DEVELOPMENT OF REAL-TIME FLUORESCENCE MULTIPLEX LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY FOR SIMULTANEOUS DETECTION OF *Streptococcus pneumoniae* AND *Haemophilus influenzae*

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DEVELOPMENT OF REAL-TIME FLUORESCENCE MULTIPLEX LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY FOR SIMULTANEOUS DETECTION OF *Streptococcus pneumoniae* AND *Heamophilus influenzae*

ABSTRACT

Pneumonia is one of the leading causes of death in children worldwide, accounting for 19% of all deaths of children under the age of five years old. Rapid identification of the causative agents is crucial to ensure prompt treatment is given to reduce infection-related morbidity and mortality rate among hospitalized patients. Current conventional laboratory culturing methods are time consuming and lack of sensitivity and specificity. The loop-mediated isothermal amplification (LAMP) method is an alternative amplification technique which amplifies the genes of interest at constant temperature. It is rapid, highly specific and efficient. Hence, the objective of the study was to develop and evaluate the specificity and sensitivity of two monoplex LAMP assays targeting *Streptococcus pneumoniae* and *Haemophilus influenzae*. The developed monoplex LAMP assays were further combined and optimized into a real-time multiplex fluorescent LAMP (mf-LAMP) assay for simultaneous differential detection of the two pathogens. Two sets of species-specific primers targeting *lytA* (autolysin) gene from *S. pneumoniae* and *pal* (outer membrane protein P6) gene from *H. influenzae* were used and their specificity and sensitivity were evaluated against 59 targeted and non-targeted bacterial strains. In monoplex LAMP assay, positive amplification for *S. pneumoniae* was found between 30 min – 35 min with DNA concentration ranged between 108 ng-1500 ng per reaction while 17 min - 20 min for *H. influenzae* strains with DNA concentration between 120 ng – 400 ng per reaction. The detection limit for each of the LAMP assay was also found 6.6 ng per reaction for *S. pneumoniae* and 32.7 ng per reaction for *H. influenzae*. Thereafter, a real-time mf-LAMP was constructed by combining these two sets of ten primers in a single reaction with an additional

fluorescent-labelled primer termed FIP (Forward Inner Primer) to generate a single-step, closed-tube real-time DNA amplification by detection of fluorescent signal. The mf-LAMP assay was further optimized and validated using 20 strains of *S. pneumoniae*, 20 strains of *H. influenzae* and 19 non-targeted strains including *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and methicillin-sensitive *Staphylococcus aureus*. This newly developed mf-LAMP assay showed 100% specificity toward both targeted strains with no cross-reaction with non-targeted strains. However, the time for positive amplification were delayed, 40 - 45 min for *S. pneumoniae* and 33 - 39 min for *H. influenzae* detection compared to monoplex LAMP assays. The detection limit was 66 pg per reaction for *S. pneumoniae* and 32.7 pg per reaction for *H. influenzae* demonstrating 100 and 1000 time higher sensitivity in detection of *S. pneumoniae* and *H. influenzae* respectively compared to monoplex LAMP assays. Moreover, the detection limit for mf-LAMP was equivalent to conventional PCR. Although the cost for generating fluorescent labelled primers was higher, the mf-LAMP assay allows real-time detection of two pathogens in a single-step closed-tube assay that requires short preparation time and smaller amount of reagents in comparison to monoplex LAMP assay. In conclusion, the multiplex LAMP assay developed was a simple and rapid method for identification of pathogens associated with childhood pneumonia.

Keywords: Pneumonia, *Streptococcus pneumonie*, *Haemophilus influenzae*, loop-mediated isothermal amplification (LAMP), multiplex fluorescent LAMP, Polymerase Chain Reaction (PCR), autolysin, outer membrane protein P6

PEMBANGUNAN UJIAN MULTIPLEKS REAL-TIME LOOP-MEDIATED ISOTHERMAL AMPLIFICATION YANG BERPENDAFLOUR BAGI PENGESANAN SERTA PEMBEZAAN SERENTAK PATOGEN *Streptococcus pneumoniae* DAN *Haemophilus influenzae*

ABSTRAK

Radang paru-paru merupakan salah satu punca utama kematian kanak-kanak di seluruh dunia, iaitu 19 % daripada semua kematian kanak-kanak di bawah umur lima tahun. Pengesanan patogen dengan cepat adalah sangat penting bagi memastikan rawatan segera diberi bagi mengurangkan kadar morbiditi dan kematian berkaitan jangkitan kuman di kalangan pesakit hospital. Pengesanan patogen kini dengan menggunakan kaedah konvensional pengkulturan mengambil masa yang lama dan kurang sensitif dan spesifik Aplikasi *Loop-mediated isothermal amplification (LAMP)* merupakan teknik aplikasi alternatif yang mengamplifikasi gen kajian pada suhu yang tetap. Teknik gen amplifikasi ini adalah cepat, sangat spesifik dan efisien. Oleh itu, objektif bagi kajian ini adalah untuk membangunkan dan menilai spesifisiti dan sensitiviti dua ujian monopleks LAMP yang menyasarkan *Streptococcus pneumoniae* dan *Haemophilus influenzae*. Ujian monopleks yang dibangunkan ini akan digabungkan dan dioptimumkan sebagai ujian multipleks 'real-time' berpendaflour (mf-LAMP) untuk pengesanan serta pembezaan serentak dua patogen tersebut. Dua set primer spesifik kepada spesies yang menyasankan gen *lytA (autolysin)* daripada *S. pneumoniae* and gen *pal (outer membrance protein P6)* daripada *H. influenzae* telah digunakandan spesifisiti dan kekhususan mereka telah dinilai terhadap 59 strain bakteria yang sisasarkan dan tidak disasarkan. Dalam ujian monopleks LAMP, amplifikasi positif untuk *S. pneumoniae* didapati antara 30 min – 35 min dengan konsentrasi DNA dalam lingkungan 108 ng – 1500 ng setiap reaksi manakala 17 min- 20 min untuk strain H. influenza dengan konsentrasi DNA di antara 120ng – 400 ng setiap reaksi. Had pengesanan untuk setiap ujian LAMP didapati 6.6 ng setiap reaksi bagi *S. pneumoniae*

dan 32.7 ng setiap reaksi untuk *H. influenzae*. Maka ujian multipleks 'real-time' LAMP (mf-LAMP) telah dibangunkan dengan menggabungkan kedua-dua set primer yang mengandungi sepuluh primer dalam satu reaksi dengan tambahan *fluorescent-labelled primer* iaitu *FIP (Forward Inner Primer)* dari setiap set untuk menyara isyarat satu langkah, tiub-tertutup, amplikasi 'real-time' berpendaflour semasa sintesis DNA. Ujian mf-LAMP dioptimumkan dan dinilai dengan menggunakan 20 strain *S. pneumoniae*, 20 strain *H. influenzae* dan 19 strain patogen bukan sasaran termasuk *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* dan methicillin sensitif *Staphylococcus aureus*. Ujian mf-LAMP yang baru dibangunkan ini menunjukkan 100 % spesifisiti terhadap kedua-dua strain sasaran tanpa menunjukkan reaksi silang dengan strain bukan sasaran. Walau bagaimanapun, masa untuk mencapai amplifikasi positif telah dilambatkan, 40 min – 45 min untuk *S. pneumoniae* dan 33 min – 39 min untuk *H. influenzae*. Had pengesanan didapati 66 pg dalam setiap reaksi untuk *S. pneumoniae* dan 32.7 ng dalam setiap reaksi untuk *H. influenzae* menunjukkan 100 dan 1000 kali ganda lebih sensitive dalam pengesanan *S. pneumoniae* dan *H. influenzae* berbanding ujian monopleks LAMP Malahan, had pengesanan untuk mf-LAMP adalah setara dengan ujian monopleks LAMP yang mana kedua-duanya boleh mengesan sehingga 66 ng setiap reaksi untuk *S. pneumoniae* dan 32.7 pg setiap reaksi untuk *H. influenzae*. Walaupun kos untuk menjana *"fluorescent labelled primers"* adalah lebih tinggi, namun ujian mf-LAMP ini membenarkan pengesana 'real-time' dua patogen dalam ujian satu langkah, tiub-tertutup yang memerlukan masa penyediaan yang singkat dan amaun reagen yang lebih sedikit berbanding dengan ujian monopleks LAMP. Kesimpulannya, ujian multipleks LAMP yang dibangunkan ialah satu kaedah yang mudah dan cepat untuk mengenal pasti patogen yang berkaitan dengan penyakit radang paru-paru di kalangan kanak-kanak.

Katakunci: Radang paru-paru, *Streptococcus pneumonie*, *Haemophilus influenzae*, loop-mediated isothermal amplification (LAMP), multipleks LAMP yang berpendaflour, Polymerase Chain Reaction (PCR), *autolysin*, outer membrane protein P6

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LIST OF SYMBOLS AND ABBREVIATION

%	Percentage
% (w/v)	Percentage Weight/Volume
°C	Degree Celsius
CO_2	Carbon dioxide
M	Molar
µg	Microgram
mg	Milligram
$MgSO_4$	Magnesium sulphate
min	Minute
µL	Microliter
µM	Micromole
ng	Monogram
pg	Picogram
ΔRn	Normalized reporter value. Ratio of the fluorescence signal divided by the fluorescence of passive dye
sec	Second
Tris-HCl	Tris hydrochloride
U	Unit
B3	Backward Outer LAMP Primer
BIP	Backward Inner LAMP Primer
Bst	*Bacillus stearothermophilus*
CFU	Colony forming unit
Ct	Threshold cycle
et al	and others
Df	White turbidity due to by-product (magnesium pyrophosphate) from DNA amplification in LAMP assay

DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dTTP	Deoxythymidine triphosphate
dUPT	Deoxyuridine triphosphate
F3	Forward Inner LAMP Primer
FIP	Forward Inner Primer
GC	Guanine-cytosine
HLPC	High-performance liquid chromatography
HNB	Hydroxyl napthol blue
KCl	Potassium chloride
LAMP	Loop-mediated Isothermal Amplification
LF	Loop-primer Forward
LB	Loop-primer Backward
mf-LAMP	Multiplex fluorescent Loop-mediated Isothermal Amplification
MSSA	Methicillin-sensitive *Staphylococcus aureus*
NCBI	National Center for Biotechnology Information
OD	Optical density
PCR	Polymerase chain Reaction
RFU	Relative fluorescence unit
Tm	Melting temperature for biological polymers
Tt	Time needed for the turbidity reaction of sample to exceed OD_{650nm} at 0.1 in turbidimeter
USFDA	United States Food and Drug Administration
UDG	Uracil-DNA-glycosylase
UNG	Uracil N-Glycosylase
UV	Ultra-violet

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

Childhood pneumonia is a serious problem. It is the leading cause of death in children worldwide, accounting for 19 % of all deaths of children under 5 years old (Wardlaw *et al.,* 2006). In Malaysia, it is the fifth highest cause of death in Malaysian children, contributing 4 % of death under the age of five years (Musa, 2011). Childhood pneumonia often underdiagnosed or miss-diagnosed as other respiratory disease which usually occur in children, for example asthma or cyctic fibrosis. The sign and symptoms of pneumonia are often non-specific and varies among patients with different age group as well as the infectious organism involved. Thus, this has made the definitive diagnosis of pneumococcal pneumonia difficult to establish if solely based on clinical symptoms upon admission (Wardlaw *et al.,* 2006). The 'gold standard' of diagnostic method is still laboratory culturing method, although area such as genomics and proteomics are continually advancing in bacteriology diagnosis. However, there are some limitations when using the gold standard method because sometimes good quality sample are hardly available. In addition, a negative culture may sometimes present due to prior antibiotic administration. Furthermore, when the site of sample isolation varies, different outcome will be obtained; for example isolation of pathogens from sputum may represent colonization whereas isolation of pathogens from blood is lacking sensitivity (Bogaert *et al.,* 2004). Clinical suspicions are usually paired with positive culture from sample for a confirmatory result on pneumonia infection in order to improve further the diagnosis.

To date, many molecular or antigen detection methods have been developed to provide a rapid diagnosis of the disease. Compared to culture method, molecular detection methods offer higher specificity, sensitivity and detection is independent on the viability of the target and is not affected by prior administration of antibiotic. However, in spite their many advantages like above, their limitation such as the detection is not available for all species or sample type, complicated method of preparation or complex analysis of result may somehow hinder their use in many clinical laboratories or in resource poor setting. Currently, there is no report to demonstrate a simple and rapid method to detect and identify two important pneumonia causative agents, namely *Streptococcus pneumoniae* and *Haemophilus influenzae* in a single assay. Thus, in order to address the problem the aims of this research are:

(a) To develop a monoplex Loop-mediated Isothermal Amplification Assay (LAMP) for separate detection of *S. pneumoniae* and *H. influenzae.*

(b) To develop a rapid, sensitive and specific real-time multiplex fluorescence Loop-mediated Isothermal Amplification (mf-LAMP) assay for simultaneous detection of *S. pneumoniae* and *H. influenzae* by combining both monoplex LAMP assays.

CHAPTER 2

LITERATURE REVIEW

2.1 *Streptococcus pneumoniae*

S. pneumoniae was first isolated by Louis Pasteur in 1881 from France. It is an important pathogen that is responsible for community-acquired infections among young children, the elderly and in those individual with certain underlying illnesses. It is a fastidious organism that grows optimum at temperature 35-37^{o}C supplemented with 5% CO_2. It can be cultured in media containing blood or sometimes on chocolate agar. Under microscope, *S. pneumoniae* appears as Gram positive, lancet-shaped cocci and is usually occurs as pairs of cocci (diplococci), but it may also be found as single short chain. In addition, it also has alpha-hemolytic properties where it can lyse red blood cells, producing a zone of alpha-hemolysis. However, the alpha-hemolysis characteristic can only allow differentiation of *S. pneumoniae* from beta-hemolysis streptococcus but not from the commensal viridans streptococci. The only way to differentiate among these two pathogens is to incubate the bacteria for around 24 - 48 hours, colonies for *S. pneumoniae* appear to be flattened with center portion become hollow and this type of morphology cannot be seen in viridans streptococci (CDC, 2012).

S. pneumoniae are commonly found in very young children and the very old adults. The colonization rate was found to be higher in children under six years of age and generally it can cause a wide spectrum of diseases from upper respiratory tract infection to invasive pneumococcal infection or it may appear to be asymptomatic to healthy children (Bogaert *et al.,* 2004). Serious diseases that are commonly associated with this pathogen are pneumonia, meningitis, febrile bacteraemia; in less serious manifestation such as otitis media, bronchitis and sinusitis are also common for pneumococcal infection. There are about 90 different pneumococcal serotypes being

identified worldwide and different serotypes will have different propensities in causing various diseases. The distribution of *S. pneumoniae* serotypes varies by country, age, and ethnicity (CDC, 2015).

A definitive diagnosis of *S. pneumoniae* infection is usually from sample isolated from blood or other sterile body sites (CDC, 2015). However, not every pneumococcal infections may lead to bacteremia and thus sample isolated from sterile site may greatly underestimate the incidence of significant pneumococcal infection. In young children, a none invasive isolation method will be performed such as nasopharynx or sputum isolation but respiratory specimens are sometimes difficult to ascertain the diagnosis because the carriage of pneumococci in nasopharynx in young children are common (Silvermann *et al.,* 1977). Sputum specimens are also sometimes not suitable for diagnosis because the poor quality and inadequate quantity of sputum obtained from young children may hinder the accuracy of laboratory test. Besides, prior administration of antibiotic therapy in both adult and children with pneumonia has apparently further reduced the incidence of significant pneumococcal infection. In order to enhance the diagnosis of pneumococcal infection, the use of needle aspiration of the lung may probably give the most accurate diagnosis. However, this method could not be use in routine basis because it is too invasive and traumatizing to patient (Busk *et al.,* 1988).

Apart from using sampling method like needle aspiration, there are still many more confirmatory lab tests available which are non-invasive but specific to identify *S. pneumoniae*, such as Gram stain, optochin test and bile solubility test. These pathogen is optochin-sensitive and also sensitive to lysis by bile. However, those detection methods are slow and lack sensitivity. On the other hand, immunochromatographic test to detect capsular polysaccharide antigen in body fluids (Binax NOW, Binax, Portland, Maine) seems to be a promising tool that can detect

causative antigen within 15 minutes of sample inoculation, requires only minimal laboratory infrastructure and is non-labor intensive. This test was approved by USFDA for detection of C-polysaccharide cell wall antigen commonly found in all strains of *S. pneumoniae* that caused community-acquired pneumonia in adults. However, this particular test can only be used in detection of *S. pneumoniae* from adult's urine suspected with community-acquired pneumonia but could not be used in children because urine has lacks specificity in children and will give positive detection even in healthy children with normal colonization by pneumococci and other closely related Streptococcus species. (Navarro *et al.,* 2004).

At present, there are limited data available in Malaysia on the incidence of pneumococcal diseases although there was a study reported that *S. pneumoniae* was ranked at 25th among a total of 99 significant bacterial pathogens encountered in 15 hospitals, contributing an incidence rate of 75.4 per 10,000 patients for *S. pneumoniae* (Rohani *et al.,* 1999). Based on WHO estimates for Malaysia, there are 4% of the 7000 deaths among children under the age of five years old being attributed to pneumococci. This figure may translate into an incidence of overall pneumonia deaths of 10.2 out of 10,000 children ages under five years old (Wardlaw *et al.,* 2006).

2.2 *Haemophilus influenzae*

H. influenzae are categorized into two main groups: encapsulated (presence of polysaccharide capsule) strains and unencapsulated (lack of polysaccharide capsule) strains. The encapsulated strains are subdivided into six serotypes (a-f) based on their reactivity with conventional type-specific antiserum agglutination. Out of the six antigenically distinct encapsulated strains, type b was the one that causing invasive diseases, for example, pneumonia, septicemia, meningitis etc. In contrast,

unencapsulated strains of *H. influenzae,* termed non-typeable strains (NTHi) are usually associated with mild inflammatory diseases including otitis media, sinusitis, epiglottitis, and exacerbation of chronic obstructive pulmonary disease (COPD).

However, a recent report revealed that the introduction of *H. influenzae* type b (Hib) conjugate vaccine (Adam *et al.,* 2010) and *S. pneumoniae* polysaccharide conjugate vaccine (PVC) (Spijkerman *et al.,* 2012) has contribute to the emergence of NTHi that caused invasive diseases, making this under-recognized pathogen a global concern. The occurrence of invasive NTHi diseases (including pneumonia) struck predominantly in perinatal infants, children younger than 5 years old, elderly folks or individual with chronic respiratory diseases or compromised immunity. Based on the reports above, it was suggested that both encapsulated and unencapsulated strains of *H. influenzae* may have certain level of pathogenesis in childhood pneumonia. Thus, to develop a multiplex LAMP assay that is specific to both typeable & non-typeable *H. influenzae*, careful selection of target gene is a crucial to ensure specific detection of both typeable and non-typeable *H. influenzae*.

