THE EVALUATION OF LOOP MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) FOR THE DETECTION OF *Porphyromonas gingivalis* USING HEAT-TREATED SALIVA

by

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CERTIFICATE

This is to certify that the dissertation entitled "The evaluation of Loop Mediated Isothermal Amplification (LAMP) for the Detection of *Porphyromonas gingivalis* using Heat-Treated Saliva" is the bonafide record of research work done by MRS. ROSNANI BINTI CHE AWANG during the period from July 2013 to May 2014 under my supervision.

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	Degree Celsius
µg	Microgram
µL	Microlitre
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
bp	Base pair
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphates
g	Gram
H_2O	Water
kb	Kilo base
L	Litre
LAMP	Loop mediated isothermal amplification
LB	Lithium borate
mA	Miliampere
mg	Miligram
mL	Mililitre
mM	Milimolar
ng	Nanogram
PCR	Polymerase Chain Reaction
pmol	Picomol
rpm	Revolution per minute
s	Seconds
U	Units
USM	Universiti Sains Malaysia
UV	Ultraviolet
V	Volts
v/v	Volume/volume

ABSTRACT

THE EVALUATION OF LOOP MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) FOR THE DETECTION OF *Porphyromonas gingivalis* USING HEAT-TREATED SALIVA

Porphyromonas gingivalis has been implicated as a major pathogen of periodontitis. Thus far, there have been no reported studies on attempted to detect *P. gingivalis* by Loop Mediated Isothermal Amplification (LAMP) using heat-treated saliva. Therefore, this study aims to evaluate the applicability of LAMP assay for detection of *P. gingivalis* in heat-treated saliva samples. DNA of saliva samples from periodontitis patients and healthy subjects were prepared by using heat-treated method and commercial kits. The results obtained were then compared with those omit obtained by conventional PCR assay. In periodontitis patients, *P. gingivalis* was detected in 4/5 (80%) extracted DNA using commercial kit in both LAMP and PCR assays. Two out of five (40%) of heat-treated saliva of periodontis patients were positive by LAMP and none was detected by PCR assay. In healthy subjects, no sample was positive by PCR in both heat-treated saliva and extracted DNA using commercial kit. However, 2/5 (40%) of heat-treated saliva and 1/5 (20%) of extracted DNA using commercial kit were positive by LAMP assay. These findings suggested that LAMP assay was corresponded to those of conventional PCR when using extracted DNA prepared from commercial kit. However, LAMP assay using heat-treated saliva was more sensitive than the conventional PCR, suggesting the potential of LAMP assay using heat-treated saliva samples in detection of *P. gingivalis*.

Key words: Periodontitis, *P. gingivalis*, LAMP assay, PCR, heat-treated

ABSTRAK

PENILAIAN AMPLIFIKASI ISOTHERMAL PERANTARAAN GEGELUNG (LAMP) UNTUK PENGESANAN *Porphyromonas gingivalis* MENGGUNAKAN AIR LIUR OLAHAN HABA

Porphyromonas gingivalis telah dikaitkan sebagai pathogen utama dalam menyebabkan penyakit periodontitis. Sehingga kini, tiada kajian yang dilaporkan untuk mengesan *P. gingivalis* dengan amplifikasi isothermal perantaraan gegelung (LAMP) menggunakan air liur olahan haba. Oleh itu, kajian ini bertujuan untuk menilai kebolehgunaan ujian LAMP untuk mengesan *P. gingivalis* dalam sampel air liur olahan haba. DNA sampel air liur daripada pesakit periodontitis dan subjek sihat telah disediakan dengan menggunakan kaedah olahan haba dan kit komersil. Keputusan yang diperolehi kemudiannya dibandingkan dengan keputusan yang diperolehi dengan asai PCR konvensional. Untuk pesakit periodontitis, *P. gingivalis* telah dikesan dalam 4/5 (80%) menggunakan DNA yang diekstrak melalui kit komersil dalam kedua-dua ujian LAMP and PCR. Dua daripada lima (40%) air liur olahan haba pesakit periodontis menunjukkan keputusan positif oleh LAMP dan tiada sampel yang dikesan oleh asai PCR. Dalam subjek yang sihat, tiada sampel yang menunjukkan keputusan positif oleh PCR dalam kedua-dua air liur olahan haba dan DNA yang diekstrak menggunakan kit komersial. Walaubagaimanapun, 2/5 (40%) daripada air liur olahan haba dan 1/5 (20%) daripada DNA yang diekstrak menggunakan kit komersil menunjukkan keputusan positif dengan LAMP. Penemuan ini menunjukkan asai LAMP sepadan dengan PCR konvensional apabila DNA yang diekstrak daripada kit komersial digunakan. Walaubagaimanapun, asai LAMP yang menggunakan air liur olahan haba adalah lebih

sensitive daripada PCR konvensional, mencadangkan potensi ujian LAMP menggunakan sampel air liur olahan haba dalam mengesan *P. gingivalis*.

