resource areas in developing countries, universal tests must conform to the requirements set by the World Health Organization (WHO). WHO has developed the ASSURED criteria for

ideal diagnostic tests. The test must be *Affordable* by those at risk of infection, *Sensitive* (few false-negative results), *Specific* (few false-positive results), *User-friendly* (simple to perform by persons with little training), Rapid and robust, Equipment-free and Deliverable to those who need it (Peeling *et al.,* 2006; Urdea *et al.,* 2006). Current research is directed towards understanding the aetiology of non-malarial febrile illnesses in different epidemiological settings. Most of these studies employ advanced molecular and serological techniques that are expensive, time-consuming, requiring trained personnel and in most cases, electric-powered equipment (D'Acremont *et al.,* 2014b; Mahende *et al.,* 2014). Although these techniques are useful in pathogen identification, most of them are impractical to be used at point-of-care. This underscores the need to develop simple, highly sensitive, and specific tests that meet the WHO ASSURED criteria in order to reach and serve resource-limited parts of the world where they are needed most. An example of such efforts employs the use of microfluidic paper-based analytical devices (µPADs) (Mabey *et al.,* 2004; Yager *et al.,* 2006; Chin *et al.,* 2007; Martinez *et al.,* 2010). Using paper ensures cost reduction and flexibility for patterning where photolithography (Martinez *et al.,* 2008a), plotting (Bruzewicz *et al.,* 2008), ink etching (Abe *et al.,* 2008), plasma etching (Li *et al.,* 2008), cutting (Fenton *et al.,* 2009) and wax printing (Carrilho *et al.,* 2009; Lu *et al.,* 2009) have potential as patterning methods for µPADs. Paper has potential as a diagnostic platform because it is readily available and inexpensive, can wick and distribute aqueous fluids, and has been in use for a long time as an analytical platform. Paper can be modified to incorporate functional groups, can easily be transported and stored, is compatible with most printing technologies, and can easily be disposed by incineration. Paper is therefore a potential starting material for making ASSURED diagnostics (Martinez *et al.,* 2010; Phillips and Lewis, 2014).

2.4.3 Develop multiplex rapid diagnostic tests for alternative causes of febrile illness

The WHO recommends rapid diagnostics that are affordable. This means that they should be made using cheap, readily available materials and simple technology so that the overall cost of a finished product should not deter the poor from using the service. Multiplexing the tests will also help. Even in cases where simple and cheap diagnostic tests for unconventional fever aetiologies are available, most of these diseases are so unknown or overlooked in the developing world that it may imply a trial-and-error approach to arrive at the right diagnosis taking one disease at a time after malaria has been excluded. This act of sequential testing may be slow, and during this time the patient's condition may deteriorate. It may also become prohibitively expensive to most people with limited resources, since the cost of each test will

quickly reach an unaffordable range. These setbacks may be overcome if multiplexed, low-cost, rapid diagnostic tests that are capable of simultaneous detection of multiple pathogens are developed. Prototype μPADs have shown potential for multiplexing and also may have favourable performance. This type of flexibility should be utilized in designing multiplex tests for common causes of febrile illnesses in Tanzania and other low-resource countries where malarial and non-malarial febrile illnesses are common. Ongoing research provides crucial information about common causes of fever in children and adults and in different geographical and epidemiological settings. This information should be taken into consideration while planning any multiplex prototype. With all other WHO guidelines met, a diagnostic that is capable of multiplexing will greatly enhance affordability by cutting the overall cost of otherwise sequential multiple testing in non-malarial febrile patients.

2.5 Conclusion

Febrile illnesses continue to cause significant problems in developing countries, especially in children under five years of age. Despite its historical dominance, malaria parasites are not the only cause of febrile conditions; the actual contribution of malaria has been diminishing over the past decade. Continuous malaria monitoring and improvement in malaria diagnosis has uncovered significant contributions from non-malarial causes of febrile illnesses. This observation has created a new challenge since most healthcare outlets in low-resource countries, like Tanzania, are not equipped with the capacity to accurately diagnose non-malarial febrile illnesses at point-of-care. Instead, they usually rely on patient or parent history and presenting clinical signs and symptoms. Therefore, while there is still a need to develop sensitive diagnostics for malaria in order to meet new challenges resulting from low parasitemia and decline in naturally acquired immunity to malaria, there also is an urgent need to (a) create knowledge and awareness of the community regarding non-malaria fevers, and (b) intensify efforts to develop point-of-care diagnostics for non-malarial febrile illnesses in order to improve diagnosis and management of these diseases in developing countries.

CHAPTER THREE

Multiplex Paper-based designs for Point-of-Care (POC) Diagnostics[2]

Abstract

Introduction: Accurate and timely diagnosis is usually the first step towards appropriate disease management. In resource-limited settings, healthcare workers lack proper facilities to perform vital tests, and the diagnosis of disease is often determined by non-specific, physiological symptoms alone. Simple and rapid tests are needed as prerequisite tools for patient care and must conform to the criteria set out by the World Health Organization. To address the challenge of specific diagnosis for diseases that present with similar symptoms, multiplex diagnostic platforms must be designed.

Methods: Simple designs for multiplexed paper-based diagnostic platforms were developed. Laser-cutting and wax printing were used to create specific patterns on paper to guide the flow of sample and reagents towards reaction zones. Multi-arm and single strip multiplex platforms were designed and tested using Human Immunoglobulin G, Hepatitis B virus surface antigen (HBsAg), and *Helicobacter pylori* antigens.

Results and Conclusion: All designs produced promising results with the lowest limit of detection for antigens being 30 ng/ml for single strip designs. The use of glass fibre conjugate pad was found to be more sensitive compared wax-printed chromatography paper. Results from this study indicate great potential for further application in development of diagnostic low-cost paper-based diagnostic devices.

Keywords: Point-of-care diagnostics, Multiplex devices, paper-based diagnostic platforms, low-cost diagnostics, ASSURED diagnostics

[2]*International Journal of Research in Medical Sciences* (2017), **5**(11):5052-5059
DOI: http://dx.doi.org/10.18203/2320-6012.ijrms20174969

3.1 Introduction

Appropriate patient care or disease management is highly dependent on timely and accurate diagnosis, which is insufficiently available in resource-limited settings, culminating into symptomatic diagnosis and treatment most of the time (Petti *et al.,* 2006; Yager *et al.,* 2006). Simple and rapid tests are needed as prerequisite tools for patient care and must conform to the 'ASSURED' criteria set out by the World Health Organization (WHO). An ideal test must be *Affordable* by those at risk of infection, *Sensitive* (few false-negative results), *Specific* (few false-positive results), *User-friendly* (simple to perform by persons with little training), Rapid/robust, Equipment-free and Deliverable to those who need it (Mabey *et al.,* 2004; Peeling *et al.,* 2006; Urdea *et al.,* 2006).

In recent years, paper has emerged as an important substrate for fabrication of low-cost, simple diagnostics for use in limited-resource settings (Lu *et al.,* 2009; Li *et al*., 2012; Yetisen *et al.,* 2013). Desirable features of paper include its ubiquitous abundance, mechanical strength, and flexibility. Paper can wick and distribute aqueous fluids, and has been in use for a long time as an analytical platform. Also, paper can be modified to incorporate functional groups, can easily be transported and stored, is compatible with most printing technologies, and can easily be disposed by incineration (Martinez *et al.,* 2010; Phillips and Lewis, 2014). Cellulose-based materials such as chromatography and filter papers have found wide applications in making dipsticks and microfluidic paper-based analytical devices (Pelton, 2009) while nitrocellulose membranes are mostly used in Lateral flow immunoassays (Mansfield, 2009).

There are cases where multiple diseases or conditions need to be diagnosed together or differentiated, especially if observable symptoms are similar. The best example is the case of febrile illnesses in malaria-endemic regions (Bisofi and Buonfrate, 2013; Crump *et al.,* 2013; D'Acremont *et al.,* 2010; D'Acremont *et al.,* 2014; Seth *et al.,* 2015). Patients predominantly present with fever symptoms and cannot be accurately diagnosed without a specific test. When the most common aetiology of fever (malaria) is ruled out, identifying the exact cause of fever is usually done clinically in most cases (Chipwaza *et al.,* 2014a), or if resources allow, through sequential testing of the likely fever aetiologies to reach definitive diagnosis.

Designing multiplexed test devices is therefore very important for enhancing timely diagnosis as well as minimizing the cost that would otherwise be incurred if sequential testing was to be performed (Seth *et al.,* 2015). Using paper, various patterning methods have been

described and tested in previous studies. These methods include cutting (Fenton *et al.,* 2009), wax printing (Carrilho *et al*., 2009; Lu *et al*., 2009), photolithography (Martinez *et al.*, 2008a), plotting (Bruzewicz *et al.,* 2008), ink etching (Abe *et al*., 2008) and plasma etching (Li *et al*., 2008), among others. Apart from clinical applications, other point-of-need applications for multiplex paper-based devices may include their use in qualitative/semi-quantitative detection of antimicrobial residues in milk and other foods of animal origin where other testing methods are currently being applied (Ferguson *et al*., 2002; Pikkemaat *et al*., 2009) and screening for environmental contamination with heavy metals (Lewis *et al.*, 2014; Wang *et al.*, 2014). Of all the above applications, the use of these devices for point-of-care diagnosis at health outlets to facilitate patient care in the developing world remains the most compelling. In this article, simple designs for multiplexed paper-based diagnostics where a combination of laser-cutting and wax-printing was used to create channels and barriers to direct the flow of sample and reagents on paper are described. The designs were tested using Human Immunoglobulin G, Hepatitis B virus surface antigen (HBsAg), and *Helicobacter pylori*. These were selected on the basis of their availability and diversity comprising of immunoglobulin, virus and bacterial targets.

3.2 Materials and Methods

3.2.1 Antibodies and antigens

Antibodies and antigens used in this study were obtained from Arista Biologicals, Allentown, PA, USA. Conjugate antibodies used were monoclonal mouse anti *H. pylori* colloidal gold conjugate, clone 4 (CGHPY-0704), monoclonal mouse anti HBsAg colloidal gold conjugate, clone 2 (CGHBS-0702), and goat anti human IgG colloidal gold conjugate (CGIGG-0500). All conjugate antibodies were conjugated to a 40 nm colloidal gold particle at a concentration of 10 µg/ml (OD540=10). Capture antibodies used were mouse anti *H. pylori* antibody (ABHPY- 0403), Goat anti HBsAg (ABHBS-0500) and Goat anti human IgG (ABIGG-0500). Invitro purified proteins from H. pylori, recombinant HBsAg strain produced in *pichia pastoris*, and Human IgG purified by immunoaffinity from whole goat antisera, were used as antigens for *H. pylori*, Hepatitis B and IgG, respectively. There was no positive control since testing was done using purified antigens. The device platforms were designed using glass fiber pads, chromatography paper and nitrocellulose membrane. Chromatography paper and fiberglass were used as sample and conjugate pads while nitrocellulose was used as capture membrane. Three different designs are described in this paper.

3.2.2 Multi-channel multiplex designs

In the first design, conjugate pad and nitrocellulose membrane were both cut using laser cutter per the plots made on a computer using Corel 7 drawing software (Fig. 1A). In the other two designs, sketches drawn using computer program were printed on chromatography paper using Xerox 7560 solid ink (wax) printer to create hydrophobic barriers and hydrophilic chambers and channels for deposition and flow of conjugate reagents and sample (Fig. 1 B and C). Printed paper was heated in hot-air oven for 30 seconds at 150ºC with the printed side facing up to melt the wax through the paper thereby creating continuous channels and barriers. The designs were tested by assembling a device for simultaneous detection of *Helicobacter pylori* (bacteria), Hepatitis B viral antigen S (virus), and Human Immunoglobulin G. Each arm of the conjugate pad was treated with 2.5 µl of 10% BSA in PBS and allowed to dry at room temperature for 5 minutes and then at 50ºC for 10 minutes. Stock conjugate antibody (OD10) was diluted one fifth in conjugate buffer (2% w/v BSA, 10% w/v sucrose and 0.1% v/v Tween 20 in Borate buffered saline). This was followed by addition of 5µl of gold conjugate antibody to the conjugate pad, each arm with a specific antibody type. The pads were dried at room temperature for 5 minutes and then at 50^{O}C for 10 minutes as above. Dried conjugate pads were kept in sealed bags with desiccant ready for device assembly.

The nitrocellulose (capture) membranes appropriately cut to fit the design were treated with the specific capture antibodies. Capture antibodies were diluted in PBS (pH 7.4) to a concentration of 0.5 mg/ml, with 5% methanol included to enhance fixation of antibodies to the membrane. A total of 3 µl were applied to the membrane in small quantities (0.5 µl per round). The membrane was dried at room temperature for 2 minutes followed by 10 minutes at 50º C. This was followed by addition of 2 µl of blocking buffer (0.1% v/v Tween 20, 5% w/v sucrose and 1% w/v BSA) per arm, and then dried as above. Assembly of the multichannel device was accomplished by first sticking the nitrocellulose membrane on an adhesive tape/plastic backing card placed on a flat surface. At the distal end of each arm, appropriately cut absorbent filter papers were placed on top of the membrane in an overlapping fashion to serve as wicking or absorbent pads. This was followed by overlaying the conjugate (or conjugate cum sample) pad at the center position on top of the membrane. For the first design (Fig. 3A), a sample pad was placed on top of the fiberglass conjugate pad to complete the device assembly. Assembled devices were tested using a serially diluted mixture of target antigens to establish the limits of detection of each against respective antigens.

3.2.3 Single channel multiplex design

The aim was to assess the possibility of combining tests for various analytes on a single strip, comprised of a single conjugate pad and capture (nitrocellulose) membrane. In this design, gold-conjugated antibodies specificto target antigens Human immunoglobulin G, Hepatitis B, and *Helicobacter pylori* (all from Arista Biologicals, Allentown, PA, USA) were blotted on a single piece of glass fiber pad either individually or as pooled solution. In the first approach 5 µl of each gold conjugate was applied on the pad, followed by the next until all were added. The pad was dried between additions, first at room temperature for 5 minutes, followed by 2 minutes at 50ºC in an oven. After the final addition, the conjugate pad was dried for a further 10 minutes at 50ºC and kept dry until use. Alternatively, all conjugates were mixed together to make one pooled solution. About 10-15 µl of the conjugate mixture was applied to the conjugate pad and dried as above. Prior to addition of conjugates, the glass fiber pad was pre-treated with 5 µl of 10% BSA and dried as described above. Capture antibodies were diluted in PBS (pH 7.4) and 5% methanol to a working concentration of 0.5 mg/ml. A total of 3 µl of each antigen-specific capture antibody were applied in small quantities (0.5 µl per round) at specifically identified test zones on the membrane. The membrane was dried at room temperature for 2 minutes followed by 10 minutes at 50ºC. The membrane was blotted with 2 µl of blocking buffer (0.1% v/v Tween 20, 5% w/v sucrose and 1% w/v BSA) and dried as above. The two methods of conjugate pad treatment were compared by running a sample of antigen mixture at the concentration of 30 µg/ml and response was observed within 10 minutes after sample addition.

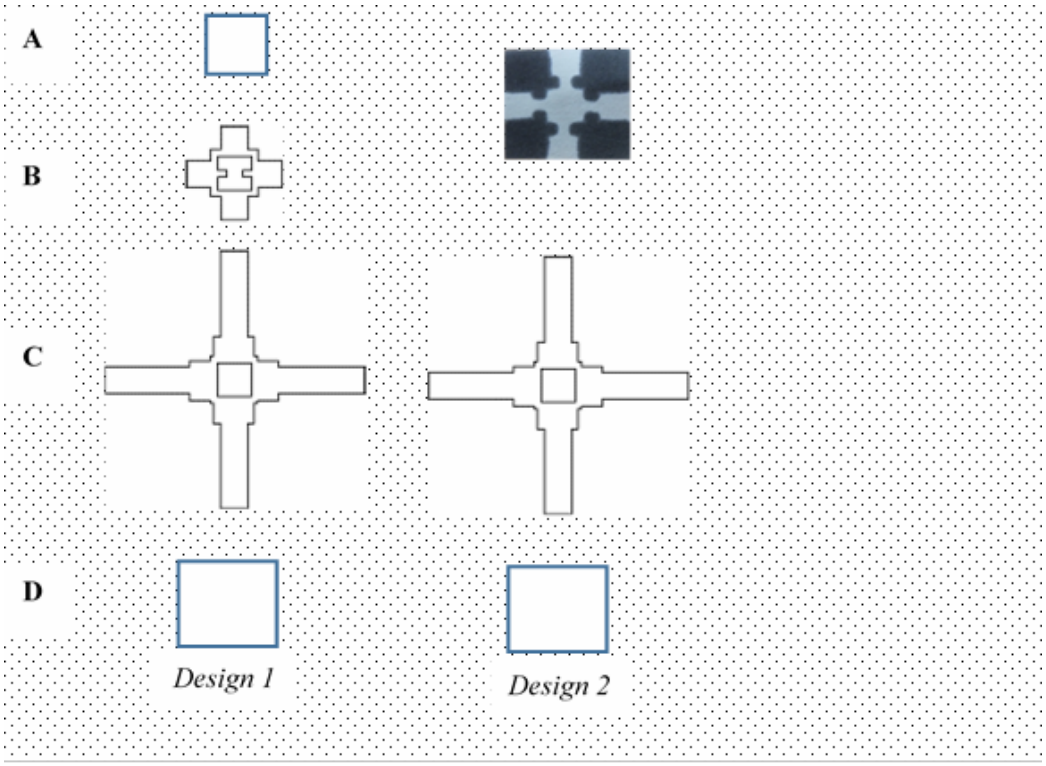


Figure 1. Components of four-channel multiplex designs (laser-cut and wax-printed)

Sample pad (A), conjugate pad (B), lateral flow nitrocellulose membrane (C), and absorbent pad (D). Note that for design 2, both the sample and conjugate pads are printed with wax on same paper.

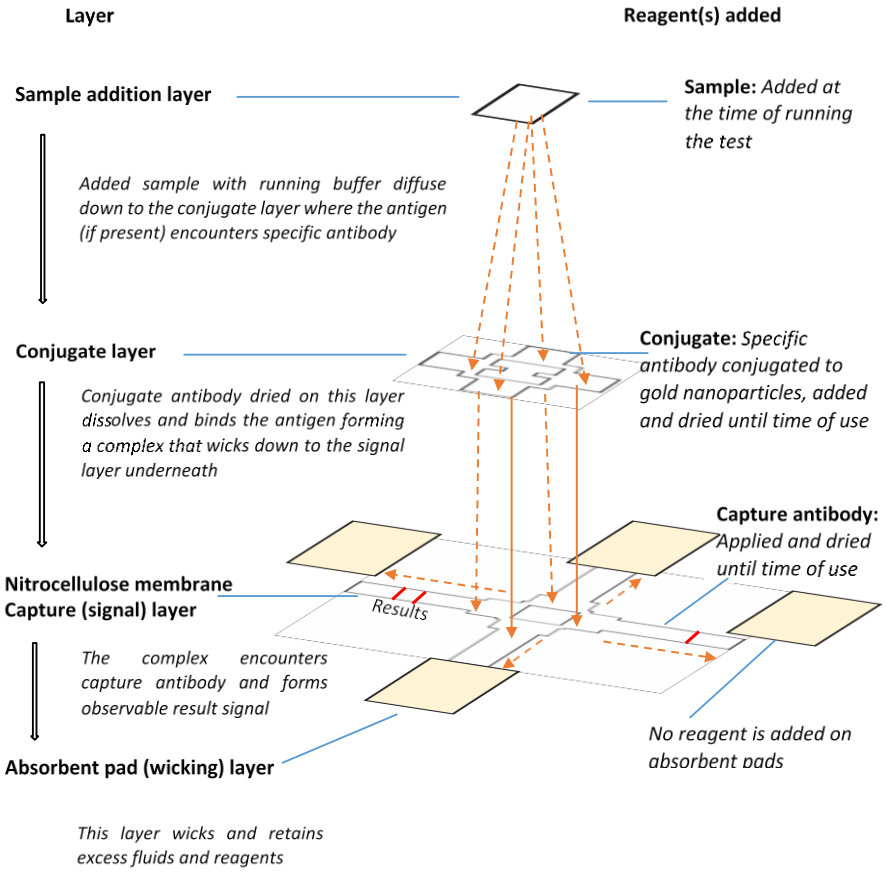
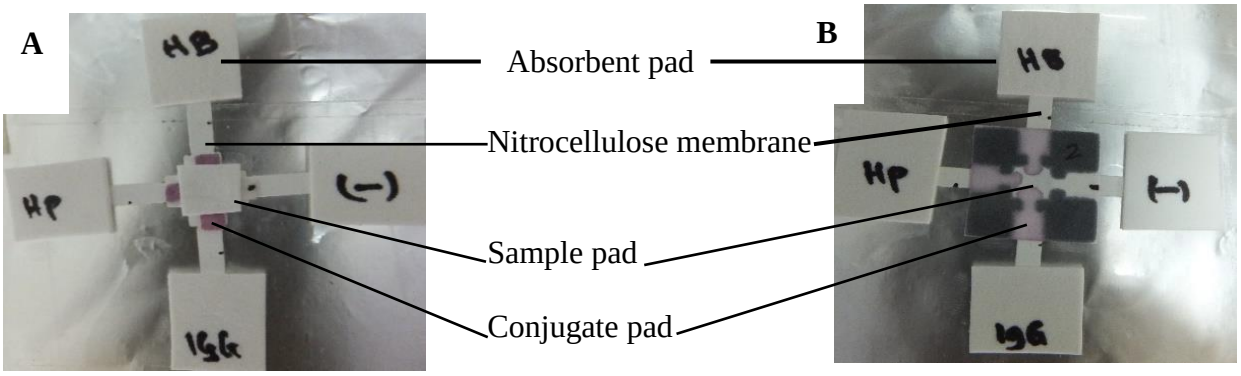


Figure 2. Schematic representation of layers of multiplex paper-based device



HB: Hepatitis B; HP: *Helicobacter pylori*; IgG: Human immunoglobulin G

Figure 3. Paper-based device assembly before sample application

A: Radial design with glass fiber conjugate pad and separate sample pad cut from Whatman filter paper. B: Conjugate and sample pads printed on chromatography paper.

