MOLECULAR POINT-OF-CARE DIAGNOSTIC FOR *TREPONEMA PALLIDUM SUBSP. PERTENUE* (YAWS)

by

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A Thesis

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Master of Science in Biomedical Engineering

Weldon School of Biomedical Engineering

West Lafayette, Indiana

May 2019

THE PURDUE UNIVERSITY GRADUATE SCHOOL
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To **Professor Yaw Adu Sarkodie**: who introduced me to yaws and ignited a passion for finding a solution to this disease.

To **Portia Mamavi Selenu Goh**: Thank you for being there and for understanding.

ACKNOWLEDGMENTS

This study was funded by a Mark and Pamela LAMP scholarship from the Weldon School of Biomedical Engineering, Purdue University as well as Grand Challenges Exploration Program-Gates (Opp150806) from the Bill and Melinda Gates foundation. I am grateful to the two organizations for making this study a reality.

I am extremely grateful to Dr. Jacqueline Linnes for the opportunity to study in her lab, I am awed by her mentorship and supervision. I have learnt so many techniques that I would not have had the opportunity to learn, had it not been for her. I am also very grateful to Dr. Arvind Raman and Dr. Herman Sintim, members of my thesis committee for their mentorship and support.

I am indebted to the members of the Linnes Lab for welcoming me to the lab and by supporting my work. I am especially grateful to Taylor Moehling and Elizabeth Phillips for taking the time to teach me all the techniques things I needed to learn in the lab. I am indeed thankful for the opportunity to have learnt and worked with such great minds.

I am grateful to the staff of the parasitology department of the Komfo Anokye Teaching Hospital namely Ernest Badu Boateng, Sylvia Karikari, Michael Odame Lartey and Nicholas Asuo Gyebi for their patience and support.

To Shirley Victoria Simpson of the Noguchi Memorial Institute for Medical Research, Frank Biney of the Asamankese who provided the yaws samples used in the study, to Moses Djan who offered support during the Sensitivity and specificity studies as well as to Elliot Eli Dogbe for providing the syphilis samples

Finally, I am indebted to both my American family and my Ghanaian family for being supportive during my stay in the US. I am grateful to the Riders, Jeff and Abby and their children for the warm dinners and the laughter, I am thankful to my friend Kusum Raghuvaran for being there for me throughout my stay. I am also indebted to Dr and Mrs Baah of PIWC Lafayette Assembly as

well as the entire PIWC family for their constant support. I am grateful to Josephine Tanye, Sean Declan Basing, Anne Marie Basing and the entire Basing family for the patience and love.

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ABSTRACT

Author: Basing, Laud Anthony W. MSBME
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Degree Received: May 2019
Title: A Molecular Point of Care Diagnostic for *Treponema pallidum subspecies pertenue*(yaws).
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Yaws is a neglected tropical disease caused by *Treponema pallidum subsp. pertenue*, which affects children living in very deprived hard to reach rural communities. Infection with yaws, if untreated could lead to gross deformities and disabilities. Yaws can be treated effectively with a single dose of azithromycin and the World Health Organization has earmarked yaws for eradication by the year 2020. The eradication of yaws however is constrained by the lack of rapid, accurate diagnosis. I sought to develop a molecular point-of-care test for the diagnosis of yaws. A Loop-mediated isothermal amplification (LAMP) assay with primers targeting the conserved gene, *tp0967*, with visual detection by lateral flow test strip was developed and optimized. The limit of detection was evaluated while 63 samples from clinical cases of yaws and 5 samples with PCR-confirmed syphilis were used to determine the sensitivity and specificity of the assay compared to the current molecular testing protocol. Reagents were dried in tubes and tested up to 14 days. The developed LAMP assay was found to be optimal when run at 65^{o}C in a water bath for 30 minutes. The limit of detection was $2.7*10^{4}$ DNA copies/ml. The sensitivity of the LAMP assay using unextracted and DNA extracted samples were 0.67 and 1.00 respectively. None of the syphilis samples tested positive in any of the assays. We show the development of a fast and sensitive LAMP assay for *yaws* detected by lateral flow test strip. Using extracted DNA, the assay sensitivity is at par with gold standard detection. The assay can be adapted to minimal sample processing required for in-field detection without DNA extraction.

CHAPTER 1. BACKGROUND

1.1 Introduction

Diagnostic tests are an essential component of patient care [1]. Despite the increased availability and increasing complexity of diagnostic tests in developed countries, developing countries that bear the majority of the burden of infectious diseases do not have access to advanced diagnostic methods and are largely dependent of clinical diagnosis or rapid point-of-care tests [2], [3]. Many laboratories in resource-limited countries (RLCs) are sparsely distributed and patients do not have access to laboratory tests due to economic reasons [4]. The laboratories that exists are hugely deprived and have challenges with electricity, equipment and human resources [5]. In most African countries, diagnostic services are provided in a tiered framework that ranges from state-of-the-art centralized laboratory settings in highly populated regions to remote Community Health Posts (CHP) that have limited resources and personnel [6]. A well implemented centralized laboratory in most African countries has the potential to achieve high throughput testing with multi-purpose platforms, often at low cost [7]. However, to date, the function of existing laboratory services in developing regions remains poor due to multiple factors including low instrument utilization rates, poor data management, supply chain issues, human resource challenges, low rates of results returned, poor quality systems, poor sample transportation systems and low-quality specimens [8].

To compound this problem appropriate tests have not yet been developed for several neglected tropical diseases (NTDs) making it difficult to distinguish between illnesses with similar symptoms [9]. A typical example of this is the diagnosis and differentiation of several diseases found in tropical countries that present with skin lesions which can be difficult to distinguish from one another even for the experienced clinician [10]. The most common of these diseases is yaws, a neglected treponemal disease which caused by the pathogen *Treponemal pallidum subsp pertenue* (TPE) [11]. The eradication of yaws, is constrained by accurate diagnosis as current available diagnostics are inadequate as they are either too expensive or they are not specific[11]. Developing a rapid test for use in endemic countries therefore offers a new solution for accurately diagnosing yaws [12].

1.2 Treponema pallidum Subsp pertenue

Treponema pallidum subspecies pertenue is a corkscrew-shaped bacterium. It is a fragile organism with a rigid, uniform and tightly wound with deep spirals with a pitch of approximately 1.0-1.5 μm [13]. It measures 6-20 micrometers (μm) long , and an average length of 10 μm and width of 0.13-0.15 μm [14]. Its genome is approximately 1.14 million basepairs (mb) and encodes about 1041 putative proteins [15]. *Treponema pallidum subsp pertenue* is deficient in a lot of metabolic activities [16]. Although TPE is able to undergo glycolysis, it does not have a Tricarboxylic Acid (TCA) cycle, lacks an electron transport chain, does not have pathways that use alternative carbon sources for generating energy as well as making nucleotides and cofactors needed in enzymatic reactions. [17]. The prevailing school of thought is that TPE derives all the major macromolecules from the host as it also lacks amino acid and fatty acid synthesis pathways while interconversion pathways may likely be used to make micro molecules. [18].

Treponema pallidum subsp pallidum (TPA) which causes syphilis and *Treponema pallidum subsp pertenue* (TPE), the causative organism for yaws were initially two different species mainly because they cause different diseases with quite different symptoms as well as the fact that they affect different epidemiological populations. [19]. With the application of DNA hybridization however, the two strains were closely related and had to be reclassified as a single species. [20][21]. DNA hybridizations reveal that *Treponema pallidum subsp pertenue* strain Gauthier and *Treponema pallidum subsp pallidum* strain Nichols are identical with only about 0.2% of genome differences [22].

1.3 Yaws

1.3.1 Pathology and Clinical Features

Treponema pallidum is very sensitive to oxygen and is unable to survive outside its host and would lose its ability to cause infections within hours of being outside its host [23] . In spite of its challenges however, the pathogen is able to cause chronic infections with different symptoms and manifestations in its host. [24]. There is nothing in the *Treponema pallidum* genome sequence that readily explains the classical yaws symptoms [22]. Despite the fact that lipopolysaccharide (LPS), the endotoxin *an important virulence factor in gram negative bacteria,* is lacking in *Treponema*

pallidum [25] the pathogen has the ability to infect and persist in several parts of the body [26]. Although the disease is known to infect some adults, it commonly infects children from 3 years to 15 years [27]. Aside the fact that children younger than 3 years are not mobile, hence less likely to come into contact with the pathogen, the age preference in humans for TPE is thought to be due to the fact that adults have immunity whilst children do not [28].

The clinical presentation of yaws has a number of similarities to that of syphilis [29]. There are two stages in yaws, early (primary and secondary) and late (tertiary) similar to the staging in syphilis [30]. This staging is largely artificial as a lot of patients do not present with classical symptoms that allow for staging. Most patients present with a mixture of symptoms that defy staging [31]. In primary yaws, a papule develops after about 9 -90 days (average 21) after inoculation at the site of inoculation. appears at the site of inoculation after about 21 days (range 9–90 days). This may then develop into either into an exudative papilloma, or degenerate to become a single, non-tender ulcer [32]. Yaws ulcers are characterized by raised edges with dirty and crusty bases but may be also be soggy and filled with treponema containing exudates [33]. Yaws lesions cover the whole body but the most common sites are the face, legs, ankles, buttocks and arms. 'Splitpapules' have been known to occur around the mouth while regional lymphadenopathy is a common occurrence [34]. As the pathogen spreads to the lymphatic and Hematogenous tissues, secondary lesions, characterized by osteoperiostitis begin to occur. This is usually a month or two after primary infection although it's been known to take up to 24 months. [28]. Bone involvement causes pain at night as well as periosteal thickening which can easily be seen. Polydactylitis is a common manifestation of secondary yaws and it often involves the phalanges of the fingers. [35]. Even after clinical symptoms have been cleared, the infection if not completely treated could pass into a lag period known as latency while tertiary yaws occurs in about 10% of untreated patients. The symptoms described for secondary yaws become exacerbated in tertiary yaws with mutilating facial ulcerations of both the palate and the nasopharynx being the most important characteristic. [34].

1.3.2 Epidemiology

Yaws is considered a disease of poverty and affects people living in warm, humid, and moist climates as conditions provide a favorable environments for the infection to spread [36]. Yaws is

very common among children between 3 and 15 years old living in endemic communities who are also considered as the reservoir for infection [37]. Yaws is most often contracted after a cut or abrasion in the lower leg [37]. Transmission is also known to occur through direct skin contact with fluids from an infected lesion [38]. With the above explanation, it is no surprise that studies have shown more males than females suffering from the disease. The prevailing explanation is that boys are more active than girls and therefore suffer more traumas and direct contact [39]. There is no evidence of the congenital transmission of yaws as children born to mothers previously or currently infected have not been shown to have the disease. [40].

1.3.3 Burden

The true global burden of yaws is unfortunately still unknown, as there is no official international system where disease prevalence is reported as is done for a number of infectious diseases. [41]. A review of documents and studies from 1950 to 2016 shows that although there are 99 countries and territories that are known to be endemic for yaws, only 15 of those countries and territories have reported recent data on yaws. This implies that even for the 15 countries and territories, the full extent of the disease burden may be lacking, it would therefore be quite inaccurate to indicate that there are 15 endemic countries as data from the remaining 84 previously endemic countries are lacking [42]. The recent data are all obtained from within Africa (Ghana, Benin, Togo, Cameroon, Cote D'Ivoire, the Democratic Republic of Congo and Central African Republic), Western Pacific Regions (Papua New Guinea, Solomon Islands, Vanuatu, Wallis and Futuna and Philippines) and South-East Asia (Indonesia and Timor-Leste) [43]. India and Ecuador, which had previously been endemic for yaws, have successfully eradicated the infection [44]. A review in *the Lancet* shows that although approximately 65,000 new cases of yaws per year occurred in 13 of the 15 currently endemic countries between 1990 and 2014 [45], almost 85% of all infections occurred in Ghana, Papua New Guinea and Solomon Islands [42]. Wallis and Futuna and Philippines only started reporting yaws cases in 2018, implying that the other previously endemic countries may likely still be endemic but are not reporting cases. Europe is currently the only World Health Organization (WHO) Region which has never reported a case of yaws since 1945 [46]. In 16 other countries, there has been references to yaws in published studies but there has been no official report of yaws endemicity in those countries and these are Myanmar, Martinique,

El Salvador, Montserrat, Guam, Bangladesh, French Guiana, Honduras, Puerto Rico, Marshall Islands, British Virgin Islands, Nauru, , Guadeloupe, New Caledonia and Nicaragua,[42].

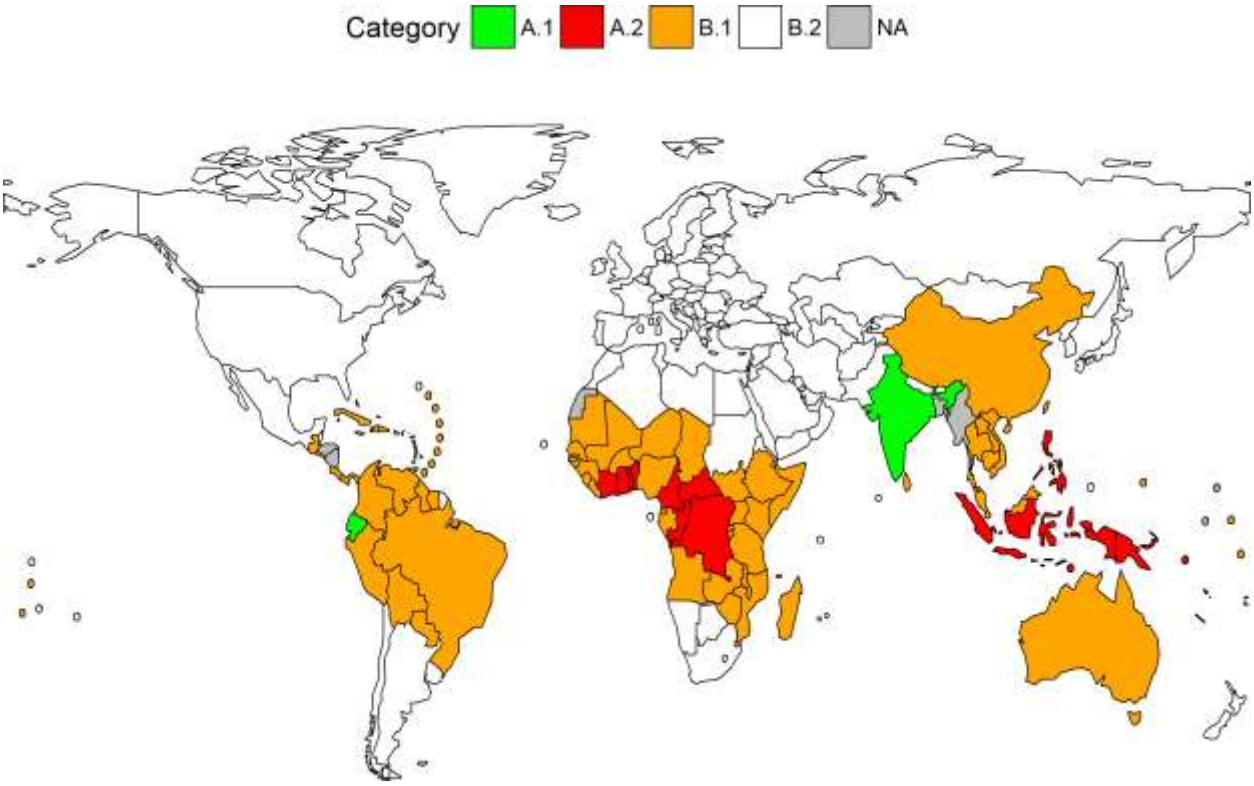

Fig 1.1: Current State of Global yaws endemicity [42]

A.1: Interrupted transmission; A.2: Currently endemic; B.1: Previously endemic (current status unknown); B.2: No history of case reports.

1.3.4 Current treatment and control

The world Health Organization's guidelines recommend that one intramuscular injection of benzathine benzylpenicillin at a dose of 1.2 Million Units (MU) for adults and 0.6 MU for children for the treatment of yaws [47]. This treatment has been shown to be highly effective and has several advantages; Penicillin works by preventing the bacteria from forming a cell wall which is required by the bacteria to survive [48]. Without it, water enters the cell due to molecular pressures and the bacteria dies. Human cells lack cell walls and so using penicillin destroys the bacteria does not affect human cells hence it is relatively safe [49]. Despite the fact that penicillin is affordable and relatively safe, a few number of patients, less than 10 in 5 million are known to have anaphylactic shock after being injected with penicillin [47]. Although Oral phenoxymethylpenicillin has been

shown to be effective in treating yaws, the major problem associated with it is the fact that patients have to take the drug for 7 to 10 days, 4 times a day creating issues of compliance [50].

In 2012, a study by Mitja et al, [51] in Papua New Guinea showed that a single-dose of oral azithromycin was as effective as benzathine penicillin in treating yaws. A study from Ghana also showed that 30 mg/kg azithromycin was non-inferior to penicillin [52]. Pilot data from Ghana, Papua New Guinea, and Vanuatu suggest that community mass treatment of yaws with 30 mg/kg azithromycin is highly effective at reducing community burden of disease [53]–[55]. Using this drug for mass drug administration in yaws endemic communities has its advantages as oral azithromycin is well tolerated and easy to administer. Azithromycin has been used successfully in community-based mass treatment programs for the treatment of trachoma and has led to the reduction of the prevalence of trachoma [56], [57].

