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"LAMP: AN APPROPRIATE TECHNOLOGY FOR POINT-OF-CARE (POC) APPLICATION IN MEDICAL DIAGNOSTICS"

BY

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Thesis

Submitted in partial fulfillment for the requirements of the degree of
Master of Science (M. Sc.)

August, 2014

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Dedication

This thesis is dedicated to my father Md. Nurul Islam and mother Mrs. Nazma Begum, who encouraged me to learn and taught me to be curious; to my husband Dr. Nazmul Alam, who inspired and supported me to pursue my research career; and my beloved daughters Sadia Naoshin and Nabeeha Nawal for their emotional support.

Abstract

In the field of medical diagnostics, point-of-care (POC) applications are simple to use, portable, easily disposable, and stable under different operating conditions. A high-throughput, automated robotic processing instrument is generally not affordable or feasible in low-resource settings that lack the necessary laboratory infrastructure. Though many methods are already established for medical diagnostics, not all of them are suitable for point-of-care diagnostics. Nucleic acid amplification methods are very sensitive and specific due to target amplification and base-pairing interactions. Over polymerase chain reaction (PCR), the isothermal amplification of DNA/RNA has recently drawn interest since it does not require a large thermal cycler. LAMP (Loop mediated isothermal amplification) is an isothermal amplification technique and considered as a robust method in terms of sensitivity, tolerance to inhibitory substances present in the real sample, and easy naked eye detection. Therefore, it is a simpler and more energy efficient approach, making it an excellent choice for POC applications. I developed a low cost plastic pouch using a plastic bag (e.g. simple re-sealable zipper storage bag) for the detection of Herpes Simplex Viruses (HSV). The LAMP method was easily incorporated into this plastic pouch and allowed the detection of 6.08 x 10^1 copies/µl of HSV-1 DNA and 0.598 copies/µl of HSV-2 DNA within 45 minutes. Since the LAMP method is less sensitive to inhibitory substances present in the real sample, we also could detect viral DNA without purifying it. The result was easily evaluated -colorimetrically using the naked eye via the addition of hydroxynaphthol blue (HNB) dye in the reaction mix. Therefore, colorimetric detection by the naked eye makes for easy result analysis. -The lack of need for expensive instruments and its low cost and portability make this invension a perfect candidate for point-of-care (POC) diagnosis both in the laboratory and in low-resource countries.

Keywords: Point-of-care (POC), LAMP, Herpes Simplex Virus-1&2, HNB dye, colorimetric detection, plastic pouch.

Résumé

Dans le domaine du diagnostic médical, les applications points de soins (PDS) sont simples à utiliser, portables, disponibles, et stables dans différentes conditions de fonctionnement. Les instruments automatiques robotisés à haut-débit de traitement ne sont généralement pas accessibles ou réalisables dans les milieux à faibles ressources qui manquent d'infrastructures dans les laboratoires. Bien que de nombreuses méthodes sont déjà établis pour le diagnostic médical, elles ne sont pas toutes adaptées pour le diagnostic aux points de soins. Les méthodes d'amplification d'acides nucléiques sont très sensibles et spécifiques en raison de l'amplification de la cible et des interactions d'appariement de bases. Au cours de la réaction en chaîne polymérase (PCR), l'amplification isotherme de l'ADN / ARN a récemment suscité de l'intérêt, car elle ne nécessite pas un thermocycleur. LAMP (Loop-mediated isothermal amplification) est une technique d'amplification isotherme, considérée comme une méthode robuste en termes de sensibilité, de tolérance avec des substances inhibitrices présentes dans l'échantillon réel, et qui permet la détection du résultat avec l'œil nu. Par conséquent, il s'agit d'une approche plus efficace et plus simple, ce qui en fait un excellent choix pour les applications de points de soins (PDS). J'ai développé un étui en plastique à faible coût en utilisant des sacs en plastique (par exemple, les sacs à fermeture zip simples) pour la détection du virus d'herpès simplex (HSV). J'ai incorporé la méthode LAMP dans cette pochette de plastique et j'été capable de détecter 6,08 x 10^1 copies / µl de HSV-1 ADN et 0.598 copies / µl de HSV-2 ADN dans 45 minutes.

Mots-clés: point de service (PDS), LAMP, virus d'herpès simplex (HSV), HNB colorant, détection colorimétrique, le sachet en plastique.

Preface

This thesis includes three chapters. The first chapter is the introductory chapter where I briefly discuss point-of-care (POC), target analytes for disease diagnosis, and methods used for POC diagnostics. Though many methods are already established for medical diagnosis, not all of them are suitable for POC applications. Nucleic acid amplification methods are very sensitive and specific due to target amplification and base-pairing interactions. The isothermal amplification of DNA/RNA has recently drawn considerable interest since in contrast to the polymerase chain reaction, it does not require a large thermal cycler. I focused on the LAMP (Loop mediated isothermal amplification) technology because it is an isothermal amplification method and considered as a robust technique in terms of sensitivity, tolerance with inhibitory substances present in the real sample and facile detection with the naked eye. LAMP technology is a simpler and more energy efficient approach, making it an excellent choice for POC applications. Accordingly, I was motivated to use LAMP in my thesis work. The second chapter encompasses a discussion of the LAMP method and importantly describes my first experimental results. I developed a low cost plastic pouch for the detection of Herpes Simplex Viruses using the LAMP method. The amplified product of LAMP could be detected in various ways, for example, colorimetrically, electrochemically, or even by the naked eye. In my work, I used visual detection facilitated by a dye binding to the product. In the third and finalchapter I described an example of an electrochemical detection of LAMP product, which was a collaborative effort work with members of our laboratory at INRS. This work has been published in the journal *Analyst* in 2013 and is entitled: "Real-time electrochemical detection of pathogen DNA using electrostatic interaction of a redox probe".

Acknowledgements

I would like to express my special appreciation and thanks to my supervisor Professor Fiorenzo Vetrone, INRS-EMT (Institut National de la Recherche Scientifique- Énergie Matériaux Télécommunications) for his guidance, support and time invested to complete this assignment. I would also like to thank Dr. Mohammed Zourob, my previous supervisor, who gave me the idea and much of the laboratory support to do my work. In my experiments, I had to work with the Herpes Simplex Virus necessarily specific biosafety facilities. This facility was not available at the INRS-EMT campus. Professor Angela Pearson, INRS-IAF (Institut National de la Recherche Scientifique-Institut Armand-Frappier), who was my former co-supervisor, allowed me to use her laboratory facilities. Without this I could not have finished my experiments. I would like to thank her for this support. Dr. Minhaz Uddin Ahmed, who was a post-doctoral fellow of INRS-EMT, taught and guided me throughout my work. I want to express my special gratitude to Prof. Marc A. Gauthier, INRS-EMT (Institut National de la Recherche Scientifique- Énergie Matériaux Télécommunications) for his guidance and support to complete my thesis writing. I also want to give special thanks to Prof. Federico Rosei, Director of INRS-EMT and Prof. Tsuneyuki Ozaki, Director of the programme Material Science for their support in persuading me to complete my degree. I would like to give special thanks to Ms. Mouna Moumene, a PhD. student of INRS-EMT, who helped me to translate the thesis summary into French. Finally, I would like to thank all the members of Biosensors, BioMEMS and Bionanotechnology laboratory (BBBL), INRS-EMT, and my family members for their support and encouragement.

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Chapter 1

Technologies for point-of-care testing (POCT) for medical diagnostics

1.1 Introduction

Infectious diseases cause 9.5 million deaths per year, almost all in developing countries. (Holzscheiter, 2010) This rate is significantly high compared to developed countries, due to the delay of diagnosis and treatment in limited resource settings. Early diagnosis of disease and treatment can have an important role in preventing the development of long-term complications or in interrupting transmission of the infectious agent. It also provides appropriate and timely care to patients, preventing nosocomial infections and providing crucial surveillance data for both emergency public health interventions and long-term public health strategies (Yager, et al. 2008). In rural areas, especially in the developing countries, laboratories cannot afford high throughput platforms. Conventional and low-sensitive technologies create delays or prevent diagnosis of the disease. Healthcare professionals, therefore, are seeking more affordable, smaller-scale, field-ready diagnostic technologies that can quickly and accurately identify pathogens for infectious diseases. To be useful, diagnostic methods must be accurate, simple and affordable for the population for which they are intended. Point-of-care (POC) diagnostics can meet these needs (Dwortzan, 2013)

In the area of medical diagnostics, POC applications are simple to use, portable, easily disposable, stable under different operating conditions (such as temperature, humidity especially in low resource area). Lab-on-a-chip (LOC) devices offer many advantages for pathogen detection such as miniaturization, small sample volume, portability, and short detection time for POC diagnosis.

1.2 Point-of-care (POC)

POC testing (POCT) is - defined as medical testing at or near the site of patient care. These are simple medical tests which can be performed at the bedside and include tests such as those found in typical medical examinations: urine dip stick tests, simple imaging such as with a portable ultrasound device, etc. (Kost, 2002).

The driving notion behind POCT is to bring the test conveniently and immediately to the patient. This increases the likelihood that the patient, physician, and care team will receive the results more quickly, which facilitates for immediate clinical management decisions to be made. POCT includes: blood glucose testing, blood gas and electrolytes analysis, rapid coagulation testing (PT/INR, Alere, Microvisk Ltd), rapid cardiac markers diagnostics (TRIAGE, Alere), screening of drug abuse, urine strip testing, pregnancy testing, fecal occult blood analysis, food pathogen screening, hemoglobin diagnostics, infectious disease testing, and cholesterol screening (TriMark Publications, 2013). Cheaper, smaller, faster, and smarter POCT devices have increased the use of POCT approaches by making testing cost-effective for many diseases.

1.3 Types of analytes

For medical diagnostics including POC diagnostics, different types of analytes are detected, for example, proteins, nucleic acids, cells, and small molecules (Chin, et al. 2007).

1.3.1 Proteins

Proteins in clinical specimens are mainly found in body fluids, *e.g.* in whole blood, saliva, urine, and other intracellular substances. These are used for clinical diagnostics and monitoring disease states .POC for detecting proteins includes both immunoassays and enzymatic assays. Clinical tests for POC include viral infections (anti-HIV antibodies, antibodies against influenza A/B virus, rotavirus antigens), bacterial infections (antibodies against *Streptococcus* A and B, *Chlamydia trachomatis*, *Treponema pallidum*), parasitic infections (histidine-rich protein 2 for *Plasmodium. falciparum*, trichomonas antigens), and noncommunicable diseases (PSA for prostate cancer, C-reactive protein for inflammation, HbA1c for plasma glucose concentration) (Ahmed et al. 2007, Chin et al. 2007, Ahmed et al. 2009)

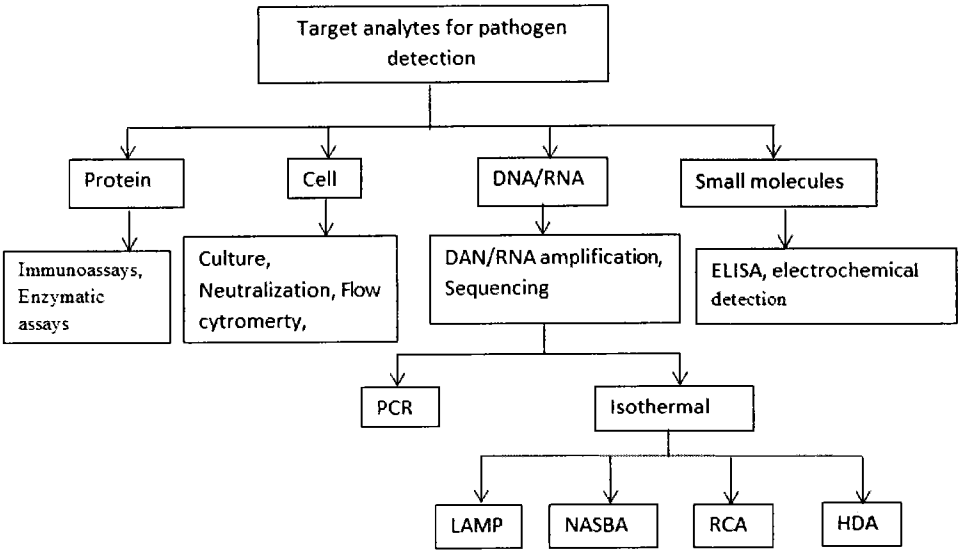

Fig1.1: Flow chart showing target analytes and their detection technologies for disease diagnosis. ELISA = Enzyme-linked immunosorbent assay; PCR = Polymerase chain reaction; LAMP = Loop mediated isothermal amplification; NASBA = Nucleic acid sequence based amplification; RCA = Rolling cycle amplification; HDA = Helicase-dependent amplification.

1.3.2 Cells

Cells are mainly blood and tissue origin. Viruses are present in body fluid in low quantities. They are cultured in living cells in the presence of culture media and analysed neutralizing assays or under a microscope to determine cell morphology. For viruses that target blood cells, cell countprovide information in diagnosing and monitoring of diseases such as anemia and HIV/AIDS. For example, CD4 cell counting is used to monitor the progression of HIV/AIDS. Cell-based POC testing is often needed for disease diagnosis and hematological analysis.

1.3.3 Nucleic acids

Clinical diagnoses can be made based on the analysis of DNA or RNA sequences. Nucleic acid detection and analysis can identify the type of infection or pathogen and disease. It can be used in prenatal diagnosis of inherited disorders, clinical disease diagnosis (genetic disease, infection, disease staging, drug resistance mutation, and pathogen presence/abundance), and forensic investigations Nucleic acid testing (NAT) offers detection that is highly sensitive (due to amplification) and specific (due to specific base pairing of complementary nucleotides).

Currently available systems are primarily used in hospitals and centralized laboratories with complex operation steps and high-cost instruments (Safavieh et al. 2012) . In order to achieve NAT for POC diagnosis, a fully integrated system is preferable; for instance, to avoid contamination issues, reduce worker steps, and deliver rapid results.

Table 1.1: Classes of assays for POC testing (adapted from: (Chin et al. 2012)

			Availability of POC products	
Class of assays	**Examples**	**Method of detection**	**Qualitative**	**Quantitative**
Chemical	Glucose, HbA1c	Direct detection	Widespread	Widespread
Immunoassays	Troponin, PSA	Signal amplification	Widespread	Limited
Nucleic acid testing	HIV viral load	Target amplification followed by signal amplification	Limited	Limited

1.3.4 Small molecules

Small molecules from body fluids are used to monitor health parameters in disease prevention. The ranges of electrolytes (Na^+, K^+, Cl^-, Ca^{2+}), general chemistries (pH, urea, glucose), blood gases (pCO2, pO2), and hematology (hematocrit) are analyzed (Chin et al. 2012). Currently, methods are based on electrochemical detection such as potentiometry, amperometry, optical sensing (Safavieh et al. 2012) and conductance (Chin et al. 2007).

1.4 Technologies used for POCT

Many methods (Table 1.1) are already established for the identification of pathogens but not all of them are suitable for POC testing. For example, ELISA (Enzyme Linked Immuno-Sorbent Assay) is a standard assay for pathogen detection in the laboratory. However, it is a multi-stepped assay that is less sensitive than other assays. This method requires an ELISA reader (Fig. 1.2) to analyze the result, which is large as well as expensive. Additionally ELISA is not well suited for use outside the sophisticated climate-controlled laboratory manned with highly trained personnel.

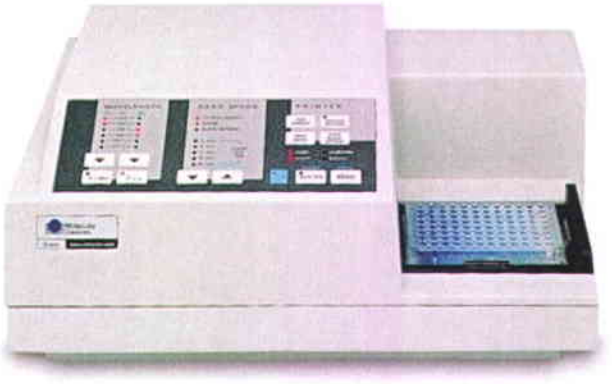

Fig. 1.2: ELISA Reader
(Source: http://www.moleculardevices.com/Products/Instruments/Microplate-Readers)

Cell culture is another standard method for the detection of bacterial or viral pathogens. To perform this method specific laboratory setup, bio safety cabinet, hepa filter (Fig. 1.3), and trained personnel are needed. Although reliable this technique takes 3-7 days to obtain results. Additionally, since live cultures of pathogens are required, biosafety rules must be strictly maintained according to their safety level. For this reason, this method is not suitable for limited resource settings such as remote clinics as well as for POC application. Therefore, a real need exists for more rapid, sensitive and specific diagnostic technologies for infectious disease to replace the time-consuming and limited culture methods (Yageret al. 2008).

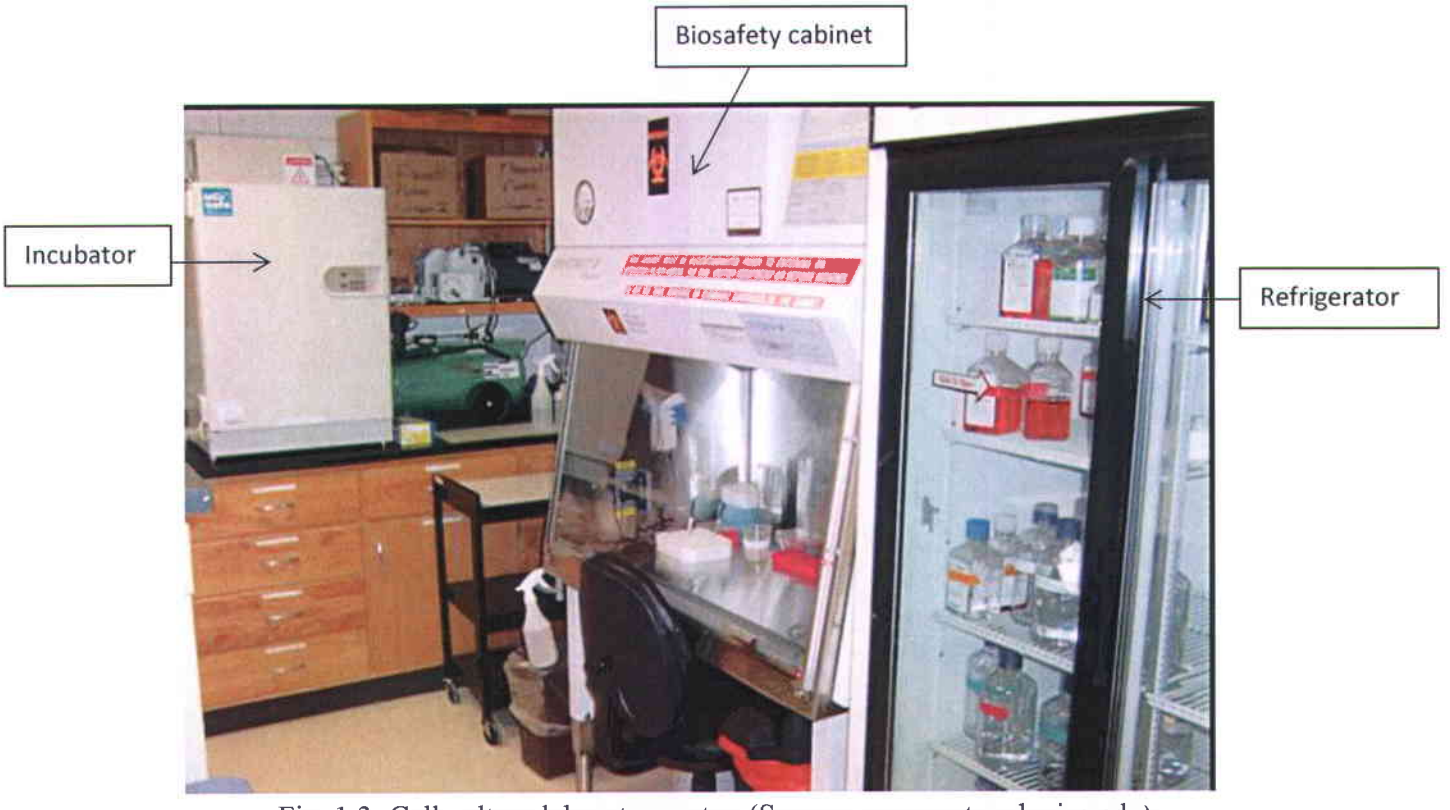

Fig. 1.3: Cell culture laboratory setup (Source: www.vetmed.wisc.edu)

A major class of POC diagnostic tests is the lateral flow test. In this test, a membrane or paper strip is used to indicate the presence of protein markers such as pathogen antigens or host antibodies. On a membrane, the addition of the sample induces a capillary action. The sample flows across the membrane, reacts with reagents that are pre-embedded in the membrane, and flows over an area that contains captured molecules. The labeled captured analytes form a visible band on the membrane which is detected by eye. (Chin et al. 2012). Lateral flow tests are used for the diagnosis of pregnancy as well as infections with streptococcus or flu or to diagnose HIV. Although the test is simple to perform, the single-flow action does not mimic the multi-step procedures of laboratory-based assays that are crucial for producing highly reproducible, quantitative, and sensitive results. Blood glucose analysis is another major class of successful POC tests. This test is also performed on membranes but is distinct from lateral flow immunoassays. It uses signal amplification by a redox enzyme, typically ending in an electrochemical readout (Price et al. 2010).

Many POC test systems are devised as easy-to-use membrane-based test strips, often enclosed by a plastic test cassette. This concept is often realized in test systems for detecting pathogens.

Recently such test systems for rheumatology diagnostics have been developed (Zhang 2004). These tests require only a single drop of whole blood, urine or saliva, and they can be performed and interpreted by any general physician within minutes.