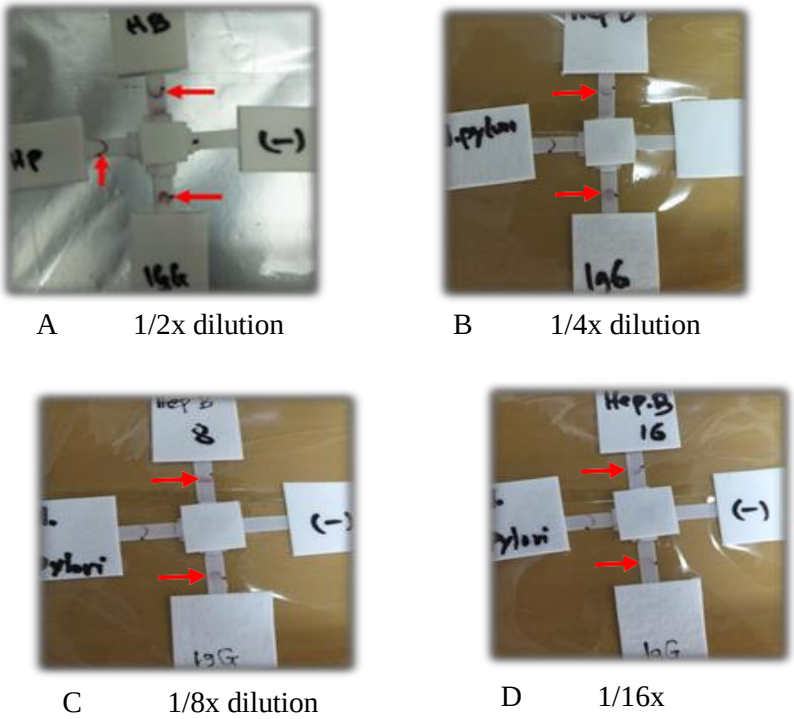
3.3 Results

3.3.1 Determining optimal conjugate dilution

To determine optimal dilution for conjugate, stock conjugates with optical density (OD) of 10 at 540 nm wavelength were diluted serially and then applied to conjugate pads. The concentration of capture antibodies on nitrocellulose membrane was fixed at 0.5 mg/ml, and 3 µl of each antibody were applied in several rounds of small quantities (0.5 µl) at test zones. The pads and membranes were dried at room temperature for 5 minutes, then at 50ºC for 10 minutes. Stock antigens were diluted to a mixture concentration of 30 µg/ml and applied to the device after assembly. The response was as shown below.

i. Using glass fiber conjugate pad

Each of the three positive arms showed response at various level of conjugate dilution. Reaction signal was clear up to the 1/8th dilution of the conjugates (Fig. 4C) but signal strength decreased upon further dilution. The highest working conjugate dilution was found to be 8-fold.



A 1/2x dilution B 1/4x dilution

C 1/8x dilution D 1/16x

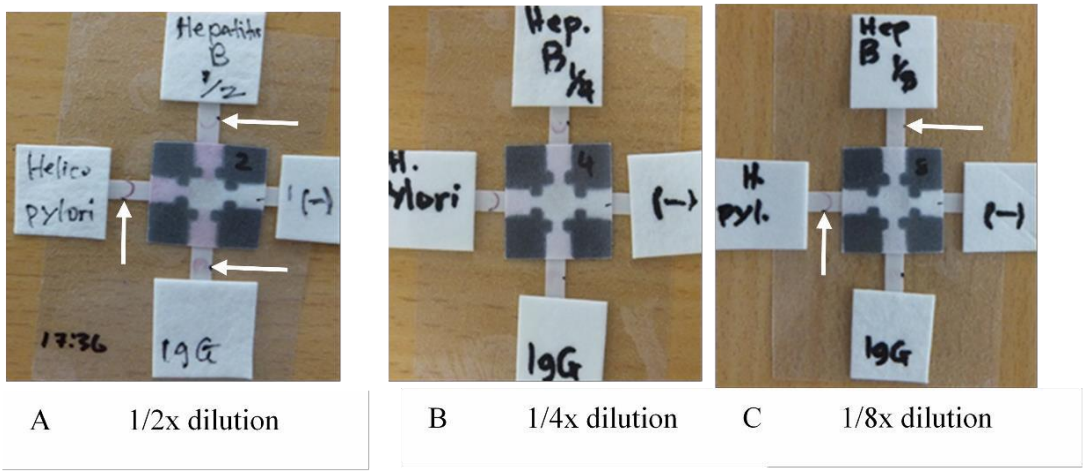
Figure 4. Determining optimal conjugatedilution for glass fiber conjugate pads

Arrows indicate positive signal. Note the decrease in signal intensity from A to D.

ii. Using chromatography paper as both sample and conjugate pad

As depicted in Fig. 5, signal was observed up to the 1/4th dilution of the conjugates (Fig. 5B). The conjugate part was found to still contain some residual conjugate even after sample and

buffer wash. Overall, it appears that the glass fiber conjugate pads offer complete conjugate release compared to chromatography paper, hence could be a better medium for multiplex rapid diagnostic devices.



A 1/2x dilution B 1/4x dilution C 1/8x dilution

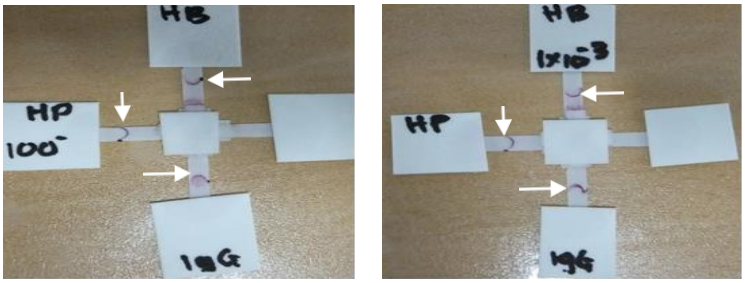
Figure 5. Determining optimal conjugate dilution for use on wax-printed chromatography paper conjugate chambers**.**

The signal intensity decreases with increasing conjugate dilution, only visible up to 4x dilution.

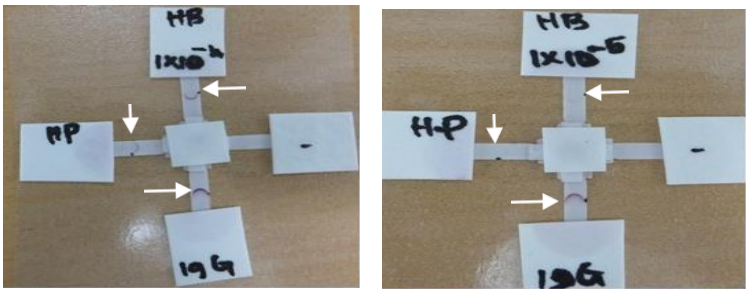
3.3.2 Determining the limit of detection

Antigens were mixed together and serially diluted to determine the limit of detection of the device. Serial dilution followed a hundred-fold initial dilution of stock antigens to approximately 300 µg/ml followed by ten-fold dilutions up to 30 ng/ml. Antigen mixture at each level of concentration was used to test the devices. Results are presented in the panel below (Fig. 6 and 7).

(i) Using glass fibre conjugate pad



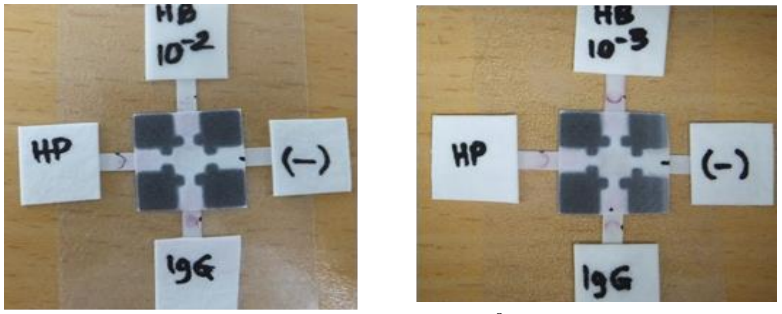
A: 1/100x dilution (approx. 30µg/ml) B: $1/10^{-3}$x dilution (approx. 3.0µg/ml)



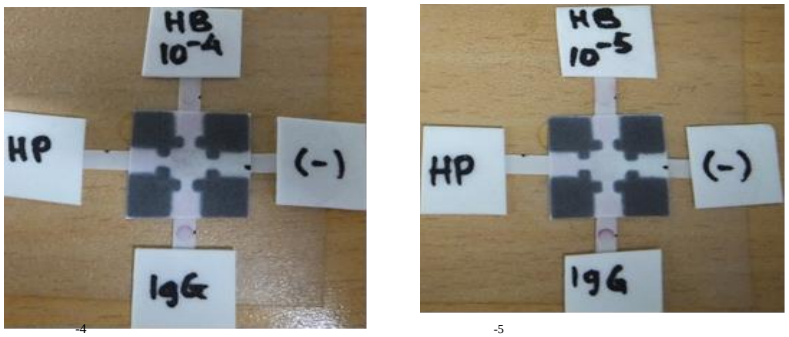
C: $1/10^{-4}$ x dilution (approx. 300ng/ml) D: $1/10^{-5}$ x dilution (approx. 30ng/ml)

Figure 6. Limit of detection for devices employing glass fiber conjugate pads

(ii) Using Chromatography paper conjugate pad



A: 1/100x dilution (approx. 30µg/ml) B: $1/10^{-3}$ x dilution (approx. 3.0µg/ml)



C: $1/10^{-4}$ x dilution (approx. 300ng/ml) D: $1/10^{-5}$ x dilution (approx. 30ng/ml)

Figure 7. Limit of detection for designs employing chromatography paper conjugate pads.

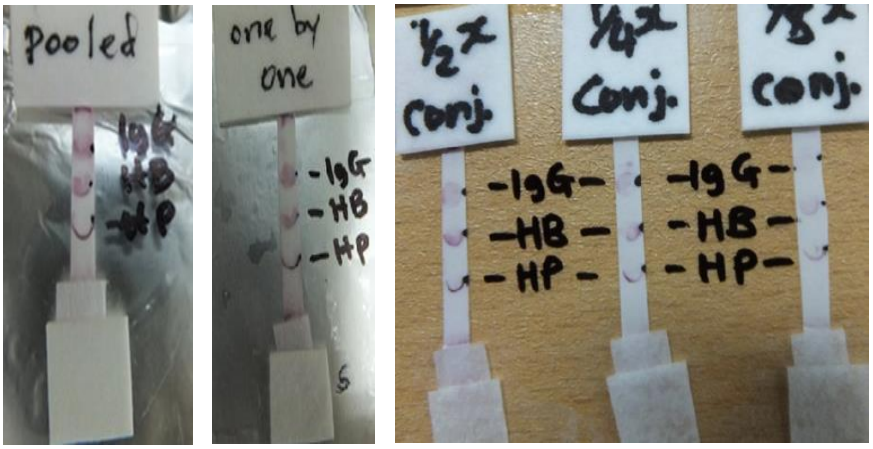
Note: HP: Helicobacter pylori; HB: Hepatitis B; IgG: Human Immunoglobulin G.

Test signal was observable for all three analytes up to the antigen concentration of 300 ng/ml

when glass fibre conjugate pad was used. In this case, only IgG showed positive signal at lower antigen concentration of 30 ng/ml (Fig. 6D). On devices made using chromatography paper as sample and conjugate pads, sensitivity was lower as the antigen limit of detection was found to be 3.0 µg/ml except for IgG which was detected even at 30ng/ml (Fig. 7D). Based on these results, the average limit of detection could be around 300 ng/ml as shown in Fig. 6. Only IgG showed response at lower concentrations.

3.3.3 Conjugate optimisation and limit of detection for single strip design

For single strip test, evaluation of optimal method for conjugate application revealed no difference in signal intensity or time taken for signal to develop, whether conjugates are pooled or added one-by-one onto the conjugate pad (Fig. 8A). Optimal conjugate dilution in this case was found to be 1/4th of the original (OD540=10) stock (Fig. 8B). Positive signal was observed shorter than five minutes since sample addition. Signal was clear at antigen concentration 300 ng/ml and above, although much lower antigen concentration (30 ng/ml) still produced visible signal (Fig. 9).



A1 A2 B1 B2 B3

Figure 8. Conjugate optimization.

Pooling of conjugate (A1), conjugate added one by one (A2), optimal conjugate dilution: 1/2x (B1), 1/4x (B2), and 1/8x (B3)

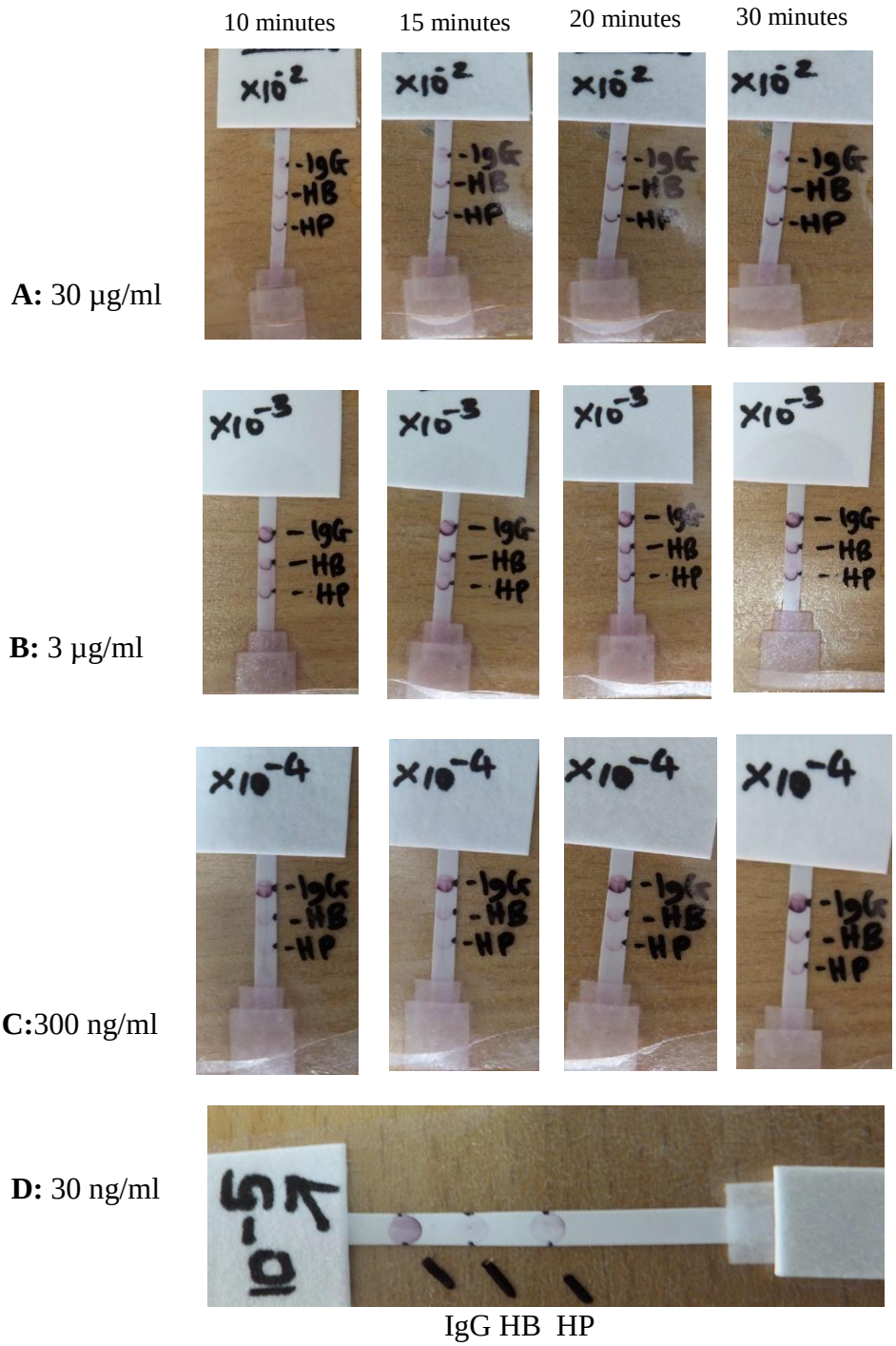


Figure 9. Limit of detection after 10, 15, 20 and 30 minutes.

Note significant signal visible up to 30 ng/ml (IgG: Immunoglobulin G; HB: Hepatitis B HP: *H. pylori*)

3.4 Discussion and Conclusion

3.4.1 Discussion

Simple designs for multiplexed lateral flow immunochromatographic tests were tested in this study. Materials used have already been described and tested previously for similar purposes and results were promising (Mabey *et al.,* 2004; Yager *et al.,* 2006; Chin *et al*., 2007; Martinez *et al.,* 2010; Phillips and Lewis, 2014). Different designs were compared with respect to their potential as diagnostic prototypes.

The study has demonstrated the possibility of using paper devices to simultaneously diagnose or detect multiple analytes in fluids. These may be applied in detection of disease-causing pathogens in people seeking healthcare at health facilities especially in developing countries where resources for advanced diagnosis may be limited (Urdea *et al*., 2006). One example where multiplexed devices could be of great importance is in the diagnosis of non-malarial febrile illnesses, mostly observed in malaria-endemic tropical countries. In this case, specific identification of febrile aetiologies has proven to be a great challenge, forcing clinicians to resort to clinical diagnosis or symptomatic treatment (Lewis *et al.,* 2013), or encouraging a trial-and-error sequential diagnosis of all possible causes of fever once malaria, the most common cause has been ruled out. Simple diagnostic devices that can help differentiate between malarial and non-malarial fevers simultaneously will greatly facilitate timely management of patients with fever not due to malaria. Other areas of application include detection of antimicrobial residues in animal-derived foods and detection of environmental contaminants in soil and water.

Two multi-arm designs were tested in this study, one that uses glass fiber conjugate pads and the one that uses wax-printed chromatography paper as conjugate pad. Although both designs could show satisfactory test signal results, fiberglass was superior over chromatography paper, as the former afforded complete conjugate release as compared to the later when compared back-to-back. This makes fiberglass a better conjugate pad although chromatography paper offers more versatility and simplicity for multiplexing due to its wax-patterning potential. Apart from the above two designs, a single strip multiplex design was also tested using similar analytes and proved to be simple, sensitive and even more convenient than the other two. In this case, there was no difference in signal response whether the conjugate antibodies were pooled before being applied to the conjugate pad or added to the conjugate pad individually (Fig. 8A). One common feature for all designs is that despite being able to detect multiple

analytes, and with potential for further multiplexing, testing is done through one step only, i.e. the operator needs to add the sample and some buffer, without any more technical requirement. Therefore, such tests can ideally be performed by persons with no advanced training and is thus useful for developing countries where resources are limited.

The generalized limit of detection for tested antigens was found to be 300 ng/ml for multi-arm designs, and 30 ng/ml for single-strip device. There were variations in the LOD for different antigen targets where for the multi-arm design, the LOD for *H. pylori* and Hepatitis B antigens was found to be 300 ng/ml while IgG was still detectable at 30 ng/ml. for the single strip design, LOD for *H. pylori* and Bepatitis B antigens was found to be 30 ng/ml while that for IgG was <30 ng/ml. However, lower detection limits can still be achieved if the methodology is slightly modified. Other paper devices tested using different analytes indicated more sensitivity as shown by comparatively lower limits of detection (Lewis *et al.*, 2013; Schonhorn *et al*., 2014). The most probable reason for reduced sensitivity in this study could be poor concentration of capture antibodies at the nitrocellulose membrane reaction zone due to dispersion of antibody solution around the point of application using handheld pipette. The half moon and dot signals resulted from the way the capture antibodies were applied to the nitrocellulose membrane i.e. using a handheld pipette. The shape of the signal (dot or half-moon depended on the flow of the antibodies immediately after addition to the membrane. This resulted in non-uniformity of the signal observed. This problem could be overcome if a special lateral flow reagent dispenser (LFRD) or material printer is used to draw compact lines on the membrane instead of hand application. This line will have high and uniform concentration of capture antibodies resulting in a high intensity test signal. To incorporate control lines, antibody dispenser or material printer will be used. In case of single strip, an ordinary lateral flow reagent dispenser may be used by fixing the head with required number of holes that correspond to the number of lines required. However, in case of the multiple arm design the LFRD may not be helpful. In this case a material printer working via computer command is ideal since deposition of antibodies for both test and control lines will be carried out as per image prepared and deposition will take place at indicated points.

3.4.2 Conclusion

This study demonstrated that a variety of multiplex designs for paper-based rapid diagnostics can be developed to improve diagnosis of common diseases and conditions in resource-limited settings. This approach promises a potential alternative to the more time-consuming and rather expensive sequential testing in circumstances where diseases presenting with similar symptoms

cannot be told apart clinically. Challenges encountered during this designing phase, like low signal will be addressed in subsequent phases by using antibody dispenser for capture antibody application on the membrane. More optimisation will be conducted to establish the maximum number of multiple analytes that could be incorporated on one device while retaining the minimum sensitivity and specificity required for point-of-need diagnostic purposes.

CHAPTER FOUR

Thread-on-a-tape diagnostic test platform for colorimetric detection of chemical analytes of diagnostic importance in resource-limited settings[3]

Abstract

Introduction: The recent need for high impact diagnostics in the developing world has stimulated research towards development of low-cost diagnostic platforms. Paper has been extensively studied as a potential matrix for microfluidic devices. Focus has also been directed towards exploring the potential of hydrophilic threads as microfluidic channels and signal substrate. In this study, threads, paper discs and mounting tape were used to fabricate and test a multiplex device platform.

Materials and Methods: Threads were treated using hot water, bovine serum albumin, sodium carbonate and sodium hydroxide to improve their wicking properties. Mounting tape, paper discs, sewing needle, and hydrophilic threads were used to fabricate a multiplex thread-on-a-tape device platform which was tested using food dyes, glucose, uric acid and bovine serum albumin.

Results: Hydrophobic threads could wick fluids following treatment with hydrophilic-inducing agents. Wicking rate on mercerized thread was 42cm/minute while the rate was 22, 16 and 9 centimeters per minute for threads treated with sodium carbonate, BSA and water respectively. Reagents, including enzymes were kept viable in dried form on paper discs. Results of the colorimetric device were ready for reading from 3 minutes of sample application.

Discussion and Conclusion: Treatment with hydrophilic-inducing agents markedly improved wicking potential of threads. Mercerization was found to induce more wicking rate compared to other agents and shorter threads wicked fluids faster than longer threads. Assembly and testing of this multiplex device gives positive indicator of the potential application of threads and tape for designing simple, low-cost prototype devices for use in low-resource settings.