Similarly to *S. pneumoniae*, there are limited data available in Malaysia on the incidence of *H. influenzae* infection. This may partly due to the small-scale, outdated studies that limiting the understanding of the current epidemiological situation. However, a study conducted by Hussain *et al.* (1998) showed that nearly one-half (48 %) of all positive cases of meningitis found in Malaysia were caused by Hib and majority of the cases (76 %) were from infant less than one year of age.

2.3 Conventional detection method versus molecular-based detection method of both pneumonia pathogens

To date, laboratory culturing method still remains as the gold standard approach for bacterial detection or identification but the entire process is time-consuming and laborious. This conventional method is now superseded by molecular diagnostic methods using species-specific DNA amplification technology, which can significantly reduce the time required to obtain results, especially for detection of fastidious organism.

Many efforts have been invested to develop high-throughput molecular-based detection and identification of pathogens such as polymerase chain reaction (PCR). PCR is one of the most established techniques but is laborious and often requires lengthy sample preparation and purification to eliminate potential inhibitors (Wang *et al.,* 2014). Furthermore, post-PCR target identification such as agarose gel electrophoresis or pyrosequencing which are time-consuming and also adding more cost into the method used.

2.4 Loop-mediated Isothermal Amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a single tube nucleic acid amplification technique that amplifies DNA under constant temperatures in contrast to conventional polymerase chain reaction (PCR) that amplifies DNA by alternating temperature in multiple cycles. LAMP reaction can be performed using only a simple heating block that supplies constant temperature ranging from 60 to 65°C. The DNA synthesis mechanism is based on enzymatic activity of *Bacillus stearothermophilus (Bst)* polymerase to provide strand separation of dsDNA. *Bst* polymerase is an enzyme that has helicase-like property to unwind DNA strands under isothermal temperature with the optimum functional temperature between 60°C to 65°C.

Inactivation of enzyme in a reaction can simply be done by heating up at high temperature above 70°C. This unique enzyme was derived from thermophile *Geobacillus stearothermophilus* (previously known as *Bacillus stearothermophilus)* a Gram-positive bacteria that was found widely in ocean sediment, hot spring and in soil (Donk, 1920)

In addition to the presence of unique *Bst* polymerase that offers high strand displacement activity in LAMP reaction, a set of highly specific primers consisting of two outer and two inner primers are also one of the essential factors to achieve a successful LAMP reaction. These four LAMP primers are specifically designed spanning six distinct region of the target gene (F3, F2, F1, B3, B2, B1) (Figure 2.1). The two outer primers are labeled as F3 and B3 whereas inner primers are labeled as FIP (forward inner primer) and BIP (backward inner primer) respectively. Each inner primers contains two distinct sequences of the target DNA where one (F2 or B2) is responsible in priming at the initial stage, and the other sequence (F1c or B1c) is for self-priming at the later stage.

During the initial stage (non-cycling stage) of a LAMP reaction, the synthesis of complementary strand of target DNA is initiated by the priming of F2/B2 region from FIP/BIP then followed by the extension of the subsequent region. Outer primer F3/B3 will then hybridize to complementary region at the target DNA, displacing the FIP linked complementary strand generated at the initial step. The displaced single stranded DNA will then form a loop at the 5' end and will serve as a template for subsequent hybridization by BIP primer. Once the BIP linked complementary strand is generated, this will result in on the formation of single stranded dumbbell DNA structure. Nucleotide will be added to the 3' end of this dumbbell structure and the subsequent extension of DNA strand will lead to the opening of the loop at 5'end, transforming the dumbbell structure into stem loop structure. This

stem loop structure is crucial initiator for subsequent cycle in cycling stage where it can facilitate exponential amplification in LAMP reaction.

In LAMP cycling stage, it is initiated by first hybridization of FIP to the loop of the stem-loop DNA structure to initiate the displacement of DNA synthesis. Under this process, a new stem-loop DNA with a stem doubled in length will be produced and the original stem-loop DNA serve as a template for the subsequent DNA synthesis. As the FIP linked complementary strand is generated, it will be displaced to form a new single stranded dumbbell DNA structure. Thus, large amount of DNA final product consisting of a mixture of stem loop DNA with alternately inverted repeats in various stem lengths will be produced as the cycle goes on because both the dumbbell and stem-loop structure will serve as a template for subsequent DNA synthesis. As the cycling reaction continues, it can give up to 10^9 copies of target within an hour reaction time. The final product is in different sized structure with various inverted repeats of the sequence on the same strand forming a cauliflower-like structure (Notomi *et al.,* 2000). There is another pair of primers term Loop primer (Forward loop primer = LF, Backward loop primer = LB) which are additional pair of primers bind specifically to and additional site at loop structure that are not accessible to internal primers. The use of loop primers in LAMP reaction not only can accelerate the duration of reaction but also able to enhance the sensitivity of the assay (Nagamine *et al.,* 2002)

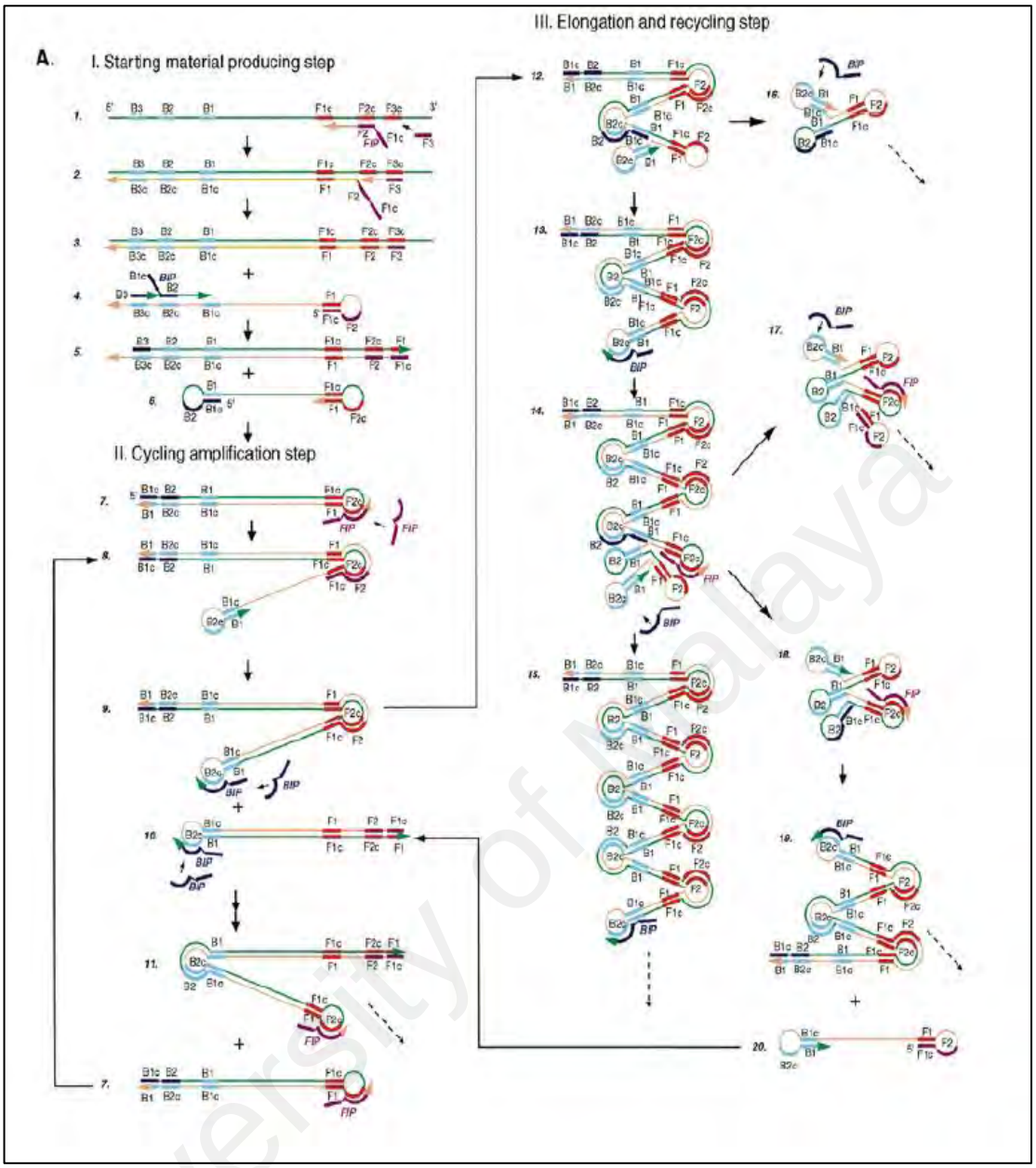

Figure 2.1: Schematic representation of complete non-cycling and cycling stages in a LAMP reaction. Adopted from Notomi *et al.* (2000).

LAMP assay was developed by Notomi *et al.* in year 2000. Ever since then, this LAMP protocol has drawn a lot of attention to many researchers ranging from the field of bacteriology, virology and parasitology due to its high efficiency in generating large amount of DNA product in shorter reaction time. Four primers that recognize six distinct regions on a target gene confers a high degree of specificity to the reaction, and it does not require sophisticated instrument as the reaction can be done at isothermal temperature by utilizing simple heat block or water bath. Measurement of

LAMP product is also rather simple as positive or negative LAMP reaction can be determine by naked eye observation for solution turbidity generated during reaction due to the accumulation of white precipitate (magnesium pyrophosphate) (Mori *et al.,* 2001). For better visibility of the detection result, some researchers used DNA intercalating dye such as SYBR Green (Parida *et al.,* 2005), Propidium iodide (Hill *et al.,* 2008), and Picogreen (Curtis *et al.,* 2008). When there is positive amplification, colour change can be observed under ambient or UV light. Furthermore, result analysis using colorimetric assay by addition of DNA intercalating dye may post a higher risk of cross-over contamination on the subsequent LAMP assay as it requires opening of tubes at the end of the reaction. Thus, another colorimetric assay using hydroxyl napthol blue (HNB) (Mori *et al.,* 2001) were designed in attempt to reduce contamination risks. HNB is a metal ion indicator which will fluoresce only when DNA is synthesized *in vitro* without the need to open reaction tube and thus reducing the potential risk of contamination. However this method shares the same limitation as other colorimetric assay whereby the assay is only for single target detection.

Other end point analysis method of detection such as gel electrophoresis (Curtis *et al.,* 2008; Curtis *et al.,* 2012), enzyme digestion or pyrosequencing (Notomi *et al.,* 2000) on LAMP product also requires opening of reaction tube for further processing and instrumentation. However, this has made the post-LAMP analysis less desirable as each time after LAMP reaction, the operator will need to carry out subsequent LAMP assay in separate facilities with separate equipment to reduce risk of cross-contamination.

2.5 LAMP assay in detection of infectious disease

In the past recent years, multiple LAMP assays have been designed to detect virus or bacteria pathogens that caused human infectious diseases. LAMP method has successfully used for specific detection of pathogenic bacteria such as Leptospira (Sonthayanon *et al.,* 2011), *Mycobacterium tuberculosis* complex, *Mycobacterium avium* and *Mycobacterium intracellulure* (Iwamoto *et al.,* 2003), *Mycobacterium tuberculosis* (Geojith *et al.,* 2011), *Staphylococcus aureus* (Lim *et al.,* 2013), methicillin-resistant *S. aureus* (Misawa *et al.,* 2007) and *S. pneumoniae* (Seki *et al.,* 2005). It was also demonstrated that LAMP has 10 to 1,000-fold-greater sensitivity than multiplex PCR with detection limit of 2 to 20 CFU per reaction and high specificity with no cross-reaction with non-target DNA for detection of *Acrobacter* species (Wang *et al.,* 2014). In addition, LAMP assay appeared to be more robust compared to detection using real-time quantitative PCR (qPCR). The unique characteristic of LAMP is high sensitivity and robust under the presence of PCR inhibitors and various incubation time make it an ideal tool for deployment in clinical setting or in developing country (Francois *et al.,* 2011).

Not only LAMP can be used to detect bacterial DNA, it has also been successfully used to detect and amplify viral DNA such as severe acute respiratory syndrome coronavirus (SARS) (Notomi *et al.,* 2004), human immunodeficiency virus-1 (Curtis *et al.,* 2012), Japanese encephalitis virus (Parida *et al.,* 2006) and dengue virus (Teoh *et al.,* 2013). Kaneko *et al.* (2005) applied LAMP method to detect herpes simplex virus (HSV) and varicella-zoster virus (VZV). The detection sensitivity is 10-fold higher than conventional PCR and can be well-applied to clinical diagnosis of HSV and VZV infection. In addition to this, detection of H5 avian influenza virus from throat swab samples collected from wild birds was first reported by Imai *et al.* (2007). The developed assay targeting H5 hemagglutinin gene for detection of H5N1 influenza virus

showed 100-fold higher sensitivity compared to RT-PCR and the primers designed showed no cross-reactivity with RNA from human influenza virus (H1N1 and H3N2).

The application of LAMP is not limited to the detection of pathogen, it also can be explored for quantification of pathogen in clinical samples to give an idea of the pathogen load. It has been reported that the end-point evaluation of turbidity (accumulation of insoluble magnesium pyrophosphate) makes it possible to quantify the initial amount of DNA template in samples (Mori *et al.,* 2004).

Majority of the current LAMP assays are for single pathogen detection. These LAMP methods could not be applied to multiple target detection as they measure total DNA. However, multiplex LAMP assay can still be performed by utilizing additional equipment or protocols in helping to achieve real-time detection of multiple targets. To date, multiplex LAMP assay has been demonstrated to amplify two targets (He *et al.,* 2011; Iseki *et al.,* 2007), three targets (Jiang *et al.,* 2012), four targets (Huy *et al.,* 2012) and the most was five targets (Tanner *et al.,* 2012) simultaneously in single reaction tube

It was demonstrated that the LAMP assay can be used for the detection of *S. pneumoniae* (Seki *et al.,* 2005) and *H. influenzae* (Torigoe *et al.,* 2007; Kim *et al.,* 2011). Each of their pathogens was done separately in a monoplex assay. The results showed that the LAMP method was more specific and sensitive than PCR for rapid detection of both pathogens in clinical samples. However, there is no LAMP assay currently available to identify these two pathogens in single tube assay. Therefore, the objective of this study was to develop a real-time fluorescence multiplex LAMP assay for the detection of two different pathogens in a single tube reaction. This can be achieved by designing species-specific primers together with fluorescently labelled primers attempted to detect these two pathogens in a single tube reaction.

2.6 Detection of Amplification by Releasing of Quencher (DARQ)

DARQ is a detection method that provides detection signal by releasing of quencher. It allows detection of multiple pathogens in a single-step, closed tube reaction, obviating the need for end-point analysis using gel electrophoresis or other approaches. DARQ technology uses a unique duplex fluorescent probe with sequence complementary to F1c section of inner primers (FIP or BIP). Firstly, 5'- end of FIP was modified with either a dark quencher or fluorophore. Modification was done at this region because it is inherent to LAMP and the sequence is unique to each target, obviating the need for probe sequence optimization. To create the probe (Fd), an oligonucleotides complementary to the flap region of F1c (partial sequence in FIP) with 3'-prime end modified with either dark quencher or fluorophore was annealed to FIP sequence, overlapping with the dark quencher or fluorophore of the FIP (Figure 2.2). Before amplification starts, this duplex primer remained its function as a LAMP primer and both fluorophore and quencher are in close proximity, thus no emission of fluorescent will be detected. Upon DNA synthesis from reverse direction, duplex primer will be separated, resulting in detection of fluorescence by releasing of quencher (Tanner *et al.,* 2012). This technology provides a method for multiplex detection of pathogens in LAMP assay without additional primer optimization or probe design. The creation of probe is hassle free by just using inherent LAMP primer sequence modified with fluorophore or quencher at its 5'region overlapped with a complementary detection sequence (Fd) , providing flexibility for LAMP system. The most essential part of this technology is that it allows real-time detection of pathogens in a single-step, closed tube reaction and at the same time maintains its specificity and high sensitivity of LAMP assay. The system is robust and adaptable to any LAMP reaction.

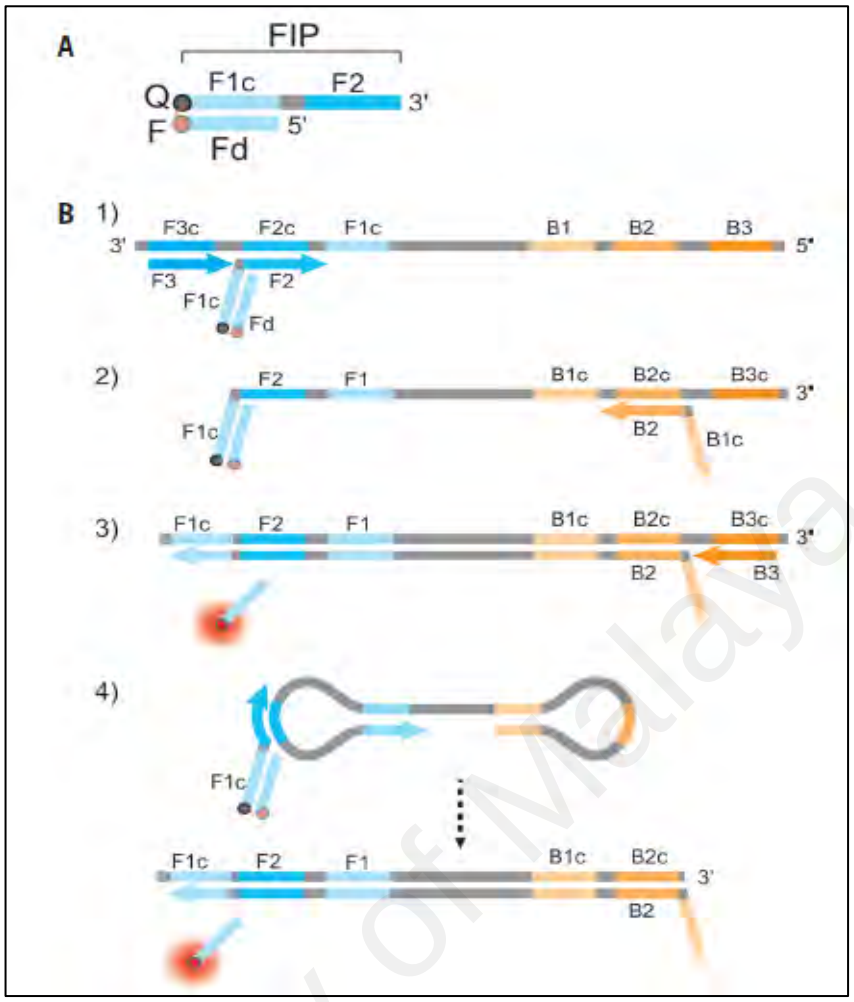

Figure 2.2: Schematic description of a DARQ probe.
(A) QFIP:Fd duplex primer annealed with a 5′-quencher FIP (F1c + F2 sequence) and a 3′-fluorophore Fd. The quencher and fluorophore moieties are represented by Q and F, respectively. (B) Outline of DARQ LAMP reactions, with core LAMP primers FIP (F1c + F2), BIP (B1c + B2), F3 and B3, and the DARQ oligonucleotide, Fd (Q, black; F, red). For clarity, LoopF and LoopB primers are not shown. (B1) LAMP initiates at the F2c sequence of the target, with the Fd probe quenched through annealing to Q-FIP. This new strand is displaced by upstream synthesis from the F3 primer. (B2) The BIP primer anneals to the B2c site in the newly synthesized strand. (B3) Synthesis from the primer annealed to the B2c sequence displaces the Fd probe. This releases the quenching resulting in a gain of signal. The newly synthesized strand is displaced by extension from the B3 primer (B4). The resulting structure undergoes exponential amplification in the LAMP reaction. Subsequent initiations at FIP give rise to additional release of Fd, resulting in exponential signal detection. Adopted from Tanner *et al.,* (2012).

