



UNIVERSITI PUTRA MALAYSIA

***MOLECULAR CHARACTERISATION OF ANTIBIOTIC RESISTANT
GENES AND DEVELOPMENT OF REAL TIME LOOP- MEDIATED
ISOTHERMAL AMPLIFICATION FOR RAPID DETECTION OF
VIRULENCE GENE (*speB*) IN GROUP A STREPTOCOCCI***

AZI SIMON ONYEMA

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By

AZI SIMON ONYEMA

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
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Doctor of Philosophy**

June 2020

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

MOLECULAR CHARACTERISATION OF ANTIBIOTIC RESISTANT GENES AND DEVELOPMENT OF REAL TIME LOOP-MEDIATED ISOTHERMAL AMPLIFICATION FOR RAPID DETECTION OF VIRULENCE GENE (*speB*) IN GROUP A STREPTOCOCCI

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June 2020

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Faculty : Medicine and Health Sciences

Group A streptococcus is one of the major human pathogens responsible for over 600 million infections annually. This “flesh-eating bug” has caused more than 600,000 deaths per annum globally. Heightened concerns have been escalating among the scientists and physicians due to its ability to cause serious invasive disease and long-term sequelae. Loop mediated isothermal amplification (LAMP) has been considered as a novel approach for its cost - effectiveness, good reliability, high sensitivity results, and can be performed in a rural setting with less sophisticated equipment. Thus, this study is aimed to develop a rapid, reliable and cost -effective diagnostic approach for the detection of streptococcal pyrogenic exotoxin B (*speB*) from GAS isolates by RT-LAMP technique. Forty-three GAS isolates were obtained from stock culture with 31 (72.1%) and 12 (27.9%) isolates from non-invasive and invasive samples respectively. Re-identification of these isolates was carried out using several conventional methods and confirmed with 16s rRNA. Antimicrobial susceptibility testing was performed using Kirby-Bauer disk diffusion method and interpreted according to CLSI guidelines. Preliminary screening for antibiotic resistance genes (*tetM*, *InuA*, *ermA*, *ermB*, *mefA*) and virulence genes, (*speB*, and *prtF1*) were performed using PCR amplification to choose variables for real time-LAMP development. Some categorical variables were tested by Chi-square analysis and *p* values less than 0.05 were considered significant. All GAS isolates (100%) were sensitive to erythromycin and azithromycin. Twenty-five (58.1%) and 7 (16.3%) of them exhibited resistance to doxycycline and clindamycin respectively. There were no inducible MLSB (iMLSB) or constitute MLSB (cMLSB) or MS phenotypes detected among GAS isolates but an L-phenotype 7 (16.3%) was observed. Regarding virulence genes, 43 (100 %) and 20 (46.5%) of GAS isolates carried *speB* and *prtF1* genes, respectively. In addition, *tetM* and *InuA* genes were detected in all doxycycline and clindamycin-resistant

isolates (100% for each). *speB*, the major cause of pathogenicity in GAS that was detected up to 100% in the preliminary gene, was used as a marker to progress with the development of real-time loop mediated isothermal amplification (RT-LAMP). ATCC 19616, (*S. pyogenes*, positive control) and no DNA template as negative control) were used after optimization of primers, time and RT- LAMP reagents for the initial development. The optimal RT- LAMP condition was obtained at 63°C for 45 min using laboratory heating block and Real-time turbidimeter machine (LA- 500 Eiken Company, Japan) respectively.

Forty- three clinical isolates of *S. pyogenes* as described earlier were also used to verify the possibility of the RT-LAMP assay in the detection *speB* gene followed by nine bacteria strains including *Streptococcus pyogenes* and other non- GAS bacteria strains (from American Type Culture Collection) and clinical isolates in evaluating the specificity and sensitivity following serial dilutions of 10^{-2} to 10^{-6} ng/μl. The detection limit of our RT-LAMP was 0.001 ng/μl of the template, showing higher sensitivity than conventional LAMP and PCR detection limit of 0 .000001 ng/μl and 0.001 ng/μL making it 100,000- folds more sensitive than PCR and 100-fold more sensitive than conventional LAMP assay. The detection rate of *speB* using RT-LAMP 100% while PCR was 93% with conventional PCR primer set.