Keywords: thread-on-a-tape, thread-based devices, diagnostics

[3] *Manuscript ready for submission*

4.1 Introduction

Accurate diagnosis is usually the first and most important step for appropriate health care in both developed and low-resource regions of the world. Rapid diagnosis is particularly ideal for point-of-care application to guide clinicians on appropriate treatment options to patients at health facilities. This is because such tests can be requested, carried out and results obtained within a short time to allow for treatment or advice to be given to the patient seeking care, contrary to highly advanced diagnostic tools that are logistically complicated and require hours or even days before test results are available for clinical use (Urdea *et al*., 2006). Therefore, although highly advanced diagnostic platforms facilitate complex diagnostic procedures and are desirable everywhere in the world, simple diagnostics are particularly useful in resource-limited settings where only essential minimum facilities for saving lives are available and/or affordable (Berkelman *et al.,* 2006). Such diagnostic platforms usually conform to the criteria put forward by the World Health Organization (WHO) regarding diagnostics for the developing world. These diagnostics must be affordable, sensitive, specific, user-friendly, robust, equipment-free, and deliverable to end users, also known in short as ASSURED diagnostics (Mabey *et al.,* 2004; Peeling *et al.,* 2006; Urdea *et al.,* 2006).

In response to this call by WHO, many research groups started working on various approaches, with microfluidic paper-based analytical devices (µPADs) taking leading role. With this approach, paper being a convenient material because of its ubiquitous and cheap availability, ease of patterning and disposal became a useful matrix for µPAD. Microfluidic channels on paper were created by plotting (Bruzewicz *et al.,* 2008), ink and plasma etching (Abe *et al.*, 2008; Li *et al.,* 2008), photolithography (Martinez *et al.,* 2008), cutting (Fenton *et al.,* 2009) and wax printing (Carrilho *et al.*, 2009; Lu *et al.,* 2009). To create these designs, a wide range of equipment was required ranging from simple plotting pen filled with hydrophobic ink (Bruzewicz *et al.,* 2008) to a more complex system requiring a computer and carbondioxide laser-cutter (Fenton *et al*., 2009). Among these patterning methods, laser-cutting and wax-printing appear to be the most convenient and thereby more promising in efforts to create ASSURED diagnostics using paper (Martinez, 2011). Despite paper being very promising for application in analytical devices, the search for more substrates is an ongoing endeavour.

As part of an ongoing effort for seeking new materials for affordable diagnostics, threads and other forms of fabric have been investigated as possible target for low-cost microfluidic devices (Li *et al.,* 2010a, b; Reches *et al.,* 2010; Bhandari *et al.,* 2011; Safavieh *et al*., 2011; Nilghaz

et al., 2012). Threads have been analyzed with respect to their wicking characteristics. Some threads such as cotton and some acrylic thread are naturally hydrophobic because their fibres contain wax on the surface and require treatment by various methods to make them hydrophilic, while other threads are hydrophilic thus requiring no treatment prior to their use. Methods that have been used to improve wicking in threads include boiling in water (Bhandari *et al.*, 2011), plasma oxidation (Li *et al*., 2010a, b; Reches *et al.*, 2010), treatment with bovine serum albumin (BSA), sodium carbonate and sodium bicarbonate (Nilghaz *et al.*, 2012) and mercerization, which involves treatment with sodium hydroxide followed by neutralization with an acid (Reches *et al.*, 2010; Nilghaz *et al.*, 2012). In various studies, threads and cloths have been found to be promising substrates for fabrication of low-cost, easily disposable and convenient rapid diagnostic devices for resource- limited countries (Nilghaz *et al.*, 2013).

To realize the potential for making ASSURED diagnostics available for use in low-income countries, continuous efforts must be directed towards search for more thread-based designs. In this work, we evaluate the potential of thread as a fluidic channel for multiplex diagnostic devices and demonstrate this concept by developing a multiplex thread-on-a-tape device for detection of health biomarkers namely uric acid (gout), glucose (diabetes) and bovine serum albumin (renal function).

4.2 Materials and Methods

4.2.1 Materials

Threads and needles were purchased from local textile dealers. Polyester sewing thread (MH®, Ningbo, China) and acrylic knitting threads (Robin®, Super Knit, Kenya) were used. Polyester thread was naturally hydrophilic and treatment attempts were made to enhance its wicking potential. Yellow acrylic knitting thread was hydrophilic while the white acrylic was completely hydrophobic. To use this thread, treatment with hydrophilic-inducing chemicals was necessary. Although yellow thread was hydrophilic, it was dropped because of poor contrast in some of the tests envisaged. Double-sided mounting tape (Fantastic®, Republic of China) was purchased from a local stationery outlet. Red, green, orange and yellow food dyes (Three Parrots, L.Liladhar and Co., India) were obtained from local food supermarkets. For specific tests, chemicals and biologicals were used. Potassium iodide (KI), Glucose oxidase (GO) and Bromophenol blue were purchased from AMRESCO, USA while Uric acid (UA), bicinchoninic acid (BCH), horseradish peroxidase (HRPO), sodium citrate, copper sulphate and citric acid were purchased from Sigma-Aldrich, USA).

4.2.2 Treatment of threads to improve fluid wicking

Six different methods were used to treat hydrophobic threads in order to improve their wetting properties. These include boiling in water, soaking in 1% Bovine Serum Albumin (BSA) in 0.05% Tween 20, boiling in Sodium Carbonate (5 mg/ml), Sodium bicarbonate (5 mg/ml), a mixture of Sodium carbonate and Sodium bicarbonate (5 mg/ml) and mercerization (treating with Sodium Hydroxide followed by neutralization with acetic acid).

(i) Hot water

In a one-liter beaker on a hot plate, water was heated to a boiling point. A length of hydrophobic acrylic thread was immersed in boiling water and heated for five minutes and thereafter allowed to cool and dry at room temperature.

(ii) Sodium carbonate (Na_2CO_3) and sodium bicarbonate

Sodium carbonate solution at a concentration of 5 mg/ml was used. About 2.5 g of sodium carbonate powder was weighed and dissolved in 500 ml of water in a 1000 ml Pyrex beaker. The solution was placed on a heat source and heated to boiling point. After boiling the thread was introduced into the hot solution and allowed to boil for a further 5 minutes after which the thread was rinsed in water and allowed to dry at room temperature. For the case of treatment with sodium bicarbonate and the mixture of sodium carbonate and sodium bicarbonate, the same approach was applied.

(iii) Mercerization

This involves a neutralization reaction between a base and an acid. About 2 g of sodium hydroxide pellets was dissolved in 200 ml of water. The thread was soaked into the basic solution for approximately 5 minutes at room temperature. The solution was poured out and approximately the same volume of glacial acetic acid was added and let to stand for five minutes. The thread was squeezed to get rid of excess solution and dried at room temperature. These treatment methods were applied to all available types of threads, including the naturally hydrophilic polyester sewing thread to assess how each method impacts on hydrophilicity and wicking properties of different threads. Tags or labels to denote treatment method applied were attached at end of the threads to facilitate easy identification. Treated threads were kept dry in sealed tubes with silica gel bags (desiccant) for humidity control.

4.2.3 Fabrication of a simple diagnostic test device format

Using threads, double-sided mounting tape, and punched paper discs, a simple design for test

device was prepared. Initially, the nature of fluid flow and mixing was evaluated with aid of food dyes (colors). Small-sized paper discs were made from filter paper or fiberglass with the aid of normal office punching machine. Discs were dipped in food dye solutions of different colors (tomato red, yellow, green and orange), allowed to dry at room temperature and kept in a sealed tube with desiccant. A device was assembled to assess the potential for paper discs to store reagents in dried form for later release during testing, and the power of hydrophilic threads to independently direct sample and reagent flow in specific directions. Using an ordinary household sewing needle, a thread was sewn from the centre of the top-side of a double-sided mounting tape to the other side and through a dyed paper disc back to the top side of the tape at the periphery. This was done for the rest of the discs as depicted in Fig. 14A and B. A second layer of tape was applied to the bottom side of the tape to laminate the discs thereby protecting impregnated reagents from physical and chemical degradation. White paper discs were placed at the top of the sample inlet thread end at the centre, as well as the signal readout thread ends at the periphery of the tape to form sample and signal pads respectively (Fig. 14C and D). In some reactions, the final reactant/reaction indicator is impregnated into this disc. With this arrangement, water applied to the sample pad flows through the threads to the discs and dissolves the dye. Dissolved dyes wick further along the threads back to the top signal discs where results will be displayed (Fig. 15). Variation of the above setup was designed by delivering sample through a single thread towards a central disc from which individual threads radiate towards specific reaction zones (Fig. 14B). Several approaches for mixing solutions flowing through independent threads were tested. These include twisting threads together as described by Li *et al.* (2010b), and making knots of two or more threads (Fig. 13).

Detection of chemical analytes in samples using an assembled prototype device

To demonstrate the application of this design, a test device was assembled for simultaneous detection of proteins, glucose, and uric acid in a sample as previously described (Martinez *et al*., 2007; Li *et al*., 2010b; Reches *et al.*, 2010).

Protein test: Indicators for protein detection were 250 mM Citric acid and 3.3 mM Tetrabromophenol blue (TBPB) or Bromophenol blue in 95% ethanol. A paper disc was soaked in the indicator solution and allowed to dry at room temperature and were later kept in sealed bag with desiccant until time of use.

Glucose test: To test for presence of glucose, Glucose oxidase (GO), horseradish peroxidase

(HRP) and potassium iodide (KI) were used. Horseradish peroxidase and GO were mixed in a ratio of 1:5 and 5 µl of the mixture was applied on a paper disc and dried at room temperature. On another paper disc 5 µl of KI was applied and dried. The discs were kept in sealed bag with desiccant until time of use.

Uric acid: Indicators for uric acid were sodium citrate, copper II sulphate and bicinchoninic acid disodium salt hydrate or 2,2-biquinoline-4,4-dicarboxylic acid disodium salt hydrate. One paper disc was dipped in a solution containing 20 mmol/L sodium citrate and 0.08% (w/v) copper sulphate while another disc was soaked into a solution of 2.56% (w/v) bicinchoninic acid disodium salt hydrate. The discs were dried at room temperature and kept in sealed bag with desiccant until time of use.

To assemble a device for testing a single analyte (e.g. glucose), a hydrophilic thread was sewn from the top-side of a double-sided mounting tape to the other side and through a GO paper disc back to the top side of the tape at the far end. A disc impregnated with dried KI was overlaid on top of the emerging thread. A plain disc was stuck on top of the thread origin to serve as sample inlet pad. To assemble a multiplex device, same approach was employed. In this case threads originated at the centre of the device and emerged at the periphery. Reagent discs embedded between the layers and on top of tape were specific to the target analyte as described above.

4.3 Results

4.3.1 Treatment of hydrophobic thread to improve wicking properties

The thread used was initially hydrophobic with no wicking tendency. Following treatment with various hydrophilicity-inducing agents, the thread could wick fluids through. Figure 10 demonstrates this transformation where treated and non-treated threads from the same yarn were twisted against each other and one end was dipped into a solution of red dye. As observed, the dye could wick through treated thread (a) only.

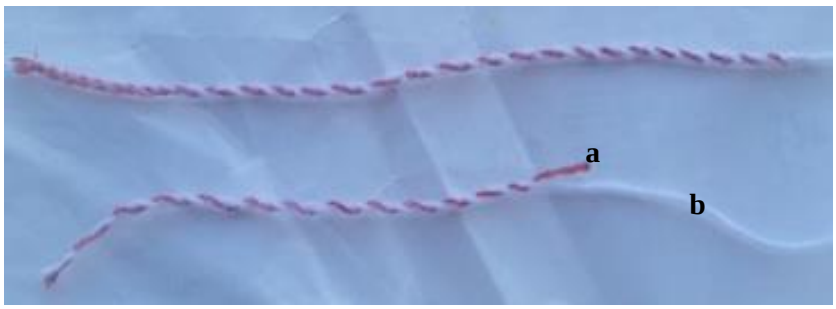


Figure 10. Treated and untreated acrylic knitting threads twisted against each other.

The thread was treated using NaOH followed by acetic acid (mercerization). One end of each of the threads was dipped into a solution of red food dye, which subsequently wicked through treated thread only.

4.3.2 Effect of different treatment methods on thread wetting/wicking properties

Mercerization (treatment with a solution of Sodium Hydroxide followed by neutralization with acetic acid) was found to have the most positive effect on wicking properties of acrylic thread followed by boiling of thread in 5mg/ml of Sodium Carbonate (Na_2CO_3). For example, it took approximately 6 and 8 minutes for dye to wick through a 30-cm thread treated with NaOH and Na_2CO_3 respectively while it took more than 20 minutes for the dye to flow through same length of thread treated by hot water only (Fig. 11).

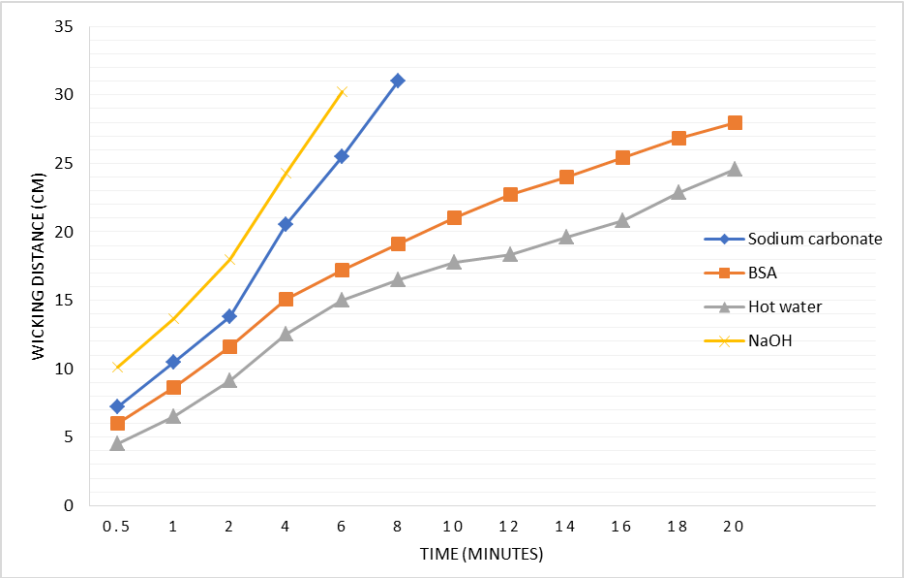


Figure 11. Effect of different treatment methods on thread wetting/wicking properties

Wicking rate was determined as the length of thread picking the dye per minute. From the graph (Fig. 12), mercerization resulted into high wicking rate compared to other treatment methods. The average wicking rate was 42 cm/min for the first 4 centimetres, but constantly dropped to 9.2 cm/min at the end of the 30 cm thread. For other treatment methods, the rate was lower, for example initial wicking rate (cm/min) for the first 4 cm was 22, 16 and 9 centimeters per minute for threads treated with sodium carbonate, BSA and water respectively. In all cases, the rate dropped constantly as the length of thread increased to 4.1 cm, 1.4 cm and 1.0 cm per minute for sodium carbonate, BSA and water respectively, at the end of 30 cm thread length.

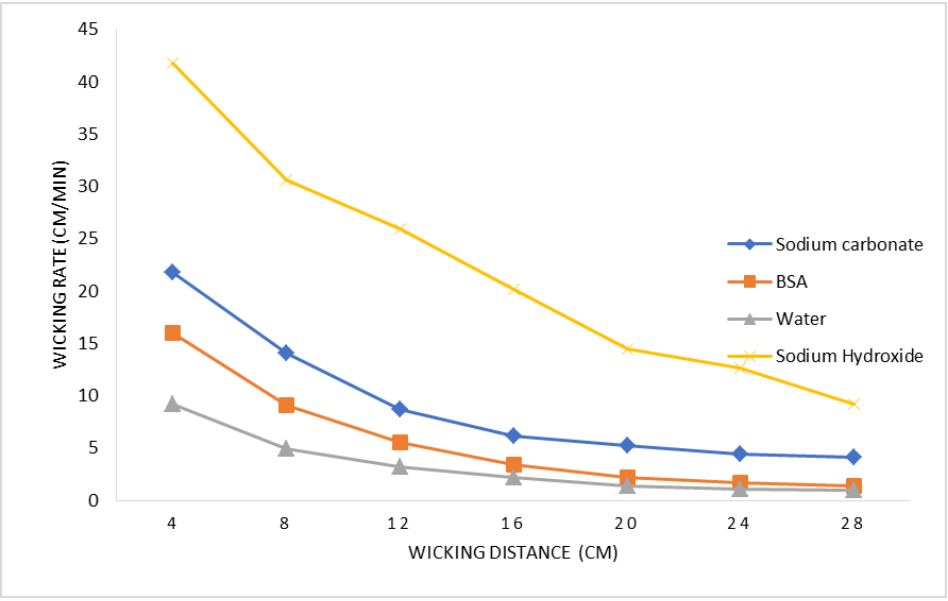


Figure 12. Impact of different treatment methods on wicking rate of acrylic thread.

Note that treatment with sodium hydroxide followed by neutralization with acetic acid produced the highest wicking rate (yellow graph, x data points) followed by hot sodium carbonate solution (blue line and blue diamond data points), BSA (red line, square data points). The rate decreased with increasing wicking distance. This implies that more efficient reagent flow and rapid results are expected when shorter threads are used in the design.

Mixing of solutions that wick through different hydrophilic threads can be achieved by twisting threads at the point where mixing is planned to take place. Alternatively, a disc through which threads pass may be used as a mixing zone for solutions being delivered through different threads. Figure 13 shows results of mixing different solutions by disc, twisting, and knotting methods. Mixing of solution was attained with all methods, however, the most efficient and fast mixing was achieved through thread twisting and knotting.

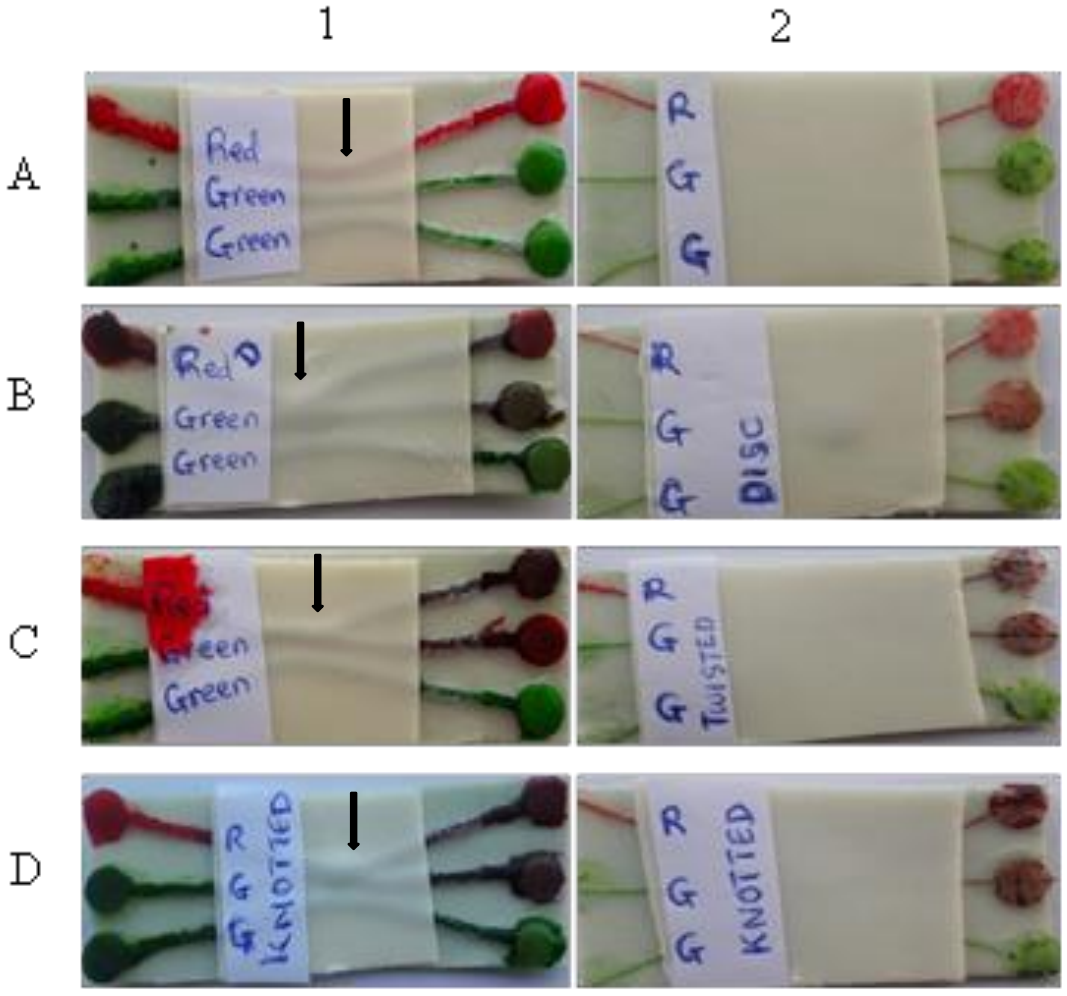


Figure 13. Mixing of solutions by disc, twisting and knotting.

A: No mixing B: mixing on a paper disc C: Mixing by twisting D: Mixing by knotting. Dyes originate on the left and flow towards the mixing zone at the middle. The right-end discs

contain mixture of dyes Panels A and B represent two different types of threads (A: Acrylic mercerized thread; B: naturally hydrophilic polyester thread. In B, C and D, top two threads are mixed while the bottom thread runs free for comparison purposes.

4.3.3 Fabrication of multi-analyte device using thread and paper discs on tape

The device was fabricated using acrylic thread that was treated by mercerization. The bottom view of the tape appears as shown in Fig. 14A below. Red, yellow/orange and green paper discs each is linked to the sample and results pads by a thread. In the current design, when water is applied to the sample disc at the top of the tape, water flows through the thread and re-dissolves the dye, which flows further up to the top of the tape to the signal/display discs (Fig. 14D). In subsequent stages, the food dye on discs will be substituted with specific reagents for each target analyte such that, a specific colorimetric signal at the results disc will be indicative of presence of target analyte, hence positive test.

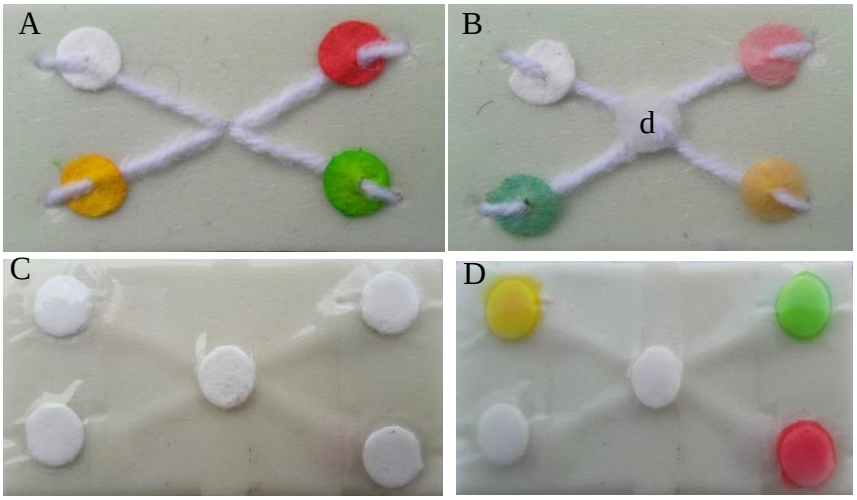


Figure 14. Assembly of the device on tape.

