# ESTABLISHMENT OF RAPID DETECTION OF *Haemophilus influenzae* TYPE B BY ISOTHERMAL AMPLIFICATION OF *bex*A GENE

By

## LEOW CHEE SIONG

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# LIST OF SYMBOLS AND ABBREVIATION

| Symbols/ Abbreviation | Definition | Symbols/ Abbreviation | Definition |
|---|---|---|---|
| bp | Base pair | °C | Degree celcius |
| *Bst* | *Bacillus stearothermophilus* | µg | Microgram |
| CBA | Chocolate Blood Agar | B3 | Backward outer primer |
| CSF | Cerebrospinal fluid | BIP | Backward inner primer |
| CFU/ml | Colony-Forming-Unit per millilitre | dATP | Deoxyadenosine triphosphate |
| DNA | Deoxyribonucleic acid | dCTP | Deoxycytidine triphosphate |
| EDTA | Ethylene diamine tetraacetic acid | dGTP | Deoxyguanosine triphosphate |
| EHEC | Enterohemorrhagic *Escherichia coli* | $dH_2O$ | Sterile distilled water |
| EIEC | Enteroinvasive *Escherichia coli* | dNTP | Deoxynucleoside triphosphate |
| EPEC | Enteropathogenic *Escherichia coli* | dsDNA | Double stranded deoxyribonucleic acid |
| ETEC | Enterotoxigenic *Escherichia coli* | dTTP | Deoxythymidine triphosphate |
| *H. influenzae* | *Haemophilus influenzae* | F3 | Forward outer primer |
| HCI | Hydrochloric acid | fg | Femtogram |
| LAMP | Loop mediated isothermal amplification | FIP | Forward inner primer |
| LOD | Limit of detection | LF | Loop forward primer |
| $MgCl_2$ | Magnesium chloride | ml | milliliter |
| min | Minute | ng | Nanogram |
| NaOH | Sodium hydroxide | pg | Picogram |
| $OD_{600}$ | Optical Density at 600nm | pmol | Picomole |
| PCR | Polymerase Chain Reaction | $T_a$ | Annealing temperature |
| rpm | revolutions per minute | *Taq* | *Thermus aquaticus* |
| s | Second | $T_m$ | Melting temperature |
| spp | Species | U/µl | Unit per microliter |
| TBE | Tris-borate-ethylenediaminetetraacetic acid | v/v | Volume/volume |
| TSB | Trypticase Soy Broth | w/v | Weight/volume |
| xg (x gravity) | Relative centrifugal force | | |

# ABSTRAK

*Haemophilus influenzae* jenis b (Hib) adalah salah satu penyebab meningitis kanak-kanak yang membawa kepada morbiditi dan mortaliti yang ketara dalam negara-negara membangun dan negara-negara mundur, di mana vaksin konjugat Hib tidak boleh diakses. Satu kaedah diagnostik yang pantas dan kos efektif sangat diperlukan oleh makmal yang sumbernya terhad. Oleh itu, tujuan kajian ini adalah untuk mewujudkan satu kaedah pengesanan yang pantas untuk *H. influenzae* jenis b dengan loop-mediated isothermal amplification (LAMP) daripada bexA gen. Satu set primer LAMP telah direka khususnya untuk mensasarkan bexA gen. In-house LAMP telah direka dan dioptimumkan dengan menggunakan DNA genom *Haemophilus influenzae* ATCC 10211 yang telah diekstrak. Selepas itu, reagen LAMP dan keadaan reaksi LAMP telah dioptimumkan. LAMP assay yang dioptimumkan telah dikajikan dengan spesificiti analisis dan sensitiviti analisis. Reaksi PCR telah dijalankan dengan menggunakan outer primers untuk memberikan perbandingan dari segi sensitiviti analisis. Keputusan spesificiti analisis yang menggunakan tiga puluh jenis non-haemophilus telah dapat menunjukkan seratus peratus spesificiti untuk pengesahan bexA gen. Had pengesahan untuk LAMP assay telah ditentukan pada 2 pg DNA dan 2 colonies-forming units (CFU) bagi setiap reaksi. Hal ini menunjukkan bahawa had pengesahan bagi LAMP dengan CFU bagi setiap reaksi adalah 100 kali ganda lebih rendah daripada had pengesahan PCR. Berdasarkan kepada keputusan yang diperolehi, LAMP mempunyai potensi untuk menjadi alat diagnostik yang mampu menghasilkan keputusan yang pantas, sensitif dan spesific dalam keadaan suhu yang tetap.