For nucleic acid amplification-based testing, polymerase chain reaction (PCR) was the first technology to be used in POC devices. However, PCR requires an expensive thermal cycler and relatively sensitive optics for real-time detection. The use of a thermal cycler and sensitive optics are not ideally suited for POC devices where the goal of POC is not only shorten the test time, but also to reduce the cost (Steel et al. 1999). Accordingly, isothermal techniques for DNA/RNA amplification represent a more promising option for nucleic acid amplification-based testing (Ho et al. 1987, Rodriguez and Bard 1990, Maruyama et al. 2002, Asiello and Baeumner 2011, Nagatani et al. 2011).

The commonly used isothermal technologies are NASBA, RCA, HDA, LAMP. Among these, LAMP has become a preferred technique for diagnosis of infectious diseases at the POC due to its rapidity, low equipment cost and robustness to inhibitors present in the clinical sample (Notomi et al. 2000, Defever et al. 2011). The LAMP assay can also be monitored in real-time (Ho et al. 1987, Ahmad et al. 2011, Ahmed et al. 2013) for quantification, and can be used to differentiate single nucleotide polymorphisms (Ikeda et al. 2007). Human health related applications that would benefit from quantitative and multiplexed POC genetic testing include measuring viral load with HIV (Shen et al. 2011), differentiation of point mutations for multiple drug resistance tuberculosis (Lee et al. 2010), or measurement of microRNA panels for diagnosing cancer (Li et al. 2011). In general, genetic testing is aimed at detecting the presence or absence of genetic markers such as pathogen-specific virulence genes, antibiotic resistance genes, or disease-specific mutations. Sequencing, the most desirable genetic analysis, is not yet available using low-cost POC devices.

To incorporate nucleic acid (NA) testing into the POC device, several formats can be employed to effect sequence-specific detection as shown in Fig. 1.4.

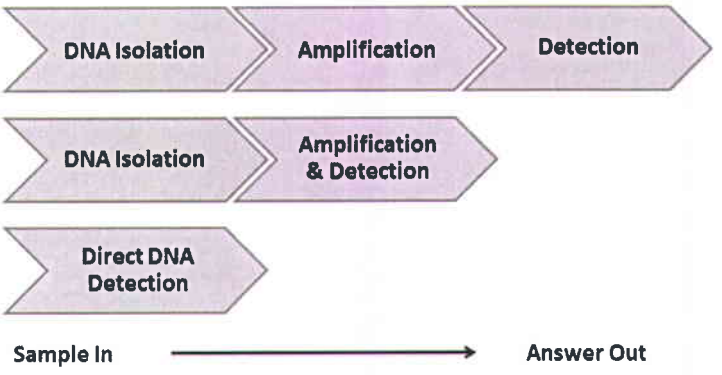

Fig. 1.4: Nucleic acid testing formats (Craw and Balachandran 2012)

For the amplification of NAs, isolation or purification of it from a clinical sample is an essential step. This is because, salt or sugar which present in clinical sample inhibit the amplification reaction. Firstly, NAs isolated from a clinical sample, then amplification followed by detection of the amplified product. For example, PCR, where NA (DNA/RNA) isolated from the clinical sample, then amplified in the thermo cycler, and then amplified product analyzed. Such methods have been well characterized, are extensively used and are widely applied across genetic determination and infectious diseases. In other format, NAs are isolated and then amplification and detection steps are performed together. Even sometimes amplification and detection steps are carried out simultaneously without prior NAs isolation. These simplified tests therefore provide a robust format for the development of further NATs. With regard to POC applications, it is essential to simplify the assay procedure and avoid the multiple procedural steps for amplification and separate detection and thereby reduces the time-to-result (Craw and Balachandran 2012).

1.4.1 Nucleic acid testing (NAT)

NAT is performed to identify specific nucleic acid sequences from clinical samples. The presence of specific NA sequences indicates the presence of infection or genetic disease, progression and prognosis or, in the case of genomic medicine, suitability for a tailored therapy. In this section, several NAT methods, such as PCR, NASBA, RCA, HDA, LAMP are described.

1.4.2 Polymerase chain reaction (PCR)

PCR is a primer-mediated enzymatic amplification of specifically cloned or genomic DNA sequences. The main purpose of the PCR is to amplify template DNA using thermo stable DNA polymerase enzyme. This enzyme catalyzes the buffered reaction in which an excess of a template DNA (oligonucleotide primer pair) and four building blocks (deoxynucleoside triphosphates ordNTPs) are used to make millions of copies of the target sequence.

Fig. 1.5: Image of a conventional PCR machine
(Source: http://biology.clc.uc.edu/fankhauser/labs/genetics/pcr/pcr_protocol.htm)

The PCR requires a repetitive series of the three fundamental steps that defines one PCR cycle. DNA amplification by the PCR is schematically outlined in Fig 1.6. There are three general steps to the process that are repeated for a number of cycles to exponentially increase the number of copies of a specific target region. The whole process is carried out in a thermo cycler (Fig. 1.5) which controls time and temperature according to the command. Genomic DNA is normally double-stranded (DS-DNA) (Kolmodin, Williams et al. 2000).

STEP 1 is to first unzip the DS-DNA, also denoted **denaturation**, into two complementary single strands of DNA by heating the reaction mix to 95 °C.

STEP 2 isolates the target region of the genomic DNA by the addition of two primers (P1 & P2), which exactly match two 20-30 unique base pair regions that flank the target region. This is known as **annealing**.

STEP 3 is initiated once time is allowed for the primers to bind to the DNA and involves heating the mix to 72-75 °C at which point a special polymerase builds the DNA strand starting at the primers and continuing in the 5′ direction. This is called **extension**. These three steps are repeated 25-40 times to produce millions of exact copies of the target region of DNA. During the second cycle of this process, extension can occur on both the original copy of genomic DNA and the newest pieces (the colored ones in the Fig. 1.6), thus subsequent extensions are quickly limited precisely to the target region **(A)**.

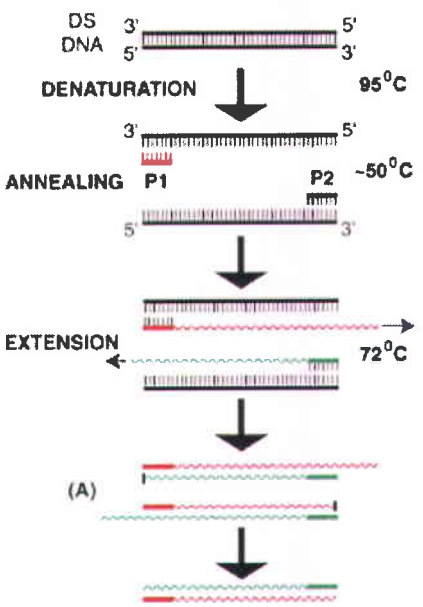

Fig. 1.6: Schematic outline of PCR steps (Source: www.flmnh.ufl.edu/cowries/amplify.html)

1.4.3 Isothermal amplifications

Novel developments in molecular biology of DNA synthesis *in vivo* demonstrate the possibility of amplifying DNA in isothermal temperature. Unlike PCR, isothermal amplification methods do not require a thermocycling machine to separate the two DNA strands and then to amplify the required fragment. DNA polymerase replicates DNA with various accessory proteins. Therefore, with identification of these proteins, we are able to develop new *in vitro* isothermal DNA amplification methods by mimicking these *in vivo* mechanisms. Though there are several

isothermal nucleic acid amplifications, in this section I will discuss the commonly used isothermal methods, such as NASBA, RCA, HDA and LAMP.

1.4.3.1 Nucleic acid sequence -amplification (NASBA)

NASBA is a method in molecular biology which is used to amplify RNA sequences. The NASBA technique has been used to develop rapid diagnostic tests for several pathogenic viruses with single-stranded RNA genomes, *e.g.* influenza A (Collins et al. 2002), foot-and-mouth disease virus (Collins et al. 2002), severe acute respiratory syndrome (SARS)-associated coronavirus (Keightley et al. 2005), Human bocavirus (HBoV) (Bohmer et al. 2009) and also parasites like *Trypanosoma brucei* (Mugasa et al. 2009). NASBA has been introduced into medical diagnostics, where it has been reported to provide more rapid results than PCR, and it can also be more sensitive.

NASBA's main advantage is that it functions at isothermal conditions - usually at a constant temperature of 41 °C. NASBA technology is based on the concerted action of three enzymes (Fig. 1.7):

- AMV Reverse Transcriptase: for cDNA synthesis
- RNase H: for degradation of the RNA in the heteroduplex RNA-DNA
- T7 RNA polymerase: for synthesis of RNA from the T7 promotor

NASBA works as follows:

1. RNA template is added to the reaction mixture and then the first primer attaches to its complementary site at the 3' end of the template.
2. Reverse transcriptase synthesizes the opposite, complementary DNA strand.
3. RNAse H destroys the RNA template from the DNA-RNA compound (RNAse H only destroys RNA in RNA-DNA hybrids, but not single-stranded RNA).
4. The second primer attaches to the 5' end of the DNA strand.
5. Reverse transcriptase again synthesizes another DNA strand from the attached primer resulting in double stranded DNA.

6. T7 RNA polymerase continuously produces complementary RNA strands of this template, which results in amplification. The amplicons (newly produced complementary RNA), however, are antisense to the original RNA template.
7. A cycle can now begin, starting with the RNA strands from the previous step, the second primer and reverse transcriptase and repeating the mechanisms of steps 1–6.

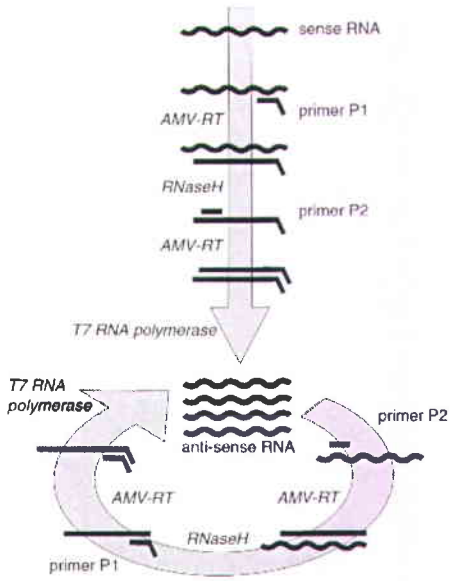

Fig. 1.7: Schematic diagram of NASBA
(Source:http://nar.oxfordjournals.org/content/30/6/e26/F2.large.jpg)

1.4.3.2 Rolling circle amplification (RCA)

RCA is another isothermal, enzymatic process mediated by certain DNA polymerases in which long single-stranded (ss) DNA molecules are synthesized on a short circular ssDNA template by using a single DNA primer. This is a process of unidirectional nucleic acid replication that can rapidly synthesize multiple copies of circular molecules of DNA or RNA. This replication process involves continual synthesis of a polynucleotide which is 'rolled off' of a circular template molecule (Fig. 1.8).

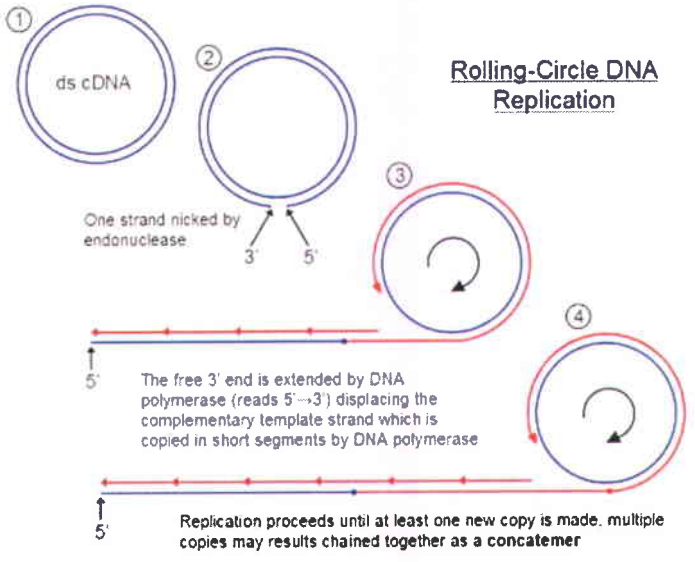

Fig. 1.8: Schematic diagram of RCA
(Source: http://cronodon.com/images/Rolling_circle.jpg)

The RCA method, traditionally used for ultrasensitive DNA detection in areas of genomics and diagnostics, has been used more recently to generate large-scale DNA templates for the creation of periodic nanoassemblies. Various RCA strategies have also been developed for the production of repetitive sequences of DNA aptamers and DNA enzymes as detection platforms for small molecules and proteins. Accordingly, RCA is rapidly becoming a highly versatile DNA amplification tool with wide-ranging applications in genomics, proteomics, diagnosis, biosensing, drug discovery, and nanotechnology (Zhao et al. 2008).

RCA uses specific DNA polymerases and primers to allow a circular piece of DNA to be replicated continuously until all reagents are exhausted. The overall reaction takes very little time compared to other methods and does not require NA purification or centrifugation. The results are easily analyzed, some cases, as little as 10 minutes (Alsmadi et al. 2003).

1.4.3.3 Helicase-dependent amplification (HDA)

Helicase-Dependent Amplification (HDA) is an isothermal amplification method of nucleic acids. Like PCR, the HDA reaction selectively amplifies a target sequence defined by two primers. However, unlike PCR, HDA uses an enzyme called a helicase to separate DNA, rather than heat (Fig. 1.9). This allows DNA amplification without the need for thermo cycling. Thus,

two types of enzymes are required to complete the reaction: a helicase (to separate the ds-DNA strand) and a DNA polymerase (to precede the reaction). Like in PCR method, DNA polymerase can be is inhibited by elements present in the clinical sample, so the NA needs to be purified before the reaction. The HDA reaction can also be coupled with reverse transcription for RNA analysis.

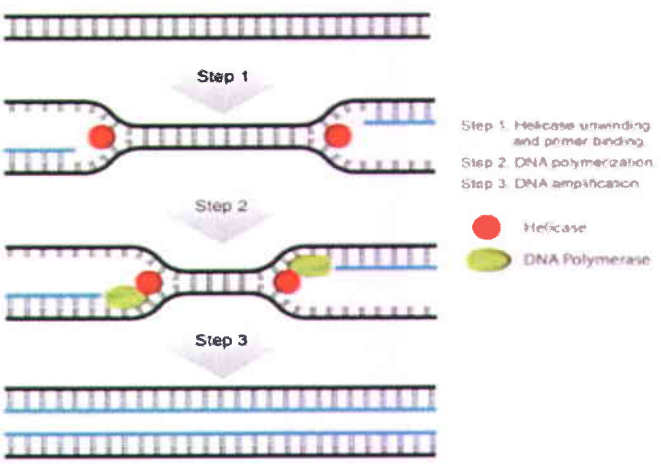

Fig.1.9: HDA Technology
(Source: https://www.neb.com/products/h0110-isoamp-ii-universal-thda-kit)

1.4.3.4 Loop-mediated isothermal amplification (LAMP)

LAMP is a simple, rapid, specific and cost-effective isothermal nucleic acid (DNA or RNA) amplification technique developed by Notomi and colleagues over a decade ago (Notomi et al. 2000). In this technology, four different specifically designed primers are used to recognize six distinct regions on the target gene. Amplification and detection of the gene can be completed in a single step, by incubating the mixture of samples, primers, DNA polymerase with strand displacement activity and the reaction can proceed at a constant temperature. It provides high amplification efficiency (10^9-10^{10} times in 15-60 minutes). Thus, the presence of the amplified product can indicate the presence of the target gene. Thus this method may be of use in the future as a low cost alternative to detect diseases. The detailed of the LAMP method is described in the Chapter 2.

1.5 Conclusion

POCT minimizes the gap between centralized laboratory diagnostics and rural healthcare service providers. Particularly in infectious diseases such as HIV/AIDS and Tuberculosis (TB), where early detection is imperative to improve disease outcome, the development of an accurate test that is simple, rapid and robust can significantly alter the epidemiology and control of the disease. An effective POCT, however, can only serve its full potential when adopted in a comprehensive programmatic context linking patients to on-site case management. Immunochromatographic lateral flow devices for detection of antibody or antigen currently dominate available POCTs, and the development of such devices has relied on the discovery and optimization of definitive biomarkers suitable for such platforms. In the future, however, there will be an increasing need to develop cost-effective POCTs that address biomarkers that are well established in laboratory settings but are not currently amenable to POCT, such as molecular tests for drug resistance in TB and viral load in HIV and viral hepatitis (Mohd Hanafiah et al. 2013).

There is no doubt that the need for POC diagnostics is crucial in developing countries and low-resource setting laboratories/hospitals in the developed countries. For example, a high-throughput, automated robotic processing instrument is generally not affordable or feasible in low-resource settings that lack the necessary laboratory infrastructure (Yager et al. 2008)

Chapter 2

Loop-mediated isothermal amplification (LAMP) for point-of-care diagnostics

LAMP technology is a robust method to identify disease within very short time. The overall cost for this method is very low compared to other existing technologies. With this motivation I used the LAMP method in my MSc thesis work for the detection of Herpes Simplex Viruses.

2.1 Objective of my thesis

To develop a low-cost tool for the detection of Herpes Simplex Viruses suitable for low resource countries.

2.2 Introduction

Herpes simplex virus (HSV) infection is a major problem in both the industrialized and developing worlds. HSV has been characterized into two different serotypes: HSV type 1 (HSV-1, named as oral herpes) is generally associated with infections in the tongue, mouth, lips, pharynx, and eyes, whereas HSV type 2 (HSV-2, named as genital herpes) is primarily associated with genital and neonatal infections (Aurelian 1992). Both HSV-1 and HSV-2 establish lifelong latency in human sensory neuronal ganglia, and subsequently reactivate. Following reactivation, each of these herpes viruses may cause significant clinical symptoms in the individual and may spread to uninfected persons. The virus can also be passed from mother to child during birth. Neonatal infection) can be very serious (Pinninti and Kimberlin 2013). Without treatment, 80% of infants with HSV infection die, and those who do survive are often carry physical damage throughout their life (Brown 2004). In one study in the United States of America (USA), four of nine infants born to women who acquired genital herpes shortly before labour developed neonatal infection, died (Brown et al. 1997). Thus the early diagnosis of the virus is important for the determination of clinical management and for an understanding of the clinical progress and prognosis.

There are many established methods for the identification of both HSV1 and HSV2 viruses. Among them, cell culture (Singh et al. 2005) and serological assays (Wald and Ashley-Morrow 2002) are standard methods of HSV diagnosis. These methods, however, require substantial time to obtain accurate final results. PCR is a highly sensitive and gold standard method for the detection of HSV DNA (Johnson et al. 2000, Gardella et al. 2010) compared to antigenic detection or cell culture methods (Koenig et al. 2001). Bedside monitoring of HSV infection, its progression could also be monitored by the real-time PCR quantitative analysis of viral DNA (Enomoto et al. 2005). This method however, has not yet become a common procedure in hospital laboratories and low-resource settings and is also not suitable for POC applications due to the requirement of specific expensive equipment (a thermal cycler), dedicated laboratory space, and specially trained technicians.

In 2000, Notomi and his colleagues reported a novel nucleic acid amplification method, he denoted as loop-mediated isothermal amplification (LAMP). In this method DNA is amplified under isothermal conditions (between 63 °C and 65 °C), which requires only simple and cost-effective equipment (*e.g.,* a heating block) suitable for use in limited resort setting areas. The technique also exhibits both high specificity and high amplification efficiency. The LAMP method uses four primers which recognize six distinct target DNA sequences, yields extremely high specificity. This method does not need to denature double strand DNA to a single strand, which is a crucial step in PCR, because the *Bst* DNA polymerase enzyme has strand displacement capacity. In contrast to PCR, there is no time lost due to temperature changes in each step. Therefore, the entire method can be conducted in a short time period (30-60 minutes). LAMP also exhibits extremely high amplification efficiency compared to PCR. As the reaction can be conducted at the optimal temperature for enzyme function, the inhibition reactions that often occur at later stages of typical PCR amplifications are less likely to occur (Enomotoet al. 2005). The LAMP method could potentially be a valuable tool for the rapid diagnosis of HSV (Reddy et al. 2011) as well as other infectious diseases in both commercial and hospital laboratories (Notomi et al. 2000).

Plastic/paper-based microfluidic devices possess many of the desired characteristics of a suitable POC viral DNA test (Fu et al. 2011, Pollock et al. 2012). These diagnostic devices are inexpensive, portable, and simple to operate, making them appropriate for low-resource settings

(Martinez et al. 2010). We developed a simple plastic pouch for the detection of both HSV-1 and HSV-2. In this device we could detect viral DNA within 45 minutes using the LAMP method. Since the LAMP reaction is less sensitive to inhibitory substances present in the clinical sample (Enomoto et al. 2005, Kaneko et al. 2005, Defever et al. 2011), this allowed us to detect viral DNA without purifying it. The final result was evaluated by the naked eye via the addition of hydroxynaphthol blue (HNB) dye in the reaction mix (Goto et al. 2009, Das et al. 2012). HNB is a metal indicator for magnesium and a colorimetric reagent for alkaline earth metal ions, which is usually purple color. With the LAMP reaction, magnesium pyrophosphate is produced as by-product, thus the amount of free magnesium ion (Mg^{+2}) is reduced in the assay mixture. As the magnesium is reduced, due to the change of P^H, the color of HNB dye changed from purple to light blue. Thus, positive reaction is indicated by a color change from purple to sky blue. The results of our colorimetric assay were further confirmed by 2% agarose gel electrophoresis.

2.3 LAMP primers:

There are four types of primers based on the six distinct regions of the target gene. The primers are denoted Forward Inner Primer (FIP), Forward Outer Primer (F3), Backward Inner Primer (BIP) and Backward Outer Primer (B3)(Notomi et al. 2000). The positions of the primers are shown in the Fig. 2.1.