A and **B:** Bottom view of the tape showing threaded paper discs soaked in red, green and yellow food dye solutions and dried. In Fig. A, each test line is represented by a continuous individual thread from the top side of the tape while in B only one thread crosses the tape from the top. Threads that form individual test arms emerge from beneath the tape and reagent distribution is bythe disc (d). In test prototypes, these food dyes will be replaced with specific reagent for specific tests. **C**: Top view of the tape showing completed test assembly. The central disc (sample pad) is used for sample injection while the four peripheral discs are used for displaying results. Reactions taking place between reagents on discs beneath the tape yield colorimetric signals, which are delivered back to the top discs through threads. **D:** Results after addition of water on the sample pad. Water flowed through threads and dissolved the food dyes

on each disc, and the respective colour solutions were delivered back to top peripheral discs via threads as shown except for the bottom left discs whose corresponding reagent discs beneath the tape were blanks (negative control).

A B C

Figure 15. Eight-channel multiplex test assembly

A: Bottom view of tape indicating discs of different colours linked to the top of tape by thin polyester sewing threads. The other end of each thread passes through the discs and goes back to the top of the tape. B: Top view of the tape indicating sample-loading disc (centre) and 8 peripheral discs for displaying results. C: Top view of the tape showing results (color discs) after application of water through the central (sample) disc. Note that two plain discs in C correspond to the two plain discs in A

4.3.4 Multiplex device for detection of Glucose, proteins, and Uric acid

Results for uric acid, glucose and protein (BSA) were obtained as documented below. Individual discs, single, dual, and multiplex thread-on-tape devices were tested, and results imaged. Colorimetric signals began to develop within 3 minutes of sample application for uric acid while it required up to 10 minutes for the glucose test to be ready. Test discs were created by impregnating specific reagents in paper discs followed by application of sample. Using these discs, lowest detectable concentrations were found to be 0.5 mM for both uric acid and glucose (Fig. 16)

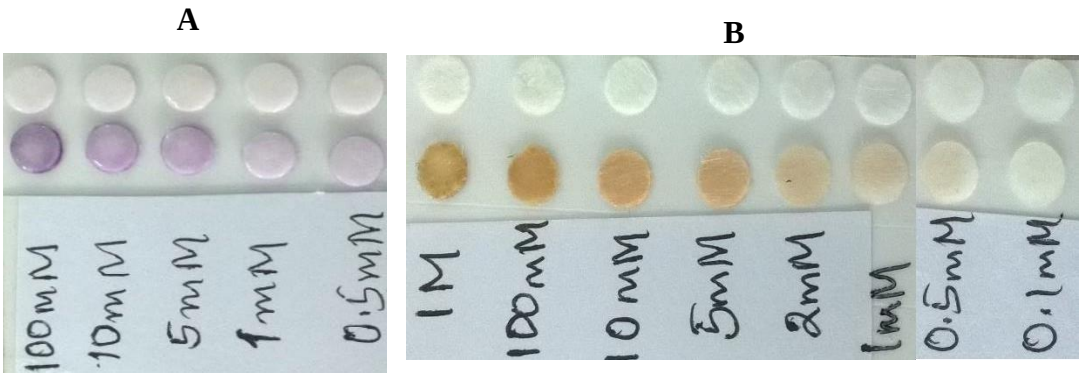


Figure 16. Test discs for Uric acid and Glucose tests.

Intensity gradient with decreasing concentration of uric acid and glucose in the solution read at 3 and 10 minutes respectively. Top rows correspond to negative control.

Thread-on-tape devices were fabricated for single, dual and multiplex tests as indicated below. In single devices for glucose (Fig. 17A) and uric acid (Fig. 17C) sample is applied to the white sample disc (on the left) and results are observed on the right onto the results disc. Figure 17B shows a dual test for glucose and protein (BSA) where the sample is applied onto the white sample disc in the middle. A more inclusive design is shown by the multiplex test (Fig. 18) where three or more targets are tested in a single run. In this case, the sample pad is located centrally while result display discs are spread radially outwards.

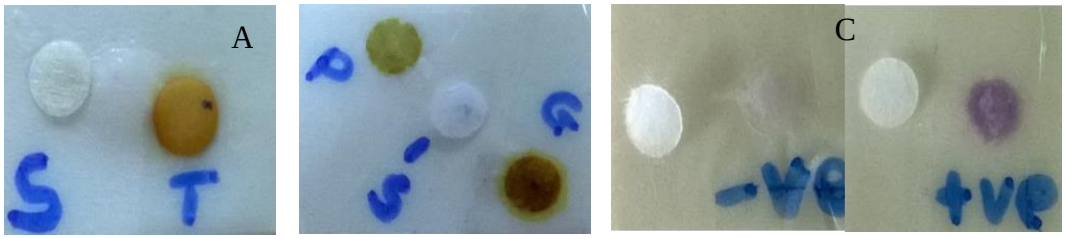


Figure 17. Thread-on-a-tape-devices (individual) after running a sample through the device

A: 5 mM Glucose test positive signal. B: Protein (BSA) and Glucose test positive signal. C: 5 mM Uric acid positive control read at 3 minutes against PBS negative control. S; sample disc, T; test disc, P; protein disc, and G; Glucose

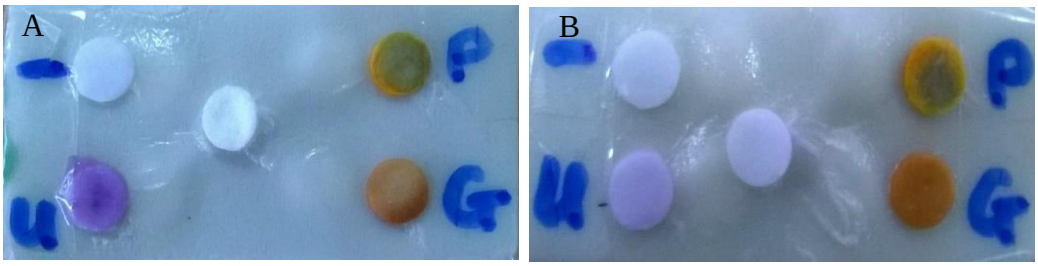


Figure 18. Multiplex device for glucose, uric acid and BSA protein.

Glucose (G), uric acid (U) and BSA protein (P) are tested on a multiplex device. White discs turn brown and purple in presence of glucose and uric acid respectively. Yellow disc turns green when a sample containing protein is added. A sample solution with varying concentrations of target analytes was added to the two devices above. The top left disc was blank to serve as negative control.

4.4 Discussion and Conclusion

4.4.1 Discussion

Hydrophilic threads have been demonstrated to be useful as conducting channels for sample and reagents in microfluidic diagnostic devices (Li *et al*., 2010a, b; Ballerini *et al*., 2011b; Nilghaz *et al.*, 2013). When available threads are hydrophobic, treatment with hydrophilizing agents renders threads hydrophilic. In this study, several approaches were applied to modify the wetting properties of threads. Figure 10 demonstrates the impact of this treatment on wicking properties of a thread that was originally hydrophobic as the dye wicks along treated thread only, when treated and untreated threads are twisted/wound around each other and dipped in a dye solution.

In this study, different approaches were compared with respect to their efficiency in inducing hydrophilicity in a hydrophobic thread. Treatment with a solution of sodium hydroxide, followed by neutralization with acetic acid produced the best results. Other treatment methods such as soaking in blocking solution like BSA or boiling in water and sodium carbonate (scouring) produced same effect although they induced lower and varying wicking efficiency. Wicking rate was more than two-folds higher for a mercerized thread compared to that treated by other methods (Fig. 11 and 12). Considering the approaches used in this study, treatment with sodium hydroxide followed by neutralization with an acid solution (mercerization) can be considered the most efficient method. The above methods have also been used previously by other study groups and found to be useful (Bhandari *et al.*, 2011; Nilghaz *et al.,* 2012). Plasma oxidation (Li *et al.,* 2010a, b; Reches *et al.*, 2010) and other methods such as boiling in sodium

bicarbonate, washing with sodium carbonate (Nilghaz *et al*., 2012) and using hand soap solution (Xing *et al.,* 2013) have been applied to enhance hydrophilicity of threads as well. These methods are a useful tool during fabrication of microfluidic thread-based device when all the available threads are naturally hydrophobic, and their comparison may help in selection of the best method to use.

The length of thread was found to be an important factor for determining the wicking properties of a modified thread. The wicking rate was found to be high on shorter threads or during the initial segment of a long thread. In this study, the wicking/flow rate dropped substantially from over 40 cm/min during the first 4 centimetres to below 10 cm/min at 30 cm for a thread treated with NaOH. To benefit from fast fluid flow and obtain rapid results from thread-based microfluidic devices, designs that utilize shorter thread segments should be considered in favour of those requiring longer threads.

Three methods for mixing of solutions were compared: discs, knotting and twisting. Knotting and twisting were efficient mixers compared to discs, which tend to mix slowly (staggered). If a design requires rapid mixing of solutions, then twists and knots should be considered. However, if staggered or slow mixing is desired, disc mixers should be considered as best choice. Knots as form of mixers for fluids wicking along hydrophilic threads were also explored by other studies where different number of knots produced different levels of resistance hence different levels of mixing and dilution of solutions (Safavieh *et al.,* 2011) The work by Ballerini *et al.* (2011b) showed that thread micromixers were capable of high quality mixing demonstrated by food dyes and acid-base neutralization reaction.

Demonstration of a microfluidic device fabrication was made using hydrophilic threads as conductors of fluids, punched paper discs as reagent storage and results display zones, and a double-sided mounting tape for binding different device parts together. These threads were initially hydrophobic before being treated by mercerization to induce their hydrophilicity. Initial demonstration with food dyes yielded promising results. Flowing along a hydrophilic thread, water wicked through the layer of tape to dissolve dyes of different colors on different paper discs, with the dye solution emerging from under the tape within two to three minutes. This shows that a complete device can be assembled in similar manner, with results being expected in less than 5 minutes. The above design was used to fabricate a POC diagnostic device for single, dual and multiplex devices for colorimetric detection of analytes of medical or clinical importance.

In low resource settings, standard diagnostics are not available for use at point of care, and only specialized health facilities have access to modern diagnostic services (Urdea *et al.,* 2006). Since individual financial status of people living in poor countries is also low, there is need to develop low-cost devices that are affordable to most people. The device platform described here fulfils qualities of low-cost diagnostics. The materials used like sewing needles, thread and mounting tape are readily available and can be purchased at reasonably low price. Thread-based multiplex prototypes can be designed to co-diagnose conditions that resemble one another symptomatically or whose diagnostic markers share common medium. For example, glucose, uric acid and some proteins can be diagnosed in urine sample. However, this combination was conveniently selected only for demonstration purposes not for clinical applications.In this multiplex format, different tests required different reaction times to complete. It took less than 3 minutes for the uric acid test to complete while the protein and glucose tests were completed after 10 minutes. If time for reading test results is supposed to be synchronized, then selection of time-compatible target analytes should be considered when designing multiplex tests.

The WHO advocates development of ASSURED diagnostics for wide use in resource-limited settings, which is based on the following criteria where an ideal diagnostic should be: affordable, sensitive, specific, user-friendly, rapid/robust, equipment-free and deliverable to end users. This design has addressed some of these criteria. Simplicity in the manufacturing process and low cost of materials required for making the diagnostic platform described here qualifies it as being potentially affordable although exact costs/expenses have not been calculated. The device requires the operator to add only sample in a one-step action and wait for results, which makes it user-friendly. Complimentary to the above simplicity, the time required for reading results is short, thus fulfilling the criterion of being rapid. The potential to be multiplexed contributes to its robustness since multiple analytes/biomarkers/pathogens can be simultaneously diagnosed together obviating the need for sequential testing. Like other simple diagnostics, this design requires no extra equipment or any sophisticated environment for reading the results. Lastly, the devices can be stored at room temperature since all reagents are kept in dried form, enabling them to be transported to different parts where they are needed for use. The proposed test device improves on the standard dipstick test as it requires relatively small sample volume as compared to the dipstick which requires the operator to use large volume of sample due to its wetting nature. The sensitivity and specificity attributes will be evaluated for each individual prototype developed using this platform and are therefore not

discussed here.

4.4.2 Conclusion

Thread can be combined with paper discs and mounting tape to fabricate low-cost microfluidic devices with potential of application as point-of-care diagnostics. Hydrophobic threads can be modified to become hydrophilic by a range of hydrophilizing procedures. The use of cheap materials such as sewing needles, office punching machine, threads, paper and tape means that the overall cost of resultant devices is likely to be low, hence affordable by people living in limited resource countries. The use of white paper discs facilitates colour display, implying that a hydrophilic thread of any colour can be applied for fabrication of such devices. Thread- on-tape microfluidic devices may present a viable possibility for designing and development of ASSURED diagnostics for the low-income parts of the world.

CHAPTER FIVE

Immunochromatographic Thread-based Test Platform for Diagnosis of Infectious Diseases[4]

Abstract

Introduction: Patterning is an important step in fabrication of multiplexed microfluidic devices. Various approaches including cutting, photolithography, wax-printing, plotting and etching have been developed and tested. Recently, using threads is increasingly being considered as an optionfabrication of low-cost microfluidic diagnostic devices. We explored the application of threads in combination with nitrocellulose membrane to fabricate multi-channel immunochromatographic diagnostic devices.

Methods: Microfluidic channels were made using hydrophilic threads and nitrocellulose membrane strips. Household sewing needle was used to weave hydrophilic thread into desired patterns through a double-sided mounting tape. Glass fiber discs were used as conjugate pads while nitrocellulose membrane was used for immobilization of capture antibodies. Patterned threads were linked to nitrocellulose membrane strips by overlapping so that reagents flowing through threads were eventually transferred to the membrane. The design was tested using IgG, *H. pylori* and Hepatitis B surface antigen.

Results and Discussion: Continuous flow was observed from hydrophilic threads to the nitrocellulose membrane and a positive signal was visualized on the membrane within 5 minutes of sample application. The observed limit of detection ranged between 30ng/ml to 300 ng/ml for *H. pylori* and Hepatitis B respectively.

Conclusion: Using thread and tape offers a promising alternative for patterning of multiplexed microfluidic diagnostic devices with potential point-of-care applications in resource-limited settings.

Keywords: immunochromatographic, thread-based diagnostics, infectious diseases, multiplex microfluidic devices, point-of-care, low-cost diagnostics

[4] *Microfluidics and Nanofluidics* (2018) **22**(4):45 https://doi.org/10.1007/s10404-018-2065-1

5.1 Introduction

Membrane-based Immunochromatographic Lateral Flow Tests (ILFTs) are the first and most widely applied point-of-care tests currently in use (Boisen *et al.*, 2016). These tests have wide applications ranging from the earliest pregnancy test strips (May, 1991) to include sperm quality testing (Matsuura *et al.*, 2014), infectious diseases diagnosis for medical and veterinary fields (Nielsen *et al*., 2008; Rohrman *et al*., 2012; Kamphee *et al*., 2015), food safety e.g. detection of mycotoxins, drug residues, food allergens, food-borne pathogens (Toldra and Reig, 2006; Shankar *et al*., 2010; Law *et al*., 2015; Weng *et al*., 2016), urinalysis for non-infectious health biomarkers e.g. detection of glucose, proteins, ketones, drugs of abuse, nitrite, bilirubin (Martinez *et al*., 2007; Li *et al*., 2010a, b; Reches *et al.*, 2010), bio-threats such as detection of anthrax, plague, tularemia, nerve agents, ricin and botulism (Shyu *et al.*, 2002; Gessler *et al.*, 2007; Zasada *et al*., 2015), environmental safety monitoring e.g. monitoring chemical or microbial contaminations in soil, water and air (Hossain *et al*., 2012; Sicard *et al.*, 2015).

Typically, a test strip consists of the following components: *Sample pad*; usually a filter paper or glass fiber pad onto which the test sample is applied, *Conjugate or reagent pad;* this contains antibodies specific to the target analyte conjugated to colored particles (usually colloidal gold particles, or latex microspheres), *Reaction membrane;* typically a hydrophobic nitrocellulose or cellulose acetate membrane onto which anti-target analyte and control antibodies are immobilized in a line that crosses the membrane to act as a capture zone or test line, and a *Wick or waste reservoir;* an absorbent pad designed to draw the sample across the reaction membrane by capillary action and collect excess reagents and buffer.

The sample pad acts as deposition site for the sample and may also help in filtration of particulate matters from the sample which passes through this pad towards distal sections of the strip. The conjugate pad holds dried detection antibodies conjugated to a reporter (like gold nanoparticles or colloidal gold) until when re-dissolved by the sample and buffer. However, the reaction membrane binds protein more strongly so that even after the sample and buffer flows through the test and control zones, immobilized capture reagents (antibodies/antigens) are not dislodged, hence forming a focal point for signal development. This format of diagnostic meets the minimum criteria set by the World Health Organization (WHO) for ideal diagnostics in developing nations which requires such diagnostics to be affordable, sensitive, specific, user-friendly, robust, equipment-free, and deliverable to end users,

abbreviated as ASSURED diagnostics (Peeling *et al.,* 2006; Urdea *et al.,* 2006).

Designing multiplexed membrane-based tests involves patterning of the sample and/or conjugate pads, and the membrane itself to create channels that direct reagents towards the multiple testing zones/points. The most common patterning methods are wax printing for conjugate and sample pads (Carrilho *et al.,* 2009; Lu *et al.,* 2009) and laser cutting for conjugate pads and detection membrane (Fenton *et al.,* 2009). Although these approaches are relatively simple to execute and are very promising in development of ASSURED diagnostics, it may still be difficult in developing countries to obtain the required equipment/machines such as laser-cutter, solid ink/wax printer, and reliable power supply to run the machines all the time.

Microfluidic thread-based analytical devices (μTADs) have recently emerged as a possible solution to patterning problems since thin threads can conduct solutions in micro volumes towards a target reaction zone. This is because threads are flexible hence can be manipulated in any form and direction without any problem, and with their small diameter, can wick and transport very small amounts of analyte (Li *et al.,* 2010a, b; Reches *et al.,* 2010).

In this study, threads were used to create channels through which sample, conjugate reagents, and reaction complexes flow, thereby obviating the need for patterning necessary for multiplexed paper-based diagnostic devices. Since the membrane is an essential part for immobilization of capture reagents, this design will allow the analyte-detector (conjugate) complex flowing through threads to be delivered to an underlying strip of nitrocellulose membrane containing immobilized capture antibodies for signal development.

5.2 Materials and Methods

5.2.1 Materials

White polyester sewing thread (MH®, Ningbo, China) and household sewing needle were purchased from local textile dealers. Mounting tape (Fantastick (FK-M485)®, China) and food dyes (Three Parrots®, L.Liladhar and Co., India) were purchased from local stationery shops and food markets respectively. Nitrocellulose membrane, glass fiber conjugate pads, sample pads and absorbent pads were all supplied by Claremont Bio Solutions (CA, USA). Detection (conjugate) and capture antibodies, and antigens (Immunoglobulin G, Hepatitis B virus and *Helicobacter pylori*) were all supplied by Arista Biologicals (Allentown, PA, USA). Conjugate antibodies used were monoclonal mouse anti *H. pylori* colloidal gold conjugate, clone 4 (CGHPY-0704), monoclonal mouse anti HBsAg colloidal gold

conjugate, clone 2 (CGHBS- 0702), and goat anti human IgG colloidal gold conjugate (CGIGG-0500). All conjugate antibodies were conjugated to a 40 nm colloidal gold particle at a concentration of 10 µg/ml (OD540=10). Capture antibodies used were mouse anti *H. pylori* antibody (ABHPY-0403), Goat anti HBsAg (ABHBS-0500) and Goat anti human IgG (ABIGG-0500). Invitro purified proteins from *H. pylori*, recombinant HBsAg strain produced in *pichia pastoris*, and Human IgG purified by immunoaffinity from whole goat antisera, were used as antigens for *H. pylori*, Hepatitis B and IgG respectively.

5.2.2 Design fabrication using food dyes

Red, yellow and green food dye solutions were prepared, and punched paper discs were immersed in the solutions and allowed to dry at room temperature. A pair of scissors was used to cut 2x10mm strips of nitrocellulose membrane. Using an ordinary household sewing needle, a thread was sewn from the centre of the top-side of a double-sided mounting tape to the lower side and through a dyed paper disc back to the top side of the tape further distal. This was done for the rest of the discs as depicted in Fig. 19A. A second layer of tape was applied to the bottom side of the tape to laminate the discs thereby protecting impregnated reagents from physical and chemical degradation. A plain paper disc was attached at the top of the sample inlet thread end at the centre to serve as sample pad. Nitrocellulose membrane strips were stuck in contact with thread ends emerging from below the tape, with thread ends overlapping the membrane strips by 1-2 mm, to facilitate transfer of solutions from threads to the membrane. At the distal ends of membrane strips, absorbent pads were attached to facilitate wicking and draining of excess solutions (Fig. 19B). To test the assembly, a few drops of water were added to the sample pad and observed for emergence of different dyes from under the tape via the thread onto the nitrocellulose membrane and absorbent pad further distal.

5.2.3 Immunochromatographic thread-based device

Glass fiber conjugate pad discs were each treated with 2.5 µl of 10% BSA in PBS and allowed to dry at room temperature for 5 minutes and then at 50ºC for 10 minutes. Stock conjugate antibody (OD10) was diluted one fifth in conjugate buffer (2% w/v BSA, 10% w/v sucrose and 0.1% v/v Tween 20 in Borate buffered saline). This was followed by addition of 5 µl of gold conjugate antibody, each with a specific antibody type (anti human IgG, anti-hepatitis B surface antigen and anti *H. pylori*). The pads were dried at room temperature for 5 minutes and then at 50ºC for 10 minutes as above. Dried conjugate pads were kept in sealed bags with desiccant ready for assembly. Capture and control antibodies were diluted in PBS (pH 7.4) to a concentration of 0.5 mg/ml. Five percent (5%) methanol was included to

enhance fixation of antibodies to the membrane. Test and control lines were drawn on nitrocellulose membrane by using Lateral Flow Reagent Dispenser and Legato Syringe Pump (Claremont Bio Solutions, CA, USA). The dispense rate was 0.25 ml/minute and voltage was set at 5 v. After dispensing, the sheet was dried at 50ºC for 30 minutes and cut into 2x10 mm strips using a pair of scissors. This was followed by addition of 2 µl of blocking buffer (0.1% v/v Tween 20, 5% w/v sucrose and 1% w/v BSA) per strip. After blocking, strips were dried as above and kept at room temperature in sealed bags with desiccant. This step was carried out for all capture antibodies (anti human IgG, goat anti Hepatitis B and mouse anti *H. pylori*) and control antibody (Goat- anti-mouse IgG).