CHAPTER 3

MATERIALS AND METHODS / METHODOLOGY

3.1 Bacterial strains

This study was approved by UMMC Medical Research Ethics Committee (MEC ID: 20146-336). Fifty nine clinical bacterial strains were used for method development. These include *S. pneumoniae* strains (n = 20), *H. influenzae* strains (n = 20) and 19 non-targeted strains of bacteria which consists of methicillin-sensitive *Staphylococcus aureus* (MSSA) (n = 4), *Escherichia coli* (n = 5), *Klebsiella pneumoniae* (n = 5) *and Pseudomonas aeruginosa* (n = 5). All bacterial strains were previously isolated from in-patients admitted to UMMC. Pure bacterial cultures were then obtained from the Diagnostic Microbiology Laboratory, UMMC. *S. pneumoniae* strains were cultured in Brain Heart Infusion (BHI) (BD BBL™ Brain Heart Infusion Broth, Canada) broth and Columbia agar supplemented with 5 % sheep blood (BD BBL™ Columbia Agar with 5 % Sheep Blood, USA) and incubated overnight in an aerobic atmosphere containing 5 % CO_2 at 37°C. Whereas for *H. influenzae* strains, they were cultured in Haemophilus Test Medium Broth and Chocolate II Agar (BD BBL™ Chocolate II Agar, USA) and were incubated at 37°C with 5 % CO_2. The rest of the non-target strains namely methicillin-sensitive *S. aureus* (MSSA), *E. coli, K. pneumoniae,* and *P. aeruginosa* were cultured on Luria-Bertani (LB) agar and in LB broth (Difco ™ LB Agar, Miller, USA) at 37 ºC for 24 hours. LB broth was prepared by mixing 5.0 g of yeast extract, 5.0 g of Sodium Hydroxide, 10.0 g of tryptone and 1 litre of distilled water. For preparation of LB agar, 15.0 g of agar was added into the mixture above to yield LB agar. Both broth and agar suspension were heated with frequent agitation and boiled for 1 min to completely dissolve the solid particles. The suspension was then autoclaved at 121 °C for 15 min.

3.2 Extraction and quantification of DNA template from bacterial strains

Genomic DNA of targeted strains *S. pneumoniae*, *H. influenzae* and non-targeted strains methicillin-sensitive *S. aureus* (MSSA), *E. coli, K. pneumoniae and P. aeruginosa* were obtained by following manufacturer's protocol for DNA extraction gram-positive and Gram negative bacteria of the DNeasy Tissue Kit (Qiagen, Valencia, CA). Briefly, for *S. pneumoniae* and methicillin-sensitive *S. aureus* (MSSA), Gram positive bacteria were subjected for cell lysis by incubation in enzymatic lysis buffer (containing 20 mM Tris-Cl, pH 8.0; 2 mM sodium EDTA; 1.2 % Triton® X-100; 20 mg/ml lysozyme) for at least 30 min at 37 °C. Next, the contaminating proteins were removed by addition of proteinase K. DNA was then obtained by addition of ethanol (96 - 100 %) to form white precipitate. This white precipitate was then transferred to DNeasy Mini spin column and spun down to obtain pure DNA sample. The DNA concentration was determined using NanoDrop spectrophotometer. DNA sample was then stored at - 20 °C until further use.

Conventional PCR (Applied Biosystems Veriti™ 96-Well Termal Cycler, USA) was used to confirm the identity of test organisms using forward & reverse primer specific to each organism (Table 3.1). Briefly, the PCR was carried out with a total volume of 25 µL containing 3 µL of DNA template, 1 X of buffer, 0.3 µM of each Froward and Reversed primers, 200 µM of dNTP mix, 1.5 µM of $MgCl_2$ and 1U of *Taq* DNA polymerase (Promega, USA). The PCR mixture was then subjected to denaturation at 94 °C for 4 min, followed by 30 cycles of 95 ˚C for 30 sec, 50 °C – 55 °C for 30 sec, 72 °C for 45 sec and a final extension at 72 °C for 10 min. PCR products were then resolved on a 1.0 % (w/v) agarose gel pre-stained in with RedSafe™ (iNtRON Biotechnology, Korea) at 100 V. The gel was then visualized under UV using Gel Doc system (BioRad, USA).

Table 3.1: Primers sequence used to confirm the identity of the bacterial species.

Primer Sequence (5' → 3')	Length	Tm	Target gene/ bacteria	Reference
recP1: GCCAACTCAGGTCATCCAGG **recP2:** TGCAACCGTAGCATTGTAAC	20 bp 20 bp	55 °C	*recP* *S. pneumoniae*	mlst.net
pal-F3: TAGAAGGTAACACTGATGAACG **pal-B3:** TACGCTAACACTGCACGA	22 bp 18 bp	50 °C	*pal* *H. influenzae*	Torigoe, *et al.,* 2006
arc1: TTGATTCACCAGCGCGTATTGTC **arc2:** AGGTATCTGCTTCAATCAGCG	23 bp 21 bp	55 °C	*arc* MSSA	mlst.net
phoA1: GTGACAAAAGCCCGGACACCATAAATGCCT **phoA2:** TACACTGTCATTACGTTGCGGATTTGGCGT	30 bp 30 bp	67 °C	*phoA* *E. coli*	Kong, *et al.,* 1999
mdh1: CCCAACTCGCTTCAGGTTCAG **mdh2:** CCGTTTTTCCCCAGCAGCAG	21 bp 20 bp	50 °C	*mdh* *K. pneumoniae*	Laure, *et al.,* 2005
oprL1: ATGGAAATGCTGAAATTCGGC **oprl2:** CTTCTTCAGCTCGACGCGACG	21 bp 21 bp	55 °C	*oprL* *P. aeruginosa*	Xu, *et al.,* 2004

3.3 Data mining and determination of targets for primer design

3.3.1 Selection of gene targeting *S. pneumoniae*

A thorough literature research was done to look for gene which is specific and conserved in *S. pneumoniae.* Autolysin *(lytA)* and pneumolysin *(ply)* are the potential targets for specific detection of *S. pneumoniae* because both were found to contribute to the pathogenesis of pneumonia. In this study, *lytA* gene was selected for subsequent gene analysis and LAMP primer design because it was reported that this virulence gene was present in all isolates irrespective of site of isolation and types of pneumococcal infection (Sourav *et al.,* 2010). Furthermore, this gene can distinguishing *S. pneumoniae* from genotypically similar species such as atypical *Streptococcus mitis* and *Streptococcus oralis* strains which are commensals of the human cavity and also known to harbor the same *lytA* gene (Sheppard *et al.,* 2004). In addition to this, Messmer *et al.* (2004) also demonstrated that *lytA* gene is capable to specifically identify typeable and non-typable *S. pneumoniae* with no cross-reaction with atypical streptococci and viridans streptococci in comparison with *ply* gene and pneumococcal surface adhesion A (*psaA)* gene.

3.3.2 Selection of gene targeting *H. influenzae*

Two potential genes were shortlisted from literature search namely glycerol kinase (*glpK*) and protein D (*hpd)*. All of these genes were subjected to bioinformatics analysis in attempt to look for conserved regions that could be used for amplification. Alignment of individual genes was accomplished by using an open source Kalign software (http://www.ebi.ac.uk/Tools/msa/kalign/). The accession numbers of gene sequences are summarized in Table 3.2.

Table 3.2: Eight sequences for each gene (*glpK* and *hpd)* were adopted from National Centre for Biotechnology Information (NCBI) with the accession numbers as shown below to be used for multiple sequence alignment.

No.	Gene	Protein	Accession Number of gene sequences adopted from NCBI
1	*glpK*	glycerol kinase	L42023, CP000671, CP000672, CP007470, CP002276, CP002277, CP005967, FQ312002
2	*hpd*	protein D	Z35656, CP000671, Z35659, L15200, M37487, L12445, L42023, X90489

A conserved region from each gene was elucidated and subjected for LAMP primer design. Subsequently, the specificity of the newly designed LAMP primers was checked using *in silico* PCR and primer blast. To further confirm the specificity of the newly designed LAMP primer based on four targeted genes mentioned above, the outer primers termed F3 and B3 derived from each gene were evaluated using conventional PCR.

3.4 Development of LAMP assays for *S. pneumoniae* and *H. influenzae*

3.4.1 Primer design

LAMP assay requires two primer sets, where each set of primers consists of two outer primers (F3 and B3), two inner primers (FIP and BIP) that recognize a total of six distinct sequences in the target DNA and at least one loop primer (LF or LB) that increases the amplification efficiency. The primers used for detection of *S. pneumoniae* in monoplex LAMP assay were designed based on the sequences of *lytA* gene. LAMP primers targeting *lytA* gene was designed using Primer Explorer (version 4) software http://primerexplorer.jp/e/ (Primer Explorer, Eiken Chemical Co. Ltd.). This software was used to design 4 primers that target 6 distinct regions required by the LAMP method. On top of that, it can also be used to design loop primer when required. This software uses 'nearest neighbor' method for Tm calculation and it offers the advantage of installation free as it is available to be used via the web.

In this study, LAMP primer targeting *H. influenzae* was adopted directly from work done by Torigoe *et al.* (2007) *pal* (peptidoglycan-associated lipoprotein) gene which is highly conserved in most of the typeable and non-typeable *H. influenzae*. The selected LAMP primers were then assessed for specificity in *in silico* PCR and BLAST search (http://www.ncbi.nih.gov) with sequences in GenBank before use in multiplex LAMP assay.

The outer primers namely F3 and B3 from each set targeting *lytA* gene (*S. pneumoniae*) and *pal* gene (*H. influenzae*) were also tested on conventional PCR with similar condition as previously described to ensure the specificity of primers toward targeted strains. Initial denaturation at 94 °C for 4 min followed by denaturation at 94 °C for 15 sec, annealing at 50 °C (*pal* primer) or 55 °C (*lytA* primer) for 30 sec, and followed by elongation at 72 °C for 45 sec and cycle ended with final extension at 72°C

for 10 min (30 cycles). The PCR products were resolved on 1 % (w/v) agarose gel to ensure the PCR products are at desired amplicon size.

In order to achieve real-time monitoring of amplification in multiplex LAMP assay, the 5'-end of FIP was labelled with dark quencher (Q-FIP; Iowa-Black FQ) while 3'-end of the sequence complementary to sequence of F1c (Fd) was labelled with either FAM or JOE-fluorescence dyes to form a duplex primer. During amplification, this species-specific duplex primers are separated, resulting in detection of fluorescence by release of quenching. In this case, it is able to differentiate the two pathogens during real-time detection of LAMP product. Both fluorophores used were corresponding to channels in an Applied Biosystem Step-One Plus Real-Time PCR System used in this study.

3.4.2 Determination of the Threshold time (Tt) and detection limit for targeted and non-targeted strains

LAMP assay was conducted according to protocol established by Notomi *et al.* (2000). Briefly, the reaction was performed in 25 µL of a mixture containing 3 µL DNA template, 12.5 µL of 2 × LAMP buffer (20 mM Tris–HCl, 10 mM KCl, 8 mM MgSO4, 10 mM $(NH4)_2SO4$, 0.1 % Tween 20, 0.8 M Betaine, 1.4 mM dNTPs mixtures), 1.6 µM each of inner primers FIP and BIP, 0.2 µM each of outer primers F3 and B3, 0.8 µM of loop primers LF, 8 U of *Bst* DNA polymerase (New England Biolabs, UK). Optimization of monopelx LAMP assay was carried out by incubating reaction mixture at temperature 63 °C for 60 min followed by enzyme termination at 80 °C for 2 min in the Loopamp real-time turbidimeter. Positive reaction is determined when the turbidity reached 0.1 within 60 min at 650 nm. The time needed for the turbidity

reaction of sample to exceed OD_{650nm} at 0.1 is referred as threshold time (Tt). (Mori, *et al.,* 2004)

Seventeen *S. pneumoniae* clinical isolates and twenty non-targeted strains were tested using *lytA* LAMP primers whereas eighteen *H. influenzae* clinical isolates and eighteen non-targeted strains were subjected for specificity test in monoplex reaction containing only *pal* LAMP primers. The range of Tt to detect targeted strains and non-targeted strains in corresponding to the DNA concentrations were determined.

3.4.3 Sensitivity

Sensitivity of the detection method was performed. Briefly, genomic DNA at 220 ng/µL and 109 ng/µL concentration for *S. pneumoniae* and *H. influenzae* respectively were diluted serially (10-fold) and was subjected to monoplex LAMP assay.

3.4.4 Analysis of LAMP products on agarose gel

To monitor real-time LAMP amplification of both *S. pneumoniae* and *H. influenzae*, a Loopamp real-time turbidimeter (LA-500, Eiken Chemical Co.,Ltd, Tokyo, Japan) was used. This LAMP turbidimeter has the capability of monitoring the amplification in every 6 secs in the form of OD at 650 nm. For further confirmation of amplification, the resulting LAMP products were resolved using electrophoresis in 2 % (w/v) agarose gel pre-stained with RedSafe™ (iNtRON Biotechnology, Korea), and the gel was run at 80V. 1 µL of 10 x diluted SYBER Green™ I Nucleic Acid Gel stain (Invitrogen, UK) was added into LAMP products prior loading into the gel.

3.5 Optimization of Real-time Fluorescence multiplex LAMP (mf-LAMP) Assay

Optimization of mf-LAMP assay was performed on several clinical *S. pneumoniae* and *H. influenzae* strains by using Loopamp DNA amplification kit (Eiken Chemical Co. Ltd., Japan). This kit consists of reaction mix, *Bst* DNA polymerase and distilled water for 192 tests. Briefly, Q-FIP and Fd duplex primer were annealed first by heating equimolar of Q-FIP & Fd primer (50 µM Q-FIP primer and 50 µM Fd primer) to 98 °C and the mixture was slowly cooled to room temperature. An aluminium foil was wrapped around the tube containing QFIP : Fd primer mixture to minimize light exposure in order to prevent deterioration of fluorophore & quencher activity. Multiplex LAMP assay was carried out at 25 µL reaction mixture contained 12.5 µL of 2 ×LAMP buffer (20 mM Tris–HCl, 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH4)_2SO4$, 0.1% Tween 20, 0.8 M Betaine, 1.4 mM dNTPs mixtures), 1.6 µM of BIP, 0.8 µM of FIP, 0.8 µM of QFIP:Fd, 0.2 µM of F3 and B3, 0.4 µM of loop primers LF (as final primer concentration), 8 U of *Bst* DNA polymerase (New England Biolabs, UK). For this multiplex reaction, total primer concentrations were kept to 4 µM but with each set of LAMP primers having half the total concentration. Table 3.3 summarizes the concentration of LAMP primers used in the multiplex fluorescent LAMP assay.

Table 3.3: Concentration for both sets of LAMP primers (*lytA* and *pal*) in a multiplex fluorescent LAMP reaction.

LAMP primers (*lytA* gene)	**Primer concentration (µM)**	**LAMP primers (*pal* gene)**	**Primer concentration (µM)**	**Total primer concentration per reaction (µM)**
BIP	0.8	BIP	0.8	**1.6**
FIP	0.4	FIP	0.4	**0.8**
QFIP:Fd	0.4	QFIP:Fd	0.4	**0.8**
F3	0.1	F3	0.1	**0.2**
B3	0.1	B3	0.1	**0.2**
LF	0.2	LF	0.2	**0.4**
Total concentration	**2.0**	**Total concentration**	**2.0**	**4.0**

Real-time Fluorescent multiplex LAMP assay was carried out at different temperatures ranging from 63 ˚C to 65 ˚C for 60 min using Applied Biosystem Step-One Plus Real-Time PCR System which was set to collect fluorescence signals at 1 min intervals. The reaction was then terminated by enzyme inactivation at 80 ˚C for 2 min. qPCR measures DNA amplification on the exponential phase, giving an accurate data for quantification. When the fluorescence intensity of a sample reaction reaches the threshold line (threshold adjusted to Δ Rn 0.1), number of cycle need to reach the threshold will is referred as threshold cycle (C_t). In this study, 1 C_t is equivalent to 1 minute of reaction duration. Furthermore, optimization was also done by addition of reference dye (ROX-High) or by manipulating LAMP primer concentration, reaction temperature in attempt to achieve a good amplification curve.

3.5.1 Specificity

Specificity of mf-LAMP assay was evaluated using twenty strains of *S. pneumoniae*, twenty strains of *H. influenzae* and nineteen non-targeted strains including *E. coli*, *P. aeruginosa*, *K. pneumoniae* and methicillin-sensitive *S. aureus*. Each of the bacteria was tested in optimized mf-LAMP assay developed from protocol in section 3.5

3.5.2 Sensitivity

Sensitivity of the mf-LAMP assay was evaluated using 10-fold serial dilution of the genomic DNA of both targeted bacteria. Briefly, *S. pneumoniae* ATCC 49619 strain at concentration of 220 ng/µL and *H. influenzae* ATCC 49766 strain at concentration 109 ng/µL were serially diluted 10-fold up to 10^{-4}. mf-LAMP was performed on the diluted DNA templates using the optimized conditions.