These recent advances have prompted WHO to launch a roadmap targeting the eradication of yaws in endemic countries especially Ghana by the year 2020. This global strategy, called the Morges Strategy, stipulates that in areas where prevalence exceeds 10%, the entire population should be treated. In places where prevalence is 5%–10%, all children aged under 15 years and their close contacts should be treated, and in areas where prevalence is less than 5%, only household and other close contacts should be treated [58]. This strategy however requires that there are accurate diagnostic tools to support the determination of the prevalence in communities. Monitoring of antimicrobial resistance has become an essential part of the strategy since a recent study published in *The Lancet* reported five cases of resistance to azithromycin in Papua New Guinea [59].

1.4 Current methods of diagnosis

Health care workers in yaws endemic countries are faced with two major challenges when it comes to the diagnosis of yaws. First, the genomic structure of *TPE* which causes yaws has nearly 99.8% similarity with *TPA* which causes syphilis [60]. As such all of the current serological diagnostic methods that test positive for yaws also test positive for syphilis. Secondly, clinical diagnosis which is the main method of diagnosis of several yaws endemic communities is unreliable [32]. Clinical diagnosis relies on identifying lesions that are consistent with yaws. However, studies

have shown that lesions consistent with yaws are often caused by *Haemophilus ducreyi* and not *TPE* [61]. To compound this, *TPE* as well as other pathogenic treponemes are unable to be cultured *in vitro* and outside humans can only grow in rabbits, thus hindering the ability to properly study the pathogen [20],[62]. Previously, the only way to detect the organism would have been to inoculate into a rabbit and when the rabbit develops characteristic lesions after 12 days, the patient was said to be positive [63]. This is no longer in use and current methods that are used for the detection of the pathogen include microscopy, serology and PCR.

1.4.1 Microscopy

Microscopy remains the simplest and most reliable way of detecting Treponema pallidum, except that it cannot distinguish between treponemes because they are morphologically indistinguishable [64]. The two most common microscopy methods used are darkfield microscopy and the direct fluorescent antibody test [65]. Exudates from yaws lesions are collected on a slide and examined using dark field microscopy. The organism is identified based on the corkscrew morphology and its characteristic motility [64]. The direct fluorescent antibody test does not need the treponemes to be alive or to be motile. Fluorescein isothiocyanate-labelled antibody specific to pathogenic treponemes are used to stain the exudate and examined under a fluorescent microscope [66]. The major issues with these two methods are that they are highly subjective and can lead to false negative and false positives. Moreover, they require infrastructure that most endemic communities do not have [67].

1.4.2 Serology

Serology is the main method of diagnosis in several yaws endemic communities [68]. The two serological methods of diagnosis are treponemal tests and non-treponemal tests [69]. Treponemal tests like the Treponema Pallidum Particle Agglutination assay or the TPPA indirect agglutination semi-quantitatively detect antibodies against *Treponema pallidum* [70]. In the test, serum of patients is mixed with reagents containing gelatin particles which have been sensitized with Treponema pallidum antigen. The particles, in the presence of antibodies for yaws or syphilis in the patient serum would aggregate to form clumps. There would be no clumping in the absence of antibodies. [71]. This assay is very specific to *Treponema pallidum* but is unable to distinguish between the various subspecies of the organism [70]. Moreover, once a patient tests positive for

TPPA, they would remain positive for life even if they are successfully treated [72]. Non-Treponemal tests on the other hand, like the rapid plasma reagin (RPR) test, detect biomarkers which are released when the treponeme causes cellular damage [70]. Reagin is made up of Cardiolipin, lecithin and cholesterol. Cardiolipin is a virulence factor in pathogenic treponemes which mediates the obstruction of host defenses. Anti-cardiolipin antibodies found in the plasma of patients with a treponemal infection would react with reagin to cause agglutination [73]. The main problem with the RPR and other non-specific tests is that it's not specific to treponema pallidum [74]. Although nonspecific, RPR titers is the best indicator of the progression of the disease. Titers of RPR greatly reduce with treatment and in primary infections may actually fall to zero. When used alone, serological tests may provide little value but used together, these tests provide a more reliable platform for the diagnosis of treponema pallidum [32].

A rapid test, that combines both the treponemal and non-treponemal is the Dual Path Platform or the DPP test, an immunochromatographic test that uses whole blood. Initially developed to diagnose syphilis, the results of a study in Papua New Guinea shows that they can also be used in the diagnosis of yaws and has very high sensitivity and specificity [75]. The conjugate pad of DPP contains protein A conjugated to gold nanoparticles. On the Membrane is a recombinant antigen of *Treponema pallidum* on the treponemal line, a synthetic antigen for non-Treponema on the non-treponemal line and antihuman IgM antibody, on the control line [76]. Studies conducted in Papua New Guinea, Solomon Islands and in Ghana have shown sensitivities of above 90% and the specificities being between 94% and 100% [72]. The test has been designed for field use, not requiring any kind of laboratory support and it has been adopted for use in isolated rural settings [77].

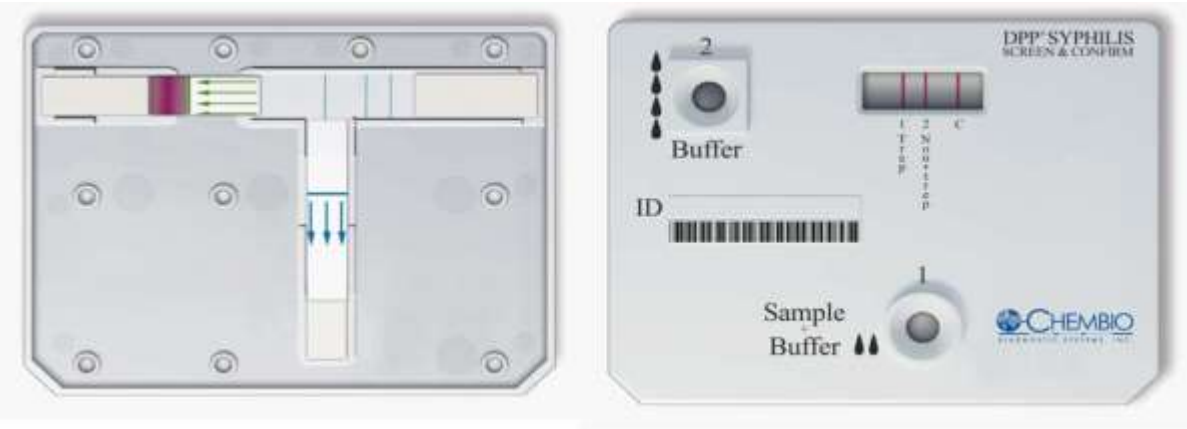

Fig 1.2: Device format of the dual lateral flow combined test [72]

1.4.3 PCR

Polymerase Chain Reaction (PCR) is a sensitive and specific method for the direct of treponemal DNA. It has sensitivities of just a few copies of treponema per PCR reaction [78]. Several gene targets have been tested with some, such as tpp*47, tpf-1, tmpA, bmp* and polA being adapted to real-time PCR [79]–[82]. PolA for instance has been used to detect treponemes in swab samples from lesions. Despite its usefulness in swab samples however, the low numbers of treponemes in blood has limited the usefulness of PCR for the diagnosis of treponema from blood samples [70], [78]. Notwithstanding this, PCR assays meant to distinguish non-venereal *Treponama pallidum* subspecies have also been developed and used for molecular detection [83].

1.4.4 Point of Care Devices

Point-of-Care Diagnostics (POCD) are diagnostic tests which are performed at the site of patient care. POCD's main advantages are a rapid turnaround time, and the timely actionable information they provide which reduces the time to treatment, prevents the need for multiple visits to the hospital or reduce the need and use of presumptive diagnosis and which leads to a change in patient management. Rapid diagnostic test kits detect analytes that are found in or extracted from clinical samples [1].

Immunoassays have been used in the diagnosis of infectious diseases since Dochez and Avery detected Pneumococcal polysaccharides in the serum and urine of lobar pneumonia patients in 1917. [84]. Interests in detecting antigens or antibodies of infectious agents led to the development of radioimmunoassay (RIA) and the enzyme linked immunosorbent assay (ELISA)[85]. These platforms still remain popular till date but the disadvantage of being time consuming, complex hence requiring trained laboratory personnel as well as the fact that they require a lot of equipment and are therefore not very appropriate for use for point-of-care.[86].

The search for POC platforms that employ the principle of immunoassay led to the development of the lateral flow immunoassay (LFIA). This is a highly versatile platform that allows the coupling

of antibodies to an epitope on the analyte of interest to a detector particle, mostly gold nanoparticles. [87]. A second antibody, the capture antibody to a second epitope on the analyte of interest is immobilized on the strip. The intensity of the test line correlates to the concentration of the analyte in the sample. [88]. LFIAs have been adapted for several point-of-care tests for infectious diseases including malaria, HIV and cryptococcal meningitis. [89]. Many of these tests have been Clinical Laboratory Improvement Amendments (CLIA) waived. This means that the tests are so simple with a low risk for an incorrect result that they can be used with minimal training at point-of-care. [90].

With the development of LFIAs, the next question of their sensitivities and analytical performance became an important area of research. The general performance of LFIAs is dependent on several factors including the concentration of the infectious agent in the sample. [91]. The Dual Path Platform (DPP), developed by Chembio Systems allows analytes and detection probes to be independently delivered using different paths. Briefly, the analyte of interest would travel along one path, binding to the immobilized capture antibodies in the test region.[92],[93]. Then a buffer is added which then pushes the detection probes to bind to the analyte and the immobilized capture antibody in the test antibody. This has been shown to have increased sensitivity over traditional LFIAs. DPP has been employed for use in the detection of syphilis, yaws, and *Candida albicans.* [94],[95].

The low sensitivities of the LFIA has turned the attention of scientists to molecular diagnostics for point-of-care. Nucleic Acid Amplification Test (NAAT) like the GeneXpert, would purify and concentrate infectious pathogens, use sonification to isolate genetic material, amplifies the genetic material and detects it. This highly sensitive platform has been used for detecting tuberculosis but still requires equipment and training [96].

Microfluidic devices provide the opportunity for a fully integrated POC device. The goal of POC for resource limited settings require a device that integrates sample processing, fluid handling, and signal generation [97].. Microfluidic devices are meant to be able to detect pathogens with a high sensitivity, be able to detect multiple analytes, be able to use very small volumes for analysis, be

able to perform fast analysis with little user input as well as make it portable for use in POC diagnostics [98], [99], [100].

The current trend in POCD is the development of smart diagnostic devices that are equipped with mobile technology. This has been seen essential to next generation POCD which includes personalized healthcare monitoring and management [101], [102], [103]. Combining mobile healthcare technologies presents an opportunity to solve the issue of access to affordable health care because currently there are more than 7.4 billion people who use mobile phones and over 70% of them are in developing countries where there is a critical need for POCD [104].

1.5 Aim

This study is aimed at developing a molecular based Point of Care diagnostic for Treponema pallidum pertenue.

1.5.1 Objective I

To develop a Loop Mediated Isothermal Amplification (LAMP) assay for treponema pallidum pertenue: The choice of LAMP as an assay for a molecular Point of Care diagnostic is informed by the fact that it does not require thermal cycling, hence overcomes the logistical challenge of using heavy equipment. Lamp is also known to produce more DNA in a relatively shorter time than PCR and most importantly, the enzymes and reagents used in Lamp are more robust and can be stored and transported in less ideal conditions than PCR. A lamp assay would therefore offer a simple, convenient and scalable method for the diagnosis of yaws.

1.5.2 Objective II

To develop a Point-of-Care diagnostic platform for use in yaws endemic countries: The ability to take the developed assay and adapt it for use on the field is what this objective seeks to achieve. Determining the sensitivity and specificity of the assay on clinical samples would validate the assay for field use. Being able to show that the assay would work under field conditions including in a water bath makes it possible for use in endemic communities.

1.5.3 Objective III

Proof of concept for size-based capture and separation: To increase the sensitivity of the of our developed device, it is important to consider a self-contained device that have very few user steps as several user steps could lead to contamination. A very is sized based separation of pathogens from cellular components in the sample to allow for concentration and easy amplification. Another area to explore is the fluid flow before and after pathogen capture. This chapter would therefore develop a proof of concept for size-based pathogen capture and separation as well as characterize the fluid flow before and after pathogen capture.

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CHAPTER 2. AN ISOTHERMAL AMPLIFICATION ASSAY FOR YAWS[1]

2.1 Introduction

2.1.1 Isothermal Amplification Techniques

Nucleic Acid amplification methods are the gold standard in the detection of several pathogens because of their sensitivity. Conventional Nucleic Acid amplification methods like the Polymerase Chain Reaction (PCR) typically make use of two oligonucleotide primers as well as DNA polymerase and cyclic heating above and below the melting point of double stranded DNA. As the temperatures move above the melting point, the strands of the DNA separate into two templates. A lowering of the temperature causes the primers to anneal to the DNA template. The elongation step then involves *the* polymerase, often Taq polymerase adding deoxyribonucleoside triphosphates (dNTPS) to the annealed primers in order to form double stranded DNA products [1]. When the temperature of the reaction is increased above the melting temperature of the products, the two strands separate and act as templates for another round of annealing and elongation when the temperature is then lowered [2]. Because PCR requires the use of thermocycling, it is impractical for use in the field [3].

Although there are PCR methods that have been used on microfluidic chips there is the need for the elimination of thermocycling and permit the amplification of nucleic acid products at a constant temperature especially in a resource limited setting. Isothermal amplification techniques provide the tools for the amplification of nucleic acid products at a constant temperature. Moreover, the enzymes used isothermal amplification are more robust hence offers an advantage over PCR [4][5]. Currently, popular isothermal amplification methods are herein discussed.

2.1.1.1 TMA/NASBA

Nucleic acid sequence-based amplification (NASBA) [6] and transcription mediated amplification (TMA)[7] are isothermal techniques which rely on producing DNA from RNA templates using the enzyme reverse transcriptase [8]. A promoter is engineered in the primer and an RNA

[1] Part of this chapter has been submitted for publication

polymerase is used to make RNA from this promoter. Only one pair of primers is needed for this amplification [9]. While one of them is a target specific sequence, the other is a promoter primer containing a 5' sequence which is recognized by the RNA polymerase [7].

During the amplification process, which usually occurs at the isothermal temperature of 41°C, double stranded DNA intermediates are formed and then the RNA polymerase starts transcription after recognizing the promoter sequence [10]. To ensure success of transcription, the first 10 sequences must be purine rich, since this will result in lowering the melting temperature thus making it easy to melt the strands thus forming open promoter complexes needed for transcription [11]. The amplification products are single stranded RNA, about 120-250 nucleotides long usually which can be detected by using probe hybridization [9],[12].

2.1.1.2 SMART

Signal Mediated Amplification of RNA Technology is an isothermal amplification method which is used for the detection of RNA and DNA [13]. It is based on the amplification of the signal in a reaction and not on the copying of target sequences [14]. The reaction involves the use of three enzymes, DNA polymerase, RNA polymerase and Bst DNA polymerase to anneal two single stranded oligonucleotide probes, to a specific target sequence [15]. The two probes made up of an extension and a template are structured such that each probe has one region that hybridizes to the target and another which is much shorter which hybridizes the other probe [1 6]. These two probes only anneal to each other in the presence of a specific target. This forms a three-way junction structure [17]. The three-way junction then permits the extension of the extension probe and the transformation of the template probe into a double stranded promoter which is functional and allows the generation of multiple copies of RNA signal. The signals are detected by ELISA and real-time fluorescent changes [18].

2.1.1.3 RCA

In Rolling circle amplification (RCA), a short DNA or RNA primer is amplified to form a long single stranded DNA or RNA. In this enzymatic isothermal method, a circular DNA template and special DNA or RNA polymerases such as the bacteriophage Ø29 DNA polymerase enzyme are used for application at a single temperature [19],[20]. This method is simple, versatile, accurate and efficient,

thus makes it easy for miniaturization and automation in the high throughput analysis [21]. Although RCA is a linear amplification method, it can be made exponential by adding reverse primers to the reaction. Because RCA is resistant to contamination and amplification errors, RCA has been used to develop sensitive diagnostic methods for a variety of targets including nucleic acids (DNA, RNA)[22].

2.1.1.4 SDA/IMDA

Strand Displacement Amplification (SDA) [23] and Isothermal Multiple Displacement Amplification (IMDA) [24] are isothermal amplification methods based on strand displacements techniques. Restriction endonucleases have the ability to nick unmodified strands of its target DNA. In SDA, a DNA polymerase which has strand displacement abilities like Bst or the *Escherichia coli* DNA polymerase 1 (exo-Klenow) enzyme initiates amplification the site of these nicks [25]. During the amplification, 10^9 copies of DNA are produced in less than an hour [26]. IMDA on the other hand is able to amplify small amounts of sample into large quantities by using the bacteriophage Ø29 DNA polymerase enzyme to amplify the strand at 30°C [27], [28]. IMDA requires the use of 6 random nucleotides which anneal to the DNA template followed by elongation mediated by the Ø29 DNA polymerase enzyme.

2.1.2 LAMP

Loop mediated Isothermal amplification (LAMP) requires the use of a DNA polymerase which aside its polymerization ability, also has the property of strand displacement [29]. LAMP, compared to PCR, is more sensitive and specific, amplifies more DNA within a short time [30] [31]. The main principle of this assay is the utilization of DNA synthesis by the DNA polymerase which has high strand displacement activity. The DNA synthesis occurs by autocycling Strand displacement and although mostly qualitative, LAMP can also be quantitative [32], [33]. LAMP is a highly sensitive and specific amplification method with high efficiency and able to detect DNA at a few copies in the reaction mixture [34]. It is very specific to the target mainly because the target sequence is recognized by four to six independent sequences and this effectively solves the problem of backgrounds often associated with amplification methods [35].