In conclusion, the improved rapidity for detection of the *speB* which contribute greater percentages in GAS virulence among other factors by the RT-LAMP technique with its specificity and sensitivity using less sophisticated equipment, simple to perform and cost-effective, is expected to be a new frontier in the reliable method for the diagnosis of *S. pyogenes* infection and will soon replace other time- consuming and costly molecular assays. The technique particularly is suitable for rural or community hospitals in developing nations with middle income level.

Keywords: Real-time Loop mediated isothermal amplification, *speB*, virulence genes, antibiotic resistance genes, Group A *Streptococcus. pyogenes*

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

CIRI-CIRI MOLEKUL GEN TAHAN ANTIBIOTIK DAN PEMBANGUNAN GELUNG MASA NYATA-AMPLIFIKASI ISTERMA BERMEDIAT UNTUK PENGESANAN PANTAS GEN VIRULENS (*speB*) DALAM KUMPULAN A STREPTOCOCCI

Oleh

AZI SIMON ONYEMA

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Streptokokus Kumpulan A adalah salah satu patogen manusia utama yang bertanggungjawab untuk lebih 600 juta jangkitan setiap tahun. "Kuman makan-daging" ini telah menyebabkan lebih daripada 600,000 kematian setahun di seluruh dunia. Kebimbangan semakin meningkat dalam kalangan saintis dan pakar perubatan kerana keupayaannya menyebabkan penyakit invasif yang serius dan sekuel jangka panjang. Penguatan pengedaran isothermal kitaran (LAMP) telah dianggap sebagai pendekatan baru disebabkan kos efektif, kebolehpercayaan yang baik, keputusan kepekaan yang tinggi, dan boleh dilakukan di luar bandar dengan peralatan kurang canggih. Oleh itu, kajian ini bertujuan untuk membangunkan pendekatan diagnostik yang cepat, boleh dipercayai dan kos efektif untuk mengesan streptokokus pyrogenik eksotoksin B (*speB*) dari isolat GAS dengan teknik RT-LAMP. Empat puluh tiga isolat GAS diperoleh daripada kultur stok dengan 31 (72.1%) daripada sampel bukan invasif dan 12 (27.9%) daripada sampel invasif.

Pengenalpastian semula isolat ini dilakukan dengan menggunakan beberapa kaedah konvensional dan dipastikan dengan rRNA 16s. Pengujian kerencatan antimikrob dilakukan dengan menggunakan kaedah penyebaran disk Kirby-Bauer dan ditafsirkan dengan garis panduan CLSI. Pemeriksaan awal untuk gen rintangan antibiotik (*tetM*, *InuA*, *ermA*, *ermB*, *mefA*) dan gen kebisaan, (*speB*, dan *prtF1*) dilakukan menggunakan amplifikasi PCR untuk memilih pembolehubah untuk pembangunan RT-LAMP. Beberapa pembolehubah kategori diuji oleh analisis Chi-square dan nilai p kurang daripada 0.05 dianggap signifikan. Semua isolat GAS (100%) adalah sensitif terhadap eritromisin dan azithromisin. Dua puluh lima (58.1%) dan 7 (16.3%) daripadanya menunjukkan daya rintangan terhadap doxycyclin dan klindamicin masing-masing. Tiada

penyebab MLSB (iMLSB) atau konstitut MLSB (cMLSB) atau fenotip MS yang dikesan dalam kalangan isolat GAS tetapi L-fenotip 7 (16.3%) pula dikesan. Mengenai gen kebisaan, 43 (100%) dan 20 (46.5%) isolat GAS masing-masing membawa gen *speB* dan *prtF1*. Di samping itu, gen *tetM* dan *lnuA* dikesan dalam semua isolat doxycyclin dan clindamycin-rintang (100% bagi setiap satu). *speB*, penyebab utama patogenesis dalam GAS yang dikesan sehingga 100% dalam gen awal, digunakan sebagai penanda untuk dimajukan dengan perkembangan penguatan isothermal (RT-LAMP) yang diselaraskan oleh kitaran masa nyata.

ATCC 19616, (*S. pyogenes*, kawalan positif) dan tiada templat DNA sebagai kawalan negatif) telah digunakan selepas pengoptimuman reagen primer, masa dan reagen RT-LAMP untuk pembangunan awal. Kondisi RT-LAMP yang optimum diperolehi pada 63 ° C selama 45 minit menggunakan blok pemanasan makmal dan mesin turbidimeter Real-time (LA- 500 Eiken Company, Japan) masing-masing.