# ABSTRACT

*Haemophilus influenzae* type b (Hib) is a common cause of childhood meningitis that leads to a significant morbidity and mortality in both developing countries and underdeveloped countries, where the successful Hib conjugate vaccine is not accessible. A rapid and cost-effective diagnostic method was highly demanded in resource-limited laboratory. Hence, the aim of this study is to establish a rapid detection method for *H. influenzae* type b by loop-mediated isothermal amplification (LAMP) of *bex*A gene. A LAMP primer set was specifically designed to target on *bex*A gene. An in-house LAMP reaction was established and optimized using extracted genomic DNA of *Haemophilus influenzae* ATCC 10211. Subsequently, both LAMP reagents and reaction conditions were optimized and the optimized LAMP assay was subjected to analytical specificity and sensitivity evaluation. PCR reaction was conducted by using outer primers to provide comparison in term of analytical sensitivity. The analytical specificity was evaluated with thirty non-haemophilus strains which showed 100% specificity for *bex*A gene identification. The limit of detection (LOD) for LAMP assay was determined at 2 pg of DNA per reaction and as low as 2 colonies-forming units (CFU) per reaction. This indicates that the detection limit of LAMP by CFU per reaction was 100 fold lower than the detection limit of PCR. Based on the result obtained, LAMP has the potential to become an excellent diagnostic tool capable to produce a rapid, highly sensitive and specific result in a single temperature condition.

# CHAPTER 1: INTRODUCTION

## 1.1 Introduction to *Haemophilus influenzae*

### 1.1.1 History

*Haemophilus influenzae* is a Gram negative coccobacillus bacteria that is capable to cause diseases ranging from non-pathogenic colonization in the respiratory tract to serious systemic infections (Dajani *et al.*, 1979). It is a facultative anaerobe bacteria. *H. influenzae* was formerly known as Pfeiffer's bacillus or *Bacillus influenzae*. It was discovered by a German bacteriologist, Richard Pfeiffer in 1892 and mistakenly thought to be the etiologic agent of influenza (Van Epps, 2006). Most of the scientists mistakenly identified *H. influenzae* as the culprit that caused the devastating 1918 pandemic of influenza based on Pfeiffer's theory (Schuchat and Messonnier, 2007). This is due to *H. influenzae* being repeatedly isolated from patients with the fatal respiratory infections, before scientists had discovered that the flu was actually originated from influenza virus in 1933 (Shope, 1931). Nevertheless, its confusing name remained in spite of no association with influenza.

### 1.1.2 Serotyping

*H. influenzae* can be classified into two main groups, which are typeable and non-typeable strains according to possession of polysaccharide capsule (Satola *et al.*, 2003a). There are six capsule serotypes (serotype a to f) that can be differentiated based on antisera that react specifically towards each of the particular serotypes (LaClaire *et*

al., 2003). It is similar to the Lancefield grouping of *Streptococcus spp.* Among six serotypes that had been identified, *H. influenzae* type b has been the most significant serotype in causing systemic infections such as meningitis and bacteremia (Musher, 1983). Meanwhile, non-typeable strains of *H. influenzae* do not produce any polysaccharide capsule and mostly inhabit in upper respiratory tract as a form of asymptomatic colonization (King, 2012). Traditionally, *H. influenzae* serotypes have been identified by slide agglutination serotyping (LaClaire *et al.*, 2003).

### 1.1.3 Epidemiology of *Haemophilus influenzae* type b

*H. influenzae* type b is a common cause of childhood meningitis that leads to a significant morbidity and mortality in both developing countries and poorest countries, where the successful *H. influenzae* type b conjugate vaccine is not accessible (Amanda and Messonnier, 2014). In 2000, there are 8 millions of severe illness cases caused by *H. influenzae* type b worldwide (Watt *et al.*, 2009). In 2000, approximately 371000 deaths in children that less than 5 years old caused by *H. influenzae* type b, mainly in both developing countries and poorest countries (Watt *et al.*, 2009). In Malaysia, 50% of the childhood bacterial meningitis caused by *H. influenzae* type b before Hib conjugate vaccine introduced in year 2002 (Hussain *et al.*, 1998). It was estimated that 38 per 100000 children in Malaysia suffered *H. influenzae* type b meningitis in the year of 1999 (Peltola, 2000).