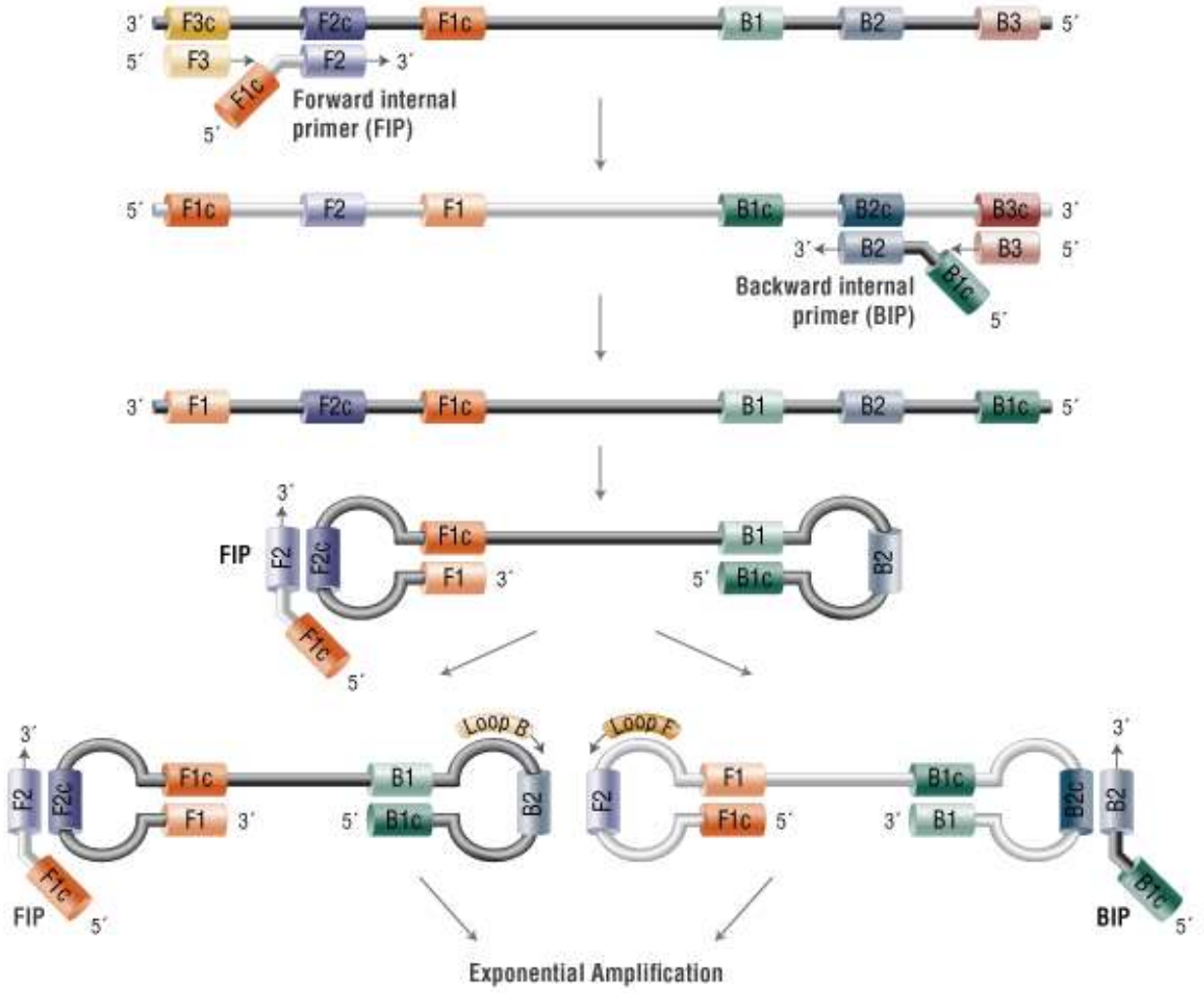


Fig 2.1: A schematic representation of the principle of LAMP [29]

2.1.2.1 Requirements of LAMP

2.1.2.1.1 Primers

The analytical performance of LAMP is reliant on developing primers that are very specific. LAMP uses a minimum of 4 primers namely F3 (Forward outer), B3 (Backward outer), FIP (Forward inner) and BIP (Backward inner) primers. The forward and backward outer primers play a major role during strand displacement while the inner primers (FIP and BIP) fold back on themselves to form loops [36]. Loop for ward (LF) and Loop backwards (LB) primers are often added in order to speed up the reaction [37]. With all 6 primers, there are 8 target regions in the gene that the primers target: the F3c, F2c, F1c and FLP which are in 3' strand and B1,

B2, B3 and BLP in the 5' strand [38]. In designing LAMP primers, the GC content must be around 50% to 60% and the inner primers should not have AT rich sequences at the ends as GC rich ends are more stable.

2.1.2.1.2 Enzymes

The DNA polymerase enzyme that has the ability to displace the strand while having a high polymerizing ability is essential to the LAMP assay. The two enzymes with these abilities are Bst polymerases which are isolated from *Bacillus stearothermophilus* and *Bsm* polymerase isolated from *Bacillus smithii*. However, Bst is more commonly used because it can maintain enzymatic activity up to 66°C, Bsm can maintain enzyme activity up to 63°C but works best at 60°C [37], [39]. *Note that new versions of Bst that NEB sells work up to 72oC and that 3.0 includes RT properties*

2.1.2.1.3 Other Components

Nucleotides required for the assay is achieved by adding dNTPs to the reaction. Whilst magnesium sulphate ($MgSO_4$) is added to complete the DNA polymerization. In the process, magnesium pyrophosphate is formed. The amount of this correlates with the amount of DNA and is used to visualize the reaction based on its turbidity [40]. To stabilize the AT and GC rich contents, betaine is added to the reaction while buffers like tween, Tris-HCl with a pH 8.8, $(NH_4)_2\ SO_4$, $MgSO_4$ and KCl may be used to stabilize the reaction. While extracted DNA is often used for LAMP, unlike PCR, the DNA extraction step is not a requirement for LAMP [41].

2.1.3 End Point Detection of LAMP

2.1.3.1 Turbidity detection

LAMP results can be read macroscopically through turbidity. Magnesium pyrophosphate is formed as a result of $MgSO_4$ interacting with the DNA. This by-product causes turbidity and gives an indication of the presence and quantity of the target DNA. This makes it a very simple technique which does not require very skilled personnel as semi-skilled personnel can easily be trained to interpret these results. When the amplified products are spun for a short

period white precipitate settles down in the bottom of the tube [42] and can help in detecting turbidity by eye. However, most often a turbidometer is used in detecting LAMP products.

2.1.3.2 Agarose Gel Electrophoresis

The amplification products can also be detected by agarose gel electrophoresis. DNA binding dyes such as ethidium bromide [43], [44], or propidium iodide [45] can be added to the amplification products and viewed using an imager. However, using gel electrophoresis to view and detect LAMP amplification products requires that the tubes be open, and this makes it easy for contamination. The use of ethidium bromide which is a highly hazardous chemical makes this process unsuitable for field use [46].

2.1.3.3 Fluorescence Detection

Gene amplification products can also be monitored real time by the use of dyes like calcein and SYBR green I. Calcein dye is a metal ion binding fluorophore that forms an insoluble salt complex called manganese- pyrophosphate as a result of manganese ion, from calcein reacting with pyrophosphate, the by- product of LAMP reaction, thus making the reaction fluorescent and could be easily be observed under UV light (365 nm) [47]. Adding SYBR green I to a reaction tube containing LAMP amplification products leads to a colour change from reddish orange to yellowish green. This fluoresces under UV light. However, when added before the reaction, the reaction is inhibited. Adding SYBR green I post amplification means that the tubes would have to be opened hence increasing the risk of contamination [48].

2.1.4 LAMP for Point-of-Care Diagnostics

LAMP is a very useful assay for point-of-care diagnostics as it is a simple and easy to perform once the reagents can be dried or incorporated into the diagnostic device, requiring only a regular laboratory water bath or heat block for reaction [47]. LAMP is found to be stable at a range of temperature, PH and elongation time [49]. LAMP is also known to tolerate trace quantities of blood, culture media, anticoagulants etc. which inhibit Taq polymerase and hence PCR. This makes it extremely useful in amplifying non-extracted samples or incompletely processed samples which would have invariably affected a normal PCR reaction. Other samples like urine and stool which hinder Taq polymerase have little effect on LAMP [41], [49]. Finally, while cold chain is essential when preparing a master mix for PCR, it is not mandated in the case of LAMP [50].

In this chapter therefore, we sought to therefore develop a robust LAMP assay that would be highly specific to yaws and would be sensitive in detecting *Treponema pallidum subsp pertenue*.

2.2 Methods

2.2.1 Primer Design

Primers for the lamp assay were designed from the conserved *hypothetical protein* gene tp0967 sequence of *the Gauthier strain of Treponema pallidum subsp. pertenue* (GenBank: HM151371.1), using Primer Explorer V5 software (http://primerexplorer.jp/e/v5_manual/index.html). All primers were assessed for specificity before use in LAMP assays by doing a BLAST search with sequences in GenBank (https://www.ncbi.nlm.nih.gov/guide/howto/design-pcr-primers/).

2.2.2 Development of the LAMP assay

The LAMP reaction was performed using an Isothermal Master Mix in a 25 µl volume, which contained 1.6µmol each of FIP and BIP, 0.2µmol each of F3 and B3 primers and 1.6µmol each of the loop primers, Loop B and F (Integrated DNA Technologies, Skokie, IL),. The Isothermal mastermix contained 10X isothermal buffer (NEB, Ipswich, MA), 1.4mM of 100mM dNTPs ((Agilent Technologies, Santa Clara, CA), 400mM of 5M Betaine (Millipore Sigma, Burlington, MA) as well as 0.2x and 1x of the florescent dyes Evagreen (VWR International, Radnor, PA) and ROX (Thermo Fisher Scientific, Waltham, MA) respectively. 0.2 µl of DNA sample and 8U of Bst Polymerase (NEB, Ipswich, MA) were the final constituents of the reaction mix. The LAMP assay was conducted under isothermal condition at 65 °C for 60 min followed by a heating and cooling step from 95 °C to 60 °C (ramping at 0.05 °C per sec) in ABI7500 real time PCR system (Applied Biosystems, Thermofisher Scientific, Waltham, MA). The amplicons were run on 2.0% Ethidium Bromide Agarose Gel by using Easy Cast B, the Bio-Rad Power System and the Azure c400 imager (Azure Biosystems, Dublin, CA).

2.2.3 Optimization of the LAMP assay

Concentrations of Betaine and MgSo4, the optimal temperature for the reaction and time were optimized for the LAMP assay. In the study, 25 µl of amplified DNA fragments were analyzed using 2.0% agarose gel electrophoresis. For determining the best reaction temperature, the reaction

mixture was incubated for 60 min at 61 °C, 63 °C, 65 °C, 67 °C, 69 °C, and 71 °C, respectively, and then terminated at a range from 95 to 60 °C (0.05 °C per sec). The concentrations of betaine (800mM, 400mM and 200mM) as well as those for MgSo4 (6mM, 2mM and 0mM) were tested. Moreover, to investigate the minimum reaction time required in a LAMP run, the reaction was performed at 65 °C and assessed at 20 min, 25min, 30 min, 35min, 40 min, 45 min, 50mins and 60 min, respectively.

2.2.4 Statistical analysis

All reactions in the study were run in triplicates with triplicate lateral flow tests per sample except the clinical sample which had one reaction per sample and one lateral flow test per sample. All data were first entered into Microsoft excel (2016) and then transferred onto GraphPad Prism version 8.00 (GraphPad Software, La Jolla, California, USA).

2.3 Results and Discussion

2.3.1 Primer design

Table 2.1**:** Primers used in the development of the assay **A**. Primer set 1 and **B.** Primer set 2

A

Set1	ID:42		dimer(minimum)dG=-2.18					
label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate[2]	Sequence
F3	802	819	18	59.5	-6.1	-5.4	0.56	TGCGAAGGCTGTAACTGC
B3	969	988	20	59.5	-4.7	-6.4	0.55	CCAGATTCTCGTACAGCGAC
FIP			39					CGGTACGCAAACCCTGCACG-TGTCAGGACTGCCAAGACA
BIP			41					GCCCTTCAGCTGGTTCCTCTT-CAAGGTCTGCACATTCATGC
F2	823	841	19	60.3	-4.9	-4.4	0.53	TGTCAGGACTGCCAAGACA
F1c	874	893	20	65.9	-5.5	-6.7	0.65	CGGTACGCAAACCCTGCACG
B2	935	954	20	59.2	-5	-5.3	0.5	CAAGGTCTGCACATTCATGC
B1c	894	914	21	64.3	-6.2	-4.7	0.57	GCCCTTCAGCTGGTTCCTCTT

[2] 5'pos: 5 prime position. 3'pos: 3 prime position. Len: Length of the primer sequence Tm: Melting time

Table 2.1 continued

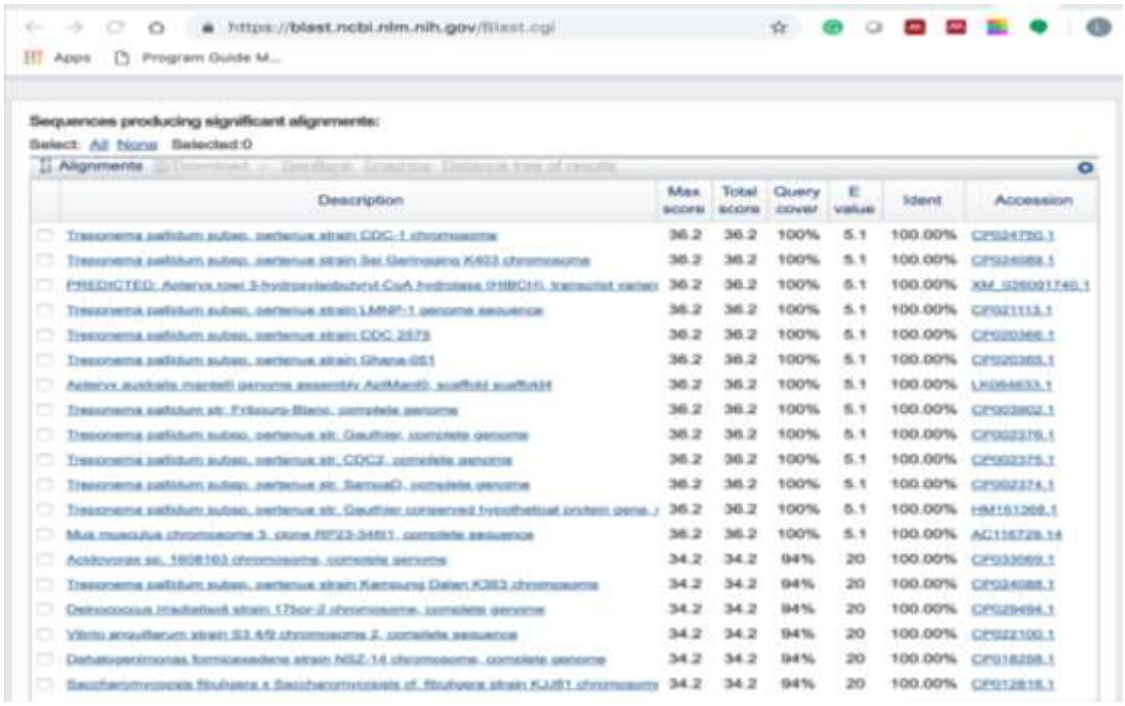
B

Set2	ID:31		dimer(minimum)dG=-2.46					
label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	761	778	18	59.1	-4.2	-6.3	0.56	AGAAGTCCTGCTGCAACG
B3	933	952	20	60.2	-5.1	-5.3	0.5	AGGTCTGCACATTCATGCTG
FIP			41					TTGTCTTGGCAGTCCTGACAGG-GTAGTACGTGCTGCACGAA
BIP			42					GTTCGGTGTACGCGACAGACAC-GCACCAAGGCTAAAGAGGAA
F2	779	797	19	59.2	-3.3	-5.4	0.53	GTAGTACGTGCTGCACGAA
F1c	821	842	22	64.3	-4.4	-5.4	0.55	TTGTCTTGGCAGTCCTGACAGG
B2	907	926	20	59.6	-6.4	-4.7	0.5	GCACCAAGGCTAAAGAGGAA
B1c	845	866	22	65.2	-5.8	-4.9	0.59	GTTCGGTGTACGCGACAGACAC

Based on the 1566 bp sequence of the conserved *hypothetical protein* gene tp0967 sequence of *the Gauthier strain of Treponema pallidum subsp. pertenue* (GenBank: HM151371.1), 2 primer sets (**Table 2.1**) were selected from several sets designed. This was because each of the primer sets designed were assessed for specificity by doing a BLAST search with sequences in GenBank. As can be seen in **Fig 2.2 A and B**, only two of the numerous primer sets identified were specific to *Treponema pallidum subsp. pertenue*. Both primer sets 1 and 2 were specific to all the major strains and subtypes of *Treponema pallidum subsp. pertenue* but most importantly, a blast of these sequences excluded *Treponema pallidum subsp. pallidum* and *Treponema pallidum subsp. endemicum* which causes syphilis and bejel respectively [51]. This is important because sub species of *Treponema pallidum* are impossible to distinguish clinically, microscopically and serologically [52]. Since the genome of these sub species differ by less than 0.2%, it is important to ensure that the primers developed would truly differentiate between these sub species [53]. The *Treponema pallidum strain Fribourg-Blanc* seen in **Fig 2.2 A and B** is an unclassified simian strain of treponema pallidum which was isolated in baboons in West Africa [54]. However, several studies have shown that this classified strain is identical to Treponema pallidum subsp pertenue and it shows all the characteristics of this sub species [53]. There has been a proposal therefore to rename this strain as *Treponema pallidum subsp pertenue strain Fribourg-Blanc*[55]

Each of the primer sets consisted of 4 primers along with two loop primers targeting six distinct regions on the gene, that is, a forward inner primer (FIP), a backward inner primer (BIP) and two outer primers (F3 and B3) and the loop primers (Loop F and Loop B).