The diluted DNA templates of *S. pneumoniae* and *H. influenzae* were used for conventional PCR assay targeting *lytA* gene and *pal* gene respectively. Briefly, the PCR reaction was carried out in a total volume of 25 µL, where each mixture contained 3 µL of DNA template, 1 x of buffer, 0.3 µM of each primer, 120 µM dNTP mix, 1.2 mM $MgCl_2$, and 1 U of *Taq* DNA polymerase (Promega, USA). PCR was performed in thermal cycler (Applied Biosystem® Veriti™ 96-well Thermal cycler, Thermo Fisher Scientific) for 30 cycles. Amplification was carried out at 94 °C for 4 min, followed by 30 cycles of 95 °C for 30 sec, annealing temperature according to different primer set (Table 3.1) for 30 sec, 72 °C for 45 sec, and a final extension at 72 °C for 10 min. The PCR amplification products were subjected for gel electrophoresis on a 1 % (w/v) agarose gel pre-strained with RedSafe™ (iNtRON Biotechnology, Korea) and visualised under UV using Gel Doc system (BioRad, CA, USA). The results of PCR were used to compare with mf-LAMP assay.

3.5.3 Validation of the products from real-time multiplex fluorescent LAMP (mf-LAMP) Assay

The resulting mf-LAMP products were also resolved using electrophoresis on 2 % (w/v) agarose gel pre-stained with RedSafe™ (iNtRON Biotechnology, Korea), and the gel was run at 80 V. 1 uL of 10 x diluted SYBER Green™ I Nucleic Acid Gel stain (Invitrogen, UK) was added into mf-LAMP products prior to loading into the gel.

CHAPTER 4

RESULTS

4.1 Bacterial strains

Fifty-nine bacterial strains comprised of Gram positive and Gram negative bacteria were successfully revived and used in this study. The number and species of the bacterial strains used in this study is presented in bar chart format as shown below in Figure 4.1

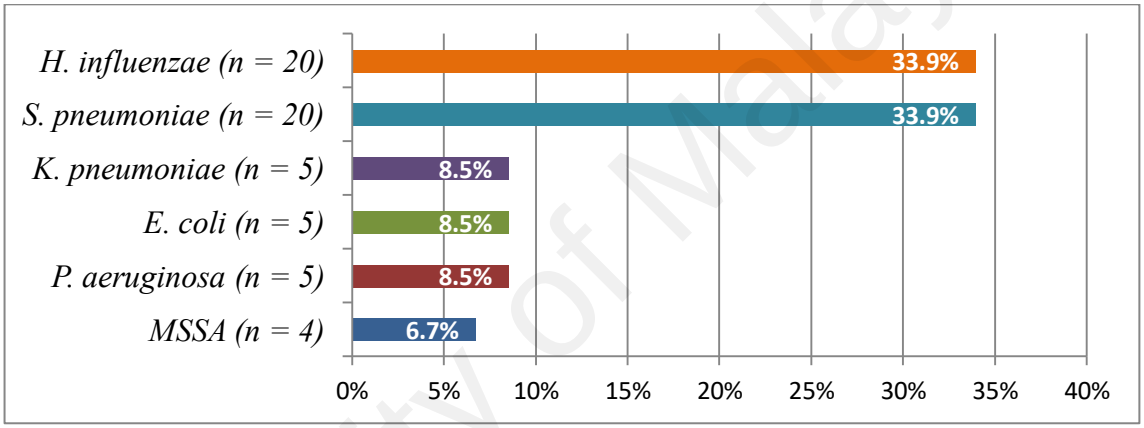

Figure 4.1: Bar chart showing the percentage of each bacterial species used in this study.

4.2 DNA extraction and quantification

Chromosomal DNA from bacterial strains was prepared using the commercial kit (DNeasy Tissue Kit; Qiagen, Valencia, CA) and each of the DAN samples was quantified in ng/µL and the purity was set to measure at 260 nm/230 nm ratio and 260 nm/28 0nm ratio. The concentration of DNA extracted, were in the range of 36 – 500 ng/µL for *S. pneumoniae* and 40 – 140 ng/µL for *H.influznae*.

4.3 Data mining and Selection of targets for primers design

4.3.1 Target gene for *Streptococcus pneumoniae*

Based on literature search, *lytA (*autolysin*), ply (*pneumolysin*), psaA* (pneumococcal surface adhesion) (Carvalho *et al*., 2007) genes were used for specific detection of *S. pneumoniae*. Among the genes, *lytA* was the most specific. Hence, this gene was selected for primers detection of *S. pneumoniae*.

Eight sequences of *lytA* for *S. pneumoniae* were retrieved from NCBI database with the accession numbers L42023, CP000671, CP005967, AY738786, AF525730, AF536050, AF322742, AY728481 and multiple sequence alignments were performed. The conserved regions were identified and used for primer design.

4.3.2 Target gene for *Haemophilus influenzae*

Based on the literature search, *hpd* gene was widely used for detection of *H. influenzae*. Hence, eight sequences from each gene were retrieved from NCBI and each set of eight sequences were subjected for multiple sequence alignment using Kalign software. Conserved regions among the eight sequences were identified and used for LAMP primer design. However, no conserved region was not found in either *hpd* or *glpK* gene, so they could not be used to design LAMP primers. Sequence of LAMP primer set targeting *pal* (OMP P6) gene in *H. influenzae* was used for development of mf-LAMP assay (Torigoe *et al.,* 2007).

4.4 Development of LAMP assays for *S. pneumoniae* and *H. influenzae*

For *S. pneumoniae*, five conserved regions along the *lytA* gene have been identified. The design of LAMP primers was accomplished by using the software Primer Explorer version 4.0 developed by Eiken Chemical Co., Ltd. A set of LAMP primers which consisted of five unique sequences (F3, B3, BIP, FIP, loop primer) were obtained. The primers sequences and binding sites are shown in Table 4.1 and Figure. 4.2

Table 4.1: LAMP primers sequences derived from *lytA* gene with the accession numbers AE007317, Gene ID 933669 of *Streptococcus pneumoniae* strain R6.

Primer	Sequence 5' → 3'	Position	Length
F3	CTGCATAGGTCTCAGCATTC	705 → 724	20 bp
B3	CGTGCAACCATATAGGCAA	892 → 910	19 bp
FIP	TCTCGCACATTGTTGGGAAC-CCCAGGCACCATTATCAAC	F2: 741 → 759 F1c: 784 → 803	F2: 19 bp F1c: 20 bp
BIP	AACCTAATTCTGGGTCTTTCCG-ACACTCAACTGGGAATCC	B2: 866 → 883 B1c: 807 → 828	B2: 18 bp B1c: 22 bp
LF	TGCATCATGCAGGTAGGACC	761 → 780	20 bp

Figure 4.2: Location of LAMP primer sequence derived from autolysin (*lytA)* gene. *lytA* gene accession numbers is AE007317, Gene ID 933669 of *S. pneumoniae* strain R6

For *H. influenzae*, conserved regions could not be identifed from *hpd* and *glpK* gene, therefore, sequence of LAMP primer set targeting *pal* (OMP P6) gene in *H. influenzae* was adopted from work done by Torigoe *et al.* (2007). The primer sequences and binding sites are shown in Table 4.2 and Figure 4.3

Table 4.2: LAMP primer sequences derived from *pal* (OMP P6) gene which was found in both typeable and non-typeable *H. influenza*. Adopted from Torigoe *et al.* (2007).

Primer	Sequence 5' → 3'	Position	Length
F3	TAGAAGGTAACACTGATGAACG	254 → 275	22 bp
B3	TACGCTAACACTGCACGA	441 → 458	18 bp
FIP	ACACCTTTACCAGCTAAATAACCTT-TGGTACACCAGAATACAACATC	F2: 277 → 297 F1c: 329 → 353	F2: 21 bp F1c: 25 bp
BIP	AGGCACAGTATCTTACGGTGA-ATATGCAGCTTCATCATGACC	B2: 409 → 429 B1c: 369 → 389	B2: 21 bp B1c: 21 bp
LF	TGCACGACGTTGGCCTAA	301 → 318	18 bp

Figure 4.3: Location of LAMP primer sequence derived from *pal* (OMP P6) gene. Pal gene accession number is L42023, Gene ID 949485 of *H. influenzae* strain Rd

To confirm the specificity of LAMP primers, two rounds screening method was employed whereby LAMP primer sequences were first subjected for *in silico* PCR analysis to check for primer specificity followed by validation using conventional PCR. In this case, LAMP outer primers (F3 and B3) were synthesized and used. Primer set that were able to amplify all targeted strains were selected to be used in subsequent LAMP assay,

For analysis in *in silico* PCR, LAMP primers targeting *lytA* genes showed 100% specificity towards all *S. pneumoniae* strains available in database while the LAMP primers targeting *pal* gene showed only 90% specificity to *H. influenzae* strains

Based on the PCR validation using outer LAMP primers (F3 & B3) derived from *lytA* gene, all sixteen clinical strains of *S. pneumoniae* were successfully detected (Figure 4.4). For *H. influenzae,* screening of LAMP primers (F3 & B3) derived from *pal* gene was not able to give 100% specificity in *in silico* PCR analysis, but the same set of primers were able to detect all seventeen clinical strains of *H. influenzae* in conventional PCR (Figure 4.5). The summary of specificity of the primers is shown in Table 4.3.

Table 4.3: LAMP outer primer sequences derived from targeted genes (*lytA* and *pal*). Outer primer (F3 and B3) sequences were synthesized and used in conventional PCR for second-round screening of clinical strains.

No.	Gene	LAMP primer set (5' → 3')	Specificity test		
			in silico PCR		Conventional PCR
			Target	Non-target	Target
Streptococcus pneumoniae					
1	*lytA*	F3: CTGCATAGGTCTCAGCATTC B3: CGTGCAACCATATAGGCAA	100%	0%	100%
Haemophilus influenzae					
2	*pal*	F3: TAGAAGGTAACACTGATGAACG B3: TACGCTAACACTGCACGA	90%	0%	100%

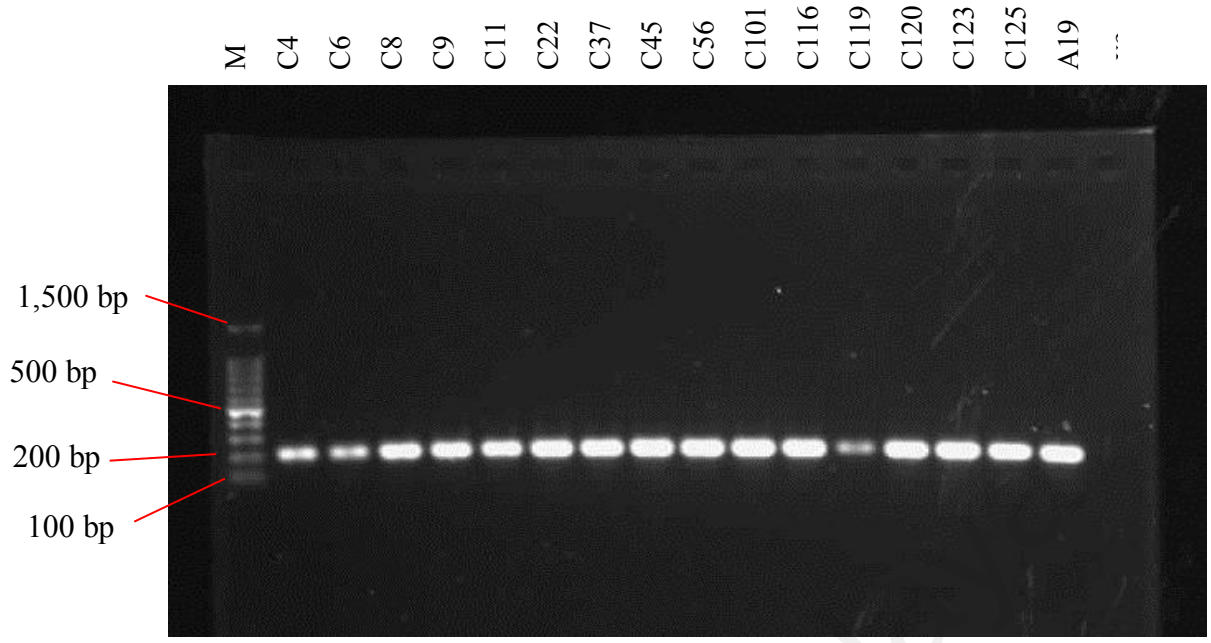

Figure 4.4: A representative electrophoretic image showing the confirmed identity of *S. pneumoniae* using conventional PCR. 16 out of 16 genomic DNAs were detected by using LAMP outer primers derived from *lytA* gene. Left to right: Lane M represent 100-bp ladder; lane 2-15 represent different *S. pneumoniae* strains; lane 16 represent *S. pneumoniae* ATCC 49169 strain; lane 17 represent non-template control. The expected amplicon size is at ~200bp (206bp) and all genomic DNAs tested showed positive for *S. pneumoniae*.

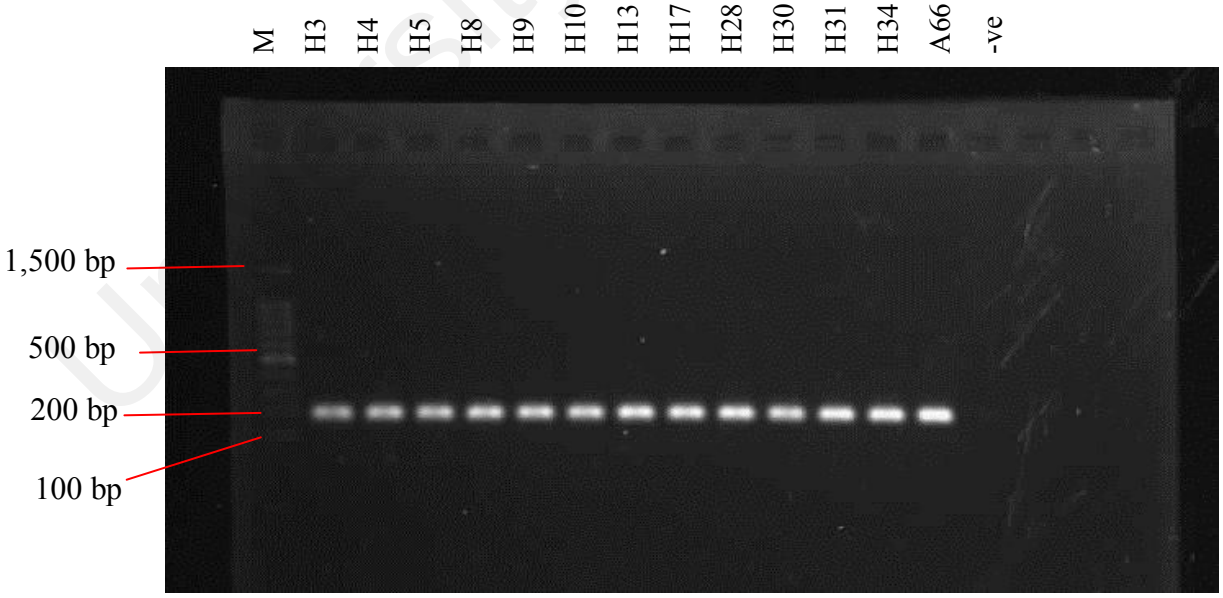

Figure 4.5: A representative electrophoretic image showing the confirmed identity of *H. influenzae* using conventional PCR. 13 out of 13 genomic DNAs were detected by using LAMP outer primers derived from *pal* gene. Lane M 100-bp ladder; lane 2-13 *H. influenzae* strains; lane 14 *H. influenzae* ATCC 49766 strain; lane 15 non-template control The amplicon size is expected to be at ~200bp (205bp) and all genomic DNAs tested showed positive for *H. influenzae*.

4.5 Validation of LAMP primers in monoplex LAMP assay

4.5.1 Determination of the Tt and detection limit for targeted and non-targeted strains

The specificity of the LAMP primer sets derived from *lytA* gene (specific for *S. pneumoniae*) and *pal* gene (specific for *H. influenzae*) were evaluated separately. Seventeen strains of *S. pneumoniae* were tested with the genomic DNA with concentration ranged from 108 – 1500 ng per reaction. Time to positive amplification (Tt) for all *the S. pneumoniae* strains were ranged between 30 - 35 min (Table 4.4). On the other hand, the amplification range for another twenty non-targeted strains which include *H. influenzae* (n=5), *E.coli* (n=5), *P. aeruginosa* (n=2), *K. pneumoniae* (n=4) and methicillin-sensitive *S. aureus* (MSSA) (n=4) were between 36 - 59 min (Table 4.4).

Table 4.4: Specificity evaluation on LAMP primer set derived from *lytA* gene specific to *S. pneumoniae* strains.

Sample Name	Bacteria species	DNA conc. (ng/µL)	DNA purity	Conventional LAMP (LAMP turbidimeter)	
				Tt*	Df**
C4		88.15	2.211	32.54	0.160
C6		186.50	2.029	34.06	0.163
C8		478.50	2.244	33.48	0.168
C9		60.00	2.273	33.42	0.153
C11		42.50	2.208	32.06	0.163
C22		268.00	2.167	32.24	0.182
C28		496.50	2.236	33.12	0.162
C37		205.00	1.647	30.48	0.165
C45	*S. pneumoniae*	69.25	2.263	33.24	0.148
C56		56.25	2.056	32.12	0.160
C101		170.00	2.125	34.12	0.156
C116		178.00	2.088	32.30	0.152
C119		168.00	2.094	32.54	0.150
C120		175.00	2.088	31.18	0.153
C123		312.00	2.016	34.30	0.154
C125		91.25	2.100	33.54	0.152
ATCC 49619		36.25	2.242	34.05	0.150
ATCC 49766	*H. influenzae*	141.50	1.931	36.0	0.167
H5	*H. influenzae*	67.50	3.000	38.0	0.153
H16	*H. influenzae*	50.0	2.833	38.48	0.115
H31	*H. influenzae*	80.0	2.615	38.06	0.158
H35	*H. influenzae*	118.0	2.474	41.54	0.144
B4	*E. coli*	58.0	2.189	0.00	0
B5	*E. coli*	54.5	2.096	0.00	0
B6	*E. coli*	111.0	2.135	0.00	0
B7	*E. coli*	67.5	2.250	0.00	0
B8	*E. coli*	71.5	2.072	0.00	0
KB4	*K. pneumoniae*	5.0	2.500	36.30	0.150
KB5	*K. pneumoniae*	3.0	1.889	47.18	0.146
KB6	*K. pneumoniae*	8.5	1.714	50.00	0.108
KB7	*K. pneumoniae*	5.5	2.200	38.24	0.173
E3	*P. aeruginosa*	40.0	2.216	39.06	0.126
E6	*P. aeruginosa*	65.0	2.117	48.12	0.132
A19	MSSA	25.0	2.632	42.12	0.143
A20	MSSA	14.50	2.417	59.24	0.137
A28	MSSA	28.5	2.478	45.48	0.173
A31	MSSA	26.0	2.737	41.48	0.153

For results presented in Table 4.4, Tt value represents time when the measurement calculated by moving average differentiation exceeds the threshold (Threshold time). Tt is the time needed for the turbidity reaction of sample to exceed OD_{650nm} at 0.1 is referred as threshold time. Whereas for Df, it is the maximum value obtained by calculating measurements by moving average differentiation (Differential calculation or level of turbidity generated)

The specificity of the LAMP primer set derived from *pal* gene (specific for *H. influenzae*) were evaluated against 18 strains of targeted *H. influenzae* clinical strains and 18 non-targeted clinical strains which include *S. pneumoniae* (n = 5), *E. coli* (n = 3), *P. aeruginosa* (n = 2), *K. pneumoniae* (n = 4) and methicillin-sensitive *S. aureus* (MSSA) (n = 4). All of the clinical strains were previously isolated from nasal cavity of in-patients admitted to a local tertiary hospital. The positive amplification for *H. influenzae* were detected at time between 17 - 21 min for all the positive clinical strains tested with genomic DNA amount ranged from 120 – 400 ng per reaction (Table 4.5). Moreover, the amplification range for 18 non-targeted strains tested in conventional LAMP assay which contained only *pal* primers was between 22 - 48 min.