Kata kunci: Periodontitis, *P. gingivalis*, LAMP, PCR, olahan haba.

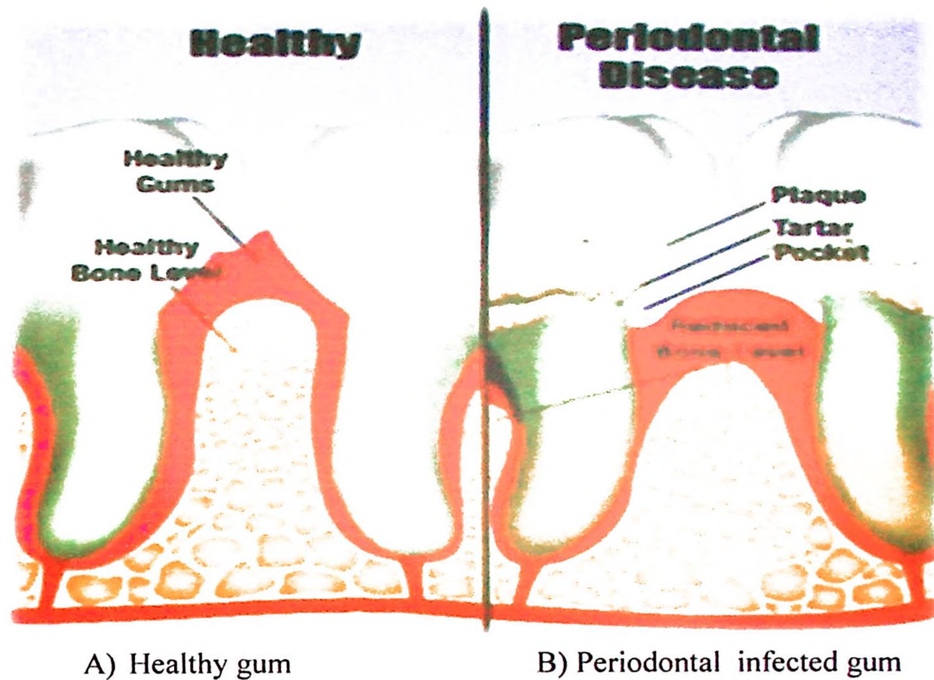
CHAPTER 1.0

INTRODUCTION

1.1 Periodontitis

Periodontitis is a common and progressive disease that will cause loss of tooth due to the loss attachment of teeth to the bone because it affects the supporting structure of the teeth (Brown *et al.*, 1996; Griffen *et al.*, 1998). Periodontitis lesions are usually associated with complex subgingival microfloras which are mainly gram-negative bacteria (Zambon and Haraszthy, 1995). The dark pigmented organism which is *Porphyromonas gingivalis* has been considered as a major pathogen of periodontitis (Ashimoto *et al.*, 1996).

In clinical features, patient with periodontitis shows alveolar bone loss, periodontal pocketing, clinical attachment loss and signs of inflammation such as redness, bleeding, swelling and probing that has been associated with the deposition of moderate to heavy calculus and plaque deposited (Flemming, 1999; Armitage and Cullinan, 2010) (Figure 1.1). The histopathological characteristics of periodontitis include loss of collagen fibers subjacent to the pocket epithelium, periodontal pocketing, location of junctional epithelium to the cemento-enamel junction, countless polymorphonuclear leukocytes in the junctional and pocket epithelium, bleeding and dense inflammatory cell infiltrate with plasma cells, lymphocytes and macrophages (Page *et al.*, 1975; Seymour and Greenspan, 1979; Flemming, 1999).



A) Healthy gum B) Periodontal infected gum

Figure 1.1: Comparison healthy gum and periodontal disease (Adapted from Dental Care of Yucaipa, 2011)

(A) Healthy gum show normal alveolar bone level and absence of deposition plaque or tartar on teeth. (B) In periodontal infected gum reduce bone level, swelling of gum, periodontal pocketing and deposition of plaque and tartar on teeth is a common observation.