5.3 Results

A four-arm design was demonstrated using food dyes, and flow of solutions was observed. This design combines both vertical and lateral flow across the layers of the device. The thread transported the solution vertically downwards from the sample pad at the top to the bottom of the tape. Then, there was a lateral movement through punched paper discs followed by a vertically upward movement of color solution back to the top layer where lateral flow occurred along the nitrocellulose membrane and absorbent pad. The time interval between addition of water sample onto the sample disc and emergence of dye solution from under the tape was approximately 1-2 minutes.

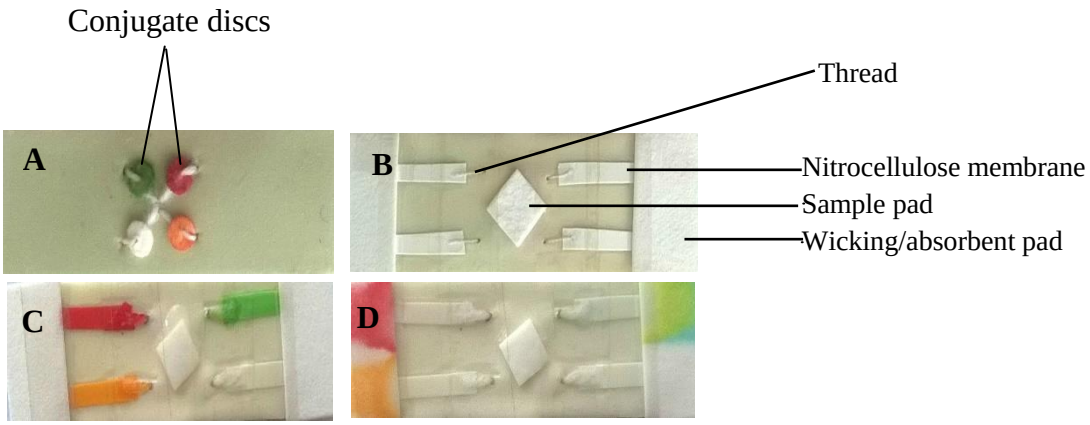


Figure 19. Reagent flow through threads and nitrocellulose membrane.

A: underside of tape indicating reagent-loaded paper discs (represented by red, green and orange dyes) B: Top view of the device after assembly. C: Flow of dyes from the middle layer of the tape through the nitrocellulose membrane at the top after addition of water (sample) D: Appearance of the membrane after all the dye has cleared out towards the absorbent pad

Assembly of device for demonstration of reagent flow using red, green and orange food dyes was done as indicated in Fig. 19A-D above. After addition of water drops at the sample pad (middle), food dyes on paper discs dissolved to subsequently flow to the top onto the nitrocellulose membrane. The membranes were clear of dyes within 3-5 minutes (Fig. 19D) with all the dyes now deposited in the wicking pads. This corresponds to the time required to drain excess sample and reagents from the membrane so that the signal can be visualized.

After demonstration with food dyes, the device was tested using antibodies to mimic actual test design for infectious disease rapid diagnostics. Colloidal gold-labelled detection antibodies were dried on glass fiber to make conjugate pads (used in place of food dye discs). With the capture antibodies immobilized on the NC membrane, introducing a sample at the top disc initiated vertical and lateral flow of reagent complexes that completed the test. A red line on the nitrocellulose membrane at a position where capture antibodies were immobilized indicated *positive* test, while a visible control line represented a *valid* test. Figure 20 represents a duo device for simultaneous detection of a bacterial and viral target antigen. *Helicobacter pylori* and Hepatitis B represented here can be substituted by any other target depending on the need and circumstances. The duo test signals began to show within the first 5 minutes after addition of 30-40 µl of sample mixture, and was ready for reading within 10 minutes. Positive and negative tests were clearly visible as described under Fig. 20 A-C.

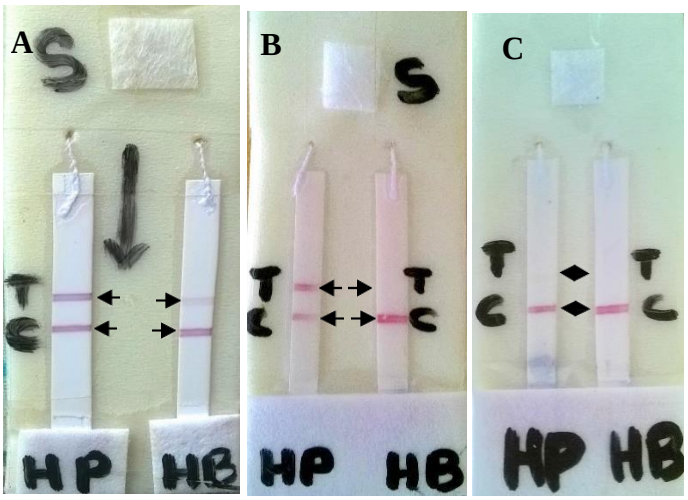


Figure 20. Duo-test device for *H. pylori* and Hepatitis B.

Note that test A is positive for both H. pylori (HP) and Hepatitis B surface antigen (HB), test B is positive for H. pylori (HP) and negative for Hepatitis B (HB) while test C is negative for both HP and HB. The sample added to test A had a mixture of both HP and HB, sample added to test B only had HP while only PBS was added to test C.

The lowest detectable antigen concentration for *H. pylori* on naked strips was 300 ng/ml with 30 ng/ml indicating a faint signal (Fig. 21A). For the case of Hepatitis B, the limit of detection for single strips was 80 µg/ml (Fig. 21B). On assembled devices, estimated limit of detection was observed to be close to the above (below 100 ng/ml and 400 ng/ml for *H. pylori* and Hepatitis B respectively), with signal intensity being slightly lower (Fig. 21C).

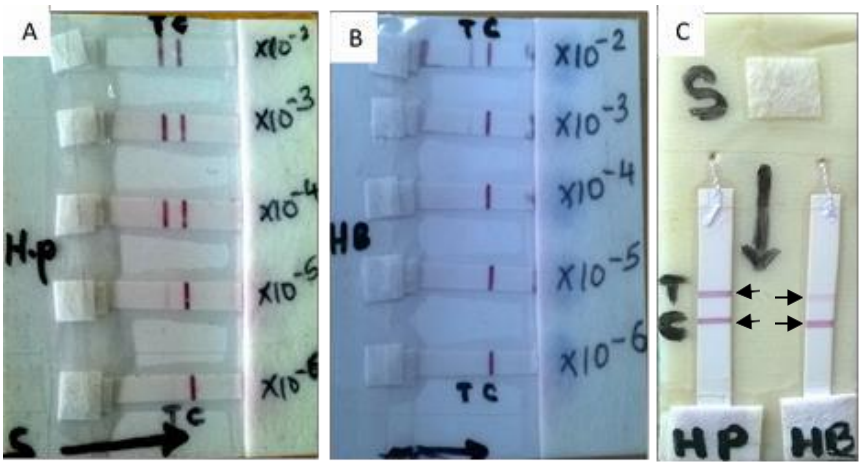


Figure 21. Detection limit for *H. pylori* and Hepatitis B strips and duo-device.

Tested concentrations on strips in Fig. 21A range from 30 µg/ml to 3 ng/ml while strips in Fig. 21 B range from 80 µg/ml to 8 ng/ml. Tested concentrations on the dual device (Fig. 21C)

were 100 ng/ml and 400 ng/ml for *H. pylori* and Hepatitis B respectively

The signal for multiplex design was read between 5-10 minutes and results were as presented in Figs. 22 and 23 below. Time for development of test results was comparable to what is usually observed in all commercial lateral flow strip tests. Also, there was no notable difference in time required by a duo test (2 strips) or multiplex test (6 strips), although slightly higher sample volume was required in case of multi-strip test ranging between 30-50 µl per multiplex run while less than 30 µl was required by single or double strip tests.

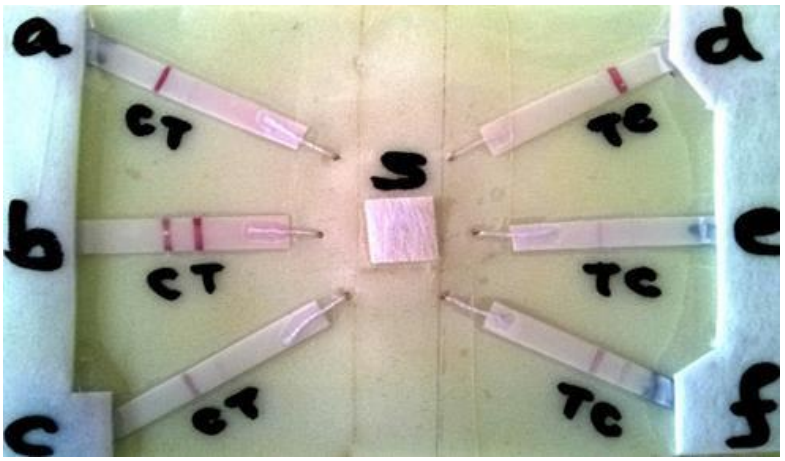


Figure 22. Multiplex immunochromatographic device with interpretation of results.

Negative results (a, d); positive results (b, f); doubtful (c); invalid (e); C=control, T=test

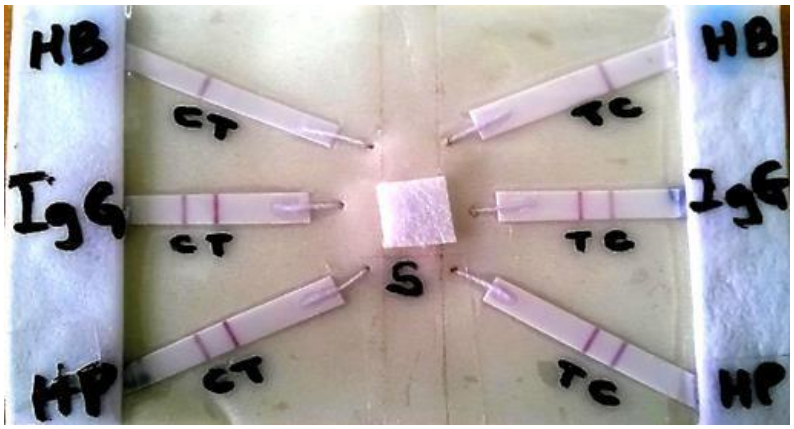


Figure 23. Six-channel multiplex immunochromatographic device.

Note that the test is positive for IgG and H. pylori (HP) but negative for Hepatitis B (HB). T: Test; C: Control

5.4 Discussion and Conclusion

5.4.1 Discussion

This study demonstrates feasibility of combining hydrophilic threads with other components of lateral flow tests to create a multiplex device that can potentially be used at point-of-care. Fabrication and assembly of this device proceeded in a three-dimensional format common for microfluidic analytical devices (Martinez *et al.,* 2008b), with the thread forming a channel that can cross multiple layers back and forth. The food dyes employed in these experiments reflected the flow of sample and reagents during actual testing. The discs were made of glass fiber using ordinary office punching machine. Polyester sewing thread used was hydrophilic, therefore, requiring no prior treatment to improve fluid wicking. It took approximately 1 minute for the dyes to emerge from under the tape. The dyes were visible as they wicked along the thread and membrane. The time taken by the dyes to clear from the membrane strips (approximately 5-10 minutes) could be equated to the time required to clear excess sample and reagents from the nitrocellulose membrane to allow for the signal to be clearly visible. Discs embedded between layers of mounting tape carry reagents that are necessary for the test. It is expected that these layers of tape help in protection of the reagent-loaded discs thereby increasing their shelf life (Fenton *et al*., 2009).

Using the above design, a device was fabricated for detection of antigens in solution. Detection antibodies conjugated to gold nanoparticles were dried on glass fiber conjugate pad. On a nitrocellulose membrane, capture antibodies were immobilized. The conjugate pads were laminated between layers of mounting tape and connected to the sample pad and nitrocellulose membrane above via hydrophilic threads. The double-sided mounting tape served as an adhesive backing for attachment of the sample pad and nitrocellulose membrane. It also served as laminate for the conjugate pads, as well as provide mechanical strength to the device. Under resource-constrained settings, this tape could be used as substitute for hard cassettes and other forms of housing (Fenton *et al*., 2009).

The designs fabricated and tested using antibodies for detection of antigens (*H. pylori*, Hepatitis B and IgG) demonstrate the potential of applying immunochromatographic thread-based platforms in diagnosis of infectious diseases. In previously described immunochromatographic assays on thread (ICAT), threads were used as both wicking (transport) and reaction surfaces (Zhou *et al.,* 2012). Both the conjugate/detection and capture antibodies were immobilized on threads or yarns. In the current design, threads are only used to facilitate wicking of solution along the test platform as detection antibodies are stored on glass fiber and capture antibodies

immobilized on nitrocellulose membrane just like on typical lateral flow strip tests. With this design, only the thread is introduced as a new component, other components such as sample loading pad, conjugate pad, nitrocellulose membrane and wicking pad remain the same.

Multiplexed platforms were made easy due to the flexibility and durability of threads (Ballerini *et al.*, 2011b). A desired number of channels was created using a sewing needle. Using threads like in the current platform facilitates multiplexing since threads can be modified to form complex networks without the need for complicated patterning. In paper-based microfluidic analytical devices, patterning of paper by various approaches such as wax-printing, laser-cutting, photolithography, etching and plotting is essential for multiplexing (Carrilho *et al.*, 2009; Lu *et al.*, 2009; Fenton *et al.*, 2009; Martinez *et al.*, 2008a; Abe *et al.*, 2008; Bruzewicz *et al.*, 2008). Using threads obviates the need for further patterning making this approach a promising alternative for designing low-cost multiplexed POC diagnostic devices. Since only simple tools such as sewing needles, threads and tape are needed during fabrication process, these platforms can simply be adopted as potential diagnostics manufacturing alternative for the developing world.

To run a six-channel device, a sample volume of approximately 30-50 µl was required. This volume is expectedly higher than what is normally required for a single test e.g. between 10-20 µl for malaria RDT (Foster *et al*., 2014) because in multiplexed designs, the sample must be distributed along multiple channels. However, this volume is comparable to what has been reported for numerous other lateral flow devices where the sample in the range of 15-200 µl has been reported (Sharma *et al.*, 2015). Threads used in this design are thin (approximately 200-300 µm in diameter), resulting in minimal fluid/sample retention during testing. Despite this fact, the flow rate was not compromised as the sample travelled across the membrane to the absorbent pad within three minutes, and signal started forming within the first 5 minutes and the test was ready within 15 minutes. This too, is comparable to most lateral flow devices reported previously, including single strip tests (Posthuma-Trumpie *et al.*, 2008).

The lowest detection limit was found to be within the range of 30-300 ng/ml, which is comparable to or vary slightly from other lateral flow tests described elsewhere (Gessler *et al.*, 2007; Jimenez *et al*., 2017), although the limit was higher for Hepatitis B surface antigen. Low sensitivity may be a result of many factors, including device assembly and storage conditions for reagents and assembled devices. Laminating the section of NC membrane with a transparent adhesive tape caused fast flow of the reaction mixture, reducing the time required to complete

the test. However, it also reduced the sensitivity of the test either by decreasing the intensity of the test line or failure to develop the test line at very low, but normally detectable analyte levels. This could probably be attributed to the fast flow rate that reduces contact time between the analyte-detector antibody complex and the capture antibody at the test and control lines. Lamination offers protection to the membrane against physical damages like scratching or wetting. Therefore, alternative protective measures that do not affect signal strength or sensitivity should be considered.

In this study, testing was done using purified antigens diluted in wash buffer (0.05% Tween 20 in PBS). Body fluids resembling this medium are such as serum, plasma, saliva and urine. However, challenges in flow dynamics, reaction time and membrane clearance time are expected when a sample with different colorimetric and viscosity properties such as blood, stool or pus is used. Evaluation of these sample media and associated flow parameters will be performed at a later stage.

Targets for this study were of diverse nature: immunoglobulins, bacterial antigen and viral antigens. This selection was intended to demonstrate that multiplex POC diagnostic devices can be designed to detect pathogens that fall into diverse taxonomic groups. This implies that viral, bacterial, protozoal and fungal pathogens can all be incorporated on one multiplex device for enhanced throughput. This is a promising prospect for addressing the challenge of diagnosing non-malarial febrile illnesses (NMFI), whose etiology has been reported to be significantly diverse, comprising of bacterial, viral, fungal, protozoal and other causes (Kiemde *et al*., 2016; Acestor *et al.,* 2012; Bisoffi and Buonfrate, 2013; Okiro and Snow, 2010; Mahende *et al.,* 2014). To enable accurate and timely diagnosis of such complex clinical presentation, multiplexed diagnostic platforms must be explored.

Most of the items used for fabrication of this device such as threads, needle, adhesive tape and papers are easily available and cheap. Conjugate and capture antibodies dried on the glass fiber and nitrocellulose membranes can remain stable for months. To run the test, the operator only applies the sample on the sample pad and observe for only a few minutes to read the results. These attributes will result in simple devices that are affordable, user-friendly, robust, equipment free, and deliverable to end users.

5.4.2 Conclusion

Multiplexed immunochromatographic diagnostic platforms can be designed using hydrophilic threads as fluid distribution channels. Using threads obviates the need for

patterning of paper that would otherwise require additional equipment like solid-ink (wax) printer or carbon dioxide laser cutter. Combining glass fiber, thread, nitrocellulose membrane and mounting tape produces a firm device that can be used at the point-of-care. More work on these platforms is needed to transform the concept into practical diagnostic solution to facilitate manufacture of low-cost devices for diagnosis of multiple illnesses at the point-of care in resource-limited settings.

CHAPTER SIX

Short-term Stability of a Thread-on-a-tape Diagnostic Device under Ambient Tropical Conditions[5]

Abstract

Introduction: Stability of rapid diagnostic tests under normal storage and transportation conditions is a key parameter that ensures storage and deliverability of diagnostics to remote areas and allows periodic delivery from central supply points. In this study, viability of thread-based colorimetric as well as immunochromatographic tests under uncontrolled storage conditions was evaluated.

Methods: Hydrophilic polyester threads were used as microfluidic channels while punched whatman paper discs were used for reagent storage and results display for colorimetric tests. Glass fiber was used for conjugate storage while nitrocellulose membrane was used for immobilization of capture antibodies for test and control lines. Glucose was used for colorimetric test device while Hepatitis B surface antigens and *H. pylori* were used for testing the immunochromatographic device. All units were produced on same day, kept at room temperature and tested periodically for 16 weeks.

Results and discussion: All tests remained viable for at least 16 weeks. However, there was visible deterioration of signal throughout the testing period suggesting loss of sensitivity with time. By end of 16^{th} week, immunochromatographic test had lost most of the signal, while it was the same for colorimetric tests on samples of low concentration.

Conclusion: Both colorimetric and immunochromatographic thread-based platforms can remain viable for a limited length of time. Precautions during preparation and storage of finished units should be taken to prolong their shelf life so as to improve their usefulness in resource-limited settings.

Keywords: Viability, stability, thread-based diagnostics, resource-limited settings

[5]*Manuscript ready for submission*

6.1 Introduction

Rapid diagnostic tests are essential tools for improving the quality of healthcare by facilitating appropriate management of the disease or condition (Leslie *et al.*, 2014; Ansah *et al.*, 2015; Johansson *et al.*, 2017). If the diagnosis is accurate and timely, treatment outcome becomes favorable. In developing countries, diagnostic systems are poor, hence most diagnosis have always been accomplished through history and clinical signs and symptoms only (Chipwaza *et al.*, 2014a). This clinical diagnostic algorithm lacks specificity and sensitivity and usually results into misdiagnoses (Sonkar *et al.*, 2016, 2017).

In recent times, rapid diagnostics for most diseases have been introduced and efforts have been made to ensure accessibility to such diagnostic services especially by the poor communities. However, the need is still high, and more options must be explored to cover all common diseases and conditions that are most prevalent in low income countries, especially Sub-Saharan Africa. Both paper-based and thread-based diagnostic devices are currently being explored as potential diagnostic options (Li *et al.*, 2010a, b; Reches *et al.*, 2010; Nilghaz *et al.*, 2012; Nilghaz *et al.*, 2013). The World Health Organization has set out criteria for diagnostics required in poor-resourced parts of the world. Such diagnostics must be ASSURED, meaning that they should be affordable, sensitive, specific, user-friendly, robust, equipment-free, and deliverable to end users (Peeling *et al.*, 2006; Urdea *et al.*, 2006). To meet the last attribute, diagnostic devices must not require special handling or storage conditions such as refrigeration since most health facilities in developing countries cannot meet this condition (Albertini *et al.*, 2012). Therefore, diagnostic devices that are being advocated must remain stable at ambient conditions for a reasonable length of time.

Temperature and humidity are among important factors affecting performance of paper- or cloth-based RDTs (Wang *et al.*, 2012). The effect of oxidation and photolysis can also cause drugs and diagnostics to deteriorate. Per the International Conference on Harmonization (ICH) and the Global Harmonization Task Force, a minimum stability for 1 year is expected at 30°C and 35% humidity in hot/dry regions, and 30°C and 65% humidity in hot/humid regions (ICH, 2003). High temperature causes denaturation of protein components of the diagnostic devices and this affects both enzyme and antigen-antibody-based diagnostics. Unfolding of binding sites on capture and/or conjugate antibodies results into loss of activity of these biomolecules, resulting into poor or no test results (Wang *et al*., 2007). Apart from this, higher temperatures have been reported to accelerate degradation through deconjugation of signal antibody–dye

complex, detachment of capture antibody from wick, and change in nitrocellulose wick flow characteristics (Bell *et al*., 2006). Exposure to moisture should always be avoided as it has been found to affect the shelf life of stored RDTs negatively (Bell *et al.,* 2006). Most of the reagents (such as conjugate antibodies) are diluted in sugar-containing buffers before being dried in inactive form on conjugate pads. If the conjugate pad is exposed to high humidity or moisture, sucrose may absorb moisture and activates the antibodies prematurely, causing the antibodies to aggregate leading to their loss of activity (Li *et al.*, 2016). Also, absorbed moisture turns the conjugate into a thick paste (syrup) thereby impairing its tendency to dissolve and release of conjugate upon sample application during testing time.