Table 4.5: Specificity evaluation on LAMP primer set derived from *pal* gene specific to *H. influenzae* strains.

Sample Name	**Bacteria species**	**DNA conc. (ng/µL)**	**DNA purity**	**Conventional LAMP (LAMP turbidimeter)**	
				Tt*	Df**
ATCC 49766		141.50	1.931	18.05	0.193
H3		62.5	2.300	19.36	0.213
H4		42.5	2.222	18.18	0.206
H5		67.5	3.000	17.42	0.207
H6		105.0	1.909	17.54	0.205
H8		57.5	2.500	18.42	0.203
H9		46.25	2.500	18.30	0.212
H10		51.25	2.571	19.24	0.213
H12	*H. influenzae*	115.0	2.286	19.48	0.197
H13		75.0	2.400	20.18	0.192
H14		52.5	2.100	19.18	0.183
H17		76.25	2.545	18.54	0.203
H28		113.0	2.471	18.24	0.207
H29		108.0	2.240	17.36	0.200
H30		110.0	2.529	18.54	0.204
H31		80.0	2.615	19.30	0.205
H34		82.5	2.400	18.12	0.206
H35		118.0	2.474	18.36	0.205
C8	*S. pneumoniae*	478.50	2.244	48.06	0.164
C28	*S. pneumoniae*	496.50	2.236	44.54	0.169
C58	*S. pneumoniae*	355.00	2.250	44.00	0.170
C62	*S. pneumoniae*	225.00	2.266	48.12	0.148
C101	*S. pneumoniae*	170.0	2.125	44.36	0.159
B3	*E. coli*	77.5	2.153	0.0	0.0
B4	*E. coli*	58.0	2.189	0.0	0.0
B5	*E. coli*	54.5	2.096	0.0	0.0
KB4	*K. pneumoniae*	5.0	2.500	22.3	0.18
KB5	*K. pneumoniae*	3.0	1.889	25.06	0.186
KB6	*K. pneumoniae*	8.5	1.714	23.54	0.187
KB7	*K. pneumoniae*	5.5	2.200	24.24	0.193
E1	*P. aeruginosa*	123.0	2.216	47.48	0.161
E2	*P. aeruginosa*	73.0	2.212	40.48	0.183
A19	MSSA	25.0	2.632	39.54	0.188
A20	MSSA	14.50	2.417	38.30	0.188
A28	MSSA	28.5	2.478	36.30	0.208
A31	MSSA	26.0	2.737	38.36	0.208

4.5.2 Detection limit of the monoplex LAMP assay

Ten-fold serial dilution of the genomic DNA of both targeted strains namely *S. pneumoniae* (strain ATCC 49619) and *H. influenzae* (strains ATCC 49766) were used to prepare DNA template. The starting concentration of DNA template for *S. pneumoniae* was 220 ng/µL. Amplification at Tt beyond 35 min was defined as negative because positive amplification range for *S. pneumoniae* was found between 30 - 35 min from section 4.5.1 (as shown in Table 4.4). Based on the Tt determined under Section 4.5.1, the detection limit for LAMP primer targeting *S. pneumoniae* was 6.6 ng per reaction.

For *pal* LAMP primer, amplification beyond 21 min was defined as negative because the positive amplification range was found to be between 17 - 21 min (as shown in Table 4.5). Hence, the detection limit was found to be 32.7 ng per reaction. The detection limit for both *lytA* and *pal* LAMP assays are summarized in Table 4.6 and Table 4.7.

Table 4.6: Detection limit of *lytA* primer in conventional LAMP assay using serial dilution of genomic DNA from *S. pneumoniae* ATCC 49619 strain.

Genomic DNA concentration		**Conventional LAMP (LAMP turbidimeter) based on *lytA* LAMP primer**	
CFU	**ng/µL**	**Tt ***	**Df ****
1.7 x 10^7	**220.0**	**28.54**	**0.187**
1.7 x 10^6	**22.0**	**29.54**	**0.166**
1.7 x 10^5	**2.2**	**33.18**	**0.181**
1.7 x 10^4	0.22	39.54	0.172
1.7 x 10^3	0.022	48.30	0.138

Table 4.7: Detection limit of *pal* primer in conventional LAMP assay using serial dilution of genomic DNA from *H. influenzae* ATCC 49766 strain.

Genomic DNA concentration		**Conventional LAMP (LAMP turbidimeter) based on *pal* LAMP primer**	
CFU	**ng/µL**	**Tt ***	**Df ****
5.7 x 10^8	**109.0**	**19.06**	**0.197**
5.7 x 10^7	**10.9**	**20.24**	**0.214**
5.7 x 10^6	1.09	21.54	0.22
5.7 x 10^5	0.109	22.0	0.22
5.7 x 10^4	0.0109	26.0	0.21

For results presented in Table 4.6 and 4.7, Tt value represents time when the measurement calculated by moving average differentiation exceeds the threshold (Threshold time). Tt is the time needed for the turbidity reaction of sample to exceed OD_{650nm} at 0.1 is referred as threshold time. Whereas for Df, it is the maximum value obtained by calculating measurements by moving average differentiation (Differential calculation or level of turbidity generated)

4.5.3 Validation of the LAMP products

The LAMP products of *S. pneumoniae* (amplified by *lytA* gene) and *H. influenzae* (amplified by *pal* gene) assays were electrophoresed. Based on visual comparison, both assays gave different ladder-like patterns (Figure 4.6). The difference in banding patterns of these two organisms were clearly from 1,500 bp and below.

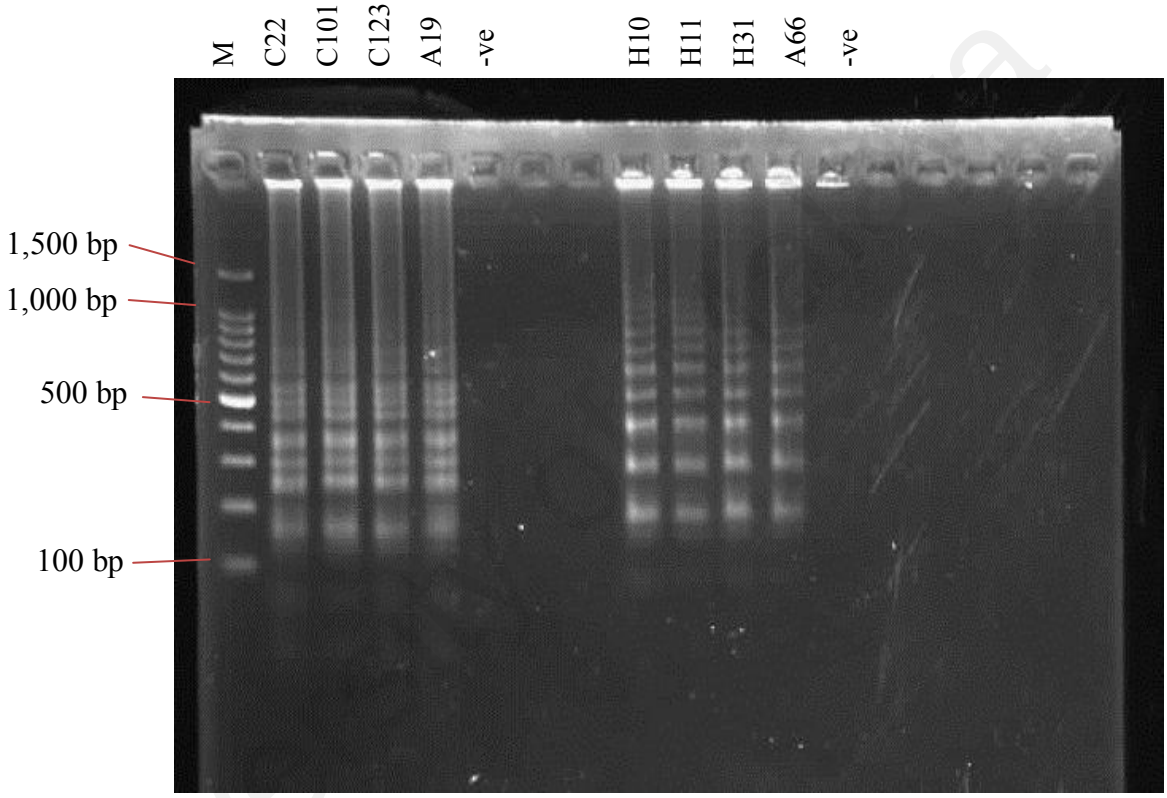

Figure 4.6: A representative gel image of the electrophoretic patterns from the LAMP amplicon. Product from monoplex LAMP assay showing the ladder-like band pattern which is the unique characteristic for amplicon generated from LAMP assay. Lane M is 100-bp ladder; lane 2-4 is *S. pneumoniae* strains; lane 5 is *S. pneumoniae* ATCC 49619 strains; lane 6 is no template control; Lane 9-11 is *H. influenzae* strains, lane 12 is *H. influenzae* ATCC 49766 strains; lane 13 is no template control.

4.6 Optimization of real-time multiplex fluorescent LAMP (mf-LAMP) assay

High quality DNA was used to optimise the mf-LAMP assay to detect *S. pneumoniae* strains. ROX^{TM} High was also added into the reaction master mix to normalize the fluorescent signal based on the recommendation of the manufacturer. In mf-LAMP assay, a positive reaction is confirmed when the Ct (threshold time) exceed ΔRn 0.1. During optimization, it was found that all *S. pneumoniae* strains tested which include ATCC 49619, C120C, C123, and C125 were successfully amplified with FAM-signal detected between 40.664 – 44.199 min. However, at this isothermal temperature (63 ºC), FAM-signal emitted from non-targeted strains (H28, H29, ATCC 49766 *H. influenzae* strains) were also found to be in the Ct range of positive amplification for *S. pneumoniae* (40.043 – 41.671 min) but they were at much lower intensity. For negative control, FAM-signal was found to be at Ct = 53.442. (Table 4.8)

On the other hand, LAMP primer targeting *pal* gene in *H. influenzae* which was labelled with JOE fluorescent dye gave positive amplification signal for all *H. influenzae* strains tested (ATCC 49766, H28, and H29) with Ct range between 28.644 – 30.456 min. Moreover, JOE-signal from non-targeted strains (*S. pneumoniae*) were between 39.502 – 41.478 min and negative control were at 41.023 min (Table 4.8). The Ct obtained for both *S. pneumoniae* and *H. influenzae* in mf-LAMP is tabulated in Table 4.8.

Table 4.8: Optimization of mf-LAMP assay at temperature 63 ºC. Result in table showing Ct reading for FAM-signal (specific to *S. pneumoniae*) and JOE-signal (specific to *H. influenzae*). Result in bold represent amplification of targeted strains.

No.	Sample name	Bacterial strain	Ct (FAM-signal) *lytA* primer	Ct (JOE-signal) *pal* primer
1	H28	*H. influenzae*	41.671	**30.456**
2	H29		40.746	**29.565**
3	ATCC 49766		40.043	**28.644**
4	C120	*S. pneumoniae*	**41.114**	40.147
5	C123		**42.003**	40.630
6	C125		**40.664**	41.478
7	ATCC 49619		**44.199**	39.502
8	Negative Control	No template	53.442	41.023

At 63 ºC isothermal reaction temperature, it was noticed that the Ct value for FAM and JOE-fluorescent dye emitted in reaction containing only genomic DNA of *S. pneumoniae* were very close to each other (Table 4.8) that makes it difficult to differentiate both pathogens in a single reaction. Hence, this multiplex LAMP assay was re-optimized by manipulating the reaction temperature in attempt to obtain a positive amplification (Ct value) which can distinguish the target from non-target strains. It was found that by raising the reaction temperature to 65 °C, positive amplification range was very clear between *S. pneumoniae* and *H. influenzae.* The positive amplification range for *S. pneumoniae* was between 41 min – 45 min and *H. influenzae* was found between 34 min to 42 min. For negative control, FAM-signal was found to be at Ct = 51.398 min whereas for JOE-signal, Ct reading was at 46.346 min where neither fluorescent signal fell within the positive range for *S. pneumoniae* and *H. influenzae* (Table 4.9). Signal detection from FAM and JOE dye are shown in Figures 4.7 and 4.8.

Table 4.9: Optimization of mf-LAMP assay at temperature 65 ºC for duration of 60 min. Result in table showing Ct reading for FAM-signal (specific to *S. pneumoniae*) and JOE-signal (specific to *H. influenzae*). Result in bold represent amplification of targeted strains

No.	Sample name	Bacterial strain	Ct (FAM-signal) *lytA* primer	Ct (JOE-signal) *pal* primer
1	H28	*H. influenzae*	51.022	**37.932**
2	H29		49.298	**41.776**
3	ATCC 49766		47.451	**34.328**
4	C120	*S. pneumoniae*	**41.171**	51.427
5	C123		**41.855**	46.366
6	C125		**41.519**	47.385
7	ATCC 49619		**44.544**	46.079
8	Negative Control	No template	51.398	46.346

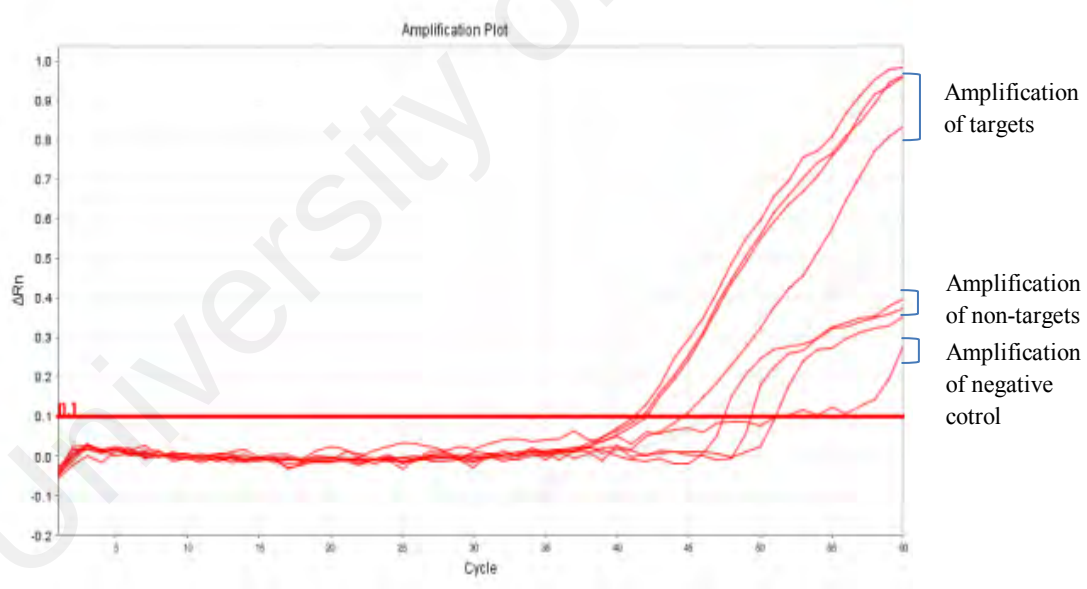

Figure 4.7: Amplification graph showing signal emitted from FAM fluorophore. FAM-fluorescent dye was covalently linked to *lytA* FIP primer. In this reaction, positive amplification of *S. pneumoniae* demonstrated earlier detection at the range of 41 - 45 min with doubled the fluorescence intensity (ΔRn 1.0) compared to non-targets.

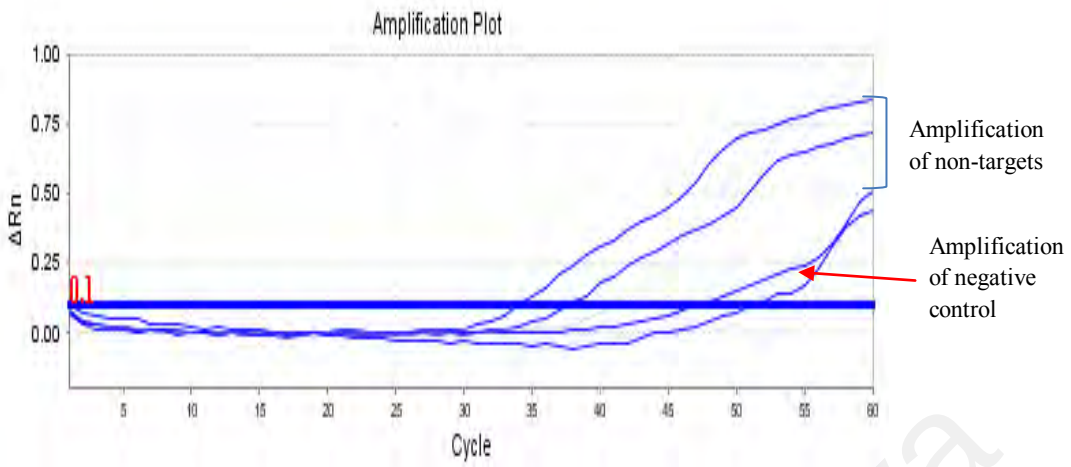

Figure 4.8: Amplification graph showing signal emitted from JOE fluorophore. JOE-fluorescent dye was covalently linked to *pal* FIP primer. In this reaction, positive amplification of *H. influenzae* demonstrated earlier detection at 34 - 42 min with the fluorescence intensity higher (ΔRn 0.75) compared to non-target.