## 1.1.4 Clinical manisfestation

Infected with *H. influenzae* type b (Hib) can result in meningitis and other severe infections such as pneumonia, bacteremia, cellulitis, septic arthritis and epiglottitis (Musher, 1996). *H. influenzae* type b meningitis could result in severe mental retardation in patients who are recover from the acute form of the disease (Sentz, 2006). Nonencapsulated strains cause otitis media, sinusitis, conjunctivitis and acute lower respiratory tract infections (Aubrey and Tang, 2003).
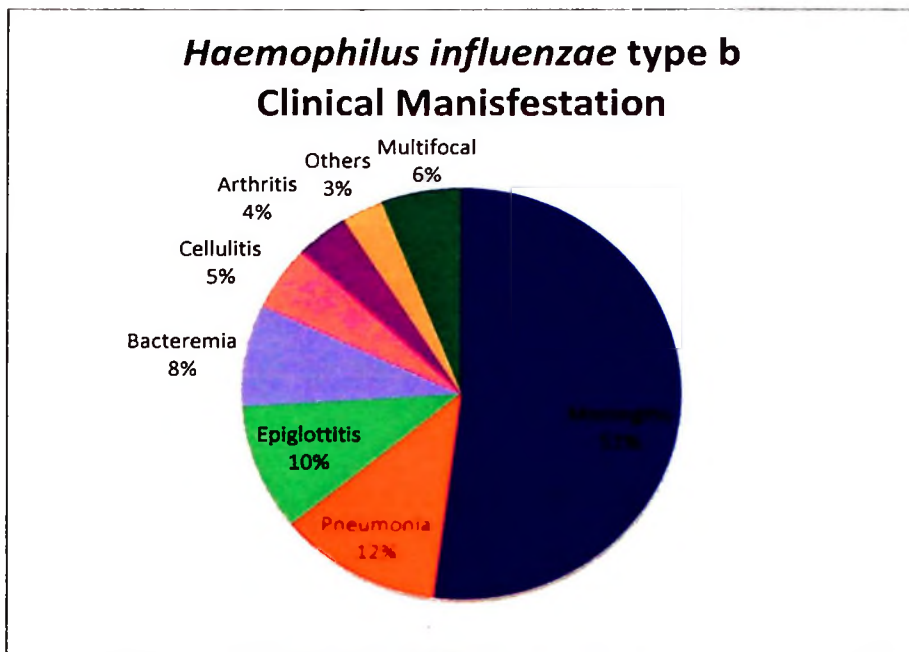
**Figure 1.1:** Worldwide spectrum of all classical (nonbacteremic pneumonia excluded) Hib diseases, taken from data for 3,931 patients in 21 studies from various parts of the world (Adapted from Peltola, 2000).

## 1.1.5 Pathogenicity

The pathogenesis of *H. influenzae* infections is not well understood, although the presence of polysaccharide capsule is known to be the major virulence factor (Musher, 1996). The outermost structure of *H. influenzae* is composed of polyribosyl-ribitol-phosphate (PRP), a polysaccharide that is responsible for virulence and immunity. In general, infections caused by *H. influenzae* type b are often systemic and life threatening. Transmission is often via respiratory secretions. In summary, the virulence factor, PRP capsule that has antiphagocytic properties can mediated attachment of bacteria cells into host epithelial cells.

### 1.1.6 *bex*A gene

All typeable strains of *H. influenzae* contain common genes for capsule production and found within *cap* loci. The *cap* loci for all serotypes consist of functionally unique regions I, II, and III. Regions I and III are common to all six capsular types and contain genes necessary for the processing and exportation of the capsular material (Satola *et al.*, 2003b). Region I genes (*bexDCBA*) code for an ATP-driven capsule export apparatus. Region II contains serotype-specific biosynthesis genes that appear to be unique to each of the six capsule types. Region III genes appear to be involved in capsule postpolymerization steps. The *bex*A gene is located in region I, which is responsible for exportation of polysaccharide capsule (Davis *et al.*, 2011).
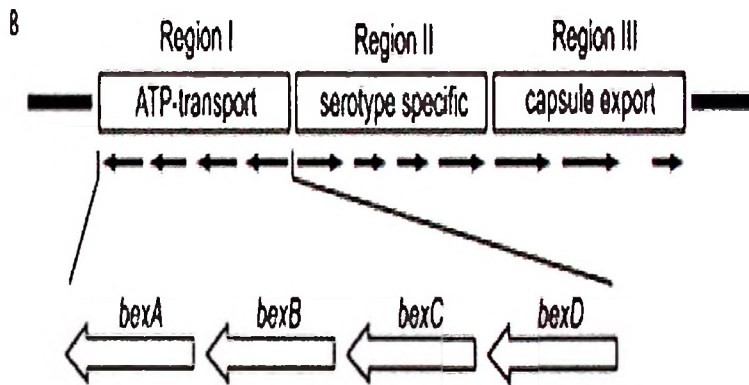
Figure 1.2: The *cap* locus divided into three regions, namely, regions I, II and III. The *bexA* gene is located within regions I (Adapted with modification from Davis *et al.*, 2001).

## 1.1.7 Treatment

Hospitalization is generally required for invasive *H. influenzae* type b patients. Antimicrobial therapy with an effective third generation cephalosporin (cefotaxime or ceftriaxone) should be begun immediately (Prasad *et al.*, 2004). The treatment course is usually 10 days. Ampicillin-resistant strains of *H. influenzae* type b are now commonly appeared. Therefore, children with life-threatening illness in which *H. influenzae* type b may be the etiologic agent should not receive ampicillin alone as initial therapy.