Although most rapid tests are designed to withstand normal storage conditions, extreme levels of temperature and humidity can result in deterioration in their activity. The degree of sensitivity to elements depends on the nature of test components, with some being highly sensitive to moisture, temperature or light (Mathews and Curtis, 1914). For example, in immunochromatographic tests, antibodies are usually sensitive to high temperature, and loss of activity may occur when tests are kept at higher temperatures for extended periods (Chiodini *et al.*, 2007; Mikhail *et al.*, 2011). In a colorimetric test for glucose, the enzymes glucose oxidase and horseradish peroxidase are sensitive to elevated storage temperatures and light (Zoldak *et al*., 2004) while potassium iodide is sensitive to light (Mathews and Curtis, 1914). Therefore, it is always important to assess each test with respect to its sensitivity to degrading elements under normal conditions of use. In this study, we assess how long thread-based colorimetric and immunochromatographic devices will maintain their viability when stored at room temperature and unregulated humidity conditions of tropical Africa where such tests are likely to be used.

6.2 Materials and Methods

Three device formats were prepared and evaluated: a thread-on-a-tape device for detection of glucose, a glucose-disc test, and an immunochromatographic thread-based device tested using *Helicobacter pylori* and Hepatitis B surface antigen.

6.2.1 Fabrication of colorimetric glucose test device

Thread-on-a-tape device for detection of sugar (glucose) was prepared and assembled following the procedure previously described (Seth *et al*., *unpublished*). Briefly, indicator reagents for glucose detection by glucose oxidase method (Glucose oxidase (GO), horseradish peroxidase (HRP) and potassium iodide (KI)) were dried on paper discs.

Horseradishperoxidase and GO (15 units/ml each) were mixed in a ratio of 1:5 and 5 µl of the mixture was applied on a paper disc and dried at room temperature. On another paper disc 5 µl of KI was applied and dried in dark. A thread from the sample inlet pad was sewn through the GO-HRP disc placed under the top layer of the double-sided mounting tape back to the top of the tape where a disc impregnated with KI was fixed. The device was kept in dark until time of use. To prepare the glucose-disc test, filter paper discs punched using ordinary office punching machine were prepared. Horseradish peroxidase and GO (15 units/ml each) were mixed in a ratio of 1:5 and 10 µl of the mixture was applied on the paper disc, followed by 10 µl of 0.6 M KI. Loaded discs were immediately kept in dark and dried at room temperature.

6.2.2 Fabrication of Immunochromatographic antigen detection test device

Glass fiber conjugate pad discs were treated with 2.5 µl of 10% BSA in PBS and dried at room temperature for 5 minutes and then at 50ºC for 10 minutes. Stock conjugate antibody (OD10) was diluted one fifth in conjugate buffer (2% w/v BSA, 10% w/v sucrose and 0.1% v/v Tween 20 in Borate buffered saline). This was followed by addition of 5 µl of gold conjugate antibody, each with a specific antibody type (anti-hepatitis B surface antigen and anti *H. pylori*). The pads were dried at room temperature, following which they were kept in sealed bags with desiccant ready for assembly. Capture and control antibodies were diluted in PBS (pH 7.4) to a concentration of 0.5 mg/ml. 5% methanol was included to enhance fixation of antibodies to the membrane. Test and control antibody lines were drawn on nitrocellulose membrane by using Lateral Flow Reagent Dispenser and Legato Syringe Pump (Claremont Bio Solutions, CA, USA). The dispense rate was 0.25 ml/minute and voltage was set at 5 v. After dispensing, the sheet was dried at room temperature for five minutes followed by 10 minutes at 50ºC and cut into 2x10 mm strips using a pair of scissors. The strips were kept at room temperature in dispensing envelops with desiccant. To assemble the device, threads from the sample inlet pad were sewn (using a needle) through the conjugate pads containing detector antibodies placed under the top layer of the double-sided mounting tape back to the top of the tape where thread ends overlapped on nitrocellulose membrane strips containing capture and control antibody lines. A second piece of tape was applied to enclose the conjugate pads. The devices were kept at room temperature until time of use.

To determine short-term stability or viability, assembled devices were subjected to normal ambient conditions common to the tropics where these devices are normally used. Some devices were tested immediately after assembly while the rest of the devices were kept in sealed bags with silica gel bags (desiccant) and kept at room temperature (25-30ºC) and

humidity between 40-54%. Testing was carried out after every three weeks to determine if stored devices maintained their viability. Sensitivity was arbitrarily defined as the test positivity. Loss in sensitivity and change in the limit of detection were evaluated to determine how long the test devices remained viable and stable under normal conditions of use. Testing was carried out for 16 weeks.

Reagent discs and devices were prepared, and complete assembly done on day one. On each testing day, test devices were retrieved from storage and tested as per standard procedure described above.

6.3 Results

Change of color was observed on the white disc upon addition of a glucose sample of given strength as observed in Fig. 24 below. The colorimetric signal was stronger at 3 weeks compared to 16 weeks, which indicate expected decrease in test sensitivity over time.

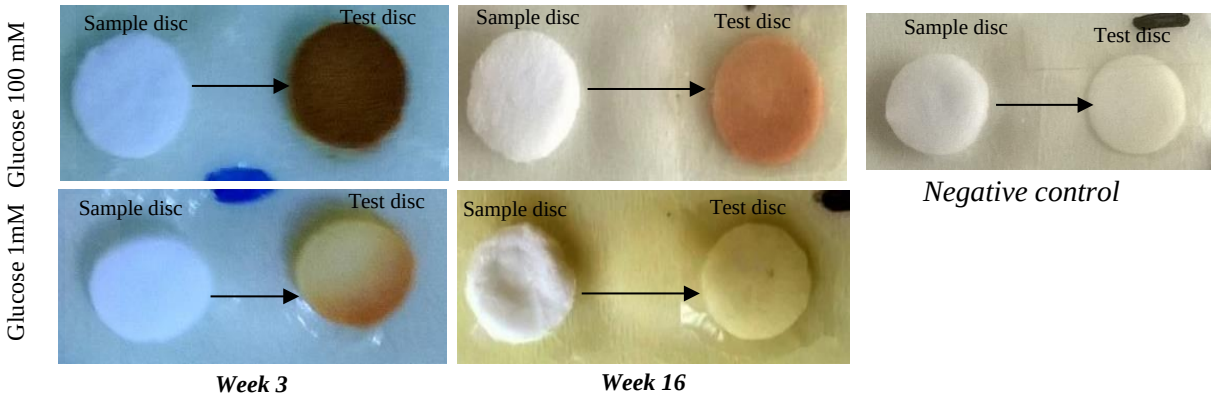


Figure 24. Thread-on-a-tape test for glucose.

Tested glucose concentration was 100 mM and 1 mM. Sample was added to the tests that had been stored at room temperature in a desiccated bag. To the negative control test, only water was applied to the sample disc.

On day one, the lowest detectable limit of glucose on glucose disc and thread-based glucose test device was less than 1 mM (Fig. 25). Testing done after 3, 6, 9, 12 and 16 weeks were also positive at 100, 10 and 1Mm glucose molar concentrations. The devices were stable for up to 16 weeks (4 months) with gradual decline in color intensity, and this time could be extended further.

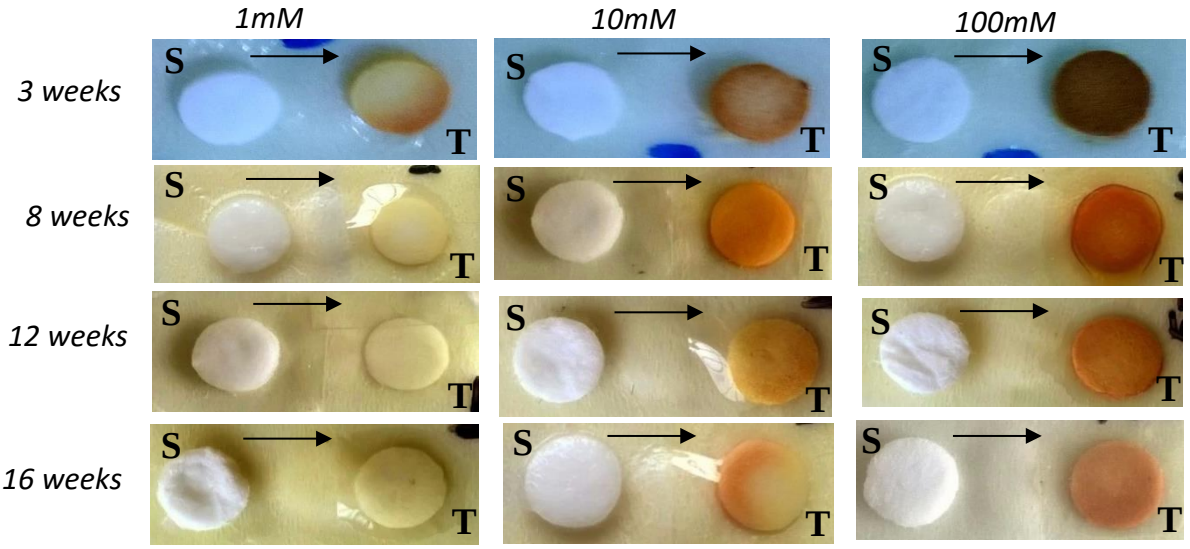


Figure 25. Results of the colorimetric thread-on-a-tape qualitative test for glucose: response up to 16 weeks.

Assembled devices were stored at room temperature in a bag with desiccant until their respective testing times.

A solution of 1 mM glucose solution remained marginally positive until end of testing time. S: sample disc; T: test disc. Arrows show the direction of sample and reagent flow from sample disc to the test disc.

Figure 26. Glucose-disc test using discs stored at ambient conditions for up to 16 weeks

Immunochromatographic devices maintained their signal strength for up to 16 weeks, during which there was systematic deterioration of the signal. The devices were not tested beyond 16 weeks, although the 16th week signal is suggestive of the end of sensitivity for the test. The

signals during earlier weeks were strongly visible as depicted in Fig. 27.

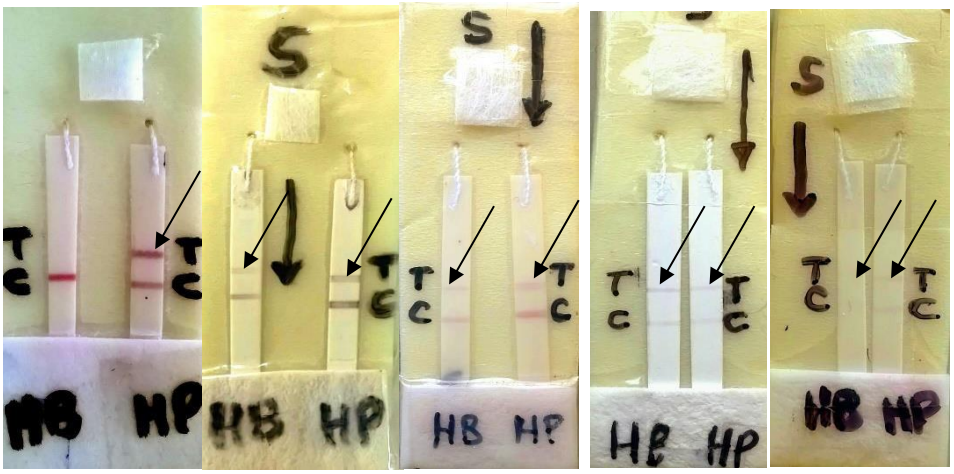


Figure 27. Immunochromatographic test device viability for up to 16 weeks.

Sample tested on day 1 was negative for HB while for the rest of the testing days, the sample mixture had 100 ng/ml (HP) and 400 ng/ml (HB). The signal is faint on the 16th week testing, suggesting the end of viability. Downward arrows indicate the direction of sample flow along the device while slanted arrow indicate the test signal. T: test; C: control.

6.4 Discussion and Conclusion

Reagents stored on discs remained viable until the end of testing time (16 weeks). Threads were also able to wick solutions through the device as effectively as freshly-made devices. This is important as it shows that thread-and-paper-based device are capable of withstanding normal storage conditions, making them potential for application in resource-limited parts of the world where they are most needed. Ability to remain viable at ambient conditions for extended periods of time facilitates deliverability of such diagnostic devices, making it possible for these devices to be delivered to remote parts of the country once or twice a year.

The limit of detection for glucose did not vary significantly between freshly made devices, or devices kept in assembled form for the whole duration of testing. This creates choice of keeping reagents in dry form on discs to be assembled into devices later or keep assembled diagnostic devices depending on convenient situation. Storing reagents in dried form on paper discs retains viability of some test components like enzymes such as glucose oxidase and horseradish peroxidase (for glucose test) which would otherwise require refrigeration. It is also possible to spot all necessary reagents on paper discs and keep them for later use or transport them to a different location where assembly will be done upon need. The glucose

disc test was designed to be a standalone test where everything is done on the disc itself, meaning that all necessary reagents are pre-loaded on a filter paper disc, which is kept dry before a sample to be tested is applied. This design is very simple in manufacturing and convenient to use. Once prepared, discs require dry storage away from direct light. Exposure to light results in photo-oxidation of potassium iodide, which is otherwise oxidized enzymatically in a positive test. Humidity/moisture re-dissolves the reagents rendering them inactive. In this study, the lowest quantity of glucose detectable on the disc for freshly made discs was < 0.1 M. The signal strength on subsequent testing interval decreased, but the sensitivity was maintained at 0.1 M throughout the testing period. Even after more than 16 weeks, the disc was still able to detect 0.1 M of glucose.

Immunochromatographic thread-based test units were all assembled on day one and kept at room temperature until testing day. There was change in signal intensity with time, with the signal almost disappearing at week 16. Despite this fact, the devices remained viable for almost 4 months under less than optimal storage conditions. Commercial diagnostic kits are individually sealed in a plastic package, and each test is opened only during testing. This keeps away moisture, which has been found to affect the viability of immunochromatographic test devices (Bell *et al.,* 2006). On the contrary, all devices in this study were placed in one bag, which was constantly being opened during each testing day thereby exposing the devices to moisture. Loss of viability could be attributed to exposure to moisture. It can therefore be expected that the viability time will be extended if ideal storage conditions are ensured.

Rapid tests have been reported to be viable for up to 12-24 months without refrigeration (Phommasone *et al.,* 2015). This attribute allows periodic distribution and storage of such tests to remote areas where cold-chain facilities are not available. In Sub-saharan Africa it is estimated that less than 30% of health facilities have reliable access to electricity (Adair-Rohani *et al.,* 2013). In remote, poorly resourced areas especially in developing countries, tests which have a shelf life exceeding 18 months at ambient conditions are favorable since they reduce the probability of wastage of expired tests (Peeling *et al*., 2010). The results reported here cover only 16 weeks, and during this time, signals had started to diminish. However, if all conditions are appropriately optimized a longer shelf-life may be achieved.

The main limitation of this study include the fact that test devices were not individually wrapped in air-tight wrappings, resulting into repeated opening and closure which allowed exposure to light and air. This limitation could have resulted into accelerated loss of sensitivity. Despite this fact, thread-on-a-tape diagnostic devices in this study were shown to

maintain viability for a considerable length of time at normal storage conditions. This is a promising step towards efforts of further developing this type of devices to meet current requirement for ASSURED diagnostics in the developing world.

CHAPTER SEVEN

General Discussion, Conclusions and Recommendations

7.1 General discussion

7.1.1 The need for appropriate diagnosis in disease management

As a prerequisite to disease management, diagnosis must be timely and accurate. Delayed diagnosis may lead to complications and deterioration of patient health, while wrong or inaccurate diagnosis may result into potentially wrong treatment, both of which compromise chances of complete recovery. To have maximum and desirable impact, diseases should be accurately and timely diagnosed. However, diagnostic facilities in low resource settings face significant limitations in terms of personnel, technology, equipment, and working environment. Most health facilities in resource-limited settings face acute shortage in terms of modern diagnostic equipment and technology, appropriately trained personnel to run the equipment (machines), and poor electricity supplyto maintain cold storage of reagents used for diagnosis (Berkelman *et al.,* 2006). These challenges have contributed to the dependence on clinical diagnosis, which has been proved to be inaccurate most of the times (McNerney, 2015; Sonkar *et al.*, 2016).

7.1.2 Low-cost designs for ASSURED Point-of-Care (POC) Diagnostics

In the present study, various designs for low-cost diagnostics were developed and tested. Both paper- and thread-based designs were discussed. The devices were designed in consideration with WHO's criteria which require diagnostics to be ASSURED, implying that they should be Affordable, Sensitive, Specific, User-friendly, Rapid/robust, Equipment-free, and Deliverable to end users (Urdea *et al*., 2006). In developing countries, health sectors are compromised in many ways to an extent that it becomes impossible to provide optimal diagnostic services (Albertini *et al.,* 2012). This study introduced simple approaches towards developing ASSURED diagnostics for the developing world. The use of paper, patterned by wax-printing and laser-cutting provided an option for producing multiplexed diagnostic platforms for infectious and non-infectious conditions (Seth and Buza, 2017). To diversify the available options, thread-based diagnostic platforms were also evaluated and found to have great potential as possible diagnostic alternatives. This study explored two thread-based designs: a thread-on-a-tape diagnostic test platform for colorimetric detection of chemical analytes and an immunochromatographic thread-based test platform for diagnosis of infectious diseases. The first design (thread-on-a-tape) uses threads to distribute and mix reagents and deliver them to a paper disc for result display. It is ideal for diagnostic tests that are based on the principle

of colorimetric reactions, where the end-point color signal is associated with presence or absence of the test substance. The second design (immunochromatographic test) uses threads to distribute and deliver bioactive molecules (antibodies, antigens and other proteins) to a membrane where further reaction results into a signal because of accumulation of a colored signal reporter molecule in presence or absence of the test substance (Seth *et al.,* 2018). Both designs have been found to be promising as potential diagnostic candidate platforms. The colorimetric platform is potentially useful for measurement of analytes of biological importance like glucose, uric acid, ketones, nitrites and others (Martinez *et al*., 2007; Li *et al*., 2010a, b; Reches *et al.,* 2010). The immunochromatographic platform will be very useful in designing multiplex diagnostics for whose principle is based on antigen-antibody reactions. This includes differential diagnosis of febrile illnesses, neonatal and maternal septicaemia, ante-natal sexually-transmitted infections (STIs) screening, and acute bacterial and viral diarrheal syndromes. Other potential applications of this platform include bio-threats detection, drug of abuse measurements, microbial and antimicrobial food safety monitoring, environmental safety monitoring, and fertility testing.

While finer details of sensitivity and specificity will be covered in subsequent studies, other aspects of the ASSURED criteria can be addressed by the designs. The simplicity of the designing process, availability and low cost of materials are necessary for production of low-cost (affordable) diagnostics. Other attributes are also considered since the described designs require no special storage or handling conditions, require no other equipment for reading the results, hence ideal for use in remote areas.

7.1.3 Technology transfer for ASSURED diagnostics manufacturing in Tanzania: The need and potential

Rapid diagnostics currently used in Tanzania are imported, mostly from Europe, America and Asia since no local company has invested in diagnostics production. Although it is not within the scope of this study, importing such diagnostics is likely to be costlier as compared to local production of diagnostic devices within the country (WHO, 2011). Local production facilitates demand-driven choice of diagnostics to be produced depending on local needs. If multiplexed ASSURED diagnostics are to be locally manufactured, such units should consider diseases and conditions that are of concern in Tanzania. As can be observed in the studies reported here, transferring the technology for diagnostics manufacturing is possible. Investment in essential equipment and training of local scientists in relevant fields is

necessary to achieve this goal. Target groups of infection may include: non- malarial febrile illnesses, zoonotic bacterial infections, causes of neonatal sepsis in hospitals, sexually-transmitted infections combined with pregnancy test, and others. To initiate the process of technology transfer, simple immunochromatographic tests may be the first aim. Here, a minimum set of equipment, supplies and biologicals required may include: antibody/antigen dispenser, laser-cutter, strip cutter, laminator, conjugate, capture and control reagents, nitrocellulose membranes, glass fiber pads, chromatography papers, and adhesive plastic backing cards. Initial investment for establishment of a diagnostics manufacturing center may be moderately high, but the gains will eventually outweigh the resources invested. Although computing the financial cost-benefit analysis was beyond the scope of this study, it can be expected that the resultant diagnostic devices will be of low cost because the materials and methods used are cheap, although this may not always be true especially where there is stiff competition from established suppliers (WHO, 2011).

7.2 Conclusion

This study has shown that diagnosis is still a challenge in resource limited parts of the world especially in Sub Saharan Africa and action is required to address this problem. Low-cost multiplex diagnostic platforms are necessary to deal with this challenge.

Paper-and thread-based diagnostics have shown to be viable options to address the challenge of diagnosis in resource-limited settings. A variety of multiplex designs for paper-based rapid diagnostics can be developed to improve diagnosis of common diseases and conditions in resource-limited settings. In this study, various such designs have been developed and tested. This approach promises a potential alternative to the more time-consuming and rather expensive sequential testing in circumstances where diseases presenting with similar symptoms cannot be clinically differentiated.

This study has also established that thread can be combined with paper discs and mounting tape to fabricate low-cost microfluidic devices with potential of application as point-of-care diagnostics. Hydrophobic threads can be modified to become hydrophilic by a range of hydrophilizing procedures. The use of cheap materials such as sewing needles, office punching machines, threads, paper and tape means that the overall cost of resultant devices is likely to be low, hence affordable by people living in resource-limited countries. Large-scale production of devices will need a new consideration and may require an engineering design to facilitate automation of the production process. The use of white paper discs facilitates

colour display, implying that a hydrophilic thread of any colour can be applied for fabrication of such devices.

This study also endeavoured to design a multiplexed immunochromatographic diagnostic platforms using hydrophilic threads avs fluid distribution channels. Using threads was found to obviate the need for patterning of paper that would otherwise require additional equipment like solid-ink (wax) printer or carbon dioxide laser cutter or other engraving machines. Combining glass fiber, thread, nitrocellulose membrane and mounting tape produces a firm device that can be used at the point-of-care situations.

In all thread-based designs, a double-sided adhesive mounting tape has been used to facilitate fixation of various components of the devices. This tape is strong thus it forms a firm backing for the device such that even in absence of a conventional backing card or cassette, the tape can provide short-term durability for these devices. Thread-based devices can remain viable at ambient conditions for a considerable length of time when necessary requirements such as protection from light and humidity are observed. Improvement on production and storage conditions will potentially extend the shelf-life of the proposed devices to suit periodic delivery to health facilities or points of use.