A.



B.

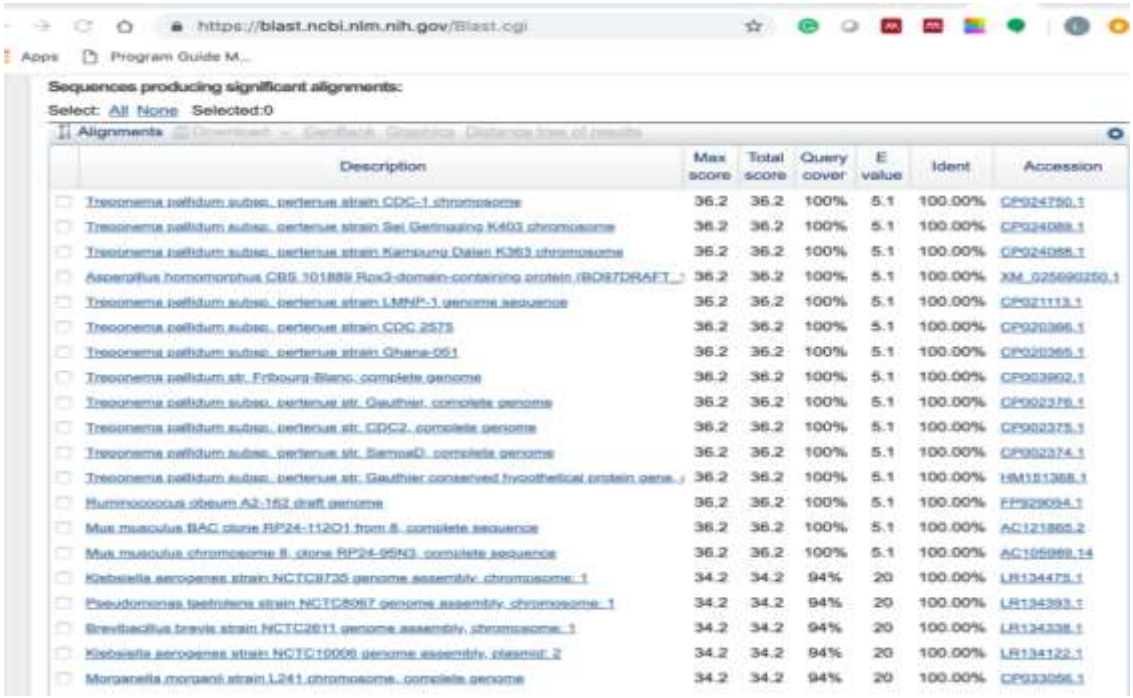


Fig 2.2: Primer blast showing the specificity of the primers to *Treponema pallidum subsp pertenue* **A.** Primer set 1 and **B.** Primer set 2

2.3.2 Development of the LAMP Assay

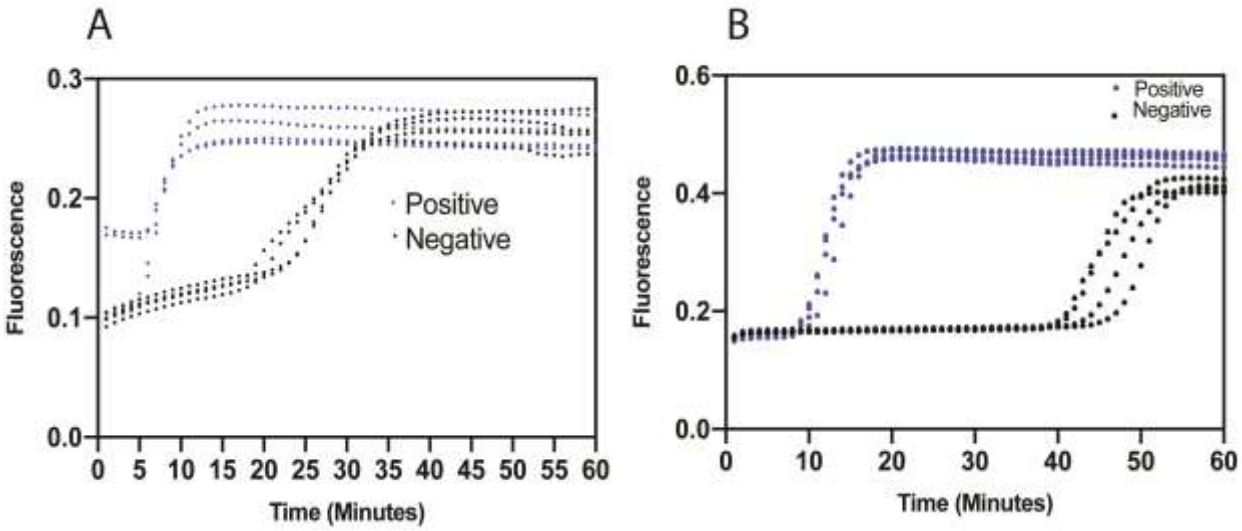


Fig 2.3: The LAMP profile for both positive samples and negative samples **A.** Primer set 1 and **B.** Primer set 2. The blue lines show the fluorescence of positive cases and the black lines shows the fluorescence of the negative cases.

DNA of *Treponema pallidum pertenue* were found to amplify using the two selected primer sets, primer set 1 and 2. While the positive control of primer set 1 amplified as early as 4 minutes, the negative controls also began to amplify within 20 minutes of the assay. On the other hand, the positive controls of primer set 2 started to amplify as early as within 9 minutes, the negative controls started to amplify after 40 minutes **(Fig 2.3)**.

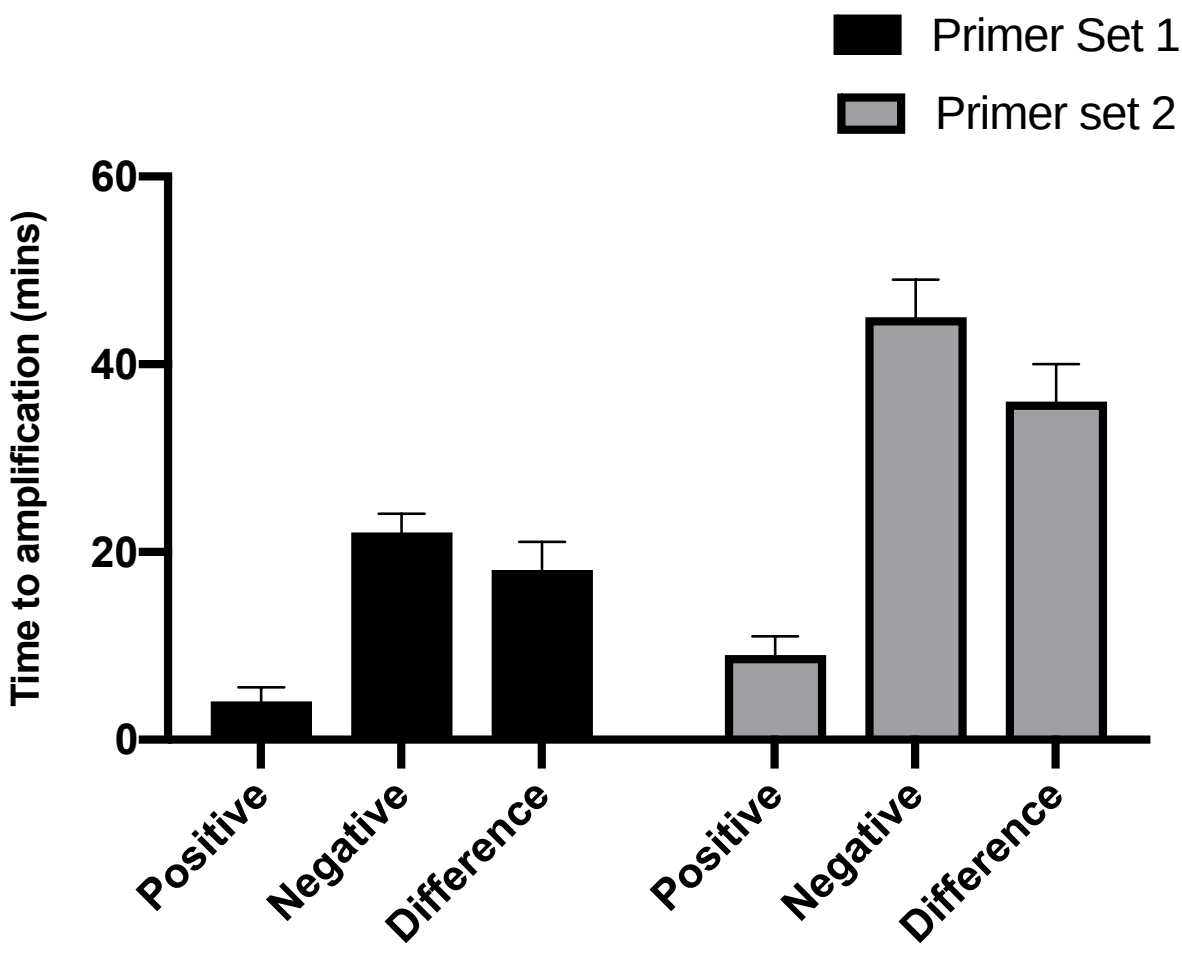


Fig 2.4: Amplification time between *Treponema pallidum subsp pertenue* DNA positive samples and negative samples in **A.** Primer set 1 and **B.** primer set 2

Fig 2.4 shows the time difference in minutes between the DNA positive and negative samples of *Treponema pallidum subsp pertenue*. While Primer set 1 had in average an earlier amplification for the positive samples of 4 minutes, the negatives also started amplifying within 20 minutes. On the other hand, the DNA positive samples of primer set 2, started amplifying around 9 minutes while the negative samples started amplifying around 42 minutes. This means that while the difference in amplification times for Primer set 1 is around 16 minutes, that for primer set 2 is around 33 minutes. Like most isothermal amplification systems, LAMP has the tendency to have false positive results in DNA negative samples [56]. The fact that there were 6 primers used in this LAMP assay targeting up to 8 different regions, the large number of primers are known to cause primer dimers and non-specific binding causing a false positive result [57]. The inner primers also

tend to form hairpin structures, and these tend to be read as positive results [58]. A longer time difference between the negative and positive samples therefore is important to be able to not only ensure consistency, but also to determine the time to stop the reaction in order to avoid these primer dimers and hairpin structure formation.

To optimize the assay, the concentrations of betaine (800mM, 400mM and 200mM) as well as those for $MgSO_4$ (6mM, 2mM and 0mM) were tested. In primer set 1, the amplification time difference between the DNA positive samples and negative samples were 14 minutes, 17 minutes and 13 minutes respectively when 2 mM $MgSO_4$ was added to the reaction containing 800mM, 400mM and 200mM of betaine. When 800 mM and 400 mM of betaine was used without $MgSO_4$, the time difference was 15 minutes and 24 minutes respectively. There was no amplification when 6 mM of $MgSO_4$ was added to the reaction containing 800 mM, 400 mM and 200 mM of betaine. A reaction containing 200 mM of betaine without $MgSO_4$ did not amplify as well as can be seen in Fig 2.5a.

On the other hand, primer set 2 had amplification time difference between the DNA positive and negative samples of 33 minutes, 30 minutes and 32 minutes respectively in reactions containing 2mM $MgSO_4$ and 800mM, 400mM and 200mM of betaine respectively. In reactions containing 800mM, 400mM and 200mM of betaine and no $MgSO_4$, the reaction time difference between the negatives and positives were 36 minutes, 33 minutes and 28 minutes respectively. As in primer set 1, there was no amplification when 6mM of $MgSO_4$ was added to the reaction containing 800 mM, 400 mM and 200 mM of betaine.

2.3.3 Optimization of the LAMP Assay

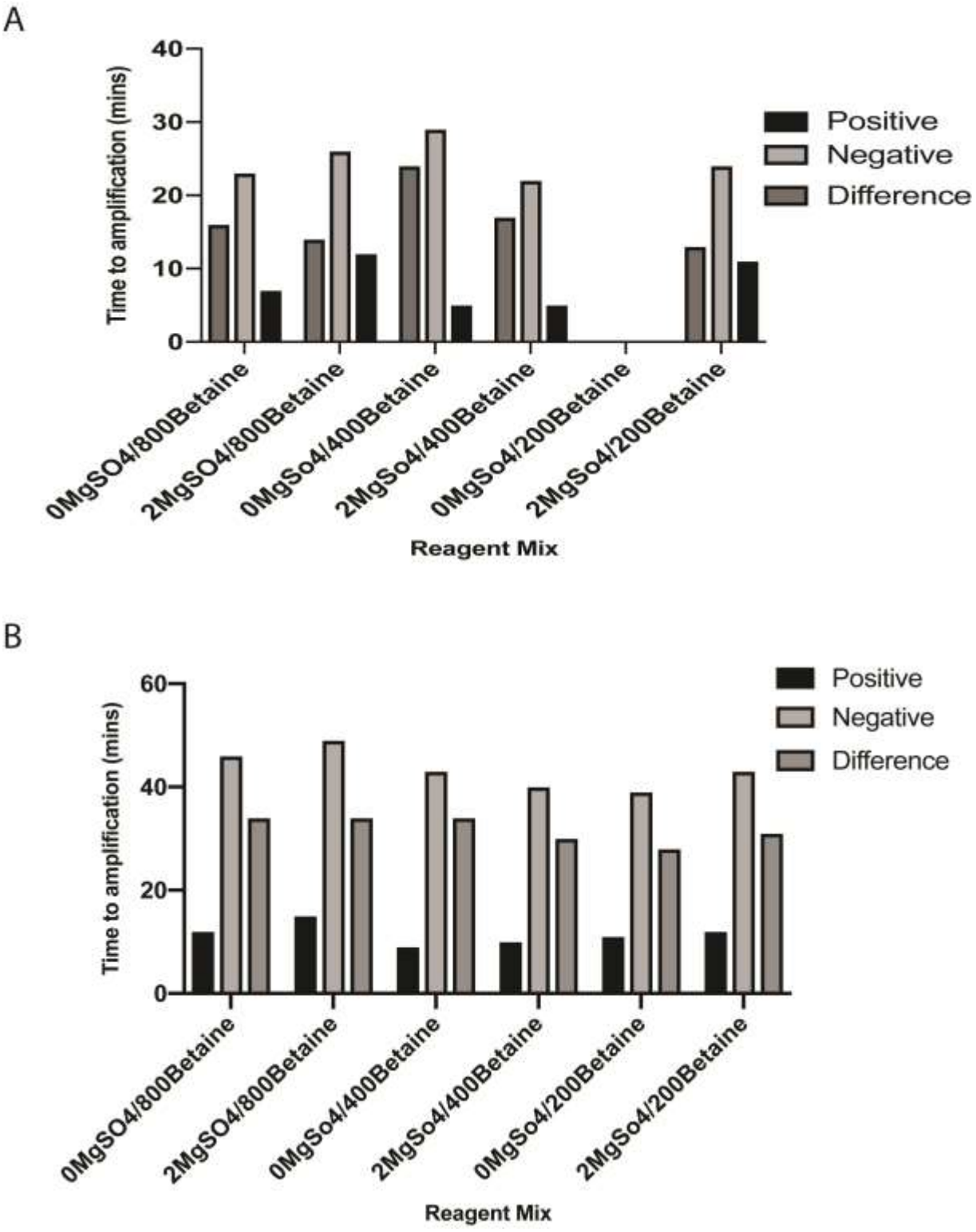


Fig 2.5: LAMP assay optimization showing the time to amplification of negative and positive samples under different reagent concentrations in **A.** Primer set 1 and **B.** Primer set 2

The optimization of the assay using these two primers showed a clear case of stability and reproducibility with primer set 2 than with primer set 1. The final LAMP assay developed for *Treponema pallidum subsp pertenue* therefore contained 800 mM of betaine with no $MgSO_4$ whilst the conditions for the yaws lamp assay was set at 65^{o}C for 30 minutes using primer set 2.

2.4 Conclusion

In this chapter, we describe the development of a LAMP assay for *Treponema pallidum subsp pertenue.* Two primer sets were selected from a set of 5, developed from the *tp0967* gene of the Gauthier strain of *Treponema pallidum subsp. pertenue* after the two primer sets had shown a specificity for strains of *Treponema pallidum subsp. pertenue* and not any other treponemes. The selected primers were shown to amplify DNA positive samples as early as 4 minutes and 9 minutes respectively. The negatives however also amplified in 20 and 42 minutes respectively for the two primer sets. Furthermore, optimization of the assay using these two primer sets showed a clear case of stability and reproducibility with primer set 2, hence conditions for the yaws LAMP assay was set at 65^{o}C for 30 minutes using primer set 2.