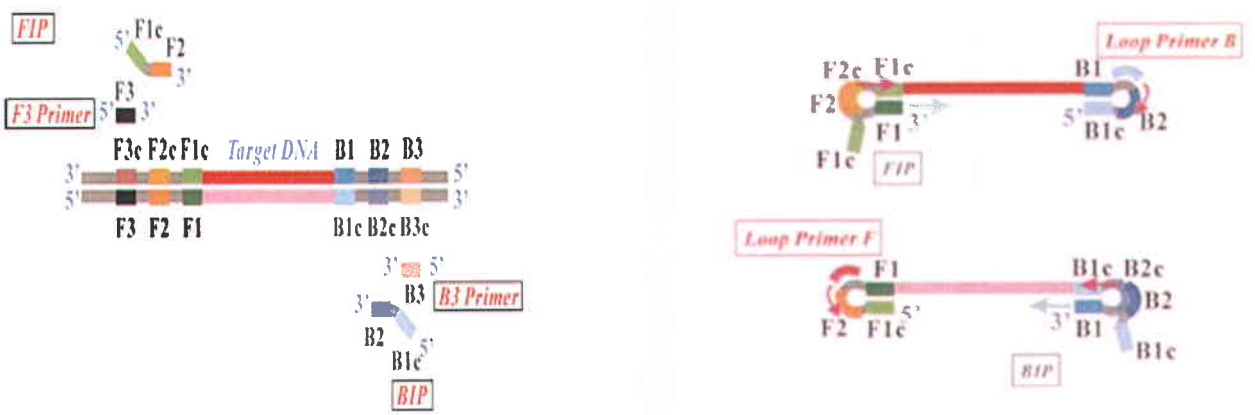

Fig. 2.1: Schematic diagram showing the position of LAMP primers in target DNA (Notomi et al. 2000)

FIP consists of the F1c region at the 5' end, which is complementary to the F1 region, and the F2 region (at the 3' end) that is complementary to the F2c region of the target DNA.

F3 Primer consists of the F3 region that is complementary to the F3c region.

BIP consists of the B2 region at the 3' end that is complementary to the B2c region, and the B1c region that is complementary to the sequence of B1 region at the 5' end.

B3 Primer consists of only B3 region that is complementary to the B3c region.

There are two other primers named **Loop Primer Forward (LPF)** and **Loop Primer Backward (LPB)** containing sequences complementary to the single-stranded loop region. These primers are used to increase the amplification rate.

2.4 Principle of LAMP

When the DNA template (the target gene) are incubated together with primers, DNA polymerase enzyme and other reagents at a constant temperature, the reaction proceed as the following steps: (see Fig. 2.2)

Step 1: FIP primers anneal to the complimentary sequence of double stranded target DNA (light pink), and then initiates DNA synthesis using the *Bst* DNA polymerase with strand displacement activity which displaces and releases a single stranded DNA. With the LAMP method, in contrast to PCR, there is no need for heat denaturation of the double stranded DNA into a single strand (Figure 2.2).

Step 2: FIP is used to produce the complementary strand of the template DNA starting from the 3' end of the F2 region of the FIP.

Step 3: The F3 Primer anneals to the F3c region, outside of FIP on the target DNA and initiates strand displacement DNA synthesis by which the FIP-linked complementary strand is released.

Step 4: A double strand is formed from the DNA strand synthesized from the F3 Primer and the template DNA strand.

Step 5: The FIP-linked complementary strand is released as a single strand because of the displacement by the DNA strand synthesized from the F3 Primer.

This released single strand forms a stem-loop structure at the 5' end because of the complementary of the F1c and F1 regions.

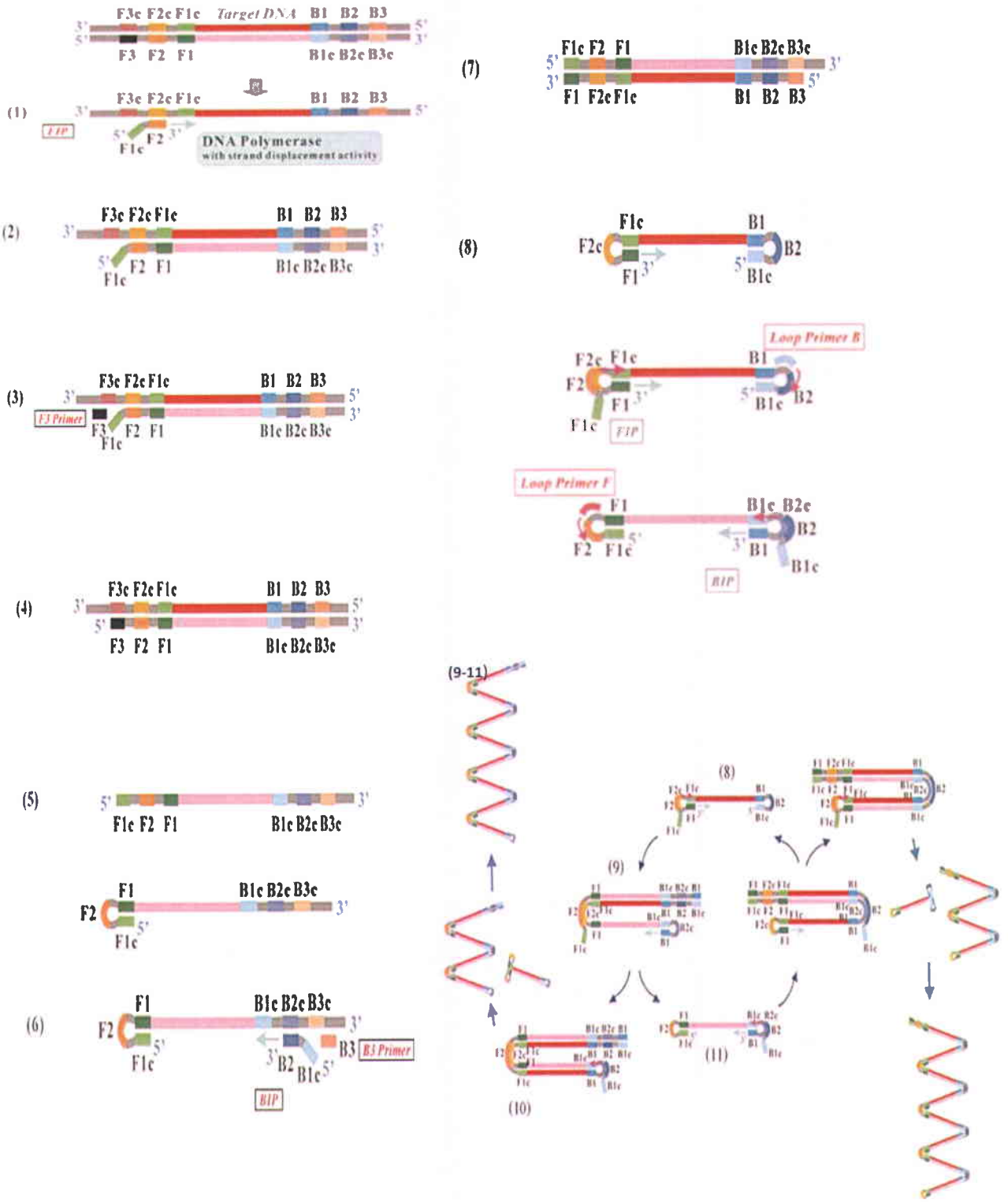

Fig. 2.2: Steps (1-11) of LAMP method to produce amplified product

Step 6: In this step BIP initiates DNA synthesis, this newly synthesized DNA strands are separated by B3 primer. Starting from the 3' end, BIP anneals to the DNA strand produced in Step (5), so that synthesis of complementary DNA takes place. As DNA synthesis proceeds, the DNA reverts from a loop structure into a linear structure. The B3 Primer anneals to the outside of the BIP and then, through the action activity of the *Bst* DNA polymerase and starting at the 3' end, the DNA synthesized from the BIP is displaced and released as a single strand before DNA synthesis from the B3 primer.

***Step 7*:** B3-primed strand displacement DNA synthesis and the DNA reverts from a loop structure into a linear structure. Double stranded linear DNA is the product at the end of this step.

Step 8: At each end complementary sequences at the same strand (*e.g.* F1, F1c and B1, B1c) forms a structure with stem-loops, which looks like a dumbbell structure

The Loop Primers containing sequences complementary to the single stranded loop region (either between the B1 and B2 regions, or between the F1 and F2 regions) on the 5' end of the dumbbell-like structure, provide an increased number of starting points for DNA synthesis for the LAMP method.

Step 9-11: After forming a dumbbell-like structure, the amplification cycle in the LAMP method begins. At this step, Loop Primer Forward (LPF) and Loop Primer Backward (LPB) bind with their complementary sequences (F2c and B2 region respectively) and primed the amplification.

At each cycle of amplification, the number and the size of the existing products increased.Thus, at the end of the reaction the products can be resolved by agarose gel electrophoresis as a ladder like band instead of single band (Fig. 2.3)

Viewing the animation of the principle of LAMP will assist the understanding of the amplification procedure. (Animation link: http://loopamp.eiken.co.jp/e/lamp/anim.html)

Fig. 2.3: Analysis of the LAMP product by gel electrophoresis. The amplification reaction was performed at 65 °C for 40 minutes. The products were resolved on a 2% agarose gel and visualized with ethidium bromide after the exposure to the UV light. Each lane shows a ladder-like band due to the combination of various sized dumbbell-shaped DNA molecule. M= 2KB marker, 1= negative control (Water), 2-6= positive control.

2.5 Materials and methods

Before developing the amplification device, the LAMP protocol was first optimized using in conventional 0.2 ml PCR tubes to amplify HSV DNA. The final result was analyzed by the color change of HNB dye from purple to light blue and further confirmed by 2% agarose gel electrophoresis.

2.5.1 Cells and Viruses

African green monkey kidney (Vero) cells were maintained in DMEM, supplemented with 5% newborn calf serum, 0.5U/ml of penicillin and 0.5ug/ml of streptomycin at 37°C and 5% CO_2. The virus strain KOS (HSV-1) (Jacobson et al. 1998) and strain HG52 (HSV-2) were used. Vero cells were infected at multiplicity of infection (MOI) of 0.01 with either HSV-1 or HSV-2 and after 3 days virus were harvested and used or DNA extraction was performed with phenol/chloroform.

2.5.2 LAMP amplification

The amplification reaction was carried out using the previously described LAMP primers for Herpes Simplex Virus type 1 (Kaneko et al. 2005) and Herpes Simplex Virus type 2 (Enomoto et al. 2005). Details of LAMP primers used in this study are listed in Table 2.1. The LAMP reaction was carried out in a total 25 µL reaction mixture containing 0.2 mM of each of the outer primers (F3/B3), 0.8 mM of each of the loop primers (LF/LB) 1.6 mM of each of the inner primers (FIP/BIP). 0.4 mM dNTPs, 0.64 M Betaine (Sigma), 3 mM $MgSO_4$, *Bst* DNA polymerase, large Fragment, 1,600 units, (8,000U/ml) (New England Biolabs), 1X ThermoPol reaction polymerase buffer (New England Biolabs Inc.) and 5 µL of double-stranded target DNA. Hydroxynapthanol blue 0.15 µL/mL was also added to the reaction mixture to visualize the amplification of HSV DNA. Mineral oil (20 µL) was added to each tube to avoid evaporation. The mixture was incubated at 65°C for 45 minutes on a heat block.

Table 2.1: Primers used for LAMP and their location in the gene

Name of primers	Sequence of primers	Name of gene	Location of target sequences
Set A:			Nucleotide position (9721-10080)
HSV1-F3	5_-CAGCCACACACCTGTGAA-3_(F3)		F3(9756-9773)
HSV1-B3	5_-TCCGTCGAGGCATCGTTAG-3_ (B3c)		B3(10008-10026)
HSV1-FIP	5_-CAGACGTTCCGTTGGTAGGTCACTTTGACTATTCGCGCACC-3_(F1c-F2)		F2(9788-9807)
HSV1-BIP	5_-CCATCATCGCCACGTCGGACTCGGCGTCTGCTTTTTGTG-3_ (B1-B2c)	UL1	B2(9969-9987)
HSV1-LPF	5_-AAATCCTGTCGCCCTACACAGCGG-3_ (LPFc)		F1(9836-9857)
HSV1-LPB	5_-CACCCCGCGACGGGACGCCG-3_ (LPB)		B1(9926-9945)
			LPF(9810-9833)
			LPB(9948-9967)
Set B:			Nucleotide position (138901-139140)
HSV2-F3	5_-GGCCTTGACCGAGGACAC-3_ (F3)		F3(138909-138926)
HSV2-B3	5_-CGACTCCACGGATGCAGT-3_ (B3c)		B3(139120-139137)
HSV2-FIP	5_-TCGACTGAGGGTGCCATGGCGTCCTCCGATTCGCCTACG-3_ (F1c-F2)		F2(138926-138945)
HSV2-BIP	5_-GCAACCACTACTCCCCCCGACCGTTTCCTCCGGCGTAA-3_ (B1-B2c)		B2(139082-139099)
HSV2-LPF	5_-GCCGACACAGGGAGGGGCGT-3_ (LPFc)	UL5	F1(138984-139004)
HSV2-LPB	5_-GATGGCCACACAAGCCGCAA-3_ (LPB)		B1(139030-139050)
			LPF(138961-138980)
			LPB(139053-139072)

2.5.3 Plastic pouch fabrication and operation

For the development of the device, a conventional reseal-able plastic bag was used (Fig. 2.4). A small plastic chamber (1.5 x 0.2 cm) was fabricated by pressing using a plastic sealer. This small chamber can hold 25 µL of reaction mixture including 5 µL of sample. A small plastic spacer was placed into the bottom of the chamber, so that the liquid can easily full and retained by the

chamber. The reaction mixture and the sample were inserted using the long-edged loading tips and, after loading, the bag was sealed. The sealed bag was kept on a heat block at 65 °C for 45 minutes to amplify the DNA. Hydroxy naphthol blue (HNB), a metal indicator for magnesium and a colorimetric reagent for alkaline earth metal ions, was added to the reaction mixture. A positive reaction is indicated by a color change from purple to light blue. After completion of the reaction, the result was analysed visually without any instrument. The visual analysis was further confirmed by 2% agarose gel electrophoresis. The pouch was punched and the reaction product was collected and loaded to agarose gel

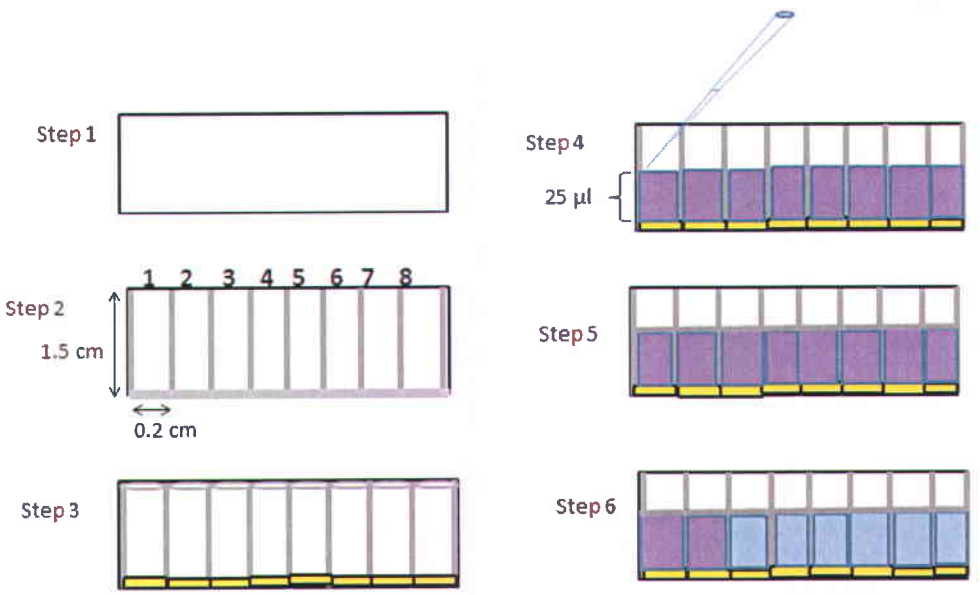

Fig. 2.4: Schematic diagram of the fabrication steps of the reaction pouch made by a plastic bag Step 1: A piece of double layered plastic bag cut in a rectangular shape; Step2: Pressed with sealer to make small chambers (L: 1.5 cm and W: 0.2 cm); Step 3: Small pieces of plastic were inserted at the bottom of each chamber which functions as spacers to ensure that the liquid inside the chamber; Step 4: Reagents and sample inserted with long-edge tips; Step 5: Pouch was sealed horizontally with sealer; Step 6: The pack was placed on the heat block for 45 min at 65°C; positive reactions turn to blue from purple.

The following figure (Fig. 2.5) is a photograph. The positive yielded a blue color after they were incubated on a heat block for 45 minutes at 65°C; negative samples purple color.

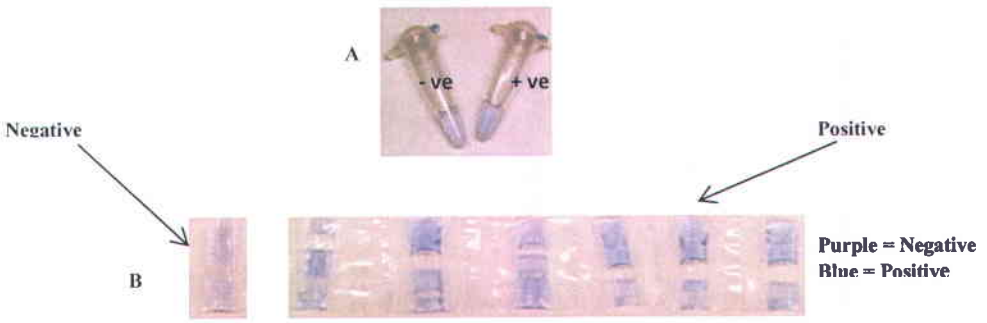

Fig. 2.5: A photograph of the reaction in an Eppendorf tube (A) or plastic (B). The purple color changed to blue in a positive reaction.

This qualitative and colorimetric LAMP assay integrated in this very simple and low cost plastic pouch has potential usefulness for the rapid diagnostic of HSV in remote clinics, field site laboratories, hospital laboratories, and also in low-resource countries.

2.5.4 LAMP in real samples

Previous reports have indicated that the LAMP assay's sensitivity is less affected iby the presence of inhibitory substances in clinical samples than is PCR (Enomoto et al. 2005, Kaneko et al. 2005, Defever et al. 2011). To evaluate these advantages, we analyzed the tolerance of lLAMP against a real virus. Viral stocks were diluted in phosphate buffered saline (PBS), distilled water, and culture medium (Dulbecco's Modified Eagle's medium or DMEM). The concentration of viral stock was 1.20×10^4 PFU/μL. We compared the results with purified DNA.

To assess the detection limit of the LAMP reaction in a real sample, virus stock was serially diluted 10-fold with PBS. The original virus stock contained 1×10^4 PFU/μL of HSV-1 and HSV-2 . The diluted viral fluids were used directly. Five microliters of each sample were added to the reaction mixture to a final volume of 25 μL, and the reaction was incubated at 65 °C for 1 h.

2.6 Results

2.6.1 Optimization of LAMP in tubes

The time, temperature, and dNTP concentrations were optimized for the LAMP assay. We performed the LAMP assay at three different temperatures (60 °C, 63 °C and 66 °C) for both HSV-1 and HSV-2 DNA. We observed that HSV-1 DNA was amplified at each of the three temperatures, whereas HSV-2 DNA was amplified at 63-66°C (Table 2.2).

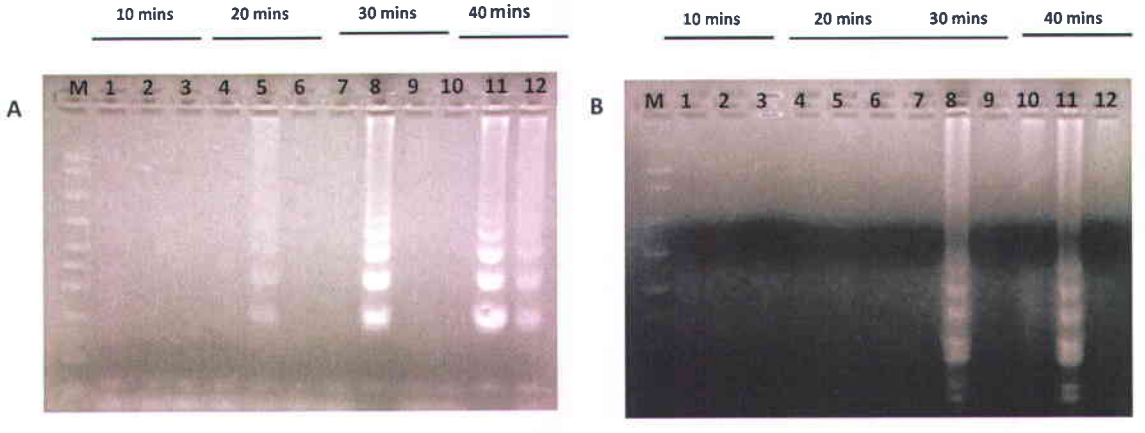

Fig. 2.6: Agarose gel analysis of time optimization of LAMP assay using HSV DNA. The assay was performed at four different times (10 mins, 20 mins, 30 mins and 40 mins).Water was used as negative control (NC) and 10 ng/µL of DNA used as positive control. **(A)** Assay using HSV-1 DNA. The lowest detection limit was observed 10 fg/µl of DNA for HSV-1 when we performed the sensitivity test. M=2 kb Marker, 1= NC; 2= 10 ng/µL DNA; 3= 10 fg/µL DNA; 4= NC; 5= 10 ng/µL DNA; 6= 10 fg/µL DNA; 7= NC; 8= 10 ng/µL DNA; 9= 10 fg/µL DNA; 10= NC; 11= 10 ng/µL DNA; 12= 10 fg/µL DNA. **(B)** Assay using HSV-2 DNA. The lowest detection limit was observed 1 fg/µL of DNA for HSV-2 when we performed the sensitivity test. M=2 kb Marker, 1= NC; 2= 10 ng/µL DNA; 3= 1 fg/µL DNA; 4= NC; 5= 10 ng/µL DNA; 6= 1 fg/µL DNA; 7= NC; 8= 10 ng/µL DNA; 9= 1 fg/µL DNA; 10= NC; 11= 10 ng/µL DNA; 12= 1 fg/µL DNA.