The condition for mf-LAMP assay was further optimized by manipulating the concentration of both sets of LAMP primers and also the final concentration of genomic DNA in the assay. This approach was taken in attempt to obtain a shorter reaction time for both LAMP primers together with distinct curve to differentiate positive detection of both pathogens. Firstly, the concentrations for primer QFIP:Fd, BIP and FIP were altered at different percentage of concentration, whereas concentration for primer F3, B3 and LF remained unchanged throughout the entire reaction. The primer in the ratio of *lytA* – 75 % : *pal* – 25 % (Table 4.10 and 4.11; Figure 4.9), *lytA* – 60 % : *pal* – 40 % (Table 4.12 and 4.13; Figure 4.10) and *lytA* – 50 % *: pal* – 50 % (Table 4.14 and 4.15; Figure 4.11) were tested. It was found that the primer ratio of *lytA* – 50 % *: pal* – 50 % gave the best result compared to the other two combination.

Table 4.10: Percentage of LAMP primer concentration used in mf-LAMP assay. Concentration of *lytA* LAMP primer was 75 % and *pal* LAMP primer is 25 %. The concentration for primer F3, B3 and LF remained the same throughout the entire reaction.

No.	Type of primer	Final LAMP primer concentration per reaction (µM). Developed by Tanner *et al.* (2012)	Trial 1 Concentration of LAMP primer (*lytA* – 75 % : *pal* – 25 %) (µM)	
			lytA	*pal*
1	QFIP:Fd	0.8	0.6	0.2
2	BIP	1.6	1.2	0.4
3	FIP	0.8	0.6	0.2
4	F3	0.2	0.1	0.1
5	B3	0.2	0.1	0.1
6	LF	0.4	0.2	0.2

Table 4.11: Ct reading for reaction containing 75 % of *lytA* LAMP primer and 25 % of *pal* LAMP primer. Result in table showing Ct reading for FAM-signal (specific to *S. pneumoniae*) and JOE-signal (specific to *H. influenzae*). Result in bold represent amplification of targeted strains.

No.	**Sample name**	**Bacterial strain**	**Ct (FAM-signal) *lytA* primer**	**Ct (JOE-signal) *pal* primer**
1	H31	*H. influenzae*	42.556	**47.808**
2	ATCC 49766		38.427	**43.305**
3	C22	*S. pneumoniae*	**36.075**	46.247
4	C120		**32.534**	44.739
5	ATCC 49619		**35.012**	40.762
6	Negative Control	No template	43.714	48.953

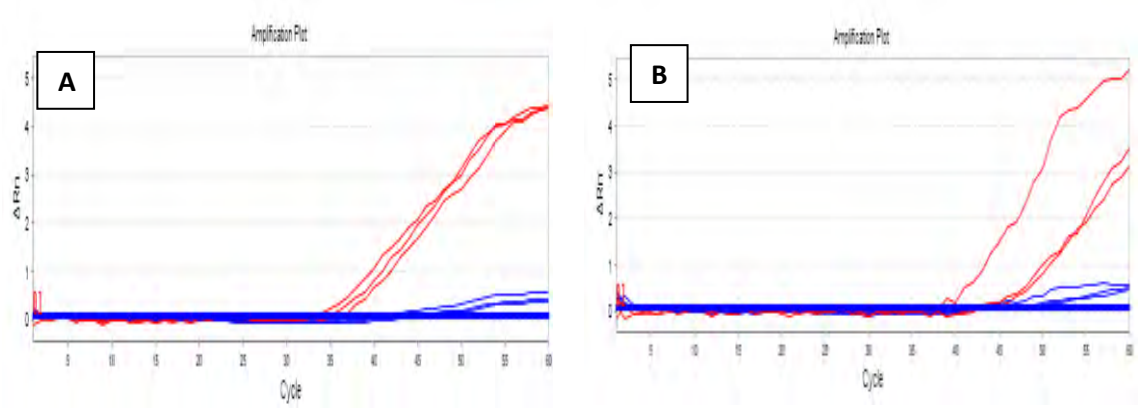

Figure 4.9: Graph showing amplification for reaction containing 75 % of *lytA* LAMP primer and 25 % of *pal* LAMP primer. Amplification curve in red represent FAM-signal and blue represent JOE-signal. In panel A, early detection time (Ct = 32 – 36 min) with high signal amplitude was observed from FAM-signal for reaction containing only genomic DNA of *S. pneumoniae* and JOE-signal from this reaction was between Ct 40-46 min with low amplitude. In panel B, the FAM-signal was rather high for reaction containing only genomic DNA of *H. influenzae*. JOE-signal which was specific to *H. influenzae* remained low.

Table 4.12: Percentage of LAMP primer concentration used in mf-LAMP assay. Concentration of *lytA* LAMP primer was 60 % and *pal* LAMP primer is 40 %. The concentration for primer F3, B3 and LF remained the same throughout the entire reaction.

No.	Type of primer	Final LAMP primer concentration per reaction (µM). Developed by Tanner *et al.* (2012)	Trial 2 Concentration of primer (*lytA* – 60 % : *pal* – 40 %) (µM)	
			lytA	*pal*
1	QFIP:Fd	0.8	0.5	0.3
2	BIP	1.6	1.0	0.6
3	FIP	0.8	0.5	0.3
4	F3	0.2	0.1	0.1
5	B3	0.2	0.1	0.1
6	LF	0.4	0.2	0.2

Table 4.13: Ct reading for reaction containing 60 % of *lytA* LAMP primer and 40 % of *pal* LAMP primer. Result in table showing Ct reading for FAM-signal (specific to *S. pneumoniae*) and JOE-signal (specific to *H. influenzae*). Result in bold represent amplification of targeted strains.

No.	Sample name	Bacterial species	Ct (FAM-signal) *lytA* primer	Ct (JOE-signal) *pal* primer
1	H31	*H. influenzae*	52.833	**49.559**
2	ATCC 49766		49.848	**45.108**
3	C22	*S. pneumoniae*	**37.755**	50.647
4	C120		**35.229**	50.847
5	ATCC 49619		**36.804**	51.798
6	Negative Control	No template	43.696	46.930

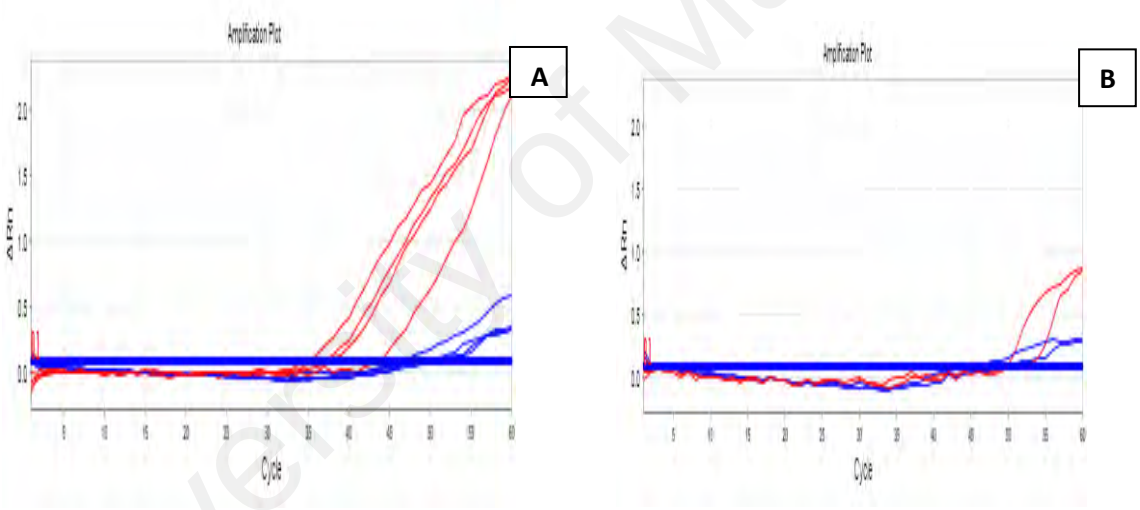

Figure 4.10: Graph showing amplification for reaction containing 60 % of *lytA* LAMP primer and 40 % of *pal* LAMP primer. Amplification curve in red represent FAM-signal and blue represent JOE-signal. In panel A, early detection time (Ct = 35 – 38 min) with high signal amplitude was observed from FAM-signal in reaction containing only genomic DNA of *S. pneumoniae* and JOE-signal in this reaction was between Ct 40 - 46 min with low amplitude. In panel B, the FAM and JOE-signal were low in reaction containing only genomic DNA of *H. influenzae*. The amplitude for JOE-signal which was specific to *H. influenzae* was reduced to insufficient levels.

Table 4.14: Percentage of LAMP primer concentration used in mf-LAMP assay. Concentration of *lytA* LAMP primer was 50 % and *pal* LAMP primer is 50 %. The concentration for primer F3, B3 and LF remained the same throughout the entire reaction.

No.	Type of primer	Final LAMP primer concentration per reaction (μM). Developed by Tanner *et al.* (2012)	Trial 3 Concentration of primer (*lytA – 50 % : pal – 50 %)* (μM)	
			lytA	*pal*
1	QFIP:Fd	0.8	0.40	0.40
2	BIP	1.6	0.80	0.80
3	FIP	0.8	0.40	0.40
4	F3	0.2	0.1	0.1
5	B3	0.2	0.1	0.1
6	LF	0.4	0.2	0.2

Table 4.15: Ct reading for reaction containing 50 % of *lytA* LAMP primer and 50 % of *pal* LAMP primer. Result in table showing Ct reading for FAM-signal (specific to *S. pneumoniae*) and JOE-signal (specific to *H. influenzae*). Result in bold represent amplification of targeted strains.

No.	**Sample name**	**Bacterial strain**	**Ct (FAM-signal) *lytA* primer**	**Ct (JOE-signal) *pal* primer**
1	H28	*H. influenzae*	51.022	**37.932**
2	H29		49.298	**41.776**
3	ATCC 49766		47.451	**34.328**
4	C120	*S. pneumoniae*	**41.171**	51.427
5	C123		**41.855**	46.366
6	C125		**41.519**	47.385
7	ATCC 49619		**44.544**	46.079
8	Negative Control	No template	51.398	46.346

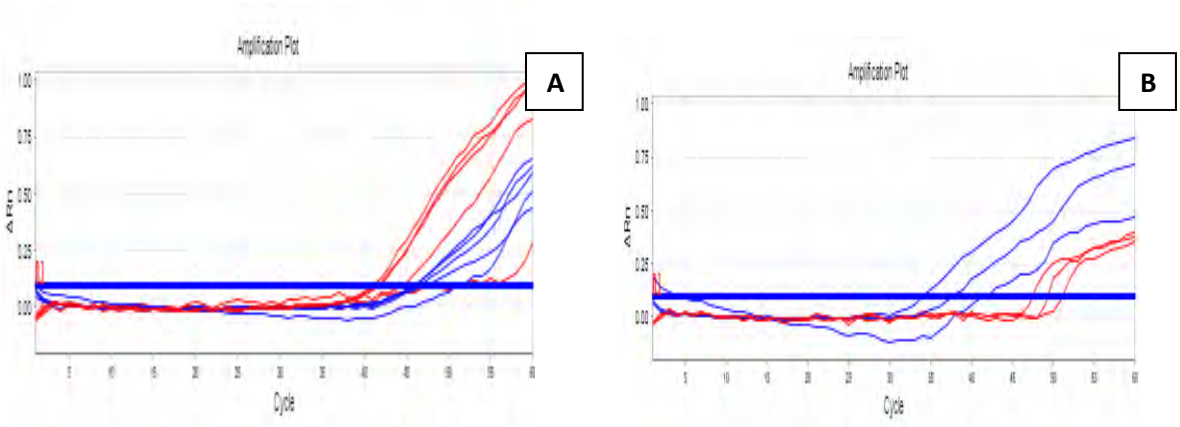

Figure 4.11: Graph showing amplification for reaction containing 50 % of *lytA* LAMP primer and 50 % of *pal* LAMP primer. In panel A, detection time was delayed to Ct = 41 – 45 min for FAM-signal in reaction containing only genomic DNA of *S. pneumoniae* and JOE-signal from this reaction was between Ct 46 - 51 min with increased in amplitude compared to lower percentage concentration ratio. In panel B, an early detection time (Ct = 34 – 42 min) with higher signal amplitude was observed in JOE-signal for reaction containing only genomic DNA of *H. influenzae*. In contrast, FAM-signal specific to *S. pneumoniae* displayed lower amplitude with delayed Ct (detection time).

4.6.1 Specificity of the real-time mf-LAMP assay using pure clinical strains

The specificity of mf-LAMP assay was determined using 20 strains of *S. pneumoniae*, 20 strains of *H. influenzae* and 19 non-targeted strains including *E. coli* (n=5), *P. aeruginosa* (n=5), *K. pneumoniae* (n=5) and MSSA (n=4). This newly developed mf-LAMP assay showed 100% specificity toward both targeted strains with no cross-reaction with non-targeted strains. The time for positive amplification for *S. pneumoniae* (DNA amount ranged from 108 ng-1500 ng per reaction) was between 40 - 45 min whereas for *H. influenzae* strains (DNA amount ranged from 120 ng – 400 ng per reaction) the positive amplification range was between 33 - 39 min. Assay specificity was also checked against non-targeted strains and the amplification range was between Ct 46 - 52 min for reaction containing only *S. pneumoniae* DNA template. On the other hand, the amplification for non-target in reaction containing only *H. influenzae* DNA template was found to be between 46 – 52 min. It was observed that

time to positive amplification (Ct) were independent to initial concentration of DNA template. An increase in amount of DNA template did not correspond to decrease (early) in Ct value. Ct value for FAM and JOE fluorescent signals for all targeted and non-targeted strains are summarised in Table 4.16 and Table 4.17

Table 4.16: Ct values for POSITIVE and NEGATIVE amplification of both pathogens (*S. pneumoniae* & *H. influenzae*) in their respective LAMP primers (*lytA* and *pal* gene).

Real-time Multiplex fluorescent LAMP assay									
Sample (S. pneumonia)	DNA conc. (ng/µL)	DNA purity	Ct (FAM-signal) *lytA* gene	Ct (JOE-signal) *pal* gene	Sample (H. influenzae)	DNA conc. (ng/µL)	DNA purity	Ct (JOE-signal) *lytA* gene	Ct (JOE-signal) *pal* gene
C4	88.15	2.211	40.829	46.190	**H1**	50.00	2.333	47.967	35.980
C6	186.50	2.029	41.050	42.877	**H3**	62.50	2.300	45.186	34.105
C7	75.00	1.600	42.369	42.005	**H8**	57.50	2.500	50.068	38.072
C8	478.50	2.244	44.005	41.573	**H9**	46.25	2.500	46.177	34.396
C9	60.00	2.273	44.038	40.908	**H10**	51.25	2.571	47.137	35.808
C11	42.50	2.208	44.161	45.881	**H13**	75.00	2.400	50.375	38.713
C22	268.00	2.167	43.134	42.458	**H14**	52.5	2.100	49.592	38.130
C28	496.50	2.236	43.409	43.399	**H15**	58.75	3.143	45.389	34.490
C37	205.00	1.647	42.536	44.539	**H16**	50.00	2.833	47.644	37.317
C45	69.25	2.263	44.156	45.237	**H18**	70.00	2.455	49.274	35.491
C58	103.25	2.056	43.356	41.540	**H20**	12.00	2.182	48.442	37.159
C59	310.00	2.240	41.332	42.024	**H23**	80.00	1.643	48.929	35.106
C61	332.00	2.293	40.413	48.903	**H28**	113.00	2.471	51.022	37.932
C62	340.00	2.215	43.227	45.529	**H30**	110.00	2.529	51.074	38.262
C116	178.00	2.088	40.873	43.437	**H31**	80.00	2.615	45.377	37.504
C119	168.00	2.094	41.726	44.448	**H34**	92.50	2.400	47.730	36.011
C120	175.50	2.088	41.171	51.427	**H43**	80.00	2.667	45.483	33.813
C123	312.00	2.016	41.855	46.366	**H46**	57.00	2.455	46.648	35.432
C125	91.25	2.100	41.519	47.385	**H48**	118.00	2.474	46.975	36.687
ATCC 49619	36.25	2.242	43.274	50.771	**ATCC 49766**	141.50	1.931	47.451	34.328

Table 4.17: Ct values for amplification of non-targeted strains in mf-LAMP assay. Primers targeting *lytA* (*S. pneumoniae*) and *pal (H. influenzae)* gene were used. All the Ct values obtained from non-target amplification showed 100% specificity with no cross-reactivity with non-targeted strains.

Multiplex LAMP assay		
Non-targeted strains	Ct (FAM-signal) *lytA* gene	Ct (JOE-signal) *pal* gene
***E. coli* B6**	Undetermined	45.642
***E. coli* B7**	Undetermined	42.247
***E. coli* B8**	Undetermined	43.855
***E. coli* B9**	Undetermined	40.943
***E. coli* B10**	Undetermined	42.605
***K. pneumoniae* KB2**	52.111	49.650
***K. pneumoniae* KB3**	Undetermined	Undetermined
***K. pneumoniae* KB4**	52.071	52.498
***K. pneumoniae* KB9**	Undetermined	45.317
***K. pneumoniae* KB10**	Undetermined	52.214
***P. aeruginosa* E1**	Undetermined	52.128
***P. aeruginosa* E4**	Undetermined	50.539
***P. aeruginosa* E5**	59.366	47.274
***P. aeruginosa* E7**	Undetermined	48.233
***P. aeruginosa* E10**	58.683	46.011
Methicillin-sensitive *S. aureus* A17	52.823	46.011
Methicillin-sensitive *S. aureus* A19	Undetermined	43.910
Methicillin-sensitive *S. aureus* A33	47.828	44.236
Methicillin-sensitive *S. aureus* A34	49.389	43.126

4.6.2 Sensitivity evaluation of multiplex fluorescent LAMP assay using pure clinical strains

Sensitivity of the multiplex LAMP assay was done using 10-fold serial dilution of genomic DNA of *S. pneumoniae* (ATCC 49619 strains) and *H, influenzae* (ATCC 49766 strains). The starting concentration of genomic DNA was 220 ng/µL for *S. penumoniae* and 109 ng/µL for *H. influenzae*. By referring to the result from specificity test above (Section 4.7), positive amplification range for *S. pneumoniae* and *H. influenzae* were found to be between 40 - 45 min and 33 - 39 min respectively. Hence, amplification beyond this range of both targeted strains was defined as negative. Base on this findings, the detection limit in this mf-LAMP assay was determined to be

at 66 pg per reaction for *S. pneumoniae* and 32.7 pg per reaction for *H. influenzae*. Table 4.18, Figure 4.12 and Figure 4.13 showing Ct value from FAM and JOE dye targeting *lytA* and *pal* gene respectively.