## 1.1.8 Prevention

*H. influenzae* type b conjugate vaccine has led to dramatic declines in incidence and prevalence of these diseases. Currently, the incidence of *H. influenzae* type b invasive diseases in developed countries has greatly decreased because of the coverage of *H. influenzae* type b conjugate vaccine (Peltola, 2000). Conjugation is the process of chemically bonding of a polysaccharide (a somewhat ineffective antigen) to a protein carrier, which is a more effective antigen. This process changes the polysaccharide from a T-independent to a T-dependent antigen and improve the immunogenicity, especially in young children (Kelly *et al.*, 2004). In addition, repeat doses of *H. influenzae* type b conjugate vaccines elicit booster responses and allow maturation of class-specific immunity with predominance of IgG antibody. The *H. influenzae* type b conjugates also cause carrier priming and elicit antibody to "useful" carrier protein. The *H. influenzae* type b conjugate vaccine had being introduced in Malaysia since 2002.

## 1.2 Diagnosis methods for *Haemophilus influenzae* type b

### 1.2.1 Conventional methods

#### 1.2.1.1 Isolation of *Haemophilus influenzae*

A Gram stain of an infected body fluid such as CSF may demonstrate small gram-negative coccobacilli suggestive of invasive Haemophilus disease. *H. influenzae* typically grows on chocolate blood agar (CBA) as mucoid colonies with mouse nest odour. CBA provide hemin (X factor) and NAD (V factor), which is necessary for the growth of *H. influenzae* (Musher, 1996). Most strains of *H. influenzae* will not grow on 5% sheep blood agar, which contains protoporphyrin IX but not NAD (Musher, 1996). Several bacterial species including *Staphylococcus aureus*, produce NAD as a metabolic byproduct. Therefore, tiny colonies of *H. influenzae* may be seen growing very close to the colonies of bacteria that are capable to produce NAD factor (Evans *et al.*, 1975). This phenomena is known as satellism. A single streak of a hemolysin-producing strain of *Staphylococcus* spp on a sheep blood agar plate has been inoculated with a suspected *H. influenzae*. The *Staphylococcus* lyses the red blood cells adjacent to the streak line, releasing hemin (X factor) and NAD (V factor), providing the necessary components for growth of *H. influenzae*. *H. influenzae* will grow adjacent to the streak line where the factors are available.

## 1.2.1.2 Slide agglutination test

All *H. influenzae* isolated from cases of invasive infections should be serotyped to determine whether or not *H. influenzae* type b is the cause of the invasive disease. Testing can be performed using a slide agglutination test. A saline control without the reagent antibodies should always be tested simultaneously alongside the patient's specimen in order to detect non-specific agglutination (Himmelreich *et al.*, 1985).

## 1.3 Molecular assay

## 1.3.1 Nucleic acid based assay

Rapid screening procedures are very useful for patient therapy and evaluating outbreak and have been developed for detection from CSF, plasma, serum, and whole blood. A PCR method for *H. influenzae* type a to f has been developed by Falla *et al* (Falla et al., 1994). PCR product was amplified for the specific capsular type for which the primers were designed. PCR have an advantage over serotyping in that problem of cross-reaction and provide a higher sensitivity and specificity over serotyping. A single multiplex PCR was developed based on *Neisseria meningitidis, Haemophilus influenzae* and *Streptococcus pneumoniae*, which together are responsible for upwards of 80% of cases of bacterial meningitis in developed and developing countries (Corless *et al.*, 2001). Nucleic acid amplification methods for the nonculture detection of *H. influenzae* in clinical specimens are designed to detect the bexA gene as a marker for *H. influenzae* based on the bexA gene sequence of the Hib Eagan strain (Van Ketel *et al.*, 1990). Outer membrane protein (P6) gene had proved to be a conserved region of *H. influenzae* and was targeted by loop mediated isothermal amplification to perform under single temperature condition (Torigoe *et al.*, 2007).

## 1.3.2 Loop-mediated isothermal amplification (LAMP)

The invention of the loop-mediated isothermal amplification (LAMP) technique a decade ago has given a new direction in point of care diagnostic tests that based on amplification of pathogen DNA. The LAMP technology amplifies DNA with high sensitivity relying on an enzyme with strand displacement activity under isothermal conditions (Njiru *et al.*, 2008). In addition, LAMP technology utilised four to six specially designed primers to recognise six to eight regions of the target DNA sequence, hence contribute to a high specificity (Notomi *et al.*, 2000). The auto-cycling reactions lead to accumulation of the target DNA and reaction by-products, such as magnesium pyrophosphate, that can allow rapid detection using real time turbidimeter (Njiru, 2012). Over the last 10 years, LAMP has been used widely in the laboratory setting to detect pathogens of medical and veterinary importance, plant parasitic diseases, genetically modified products and tumour and embryo sex identification, among other uses. However, its application under field conditions has been limited, partly due to the infancy of the technologies associated with LAMP, such as field-based template preparation methods and product detection formats