To optimize the time, the LAMP assay was carried out for 10, 20, 30, and 40 minutes at 65 °C (Fig 2.6). 10 ng/ µL of HSV DNA served as positive control and water as negative control. Another concentration of DNA was also used, which was the lowest limit for both viruses (10

fg/μL of DNA for HSV-1 and 1 fg/μL of DNA for HSV-2) and this concentration we obtained when we performed the sensitivity test (Fig. 2.7). An adequate signal for the positive control was observed at 20 minutes although a darker band was found at 30 minutes. However, the lower concentration of HSV-1 DNA (10 fg/μL) was detected at 40 minutes. In contrast the HSV-2 positive control (10 ng/μL) was visible at 30 minutes. We observed a very faint band at 40 minutes for HSV-2 DNA (1fg/μL).

2.6.2 Sensitivity and specificity of the LAMP

The sensitivity of the LAMP assay was evaluated using a series of 10-fold diluted samples of HSV DNA (10 ng/μL to 1 fg/μL) and the assay was performed at 65°C for 45 minutes. We performed the test for 45 minutes to make sure that the DNA get adequate time to amplify. This test was performed in tubes and in the plastic pouch using 5 μL of DNA at each concentration.

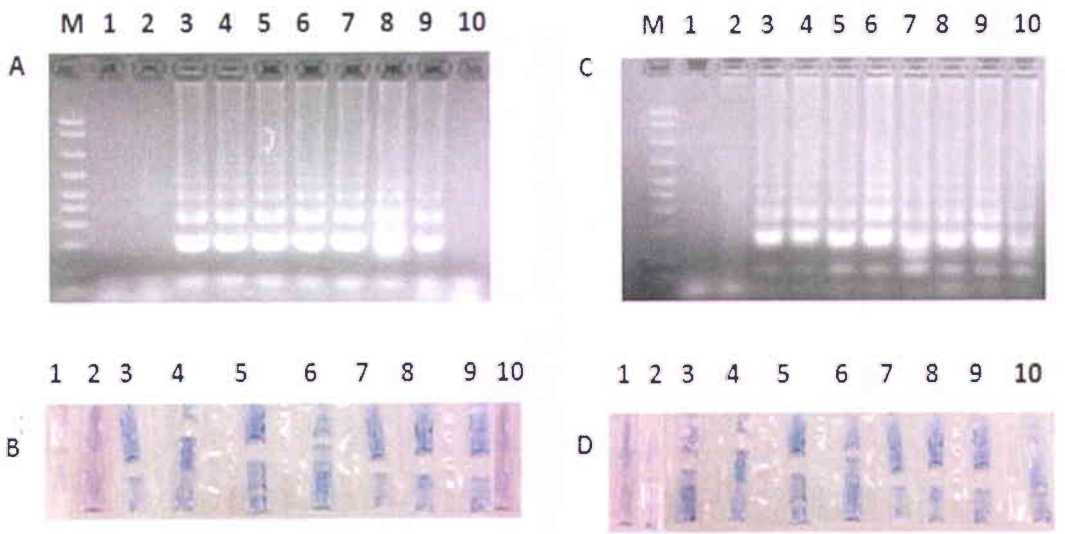

Fig. 2.7: Sensitivity of the LAMP assay for the detection of HSV DNA.
The test was performed in both tube and plastic pouch (tube images are not shown) **A:** Agarose gel electrophoretic analysis of LAMP products of HSV-1 DNA; **B:** LAMP reaction performed in plastic pouch with HSV-1 DNA, the color is due to the HNB dye. Blue indicates a positive reaction where purple indicates a negative result; **C:** Agarose gel electrophoretic analysis of LAMP products of HSV-2 DNA; **D:** LAMP reaction was performed in plastic pouch with HSV-2 DNA. In all cases M = 2kb Marker; 1 = NC (H_2O); 2 = NC (1 ng/μL *E.coli* DNA); 3 = 10 ng/μL DNA; 4 = 1ng/μL DNA; 5 = 100 pg/μL DNA; 6 = 10pg/μL DNA; 7 = 1 pg/μL DNA; 8 = 100 fg/μL DNA; 9 = 10fg/μL DNA and 10 = 1 fg/μL DNA. Total reaction volume was 25 μL including 5 μL of DNA

The LAMP products were detected by agarose gel electrophoresis and colorimetrically. The results are shown in Fig. 2.7 and summarized in Table 2.2. We observed that as little as 10 fg/μL of HSV-1 DNA could be detected in both the tube and the plastic device (Fig 2.7A, 2.7B and Table 2.2). By contrast, as low as 1 fg/μL of HSV-2 DNA was detected in both tube and plastic pouch. For negative controls both water and *E. coli* DNA were used. No band on the agarose gel (panels A and C, lanes 1 and 2) or color change (panels B and D, lanes 1 and 2) was observed in the negative controls.

2.6.3 LAMP in real samples

To verify the effect of inhibitory substances present in the real sample and to assess for the need for DNA extraction and purification, the viral stock was diluted in three different solvents: phosphate buffered saline (PBS), distilled water and culture medium (Dulbecco's Modified Eagle's medium or DMEM). The LAMP reaction was performed at 65°C for 45 min. Products were detected in eppendorf tube (Fig. 2.8 A) or on a 2% agarose gel (Fig. 2.8 B) The concentration of viral stock was 1.20×10^4 PFU/μL. We used HSV-1 (1 ng/μL) DNA as a positive control and water as negative control. A color change was observed in tubes 2-5 (panel A) and indicated that the DNA was amplified. The color did not change in tube 6 in which the virus was diluted using DMEM. This was due to the red color in DMEM. Detection using agarose gel electrophoresis (panel B) showed DNA in all solvents tested.

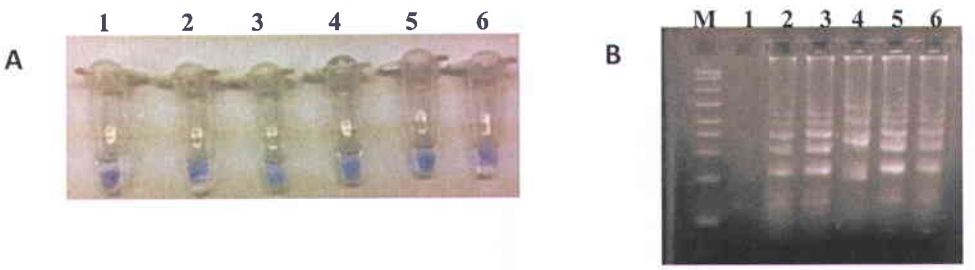

Fig. 2.8: LAMP with real samples.
LAMP products analyzed colorimetrically in tube (A) and by 2% agarose gel electrophoresis (B). M = 2 kb Marker; 1 = NC (H_2O); 2 = PC (HSV, 1 ng/μL); 3 = HSV-1 virus diluted in water; 4 = HSV-1 virus diluted in water (lane 3 and 4 are duplicate samples); 5 = HSV-1 virus diluted in PBS; 6 = HSV-1 virus diluted in DMEM. The viral concentration used in tubes 3-6 was 1.2×10^4 PFU/μL.

Next we attempted to determine the detection limit of HSV DNA in real samples using the LAMP assay performed in the plastic pouch. Viral stocks of HSV-1 (Fig 2.9A and 2.9B) and HSV-2 (Fig 2.9C and 2.9D) were serially diluted 10-fold from 10^4 pfu/μL to 10^0 pfu/μL in PBS. Purified HSV DNA was used as positive control and water was used as negative control (lane 1). Cell-line (without virus) (Lane 7) was also used to see whether cellular DNA interferes in the amplification reaction. The products were visualized in the plastic pouch (panels A and C) and by agarose gel electrophoresis (panels B and D) Lane 2-6 indicated that we could detect as little as 10^0 pfu/μL of viral load in both cases.

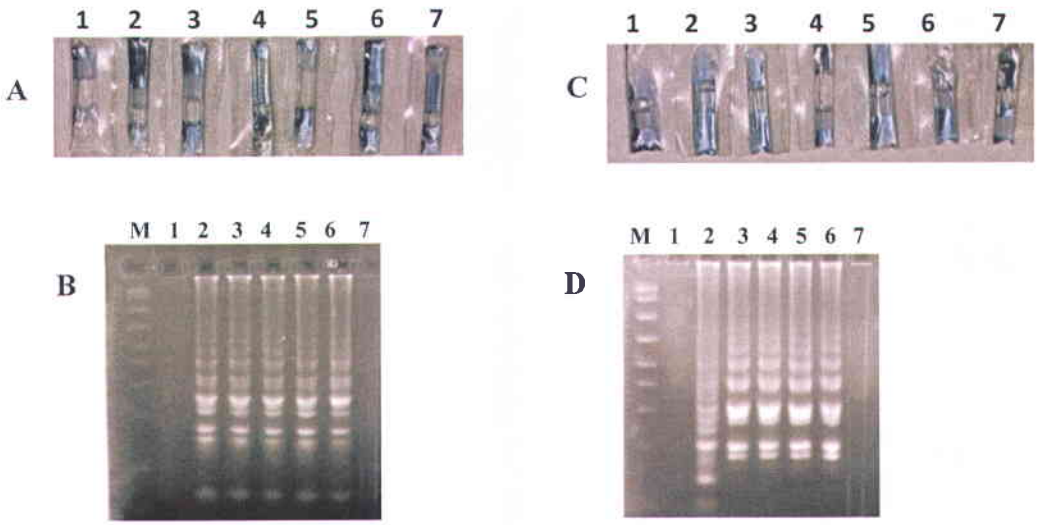

Fig. 2.9: Detection limit of HSV DNA in a real sample using the LAMP assay carried out in a plastic pouch. A: LAMP product (1-7) of HSV-1. B: Agarose gel electrophoresis of A; C: LAMP product (1-7) of HSV-2. D: Agarose gel electrophoresis of C. M= 2 Kb Marker; 1 = NC (H_2O); 2 = 10^0 pfu/μL; 3 = 10^1 pfu/μL; 4 = 10^2 pfu/μL; 5 = 10^3 pfu/μL; 6 = 10^4 pfu/μL and 7 = cells without virus.

Our findings in Figure 2.8 and 2.9 suggested that DNA extraction step could be omitted since the amplification results obtained with the positive control (purified HSV DNA) matched that obtained from unpurified DNA from viral stock. This will save time as well as cost.

Table 2.2: LAMP optimization: Time, temperature and sensitivity

Virus	Primer	Detection Time (Amplified at 10′, 20′, 30′, 40′)	Temp (Amplified at 60 °C, 63 °C, 66 °C)	Sensitivity (DNA was diluted from 10 ng/µl-1 fg/µl)
HSV-1	Set A (Table 1)	Saturation time: 50 ng/reaction: 20′-40′ 50 fg/reaction: 40′	60-66 °C	50 fg/reaction
HSV-2	Set B (Table 1)	Saturation time: 50 ng/reaction: 30′-40′ 5 fg/reaction: 40′	63-66 °C	5 fg/reaction

2.7 Discussion

To optimize the LAMP method, two different concentrations of dNTPs (0.4 mM and 1.4 mM) were used. The products of the LAMP reaction were analyzed by agarose gel electrophoresis and showed that both concentrations yielded the desired bands (data not shown). When 1.4 mM dNTP was used, the color changed from purple to blue before the addition of sample. One of the possible explanations is, when higher concentration of dNTP was added, the basic pH of the dNTP changed the color of the dye (HNB). Thus, a lower concentration of dNTP (0.4 mM) was used. The assay was carried out at 65 °C for 45 minutes at 0.4 mM dNTP concentration.

To examine see the cross reactivity of the primers used in these assays, we amplified *E. coli* DNA and water as negative control. We did not observe any color change (plastic pouch) or band (agarose gel) in *E. coli* DNA or for the negative control (Fig 2.7). This proves the specificity of the primers used in the assay as well as the lack of interference of the plastic material in the reaction. The sensitivity of the assay towards viral DNA was evaluated by carrying out the LAMP reaction in tubes and in the plastic pouch. We observed the same detection limit in both the tube and the plastic pouch which was 10fg/µL (or 50 fg/reaction since 5 µl of sample was used) for HSV-1 (Fig 2.7A, 2.7B) and 1 fg/µL or 5 fg/reaction for HSV-2 (Fig. 2.7D, Fig. 2.7D). These findings suggested that the tube and the plastic pouch function comparably. The detection limit of real virus was 10^0 pfu/µL of viral concentration in both cases (Fig 2.9A and 2.9B for HSV-1 and Fig 2.9C and 2.9D for HSV-2). We noted that the assay

conducted with the cells lacking the virus (Lane 7) did not result in a color change, which prove that the cellular DNA does not interfere in amplification reaction.

The LAMP method has been incorporated in different kinds of POC devices to detect infectious diseases (Fang et al. 2010, Fang et al. 2011). These devises need complex mechanical micro fabrication steps. Paper or plastic-based devices are comparatively cheap and easy to handle. Some other paper-plastic based devices are available (Rohrman and Richards-Kortum 2012), but they need sample processing and result analysis steps. Roskos and colleagues coupled isothermal amplification with NALF (Nucleic Acid Lateral Flow) (Roskos et al. 2013). They developed a cartridge which consisted of a reaction pouch and a pump pouch. Several steps were required to fabricate the device, in addition to a sample processing step. The final result was analyzed by a lateral flow assay.

The plastic pouch reaction vessel is very simple and does not require any mechanical micro fabrication steps. The only laboratory instruments required to perform the assay are a micropipette, pipette tips, and a heating block. The attractive feature here is to incorporate the LAMP technique. Because the LAMP is less affected by the other elements present in the real sample, we do not need to process or purify the samples. The time consuming the sample processing steps, which typically take almost one hour, can be omitted.

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Another advantage is the use of the HNB dye, which yields a color change after the reaction. HNB, a metal indicator for free magnesium and a colorimetric reagent for alkaline earth metal ions, was added to the reaction mixture which is usually purple color. When the LAMP reaction proceeds, magnesium pyrophosphate is produced, thus reduces the amount of free magnesium ion (Mg^{+2}) in the reaction mixture. Accordingly, the color of the HNB dye changes from purple to light blue, indication of a positive reaction. Therefore, after completion of the reaction, the result can be analyzed visually without any instrument. We do not need to perform gel electrophoresis or to connect with other analyser which could save another hour. Our results can be obtained in only 45 minutes. With this plastic pouch, only qualitative analysis is possible which its limitation seems. The device is lightweight, small, and easy to make. Therefore, I believe all these together can make it a perfect candidate for POC diagnosis both in the laboratory and in low-resource settings.

Further improvement and modification is needed to use this plastic device in the field. We are planning to lyophilize the primers in paper then place inside the plastic bag, which will reduce reagent addition steps and also allow for storage at room temperature. By changing the pattern of sealing by the sealer, we can modify the design of this device. For example, to make a multiplexed device, we will make one sample loading hole, so the sample will move to different chambers where different lyophilized primer sets are already stored. In this manner, more than one virus could be detected in the same device. We are also planning to improve the material quality and design to make a perfect device for POC application.

2.8 Conclusion

We have developed a plastic device that allows the detection of 10^0 pfu/μL of herpes simplex virus 1 and 2 (HSV-1 and -2) by loop-mediated isothermal amplification within 45 minutes. With this pouch there is no need for prior sample purification. In addition, colorimetric detection by eye makes analysis simple. This device is easy to handle and portable, without the need for expensive instruments. It is also low cost, which it a perfect candidate for point of care diagnosis both in the laboratory and in low-resource countries.

Chapter 3

Real-time detection of LAMP product

LAMP products can be detected by different techniques, for example, colorimetrically, by agarose gel electrophoresis, electrochemically, or even by the naked eye. In the previous chapter, the colorimetric detection of LAMP products was investigated. In this chapter, the electrochemical detection of LAMP product is described. The following work outlines the real-time monitoring of LAMP-amplified products using a redox probe as performed by our group at INRS. This work has been published in *Analyst, 2013* where I am cited as a co-author. My contribution to the publication includes carrying out bacterial preparations, DNA purification and all of the LAMP reactions.

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PAPER

Real-time electrochemical detection of pathogen DNA using electrostatic interaction of a redox probe†

Cite this: *Analyst*, 2013, **138**, 907

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Abstract

"Electrostatic redox probes interaction has been widely rendered for DNA quantification. In this report our group has established a proof-of-principle by using the ruthenium hexaamine molecule $[Ru(NH_3)_6]^{3+}$ as a redox probe. We utilized real-time electrochemical monitoring of a loop-mediated isothermal amplification (LAMP) amplicon of target genes of *Escherichia coli* and *Staphylococcus aureus* by square wave voltammetry (SWV). Ruthenium hexaamine interaction with free DNAs in solution without immobilization onto the biochip surface enabled us to discard the time-consuming overnight probe immobilization step in DNA quantification. We have measured the changes in the cathodic current signals using screen printed low-cost biochips both in the presence and the absence of LAMP amplicons of target DNAs in the solution-phase. By using this novel probe, we successfully carried out the real-time isothermal amplification and

detection in less than 30 min for *S. aureus* and *E. coli* with sensitivity up to 30 copies μL^{-1} and 20 copies μL^{-1}, respectively. The cathode peak height of the current was related to the extent of amplicon formation and the amount of introduced template genomic DNA. Importantly, since laborious probe immobilization is not required, and both the *in vitro* amplification and real-time monitoring are performed in a single polypropylene tube using a single biochip, this novel approach could avoid all potential cross-contamination in the whole procedure".

3.1 Introduction

It was discussed in the previous chapters that nucleic acid (NA) diagnostics help to diagnose disease. It is also useful in drug target identification, to study RNA interference and genotypes, as well as analysis of single nucleotide polymorphism (SNP). Most NA assays, however, are difficult to employ in practice, especially in resource-limited settings, owing to the requirement of sophisticated and expensive analytical setups. Real-time polymerase chain reaction (RT-PCR) or real-time quantitative PCR (RT-qPCR) are commonly used in the diagnosis of infectious diseases, quantitatively and with great specificity (Espy et al. 2006). These real-time assays require specialized fluorescent probes or DNA binding dyes as well as a bulky and expensive monitoring system, which make it difficult to use at resource-limited settings. Isothermal amplification and its real-time monitoring have attracted significant interest for pathogen detection (Asiello and Baeumner 2011, Nagatani et al. 2011). On the other hand, the integration of a laser diode, photo diode, and filter onto a monolithic chip always requires expensive fabrication protocols (Lui et al. 2009, Zhang and Ozdemir 2009) and therefore becomes an obstacle of fluorescent-based methods to provide low-cost portable devices. On the other hand, electrochemical methods are faster, low cost, simple, and can be applied in a miniaturized format more readily than optical methods (Liu et al. 2004, Ferguson et al. 2009). Studies show that electrochemical methods can also avoid requirements for complex instrumentation, high-power supply, calibration, and optimization. These prominent advantages make electrochemical detection a reliable and robust method for NA detection.

In the majority of electrochemical biosensors, ssDNA probes are immobilized onto the electrode surface through pre-treatment of the electrode, for which different reported techniques are used. Immobilization, irrespective of technique, requires modification of NAs or electrodes using, for example, nanoparticles, the streptavidin–biotin system, conducting polymers etc. (Castaneda et

al. 2007). The probe and target DNA hybridization yields a measurable voltammetric, chronopotentiometric, or impedimetric electrochemical signal through their interaction with a redox active compound. Among the redox active compounds, prominent are metal complexes, such as ruthenium bipyridine (Yang et al. 2002), ruthenium hexamine (Zhang et al. 2006, Zhang, Song et al. 2007), cobalt phenanthroline (Kerman et al. 2002), organic dyes, methylene dye (Boon and Barton 2003), and Hoechst 33258 (Kobayashi et al. 2001). However, all of these compounds require a laborious and time consuming probe immobilization step for practical application. To overcome such limitations, solution phase electrochemical DNA detection was first described by Bard and co-workers (Rodriguez and Bard 1990). Electrochemical sensing on PCR amplified DNA using Hoechst 33258 as the redox mediator was performed previously (Ahmed et al. 2007, Ahmed et al. 2009, Safavieh et al. 2012). In this work Ahmed and colleagues used Hoechst 33258 as a redox reporter to detect a LAMP amplicon (Ahmed et al. 2009, Safavieh et al. 2012). However, Hoechst 33258 is effective only in end-point DNA sensing, as it significantly inhibits the polymerase enzyme activity to limit DNA amplification and sensing in the solution phase. Tamiya and co-workers have also shown methylene blue (MB)-based real-time detection of LAMP products at different time intervals using RNA of influenza AH1pdm as a model target (Defever et al. 2011). An ideal redox mediator should be chemically stable, non-interfering with DNA amplification strategies over the optimum range, should preferentially bind to the target ds-DNA amplicon, and should be electrochemically active (Zhang and Tang 2004). In this study, a multiply charged transition metal cation, hexaamine ruthenium(III) – $Ru(NH_3)Cl_3$ or RuHeX has been used – due to its ideal electrochemical behaviour that enables rapid detection of LAMP amplicons (Fig. 3.1A and B) (Ho et al. 1987, Steel et al. 1999). RuHex lacks intercalating ligands and binds electrostatically with the anionic DNA backbone as suggested by Rich and colleagues (Ho et al. 1987).