Table 4.18: Detection limit of both *lytA* and *pal* primer in newly constructed mf-LAMP assay. Sensitivity of mf-LAMP assay was analysed using serial dilution of genomic DNA from *S. pneumoniae* ATCC 49619 and *H. influenzae* ATCC 49766 strain.

DNA concentration (*S. pneumoniae*) ATCC 49619		**Ct (FAM-signal) *lytA* primer**	**Ct (JOE-signal) *pal* primer**	**DNA concentration (*H. influenzae*) ATCC 49766**		**Ct (FAM-signal) *lytA* primer**	**Ct (JOE-signal) *pal* primer**
CFU	**ng/µL**			**CFU**	**ng/µL**		
1.7×10^7	220.0	**43.274**	50.771	5.7×10^8	109.0	44.952	**35.400**
1.7×10^6	22.0	**43.436**	48.006	5.7×10^7	10.9	45.802	**36.070**
1.7×10^5	2.2	**43.100**	46.128	5.7×10^6	1.09	46.329	**36.679**
1.7×10^4	0.22	**43.423**	48.972	5.7×10^5	0.109	46.455	**36.779**
1.7×10^3	0.022	**44.080**	47.670	5.7×10^4	0.0109	48.272	**37.896**

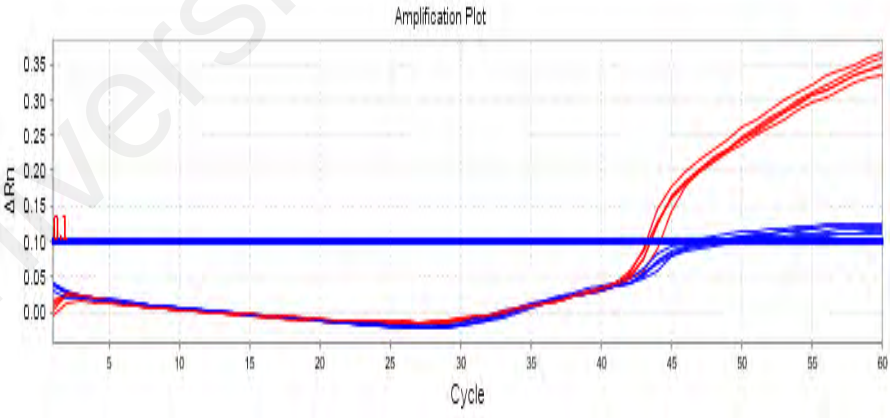

Figure 4.12: Amplification curve for 10-fold serial-dilution of *S. pneumoniae* ATCC 49619 strain. Red colour curve represents FAM-fluorescent signal (labelled in *lytA*-LAMP primer specific to *S. pneumoniae*); whereas blue colour amplification curve represents JOE-fluorescent signal (labelled in pal-LAMP primer specific to *H. influenzae*).

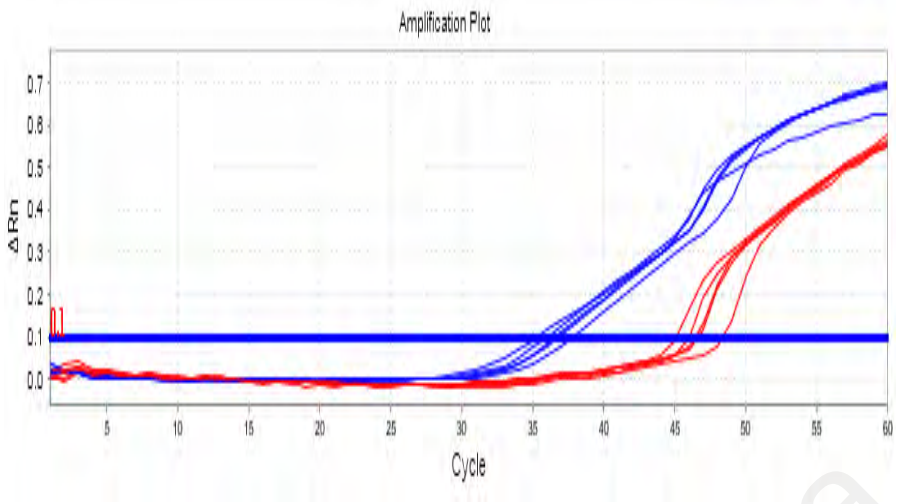

Figure 4.13: Amplification curve for 10-fold serial-dilution of *H. influenzea* ATCC 49766 strain. Blue colour curve represents JOE-fluorescent signal (labelled in pal-LAMP primer specific to *H. influenzae*); whereas red colour amplification curve represents FAM-fluorescent signal (labelled in *lytA*-LAMP primer specific to *S. pneumoniae*).

Sensitivity of the mf-LAMP assay was further compared with conventional PCR assay using both ATCC strains. The detection limit in mf-LAMP assay for *S. pneumoniae* was found to be the same as in conventional PCR (66 pg per reaction; Figure 4.14). On the other hand, both multiplex LAMP assay and conventional PCR can detect up to 32.7 pg per reaction of *H. influenzae* (Figure 4.15). When comparing the detection limit established in monoplex LAMP assay, mf-LAMP assay demonstrated 100 time higher sensitivity in detection of *S. pneumoniae* (Table 4.6) and 1000 higher sensitivity for detection of *H. influenzae* (Table 4.7).

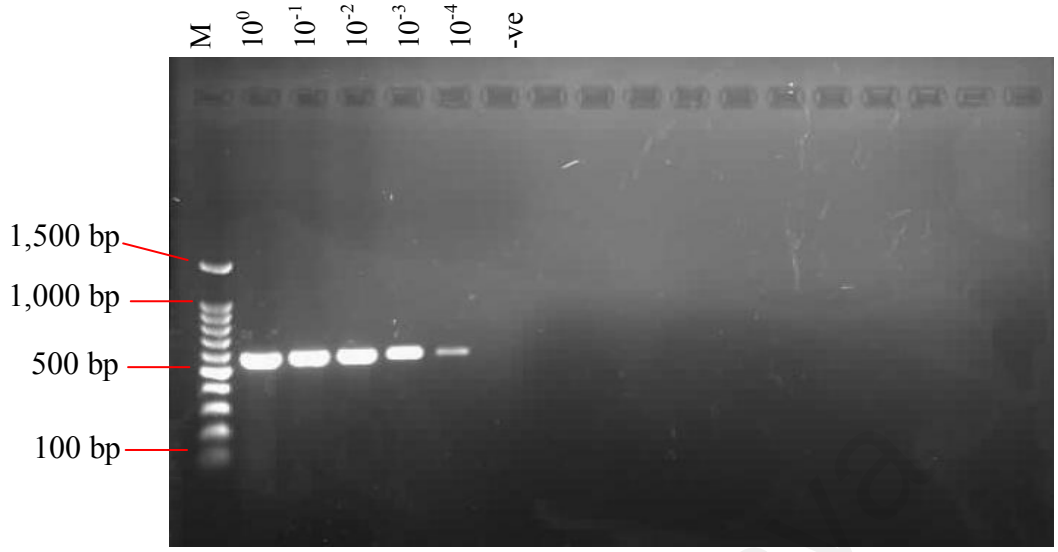

Figure 4.14: Sensitivity of the *recP* primer to detect 10-fold serial dilution of genomic DNA of *S. pneumoniae* (ATCC49619). Starting concentration (10^0) is 220 ng/µL per reaction and PCR is able to detect up until 10^{-4} dilution (66 pg per reaction). Lane M is 100 bp DNA ladder marker; Lane 2 is 10-fold DNA dilution from 220 ng/µL, 22 ng/µL, 2.2 ng/µL, 0.22 ng/µL & 0.022 ng/µL; Lane 7 is negative control (Ultra-Pure water). PCR assay was performed using *recP* primer with expected amplicon size around 571 bp.

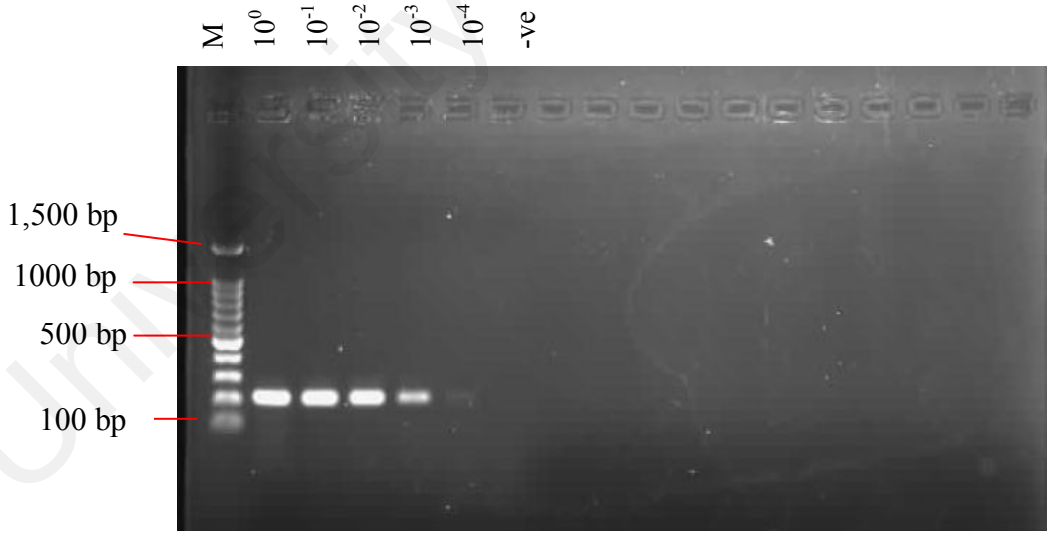

Figure 4.15: Sensitivity of *pal* primer to detect 10-fold serial dilution of genomic DNA of *H. influenzae* (ATCC 49766). Starting concentration (10^0) is 109 ng/µL per reaction and PCR is able to detect up until 10^{-4} dilution (32.7 pg per reaction). Lane M is 100 bp DNA ladder marker; Lane 2 is 10-fold DNA dilution from 109 ng/µL, 10.9 ng/µL, 1.09 ng/µL, 0.109 ng/µL & 0.0109 ng/µL; Lane 7 is negative control (Ultra-Pure water). PCR assay was performed using *pal* outer primer (F3/B3) with expected amplicon size around 205 bp.

4.6.3 Validation of the mf-LAMP results

To further confirm the positive amplification of targeted gene in mf-LAMP assay, the products were subjected for agarose gel electrophoresis on 2 % (w/v) agarose gel pre-stained with RedSafe™ (iNtRON Biotechnology, Korea), and the gel was run at 80 V. It was noticed that the banding pattern inside the ladder is less clear compared to banding pattern on gel in monoplex assay (Figure 4.6). The difference in banding patterns were able to be identified from 1,500 bp and below.

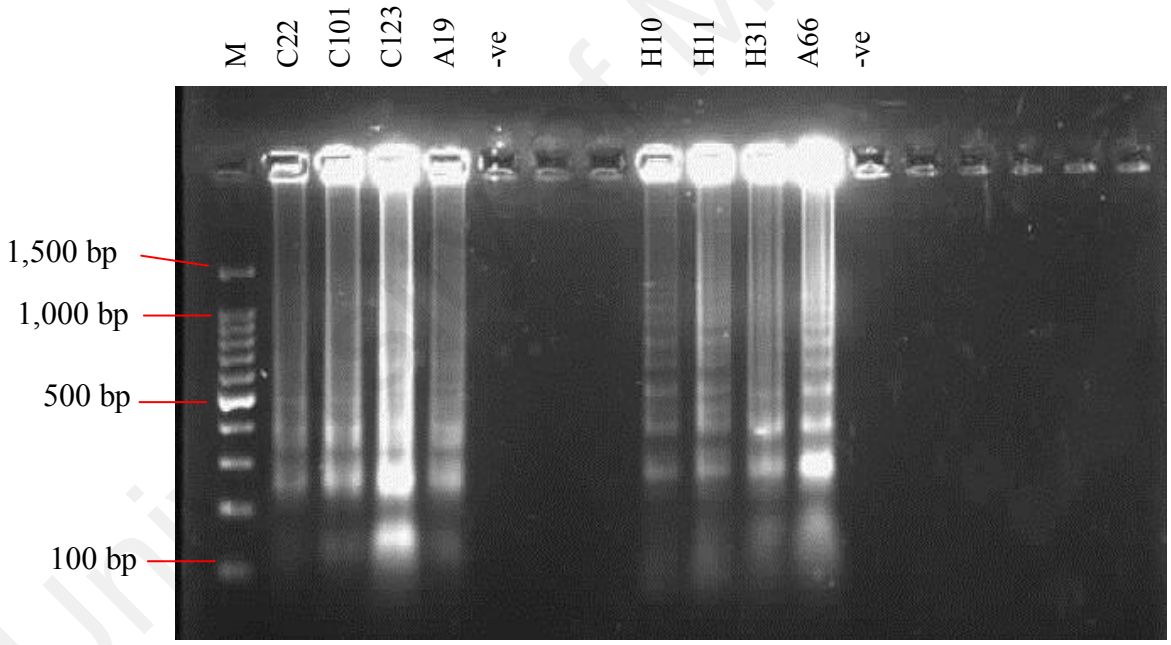

Figure 4.16: A representative gel image of the electrophoretic pattern of the mf-LAMP amplicon. Result of mf-LAMP product on 2 % (w/v) agarose gel showing the ladder-like band pattern which is the unique characteristic for product generated from LAMP assay. Lane M is 100-bp DNA ladder; lane 2 - 4 are *S. pneumoniae* strains; lane 5 is *S. pneumoniae* ATCC 49619 strains; lane 6 is negative control (Ultra-Pure Water); Lane 9-11 are *H. influenzae* strains; lane 12 is *H. influenzae* ATCC 49766 strains and lane 13 is negative control (Ultra-Pure Water).

CHAPTER 5

DISCUSSION

5.1 Development of mf-LAMP for detection of *Streptococcus pneumoniae* and *Haemophilus influenzae*

S. pneumonia remains as one of the most important pathogens in childhood pneumonia. In addition, fatal diseases such as septicaemia, meningitis or less fatal sinusitis, otitis media in children are also caused by *S. pneumoniae* infection. Children with pneumococcal infection usually possess non-specific clinical presentations that are similar to other febrile illnesses caused by *S. aureus, M. pneumoniae, Neisseria meningitides, E. coli, Salmonella enterica serovar* Typhi and *Leptospira,* thus making it crucial for the etiological diagnosis of these diseases (Chheng *et al*., 2013).

There are four methods to identify *S. pneumonia* infection in diagnostic laboratory, ie. phenotypic characterization of colonies, optochin sensitivity test, bile solubility test and serology agglutination test. Non-pneumococcal streptococci are usually optochin resistant or bile insoluble or do not have alpha-haemolytic property when it is cultured on blood agar plate. While these classical techniques allow detection of the majority of pneumococcal isolates, several closely related streptococci, typically *S. mitis* or *oralis* (viridans group streptococci-VGS) could not be correctly identified because they shared the common genetic ancestry (Whatmore *et al.,* 2000).

In fact, many PCR-based assays have been established for detection of *S. pneumoniae* using primers specific to gene encoding rRNA, *pneumolysin* (*ply*), *autolysin* (*lytA*), pneumococcal surface antigen A (*psaA*) and manganese-dependent superoxide dismutase (*sodA*) with various degrees of success. However, non-

pneumococcal strains such as *S. mitis* and *S. oralis* harboring the same virulence genes (Whatmore *et al.,* 2000), further complicates the identification of *S. pneumoniae*.

Pneumolysin (*ply*) and autolysin (*lytA*) genes are well-characterized virulence genes specific for detection of *S. pneumoniae*. Both genes are responsible in pathogenesis for *S. pneumoniae* infection. However, *lytA* gene appeared to have higher level of specificity (100 % specificity) in comparison to other genes responsible in pneumococcal infection such as *ply* (81 % specificity) and *psaA* (98 % specificity) which has been demonstrated by Carvalho *et al*. (2007) in real-time PCR assay. Furthermore, the restricted allelic variation in this gene making it an ideal target for specific identification of *S. pneumoniae* in clinical and epidemiological studies (Whatmore & Dawson, 1999). In fact, another studies also demonstrated 100 % specificity in real-time PCR to distinguish *S. pneumoniae* from its close relative mitis group. Although many reports have reported that the presence of *lytA* gene in many of the closely related mitis group strains, however based on the phylogenetic trees constructed by comparing the *lytA* gene sequences between *S. pneumoniae, S. mitis* and *S. pseudopneumoniae*, the differences in the nucleotide sequences of the *lytA* gene are found to be greater between species than within species (Greve *et al.,* 2012).

Alongside with this finding, another report also demonstrated that the difference of *lytA* alleles between typical and atypical streptococci resided in 102 nucleotide positions thus making *lytA* gene well conserved in all *S. pneumoniae* strains (Llull *et al.*, 2006). On the other hand, *lytA* gene was found in *S. pneumoniae* from all sites of isolation including both invasive (pneumoniae, meningitis, sepsis, facial palsy and sinusitis) and non-invasive ocular infections (endophthalmitis, keratitis, dacryocystitis, and lacriml abscess) in comparison to (*ply* gene where it was absence in the later (Sourav *et al.,* 2010). This signifies that regardless of the sites of isolation or kinds of infection, *lytA* gene is an obligate necessity for pneumococcal pathogenesis.

The use of *lytA* gene in LAMP assay targeting *S. pneumoniae* has been described before (Seki *et al.*, 2005) and 100 % specificity of the LAMP primers targeting *lytA* gene was reported. In the present study, different region was used for the primers design and a loop primers was designed and incorporated into the LAMP reaction. The incorporation of loop primer into a reaction is beneficial especially in multiplex setting to accelerate the duration of reaction and to enhance the sensitivity of the assay (Nagamine *et al*., 2002). Thus, based on all the literature search above, autolysin (*lytA)* gene was selected as specific target for design of LAMP primers and fluorescent probe to be tested in monoplex LAMP and mf-LAMP assays.