## 1.4 Rationale of study

A rapid and cost-effective diagnostic method was highly demanded in resource-limited laboratory. This is because *H. influenzae* vaccination coverage is 42% in developing countries and only 8% in the poorest countries (Morris *et al.*, 2008). Routine laboratory diagnosis of *H. influenzae* relied on conventional methods, since they are the gold standard (Abdeldaim and Herrmann, 2013). Both biochemical test and culture methods are time-consuming and the interpretation was vary among technician (Torigoe *et al.*, 2007). Molecular methods such as PCR can provide a rapid detection of *H. influenzae*. There was a LAMP assay that target on *H. influenzae* outer membrane protein and proved to be specific on *H. influenzae* (Torigoe *et al.*, 2007). However, *H. influenzae* type b based LAMP assay was not established and therefore this study can provide a solution to the puzzle. Besides that, expensive PCR assay limit its usage in developing countries. As alternative, LAMP can replace PCR as an excellent diagnostic tool in providing a rapid, highly sensitive and specific result based on a single temperature condition that is suitable for resource-limited laboratory.

Although, Outer Membrane Protein (OMP) P6 gene is highly conserved among strains of *H. influenzae*, it is not specific to *H. influenzae* type b and hard to differentiate from *Haemophilus haemolyticus* (Chang et al., 2010). Another gene, 16s rRNA gene was not suitable to use as priming target sites due to close sequences homology between *H. influenzae* and *Haemophilus parainfluenzae* (Torigoe *et al.*, 2007). On another hand, *bex*A gene was targeted in PCR to detect *H. influenzae* type b because *bex*A gene is highly conserved among typeable strain of *H. influenzae* (Satola *et al.*, 2003a). The failure to detect *bex*A gene in Hi type e and f by real time PCR, suggested that it is very specific to *H. influenzae* type b (Sam and Smith, 2005).

LAMP is highly specific since it utilised six different primers and it is faster than PCR (Notomi *et al.*, 2000). To add in as advantages, LAMP assay has higher sensitivity and specificity rates when compared with PCR assay (Das *et al.*, 2012). The establishment of rapid detection method to replace current conventional method can reduce time taken for diagnosis and thus help in identification of *H.influenzae* type b specifically (Speers, 2006). It is important when come to a life-threatening situation, for example, patient with meningitis and bacteremia. Discovery of a cost-effective method in detecting *H.influenzae* type b especially in developing countries and underdeveloped countries will help to reduce cost taken for diagnosis and easily to perform even without requirement of thermal cycler.

## 1.5 Objectives of study

### 1.5.1 General objective

This study is to establish a rapid detection method for *H. influenzae* type b by LAMP of *bex*A gene.

### 1.5.2 Specific objectives

1. To design primers for LAMP of *bex*A gene in detecting *H. influenzae* type b.

2. To optimize the LAMP reaction of *bex*A gene in detecting *H. influenzae* type b.

3. To evaluate the analytical sensitivity and analytical specificity of LAMP by *bex*A gene in detecting *H. influenzae* type b.

## 1.6 Overview of study

LAMP primer design

↓

Target on conserved region, *bex*A gene

↓

**Optimization of LAMP reaction**

↓

Optimization criteria (LAMP reagents and LAMP reaction condition)