7.3 Recommendations

More work on these platforms is needed to transform the concept into practical diagnostic solution to facilitate manufacture of low-cost devices for diagnosis of multiple illnesses at the point-of care in resource-limited settings. Prototype devices should be developed and tested. During initial testing, clinical samples should be tested to establish the sensitivity, specificity and predictive values of the tests. Initial targets may involve multiplexed diagnostics for non-malarial febrile illnesses, neonatal and maternal septicemia, bacterial and viral diarrheal syndrome, antimicrobial residues, and environmental monitoring.

Research and training in diagnostics should focus on both vertical and horizontal technology transfer to facilitate acquisition of knowledge and expertise in diagnostics designing and development by local scientists. Higher learning and research institutions should aim at training local biotechnologists and medical scientists who will spearhead the envisioned technology transfer and adoption.

Emphasis should be put on development and growth of local diagnostic industry to facilitate local production of diagnostics, especially low-cost diagnostic platforms. Local start-ups may

team up with established manufacturers abroad as they establish themselves. Implementation of this will ensure that the highly needed rapid diagnostics are available and affordable, contrary to the existing situation where most, if not all diagnostics are imported from other countries.

In order for these recommendations to be realised, a strong commitment is required on part of the government, pivate sector, individual scientists and business entities. The government should dedicate more resources towards development of the diagnostic industry in collaboration with the private sector. It is through this kind of collaboration and commitment that such ambitions will be realised.

REFERENCES

Abe, K., Suzuki, K. and Citterio, D. (2008). Inkjet-printed microfluidic multianalyte chemical sensing paper. *Analytical Chemistry*. **80:** 6928-6934.

Acestor, N., Cooksey, R., Newton, P. N., Menard, D., Guerin, P. J., Nakagawa, J., … and Bell, (2012). Mapping the aetiology of non-malarial febrile illness in Southeast Asia through a systematic review-terra incognita impairing treatment policies. *PLoS One.* **7**: p e44269.

Acharya, T., Daar, A. S., Thorsteinsdóttir, H., Dowdeswell, H. E. and Singe, P. A. (2004). Strengthening the Role of Genomics in Global Health. *PLoS Medicine.* **1**(3)

Adair-Rohani, H., Zukor, K., Bonjour, S., Wilburn, S., Kuesel, A. C., Hebert, R. and Fletcher, R. (2013). Limited electricity access in health facilities of sub-Saharan Africa: a systematic review of data on electricity access, sources, and reliability. *Global Health Science and Practice*. **1**:249–61

Agwu, E., Ihongbe, J. C., Okogun, G. R. and Inyang, N. J. (2009). High incidence of co-infection with Malaria and Typhoid in febrile HIV infected and AIDS patients in Ekpoma, Edo State, Nigeria. *Brazilian Journal of Microbiology.* **40:** 329-332.

Albertini, A., Lee, E., Coulibaly, S. O., Sleshi, M., Faye B., Mationg, M. L., … and Bell, D. (2012). Malaria rapid diagnostic test transport and storage conditions in burkina faso, senegal, ethiopia and the philippines. *Malaria Journal*. **11**:406.

Aluwong, T. and Bello, M. (2010). Emerging diseases and implications for Millennium Development Goals in Africa by 2015 - an overview. *Veterinaria Italiana.* **46**(2):137-145.

Ansah, E. K., Narh-Bana, S., Affran-Bonful, H., Bart-Plange, C., Cundill, B., Gyapong, M. and Whitty, C. J. M. (2015). The impact of providing rapid diagnostic malaria tests on fever management in the private retail sector in Ghana: a cluster randomized trial. *BMJ*. 350: h1019

Ashton, R. A., Kefyalew, T., Tesfaye, G., Counihan, H., Yadeta, D., Cundill, B., … and Kolaczinski, J. H. (2010). Performance of three multi-species rapid diagnostic tests for diagnosis of Plasmodium falciparum and Plasmodium vivax malaria in Oromia Regional State, Ethiopia. *Malaria Journal.* **9**: 297.

Babiker, H. A. and Schneider, P. (2008). Application of molecular methods for monitoring transmission stages of malaria parasites. *Biomedical Materials.* **3**, p 034007.

Babiker, H. A., Schneider, P. and Reece, S. E. (2008). Gametocytes: insights gained during a

decade of molecular monitoring. *Trends in Parasitology.* **24**: 525-530.

Ballerini, D. R., Li, X. and Shen, W. (2011a). An inexpensive thread-based system for simple and rapid blood grouping. *Analytical and Bioanalytical Chemistry.* **399**:1869–1875

Ballerini, D. R., Li, X. and Shen, W. (2011b). Flow control concepts for thread-based microfluidic devices. *Biomicrofluidics*. **5**, 014105; doi: 10.1063/1.3567094

Baltzell, K., Elfving, K., Shakely, D., Ali, A. S., Msellem, M., Gulati, S. and Mårtensson, S. (2013). Febrile illness management in children under five years of age: a qualitative pilot study on primary health care workers' practices in Zanzibar. *Malaria Journal.* **12:** p 37.

Bell, D. and Peeling, R.W. (2006). Evaluation of rapid diagnostic tests: malaria. Nat Revieds Microbiology. 4:S7–20. doi: 10.1038/nrmicro1525

Berkelman, R., Cassell, G., Specter, S., Hamburg, M. and Klugman, K. (2006). The "achilles heel" of global efforts to combat infectious diseases. *Clinical Infectious Disease*. **42**:1503–1504. doi: 10.1086/504494

Bhandari, P., Narahari, T. and Dendukuri, D. (2011). 'Fab-Chips': a versatile, fabric-based platform for low-cost, rapid and multiplexed diagnostics. *Lab on a Chip*. **11**(15): 2493.

Biggs, H. M., Bui, D. M., Galloway, R. L. Stoddard, R. A., Shadomy, S. V., Morrissey, A. B., … and Crump, J. A. (2011). Leptospirosis among hospitalized febrile patients in northern Tanzania. *American Journal of Tropical Medicine and Hygiene.* **85**: 275-281.

Biggs, H. M., Hertz, J. T., Munishi, O. M Galloway, R. L., Marks, F., Saganda, W., … and Crump, J. A. (2013). Estimating leptospirosis incidence using hospital-based surveillance and a population-based health care utilization survey in Tanzania. *PLoS Neglected Tropical Diseases.* **7**, p e2589.

Biggs, H. M., Lester R., Nadjm, B., Mtove, G., Todd, J. E., Kinabo, G. D., … and Crump, J. A. (2014). Invasive Salmonella infections in areas of high and low malaria transmission intensity in Tanzania. *Clinical Infectious Diseases.* **58**: 638-647.

Bisoffi, Z. and Buonfrate, D. (2013). When fever is not malaria. *Lancet Global Health* **1**: e11- e12.

Boisen, M. L., Oottamasathien, D., Jones, A. B., Millett, M. M., Nelson, D. S., Bornholdt, Z. A., … and Pitts, K. R.; Viral Hemorrhagic Fever Consortium. (2016). Field Validation of the ReEBOV Antigen Rapid Test for Point-of-Care Diagnosis of Ebola Virus Infection. *Journal of Infectious Diseases*. **15**; 214 (suppl 3):S203–S209. doi: 10.1093/infdis/jiw261

Brouqui, P., Rolain, J. M., Foucault, C. and Raoult, D. (2005). Short report: Q fever and Plasmodium falciparum malaria co-infection in a patient returning from the Comoros archipelago. *American Journal of Tropical Medicine and Hygiene.* **73**: 1028-1030.

Bruce-Chwatt, L. (1984). DNA probes for malaria diagnosis. *Lancet*. **1**, p 795.

Bruzewicz, D. A., Reches, M. and Whitesides, G. M. (2008). Low-cost printing of poly (dimethylsiloxane) barriers to define microchannels in paper. *Analytical Chemistry.* **80**: 3387-3392.

Carr, G. M. and Neary, J. P. (2008). Water Quality for Ecosystem and Human Health Publisher: United Nations Environment Programme, Global Environment Monitoring System/Water Programme, 2008, ISBN 9295039513, 9789295039513

Carrilho, E., Martinez, A. W. and Whitesides, G. M. (2009). Understanding wax printing: a simple micropatterning process for paper-based microfluidics. *Analytical Chemistry.* **81:** 7091-7095.

Chandramohan, D., Jaffar, S. and Greenwood, B. (2002). Use of clinical algorithms for diagnosing malaria. *Tropical Medicine and International Health.* **7**: 45-52.

Chappuis, F., Alirol, E., D'Acremont, V., Bottieau, E. and Yansouni, C. P. (2013). Rapid diagnostic tests for non-malarial febrile illness in the tropics. *Clinical Microbiology and Infection.* **19:** 422-431.

Chin, C. D., Linder, V. and Sia, S. K. (2007). Lab-on-a-chip devices for global health: past studies and future opportunities. *Lab on a Chip*. **7:** 41-57.

Chiodini, P. L., Bowers, K., Jorgensen, P., Barnwell, J. W., Grady, K. K, Luchavez, J., … and Bell, D. (2007). The heat stability of Plasmodium lactate dehydrogenase-based and histidine-rich protein 2-based malaria rapid diagnostic tests. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. **101**(4):331-7

Chipwaza, B., Mugasa, J. P., Mayumana, I., Amuri, M., Makungu, C. and Gwakisa, P. S. (2014b). Self-medication with anti-malarials is a common practice in rural communities of Kilosa district in Tanzania despite the reported decline of malaria. *Malaria Journal.* **13**: 252.

Chipwaza, B., Mugasa, J. P., Mayumana, I., Amuri, M., Makungu, C. and Gwakisa, P. S. (2014a). Community knowledge and attitudes and health workers' practices regarding non-malaria febrile illnesses in eastern Tanzania. *PLoS Neglected Tropical Diseases.***8**: e2896.

Cohee, L. M., Kalilani-Phiri, L., Boudova, S., Joshi, S., Mukadam, R., Seydel, K. B., … and Laufer, M. K. (2014). Submicroscopic malaria infection during pregnancy and the

impact of intermittent preventive treatment. *Malaria Journal.* **13:** p274.

Coleman, R. E., Sattabongkot, J., Promstaporm, S., Maneechai, N., Tippayachai, B., Kengluecha, A., … and Khuntirat, B. (2006). Comparison of PCR and microscopy for the detection of asymptomatic malaria in a Plasmodium falciparum/vivax endemic area in Thailand. *Malaria Journal.* **5**: p 121.

Craig, J. C., Williams, G. J., Jones, M., Codarini, M., Macaskill, P., Hayen, A., … and McCaskill, M. (2010) The accuracy of clinical symptoms and signs for the diagnosis of serious bacterial infection in young febrile children: prospective cohort study of 15 781 febrile illnesses. *BMJ.* **340**: 1594.

Crump, J. A. (2012). Typhoid Fever and the challenge of nonmalaria febrile illness in sub-saharan Africa. *Clinical Infectious Diseases.* **54**: 1107-1109.

Crump, J. A., Morrissey, A. B., Nicholson, W. L., Massung, R. F., Stoddard, R. A., Galloway, R. L., … and Bartlett, J. A. (2013). Etiology of severe non-malaria febrile illness in Northern Tanzania: a prospective cohort study. *PLoS Neglected Tropical Diseases.* **7**: e2324.

D'Acremont, V., Lengeler, C. and Genton, B. (2010). Reduction in the proportion of fevers associated with Plasmodium falciparum parasitaemia in Africa: a systematic review. *Malaria Journal.* **9**: 240.

D'Acremont, V., Kaiser, L., and Genton, B. (2014a). Causes of fever in outpatient Tanzanian children. *New England Journal of Medicine.* **370**: 2243-2244.

D'Acremont, V., Kilowoko, M., Kyungu, E., Philipina, S., Sangu, W., Kahama-Maro, J., … and Genton, B. (2014b). Beyond malaria--causes of fever in outpatient Tanzanian children. *New England Journal of Medicine.* **370:** 809-817.

Doolan, D. L., Dobano, C. and Baird, J. K. (2009). Acquired immunity to malaria. *Clinical Microbiology Reviews.* **22**: 13-36,

Douglas, A. D., Andrews, L., Draper, S. J., Bojang, K., Milligan, P., Gilbert, S. C., … and Hill, A. V. (2011). Substantially reduced pre-patent parasite multiplication rates are associated with naturally acquired immunity to Plasmodium falciparum. *Journal of Infectious Diseases.* **203**: p 1337-1340.

Dowling, M. A. and Shute, G. T. (1966). A comparative study of thick and thin blood films in the diagnosis of scanty malaria parasitaemia. *Bulletin of the World Health Organization*. **34**: 249-267.

Fenton, E. M., Mascarenas, M. R., Lopez, G. P. and Sibbett, S. S. (2009). Multiplex lateral-flow test strips fabricated by two-dimensional shaping. *ACS Applied Materials and*

Interfaces. **1:** 124-129.

Ferguson, J. P., Baxter, G. A., McEvoy, J. D., Stead, S., Rawlings, E. and Sharman, M. (2002). Detection of streptomycin and dihydrostreptomycin residues in milk, honey and meat samples using an optical biosensor. *Analyst*. **127:** 951-956.

Font, F., Alonso, G. M., Nathan, R., Kimario, J., Lwilla, F., Ascaso, C., … and Alonso, P. L. (2001). Diagnostic accuracy and case management of clinical malaria in the primary health services of a rural area in south-eastern Tanzania. *Tropical Medicine and International Health* **6**: 423-428.

Forney, J. R., Wongsrichanalai, C., Magill, A. J., Craig, L. G., Sirichaisinthop, J., Bautista, C. T., … and Gasser, R.A. Jr. (2003). Devices for rapid diagnosis of Malaria: evaluation of prototype assays that detect Plasmodium falciparum histidine-rich protein 2 and a Plasmodium vivax-specific antigen. *Journal of Clinical Microbiology.* **41**: 2358-2366.

Foster, D., Cox-Singh, J., Mohamad, D. S. A., Krishna, S., Chin, P. P. and Singh, B. (2014). Evaluation of three rapid diagnostic tests for the detection of human infections with *Plasmodium knowlesi. Malaria Journal.* **13**:60 **DOI:** 10.1186/1475-2875-13-60

Gessler, F., Pagel-Wiederb, S., Avondetc, M. and Bohnel, H. (2007). Evaluation of lateral flow assays for the detection of botulinum neurotoxin type A and their application in laboratory diagnosis of botulism. *Diagnostic Microbiology and Infectious Disease.* **57**:243 – 249

Gething, P. W., Smith D. L., Patil, A. P., Tatem, A. J., Snow, R. W. and Hay, S. I. (2010a). Climate change and the global malaria recession. *Nature.* **465**: 342-345.

Gething, P. W., Kirui, V. C., Alegana, V. A., Okiro, E. A., Noor, A. M. and Snow, R. W. (2010b). Estimating the number of paediatric fevers associated with malaria infection presenting to Africa's public health sector in 2007. *PLoS Medicine.* **7**, p e1000301.

Gething, P. W., Patil, A. P., Smith, D. L., Guerra, C. A., Elyazar, I. R., Johnston, G. L., Tatem, A. J. and Hay, S. I. (2011). A new world malaria map: Plasmodium falciparum endemicity in 2010. *Malaria Journal.* **10**: p 378.

Gonzalez, A., Estala, L., Gaines, M. and Gomez, F. A. (2016). Mixed thread/paper-based microfluidic chips as a platform for glucose assays. *Electrophoresis.* 2016 May3. doi: 10.1002/elps.201600029.

Hay, S. I., Guerra, C. A., Gething, P. W., Patil, A. P., Tatem, A. J., Noor, A. M., … and Snow, R. W. (2009) A world malaria map: Plasmodium falciparum endemicity in 2007. *PLoS Medicine.* **6**: p e1000048.

Hossain, S. M. Z., Ozimok, C., Sicard, C., Aguirre, S. D., Ali, M. M., Li, Y. F. and Brennan, J. D., (2012). Multiplexed paper test strip for quantitative bacterial detection. *Analytical and Bioanalytical Chemistry.* **403** (6): 1567-1576.

ICH (2003). ICH harmonized tripartite guideline: Stability testing of new drug substances and products Q1A(R2): Current Step 4 version. Date accessed: 2017, http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q1A_R2/Step4/Q1A_R2_Guideline.pdf.

Iqbal, J., Muneer, A., Khalid, N. and Ahmed, M. A. (2003). Performance of the OptiMAL test for malaria diagnosis among suspected malaria patients at the rural health centers. *American Journal of Tropical Medicine and Hygiene.* **68**: 624-628.

Ishengoma, D. R., Rwegoshora, R. T., Mdira, K. Y., Kamugisha, M. L., Anga, E. O., Bygbjerg, I. C., … and Magesa, S. M. (2009). Health laboratories in the Tanga region of Tanzania: the quality of diagnostic services for malaria and other communicable diseases. *Annals of Tropical Medicine and Parasitology.* **103**: 441-453.

Ishengoma, D. R., Derua, Y. A., Rwegoshora, R. T., Tenu, F., Massaga, J. J., Mboera, L. E. and Magesa, S. M. (2010). The performance of health laboratories and the quality of malaria diagnosis in six districts of Tanzania. *Annals of Tropical Medicine and Parasitology.* **104**: 123-135.

Ishengoma D. S., Francis, F., Mmbando, B. P., Lusingu, J. P. A., Magistrado, P., Alifrangis, M., … and Lemnge, M. M. (2011). Accuracy of malaria rapid diagnostic tests in community studies and their impact on treatment of malaria in an area with declining malaria burden in north-eastern Tanzania. *Malaria Journal.* **10:** p 176.

Ishengoma, D. S., Mmbando, B. P., Segeja, M. D., Alifrangis, M., Lemnge, M. M. and Bygbjerg, I. C. (2013). Declining burden of malaria over two decades in a rural community of Muheza district, north-eastern Tanzania. *Malaria Journal.* **12:** 338.

Jimenez, A., Rees-Channer, R. R., Perera, R., Gamboa, D., Chiodini, P. L., González, I. J., … and Ding, X. C. (2017). Analytical sensitivity of current best-in-class malaria rapid diagnostic tests. *Malaria Journal.* 2017, **16**:128 https://doi.org/10.1186/s12936-017-1780-5

Johansson, E. W., Hildenwall, H., Nsona, H., Buj de Lauwerier, V. and Peterson, S. S. (2017). Do rapid diagnostic tests improve overall quality of care? *BMJ* .**357**: j2290

Kamphee, H., Chaiprasert, A., Prammananan, T., Wiriyachaiporn, N., Kanchanatavee, A. and Dharakul, T. (2015). Rapid molecular detection of multidrug-resistant tuberculosis by PCR-nucleic acid lateral flow immunoassay. *PLos One* **10**: e0137791

Karl, S., Gurarie, D., Zimmerman, P. A., King, C. H., St. Pierre, T. G. and Davis, T. M. E. (2011). A sub-microscopic gametocyte reservoir can sustain malaria transmission. *PLoS One.* **6**: p e20805.

Keong, B. C. and Sulaiman, W. (2006) Typhoid and malaria co-infection - an interesting finding in the investigation of a tropical Fever. *Malaysian Journal of Medical Sciences.* **13**: 74-75.

Kiemde, F., Spijker, R., Mens, P. F., Tinto, H., Boele, M. and Schallig, H. D. (2016). Etiologies of non-malaria febrile episodes in children under 5 years in sub-Saharan Africa. *Tropical Medicine and International Health*. **21**(8):943–55.

Koczula, K. M. and Gallotta, A. (2016) Lateral flow assays. *Essays in Biochemistry*. **60**(1): 111–120

Kunda, J., Fitzpatrick, J., Kazwala, R., French, N. P., Shirima, G., MacMillan, A., … and Cleaveland, S. (2007). Health-seeking behaviour of human brucellosis cases in rural Tanzania. *BMC Public Health* 7, p 315.

Law, J. W. F., Ab Mutalib, N. S., Chan, K. G. and Lee, L. H. (2014). Rapid methods for the detection of foodborne bacterial pathogens: principles, applications, advantages and limitations. *Frontiers in Microbiology*. **5**:770.

Leslie, T., Mikhail, A., Mayan, I., Cundill, B., Anwar, M., Bakhtash, S. H., … and Rowland, (2014). Rapid diagnostic tests to improve treatment of malaria and other febrile illnesses: patient randomised effectiveness trial in primary care clinics in Afghanistan. *BMJ*. **348**: g3730 (Published 19 June 2014)

Lewis, G. G., Robbins, J. S. and Phillips, S. T. (2013). Point-of-care assay platform for quantifying active enzymes to femtomolar levels using measurements of time as the readout. *Analytical Chemistry*. **85:** 10432-10439.

Lewis, G. G., Robbins, J. S. and Phillips, S. T. (2014). A Prototype Point-of-Use Assay for Measuring Heavy Metal Contamination in Water Using Time as a Quantitative Readout. *Chemical Communication.* **50**: 5352– 5354.

Li, J., Collins, W. E., Wirtz R. A., Rathore, D., Lal, A. and McCutchan, T. F. (2001). Geographic subdivision of the range of the malaria parasite Plasmodium vivax. *Emerging Infectious Diseases.* **12**: 35-42.

Li, W., Prabakaran, P., Chen, W., Zhu, Z., Feng, Y. and Dimitrov, D. S. (2016). Antibody Aggregation: Insights from Sequence and Structure. *Antibodies*. ***5***(3): 19

Li, X., Tian, J., Nguyen, T. and Shen, W. (2008). Paper-based microfluidic devices by plasma treatment. *Analytical Chemistry*. **80:** 9131-9134.

Li, X., Tian, J. and Shen, W. (2009). Thread as a Versatile Material for Low-Cost Microfluidic Diagnostics. *ACS Applied Materials and Interfaces*. **2**(1):1–6

Li, X., Ballerini, D. R., Tian, J. and Shen, W. (2010a). Thread as a substrate for low-cost point-of-care diagnostics. *WIT Transactions on Ecology and the Environment*. **154**: 233 – 244. Doi10.2495/CHEM110221

Li, X., Tian, J. and Shen, W. (2010b). Thread as a Versatile Material for Low-Cost Microfluidic Diagnostics. *ACS Applied Materials and Interfaces*. **2**(1): 1

Li, X., Ballerini, D. R. and Shen, W. (2012). A perspective on paper-based microfluidics: Current status and future trends. *Biomicrofluidics*. **6**(1)**:** 11301-11301-13.

Lu, Y., Shi, W., Jiang, L., Qin, J. and Lin, B. (2009). Rapid prototyping of paper-based microfluidics with wax for low-cost, portable bioassay. *Electrophoresis.* **30**: 1497-1500.