Empat puluh tiga isolat klinikal *S. pyogenes* seperti yang dijelaskan sebelum ini juga digunakan untuk pengesanan asai RT-LAMP dalam pengesanan gen *speB* diikuti oleh sembilan talian bakteria termasuk *Streptokokus pyogenes* dan talian bakteria lain yang bukan GAS (dari Koleksi Kultur Jenis Amerika) dan klinikal isolate dalam menilai kekhususan dan kepekaan berikut pelarutan bersiri 10^{-2} hingga 10^{-6} ng / μ l. Had pengesanan RT-LAMP kami ialah 0.001 ng / μ l mengikut templat, menunjukkan kepekaan yang lebih tinggi daripada had pengesanan LAMP dan PCR 0.001 ng / μ l dan 0.000001 ng / μ l menjadikannya 100,000 kali lebih sensitif daripada PCR dan 100-fold lebih sensitif daripada ujian LAMP biasa. Kadar pengesanan *speB* menggunakan RT-LAMP 100% manakala PCR adalah 93% dengan set primer PCR konvensional.

Kesimpulannya, peningkatan kecepatan untuk pengesanan *speB* yang menyumbang lebih besar peratusan dalam kebisaan GAS di antara faktor-faktor lain melalui teknik RT-LAMP dengan kekhususan dan kepekaannya dengan menggunakan peralatan yang kurang canggih, mudah untuk dilaksanakan dan berkos efektif, adalah dijangka menjadi perbatasan baru dalam kaedah yang boleh dipercayai untuk diagnosis jangkitan *S. pyogenes* dan tidak lama lagi akan menggantikan ujian molekul lain yang memakan masa dan mahal. Teknik ini amat sesuai untuk hospital luar bandar atau komuniti di negara-negara sedang membangun dengan tahap pendapatan menengah.

Kata kunci: Penguatan pengedaran isothermal kitaran masa nyata, *speB*, gen kebisaan, gen rintang antibiotik, *Streptokokus pyogenes* kumpulan A

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CHAPTER 1

INTRODUCTION

1.1 Background of study

Group A *Streptococcus* (GAS) otherwise known as a “flesh-eating bug” is one of the major human pathogens responsible for over 600 million infections and about 517, 000 deaths annually (Barnett et al., 2018; Suarez-Arrabal et al., 2019). There have been heightened concerns among the scientists and physicians due to the ability of this pathogen to produce fatal toxins. Among them is known as streptococcal pyrogenic exotoxin B (*speB*), a powerful cysteine proteinase (Nelson Daniel et al., 2011), which is predominantly found in GAS and one of the major virulent factors in GAS pathogenesis (Commons et al., 2014).

Furthermore, there have been an increasing data on serious invasive GAS diseases and its long term sequelae such as rheumatic heart disease (RHD), acute rheumatic fever (ARF) and acute post-streptococcal glomerulonephritis (APSGN) that have been reported in resource-poor or limited settings and developing countries (Arias-Constanti et al., 2018).

Apart from *speB*, other virulent factors have been found to contribute to its pathogenicity such as M protein, streptokinase and fibronectin- binding protein F1 adhesin (*prtF1*) and others, (Rohde & Cleary, 2016). *prtF1* in particular, has played a pivotal role in the internalization of *speB* into respiratory epithelia which has been linked to antibiotic resistance in GAS due to its ability to block entry of antimicrobial agents. (Chiang-Ni & Wu, 2008; Neeman et al., 1998; Strus et al., 2017).

Meanwhile, due to the global economic recession ravaging many countries and adverse health effects associated with GAS infection, there have been calls for reliable, fast and cost-effective method for the detection of *Streptococcus pyogenes* and loop-mediated isothermal amplification (LAMP) seems to have great potential in solving these challenges.

The performance of LAMP which is a nucleic acid-based detection has recently been recognized (Notomi et al., 2000). It has been considered as a novel approach for its cost- effectiveness, reliability, high sensitivity of results, and more so, that it can be performed in a rural setting without recourse to sophisticated equipment or waiting for samples to be transported from rural areas to laboratories in the cities (Wong et al., 2018). The importance of this

technique is further emphasized in the fact that it relies on auto cycling strand displacement DNA synthesis under isothermal conditions. In addition, it utilizes a set of four to six specifically designed primers to hybridize to six to eight different parts of the target DNA sequence, the feature that makes this detection technique specific to gene being sought among other merits (Notomi et al., 2000).