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CHAPTER 3. A POINT-OF-CARE PLATFORM FOR YAWS IN ENDEMIC COMMUNITIES[3]

3.1 Introduction

3.1.1 Paper Based Devices

Most microfluidics devices are made of silicon or glass or polydimethylsiloxane) [1][2] (PDMS) and polymethylmethacrylate (PMMA). However, paper-based devices are currently seen as the most promising for resource limited countries because they fit the World Health Organization's ASSURED criteria (**A**ffordable, **S**ensitive, **S**pecific, **U**ser-friendly, **R**apid and Robust, **E**quipment-free, and **D**eliverable to end-users) [3][1][4].

Dipstick assays are mostly designed to detect metabolites in urine [5], [6]. These metabolites like glucose, proteins and ketones from patients with some sort of nephrotic or metabolic disease are able to indicate some sort of pathology through a color change which is read visually by the user [7]. Dipsticks are easy to manufacture, and they are simple to design and very convenient [8].

Lateral flow test strips are typically composed up of a sample pad, conjugate pad, nitrocellulose membrane and an absorbent pad and often strengthened by a backing card [9]. The backing card provides mechanical support to all the components while the absorbent pad based on capillary effect provides the driving force for the fluid flow [10]. The nitrocellulose membrane provides the platform for the reactions and their detection to occur [9]. Capture molecules like antibodies are striped onto the nitrocellulose membranes as test and controls lines. These capture molecules are deposited in the membranes by electrostatic interactions, hydrogen bonds and/or hydrophobic forces [11].

The sample to be analyzed is applied onto the sample pad which is pretreated with buffers to improve the performance as well as the compatibility with other components of the assay [12]. The sample moves along the sample pad onto the conjugated pad. The conjugated pad contains a label which is often chosen based on its structural, optical or/and its optical properties an example

[3] Part of this chapter has been submitted for publication

of which is gold nanoparticles (AuNPs) (Fig 3.1) [13]. There are two lateral flow formats, the sandwich format and the competitive format. In the sandwich format, the label is first conjugated to a reporter antibody to form a conjugated particle complex [14]. The analyte of interest in the sample then reacts with the conjugated particle complex and then the complex migrates along the fluid flow from the conjugation pad to the nitrocellulose membrane where the complexes would be captured at the test line via the interaction between the analyte complex and the capture antibody. The excess conjugated particle complex is then captured at the control line [15]. In the competitive format, however, the conjugated particles react with the capture antibodies deposited at both the test and control lines. The analyte then competes for the binding sites with the capture antibodies at the test line, leading to a non-aggregation of conjugated particles at the test line. In the absence of analyte, conjugated particles are then captured at both test and control lines. In general, the sandwich format is used for analytes which has multiple antigen epitopes, while competitive format are used for analytes with a single antigen epitope [12].

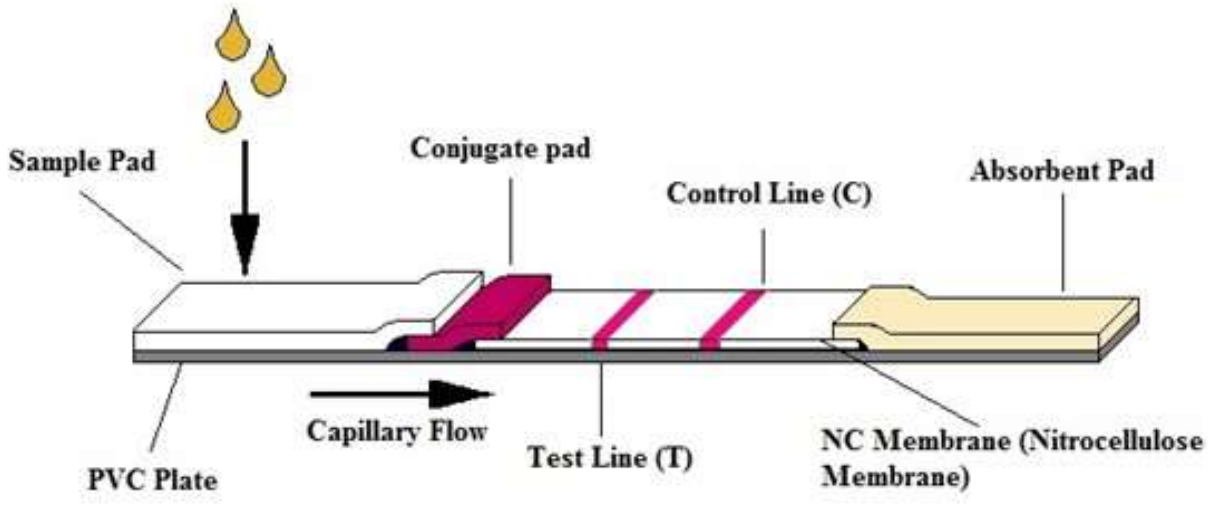

Fig 3.1: A lateral Flow test strip showing the various components [13]

The porous and hydrophilic nature of paper makes it an excellent platform for fabricating microfluidic devices [16]. Both 2-dimensional and 3-dimensional microfluidic paper-based analytical devices (μPADs) are designed to enhance capillary force in order to drive fluid movement. In 2D μPADs, techniques like cutting, photolithography, plotting, inkjet etching, plasma etching, wax printing, etc. are used to make microchannels on paper by forming patterning

the physical or chemical hydrophobic boundaries of the paper [17]. On the other hand, to produce 3D μPADs , layers of paper are put together to create channels such that each of these channels are connected to each other[18]. The size of these channels, the characteristics of the paper as well as the humidity and temperature affect how fluid flows through these channels[18]. Molecules like antibodies and detector particles can be immobilized on the paper while techniques like sample extraction, heating and reagent drying can be incorporated in a 3D μPAD. [19][20]. These μPAD are advantageous in their ability to add complex network of channels hence increasing the functionality of the device. [18].

3.1.2 Paper Based POC test for yaws

The major constraint to utilization of the LAMP assay and other molecular based assays have been with high cost of accessory instrumentation as well as well as expertise needed to run these assays. Although LAMP is convenient for use in yaws endemic communities via heating with a water bath, there is still the challenge of ensuring that personnel with expertise runs these tests [21].

In Ghana in particular, children with yaws are often found living in communities and farmlands that are hard to reach [22]. Case searches for yaws are often done at central locations where a high number of children with yaws like lesions can be found [23]. These are mostly Community-Based Health Planning and Services (CHPS) compounds located in a particular locality. Samples of children who are on farms, hard to reach areas and cottages nearby are then taken by a community health worker on a motorbike (Fig 3.1). Unfortunately, most yaws endemic communities only have semi-skilled personnel and to ensure that the assay would be useful for yaws eradication purposes, it is essential to couple the assay on a microfluidic POC platform that would offer the convenience of being used by semi-skilled personnel.

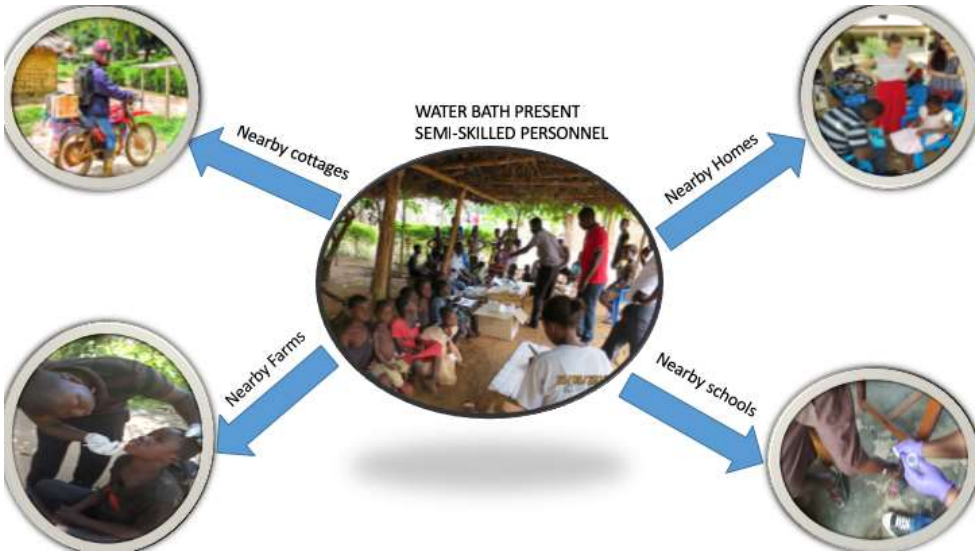

Fig 3.2: A typical case search in yaws endemic countries.

Health workers are often located at a central point, most often CHP compounds. Samples of Patients with yaws suspected lesions are then taken from nearby farms, cottages, homes and schools to the central location for testing.

3.2 Methods

3.2.1 Limit of detection

To determine the lower limit of detection of the lamp assay, 6 ten-fold dilutions of stock DNA of a concentration of 2.8 x 10^8 was prepared using molecular grade water and tested using labelled primers and the same conditions as described above for LAMP. In all, 10 µl each of reaction mixture were pipetted onto a lateral flow test strip (catalog no. D003-03, USTAR Biotechnologies, Hangzhou, China) followed by the addition of 40 µl of commercial wash buffer. The primers are labeled with FAM and Biotin. The gold nanoparticles are conjugated with streptavidin while the test line is comprised of antifluorescein antibodies. The control line is composed of antistreptavidin antibodies. The amplification products would react with the gold nanoparticle complex, flow down the nitrocellulose membrane to react with antibodies on the test and control lines. A visible pink line on the control line only (1 line) shows a negative result while a visible pink line on both the test and control lines (2 lines) shows a positive result.

The strips are then scanned using an Epson V850 Pro scanner (Long Beach, CA) after the tests have dried (30 minutes after sample application). Using a custom MATLAB script, the average grey scale pixel intensities of the test line subtracted from the background signal intensity were calculated as

$$I_{backgroundsubtracted} = \frac{I_{raw} - I_{background}}{0 - I_{background}}$$

where 0 is black, the lowest pixel intensity [23]. To determine the limit of detection of *Treponema pallidum pertenue*, 3 test strip replicates were analyzed for statistical significance. The intensities of the test line were compared to the average of the intensities of the negative control.

3.2.2 Clinical Samples

Clinical samples of *Treponema pallidum subsp pertenue* were obtained from the Noguchi Memorial Institute for Medical Research and from the Asamankese District Hospital Laboratory both in Ghana. The clinical samples were obtained from swab samples in Assay Assure transport media (Sierra molecular coporation, Navada, USA) and stored at -20^{o}C.

3.2.3 Sample Preparations

Each of the 63 *Treponema pallidum subsp pertenue* samples were divided into two equal parts as shown in Fig 3.2 and DNA was extracted from one part whiles the other part was analyzed without DNA extraction. The PCR assay was visualized on a 2.0% agarose gel while the LAMP assay was visualized both on the gel and on a lateral flow test strip.

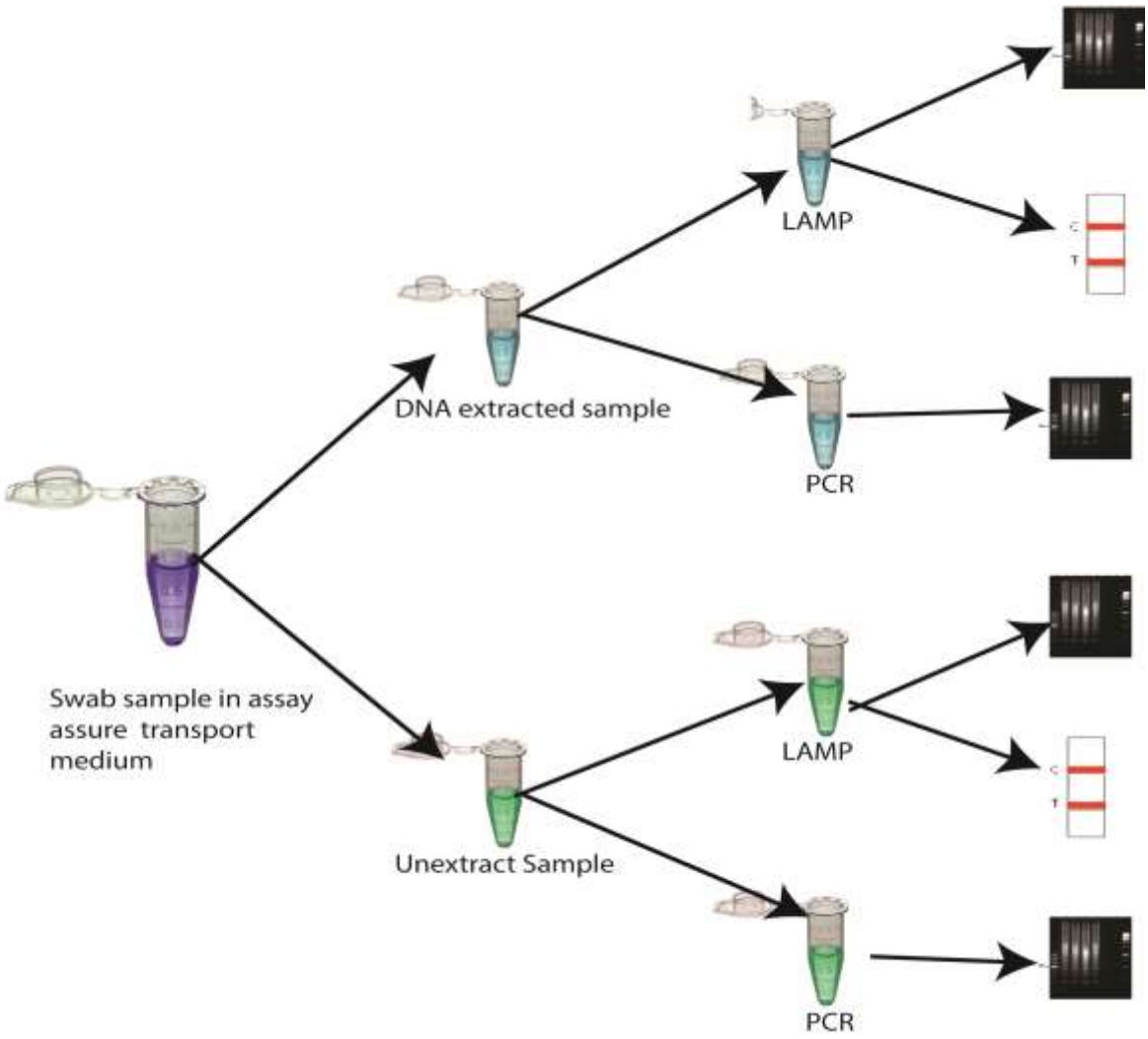

Fig 3.3: A schematic representation of sample preparation for Sensitivity and Specificity testing

3.2.4 DNA extraction

DNA was extracted using the DNA extraction Kit (Qiagen). Briefly, Cell lysis was achieved by adding 20 µl proteinase K (20 mg/ml) to 200µl Assay Assure containing sample, incubated at 55ºC in the thermomixer for 4 hours (or overnight) until complete lysis. After lysis, the DNA was processed according to the manufacturer's instructions.

3.2.5 Real Time PCR

Real-time PCR was run using a protocol as described by Chi et al, [24] using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA). Briefly, a reaction of 30 µl volume consisted of 15.0 µl Environmental Master Mix (2×), 3.0 µl Assay Mix (10×) (both from

Invitrogen, Grand Island, NY), and 5.0 µl DNA template, 7.0 µl distilled water. The amplification parameters were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60 °C for 1 min, Fluorescein (FAM) and Carboxy fluorescein (VIC) dyes were selected as detectors following the procedure by the manufacturer (ThermoFisher scientific, Waltham, MA).

3.2.6 Sensitivity and Specificity of the assay:

To determine the specificity and sensitivity of the LAMP assay, DNA templates of 63 samples were tested by LAMP with the CDC real-time PCR assay as the gold standard.

3.2.7 Validating the device for use in RLCs:

3.2.7.1 Device design

Based on the setting for case search in yaws endemic areas as shown in Fig 3.2, the yaws device was designed to be simple yet effective for the diagnosis of yaws in CHP compounds. The 8.5 mm x 3 mm base of the plastic housing of the device as well as the 7 mm x 3 mm plastic cover of the device were both designed in Solidworks 2017. The device was 3D printed on a Fortus 380c printer (Stratasys, Eden Prairie, MN).

3.2.7.2 Drying reagents

The reagents, as well as the conditions for drying, were adapted from Phillips *et al*, [25] briefly, the yaws LAMP reagents were streaked on polyethylene terephthalate film (Apollo, Lake Zurich, IL) and were dried and stored at room temperature. The primer mix which contained 0.2 µM of F3 and B3, 1.6 um of FIP, BIP, LB and LF primers as well as 2 M sucrose, 50% glycerol and 0.1% Triton X-100 were deposited using by hand. This was then dried for 60 minutes in a biosafety cabinet at room temperature under air flow. The enzyme mix contained 2 M sucrose, 1.4 mM dNTPs and 8 U Bst 3.0 polymerase. This was pipetted directly on top of the dried primer mix and dried for another 60 minutes. A 25 µl reaction corresponded to a 0.5 cm x 0.5 cm piece of the polyethylene terephthalate film. This was cut and deposited in a clean microcentrifuge tube and stored with silica gel desiccant (Uline, Pleasant Prairie, WI) at room temperature for 14 days. The dried reagents were rehydrated with a rehydrating mix comprising isothermal amplification buffer, betaine and MilliQ water. 20ul of sample was added to the reconstituted reagents and run on a simple water bath at 65ºC for 30 minutes. 20 µl of sample and water were added in triplicates to

the reconstituted reagents and run on a simple water bath at 65ºC for 30 minutes. The amplification products were then run on a lateral flow test strip in the device.