Although there was a similar work done (Seki *et al.,* 2005) using *lytA* gene to detect *S. pneumoniae* in LAMP assay, the published LAMP primer sequences were not adopted in this study as the LF or LB primer, termed loop primer was not available. Inclusion of loop primer in LAMP reaction can accelerate the LAMP reaction by reducing the detection time to less than half of the original LAMP method. Thus, in order to achieve a detection time of less than 1 hour in multiplex LAMP assay, a new set of LAMP primer targeting *lytA* gene in *S. pneumoniae* was designed with loop primer included.

glpK gene is one of the housekeeping gene found in *H. influenzae* that is responsible in the synthesis of enzyme glycerol kinase for catalyzing glycerol uptake and metabolism. This gene is required for maintenance of basic cellular function and is expressed in normal and patho-physiological conditions, making this gene a potential target for amplification. On the other hand, Protein D encoding gene (*hpd)* is a well-characterized gene and many researches has reported that *hpd* as one of the well preserved gene among encapsulated and non-encapsulated *H. influenzae* strains. (Janson

et al., 1993; Song *et al.*, 1995; Duim *et al.*, 1997). When protein D was tested in Western blot using three different monoclonal antibodies directed against human myeloma immunoglobulin D 4490, Protein D with the molecular weight of 42 kDa was found in all 127 *H. influenzae* strains which comprised of serotypes a-f and non-typeable strains (Akkoyunlu *et al.*, 1991). In a research published by Janson *et al.* (1993), it was described that the nucleotide and amino acid sequences in gene encoding protein D from *H. influenzae* b strain were found to be highly homologous to the protein D gene originates from the non-typeable *H. influenzae* strain 772 as there were only 2 out of 364 amino acids varies when the two *hpd* gene were compared

In this research, two-round screening strategy was employed to select target gene for LAMP primer design. A detailed discussion on two-round screening was described under Section 4.4. As a result, LAMP primers derived from *lytA* gene targeting *S. pneumoniae* demonstrated 100 % specificity towards all *S. penumoniae* strains tested, however, LAMP primers derived from *glpK* and *hpd* gene failed to give 100 % specificity to *H. influenzae* clinical strain during the two-round screening process. Therefore, LAMP primers targeting *pal* gene designed in work published by Torigoe *et al*. (2007) were employed. LAMP primer set for the *pal* gene was shown to be highly specific to all strains of *H. influenzae* including typeable and non-typeable strains during the two-round screening process. Besides, this set of primer also able to distinguish *H. influenzae* from genotypically similar *H. parainfluenzae* strains, which are also commensals of the human oral cavity.

LAMP primers were designed based on four essential factors, for example the melting temperature (Tm), stability at the end of each primer, GC content and secondary structure. The Tm for each primer is affected by the sequence and length of the primer, thus the sequence and length of the primers were carefully chosen so that the Tm fell within certain ranges. For example, the Tm values for F2 and B2 region in

FIP and BIP were set to fall between 60 °C and 65 °C which is the optimal temperature for *Bst* polymerase. Another segment of sequence in FIP and BIP termed F1c and B1c, the Tm values were slightly higher than those in F2 and B2 in order to facilitate the immediate formation of looped out structure after the single stranded DNA was released from the template. Furthermore, the Tm for outer primers (F3 and B3) were set lower than F2 and B2 region to ensure the DNA synthesis occur first from the inner primer (FIP and BIP) rather than from the outer primers (F3 and B3). To obtain a good primer, the GC content of LAMP primers was also set between 40 % and 65 %. Stability at the end of primers also play an important role to ensure the DNA synthesis is carry out at isothermal temperature. The 3'end of F2/B2, F3/B3and LF/LB sequences and 5'end of F1c/B1c sequence were designed to have free energy -4 kcal/mol or less.

The sequence length of LAMP inner primer, particularly FIP and BIP are usually long (28 – 45 bp) and shall not form secondary structure during DNA synthesis. Besides, the 3'ends of primers shall not form complementary to prevent formation of primer dimers. In addition, the efficiency of LAMP reaction is also dependent of the size of amplicon. It was demonstrated that (Notomi *et al*., 2000) size of amplicon within 130 to 200 bp gives the best amplification in comparison with amplicon size more than 500 bp. Thus, the size of targeted DNA shall be kept below 300 bp to ensure good efficiency in DNA synthesis.

Once the specificity of LAMP primer sets were confirmed, they were then incorporated into monoplex LAMP assay to evaluate its specificity and sensitivity. At isothermal temperature of 63 °C, both primer sets derived from *lytA* gene and *pal* gene showed 100 % specificity to all targeted strains with no-cross reactivity with non-targeted strains. The detection of both pathogens were achieved within 60 min of reaction time and the positive detection range for *S. pneumoniae* was determined to be at 30 - 35 min and 17 - 20 min for *H. influenzae*. The concentration of genomic DNA

tested was in the range of 108 – 1500 ng per reaction for *S. pneumoniae* and 120 – 400 ng per reaction for *H. influenzae*. This indicates that the newly designed LAMP primers for both assays were able to detect complementary sequence in target gene with the initial DNA concentration as low as 108 ng and amplification efficiencies of both monoplex assays were maintained when the genomic DNA concentration was raised to 1500 ng per reaction. The sensitivity of the primers in monoplex LAMP assays was determined as 6.6 ng per reaction for *S. pneumoniae* and 32.7 ng for *H. influenzae.*

The specificity of the LAMP reaction was further confirmed by electrophoresis of positive LAMP amplicon and typical ladder pattern smear were observed on 2 % (w/v) agarose gel from the target genes of *lytA* and *pal* (Figure 4.6), suggesting a successful amplification of specific target. This finding was in agreement with work published by Nyan *et al*. (2015) where the presence of specific LAMP primer-sets in multiplex assay contributed to the unique ladder-like banding patterns then enabled pathogen identification. The differences in the banding pattern can be observed through the size of the bands within the patterns, laddering shift or by the group-formation of the bands of the detected target. (Figures 4.6 and 4.16)

In this study, it was observed that mf-LAMP assay performance could be carried out at several reaction temperature ranging from 63 – 65 °C within 60 min of reaction time. However, optimal reaction was found at 65 °C isothermal temperature because the positive amplification range for both targeted strains were distinct, enable the differentiation of two pathogens in a single run. This is in agreement with Rittié and Perbal (2008) that *Bst* Polymerase is active at an optimal temperature of 65 °C.

During the optimization process, it was noticed that at isothermal temperature of 65 °C, amount of genomic DNA template in the range between 327 ng – 660 ng per reaction gave an optimal condition for mf-LAMP assay. Amount beyond this range reduced the amplification efficiency by delaying the time of positive detection.

In the present mf-LAMP assay, the optimal primer concentrations with ratio of 50 % *lytA* LAMP primer : 50 % *pal* LAMP primers gave the best results. Furthermore, Tanner *et al*. (2012) suggested that equimolar of standard FIP and QFIP:Fd duplex primer in multiplex fluorescent LAMP assay could give high fluorescent signal with acceptable speed of amplification .

In addition to that, it was demonstrated by Tanner *et al*. (2012) that lower amount of QFIP:Fd duplex primer (25 % or 10 % of QFIP) has also provided faster amplification times but the fluorescent signal for this combination was too low for detection based on the result published by Tanner *et al*. (2012). Therefore, in this study, a mix balance of standard FIP and QFIP:Fd primer concentration (50 % : 50 %) was used to provide high signal with acceptable speed of amplification of the mf-LAMP assay; overall result suggests that the concentration for all the LAMP primers involved namely QFIP:Fd, FIP, BIP, F3 B3 and LF must be in equimolar for both sets of primers when used in multiplex assay. However, it has to be reminded that the total primer concentration in a reaction must be kept to 4.4 μM regardless of the number of targets and it has to be adjusted to 1/*n* where *n* represents the number of target used in a multiplex reaction (Tanner *et al*., 2012).

Tanner *et al*. (2012) has reported a high non-template signal in the absence of DNA template in a quadruplex reaction targeting bacteriophage λ, *C. elegans, E. coli,* and Hela (BRCA1) DNA. The non-template signal was found to be high for *E. coli* and bacteriophage λ primer sets but negative for *C. elegans* and HeLa primer sets. Thus, in this study, we have incorporated an inert reference dye termed ROX^{TM} High into this mf-LAMP reaction, attempted to normalize the fluorescent fluctuation from well-to-well and to increase the detection precision (Malkki *et al*., 2012). ROX^{TM} High passive dye was chosen because it is compatible to the qPCR system used in this research (Applied Biosystem Step-One Plus Real-Time PCR System).

Fluorophores have been traditionally used in quantitative PCR (qPCR) applications for detection and quantification of pathogen load in specimens. The properties of these fluorescent dyes that are able to absorb and emit light during hybridization (excitation) were employed in this study for detection and measurement of fluorescence for amplified target DNA. In this study, we employed two different fluorophores (FAM and JOE) for detection of target pathogens. FAM fluorophore was covalently liked to fluorogenic probe specific to sequence in *lytA* gene found in *S. pneumoniae*, while JOE fluorophore was linked to probes specific to *pal* gene in *H. influenzae*. It was observed that amplified targeted DNA demonstrated higher Relative Fluorescence units (RFUs), indicating positive amplification, whereas for non-target DNA and negative control revealed lower RFUs. This observation is in agreement with the work published by Nyan *et al*. (2015). In this study, a higher fluorescent intensity was corresponded to positive amplification of targeted gene (Figure 4.11) was consistently observed. This feature can potentially be used to quantify amplicons and correlate to the pathogen load in an infected sample. However, further study and optimization are warranted.

Positive amplification for *S. pneumoniae* and *H. influenzae* in monoplex were found to be 30 - 35 min and 17 - 20 min respectively. However, when two sets of LAMP primers were combined into a multiplex assay, the detection time for both pathogens have been delayed (from 30 - 35 min to 40 - 45min for *S. pneumoniae*; from 17 - 20 min to 33 - 39 min for *H. influenzae*). This phenomenon may be due to both sets of primers are competing for the enzyme during DNA synthesis. In the present study, the volume of *Bst* DNA polymerase and LAMP enzyme buffer was kept the same as monoplex assay (12.5 μL), however with additional set of LAMP primer in multiplex assay, making a total of ten primer sequences, enzyme and substrate competition is much greater compared to monoplex assay. Furthermore, the time to positivity is also

largely dependent on the design of primers (including GC content rather than target sequence length), fine-tuning on one of these factors may help to shorten the detection time in multiplex assay while keeping the concentration of DNA polymerase and LAMP buffer constant (Notomi *et al*., 2000).

In this study, the detection of targeted strains was limited to less than 45 min to prevent any false-positive signals. In a report published by Francois *et al*. (2011), the performance of LAMP reaction can be affected by the amplification time whereby false-positive was found at negative control when amplification time was prolonged to 180 min. Thus, extensive optimization on primer design, primer concentration, amplification temperature, and duration of reaction are needed to ensure signal can be detected as soon as the reaction starts, to avoid prolong duration of amplification.

Specificity of mf-LAMP assay was further confirmed by the distinguishing ladder-like banding-pattern in 2 % (w/v) agarose gel (Figure 4.16). The differences in the banding patterns generated by each primer-set are distinguishable by the position of ladder-shift, the size of the bands and patterns, as well as the group-formation of bands relative to molecular marker. Similar pattern also appeared in gel loaded with products from amplification by monoplex LAMP assays but the bands were more distinctive as compared to bands obtained from mf-LAMP assay (Figure 4.6).

In the present study, the monoplex and multiplex LAMP assay specifically amplified only *S. pneumoniae* and *H. influenzae*, giving 100 % specificity with no cross-reactivity observed in other non-targeted bacterial species namely *K. pneumoniae, E. coli, P. aeruginosa* and MSSA. This result demonstrates that the mf-LAMP assay has high specificity and is efficient for simultaneous amplification of *S. pneumoniae* and *H. influenzae* in a single reaction tube. It was noticed that the detection limit for using mf-LAMP assay in 60 min reaction time were 66 pg for *S. pnuemoniae* strains and 32.7 pg for *H. influenzae* strains, demonstrating 100 times higher sensitivity

in detecting of *S. pneumoniae* and 1000 sensitivity for *H. influenzae* detection. Moreover, the detection

limit for mf-LAMP was comparable with conventional PCR when the lowest concentration tested were 66 pg per reaction for *S. pnuemoniae* and 32.7 pg per reaction of *H. influenzae*.

In this study, it was also found that the time to positive amplification (Ct) were independent to initial concentration of DNA template. This finding was similar to a study done by Francois *et al*. (2011), where LAMP assay was found to have poor linearity in comparison to real-time PCR which gave linear quantification ability in DNA amplification. Although there were reports demonstrated good linear relationship in multiplex LAMP assay (Liu *et al*., 2017), the additional optimization and enhancement could further improve its quantification ability for clinical application.

5.2 Advantage of real-time multiplex fluorescent LAMP assay

Loop-mediated isothermal amplification (LAMP), which amplifies genomic DNA with high specificity, efficiency and rapidity under constant temperature, has continued to attract the attention of many researches from many fields. One of the most attractive features of LAMP is its high specificity that requires a set of six primers spanning 8 distinct region of the target gene to initiate an amplification (Notomi *et al*., 2000). No amplification will occur unless all the target genes are available. Secondly, LAMP assay offers high amplification efficiency due to no time loss of thermal change as the reaction is carried out in isothermal condition. In multiplex LAMP, same concept was applied with additional advantage by providing a platform for multiple detection of pathogen in single tube that can be achieved within 1 hour of reaction time (Mahony *et al*., 2013). In the present study, we employed closed-tube system to further increase the

specificity by avoiding the opening of reaction tube for post-LAMP analysis, thus preventing cross-contamination which is very prone in LAMP assay.

Monoplex LAMP assay can only allow detection of single a pathogen, making multiple detections impossible in the singleplex setting. In order to realise the possibility of multiplex in LAMP, we incorporate fluorogenic LAMP assay into real-time PCR. There was a study by Lin *et al*. (2013) demonstrated that Taqman real-time PCR was more sensitive than LAMP assay. Thus, the integration of multiplex fluorogenic LAMP assay into real-time PCR, would greatly reduce post-analysis time as the positive amplification can be visualized by real-time monitoring of the Ct value and the result can be confirmed within 90 min (staring from DNA extraction to identity confirmation) as compared to monoplex LAMP.

In the present study, a specific sequence of LAMP primer was covalently linked to a fluorophore to construct a fluorescent-labelled species-specific primer which allows specific amplification of targeted gene while at the same time allow real-time monitoring of DNA amplification. The advance optical detection in real-time PCR coupled with fluorescent-quencher chemistry allows a higher sensitivity for detection of targeted gene when compared to conventional PCR assay (Mackay, 2004).

Moreover, this newly designed mf-LAMP assay is more efficient and economical because total reaction volume used was equivalent (25 μL) to the amount used in monoplex LAMP assay but the former can detect two pathogens in single assay with the detection time achieved within 60min obviating the need for post-analysis. Furthermore, primer concentrations in reaction were adjusted to half compare to the concentration used in monoplex LAMP assay. By doing this, a considerable time, effort and cost can be saved especially on the cost to synthesize fluorescent labelled primers and cost to purchase LAMP reaction kit.

5.3 Limitation and Future Direction

In the present study, the results were based on laboratory findings. There was no clinical samples applied in this study to evaluate its clinical application in related field. Although the quantification ability could not establish in current mf-LAMP assay, however, it was believed further optimization and enhancement from exiting mf-LAMP assay could realize its quantification ability for clinical application. Quantification of gene copy number can be accomplished easily by plotting a standard curve with a known concentration of genomic DNA against time of positivity to allow the estimation of the concentration of a clinical sample by extrapolating the time of positivity from the standard curve. By having the ability to quantify the amplified gene, this mf-LAMP assay can be an added advantage to differentiate infection from colonization in children by detecting bacterial load in the former. This is because the nasopharyngeal colonization rates are higher in children from developing country

However, this new fluorogenic LAMP assay required expensive real-time PCR thermocycler to run the assay and also expensive fluorescent probe with additional two long inner primers (FIP and BIP) that need to be synthesized in HLPC grade purity. All of the above will incur a higher initial starting cost and may eventually lead to restriction in certain laboratory with limited financial support. On the other hand, LAMP reagents might not be readily available in some countries and may further restrict the application of this multiplex LAMP assay in local setting.

There are a few components in LAMP reaction mix which are sensitive to light (fluorophores) and vigorous vortexing (*Bst polymerase*) that will make the preparation of the reaction mix more challenging. Thus, highly trained personnel is mandatory to involve in the preparation and carry out the run to avoid extensive exposure of reagents to lights and also to prevent damage to *Bst* polymerase by vigorous

vortexing which may result in reduced its amplification activity. However, this may be a burden to certain laboratories especially those at resource-poor countries

In addition, as LAMP assay is vulnerable to carry-over contamination, there is a need to prevent and overcome the contamination problem. For instance, the amplicon from previous LAMP runs are always a major source of contamination to the subsequent LAMP assay (Hsieh *et al.*, 2014). Contamination problem need to be resolved within short period of time to prevent accumulation of aerosolized amplification products to contaminate equipment, laboratory reagents and ventilation system. Thus, special precaution should be taken each time for the preparation before each run. In this study, while setting up the LAMP reaction, universal contamination precautions were undertaken to ensure that no amplicon contamination of molecular reagents occurred. These precautions included the physical separation of all the procedure manipulation from sample preparation, amplification reactions, and post-amplification processing, use of filter pipette tips, changing glove and extensive surface cleaning using hypochlorite and UV irradiation.

In the cases of false-positive signals in non-target and negative control sample, UNG-dUPT system which originally used to prevent carry over contamination in PCR could be utilized to completely eliminate risk of contamination (He *et al.*, 2010). The use of dUTP (deoxyuridine triphosphate) to replace dTTP (Deoxythymidine triphosphate) in deoxyribonucleotide mix in LAMP reaction and pre-incubation of sample with enzyme UNG (Uracil N-Glycosylase) which caused degradation of uracil in reaction mix will help to prevent contamination from previously amplified products (He *et al.*, 2010). For instance, Hsieh *et al.* (2014) had demonstrated a complete elimination of contamination in LAMP assay by incorporation of uracil-DNA-glycosylase (UDG) digestion enzyme in a one-step, closed vessel reaction.

The data from this study has revealed the potential application of this multiplex fluorogenic LAMP assay for simultaneous detection, identification and quantification of amplicon of multiple gene targets in a single-closed tube reaction. This assay has demonstrated plausible diagnostic specificity and sensitivity of assay that is useful for point-of-care diagnostics, preliminary screening of pneumonia infection, and large-scale epidemiological surveillance of pneumonia infection among children. However, further study and inclusion of more clinical samples shall be done to validate the feasibility and acceptability of assay in related field. In addition, internal control should be included to enhance he specificity of the assay. This approach is crucial as the various types of clinical samples are usually presence with several potential inhibitors or other unpredictable composition that somehow may affect the reliability of assay.

CHAPTER 6

CONCLUSION

In conclusion, we have successfully developed a rapid, specific and sensitive real-time multiplex fluorogenic LAMP assay that simultaneously detect *S. pneumoniae* and *H. influenzae* in 45min in a close-tube system. It is faster and more convenient compared to monoplex LAMP or conventional PCR as it does not require post-LAMP analysis. Further improvement shall be warranted so that this assay can be used as a screening tool in pneumonia suspected patient so that early intervention can be implemented in order to reduce infection-related morbidity and mortality rate among hospitalized patients

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