Additionally, it is hoped that LAMP will be the frontier in the point of care testing which will enhance timely detection of GAS in Malaysian hospitals and other related areas for optimal management of GAS infections thus reducing the burden of this deadly disease. This will also be supporting the 2017-2021 action plan proposed by Ministry of Health Malaysia and Ministry of Agriculture and Agro-Based Industry Malaysia on antimicrobial resistance in the area of accurate diagnosis for proper antibiotic treatment both in humans and agriculture (N Misol Jr, 2018).

1.2 Problem statement

Time - consuming, expensive and unsatisfactory results obtained with bacterial detection techniques such as cultural, immunological, serological, biochemical and even some molecular methods like conventional polymerase chain reaction (PCR) has complicated the eradication of pathogenic organisms thereby calling for a rapid attention and alternative better approach to rule out inadequate sensitivity, false negative and unreliable results associated with them (Lauri & Mariani, 2009). Resource poor countries cannot afford expensive equipment for molecular detection of GAS and those that can barely afford, long time taken for conventional detection of GAS complicates eradication compare to LAMP.

Many developing countries cannot afford a lot of sophisticated equipment required for accurate molecular detection leaving the question, should invasive bacterial pathogens such GAS and its menace be continually left misdiagnosed, wrongly treated thereby increasing antibiotic resistance which consequently leads to high virulence in such areas? On the other hand, GAS has now shown resistance to initial drugs of choice such as B lactams, tetracyclines, macrolides (Cattoir, 2016) and currently, lincosamides which was adjudged to be in the panel of last resort in streptococcal related infection treatment due to its ability to reduce toxin production (Smieja, 1998).

Similarly, doxycycline has been widely used in agriculture, animals, and various human diseases such as parasitic diseases thereby leading to high resistance rates as documented and the ability to exhibit cross-resistance with erythromycin due to cross species transfer (Andreoni et al., 2017; Mohammed Kalgo et al., 2018).

In middle- and low-income nations, wrong diagnosis is common due to lack of power supply and technologies for molecular detection. Consequently, asymptomatic cases also abound of *Streptococcus pyogenes* even though that low pathogenicity was recorded in some cases, there have been reports of switching from non-invasive to invasive condition using its molecular arsenals especially during immunocompromised status or other health issues (Barnett et al., 2018). Thereby, compounding the issue of virulence unless proper and early detection is commenced. Thus, with RT-LAMP development, the present study will provide a useful diagnostic tool by combing turbidimetric (real-time) and colorimetric (visual, under natural light and UV), and gel electrophoresis detection approaches to detect *speB*, a biological marker due to its predominance and a major cause of pathogenesis in GAS in less than one hour which will immensely contribute to the patient's welfare in developing and developed countries in general and Malaysia in particular (Suarez-Arrabal et al., 2019; World Health, 2005).

1.3 Significance of the study

The present study tries to develop the diagnostic approach that conforms with WHO's recommendations for point-of-care testing which include being robust and rapid, affordable, user friendly, sensitive, specific, and above all, being sophisticated equipment free. The importance may include also quick availability of test results to clinicians for better patients' management while maintaining high precision and yet at inexpensive rate.

1.4 Objectives

1.4.1 General Objectives

To molecularly characterize clinical isolates for the purpose of developing a sensitive, reliable and cost- effective detection method for diagnostic purposes of Group A streptococci (GAS).

1.4.2 Specific objectives

1. To determine the antibiotic susceptibility patterns of Group A streptococci (GAS) by Kirby – Baur Method for resistant or virulent determinant for RT-LAMP.
2. To determine the antibiotic resistance and virulence determinants among GAS clinical isolates for a variable to develop RT-LAMP.

3. To optimize reaction conditions of conventional and real-time loop mediated isothermal amplification (reagents, temperature, primers and time).
4. To develop real-time loop mediated isothermal amplification for the detection of streptococcal pyrogenic exotoxin B (*speB*) gene in GAS clinical isolates.



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