In this report, we have shown the detection of DNA by a solution-based electrochemical assay without any immobilization of probes, using RuHex as a redox molecule.

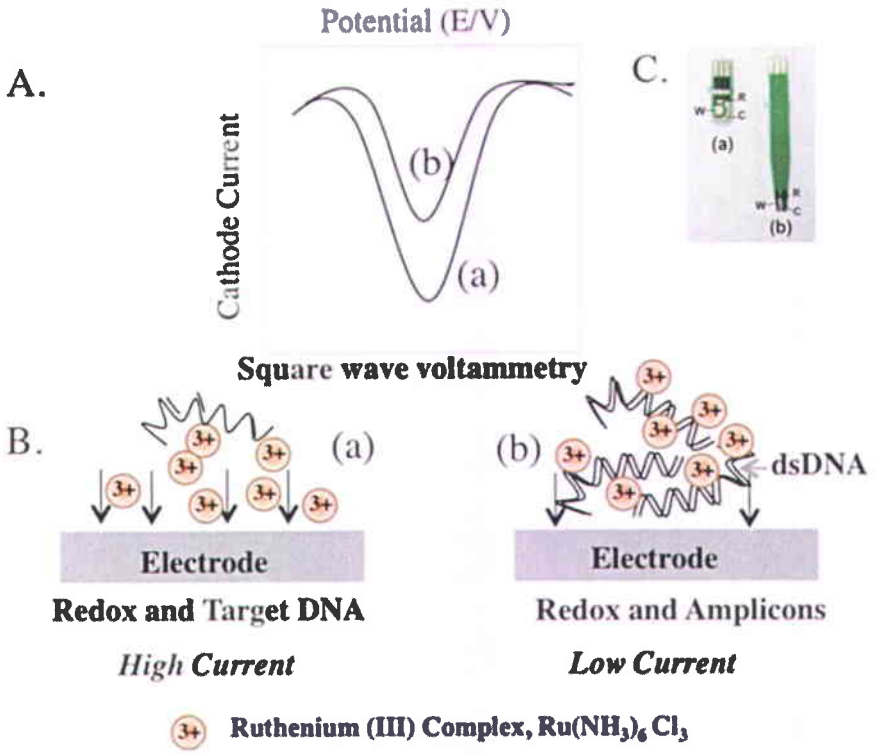

Fig. 3.1 (A) Scheme of SWV (square wave voltammetry) based-electrochemical detection of DNA and LAMP products, in the absence (a) and the presence (b) of dsDNA; (B) principle of the real-time electrochemical monitoring of the LAMP amplicon during the reaction process using a DNA electrostatic binder as a redox probe, (a) the redox and the target DNA before amplification which produced high current, (b) the redox and the amplicons after amplification which produced the low current as observed by SWV. (C) Photograph of two types of biochips or screen-printed electrodes, (a) type A for initial optimization and end point measurement, (b) type B for real-time electrochemical monitoring compatible for insertion into the 200 µL microtubes.

Next, we performed end-point and real-time detection of the LAMP amplified product. LAMP was performed using template genomic DNA (gDNA) specific for pathogenic microorganisms. The electrostatic binding of RuHex with the amplicon slows its diffusion on biochip surfaces and causes a reduction in peak current intensity. Chemically stable RuHex binds specifically to the double-stranded amplicons without inhibiting LAMP, and it is electrochemically detectable concurrently during isothermal amplification. To the best of our knowledge, we are the first to report this method with a RuHex redox molecule.

3.2 Materials and methods

3.2.1 Reagents and chemicals

Hexaaminеruthenium(III)-Ru(NH_3)$_3$ Cl_3 (RuHex) was purchased from Sigma-Aldrich (MO, USA). We prepared 200 μM RuHex solutions in H_2O and preserved the solution at 4 °C. Tris–HCl (pH 7.4) was prepared using Trizma base purchased from Bioshop Canada (Ontario, Canada). Deoxyribonucleic acid sodium salt from salmon testes was purchased from Sigma-Aldrich (MO, USA). All chemicals were of pure analytical grade. All solutions were prepared and diluted using ultra-pure water (18.3 M Ω).

3.2.2 Bacteria preparation and DNA extraction

E. coli was grown overnight (12 h) in 2% LB broth media. *E. coli* DNA was extracted using GenElute^{TM} DNA extraction kit from Sigma-Aldrich (MO, USA). Prof. Monique Lacroix from INRS Armand- Frappier (Montreal, Canada) kindly provided us with *Staphyloccocus aureus* and the DNA extraction from this pathogen was performed using the same kit. The concentration and purity of DNA were estimated by spectrophotometry (Nanodrop 2000c, USA).

3.2.3 LAMP reaction

The sequences of the LAMP primers for *E. coli* and *S. aureus* species are shown in Table 3.1 and were designed using Primer ExplorerV 3.0 from Eiken Chemical Co. Ltd. Japan. Six primers—loop forward (LF), loop backward (LB), forward inner primer (FIP), backward inner primer (BIP), forward outer primer (F3) and backward outer primer (B3)—were required to amplify target DNA of each species. The real-time LAMP was performed in a total of 50 μL reaction volume (of which 25 μL reaction mixture was used for end point-detection) containing 1.6 μM of each of FIP and BIP, 0.8 μM of each of LF and LB, 0.2 μM of each of F3 and B3 primers, 16 U of *Bst DNA polymerase* large fragment (New England Biolabs, Beverly, MA, USA), 0.4 mM of each of dNTPs, 2.5 μL of 10 × thermopol buffer (New England Biolabs, Beverly, MA, USA), 3.0 mM of $MgSO_4$, 0.64 M of betaine (Sigma- Aldrich) and 10 μL of target DNA. For real-time detection 15 μM of RuHex was added to the master mix, whereas for end point detection, the redox probe was added at the end of isothermal amplification along with 10mMTris–HCl buffer. The mixture in each tube was incubated at 65 °C (*E. coli*) and 66 °C (*S. aureus*) for 40 minutes

using a light-weight mini dry bath (Benchmark Scientific, China). In order to avoid evaporation, 20 μL of mineral oil were added to cover the solution. For agarose gel electrophoresis, 5 μL of LAMP product was used for loading in the presence of dye (NEB, USA) and 1× TBE buffer, pH 8.0. The agarose gel was pre-stained with Safe-T-Stain and following electrophoresis the image was captured for reference.

3.2.4 Electrochemical detection

Electrochemical experiments we performed using cyclic voltammetry (CV) and square wave voltammetry (SWV) methods. We used the compact Autolab system PGSTAT 101 (Metrohm, The Netherlands). The potentiostat was connected to a computer using NOVA v1.6. software. The measurements were repeated at least three times, and all the experiments were performed at room temperature (22–27 °C). The measurement conditions for SWV were: frequency = 25 Hz; amplitude = 49.5 mV, scan rate = 48.75 mV s^{-1}; step potential = 1.95 mV. The current responses from the reaction mixture including RuHex were recorded scanning in the range of -0.1 to -0.5 V. For initial dose response curve determination and end-point detection of loop amplicon type A, a horizontally placed disposable biochip was covered with the LAMP amplicon -buffer-RuHex mixture. However, for real-time monitoring, the type B biochip was inserted vertically into the polypropylene tube and scanned concurrently up to 40 min while amplifying.

Table 3.1 Primer sequences, target regions, and individual target gene of *S. aureus* and *E. coli* bacteria

Primer Type	Primer sequence (5′–3′)	Target regions	Gene name
Ecoli-F3	CTG CTG GGT GGT CAG GTA	48–65	*Tuf*
Ecoli-B3	GGA TTT TCG CTT CCC ACT CT	236–255	AX110239.1
Ecoli-LF	TGT CGC ATT TGT TCA GGA ACA	85–105	
Ecoli-LB	CGA CGA CAC TCC GAT CGT T	182–200	
Ecoli-FIP	AGC AGC TCT TCG TCA TCA ACC CAG GCG TTC CGT ACA TCA TCG		
Ecoli-BIP	TGT CTC AGT ACG ACT TCC CGG GCG CTT TCA GAG CAG AAC CAC		
Staph-F3	CTG GTG CAT TTG GGA CAT	344–361	*Catalase*
Staph-B3	TTG TTC TAG GAT CTC GTT TCA C	631–652	AJ000472.1
Staph-LF	CTA CAG TAG AGA AAC GGG CAA	457–477	
Staph-LB	CTG AAG AAG GGA ACT GGG ATT	521–541	
Staph-FIP	TCA GCA GCA CCA CGT TCT CAG GTA AGC AAA CCG AAA TGT		
Staph-BIP	GGA GCG TGA CAT TCG AGG ATT ACT GGT GTG TTA TTC CCT ACT A		

Disposable biochips used here come in two sizes (type A and B) and contain a three-electrode system (Fig. 3.1C.), including carbon-based working and counter electrodes and an Ag/AgCl reference electrode. The chips had a barrier to help keep the reaction mixture on the working electrode. The overall outer dimensions for type A and B electrodes are 12.5 mm × 4 mm × 0.3 mm and 30 mm × 4 mm × 0.3 mm, respectively. The area of the working electrode was 1.96 mm^2 for type A whereas for type B the area was 1.38 mm^2. Disposable electrochemical printed (DEP) chips were purchased from Biodevice Technology, Co. (Ishikawa, Japan).

3.3 Results and discussion

LAMP is more robust, sensitive, and rapid while RuHex, as a redox mediator, possesses high binding affinity for DNA (1.2 x $10^6 M^{-1}$), which allowed using it for electrochemical solution phase DNA sensing using LAMP amplicon. The electrostatic interaction of the redox cations ruthenium (III) hexamine and cobalt(III) tris (2′,2′- bipyridine) in solution and surface state DNA have been reported earlier using normal pulse voltammetry. In this study, at the beginning, cyclic voltammetry (CV) was performed on free RuHex and the RuHex-dsDNA in order to determine the individual electrochemical reaction rate. CV spectra on 100 mV s^{-1} of 15 μM RuHex in the absence and presence of DNA are shown in Fig. 3.2A. A decrease in the peak current with positive shift of both anodic and cathodic peaks in the presence of DNA were observed here, which was a reflection of an earlier report (Maruyama et al. 2002). The CV of free RuHex (Fig. 3.2A (a)), on the other hand, showed the occurrence of reduction at a cathodic potential E_{pc} of -0.280 V *vs.* Ag– AgCl on the type A biochip. Oxidation occurred at -0.210 (E_{pa}) upon scan reversal. The usual separation of the anodic and cathodic peak potentials (ΔEp = 70 mV) indicates a quasi-reversible one-electron redox reaction of RuHex. The value of ΔEp in the presence of DNA was 59 mV, showing that reversibility of the electron-transfer process was maintained or even improved under these conditions. Fig. 3.2B displays plots of cathode peak currents, i_{pc} vs. the square roots of the scan rates ($v^{1/2}$). The linearity of the plots in Fig. 3.2B indicates diffusion-based current signal and that there is no significant contribution from physical absorption of RuHex in 10 mM tris-buffer or RuHex-dsDNA onto the electrode surface. The lower slope of the i_{pc}–$v^{1/2}$ plot of RuHex-DNA compared with that of free RuHex indicates the occurrence of significant reduction in the apparent diffusion coefficients upon formation of the RuHex-DNA complex. Based on this slope at a 100 mV s^{-1} scan rate using 15 μM of RuHex, the

apparent diffusion coefficients are estimated to be 1.64×10^{-30} cm^2 s^{-1} and 3.18×10^{-31} cm^2 s^{-1} for free RuHex and DNA-bound RuHex, respectively.

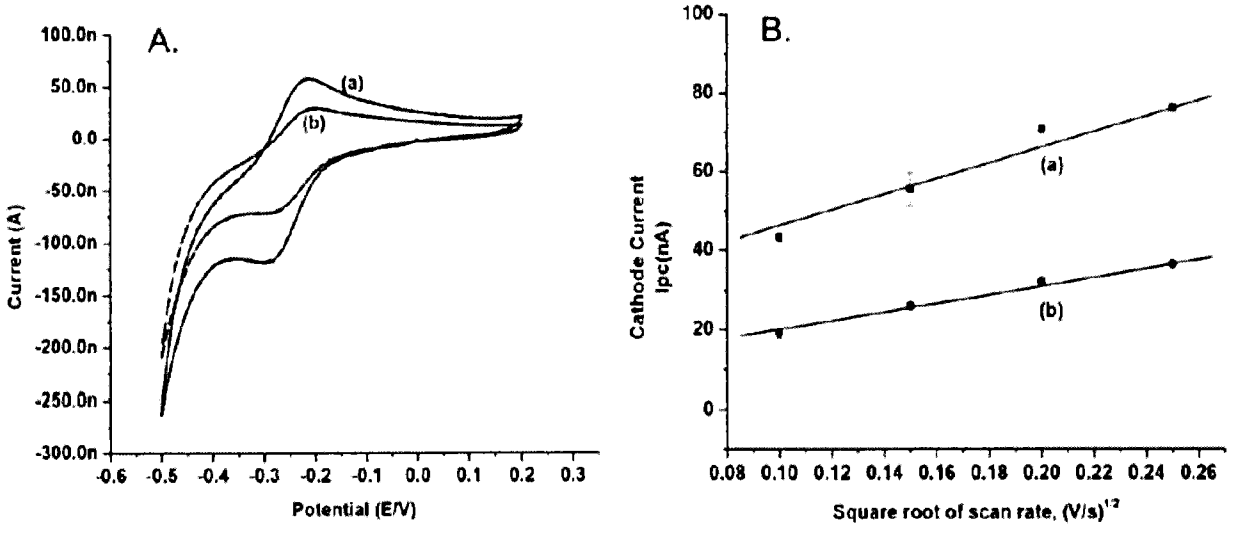

Fig. 3.2: (A) Cyclic voltammograms of RuHex at 100 mV s^{-1} scan rate, without (a) and with (b) 10 ng μL^{-1} of salmon testes DNA in 10mMtris buffer. (B) Plots of cathodic peak currents vs. square roots of scan rates recorded with the free RuHex (a) and RuHex-dsDNA complex (b). The measurement conditions were same as for (A). Error bars indicate the standard deviation of at least three replicated measurements.

3.3.1 Dose–response curve and chronocoulometric test

The DNA concentration was optimized using RuHex with a type A electrode. Noted was an increasingly slower diffusion onto the electrode surface with increasing DNA concentration in 10 mM tris-buffer. Here, salmon dsDNA was quantified from 1–10 ng μL^{-1} (Fig. 3.3) using SWV at ambient temperatures (23–27°C). SWV was chosen for endpoint and real-time detection of DNA, due to its faster signal acquisition and increased sensitivity compared with other electrochemical methods. The charge compensation by RuHex due to DNA in solution was also tested. There is an increase of intercept due to the charge associated with the RuHex in solution compared to treatment with buffer (Q_{Buffer}) only.

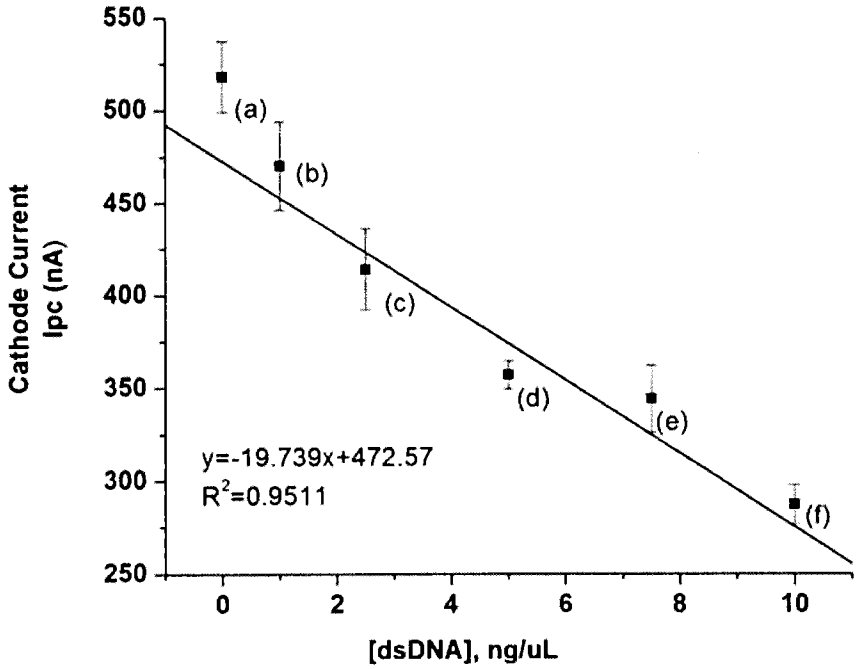

Fig. 3.3: Dose–response curve for the determination of salmon dsDNA, (0–10 ng μL^{-1}) using 15μM RuHex: (a) 0 ng μL^{-1} (b) 1 ng μL^{-1} (c) 2.5 ng μL^{-1} (d) 5 ng μL^{-1} (e) 7.5 ng μL^{-1} (f) 10 ng μL^{-1} DNA. Throughout the experiments 15 μM of RuHex was used. SWV was used as a detection mode transducer. Error bars indicate the standard deviation of at least three replicated measurements.

Upon addition of salmon dsDNA, a decrease in the intercept is observed due to RuHex and DNA electrostatic binding and charge compensation on the electrode surface. The charges of excess RuHex (or unbound RuHex) on the electrode surface were determined in the latter case, which is close to the intercept obtained by Tris-buffer.

3.3.2 End point LAMP amplicon detection

In order to determine the reproducibility of these results, LAMP reaction was carried out for 40 min (up to the end-point). Previously, LAMP amplicons diluted 4x and 5x were detected using disposable biochips with Hoechst 33258 as the redox molecule and linear sweep voltammetry (Ahmed et al. 2009, Asiello and Baeumner 2011). In the current study, however, using RuHex as a probe was much more sensitive; a 25x diluted LAMP amplicon could be detected after a 40 min amplification reaction (Fig. 3.4A and B). Capitalizing on the electrostatic interaction of RuHex with amplicon allowed us here to perform real-time analysis of *E. coli* and *S. aureus*-

specific gDNA (genomic DNA) by providing rapid detection of amplicons with a higher signal to noise ratio, even with a minute amount of sample. In each of the end-point measurements using *E. coli* and *S. aureus* gDNA (1000 fg μL^{-1}, 100 fg μL^{-1}, 10 fg μL^{-1}) as templates, 25 µL of the LAMP solution was placed onto the chip surface and the cathode current was recorded(Fig. 3.4A and B). The 25 µL mixture, consisting of 25× diluted LAMP amplicon, 15 µM of RuHex, and 10 mM Tris, was used to cover the horizontally placed electrodes. Concurrently, 5 µL aliquots of reaction solutions (1× and 25× diluted LAMP amplicons) were electrophoresed through a 2% agarose gel containing staining dye (Safe-T-Stain ™, Canada) and the products were visualized by UV illumination (figure not shown here). In the case of the *E. coli* primer based reaction, at 40 min, 1× diluted amplicons were clearly visible in the gel compared to the very faint gel image for 25× diluted amplicons. This was due to the low resolution data obtained via an agarose gel compared to electroanalysis as shown in Fig. 3.4B. While typical gel analysis took approximately 40 min (at 80.0 V) to complete, the highly sensitive voltammetry (*e.g.* SWV) measured the amplicon in less than 20 s per sample. The sensitivity of LAMP reactions for both *S. aureus* (Fig 3.4A) *E.coli* (Fig. 3.4B) was 100 fg μL^{-1}, using our end-point electrochemical measurement; 10 fg $\mu L^{-1}$of template DNA could not amplified. Due to a lack of amplicon, no electrostatic interaction was performed with an equal amount of RuHex and thus a high cathode current signal was obtained in both cases (Fig. 3.4A and B). The cross reactivity of isothermal loop-primers was evaluated by using 100 fg $\mu L^{-1}$of *S. aureus* gDNA with *E. coli* primer (Fig. 3.4A) and *E. coli* gDNA with *S. aureus* primers (Fig. 3.4B) by using both agarose gel and electrochemical sensors. We did not detect any cross-reactivity. Non target control (NTC) or water was also used to prove the absence of non-specific amplification or formation of potential primer dimers. As mentioned previously, due to the absence of amplicon in NTC, free RuHex yielded a higher cathode current on the electrode surface using SWV and showed no ladder-type band by agarose gel analysis.

3.3.3 Real-time LAMP measurements

In the next experiment, the LAMP reaction in real-time was detected using the same electrode (type B, Fig. 3.1C (b)) as reported previously (Nagatani et al. 2011). This one electrode chip was used for the detection of each target type in real-time up to 40 min of isothermal amplification at 66°C (Fig. 3.5 and 3.6).