3.3 Results and Discussion

3.3.1 Limit of Detection

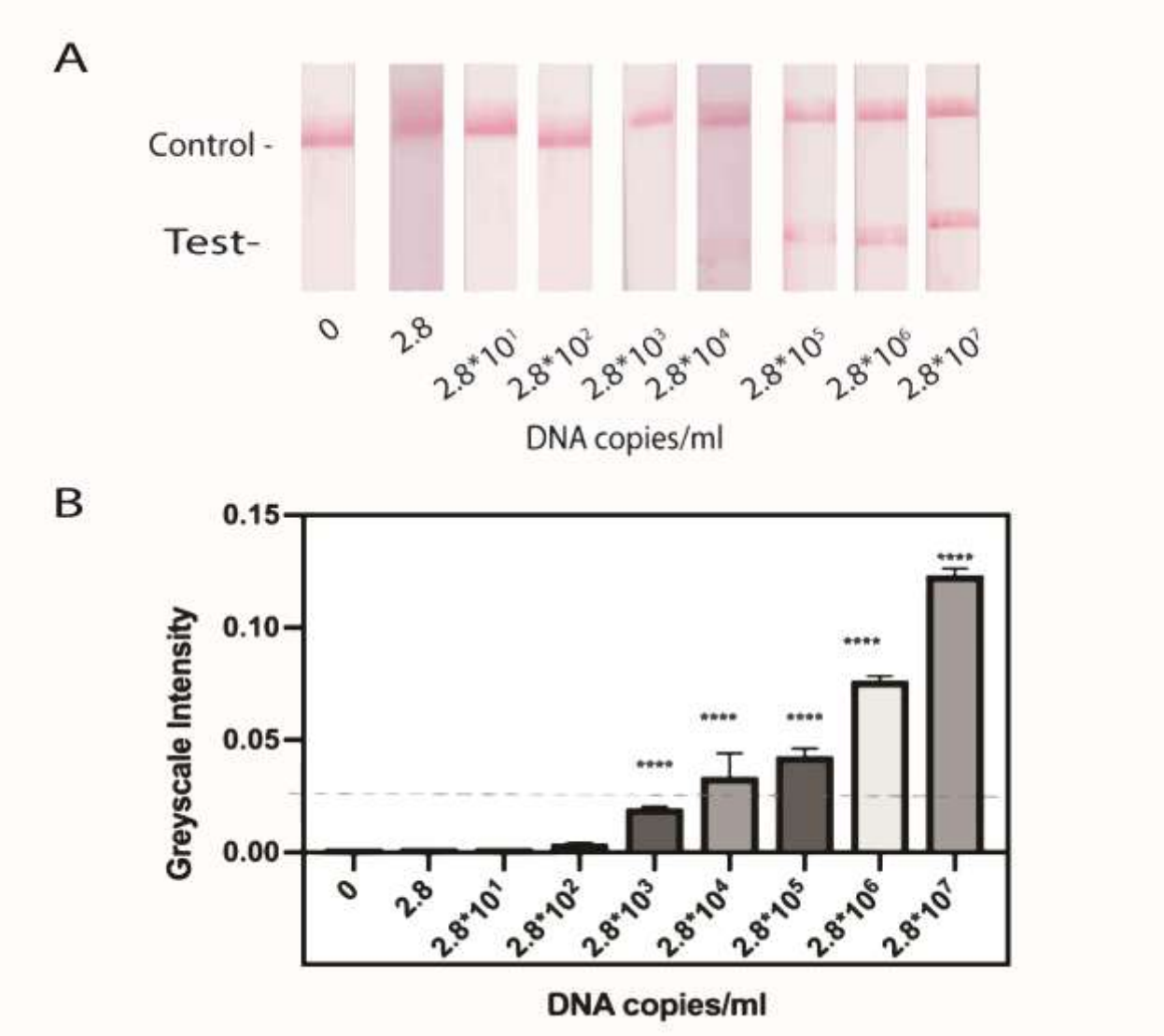

Fig 3.4 A**)** Results of the limit of detection performed on the lateral flow test strips.

The reaction of 2.8 x 104 DNA copies/ml was visible to the naked eye. B) Background subtracted line intensities (A.U.) of each of the lateral flow test strips in B at each concentration of DNA. **** indicates p < -.005 compared to 0 copies/ml

Quantification of the background subtracted LFA test lines resulted in a statistically significant difference of 2.8 x 10^3 copies/ml compared to the controls without DNA (Fig 2b). While statistically significant at 2.8 x 10^3 copies/ml, a study by Phillips *et al.* [26] indicates that only the $2.8x10^4$ copies/ml would be visible to the human eye, hence the limit of detection was determined to be $2.8x10^4$ copies/ml. The limit of detection of the yaws LAMP assay was found to be between 2.8 x 10^3 to 2.8 x 10^4 copies/ml or about 2-20 copies/reaction involving 2 µl of sample. This limit of detection was found to be lower than the CDC_PCR assay which had an analytical sensitivity of 10-100 copies per reaction involving 10 µl sample or $1.0x10^5$ to $1.0x10^6$ copies/ml [27]. This result is expected as lamp assays are known to be more sensitive than conventional PCR [28].

3.3.2 Sensitivity and Specificity

Out of the 63 samples evaluated with the gold standard (CDC_PCR), LAMP using extracted DNA samples and LAMP using unextracted samples, 12, 14 and 8 samples were found to be positive, respectively (Table 3.1).

Table 3.1: Sensitivity and Specificity of the LAMP assay compared to the gold standard CDC_PCR assay.

LAMP (Extracted DNA)	**PCR Results**			**LAMP (Unextracted DNA)**	**PCR Results**		
	Negative	**Positive**	**Total**		**Negative**	**Positive**	**Total**
Negative	49	0	51	**Negative**	51	4	55
Positive	2	12	14	**Positive**	0	8	8
Total	51	12	63	**Total**	51	12	63

Table 3.1: The sensitivity and specificity of the lamp assay using extracted DNA and Unextracted DNA compared to the CDC_PCR assay. Using the extracted DNA samples, the calculated Positive Predictive Value (PPV) was found to be 0.86 with a sensitivity of 1.00 and a specificity of 0.96. With the Unextracted DNA samples, the PPV was 1.00, with a sensitivity of 0.67 and a specificity of 1.00

Table 3.2: All of the 5 PCR confirmed syphilis samples tested negative using the CDC_PCR assay and the LAMP assay specific for yaws

LAMP Results (Extracted DNA)	**PCR Results**		
	Negative	**Positive**	**Total**
Negative	5	0	5
Positive	0	0	0
Total	5	0	5

Twelve (12) out of 63 deidentified samples tested with the CDC_PCR assay were positive for *Treponema pallidum subsp. pertenue* DNA. This is consistent with data from the World Health Organization which states the prevalence of yaws in Ghana from 0.7% to 20% in some communities. The result is also consistent with Agana-Nsiire *et al.* which described the prevalence from 2% to 19.5% in Ghana [22]. These results were also high because these samples were taken from patients with lesions which had a high suspicion of yaws. When these same DNA extracted samples were run using the Lamp assay, 14 out of the 63 were found to be positive. This is possibly because the limit of detection of the CDC_PCR assay is higher than that of the LAMP assay. It is possible that two discordant samples may have been below the limit of detection of the CDC assay. Furthermore, a study by Marks *et al.*, in 2017 [29] showed that 22% of samples previously shown to be negative by the CDC assay turned out to be positive when whole genome sequencing was used due to mutations in the PCR primer-binding sites targeted by the CDC assay. The CDC assay does not detect all isolates of *Treponema pallidum subsp pertenue*, therefore other molecular based tests, such as the LAMP assay described herein, may be necessary in the fight against yaws.

In order to increase the convenience of performing the assay in low-resource health facilities in yaws endemic districts, we explored the possibility of using the LAMP assay on samples that had been taken directly from swab samples in transport medium. While other studies [16], [30]–[34] integrated the extraction step into their devices, we explored the possibility of bypassing the extraction step altogether, thereby speeding the detection process and removing the need for a bulky device as well as eliminating the multiple hands-on steps associated with an integrated extraction. In most yaws endemic districts, DNA extraction kits and reagents are largely

unavailable. Bypassing the DNA extraction step is essential in making a LAMP based rapid test that is applicable to yaws eradication programs in endemic communities. When these unextracted samples were tested with LAMP, 8 out of 63 samples were found to be positive. Reasons why the other previously amplified samples did not amplify in this experiment remain unknown. However, it is suspected that because the DNA extraction process gives rise to highly concentrated elution of DNA as well as high DNA yields, it was easier to detect DNA in the extracted samples while it was more difficult to detect DNA in unextracted samples with low DNA concentrations. While using LAMP on extracted DNA samples was found to have a sensitivity of 100%, it can only be performed in settings with equipment to adequately extract DNA, thereby introducing a lag time between sample collection and treatment. Many yaws eradication programs suffer from loss to follow-up from sample collection to treatment [35]–[37]. Many people living in yaws endemic communities move around for agricultural purposes and may have left communities that were previously screened. While the unextracted samples have a sensitivity of only 67%, this method offers the convenience of same day sample to answer testing as well as treatment. Due to the minimal sample handing requirements, and rapid time-to-result (30 minutes for the lamp assay and 15 minutes for the lateral flow test strips),

3.3.3 Validating the device for use in Resource Limited Countries

To be able to detect the amplified products in yaws endemic district health facilities, we explored the possibility of using dried reagents that could be stored at room temperature. Being able to dry the LAMP reagents and being able to store these reagents provide a solution to a very major logistical challenge when it comes to molecular point of care in resource limited countries. Because of cold chain requirements for reagents, most of these technologies are not deployed since most of those who need it do not really have the resources to keep these reagents refrigerated. As can be seen in Fig 3.5, we had consistent results between day 0 and day 14 (n=3) indicating that the dried reagents could provide consistent results in endemic communities up to 7 days on the field. Since Most endemic countries have reference laboratories in the big cities, the reagents can be dried and packaged and then deployed on the field when there are yaws case search activities.

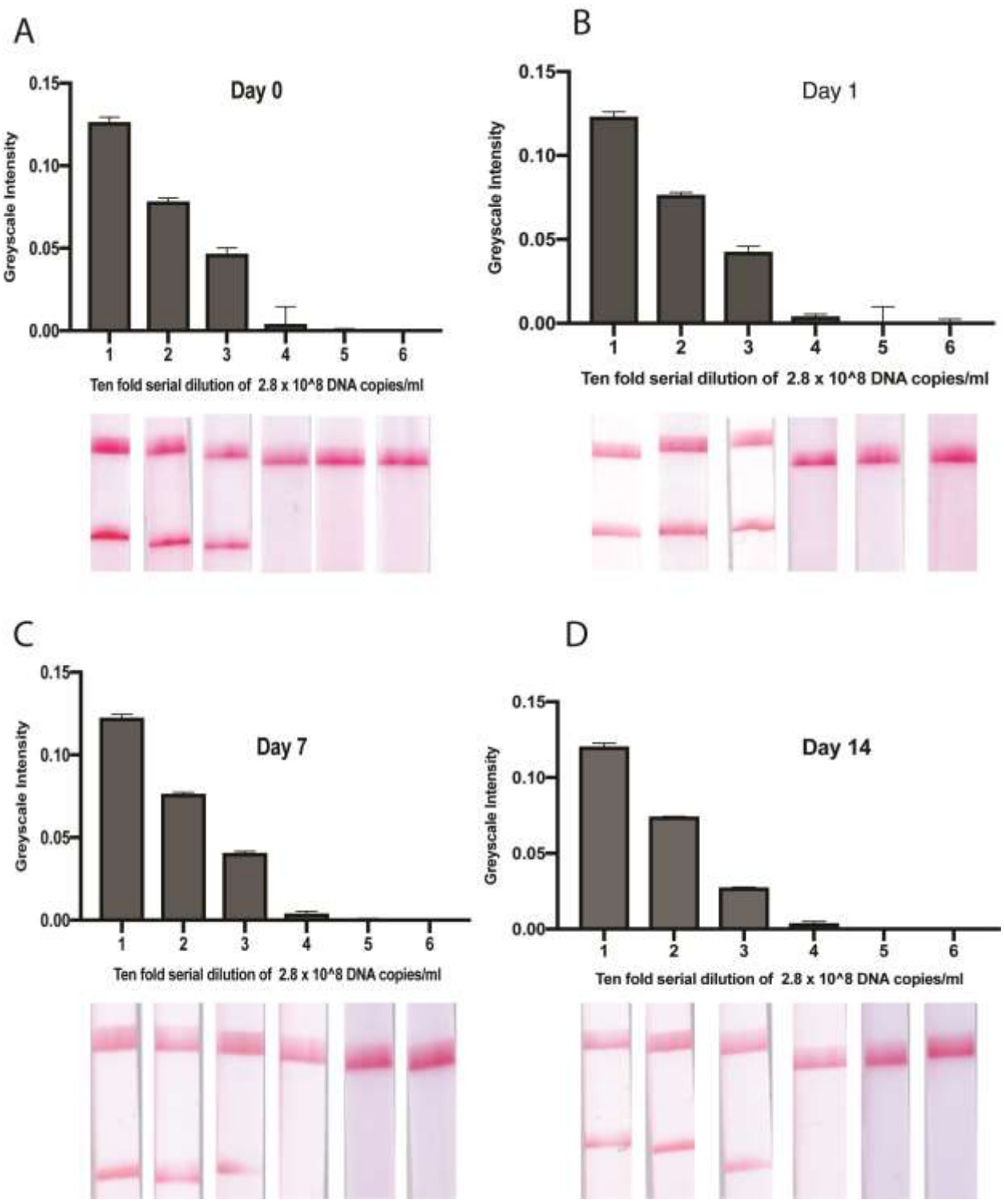

Fig 3.5: Results of amplification with dried reagents using a water bath on lateral flow strips.

These amplified DNA products were then tested on lateral flow test strips. While we ran the samples on agarose gels as controls, this method is not likely to be applicable to yaws endemic communities and greatly increases both the infrastructure and the assay time to result. Running these samples on a lateral flow strips therefore provides a convenient and effective way of detecting *Treponema pallidum pertenue* in the resource limited settings of yaws endemic communities. The downside to a field-based test however is the risk of contamination which can be mitigated by properly training users and ensuring *what other steps can be taken to mitigate (not opening tubes, performing LFIAs away from sample addition, etc.)*. The developed device which contains the lateral flow strip in a plastic housing (Figure 3.6) is meant to not only help reduce contamination, but also to ensure the accuracy of the test and prevent the wastage of the sample by directing the user to apply the sample onto the correct sample pad site. The device also protects the strip from being mishandled or damaged during transport to remote testing sites.

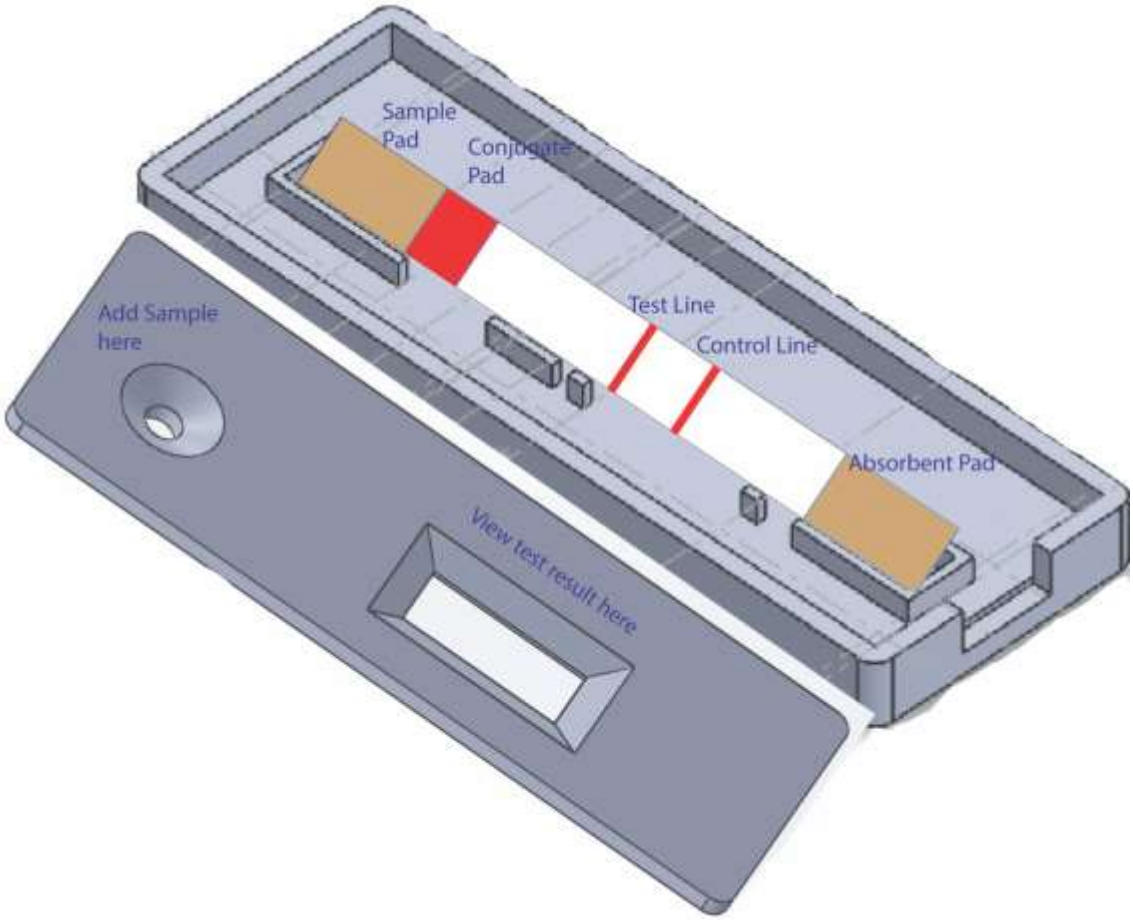

Fig 3.6: Schematic representation of the device.