Luxemburger, C., Nosten, F., Kyle, D. E., Kiricharoen, L., Chongsuphajaisiddhi, T. and White, J. (1998). Clinical features cannot predict a diagnosis of malaria or differentiate the infecting species in children living in an area of low transmission. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* **92**: 45-49. .

Mabey, D., Peeling, R. W., Ustianowski, A. and Perkins, M. D. (2004). Diagnostics for the developing world. *Nature Reviews Microbiology*. **2:** 231-240.

Mahende, C., Ngasala, B., Lusingu, J., Butichi, A., Lushino, P., Lemnge, M. and Premji, Z. (2014). Aetiology of acute febrile episodes in children attending korogwe district hospital in north-eastern Tanzania. *PLoS One.* **9:** p e104197

Mansfield, M. A. (2009). Nitrocellulose Membranes for Lateral Flow Immunoassays: A Technical Teatise. In *Lateral Flow Immunoassays. Edited by Wong RC, Tse HY.* New York: Humana Press. Springer. pp. 95-113.

Martinez, A. W., Phillips, S. T., Butte, M. J. and Whitesides, G. M. (2007). Patterned Paper as a Platform for Inexpensive, Low Volume, Portable Bioassays. *Angewandte Chemie International Edition.* **46**(8): 1318–1320. doi:10.1002/anie.200603817.

Martinez, A. W., Phillips, S. T., Wiley, B. J., Gupta, M. and Whitesides, G. M. (2008a). FLASH: a rapid method for prototyping paper-based microfluidic devices. *Lab on a Chip*. **8**: 2146- 2150.

Martinez, A. W., Phillips, S. T. and Whitesides, G. M. (2008b). Three-dimensional microfluidic devices fabricated in layered paper and tape. *PNAS* **105** (50) 19563- 19564; doi:10.1073/iti05008105

Martinez, A. W., Phillips, S. T., Whitesides, G. M. and Carrilho, E. (2010). Diagnostics for the

developing world: microfluidic paper-based analytical devices. *Analytical Chemistry*. **82:** 3-10.

Martinez, A. W. (2011). Microfluidic paper-based devices: From POCKET to paper-based ELISA. *Bioanalysis*. **3**(23): 2589–2592.

Mathews, J. H. and Curtis, H. A. (1914). The Photolysis of Potassium Iodate. *Journal of Physical Chemistry*. *18* (8): 641–652

Matsuura, K., Chen, K., Tsai, C., Li, W., Asano, Y., Naruse, K. and Cheng, C. (2014). Paper-based diagnostic devices for evaluating the quality of human sperm. *Microfluidics and Nanofluidics*. **16**: 857–867

May, K. (1991) Home tests to monitor fertility. *American Journal of Obstetrics and Gynecology*. **165**: 2000–2002

Mayxay, M., Castonguay-Vanier, J., Chansamouth, V., Dubot-Pérès, A., Paris, D. H., Phetsouvanh, R., … and Newton, P. N. (2013. Causes of non-malarial fever in Laos: a prospective study. *Lancet Global Health*. **1**: e46-e54.

Mboera, L. E. G., Mazigo, H. D., Rumisha, S. F. and Kramer, R. A. (2013). Towards malaria elimination and its implication for vector control, disease management and livelihoods in Tanzania. *Malaria World Journal.* **4** (19).

McCombie, S. C. (2002). Self-treatment for malaria: the evidence and methodological issues. *Health Policy and Planning.* **17**: 333-344.

McNerney (2015). Diagnostics for Developing Countries. *Diagnostics.* **5**(2): 200-

Minja, D. T., Schmiegelow, C., Oesterholt, M., Magistrado, P. A., Boström, S., John, D., … and Lusingu, J. P. A. (2012). Reliability of rapid diagnostic tests in diagnosing pregnancy-associated malaria in north-eastern Tanzania. *Malaria Journal.* **11**: 211.

Mmbando, B. P., Vestergaard, L. S., Kitua, A. Y., Lemnge, M. M., Theander, T. G. and Lusingu, J. P. (2010). A progressive declining in the burden of malaria in north-eastern Tanzania. *Malaria Journal.* **9**:216

Mockenhaupt, F. P., Rong, B., Till, H., Eggelte, T. A., Beck, S., Gyasi-Sarpong, C., … and Bienzle, U. (2000). Submicroscopic Plasmodium falciparum infections in pregnancy in Ghana. *Tropical Medicine and International Health*. **5**: 167-173.

Mouatcho, J. C. and Goldring, J. P. (2013). Malaria rapid diagnostic tests: challenges and prospects. *Journal of Medical Microbiology.* **62**: 1491-1505.

Msangeni, H. A., Kamugisha, M. L., Sembuche, S. H., Malecela, E., Akida, J. A., Temba, F. F., ... and Lemnge, M. M. (2011). Prospective study on severe malaria among in-patients at Bombo regional hospital, Tanga, north-eastern Tanzania. *BMC Infectious*

Diseases. **11:** p 256.

Mubi, M., Janson, A., Warsame, M. Mårtensson, A., Källander, K., Petzold, M. G., … and Björkman, A. (2011). Malaria rapid testing by community health workers is effective and safe for targeting malaria treatment: randomised cross-over trial in Tanzania. *PLoS One.* **6**: p e19753.

Muhe, L., Oljira, B., Degefu, H., Enquesellassie, F. and Weber, M. W. (1999). Clinical algorithm for malaria during low and high transmission seasons. *Archives of Diseases in Childhood*. **81**: 216-220.

Murray, C. K., Bell, D., Gasser, R. A. and Wongsrichanalai, C. (2003). Rapid diagnostic testing for malaria. *Tropical Medicine and International Health*. **8**: 876-883.

Nicastri, E., Bevilacqua, N., Sane, S. M., Paglia, M. G., Meschi, S., Ame, S. M., ... and Ippolito, G. (2009) Accuracy of malaria diagnosis by microscopy, rapid diagnostic test, and PCR methods and evidence of antimalarial overprescription in non-severe febrile patients in two Tanzanian hospitals. *American Journal of Tropical Medicine and Hygiene.* **80**: 712-717.

Nielsen, K., Yu, W. L., Kelly, L., Bermudez, R., Renteria, T., Dajer, A.,… and de Eschaide, S. T. (2008). Development of a lateral flow assay for rapid detection of bovine antibody to Anaplasma marginale. *Journal of Immunoassay Immunochemistry*. **29**, 10–18

Nilghaz, A., Wicaksono, D. H. B., Gustiono, D., Abdul-Majid, F. A., Supriyanto, E. and Abdul Kadir, M. R. (2012). Flexible microfluidic cloth-based analytical devices using a low-cost wax patterning technique. *Lab on a Chip*. **12**(1): 209.

Nilghaz, A., Ballerini, D. R. and Shen, W. (2013). Exploration of microfluidic devices based on multi-filament threads and textiles: A review. *Biomicrofluidics* **7**: 051501

Ochola, L. B., Vounatsou, P., Smith, T., Mabaso, M. L. and Newton, C. R. (2006). The reliability of diagnostic techniques in the diagnosis and management of malaria in the absence of a gold standard. *Lancet Infectious Diseases.* **6**: 582-588.

Okell, L. C., Ghani, A. C., Lyons, E. and Drakeley, C. J. (2009). Submicroscopic infection in Plasmodium falciparum-endemic populations: a systematic review and meta-analysis. *Journal of Infectious Diseases.* **200**: 1509-1517.

Okiro, E. A. and Snow, R. W. (2010). The relationship between reported fever and Plasmodium falciparum infection in African children. *Malaria Journal.* **9**: p 99.

Oliveira-Ferreira, J., Lacerda, M. V., Brasil, P., Ladislau, J. L. B., Tauil, P. L. and Daniel-Ribeiro, C. T. (2010). Malaria in Brazil: an overview. *Malaria Journal.* **9**: p 115.

O'Meara, W. P., Mangeni, J. N., Steketee, R. and Greenwood, B. (2010). Changes in the burden of malaria in sub-Saharan Africa. *Lancet Infectious Diseases.* **10**: 545-555.

Pang, T. and Peeling, R. W. (2007). Diagnostic tests for infectious diseases in the developing world: two sides of the coin. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* **101:** 856-857.

Pattanasin, S., Proux, S., Chompasuk, D., Luwiradaj, K., Jacquier, P., Looareesuwan, S. and Nosten, F. (2003) Evaluation of a new Plasmodium lactate dehydrogenase assay (OptiMAL-IT) for the detection of malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* **97**: 672-674.

Payne, D. (1988). Use and limitations of light microscopy for diagnosing malaria at the primary health care level. *Bulletin of the World Health Organization.* **66**: 621-626.

Peeling, R. W., Holmes, K. K., Mabey, D. and Ronald, A. (2006) Rapid tests for sexually transmitted infections (STIs): the way forward. *Sexually Transmitted Infections.* **82 Suppl 5**: p v1-v6.

Peeling, R. W., Smith, P. G. and Bossuyt, P. M. (2010). A guide for diagnostic evaluations. *Nature Reviews Microbiology*. **4(Suppl.**): S2–6

Pelton, R. (2009). Bioactive Paper Provides a Low-cost Platform for Diagnostics**.** *Trends in Analytical Chemistry* 9 A.D., **28** (8)**:** 925-942.

Petti, C. A., Polage, C. R., Quinn, T. C., Ronald, A. R. and Sande, M. A. (2000). Laboratory Medicine In Africa: A Barrier to Effective Health Care. *Clinical Infectious Diseases*. **42:** 377-382.

Phillips, S. T. and Lewis, G. G. (2014). The expanding role of paper in point-of-care diagnostics. *Expert Review of Molecular Diagnostics.* **14**: 123-125.

Phommasone, K., Sengvilaipaseuth, O., de Lamballerie, X., Vongsouvath, M., Phonemixay, O., Blacksell, S. D., … and Dubot-Pérès, A. (2015). Temperature and the field stability of a dengue rapid diagnostic test in the tropics. *American Journal of Tropical Medicine and Hygiene*. **93**: 33–39.

Pikkemaat, M. G., Rapallini, M. L. B. A., Oostra-Van Dijk, S. and Elferink, J. W. A. (2009). Comparison of three microbial screening methods for antibiotics using routine monitoring samples. *Analytica Chimica Acta*. **637:** 298-304.

Pinkevych, M., Petravic, J., Chelimo, K., Kazura, J. W., Moormann, A. M. and Davenport, M. P. (2012). The dynamics of naturally acquired immunity to Plasmodium falciparum infection. *PLoS Computational Biology*. **8**: p e1002729.

Posthuma-Trumpie, G. A., Korf, J. and van Amerongen, A. (2009). Lateral flow (immuno) assay: its strengths, weaknesses, opportunities and threats. A literature survey. *Analytical and Bioanalyitical Chemistry*. **393**(2):569–582

Prabhu, M., Nicholson, W. L., Roche, A. J., Kersh, G. J., Fitzpatrick, K. A., Oliver, L. D., … and Crump, J. A. (2011). Q-fever, spotted fever group, and typhus group rickettsioses among hospitalized febrile patients in northern Tanzania. *Clinical Infectious Diseases.* **53:** e8-15.

Punjabi, N. H., Taylor, W. R., Murphy, G. S., Purwaningsih, S., Picarima, H., Sisson, J., ... and Richie, T. L. (2012). Etiology of acute, non-malaria, febrile illnesses in Jayapura, northeastern Papua, Indonesia. *American Journal of Tropical Medicine and Hygiene.* **86**: 46-51.

Reches, M., Mirica, K. A., Dasgupta, R., Dickey, M. D., Butte, M. J. and Whitesides, G. M. (2010). Thread as a Matrix for Biomedical Assays *ACS Applied Materials and Interfaces.* **2**(6): 1722.

Reyburn, H., Mbatia, R., Drakeley, C., Carneiro, I., Mwakasungula, E., Mwerinde, O., … and Whitty, C. J. M. (2004). Overdiagnosis of malaria in patients with severe febrile illness in Tanzania: a prospective study. *BMJ*. **329**: p 1212.

Reyburn, H. (2010). New WHO guidelines for the treatment of malaria. *BMJ* **340**: p c2637.

Richards, J. S., Stanisic, D. I., Fowkes, F. J., Tavul, L., Dabod, E., Thompson, J. K.,… and Beeson, J. G. (2010). Association between naturally acquired antibodies to erythrocyte-binding antigens of Plasmodium falciparum and protection from malaria and high-density parasitemia. *Clinical Infectious Diseases.* **51**: e50-e60.

Rohrman, B. A., Leautaud, V., Molyneux, E. and Richards–Kortum, R. R. (2012) A lateral flow assay for quantitative detection of amplified HIV-1 RNA. *PLoS One*. **7:** e45611

Rutta, A. S., Francis, F., Mmbando, B. P., Ishengoma, D. S., Sembuche, S. H., Malecela, E. K., … and Lemnge, M. M. (2012). Using community-owned resource persons to provide early diagnosis and treatment and estimate malaria burden at community level in north-eastern Tanzania. *Malaria Journal.* **11:** p 152.

Safavieh, R., Zhou, G. Z. and Juncker, D. (2011). Microfluidics made of yarns and knots: from fundamental properties to simple networks and operations *Lab on a Chip*. **11**(15): 2618.

Santana-Morales, M. A., Afonso-Lehmann, R. N., Quispe, M. A., Reyes, F., Berzosa, P., Benito, A., ...and Martinez-Carretero, E. (2012). Microscopy and molecular biology

for the diagnosis and evaluation of malaria in a hospital in a rural area of Ethiopia. *Malaria Journal.* **11**: p 199.

Schonhorn, J. E., Fernandes, S. C., Rajaratnam, A., Deraney, R. N., Rolland, J. P. and Mace, C. R. (2014). A device architecture for three-dimensional, patterned paper immunoassays. *Lab on a Chip*. **14:** 4653-4658.

Schoonman, L. and Swai, E. S. (2009). Risk factors associated with the seroprevalence of leptospirosis, amongst at-risk groups in and around Tanga city, Tanzania. *Annals of Tropical Medicine and Parasitology.* **103**: 711-718.

Seth, M., Mdetele, D., Phillips, S. T. and Buza, J. (2015). Challenges in Diagnosis of Febrile Illnesses in Tanzania in the Era of Declining Malaria Epidemiology. *American Journal of Research Communication*. **3**(5)**:** 88-110.

Seth, M. and Buza, J. (2017). Multiplex paper-based designs for Point-of-Care Diagnostics. *International Journal of Research in Medical Sciences*. **5**(11):5052-5059.

Seth, M., Mdetele, D. and Buza, J. (2018). Immunochromatographic thread-based test platform for diagnosis of infectious diseases. *Microfluidics and Nanofluidics* **22**(4)**:**45

Shankar, B. P., Manjunatha Prabhu, B. H., Chandan, S., Ranjith, D. and Shivakumar,V. (2010). Rapid Methods for detection of Veterinary Drug residues in Meat. *Veterinary World*. **3**(5):241-246

Sharma, S., Zapatero-Rodríguez, J., Estrela, P. and O'Kennedy, R. (2015). Point-of-Care Diagnostics in Low Resource Settings: Present Status and Future Role of Microfluidics. *Biosensors*. **5**: 577-601; doi:10.3390/bios5030577

Shyu, R. H., Shyu, H. F., Liu, H. W. and Tang, S. S. (2002). Colloidal gold-based immunochromatographic assay for detection of ricin. *Toxicon*. **40**: 255–258.

Sicard, C., Gien, C., Aubie, B., Wallace, D., Jahanshahi-Anbuhi, S., Pennings, K., … and Fillipe, C. D. (2015). Tools for water quality monitoring and mapping using paper-based sensors and cell phones. *Water Research*. **70**:360–369.

Snounou, G. (1993). Population dynamics of human malaria parasites. *Parassitologia* **35 (Suppl)**: 113-116.

Snounou, G., Pinheiro, L., Goncalves, A., Fonseca, L., Dias, F., Brown, K. N. and Rosario, V. E. (1993). The importance of sensitive detection of malaria parasites in the human and insect hosts in epidemiological studies, as shown by the analysis of field samples from Guinea Bissau. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* **87:** 649-653.

Snow, R. W., Eckert, E. and Teklehaimanot, A. (2003). Estimating the needs for artesunate-

based combination therapy for malaria case-management in Africa. *Trends in Parasitology.* **19**: 363-369.

Snow, R. W., Guerra, C. A., Noor, A. M., Myint, H. Y. and Hay, S. I. (2005). The global distribution of clinical episodes of Plasmodium falciparum malaria. *Nature.* **434**: 214- 217.

Sonkar, S. C., Washnik, K., Mishra, P. K., Kumar, A., Mittal, P. and Saluja, D. (2016). Comparative analysis of syndromic and PCR-based diagnostics assay reveals misdiagnosis/ overtreatment for trichomoniasis based on subjective judgment in symptomatic patients. *Infectious Diseases of Poverty.* **5**:42.

Sonkar, S. C., Washnik, K., Kumar, A., Sharma, V., Mittal, P., Mishra, P. K., ... and Saluja, D. (2017). Evaluating the utility of syndromic case management for three sexually transmitted infections in women visiting hospitals in Delhi, India. Scientific Reports. **7**: 1465.

Toldra, F. and Reig, M. (2006). Methods for rapid detection of chemical and veterinary drug residues in animal foods. *Trends in Food Science and Technology*. **17**: 482–489

Uneke, C. J. (2008). Concurrent malaria and typhoid fever in the tropics: the diagnostic challenges and public health implications. *Journal of Vector Borne Diseases*. **45**: 133-142.

Urdea, M., Penny, L. A., Olmsted, S. S., Giovanni, M. Y., Kaspar, P., Shepherd, A., ... and Burgess, D. C. H. (2006) Requirements for high impact diagnostics in the developing world. *Nature*. **444 Suppl 1**: 73-79.

Ursing, J., Rombo, L., Rodrigues, A., Aaby, P. and Kofoed, P. E. (2014). Malaria transmission in Bissau, Guinea-Bissau between 1995 and 2012: malaria resurgence did not negatively affect mortality. *PLoS. One.* **9**: p e101167.

Van Den Ende, J., Jacobs, J. and Bisoffi, Z. (2010). Utility of point-of-care malaria rapid diagnostic tests. *American Journal of Tropical Medicine and Hygiene.* **83:** 207.

Wang, H., Li, Y. J., Wei, J. F., Xu, J. R., Wang, Y. H. and Zheng, G. X. (2014). Paper-based three-dimensional microfluidic device for monitoring of heavy metals with a camera cell phone**.** *Analytical and Bioanalalytical Chemistry*. **406:** 2799-2807.

Wang, J., Yiu, B., Obermeyer, J., Filipe, C. D. M., Brennan, J. D. and Pelton, R. (2012). Effects of Temperature and Relative Humidity on the Stability of Paper-Immobilized Antibodies. *Biomacromolecules*. **13:** 559−564

Wang, W., Singh, S., Zeng, D. L., King, K. and Nema, S. J. (2007). Antibody Structure, Instability, and Formulation. *Journal of Pharmaceutical Sciences.* **96:** 1−26.

Weng, X., Gaur, G. and Neethirajan, S. (2016). Rapid Detection of Food Allergens by Microfluidics ELISA-Based Optical Sensor. *Biosensors*. **6**(2): 24.

Whitesides, G. M. (2009). A lab the size of a postage stamp, TEDX Boston. http://www.ted.com/talks/george_whitesides_a_lab_the_size_of_a_postage_stamp.html

WHO (1988). Malaria diagnosis: memorandum from a WHO meeting. *Bulletin of the World Health Organization.* **66**: 575-594.

WHO (2009). World Malaria Report 2009. Geneva (Generic Report).

WHO (2011). IMAI district clinician manual: hospital care for adolescents and adults: guidelines for the management of illnesses with limited resources. *World Health Organization*.

Wongsrichanalai, C. and Miller, R. S. (2002). Malaria rapid tests: a public health perspective. *Lancet* **359**: 1781

Wongsrichanalai, C., Arevalo, I., Laoboonchai, A., Yingyuen, K., Miller, R. S., Magill, A. J., ... and Gasser, R. A. Jr. (2003a). Rapid diagnostic devices for malaria: field evaluation of a new prototype immunochromatographic assay for the detection of Plasmodium falciparum and non-falciparum Plasmodium. *American Journal of Tropical Medicine and Hygiene.* **69:** 26-30.

Wongsrichanalai, C., Murray, C. K., Gray, M. Miller, R. S., McDaniel, P., Liao, W. J., ... and Magill, A. J. (2003b). Co-infection with malaria and leptospirosis. *American Journal of Tropical Medicine and Hygiene.* **68:** 583-585.

Wongsrichanalai, C., Barcus, M. J., Muth, S., Sutamihardja, A. and Wernsdorfer, W. H. (2007). A review of malaria diagnostic tools: microscopy and rapid diagnostic test (RDT). *American Journal of Tropical Medicine and Hygiene.* **77**: 119-127.

Xing, S., Jiang, J. and Pan, T. (2013). Interfacial microfluidic transport on micropatterned superhydrophobic textile. *Lab on a Chip*. **13**(10): 1937

Yager, P., Edwards, T., Fu, E. Helton, K., Nelson, K., Tam, M. R. and Weigl, B. H. (2006). Microfluidic diagnostic technologies for global public health. *Nature*. **442**: 412-418.

Yang, Y., Noviana, E., Michael, P., Nguyen, M. P., Brian, J., Geiss, B. J, … and Henry, C. S. (2017) Paper-Based Microfluidic Devices: Emerging Themes and Applications. *Analytical Chemistry.* **89(**1): 71-91

Yetisen, A. K., Akram, M. S. and Lowe, C. R. (2013). Paper-based microfluidic point-of-care diagnostic devices. *Lab-on-a-Chip*. **13**(12): 2210-2251.

Yukich, J., D'Acremont, V., Kahama, J., Swai, N. and Lengeler, C. (2010). Cost savings with

rapid diagnostic tests for malaria in low-transmission areas: evidence from Dar es Salaam, Tanzania. *American Journal of Tropical Medicine and Hygiene*. **83**: 61-68.

Zasada, A. A., Formińska, K., Zacharczuk, K., Jacob, D. and Grunow, R. (2015). Comparison of eleven commercially available rapid tests for detection of *Bacillus anthracis*, *Francisella tularensis* and *Yersinia pestis*. *Letters in Applied Microbiology*. **60**: 409–413.

Zhou, G., Mao, X. and Juncker, D. (2012). Immunochromatographic Assay on Thread. *Analytical Chemistry*. 84 (18): 7736–7743.

Zoldák, G., Zubrik, A., Musatov, A., Stupák, M. and Sedlák, E. (2004) Irreversible Thermal Denaturation of Glucose Oxidase from *Aspergillus niger* is the Transition to the Denatured State with Residual Structure. *Journal of Biological Chemistry*. **279**: 47601–47609.