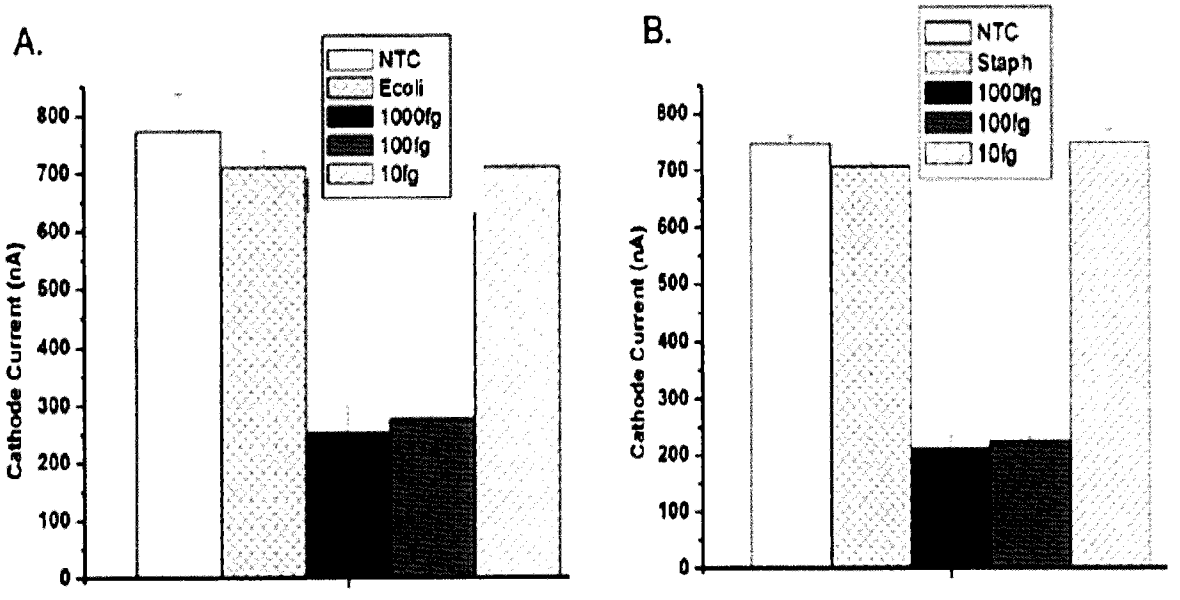

Fig. 3.4 SWV behaviour of 15 μM RuHex with type A electrode for end point detection of *S. aureus* (A) and *E. coli* (B) using their specific loop-primers as outlined in Table 3.1. A. NTC: non-target control, *E. coli*: *E. coli* gDNA (100 fg μL^{-1}) was used as a template to observe cross-reactivity with *S. aureus* primers, 1000 fg: 1000 fg μL^{-1} of *S. aureus* gDNA, 100 fg: 100 fg μL^{-1} of *S. aureus* gDNA, and 10 fg: 10 fg μL^{-1} of *S. aureus* gDNA were used as template. B. NTC: non-target control, Staph: *S. aureus* gDNA (100 fg μL^{-1} was used as a template to observe cross-reactivity with *E. coli* primers, 1000 fg: 1000 fg $\mu L^{-1}$of *E. coli* gDNA, 100 fg: 100 fg μL^{-1} of *E. coli* gDNA, and 10 fg: 10 fg μL^{-1} of *E. coli* gDNA were used as templates. Detection was performed after 40 min. of reaction after diluting the amplicon to 25×. Error bars indicate the standard deviation of at least three replicated measurements.

In this study, electrodes were inserted into 0.2 mL micro tubes where the *E. coli* and *S. aureus* specific genes were amplified by LAMP while SWV was conducted simultaneously from 0 to 40 min. RuHex pre-dissolved in water was added to the LAMP master mix while preparing the reaction components for isothermal amplification.

A straight line connecting the two ends of a cathode current signal was used as the baseline to determine the peak height of the measured SWV data using RuHex (Welch. et al. 1996). Since the master mix cannot reach 65 °C and 66 °C at 0 min, the peak height was taken at 5 min as the standard. The comparison between the ratios of peak heights as calculated from the voltammo-grams of *S. aureus-LAMP and E. coli-* LAMP and is shown in Fig. 3.5 and 3.6 respectively,

along with their quantitative analysis at different thresholds. The peak height ratios were always in good agreement with the results from typical gel electrophoresis. The ratio of peak height decreased dramatically at about 25 min for *S. aureus* (10 pg μL^{-1}, 3.1 × 10^3 copies μL^{-1}) and about 20 min for *E. coli* (10 pg μL^{-1}, or 2.0 × 10^3 copies μL^{-1}), respectively. The slow amplification and then real-time electrochemical detection of *S. aureus* compared to *E. coli* may be due to the copy numbers of the target genes (*Catalase, Tuf*) of both species. As known, the *E. coli* genome size is 4.6 Mb, which is much higher compared with the mere 2.9 Mb for *S. aureus*.

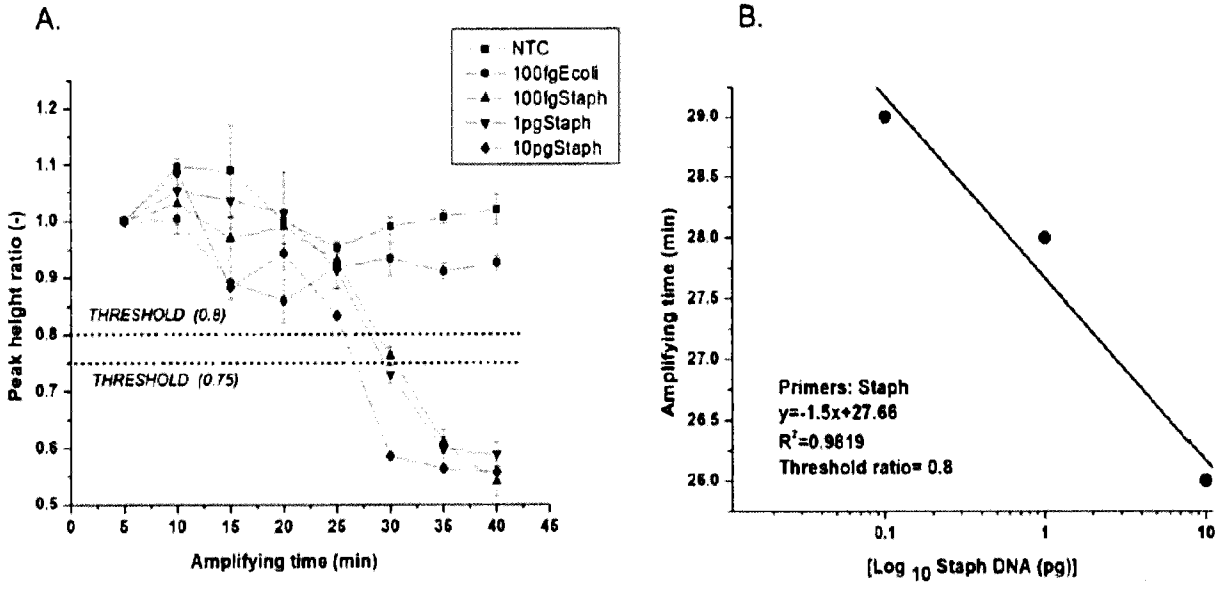

Fig. 3.5: (A) Real-time quantitative monitoring of LAMP amplicon by the ratio of peak height for different concentrations of *S. aureus* gDNA during 5 to 40 min amplifying time at 66 °C. (B) Standard curve calculated from (A) with the threshold ratio of 0.8. Threshold reaction times are plotted against the $\log_{10}$ of the *S. aureus* gDNA. The linear regression line, and the equation and R^2 values are shown.

3.3.4 Real-time quantitative detection

In the electrochemical sensing, only one chip for up to 24 scans in SWV was used successfully without observing any significant reduction of the ratio between the peak heights for the non-template controls. Usually, if the reaction proceeds with specific targets for its complementary primers, the amount of amplified DNA increases leaving less free RuHex in solution that results in the low ratio of peak heights. A greater reduction in peak height confirms the amplification of

target species even in the presence of other reactants of LAMP. Interestingly, while working with *E. coli* primers and its associated DNA template, a faster reaction rate was found compared to *S. aureus*, and it reached saturation at around 30 min (Fig. 3.6). After a certain time in the formation of the final product (dsDNA), slow dissociation-induced diffusion of bonded RuHex caused a negligible increase in the peak ratio which gradually became stable. It can be hypothesized that this was a temporary heat-induced dissociation of RuHex which occurred after completion of the LAMP reaction. Different concentrations of DNA template were also amplified and analyzed by LAMP, and then simultaneously measured up to 40 min using SWV for quantitative analysis of *S. aureus* and *E. coli* target DNA. A gradual reduction increased the differences in the ratios, which indicated that the number of LAMP amplicons formed and the rate of LAMP amplification are different.

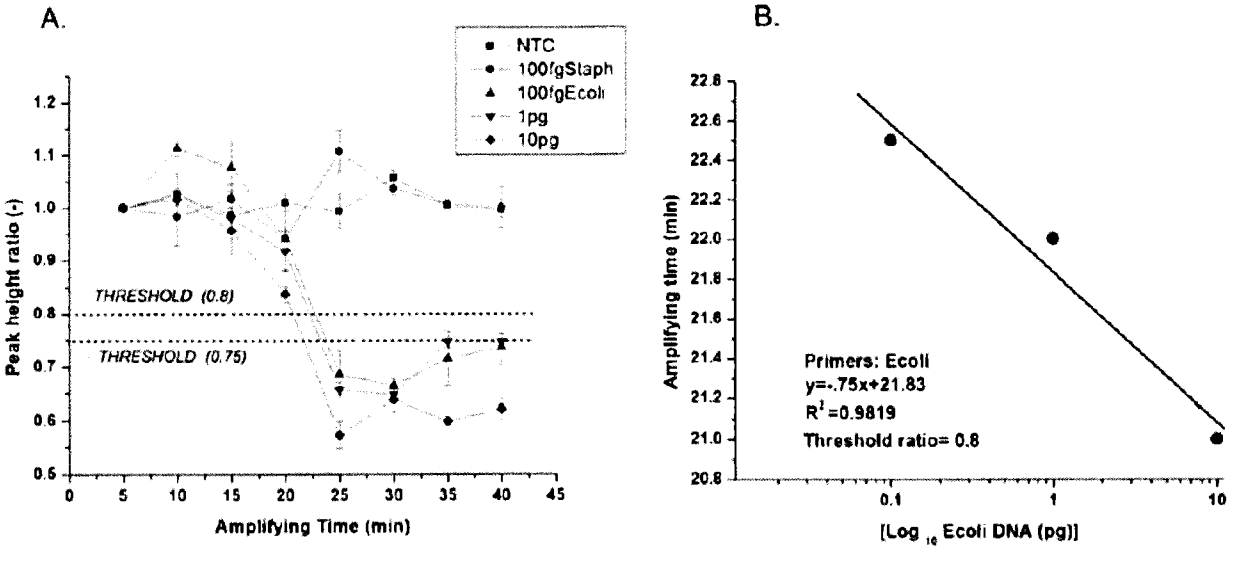

Fig. 3.6 (A) Real-time quantitative monitoring of LAMP amplicon by the ratio of peak height for different concentrations of *E. coli* gDNA during 5 to 40 min amplifying time at 65 °C. (B) Standard curve calculated from (A) with the threshold ratio of 0.8. Threshold reaction times are plotted against the Log_{10} of *E. coli* gDNA. The linear regression line and the equation and R^2 values are shown.

The standard curve based upon the amounts of *S. aureus* DNA showed a linear relationship between $\log_{10}$ Staph DNA concentration and the threshold amplifying time, and the slope of the curve is -1.5 and the square of the correlation coefficient (R^2) after linear regression is 0.9819 (Fig. 3.5B).

During *E. coli* detection and analysis, the same threshold ratios, 0.75 and 0.80, were chosen within the range of linear reduction ratios and compared (Fig. 3.6B). For the threshold at 0.8, the ratio for 100 fg μL^{-1}, 1 pg $\mu L^{-1}$and 10 pg $\mu L^{-1}$of *E. coli* template DNA begins to cross with the threshold ratio at 22.5, 22 and 21 min, respectively. The standard curve based upon the amounts of *E. coli* DNA showed a linear relationship between $\log_{10}$ *E. coli* DNA concentration and the threshold amplifying time 0.75, the slope to the curve became -0.75 and the square of the correlation coefficient (R^2) after linear regression was 0.9819 (Fig. 3.6B). With the threshold ratio taken at 0.75, the standard curve showed a linear relationship between $\log_{10}$ *E. coli* DNA concentration and the threshold amplifying time, and the slope of the curve was -0.65 and the square of the correlation coefficient (R^2) after the linear regression was 0.9285. As with *S. aureus*, here also, the threshold ratio at 0.8 was taken for quantitative analysis and detection due to a higher correlation coefficient. The stability of the RuHex complex during electrochemical analysis has also been observed by analysing the relative standard deviation (RSD) of the measured data using salmon dsDNA and loop amplicons at 65 °C. The data shows stable behaviour of the RuHex complex for both salmon dsDNA and real-time loop amplicons detection in solution.

3.4 Conclusion

The overall merit of the specific, fast, and simple DNA or amplicon detection method based on the SWV of cationic metal complex RuHex on the sensor surface was evaluated by using *S. aureus* and *E. coli* genes as model targets. As discussed, the approach does not require time-consuming probe immobilization or overnight pre-treatment prior to analysis, which makes the method worthwhile in terms of cost, speed and ease-of-use. The real-time LAMP amplicon detection using RuHex as a redox indicator, as we conclude, stands out to be a compelling tool offering high sensitivity and selectivity. The developed sensor successfully carried out the real-

time isothermal amplification and detection of as little as 30 copies $\mu L^{-1}$and 20 copies μL^{-1} for *S. aureus* and *E. coli* respectively, in less than 30 minutes.

In summary, experiments carried out for the detection of virus (Herpes Simplex Virus 1 and 2, Chapter 2) and bacteria (*S. aureus* and *E. coli,* Chapter 3) using LAMP technology yielded robust results. Colorimetric (qualitative) and electrochemical (real-time and quantitative) detection of LAMP product described here not only can reduce total assay time but also the total cost of disease diagnostics. These detection methods could be used for any other virus/bacteria-causing diseases, tumor gene detection, drug target identification, as well as in single nucleotide polymorphism (SNP) analysis. Taken together, I believe LAMP technology can be a perfect candidate for point-of-care diagnosis in both laboratory and low-resource settings.

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Résumé en Français

1. Introduction

Les maladies infectieuses causent 9,5 millions de décès par an, la quasi-totalité dans des pays en voie de développement. Ce taux est assez élevé en raison du délai entre le diagnostic et le traitement dans ces pays. Un diagnostic précoce et le traitement de la maladie peuvent avoir un rôle important pour prévenir le développement de complications à long terme ou à interrompre la transmission de l'agent infectieux. Il fournit également des soins appropriés et en temps opportun aux patients, ce que aide à prévenir des infections nosocomiales (Yager, Domingo et al. 2008). Dans les zones rurales, en particulier dans les pays en voie de développement, les laboratoires n'ont pas accès à des équipements permettant l'analyse haut débit d'échantillons. Les technologies classiques et peu sensibles font retard ou non diagnostiquée de la maladie. Par conséquent, les professionnels de la santé sont à la recherche de technologies de diagnostic plus abordables à plus petit échelle, disponibles sur le terrain et qui peuvent identifier rapidement et précisément les agents pathogènes des maladies infectieuses. Les méthodes de diagnostic doivent être précises, simples et abordables pour la population à laquelle elles sont destinées. Les points de soins de diagnostics doivent répondre à ces besoins.

Dans la première partie de ma thèse, je discute brièvement du point de soins et les technologies utilisées pour les diagnostics des points de soins. Bien que de nombreuses méthodes soient déjà établies, elles ne sont pas toutes adaptées pour les diagnostics en points de soins. Les méthodes d'amplification d'acides nucléiques sont très sensibles et spécifiques en raison de l'amplification de la cible et des interactions d'appariement de bases. Une petite quantité d'agent pathogène infectieux peut être détecté en utilisant ces méthodes. Au cours de la réaction en chaîne de la polymérase (PCR), l'amplification isotherme de l'ADN / ARN a récemment suscité de l'intérêt, car elle ne nécessite pas de thermocycleur. LAMP (Loop mediated isothermal amplification) est une technique d'amplification isotherme, considérée comme une méthode robuste en termes de sensibilité, de tolérance avec des substances inhibitrices présentes dans l'échantillon réel, at la facilité de la détection à l'œil nu. Par conséquent, il s'agit d'une approche plus efficace et plus simple, ce qui en fait un excellent choix pour les applications de points de soins (PDS). Par conséquent, j'ai été motivée à utiliser LAMP dans mon travail de thèse. Dans la deuxième partie,

je décris mon travail de thèse où j'ai développé un étui (sachet) en plastique à faible coût pour la détection des virus de l'herpès simplex selon la méthode LAMP. Le produit amplifié de LAMP peut être détecté de différentes manières, par exemple, par détection colorimétrique, par détection électrochimique, ou visuellement par l'œil nu. Dans mon travail, j'ai utilisé la détection colorimétrique. Dans la troisième partie j'ai inclus un résumé d'une détection électrochimique du produits LAMP qui a été fait par notre équipe dans notre laboratoire à l'INRS: "Real-time electrochemical detection of pathogen DNA using electrostatic interaction of a redox probe". Ce travail a été publié dans the journal *Analyst* en 2013 (Ahmed, Nahar et al. 2013).

1.1 Les Technologies utilisées pour le PDS

Les examens médicaux réalisés au chevet du patient, ou tout près de son site de traitement, sont considérés comme étant des tests de points de soins PDS ou "point-of-care technology (POCT)". Dans le domaine du diagnostic médical, les applications des points de service ou de soins (PDS) sont simples à utiliser, portables, facilement disponibles, stables dans différentes conditions de fonctionnement (comme la température, l'humidité surtout dans les zones pauvres et isolées).
Les appareils lab-on-a-chip (LOC) offrent de nombreux avantages pour la détection d'agents pathogènes tels que la miniaturisation, le petit volume de l'échantillon, la portabilité, et le court temps de détection et de diagnostic au point de soins. La nécessité du diagnostic au point de soins est cruciale dans les pays en développement ainsi que la ou les ressources des laboratoires sont faibles. Par exemple, un instrument de traitement automatique, robotisé à haut débit n'est généralement, pas accessible dans les milieux à faibles ressources qui manquent d'infrastructures aux laboratoires. Les méthodes de détection des pathogènes dépendent des analytes ciblés tels que les acides nucléiques, les protéines et les cellules entières.

De nombreuses méthodes sont déjà établies pour l'identification d'agents pathogènes, mais pas toutes sont adaptés comme correspondant à des tests PDS. Par exemple, ELISA (Enzyme Linked Immuno Assay) est un type de dosage standard pour la détection de pathogènes dans le laboratoire. Cependant, il s'agit d'un dosage multi-étape qui est moins sensible que d'autres dosages. Il a besoin d'un lecteur ELISA pour analyser le résultat, un instrument qui est volumineux et coûteux. Bien que l'ELISA soit une excellente méthode, elle n'est pas bien adaptée à une utilisation à l'extérieur. En effet, pour effectuer ce test, un laboratoire sophistiqué

ayant une température contrôlée avec un personnel hautement qualifié, ainsi que le lecteur pour l'analyse des résultats sont nécessaires (Yager, Domingo et al. 2008).

La culture cellulaire est une autre méthode de référence pour la détection d'agents pathogènes bactériens ou viraux. Cependant, une hotte de sécurité biologique muni d'un filtre HEPA, ainsi qu'un personnel qualifié, sont nécessaires.
Bien que ce soit une technique fiable, cela prend 3-7 jours pour obtenir le résultat. Puisque dans cette méthode, les pathogènes vivants sont cultivés, les règles de biosécurité sont strictement maintenues en fonction de leur niveau de sécurité. Pour cette raison, cette méthode ne convient pas pour les pays à ressources limitées, comme par exemples les cliniques éloignées des centres urbains, ainsi que pour l'application de PDS. Pour ces raisons, il y'a un réel besoin de technologies de diagnostic rapides, sensibles et spécifiques pour les maladies infectieuses afin de remplacer les méthodes de culture qui sont fastidieuses et limitées (Yager, Domingo et al. 2008) .

Une grande classe de tests de diagnostiques PDS consiste en l'essai d'écoulement latéral. Dans cet essai, une membrane ou une bande de papier est utilisée pour indiquer la présence de marqueurs protéiques tels que des antigènes d'agents pathogènes ou les anticorps de l'hôte.
Sur une membrane, l'addition de l'échantillon induit une action capillaire. L'échantillon s'écoule au travers de la membrane, réagit avec les réactifs qui y sont déjà incorporés, et s'écoule sur une zone qui contient des molécules de capture. Les analytes marqués capturés sont interprétés à l'œil nu via la formation d'une bande visible (Chin, Linder et al. 2012). Les tests à écoulement latéraux sont utilisés pour le diagnostic de la grossesse, \les infections avec le streptocoque, la grippe, ou pour diagnostiquer le VIH. Bien que le test soit simple à réaliser, l'action du flux simple ne reproduit pas les procédures multi-étapes de laboratoire, qui sont essentielles pour l'obtention de résultats reproductibles, quantitatifs et sensibles. Les tests de glycémie sont une autre grande classe de tests au PDS. Ce test est également réalisé sur des membranes, mais est différent du test immunologique d'écoulement latéral. Il utilise une amplification du signal par une enzyme d'oxydoréduction, se terminant généralement dans un affichage électrochimique.

Pour le test d'amplification d'acides nucléiques, la réaction en chaîne de la polymérase (PCR) a été la première à être utilisée dans des dispositifs de PDS. Cependant, la PCR nécessite des

cycleurs thermiques coûteux et une optique relativement sensible pour la détection en temps réel. Les deux cycles thermiques et optiques ne sont pas bien adaptés pour un appareil au PDS parce que le but de PDS est non seulement de réduire le temps de test, mais aussi le coût (Stedtfeld, Tourlousse et al. 2012). Au cours de la PCR, l'amplification isotherme de l'ADN / ARN a récemment suscité de l'intérêt, car elle ne nécessite pas de thermocycleur. Par conséquent, il s'agit d'une approche efficace et plus simple, ce qui en fait un excellent choix pour les applications de PDS.

Les méthodes pour l'amplification isotherme comprennent : boucle amplification isotherme facilitée (LAMP), l'amplification dépendante de la hélicase (HDA), l'amplification d'acide nucléique par séquence (NASBA), l'amplification par recombinase polymérase (APR) et l'amplification de cercle roulant (RCA).