↓

Evaluation on analytical sensitivity of *H. influenzae* type b LAMP assay

↓

Evaluation on analytical specificity of *H. influenzae* type b LAMP assay
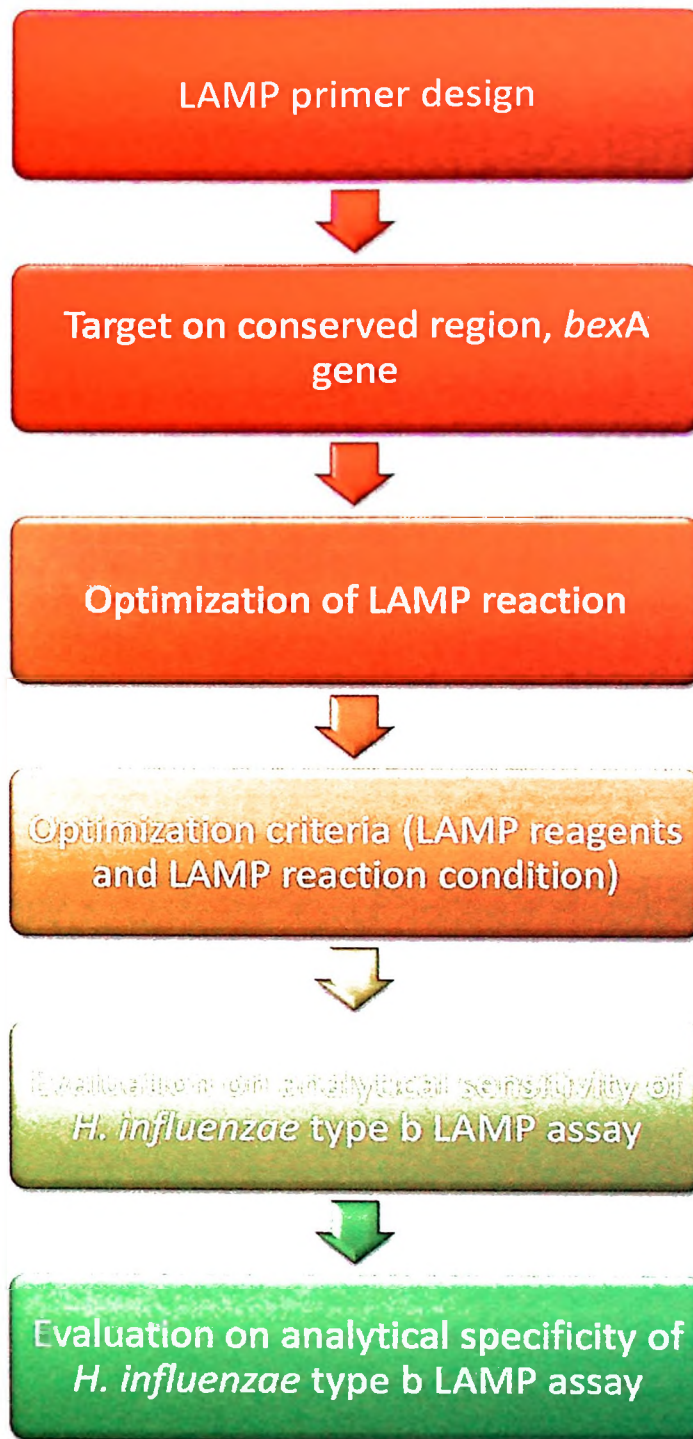
# CHAPTER 2: MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 List of chemical and reagent

| NO | Name | Company |
|----|------|---------|
| 1 | 10 mM Deoxynucleotide Triphosphates (dNTPs) mix | Thermo Scientific, USA |
| 2 | 10X Isothermal amplification buffer | New England Biolabs, USA |
| 3 | 10X *Taq* buffer with $(NH_4)_2SO_4$ | Fermentas, USA |
| 4 | 25 mM Magnesium chloride ($MgCl_2$) | Fermentas, USA |
| 5 | 5 U/μl *Taq* DNA polymerase (recombinant) | Fermentas, USA |
| 6 | 8000 U/ml *Bst* 2.0 WarmStart® DNA polymerase | New England Biolabs, USA |
| 7 | Absolute Ethanol | HmbG, Germany |
| 8 | Agarose, molecular biology grade | 1st base, Singapore |
| 9 | Betaine | Sigma-Aldrich, Germany |
| 10 | Boric acid, molecular biology grade | Sigma-Aldrich, Germany |
| 11 | Chocolate blood agar (CBA) | Thermo Scientific, USA |
| 12 | EDTA, molecular biology grade | Merck, Germany |
| 13 | Ethidium Bromide | Ultrapure™, USA |
| 14 | GeneRuler™ 100 bp plus DNA ladder | Fermentas, USA |
| 15 | Glycerol | Merck, Germany |
| 16 | Loopamp® DNA amplification kit | Eiken Chemical, Japan |
| 17 | Magnesium sulphate ($MgSO_4$) | Sigma-Aldrich, Germany |
| 18 | Mineral oil | Sigma-Aldrich, Germany |
| 19 | NucleoSpin® Tissue (50 prep) kit | Macherey-Nagel, Germany |
| 20 | Orange G sodium salt | Sigma-Aldrich, Germany |
| 21 | Protect® microorganism preservation system (Cryobeads) | Thermo Scientific, USA |
| 22 | Sodium Chloride | Merck, Germany |
| 23 | Sterile distilled water (PCR water) | Gibco®, USA |
| 24 | Tris base, molecular biology grade | Promega, USA |
| 25 | Trypticase soy broth | Merck, Germany |
| 26 | Ultra pure water | Gibco®, USA |
| 27 | Wellcogen Haemophilus influenzae kit | Thermo Scientific, USA |

## 2.1.2 List of software used

| NO | Name | Company |
|----|------|---------|
| 1 | BLAST® | NCBI, USA |
| 2 | GenDoc 2.7 | NRBSC, USA |
| 3 | Primer Explorer v4 | Biken, Japan |
| 4 | Vector NTI Advance 9 | Life technologies, USA |