Putting all the tested components together, the dried reagents, lateral flow strip and device can be used easily in any yaws endemic country as described in Figure 3.7. It is recommended that this LAMP assay be used on unextracted DNA samples on the field in endemic communities. However, negative samples with a high index of suspicion can then be sent to reference labs for extraction and retesting.

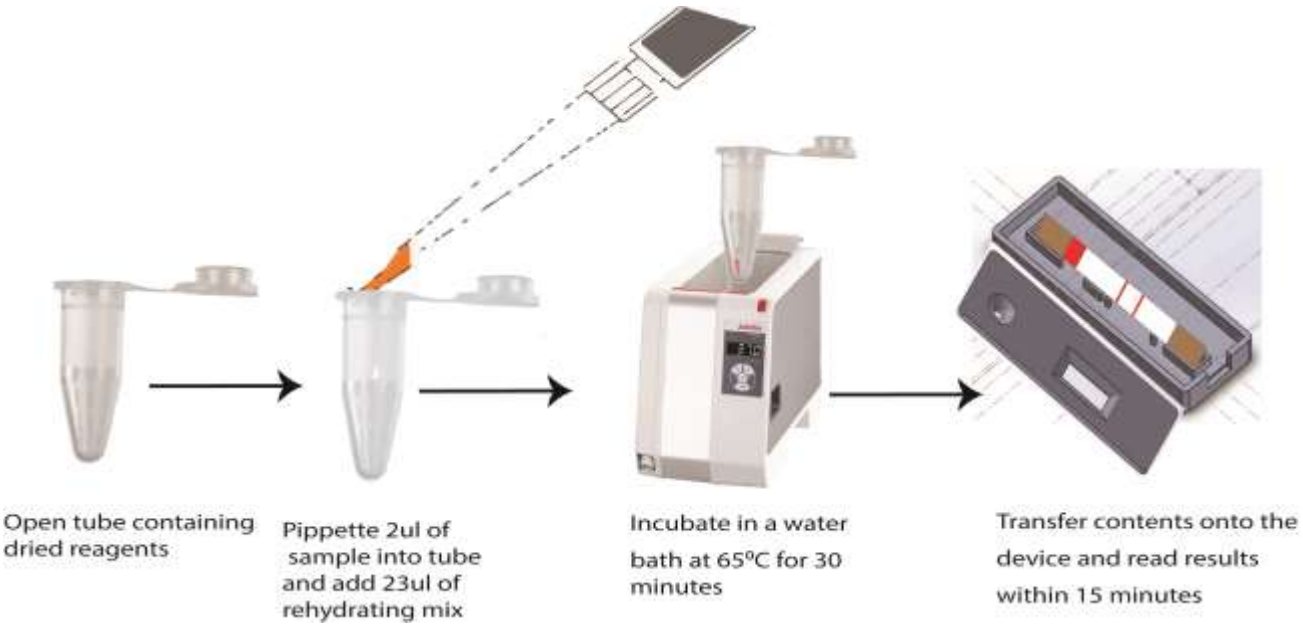

Fig 3.7: An Integrated testing protocol for the Yaws Kit

3.4 Conclusion

Herein, we have shown the development of a fast and sensitive LAMP assay for *Treponema pallidum pertenue* as detected by lateral flow test strips. This new assay provides a sensitive and convenient same day sample-to-answer testing and provides an important tool for the global yaws eradication effort. Using extracted DNA, the assay sensitivity is at par with gold standard detection. The assay can be adapted to minimal sample processing required for in-field detection without DNA extraction.

3.5 References

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CHAPTER 4. PROOF OF CONCEPT FOR SIZE BASED PATHOGEN CAPTURE[4]

4.1 Introduction

4.1.1 The Lateral Flow test strip

4.1.1.1 Sample Pad

The availability of several sample types in which pathogens or analytes can be found has necessitated that there should be several types of sample pads which would ensure the uniform distribution as well as control the flow rate of the sample [1]. Sample pads are often made with either cellulose fibers or woven meshes depending on the sample type [2]. While woven meshes are expensive, have low bed volume, retain small volume samples and have good sample distribution properties, cellulose fibers are relatively cheap and have a large bed volume hence are used when large amounts of reagents and blocking buffers are needed [3]. These blocking fluids made up of proteins, buffer salts and detergents are used to pretreat the sample pad to decrease sample viscosity, ensure that the sample is able to solubilize the detector reagents, prevent nonspecific binding and chemically modify the sample to ensure that it is able to bind with both the detector reagents and the capture reagents [4].

4.1.1.2 Conjugate Pad

The conjugate pad stores the detector reagents until the liquid sample expected to contain the pathogen of interest interacts with these detection reagents [5]. After the interaction, it is expected that there will be a uniform flow of these reagents and test sample to the detection membrane [6]. The most popular conjugate pads are made of cellulose or glass fiber and are specifically chosen not only because of the non-woven nature but also because of their consistent flow characteristics, low nonspecific binding and consistent bed volume [7]. Other major characteristics include consistent compressibility and low extractables. These characteristics are essential in a good conjugate pad as consistent flow characteristics prevent the detector reagents from contaminating the membrane with streaks leading to reduced signal at the test and control lines [8]. It is important to have low nonspecific binding to ensure that the pad does not react with the detection reagents

[4] This chapter has been published.

thereby reducing the sensitivity and specificity. When applying the reagents to the conjugate pad, a consistent bed volume ensures the distribution of the reagents to all parts of the conjugate pad to prevent variable signal intensities [9].

4.1.1.3 Membrane

The membrane binds to capture reagents at the test and control lines of lateral flow test strips. The nature of the membrane is important as this affects the capillary flow properties which in turn also affects how reagents are deposited on the membrane as well as the sensitivity and specificity of the assay [10]. The chemical and physical nature of the membrane also affects the consistency of the test line [11]. Nitrocellulose has been the membrane of choice for lateral flow tests because of the strong dipole to dipole interactions between the nitrate ester and the peptide bonds of the protein. In choosing a membrane, another important characteristic of the membrane is its pore size [12]. The pore size of the membrane is directly proportional to the flow rate, while the limit od detection of the pathogen to be detected in the sample is inversely proportional to square root of the flow rate [11]. This implies that as the slower the flow, the higher the sensitivity. To increase the sensitivity, chaotropic agents like polyvinyl alcohol (PVA), Triton X-100, Tween 20, polyethylene glycol (PEG), glycerine and polyvinylpyrrolidone (PVP) can be used to reduce background noise and inhibit nonspecific binding. However, these should be added only after the capture reagents are immobilized as they also have the ability to effect the interactions between the protein and nitrocellulose hence affecting the development of the signal [13].

4.1.1.4 Absorbent Pad

The absorbent pad, which is often made up of non-woven cellulose fibers, acts as the waste pad to absorb excess fluid in the reaction [14]. The absorbent pad, also known as the wicking pad, plays the important role of keeping a uniform capillary flow throughout the membrane ensuring that the flow is in the right direction at the appropriate flow rate [12]. This ensures that the fluid does not flow back into the reaction, hence affecting the sensitivity and specificity of the reaction [15].

4.1.2 Microfluidic paper-based devices (μPADS)

Lateral Flow Immunoassays are designed to be inexpensive, simple to use, and easy to manufacture and often operated without sample preparation and without any further steps aside the application of the sample at the sample pad [13]. This simplicity, however, presents issues of

small sample volumes, low sensitivity and difficulty multiplexing [16]. Microfluidic Paper-based Analytical Devices, or μPADs, offer an improvement over Lateral flow strips as used alone or in combination with lateral flow test strips, the present an opportunity to increase the sensitivity of the test [17]. Hydrophobic barriers are often created on sheets of cellulose which is hydrophilic in order to form microliter sized capillary channels [18]. These μPADS have the ability to distribute fluid both laterally and vertically and hence are able to perform complex reactions using very little reagents and fluids hence further lowering the cost of these devices [19].

4.1.2.1 Fluid flow in μPADS

In characterizing fluid flow in porous media, especially in microfluidic devices including LFIAs, it is important to examine both the macroscale and microscale flow characteristics associated with these devices if we are to improve on the sensitivity and specificity associated with them [20]. On the macroscale level, mass transport of reactants through a lateral flow device is important to its function [21]. Once a sample is deposited on the sample pad, movement of the sample through the conjugate pad to the test and control lines occurs through diffusion, advection and kinematic dispersion [22]. In advection, the reactants move by the bulk flow of the fluid as it moves from the sample pad to the absorbent pad through the conjugate bad and nitrocellulose membrane [23]. The transport of small diameter reactants is usually dominated by diffusion. These small diameter reactants diffuse by Brownian motion from a region of a higher concentration to a region of lower concentration [24]. However, the porous nature of the paper used in fabricating these devices reduces the diffusion coefficient, thus reducing the effective diffusion [24].

The effective diffusion of a reactant r is given as

$$D_{eff} = \frac{\in}{\tau} D_r$$

Where $\in$ = shows the porosity of the paper τ is the tortuosity of the paper and D_r is the free solution diffusion coefficient of the reactant. The tortuosity of the paper is often calculated as $\tau = \in^{\frac{1}{3}}$ [25]

Paper has heterogeneous porosity and induces the spread of reactants along the direction of flow. This is called Kinematic dispersion and leads to variable velocities in the microscopic pores of the paper [26].

In characterizing microscale flow, it is important to characterize it based on whether it's wetted paper or not. The one-dimensional fluid flow in an unwetted porous paper follows the Lucas Washburn equation:

$$L = \sqrt{\gamma D t\ 4\mu}$$

where L = the distance moved by the fluid front, γ = the effective surface tension of the liquid, D = the average pore diameter, t = time, and μ = the viscosity of the liquid. For water the surface tension at room temperature is estimated to be 0.0728 N/m and the viscosity 1.002 x 10-3 Ns/m2. This is based on the assumption that the paper has a constant cross section and the fluid is from a non-limiting source [27].

When the paper is already wetted as is seen most of the time in cases where the sample is applied and a buffer is included, the equation follows Darcy's law

$$Q = -\kappa WH\ \mu L\ \Delta P$$

where Q = the volumetric flow rate, κ = the permeability of the paper to the fluid, μ = the viscosity of the fluid, WH = the area of the channel perpendicular to flow, and ΔP = the pressure difference along the fluid flow direction over the length L [28].

Fluid flow from a channel with a larger width to a channel with a smaller width (Abundant flow) does not affect the rate of flow as the flow from the larger channel acts as a non-limiting source for the smaller channel. On the other hand, a flow from a smaller channel into a larger flow constricts the flow and limits the volumetric flow rate that can flow into the larger channel even though the larger channel has the capacity to have more liquid volume [29].

4.1.2.2 Characterizing Fluid Flow using μPIV

While there are several existing methods to characterize fluid flow, particle image velocimetry (PIV) offers a new and accurate way of characterizing fluid flow in these devices. PIV is a

technique which maps the instantaneous velocity field of macroscopic flows by periodically suspending and illuminating tracer particles in the flow [30]. PIV is an non-intrusive, *in situ* technique that can be used to characterize two-dimensional or three-dimensional flows as well as multiphase flows [31]. PIV requires the use an optically transparent medium which enables the tracer particles to be illuminated constantly by a sheet of pulsed light. Several image pairs taken at a constant time interval are then captured by a camera to record the displacement [32]. This record of displacement is then scaled to the flow velocity. Micro-PIV (μPIV) is a modified form of PIV which measures fluid velocities in microfluidic devices that have spatial resolutions on the order of tens of microns [33]–[35]. Adapting PIV to microscale level comes with its own challenges. The right sized tracer particles are essential because these particles must be large enough to enable imaging, minimize errors with Brownian motion and achieve sufficient special resolution of the microscope in order to be able to make useful measurements, but also small enough not to clog the device. [36] With a combination of experimental methods and interrogation algorithms, these problems can be solved and the fluid flow in these devices characterized [37].

4.1.3 Pathogen Capture in Porous Media

In the diagnosis of infectious diseases, assays that target the molecular structure of the organism especially its DNA or RNA have been known to offer much more sensitivity and specificity than the traditional immunological targets that traditional lateral flow devices use [38]. The LAMP assay discussed in Chapter 2, offers a viable method for use with lateral flow test strips as discussed in Chapter 3. Current diagnostics which require multiple user steps are prone to challenges such as contamination [39]. There has been a great deal of studies into self-contained diagnostic platforms [39]. These self-contained devices would have multiple steps of nucleic acid extraction, amplification and then detection on the same device with minimal user interference. Paper based devices offer the platform for this self-contained device [40]. A study by Linnes *et al*, showed that Polyethersulfone (PES) improved isothermal nucleic acid amplification compared to other porous materials hence offers an optimal support for rapid molecular diagnostics for use in POC applications [41].

PES has been used in ultrafiltration (UF) and microfiltration (MF) membranes because of its unique properties of hydrolytic, thermal and mechanical strength in extreme environments [42]. A

characteristic of commercial PES which makes it ideal for use in biological system is its low protein binding and hydrophilic properties.

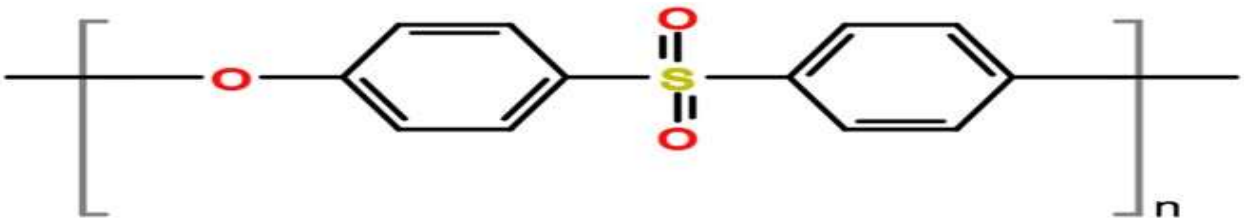

Fig 4.1: Chemical Structure of Polyethylenesulphone [43]

Other properties that make PES unique is the fact that it is strong and durable and easily handled and has uniform pore structure [44]. Combined with the ability to offer improved nucleic acid amplification, PES offers a viable platform for pathogen extraction and amplification in self-contained microfluidic devices [45].

Body fluids like blood, urine and swab samples which are used for pathogen detection are hardly homogenous [46]. They are made up of several cellular components of varying sizes. While blood is composed of red blood cells (8μm) and white blood cells (12-17μm), Urine is made up of white blood cells and epithelial cells (12-21μm). Swab samples are usually composed of white blood cells, dead skin cells etc. [47]. Most pathogens however are much smaller than the components of these body fluids as viruses are often in the size range of 20-400 nm and bacteria are 0.2-2 μm [48]. It is therefore theoretically possible to separate the various components of the samples based on the size, allowing the separation and amplification of the pathogen [49]. Sized based separation has been used in several situations especially in circulating tumor cells [50]. Blood separation membranes like MF1(GE Lifesciences, Marlborough MA), which is a glass fiber filter are already commercially available. PES is also known to come in different sizes and so using PES, it is possible to capture large sized cellular components while allowing small sized pathogens to flow through the larger sized PES and be captured in the smaller sized PES for amplification.

This chapter explores the possibility of sized based separation in PES while characterizing the fluid flow before and after pathogen capture in the PES.

4.2 Methods

4.2.1 Vertical Separation and capture

Fluorescent nanoparticles (Bangs Laboratories, Fishers, IN) of sizes 0.11 µm in dragon green (representing the pathogen) and 7.32 µm in suncoast yellow (represening the cellular components of the sample) were used in the vertical separation in order to quantify the capture efficiency of the membranes used in the study. The nanoparticles were diluted as per the manufacturer's instructions. The nanoparticle solutions were serially diluted and measured using a Spectramax M5 microplate reader (Molecular Devices LLC, San Jose, CA) at excitation of 480 nm for the 0.11 µm and 540 nm for the 7.32 µm nanoparticles. A calibration curve was then drawn to correlate the nanoparticle concentration to the fluorescence.

One hundred fifty (150) µL of the 0.11 µm and 7.32 µm nanoparticles was pipetted into a spin column which had been prepared to contain either the MF1 (GE Healthcare, Chicago, IL)), the 0.1 µm PES membrane (Sterlitech, Kent, MA) or the 0.22 µm PES membrane (Millipore Sigma, Burlington, MA). The spin column was prepared by punching a 7 mm single hole in the membrane and sandwiching it between two O-rings. This was then placed in a miniprep spin column (Qiagen, Hilden, Germany). The spin column was was then placed in a collection tube as shown in Figure 4.2. The tubes were centrifuged at 0.6 rcf for 60 minutes. The fluorescence of the elution was measured and correlated to concentration on the calibration curve. Nanoparticles which had not passed through any membrane was used as a baseline and used to calculate the proportion of nanoparticles that had passed through the membranes.