Parmi les technologies isothermes, LAMP est devenue une technique préférée pour le diagnostic des maladies infectieuses aux PDS, en raison de sa rapidité, le faible coût de l'équipement et de la robustesse aux inhibiteurs présents dans l'échantillon clinique (Kaneko, Kawana et al. 2007, Mori and Notomi 2009). Le test de LAMP peut également être suivit en temps réel (Mori, Nagamine et al. 2001, Ahmad, Seyrig et al. 2011, Ahmed, Nahar et al. 2013) pour la quantification, et peut être utilisée pour différencier les polymorphismes d'un nucléotide simple (Ikeda, Takabe et al. 2007). Les applications reliées à la santé humaine qui pourraient bénéficier des avantages quantitatives multiples des tests PDS génétiques comprennent la mesure de la charge virale du VIH (Shen, Sun et al. 2011), la différenciation des mutations ponctuelles de multiples tuberculoses pharmaco-résistantes (Lee, Chen et al. 2010), ou la mesure des panneaux de microARN pour le diagnostic de cancer (Li, Li et al. 2011). D'une manière générale, les tests génétiques sont destinés à détecter la présence ou l'absence de marqueurs génétiques tels que les gènes de virulence spécifique des agents pathogènes, des gènes de résistance aux antibiotiques, ou des mutations spécifiques d'une maladie. Le séquençage, l'analyse génétique le plus souhaitable, n'est pas encore disponible en utilisant les instruments ou les dispositifs PDS à faible coût.

Pour incorporer les essais NA pour le dispositif de PDS, plusieurs formats peuvent être utilisés pour effectuer une détection spécifique de la séquence telle que montrée ci-dessous (figure 1) (Craw and Balachandran 2012).

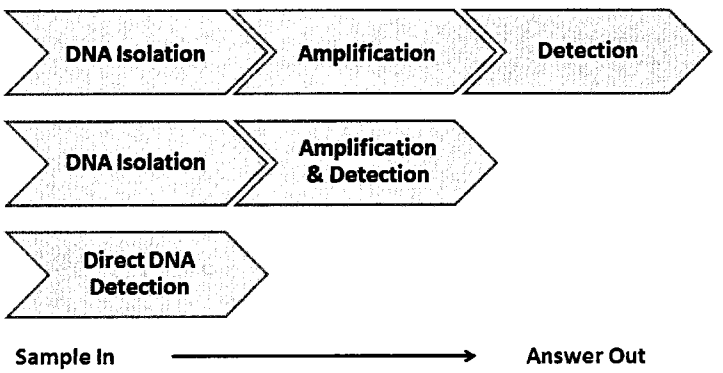

Figure 1: Formats de tests d'acides nucléiques
Source: (Craw and Balachandran 2012)

Pour l'amplification de l'acide nucléique (NA), l'isolement ou la purification de l'échantillon clinique est une étape essentielle. Ceci est dû au fait que dans les échantillons cliniques, les lipides, le sel ou le sucre inhibent la réaction d'amplification. Dans le premier format (Fig. 1) l'isolement du NA à partir d'un échantillon clinique est suivi par l'amplification et la détection du produit amplifié. Par exemple, la PCR, où le NA (ADN / ARN) est isolé à partir de l'échantillon clinique et ensuite amplifié dans le cycleur thermique, puis les produits amplifiés sont analysés. Ces méthodes ont été bien caractérisées, largement utilisées, et largement appliquées dans la détermination génétique et les maladies infectieuses. Dans d'autres essais l'amplification et la détection se font ensemble, ce qui contribue au format robuste pour le développement d'autres tests NA. En ce qui concerne les applications de PDS, il est essentiel de simplifier la procédure d'essai et d'éviter les procédures multi-étapes pour l'amplification et la séparation et donc de réduire le temps d'obtention des résultats (Craw and Balachandran 2012).

1.2 Loop mediated isothermal amplification (LAMP)

LAMP est une technique d'amplification isotherme d'acides nucléiques (ADN ou ARN) simple, rapide, précise et efficace, qui a été mise au point par Notomi et al. Dans cette technologie, quatre amorces différents et spécifiquement conçus sont utilisés pour reconnaître six régions

distinctes sur le gène cible. L'amplification et la détection du gène peuvent être effectuées en une seule étape, en incubant l'échantillon, les amorces, l'ADN polymérase ayant une activité de déplacement de brin. Le processus de réaction se déroule à une température constante (60 ° C-65 ° C) et offre une efficacité d'amplification élevée (10^9-10^{10} fois en 15-60 minutes). La présence de produits amplifiés peut indiquer la présence du gène cible. Cette méthode peut être utile à l'avenir comme une alternative à faible coût pour détecter certaines maladies.

2. Objectif de ma thèse:

Le développement d'un outil à faible coût approprié pour les pays à faibles ressources pour la détection du virus herpès simplex.

La technologie LAMP est une méthode robuste pour identifier une maladie dans très peu de temps. Le coût global de cette méthode est très faible en comparaison avec d'autres technologies existantes. Avec cette motivation, j'ai utilisé cette méthode LAMP dans ces travaux pour la détection du virus de l'herpès simplex (HSV-1 et HSV-2).

2.1 Introduction

Le virus de l'herpès simplex (HSV) est un problème majeur dans les pays industrialisés et en développement. HSV été caractérisé par deux stéréotypes différents: le HSV de type 1 (HSV- 1, nommé comme l'herpès oral) est généralement associé à des infections de la langue, la bouche, des lèvres, du pharynx et des yeux, tandis que le HSV de type 2 (HSV- 2, nommé comme l'herpès génital) est principalement associée à des infections génitales et néonatales (Aurélien 1992). Les deux virus de l'herpès (HSV-1 et HSV-2) peuvent établir une latence permanente dans les ganglions sensoriels et neuronaux humain, qui peuvent être réactivés ultérieurement. Après la réactivation, chacun des virus de l'herpès peut provoquer des symptômes cliniques importants chez l'individu et peuvent se propager à des personnes non infectées. Le virus peut également être transmis de la mère à l'enfant pendant l'accouchement. L'infection néonatale (Rudnick and Hoekzema 2002, Pinninti and Kimberlin 2013, Pinninti and Kimberlin 2013) peut être très grave. Sans traitement, 80 % des nourrissons infectés par le HSV meurent, et ceux qui survivent sont souvent porteurs de séquelles et dommages physiques tout au long de leur vie

(Brown 2004). Dans une étude aux États-Unis d'Amérique (USA), quatre des neuf enfants pourraient développer l'herpès néonatal si la mère a l'herpès génital, dont l'un est mort (Brown, Selke et al. 1997). Ainsi, le diagnostic précoce du virus est important pour la détermination de la gestion clinique et à la compréhension de l'évolution clinique et le pronostic.

De nombreuses méthodes sont déjà établis pour l'identification des virus HSV1 et HSV2. Parmi ceux-ci, la culture cellulaire (Singh, Preiksaitis et al. 2005) et les tests sérologiques (Wald and Ashley-Morrow 2002) sont les méthodes standards de diagnostic du virus de l'herpès simplex (HSV). Ces méthodes nécessitent toutefois beaucoup de temps pour obtenir les résultats finaux. La PCR est une méthode de dosage très sensible pour la détection de l'ADN du HSV (Johnson, Nelson et al. 2000, Gardella, Huang et al. 2010) par rapport à la détection antigénique ou des procédés de culture des cellules (Koenig, Reynolds et al. 2001). Le suivi de l'infection HSV et sa progression peut être contrôlée par l'analyse quantitative de l'ADN viral via la PCR en temps réel (Enomoto, Yoshikawa et al. 2005). Cependant, cette méthode n'est pas encore devenue une procédure courante dans les laboratoires hospitaliers et les milieux à faibles ressources, et ne convient pas pour des applications de PDS en raison de l'exigence d'un matériel coûteux et spécifique (un cycleur thermique), des espaces de laboratoire et des techniciens formés.

La technologie LAMP est une méthode robuste pour identifier la maladie dans très peu de temps. Le coût global de ce travail est très faible comparé à d'autres technologies existantes. Le seul équipement nécessaire est un bloc thermique. La technique présente à la fois une haute spécificité et une efficacité d'amplification à haut rendement en raison de l'utilisation de quatre amorces qui reconnaissent les six séquences distinctes de l'ADN cible. Contrairement à la PCR, il n'y a pas de temps perdu à cause des changements de température à chaque étape. Ainsi, l'ensemble du procédé peut être réalisé en peu de temps (de 30 à 60 minutes). Comme la réaction peut être conduite à la température optimale pour la fonction enzymatique, les réactions d'inhibition qui se produisent souvent à des étapes ultérieures d'amplifications typiquement observées pour la PCR sont moins susceptibles de se produire (Enomoto, Yoshikawa et al. 2005). Ainsi, cette méthode pourrait être un outil précieux pour le diagnostic rapide du HSV (Reddy, Balne et al. 2011) ainsi que d'autres maladies infectieuses dans des laboratoires commerciaux et hospitaliers (Mori and Notomi 2009).

Les dispositifs microfluidiques sur support plastique/papier comprennent de nombreuses caractéristiques souhaitées appropriées d'un test de l'ADN viral au point de soins (Fu, Ramsey et al. 2011, Pollock, Rolland et al. 2012). Ces dispositifs de diagnostic sont peu coûteux, portables et simples à utiliser, ce qui les rend appropriés pour les pays à faibles ressources (Martinez, Phillips et al. 2010). Nous avons développé un simple étui en plastique pour la détection de HSV-1 et HSV-2. Dans ce dispositif, nous pouvons détecter l'ADN viral en 45 minutes en utilisant la méthode LAMP. Puisque la méthode LAMP est moins sensible aux substances inhibitrices présentes dans l'échantillon réel (Enomoto, Yoshikawa et al. 2005, Kaneko, Iida et al. 2005), nous avons également pu détecter l'ADN viral sans le purifier. Le résultat final a été évalué à l'œil nu par l'addition du colorant bleu hydroxynaphthole (HNB) dans le mélange réactionnel. (Goto, Honda et al. 2009, Das, Babiuk et al. 2012). Les résultats ont été confirmés par électrophorèse sur gel d'agarose à 2%.

2.2 Matériaux et Méthodes

Avant de développer le dispositif d'amplification, nous avons optimisé le protocole de LAMP dans des tubes de PCR de 0,2 ml classiques pour amplifier l'ADN de HSV. Le résultat final a été analysé par le changement de couleur du colorant HNB du pourpre au bleu clair et en outre confirmé par électrophorèse sur gel d'agarose à 2%.

2.2.1 L'amplification LAMP

La réaction de LAMP dans ce travail a été effectuée dans un flacon de 25 ml, contenant 0,2 mM de chacun des amorces externes (F3/B3), 0,8 mM de chacun de des amorces de boucle (LF / LB), 1,6 mM de chacun des amorces internes. La réaction d'amplification a été réalisée en utilisant chacun des amorces LAMP préalablement décrits pour le virus de l'herpès simplex 1(Kaneko, Iida et al. 2005) et 2 (Enomoto, Yoshikawa et al. 2005). Les détails des amorces LAMP utilisés dans cette étude sont présentés au tableau 1. 0,4 mM dNTPs, 0.64 mM bétaïne (Sigma), 3 mM $MgSO_4$, Bst polymérase (grand Fragment, 1600 unités, 8000 U / ml, New England Biolabs), le tampon de réaction polymérase 1X ThermoPole (New England Biolabs Inc.) et 5 µl de l'ADN double-brin cible. 0,15 µl / ml du bleu d'hydroxynapthanole a été

également ajouté au mélange réactionnel pour visualiser l'amplification de l'ADN du HSV. 20 µl d'huile minérale ont été ajoutés à chaque tube pour éviter l'évaporation d'eau lorsque le tube est placé sur le bloc chauffant. Le mélange a été incubé à 65 °C pendant 45 minutes sur le bloc chauffant.

Tableau 1: Amorces utilisés pour LAMP et position sur le gène.

Nom des amorces	Sequence des amorces	Nom du gène	Localisation des séquences cibles
Set A:			Nucleotide position (9721-10080)
HSV1-F3	5_-CAGCCACACACCTGTGAA-3_(F3)		F3(9756-9773)
HSV1-B3	5_-TCCGTCGAGGCATCGTTAG-3_(B3c)		B3(10008-10026)
HSV1-FIP	5_-CAGACGTTCCGTTGGTAGGTCACTTTGACTATTCGCGCACC-3_(F1c-F2)		F2(9788-9807)
HSV1-BIP	5_-CCATCATCGCCACGTCGGACTCGGCGTCTGCTTTTTGTG-3_(B1-B2c)	UL1	B2(9969-9987)
HSV1-LPF	5_-AAATCCTGTCGCCCTACACAGCGG-3_(LPFc)		F1(9836-9857)
HSV1-LPB	5_-CACCCCGCGACGGGACGCCG-3_(LPB)		B1(9926-9945)
			LPF(9810-9833)
			LPB(9948-9967)
Set B:			Nucleotide position (138901-139140)
HSV2-F3	5_-GGCCTTGACCGAGGACAC-3_(F3)		F3(138909-138926)
HSV2-B3	5_-CGACTCCACGGATGCAGT-3_(B3c)		B3(139120-139137)
HSV2-FIP	5_-TCGACTGAGGGTGCCATGGCGTCCTCCGATTCGCCTACG-3_(F1c-F2)		F2(138926-138945)
HSV2-BIP	5_-GCAACCACTACTCCCCCCGACCGTTTCCTCCGGCGTAA-3_(B1-B2c)		B2(139082-139099)
HSV2-LPF	5_-GCCGACACAGGGAGGGGCGT-3_(LPFc)	UL5	F1(138984-139004)
HSV2-LPB	5_-GATGGCCACACAAGCCGCAA-3_(LPB)		B1(139030-139050)
			LPF(138961-138980)
			LPB(139053-139072)

2.2.2 Fabrication et fonctionnement des étuis en plastique :

Pour la mise au point du dispositif, un sac en plastique ordinaire (polypropylène) a été utilisé. Une petite chambre en plastique (1,5 x 0,2 cm) a été faite par en utilisant un appareil chaud pour sceller le plastique (Fig. 2). Cette petite chambre peut contenir 25 µl de mélange réactionnel comprenant 5 µl d'échantillon. Un petit morceau de plastique a été introduit dans la chambre de sorte que le liquide peut facilement rester à l'intérieur. Le mélange réactionnel et l'échantillon ont été insérés à l'aide longs pipettes et ensuite scellés. Ensuite, le dispositif a été maintenu sur un bloc chauffant à 65 °C pendant 45 minutes pour amplifier l'ADN. Le bleu d'hydroxy naphthanol (HNB), qui est un indicateur de métal spécifique au magnésium et aussi un réactif colorimétrique pour les alcalino-terreux, est alors ajouté.

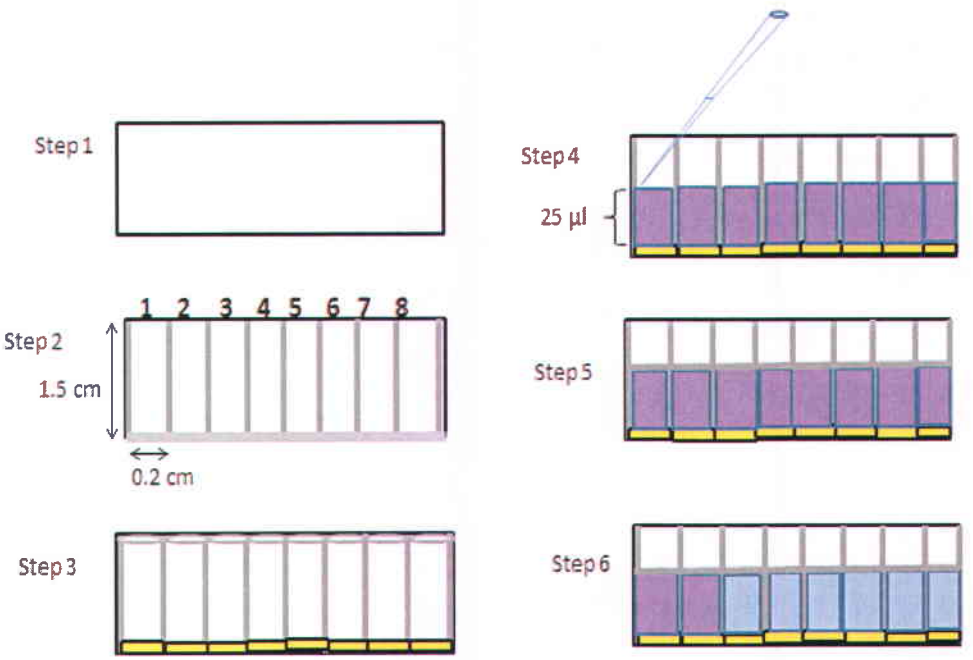

Figure 2: Diagramme schématique des étapes de fabrication des étuis faites à partir d'un sac en plastique. Étape 1: des pièces de formes rectangulaires ont été coupées à partir d'un sac en plastique; Étape 2: ces pièces ont été pressées et scellées à chaud apour faire de petites chambres ou étuis (1,5 cm de longueur et 0,2 cm de largeur); Étape 3: petites espèces de plastique (de couleur jaune) ont été insérées à l'intérieur de chaque chambre de sorte que le liquide à l'intérieur de la chambre peut rester facilement; Étape 4: Réactifs et échantillon insérées avec une pipette; Étape 5: les chambres sont scellées horizontalement avec un scellant; Étape 6: l'ensemble est placé sur le bloc thermique pendant 45 min à 65 °C. Les réactions positives sont indentifées par un changement de couleur du violet vers le bleu.

Une réaction positive est indiquée par un changement de couleur du violet au bleu ciel. Ainsi, après achèvement de la réaction, le résultat a été analysé visuellement sans aucun instrument. Le résultat a été confirmé par électrophorèse sur gel d'agarose à 2%.

Ce test qualitatif LAMP avec détection colorimétrique, intégré dans ce très simple sachet en plastique à faible coût, a un grand potentiel pour le diagnostic rapide du HSV dans les cliniques éloignées, les laboratoires sur champs, les laboratoires hospitaliers et également dans les pays à faibles ressources.

2.2.3 LAMP d'un échantillon réel

Des recherches précédentes ont rapporté que le test LAMP est moins affecté en termes de sensibilité par la présence de substances inhibitrices dans des échantillons cliniques par rapport à la PCR (Enomoto, Yoshikawa et al. 2005, Kaneko, Iida et al. 2005). Pour évaluer ces avantages, nous avons analysé la tolérance de LAMP aux virus réel. Un volume viral a été dilué soit dans une solution tampon de phosphate salin, de l'eau distillée, ou dans un milieu de culture (Dulbecco's Modified Eagle's medium or DMEM). La concentration du stock virale était de 1,20 $\times 10^4$ PFU / µl. (Figure 5) On a comparé ce résultat avec de l'ADN purifié.

Pour voir la limite de détection d'ADN dans un échantillon réel, le stock de virus a été dilué en série avec un tampon phosphate salin (PBS). (Figure 6) Le stock de virus d'origine contenait 1 x 10^4 UFP / µl de HSV-1 (Fig. 6A, 6B) et HSV-2 (figure 6C, 6D). Les fluides viraux dilués ont été utilisés directement comme échantillons sans extraction d'ADN. Cinq microlitres de chaque échantillon ont été ajoutés au mélange réactionnel à un volume final de 25 µl, et la réaction a été effectuée à 65 °C pendant 1 h.

2.3 Résultats

2.3.1 Optimisation du LAMP dans les tubes

Nous avons optimisé le temps, la température et la concentration du dNTP pour le test LAMP. Nous avons fait le test LAMP à trois températures différentes (60 °C, 63 °C et 66 °C) à la fois pour l'ADN lu HSV-1 et HSV-2. Nous avons trouvé que l'ADN du HSV-1 a été amplifié à chacune des trois températures, alors que l'ADN du HSV-2 a été seulement amplifié à 63-66 ° C (tableau 2).