## 2.1.3 List of consumables

| NO | Name | Company |
|----|------|---------|
| 1 | 0.5 ml microcentrifuge tubes | Axygen Scientific, USA |
| 2 | 1.5 ml microcentrifuge tubes | Axygen Scientific, USA |
| 3 | 2 ml microcentrifuge tubes | Axygen Scientific, USA |
| 4 | 50-2000 µl RNase-/DNase-/Protein-free UVette cuvettes | Eppendorf, Germany |
| 5 | Non filter tips 10 µl, 20 µl, 100 µl, 200 µl, 1000 µl | Axygen Scientific, USA |
| 6 | Sterile Filter tips 10 µl, 20 µl, 100 µl, 200 µl, 1000 µl | Axygen Scientific, USA |

## 2.1.4 List of apparatus and equipment

| NO | Name | Company |
|----|------|---------|
| 1 | 500 V Power supply | Major Science, USA |
| 2 | BioPhotometer | Eppendorf, Germany |
| 3 | Centrifuge 5424 | Eppendorf, Germany |
| 4 | ChemiImager™ 5500 | Alpha Innotech, USA |
| 5 | Cyber pH meter | Eutech Instruments, Singapore |
| 6 | Electronic balance | A&D, Japan |
| 7 | Heating Block | Cole-Parmer, USA |
| 8 | Incubator 37 °C with enriched carbon dioxide ($CO_2$) | Forma Scientific, USA |
| 9 | Laminar flow cabinet | ESCO, USA |
| 10 | Microcentrifuge | Denver Instrument, USA |
| 11 | Microwave oven | Panasonic, Japan |
| 12 | Owl™ gel electrophoresis system | Thermo Scientific, USA |
| 13 | peqSTAR 2X Thermocycler | Peqlab, Germany |
| 14 | PIPETMAN pipette P2, P10, P20, P100, P200, P1000 | Gibson®, USA |
| 15 | ScanSpeed 1730R centrifuge | LaboGene™, Denmark |
| 16 | Thermolyne Cimarec 2 Hot plate/stirrer | Scientific support, USA |
| 17 | Ultra low temperature freezer | Sanyo, Japan |
| 18 | Vortex mixer | IKA®, China |
| 19 | Water bath | Techne, UK |
| 20 | Water deionizer | ELGA, USA |

## 2.2 Methods

### 2.2.1 Reagent preparation

#### 2.2.1.1 Tris Borate EDTA (TBE) 10X stock solution

The preparation of 10X TBE stock solution was started by dissolving 54 g of TRIS base and 27.5 g of boric acid into 400 ml of distilled water. Then, a total volume of 20 ml EDTA (at pH 8) was added into the solution. The mixture was dissolved with magnetic stirrer. The final volume of the buffer solution was made up to 500 ml distilled water.

#### 2.2.1.2 Tris Borate EDTA (TBE) 0.5X working solution

In order to prepare 0.5X TBE working solution, 50 ml of 10X TBE stock solution was mixed with 950 ml of distilled water. The working solution was ready to use for preparation of agarose gel.

#### 2.2.1.3 Eighty percent Glycerol solution

Eighty percent glycerol was prepared by diluting 100% glycerol with distilled water. Eight millilter of 100% glycerol was added with 2 ml of distilled water in a 15 ml falcon tube and it was vortex until the solution was mixed evenly. The prepared 80% glycerol solution was stored in 4°C and ready to use.

### 2.2.1.4 Orange G loading dye (6X)

Orange G loading dye (6X) was prepared by adding 0.2% orange G Dye, 3.0 ml 60% glycerol, 0.6 ml EDTA (6 mM) into 1.4 ml distilled water until reach to a final volume of 5 ml. The mixture was vortex until the dye is completely dissolved and stored at -20°C.

### 2.2.1.5 One hundred base pairs (bp) plus DNA ladder working solution

One hundred base pairs (bp) plus DNA ladder working solution was prepared. A total volume of 10 µl Orange G dye and 10 µl of 100bp plus DNA ladder stock solution were measured. The mixture was diluted with 40 µl sterile distilled water to a final volume of 60 µl.

### 2.2.1.6 One hundred millimolar (100 mM) Magnesium sulphate ($MgSO_4$) solution

The preparation of 100 mM $MgSO_4$ solution was started by weighting 246.47 g of $MgSO_4$ powder and dissolved in 80 ml distilled water. It was dissolved by magnetic stirrer. The volume of $MgSO_4$ solution was added to a final volume of 100 ml by using distilled water.

### 2.2.1.7 Five molar (5 M) Betaine solution

Five molar betaine solution was prepared from 2.93 g of betaine powder. The betaine powder was dissolved in 5 ml sterile distilled water. Filter tips were used when preparing betaine solution. The mixture was vortex and aliquoted into different 1.5 ml microcentrifuge tube for future use.

### 2.2.1.8 Tryticase Soy Broth (TSB)

TSB was prepared by dissolving 6 g TSB powder into 150 ml distilled water. The mixture was stirred by using magnetic stirrer and the pH 7.3 was adjusted to using 1 M HCl and 1 M NaOH. The final volume was made up to 200 ml distilled water. The medium was aliquoted into universal bottle and sent to autoclave.