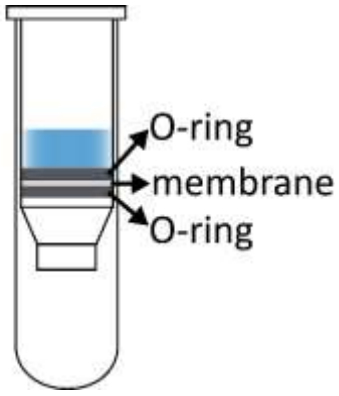

Fig 4.2**.** A schematic representation of the Vertical flow filtration setup with the membrane of interest compressed between two O-rings.

4.2.2 Lateral Separation and Capture

To show than sample components and pathogens can be captured based on size, the 0.1 µm and 0.22 µm PES (2.5 cm x 1 cm) were overlapped with the MF1 membrane (1 cm x 1 cm) into a membrane strip (Figure 4.3) on a microscope slide. Thirty (30) µL of a nanoparticle mixture containing equal parts of a 1 in 100 dilution of the 0.11 µm nanoparticles and 1 in 10 dilution of the 7.32 µm nanoparticles was pipetted into the membrane strip followed by a 30 µL PBS wash. The membrane strip was then imaged at 10X magnification with an inverted Axio Observer Z1 Fluorescent microscope and ZenPro software (Carl Zeiss Microscopy, Thornwood, NY) using the Rhodamine filter for the 7.32 µm nanoparticles and the Alexa Fluor 488 filter for the 0.11 µm nanoparticles

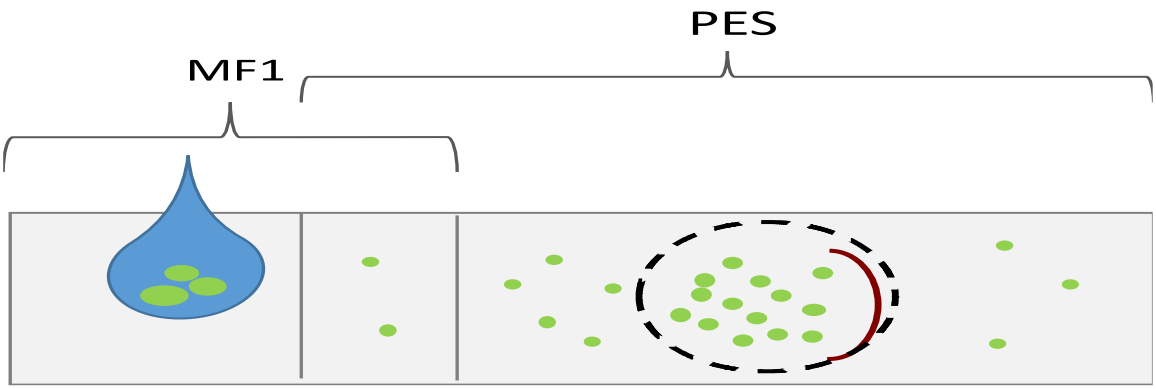

Fig 4.3: A schematic representation of the lateral flow bead separation set up

4.2.3 Characterizing Fluid Flow using µPIV

In characterizing the fluid flow in PES before and after pathogen capture, a 2.5 cm x 1 cm piece of 5.0 µm PES was overlapped with the MF1 membrane (1 cm x 1 cm) as shown in Figure 4.4. A 60 µL solution of 400 nm green fluorescent nanoparticles (ThermoFisher scientific, Waltham, MA) diluted to 0.02% was pipetted onto the MF1 and a 10 second video showing the nanoparticles as they flowed into the edge and middle of the PES was taken using a with a Zeiss Inverted Axio Observer microscope with an Axiocam 503 mono CCD camera and 40X objective.

In the second set up, a 100 µL solution was prepared containing equal parts of the 400 nm nanoparticles diluted to 0.02% and 7.32 µm nanoparticles diluted to 0.01%. Thirty (30) µL of the nanoparticle solution was pipetted onto the assembled MF1 membranes, followed by a 30 µL PBS

wash and again a 10 second video taken as described above. Each of the videos had a frame rate of 40 frames per second, a 2x2 pixel binning and a 20ms exposure.

Each of the videos taken were converted into a sequence of 8-bit bmp image files and analyzed using EDPIV software. In the analysis, central window shifting was done with a window size of 128 pixels and a grid size of 64 pixels. A central difference interrogation was also done with 4-pixel fit, and 5 primary iterations. The the results from the primary iterations were interpolated onto a 64 x 64 window with a 32 x 32 grid with a 31-pixel search radius.

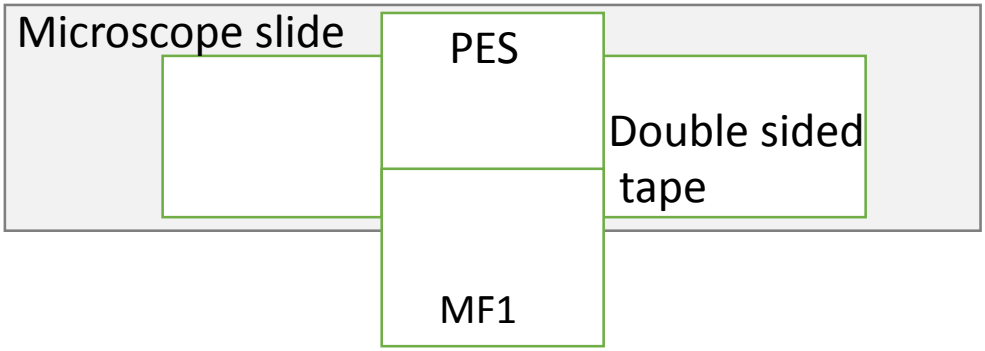

Fig 4.4: Schematic representation of the set up for the PIV study

4.3 Results and Discussion

This study was based on the hypothesis that it is possible to separate the various components of body fluids including pathogens in paper based on size. In this study, the 0.1 µm nanoparticles represented the pathogen while the 7.32 µm particles represented the cellular components found in body fluids. As can be seen in Table 4.1, 86% of the 0.1 µm were captured in the 0.1 µm PES while only 30% was captured in the MF1. This means that using the 0.1 µm PES membrane, it is possible to for 70% of the beads to flow through the MF1 membrane into the PES where 86% of these could be captured for amplification. In this scenario, it is possible then to concentrate the pathogens in the 0.1 µm PES membrane for amplification. The 30% loss of the pathogen in the MF1 is a cause for worry as it reduces the sensitivity especially in a quantitative analysis, however this concern can be factored when considering the volumes of samples to use in order to ensure the capture of enough particles. In the 022 µm PES, 47.6% of the 0.1 µm beads passed through. Although the size of the membrane is larger than the size of the beads (0.22 µm vs 0.1 µm), nearly

half of the beads were captured by the membrane and this may be as a result of many factors. It is possible that membrane tortuosity may have played a role in limiting migration of the particle. Furthermore, membranes are heterogenous and the actual pore sizes of the membrane may be different from the nominal pore size of 0.22 µm and could account for the particles being trapped. Finally, it is possible that the surface chemistries of the membrane may have created attractive forces that cause the nanoparticles to stick to the membranes.

The 7.32 µm beads representing the cellular components of body fluids were effectively captured by all the membranes including the MF1. This means that, the MF1 would be effective in serving as a cell capture membrane, capturing all the cellular components of the body fluid while allowing the pathogen flow through the MF1 into the PES membrane for amplification.

Table 4.1**.** Efficiency of membrane capture of fluorescent nanoparticles. n=3

Particle size	**Membrane**	**Fluorescence (RFU)**	**Approx. Concentration (particles/mL)**	**Particles Captured**
0.11 µm	None	4007.6 ± 165.0	4777.1 ± 1100.7	**0%**
	MF1	2810.9 ± 193.0	3532.5 ± 2470.0	**30.0%**
	0.1 µm PES	557.8 ± 72.0	670.8 ± 91.5	**86.0%**
	0.22 µm PES	1986.8 ± 103.2	2084.1 ± 169.5	**47.6%**
7.32 µm	None	275.1 ± 12.2	383.0 ± 19.3	**0%**
	MF1	2.3 ± 1.3	4.8 ± 2.0	**98.6%**
	0.1 µm PES	0.8 ± 0.1	2.9 ± 0.2	**99.1%**
	0.22 µm PES	0.9 ± 0.2	5.6 ± 1.1	**81.9%**

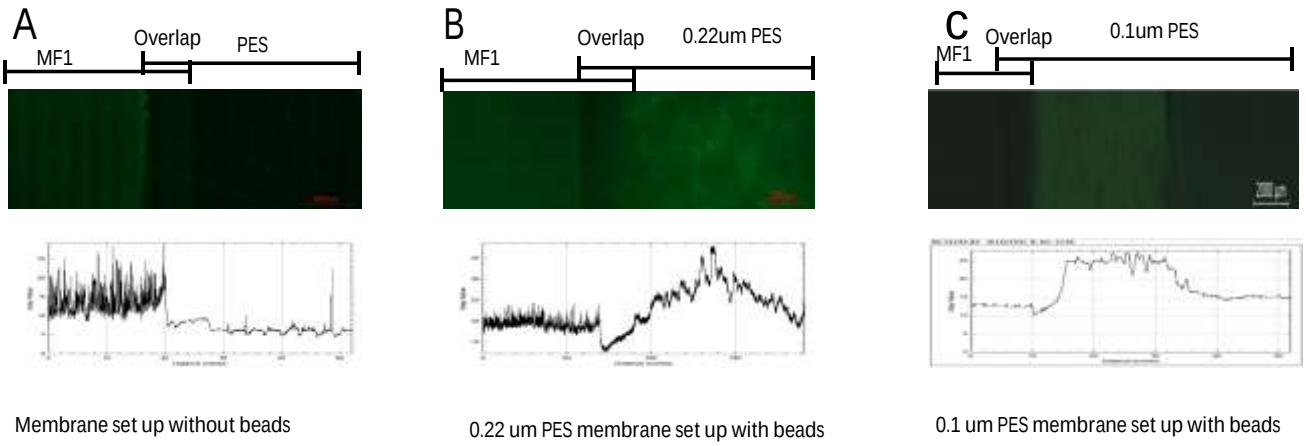

Fig 4.5: Particle capture and separation in lateral flow.

A. in the MF1/PES membrane set up without fluorescent nanoparticles **B.** An MF1/022 µm PES Membrane set up with fluorescent particles **C.** An MF1/0.1 µm PES Membrane set up with fluorescent particles.

In Figures 4.5 the particle capture and separation were studied in a lateral flow format under a fluorescent microscope. We show in this figure the pathogen capture and separation in both the 0.1 µm PES and the 0.22 µm PES. In both figures, A) shows the membrane set up without beads and B) shows the membrane set up with 0.11 µm green fluorescent beads. Just like the vertical separation, the lateral separation in the 0.1 µm PES shows all the beads being captured midway through the beads. The implication is that the pathogen can be concentrated and amplified using the 0.1 µm PES. On the other hand, the nanoparticle beads were all over the 0.22 µm PES. This also correlated with the vertical separation which showed less than 50% of the beads being captured. However, this can be utilized in developing devices as the size of the 0.22 µm membrane can be increased or reduced based on the size of the amplification window that is needed in the device.

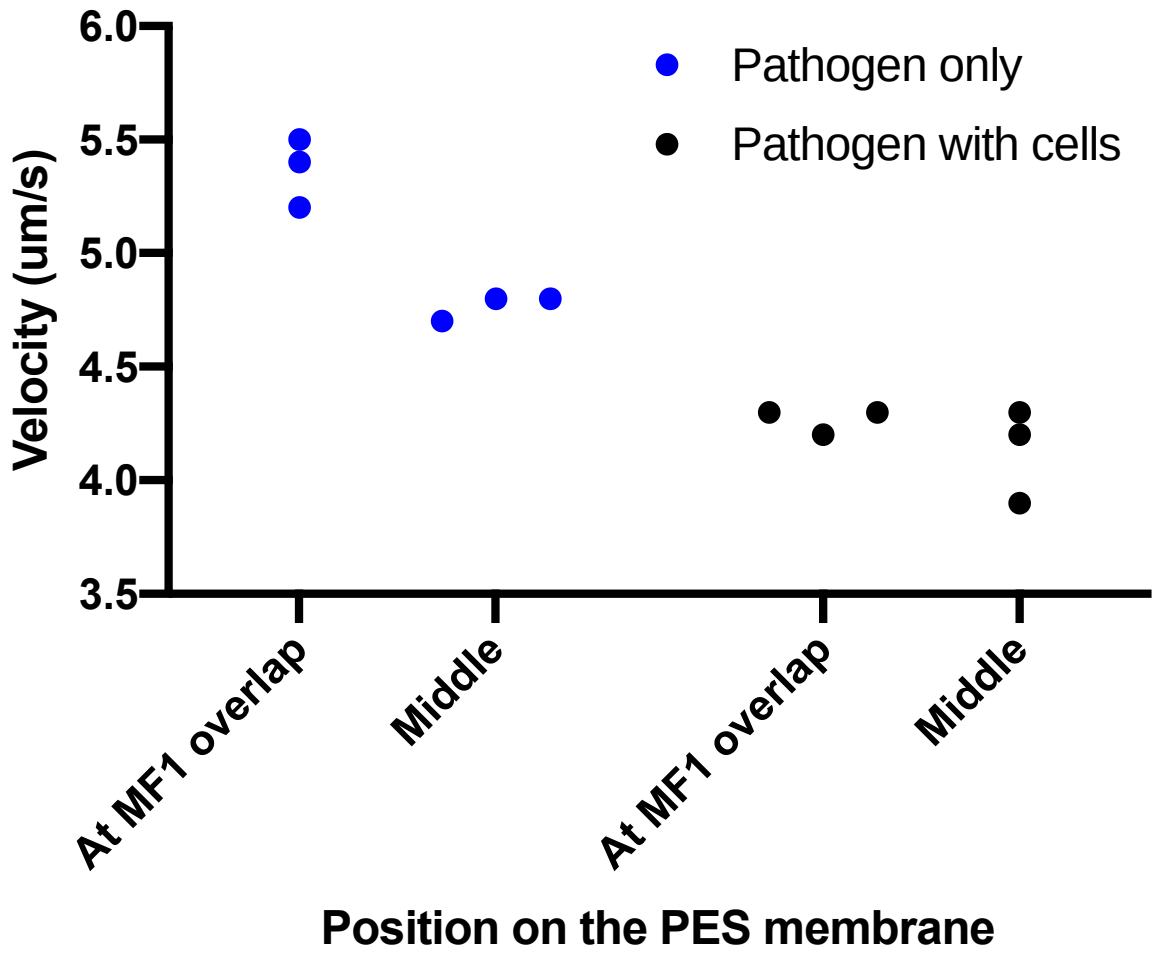

Fig 4.6 shows the velocity of flow before and after capture using the PIV analysis.

In figure 4.6, we see the velocity of the particles before and after capture. A two-way ANOVA was conducted to determine significant difference between the fluid flow before capture and after capture as well as the significant difference between the MF1/PES overlap and the middle of the PES membrane. The analysis shows that while there was no significant difference between the movement of the particles and hence the fluid at the overlap and the middle of the PES, there was a significant difference between the fluid flow before capture and after capture. This implies that there is a significant reduction in the velocity of the fluid flow after capture. Fluid flow is known to affect sensitivity of LFIA. A lower flow velocity increases the contact time of the analyte with the capture and detector probes. A lower velocity in the PES especially in the 0.22 μm PES membrane would be advantageous as it allows for more sensitivity but an adequate velocity to

ensure that sample to answer is achieved within a reasonable time frame. With the 0.1 μm PES, a lower flow velocity may mean sample to answer may take too long.

The MF1before the capture (pathogen only) is open and so allows the pathogen to flow directly onto the PES. With pathogen capture (pathogen with cells), because the cells are bigger, they get trapped within the PES. By being trapped in the PES, it becomes quite difficult for the smaller sized pathogens to flow out hence it takes longer to move into the PES. The implication for this is that sample processing would take longer, thereby affecting the total sample to answer time. For instance, with the current yaws assay, it takes about 45 minutes (Amplification and testing on the lateral flow strip). Adding a sample extraction step using size-based extraction would increase that time but it would still be same day and would be better than the current protocol where samples take up to a few weeks. It would therefore be a tradeoff between time to answer and an increased quality of results and it is worth exploring the integration of size-based pathogen extraction into the assay and device.

4.4 Conclusion

We show in this chapter that size based separation is feasible and can be applied to devices to ensure the separation and concentration of pathogens. We show that the 0.1 μm offers better separation and concentration than the 0.22 μm PES, although the 0.22 μm PES can be used in devices depending on the size of membrane needed for amplification. In this chapter, we have shown that the MF1 offers a convenient way of separating large sized particles including red blood cells in blood.

We have also shown that while there is no significant change in the direction of flow as well as the distance of the flow from the MF1/PES overlap, there is a significant difference in the flow velocity before and after pathogen capture. The implication is that while sized based pathogen separation and capture is feasible and has the ability to concentrate pathogens for amplification, it also has the possibility of reducing fluid flow velocity which could either be an advantage or disadvantage depending on the purpose of the assay, the time required for amplification and the size of the PES membrane being used in the amplification.

4.5 References

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