Pour optimiser le temps, nous avons effectué l'essai pendent 10, 20, 30 et 40 minutes à 65 ° C (figure 3). Nous avons utilisé 10 ng d'ADN / réaction comme contrôle positif et de l'eau comme contrôle négatif. Nous avons également utilisé une autre concentration d'ADN inferieure à la limite de détection pour les deux virus (10 fg / µl d'ADN de HSV-1 et 1 fg / µl d'ADN de HSV-2). Dans le cas de HSV-1, nous avons constaté que le contrôle positif était positif à 20 minutes alors que la meilleure bande a été trouvée à 30 minutes. Cependant, la concentration faible de

l'ADN de HSV-1 (10fg / µl) a été détectée à 40 minutes. D'autre part, le témoin HSV-2 positif était positif à 30 minutes

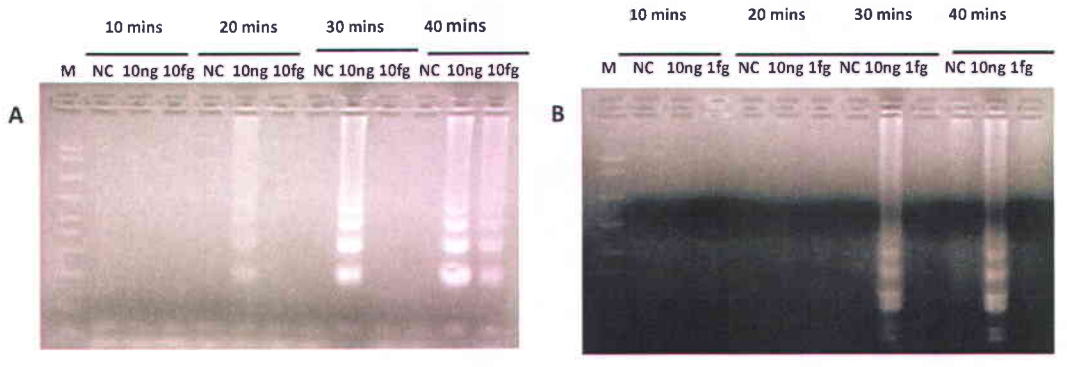

Figure 3: L'analyse du gel pour l'optimisation du temps de l'ADN du HSV.
L'eau a été utilisée comme témoin négatif (NC) et 10 ng / µl d'ADN a été utilisée comme contrôle positif. 10 fg / µl d'ADN de HSV-1 a été utilisée et a été la plus faible limite de détection pour le HSV-1 (A). Pour le HSV-2, 1 fg / µl d'ADN qui a été utilisée et a était notre limite de détection la plus faible du HSV-2 (B). La ligne du haut représente le temps d'exécution en minutes

2.3.2 Sensibilité et Spécificité LAMP

La sensibilité de l'ADN viral a été évaluée en utilisant de l'ADN de HSV diluée 10 fois en série à partir de 10 ng / µl à 1 fg / µl et le dosage de LAMP a été effectué à 65 °C pendant 45 minutes. Nous avons fait ce test dans des tubes et des dispositifs en plastique avec 5 µl d'ADN de chaque concentration. Nous avons constaté que 10 fg / µl d'ADN de HSV-1 a été détectée à la fois dans le tube et le dispositif en plastique (figure 4A, 4B et tableau 2). D'autre part une 1 fg / µl d'ADN de HSV-2 a été détectée à la fois dans le tube et le dispositif de plastique. (Fig. 4C, 4D et tableau 2). L'eau et l'ADN d'*E.coli* ont été utilisées comme contrôles négatifs. Nous n'avons pas vu de changement de couleur ou de la bande (sur gel) dans les contrôles négatifs.

2.3.3 LAMP dans l'échantillon réel

Pour vérifier l'effet des substances inhibitrices présentes dans l'échantillon réel et la nécessité de l'extraction de l'ADN, nous avons dilué le stock viral dans un tampon phosphate salin (PBS), de l'eau distillée et le milieu de culture (Dulbecco's Modified Eagle's medium or DMEM). La

concentration du stock virale était de 1,20 × 10^4 PFU / µl. Nous avons utilisé l'ADN comme contrôle positif et de l'eau comme contrôle négatif.

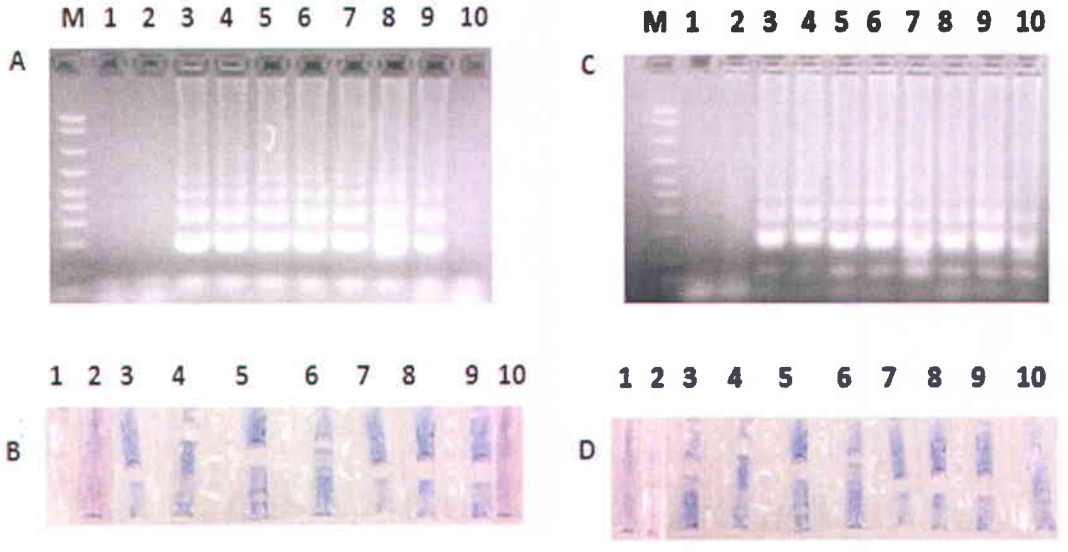

Figure 4: Sensibilité de l'ADN du VHS en utilisant un test LAMP.
Le test a été effectué dans le tube et le dispositif en plastique (les images du tube ne sont pas représentées) A: analyse du gel Agarose du produit LAMPE de l'ADN du HSV-1; B: LAMP dans le dispositif en plastique avec l'ADN du HSV-1, la couleur est due au colorant HNB. La couleur bleue indique la réaction positive tandis que la couleur pourpre indique le résultat négatif; C: analyse du gel Agarose du produit LAMPE de l'ADN du HSV-2; D: LAMP dans le dispositif en plastique avec l'ADN du HSV-2. Dans tous les cas M = 2 kb Markeur; 1 = NC (H_2O) ; 2 = NC (1 ng / µl E.Coli ADN); 3 = 6,08 x 10^7 copies / µl ADN; 4 = ADN de 1 ng; 5 = 100 pg d'ADN; 6 = ADN de 10 pg; 7 = 1 pg d'ADN; 8 = 100 fg ADN, 9 = 10fg ADN et 10 = 1 fg ADN. Le volume total des réactifs était de 25 µl dont 5 µl d'ADN

En utilisant DMEM, nous avons vu la bande sur l'analyse de gel, mais pas de changement de couleur. Ceci est dû à la présence de la couleur rouge dans le DMEM. Après vérification de la nécessité de l'extraction de l'ADN, nous avons essayé de trouver la limite de détection de l'ADN du HSV en utilisant des échantillons réels dans le dispositif en plastique. Nous avons dilué en série de HSV-1 (Figure 6A et 6B)

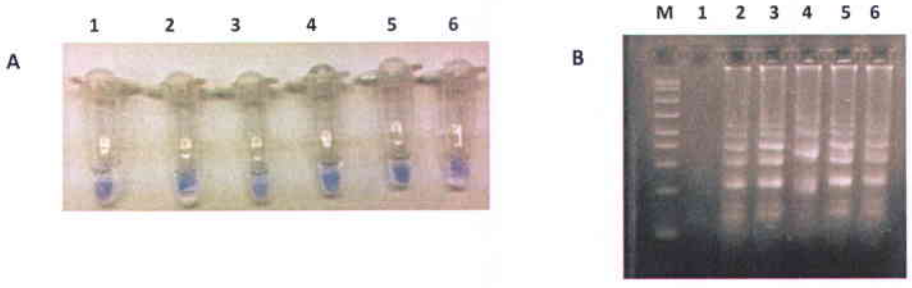

Figure 5: LAMP dans l'échantillon réel.
Produits LAMP dans le tube (A) et sur le gel d'agarose à 2% (B). M = Marqueur; 1 = NC (H_2O) 2 = PC (HSV1, 1 ng / µl); 3 = virus HSV1 dilué dans l'eau; 4 = virus HSV1 dilué dans l'eau; 5 = virus HSV1 dilué dans du PBS; 6 = virus HSV1 dilué dans du DMEM. 1,2 x 10^4 PFU / µl de virus ont été utilisés ici.

et une solution virale dix fois diluée du HSV-2 (figure 6C et 6D) à partir de 10^4 pfu / µl à 10^0 pfu / µl. L'eau a été utilisée comme contrôle négatif et seules les cellules, sans virus (Lane 7) ont été utilisés pour voir si l'ADN cellulaire interfère dans l'amplification. Nous avons pu détecter jusqu'à 100 pfu / µl de concentration virale dans les deux cas.

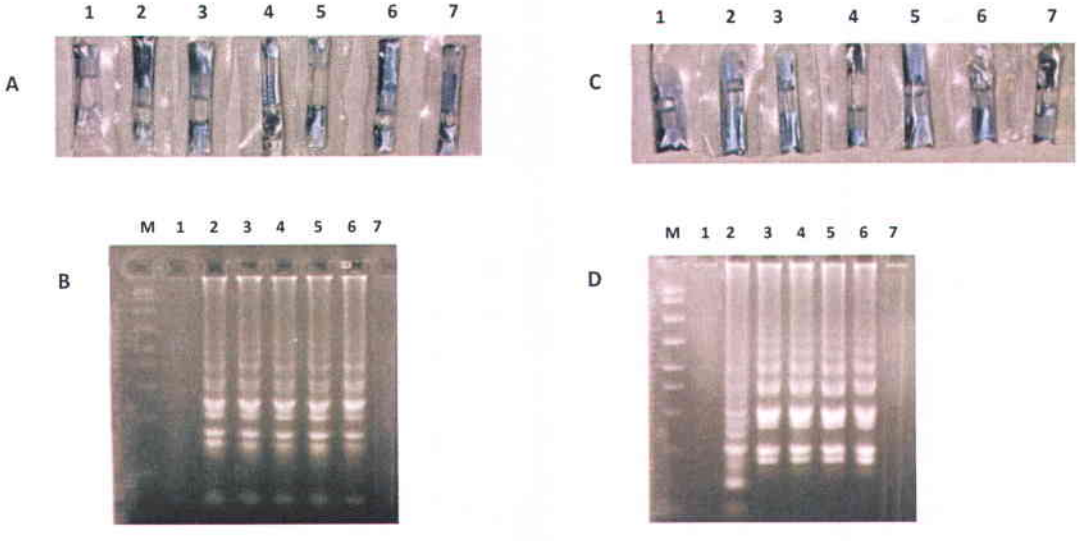

Figure 6: Limite de détection du HSV DNA dans un échantillon réel sans purification d'DNA dans le dispositif en plastique. **(A)**: produit LAMP (1-7) du HSV-1. **(B)**: électrophorèse sur gel d'agarose de A; C: produit LAMP (1-7) du HSV-2. D: électrophorèse sur gel d'agarose de C. M= 2 Kb Marqueur; 1= NC (H_2O); 2= 10^0 pfu/µl; 3=10^1 pfu/µl; 4=10^2 pfu/µl; 5= 10^3 pfu/µl; 6= 10^4 pfu/µl and 7= cellules sans virus.

Ces résultats suggèrent que l'étape d'extraction d'ADN pourrait être omise, ce qui fera gagner du temps ainsi que diminuer le coût.

Table 2: optimisation du LAMP: temps, Température et sensibilité

Virus	amorces	Temps de détection (amplifié a 10′,20′,30′, 40′)	Temp (amplifié a 60°C, 63°C, 66°C)	Sensibilité (l'ADN a ete diluée diluted 10 fois de 10ng/µl-1fg/µl)
HSV-1	Set A (Table 1)	Temps de saturation: 50ng/réaction: 20′-40′ 50fg/réaction: 40′	60-66°C	50fg/réaction
HSV-2	Set B (Table 1)	Temps de saturation: 50ng/réaction: 30′-40′ 5fg/réaction: 40′	63-66°C	5fg/réaction

2.4 Discussion

Pour optimiser la méthode LAMP, nous avons utilisé deux concentrations différentes de dNTP (0,4 mM et 1,4 mM). Le gel des deux concentrations a montré la bande désirée (données non présentées). Lorsque 1,4 mM dNTP a été utilisé, la couleur a changé, en passant du mauve au bleu avant l'addition de l'échantillon. L'une des explications possibles est que l'ajout d'une concentration élevée du dNTP modifie le pH et ainsi la couleur du colorant (HNB). Ainsi, une plus faible concentration de dNTP (0,4 mM) a été utilisée. Enfin, nous avons décidé de continuer l'essai à 65 °C pendant 45 minutes concentration de 0,4 mM du dNTP.

Pour étudier la réactivité croisée des amorces utilisées dans ces tests, nous avons utilisé l'ADN du *E. coli* ainsi qu'un contrôle négatif (eau). Nous n'avons pas vu de changement de couleur ou de bandes (sur gel) dans l'ADN du *E. coli* et le contrôle négatif (figure 4). Cela prouve la spécificité de ces amorces utilisées dans le dosage, ainsi que l'absence d'interférence du matériau plastique dans la réaction.

La sensibilité du test vers de l'ADN viral a été évalués dans des tubes ainsi que dans des dispositifs en plastique. Nous avons trouvé la même limite de détection à la fois dans le tube et le dispositif de plastique pour les deux virus (figure 4A, 4B, 4C). Celle-ci est de 10 fg/µl et 50 fg/réaction (étant donné que 5 µl d'échantillon ont été utilisés) pour HSV1 et 1 fg/µl 5 fg/réaction pour HSV2. Ces résultats suggèrent que le tube et le dispositif en plastique fonctionnent de la même manière. La limite de détection du virus réel a était de 10^0 pfu / µl de concentration virale dans les deux cas (figure 6A et 6B pour HSV-1 et la figure 6C et 6D pour HSV-2). Les cellules sans virus ont également été utilisées pour voir si l'ADN cellulaire interfère dans l'amplification. Nous avons vu que les cellules sans virus (Lane 7) ne changent pas la couleur ce qui prouve que l'ADN cellulaire n'intervient pas dans la réaction d'amplification.

La méthode LAPM est incorporée dans différents types de dispositif de point de soins (PDS) pour détecter les maladies infectieuses (Fang, Liu et al. 2010, Fang, Chen et al. 2011). Ces dispositifs nécessitent des étapes complexes de microfabrication. Les dispositifs à base de papier-plastique sont relativement peu coûteux et faciles à manipuler. D'autres dispositifs à base de papier-plastique sont disponibles (Rohrman and Richards-Kortum 2012), mais ils ont besoin de d'étapes de traitement des échantillons et analyse des résultats. Roskos K. et al ont couplé l'amplification isotherme avec NALF (flux latéral des Acides Nucléiques) (Roskos, Hickerson et al. 2013). Ils ont développé une cartouche qui consiste en des poches de réaction et des poches pompes. Il faut plusieurs étapes pour la fabrication du dispositif, ainsi qu'une étape de traitement des échantillons. Le résultat final a été analysé par dosage à écoulement latéral. Le sachet en plastique discuté ici est très simple et n'a pas besoin de toutes les étapes de microfabrication. Les seuls instruments de laboratoire nécessaires pour réaliser le dosage sont des micropipettes, embouts de pipette, et un bloc thermique. Comme la LAMP est moins affectée par les autres éléments présents dans l'échantillon réel, nous n'avons pas besoin de traiter ou purifier les échantillons. Nous pouvons donc ignorer les étapes de traitement des échantillons qui durent presque 1 heure.

Un autre avantage est l'utilisation du colorant HNB qui change sa couleur avant et après la réaction. Le bleu hydroxynaphtole (HNB), un indicateur de métal pour le magnésium et un réactif colorimétrique pour les ions de métaux alcalino-terreux, a été ajouté au mélange

réactionnel qui est généralement de couleur violette. Lorsque la réaction de LAMP est produite, le pyrophosphate de magnésium est produit comme sous-produit, de sorte que la quantité des ions libres de magnésium (Mg^{+2}) est réduite dans le mélange réactionnel. Comme le magnésium est réduit, la couleur du colorant HNB change du violet au bleu clair. Une réaction positive est indiquée par un changement de couleur du violet au bleu ciel. Ainsi, après achèvement de la réaction, le résultat peut être analysé visuellement sans aucun instrument. Nous n'avons pas besoin de faire l'électrophorèse sur gel ou de se connecter à d'autres appareils d'analyse pour traiter le résultat. Donc, nous pourrions économiser encore 1 heure ici. Dans seulement 45 minutes, nous avons obtenu le résultat final. Le dispositif est léger, petit et facile à construire. Par conséquent, je crois que tout cela fait de notre dispositif un candidat idéal pour le diagnostic en point de soins (PDS) en laboratoires et dans les milieux à faibles ressources.

Poursuite de l'amélioration et la modification du dispositif est nécessaire pour pouvoir l'utiliser dans le domaine réel. Nous prévoyons lyophiliser les amorces en papier, puis les placer à l'intérieur de la poche plastique, ce qui permettra de réduire réactifs étapes d'addition et permettra également de stocker à température ambiante. En changeant le motif de la fermeture par le scellant, nous pouvons modifier la conception de ce dispositif. Pour créer un périphérique multiplexé, nous ferons un échantillon de chargement, de sorte que l'échantillon puisse passer dans différentes chambres où différents jeux d'amorces lyophilisés s'y retrouvent déjà. Ainsi, plus qu'un virus peut être détecté dans un même dispositif.

3. Détection électrochimique par LAMP

La détection des produits de LAMP peut se faire de plusieurs façons : par la méthode colorimétrique, l'électrophorèse sur gel d'agarose, électrochimique ou même par l'œil nu. Dans le chapitre précédent l'exemple de détection colorimétrique de produits LAMP a été discuté. Dans ce chapitre la détection électrochimique de produits LAMP sera décrite. Les travaux suivants ont été effectués par notre groupe à l'INRS et représentent le suivi en temps réel du produit LAMP amplifié par une sonde redox. Ce travail a été publié dans le journal *Analyst, 2013* où j'étais l'un des co-auteurs.

Analyst RSCPublishing

PAPER

Cite this: Analyst, 2013, 138, 907

Real-time electrochemical detection of pathogen DNA using electrostatic interaction of a redox probe†

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3.1 Résumé

Redox électrostatique interaction sonde a été largement utilisé pour quantification de l'ADN. Dans cet article, notre groupe a établi une preuve de principe en utilisant la molécule ruthénium hexaamine [Ru $(NH_3)^6]^3$ $^+$. Notre groupe a appliqué cette méthode pour le controle électrochimique en temps réel d'une l'amplification isotherme facilitée en boucle (LAMP) amplicon des gènes cibles de l'Escherichia coli et le Staphylococcus aureus par voltamètrie a vague carré (SWV). L'interaction du ruthénium hexaamine avec l'ADN libre en solution sans avoir été immobilisée sur la surface de la biopuce nous a permis d'éliminer l'étape de l'immobilisation de la sonde par incubation prolongée pour la quantification de l'ADN .

Nous avons mesuré les changements du courant cathodique en utilisant des biopuces peu coûteux du tpe screen-printed, en présence et en absence d'amplicons LAMP de l'ADN cible. En utilisant cette nouvelle sonde, nous avons bien mené l'amplification isotherme en temps réel ainsi que la détection en moins de 30 min pour S. aureus et E. coli avec une sensibilité jusqu'à 30 copies μL^{-1} et 20 copies μL^{-1}, respectivement. L'intensité du pic cathodique est liée à la formation de l'amplicon et de la quantité de gabarits génomiques d'ADN introduits. Surtout, puisque l'immobilisation laborieuse de la sonde n'est pas nécessaire du tout, et surtout que l'amplification in vitro et le suivi en temps réel sont réalisés dans un tube en polypropylène unique à l'aide d'une seule biopuce, cette nouvelle approche pourrait éviter tout risque de contamination croisée dans l'ensemble de la procédure.

4. Conclusion

POCT minimise l'écart entre les diagnostics de laboratoires centralisés et les services de santé en milieu rural. En particulier pour les maladies infectieuses telles que le VIH / SIDA et la tuberculose, où la détection précoce est impératif d'améliorer les soins de ces maladies, la réalisation d'un test précis, simple, rapide et robuste peut modifier considérablement l'épidémiologie et le contrôle de la maladie. Les dispositifs immuno-chromatographique à flux latéraux pour la détection d'anticorps ou d'antigène dominent actuellement les TPDSs disponibles, et le développement de ces dispositifs est appuyée sur la découverte et l'optimisation des biomarqueurs définitives appropriés pour ces plates-formes. Dans l'avenir, cependant, il y aura un besoin croissant de développer des TPDSs rentables utilisant des biomarqueurs qui sont bien établis dans les laboratoires, mais ne sont pas actuellement prêts pour les points de soins, tels que les tests moléculaires pour la résistance aux médicaments dans la tuberculose et la charge virale du VIH et de l'hépatite virale (Mohd Hanafiah, Garcia et al. 2013).

Il ne fait aucun doute que la nécessité des diagnostics aux points de soins est cruciale dans les pays en développement et les laboratoires hospitaliers à faibles ressources dans les pays développés. Par exemple, un instrument, de traitement automatisé et robotisé à haut débit n'est généralement pas accessible ou réalisable dans les milieux à faibles ressources qui manquent de d'infrastructure de laboratoire nécessaires (Yager, Domingo et al. 2008).

Nous avons mis au point un étui en matière plastique qui permet la détection de 10^0 pfu / µl du virus herpès simplex 1 et 2 par l'amplification isotherme facilitée en boucle dans un délai de 45 minutes. Dans ce dispositif, il n'est pas nécessaire de préalablement purifier l'échantillon. De plus, la détection colorimétrique à l'œil nu rend facile l'analyse des résultats. Ce dispositif est facile à manipuler, portable, de faible coût et n'exige pas des d'instruments coûteux. Ces avantages en font un candidat idéal pour le point diagnostic dans les PDS en laboratoire et dans les pays à faibles ressources.

Enfin, je tiens à conclure que les expériences effectuées pour la détection des virus (herpès simplex virus) et bactéries (*S. aureus et E. coli*) utilisant la technologie LAMP ont donné des résultats robustes. La détection colorimétrique (qualitative) et électrochimique (temps réel et

quantitative) de produits LAMP décrit ici servent non seulement à gagner du temps, mais aussi de réduire le coût total de diagnostic et traitement maladie. Ces procédés de détection peuvent être utilisés pour d'autres virus / bactéries provoquant la maladie, la détection des tumeurs de gènes, l'identification des cibles de médicaments, ainsi que dans polymorphisme de nucléotide unique (SNP) analyse. Par conséquent, je crois que toutes ces choses ensemble peuvent rendre la technologie LAMP un candidat idéal pour le point de service (PDS) diagnostic dans les deux milieux à faibles ressources et de laboratoire.

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