### 2.2.2 Selection of bacteria strains

Two *H. influenzae* strains and 30 non-*Haemophilus* strains were used in this study. All of the bacteria strains were obtained from stock culture laboratory, Department of Medical Microbiology and Parasitology, Hospital Universiti Sains Malaysia. Two reference *H. influenzae* strains, which are *H. influenzae* ATCC 10211 (serotype b) and *H. influenzae* ATCC 49247 (non-typeable) were used as positive and negative control, respectively. Thirty non-*Haemophilus* strains were used to evaluate specificity of the *H. influenzae* type b LAMP assay and were listed in Table 2.1.

**Table 2.1: Thirty non-Haemophilus strains were used to evaluate specificity of LAMP and obtained from stock culture laboratory, Department of Medical Microbiology and Parasitology, Hospital Universiti Sains Malaysia.**

| Strains | Species/ Serotype |
|---|---|
| **Gram negative bacteria (n=11)** | *Aeromonas hydrophila* |
| | Enterohemorrhagic *Escherichia coli (EHEC)* |
| | Enteroinvasive *Escherichia coli (*EIEC) |
| | Enteropathogenic *Escherichia coli* (EPEC) |
| | Enterotoxigenic *Escherichia coli* (ETEC) |
| | *Klebsiella pneumoniae* |
| | *Moraxella catarrhalis* |
| | *Neisseria meningitidis* |
| | *Salmonella typhimurium* |
| | *Shigella flexneri* |
| | *Yersinia enterocolitica* |
| **Gram positive bacteria (n=19)** | *Bacillus subtilis* |
| | *Corynebacterium diphtheriae* |
| | *Enterococcus faecalis* |
| | *Enterococcus faecium* |
| | *Enterococcus gallinarum* |
| | *Listeria monocytogenes* |
| | *Staphylococcus aureus* |
| | *Staphylococcus epidermidis* |
| | *Staphylococcus saprophyticus* |
| | *Streptococcus* Group A |
| | *Streptococcus* Group B |
| | *Streptococcus* Group F |
| | *Streptococcus* Group G |
| | *Streptococcus sanguis* Group H |
| | *Streptococcus mitis* (Non-groupable) |
| | *Streptococcus mutans* (Non-groupable) |
| | *Streptococcus oralis* (Non-groupable) |
| | *Streptococcus pneumoniae* (Non-groupable) |
| | *Streptococcus viridians* (Non-groupable) |

## 2.2.3 Culture conditions and preservation of *H. influenzae*

All strains of *H. influenzae* were grown on chocolate blood agar (CBA) and incubated in a 5% $CO_2$ enriched incubator at 37°C for 18 to 24 hours (Turk, 1964). For long term storage of *H. influenzae* strains, two preservation methods were utilized.

Cryobeads was used to preserve fastidious organism such as *H. influenzae*. Colonies were harvested from overnight culture plate and resuspend in the provided vial. The vial was shook vigorously for 30 seconds and the vial liquid was removed. The vial was then stored in -80°C and ready for future retrieval.

Preparation of glycerol stock that consists of TSB culture and 25% glycerol was preferred for *H. influenzae* (Aulet de Saab *et al.*, 2001). Eighty percent glycerol was prepared earlier. A fresh overnight TSB culture was acquired and subjected to centrifugation at 13,600 xg for 3 minutes. Then, the supernatant was discarded. The pellet was resuspended with 500 µl new and fresh TSB solution. After adding 247.5 µl of TSB culture with 112.5 µl 80% glycerol, a final concentration of 25% glycerol was achieved. The suspension was pipetted up and down until homogenous solution can be seen. The glycerol stock was then stored in -80°C and available for future retrieval.

### 2.2.4 Design of LAMP primers

The LAMP primers were specifically designed to target on *bex*A gene of *H. influenzae* type b. The published sequences from database (Genbank accession number EF490496, M19995 and X54987) were downloaded into Vector NTI® software and aligned to locate particular consensus region. After that, LAMP primers were designed based on the consensus sequence by using Primer Explorer V4 software. The designed primers included 2 outer primers (F3 and B3), 2 inner primers (FIP and BIP) and 1 loop forward primer (LF). The primer sequences were subjected to BLAST search to check for any unspecific primer binding in other species than *H. influenzae* type b.

### 2.2.5 Preparation of LAMP primer

All synthesized primer sets in lyophilized form were reconstituted in order to prepare primer stock solutions. The lyophilized primers that were kept in tubes were centrifuged at 13, 600 xg for 3 minutes. Then, one hundred microliter of sterile distilled water was added into each tube to reconstitute the primers. The mixture was then vortex for 5 to 15 minutes to ensure the primers dissolved completely. The primer stock solutions were briefly centrifuged and kept in -20°C for future use. The concentrations of primer stock solutions were calculated based on data sheet provided by the