The pathogenesis of periodontitis begins when these bacteria enter and adhere to the oral cavity through the saliva transmission of infected individuals (Greenstein and Lamster, 1997). The oral cavity provides multiple adhesive interactions to enable *P. gingivalis* to bind microbial cells, host cells, matrix components, saliva and crevicular fluid components (Theilade, 1990). Generally, oral surface is coated with pellicle that is mostly composed of salivary molecules which is absorbed on tooth surface function as receptors for bacterial adherence. Besides that, *P. gingivalis* can also adheres to early plaque organisms such as streptococci and *Actinomyces naeslundii* (Goulbourne and Ellen, 1991). The two types of adhesion molecules of *P. gingivalis* are fimbriae and hemagglutinin. Fimbriae are the major adherence-mediating structures which mediate adherence of *P. gingivalis* to oral substrates and molecules including salivary molecules such as proline rich glycoprotein, proline rich proteins, statherin, oral epithelial cell, fibrinogen, lactoferrin, fibronectin and early plaque bacteria (Lamont *et al.*, 1993; Amano *et al.*, 1996), whereas hemagglutinins, expressed on bacteria cell surface may promote colonization by mediating the binding of bacteria to human cell receptors. The binding of bacteria cells to erythrocytes will serve their nutritional function as they need haem for their growth (Lepine and Progulske-Fox, 1996). After that, they will then interact with the epithelia cells in which epithelial cells play their role as mechanical barrier and as a sensor of microbial infection (Kagnoff and Eckmann, 1997).

In order to interact with the epithelial cells, fimbriae of the bacteria will bind to the epithelial cells receptor and trigger the activation of eukaryotic protein which involved in signal transduction (Weinberg *et al.*, 1997). *P. gingivalis* also secretes proteins into host cell cytoplasm. The invasion of *P. gingivalis* on the host cells increased the calcium ions in cytosolic cell and impaired the activation of matrix metalloproteinase (MMP), which consequently impaired the extracellular matrix repair

and alters tissue integrity (Lamont and Jenkinson, 1998). The invasion also impaired host defense by inhibiting the secretion of interleukin-8 (IL-8) from gingival epithelial cell (Darveau *et al.*, 1998). In the host cells, *P. gingivalis* secretes toxin called proteinase that causes loss of alveolar bone, host tissue integrity and other supporting periondontal tissue (Lamont and Jenkinson, 1998).

The objective of the treatment is to control the infection (National Institute of Health, 2011). There are two types of periodontal treatment non-surgical and surgical treatment (American Dental Association, 2005). Nonsurgical treatment involves deep cleaning and medication. Deep cleaning method which is also known as scaling and root planning function to remove deposited tartar and plaque on teeth and root surface. Medication is used along with scaling and root planning that help control pain, infection and for healing. Medications that are currently used are antibiotic microsphere, enzyme suppressant, antimicrobial mouth rinse, oral antibiotics and antiseptic "chip" (National Institute of Health, 2011). However, the intrinsic activity of the antimicrobial is not the only factor that influences the successfulness or failure of medication but also depends on the location of infection, presence of foreign material and clinical status of patients (Slots, 2002). Surgery treatment such as flap surgery, splints, bite guards, bone and tissue grafting are needed in the presence of periodontal pocket and loss supporting tissue to prevent tooth loss (American Dental Association, 2005).

1.2 Prevalence of Periodontitis in Malaysia

Periodontitis disease is one of the worldwide major dental diseases with high prevalence rate (Papapanou, 1999). Surveys done between 1999-2004 reported that 8.52% of adults, between the age of 20 to 64, have periodontal disease and the number increased with current smokers, people with low education level and poor (National Institute of Dental and Craniofacial Research, 2014). Factors such as excessive alcohol consumption, tobacco use, stress and poor hygienic practices were showed to increase the risk of periodontal disease.

In Malaysia, tooth loss has become a public health problem among adults and elderly, in which 76.9% of adults age 35-44 had at least 20 teeth and only 23.9% in the 60-70 age group (Oral Health Division, 2012). According to National Oral Health Survey of Adults (NOSHA), the percentages of moderate and severe periodontal disease have increased from 23% in 1990, to 26.3% in 2000 and 47% in 2001 (NOSHA, 1990, 2000, 2001). In the 2000 Adult Dental Health Survey, 40% of elderly age from 65 year old and above had loss their natural teeth (Oral Health Divison, 2004), which was high as compared to 21% in Singapore, 16% in Thailand and 24% in Indonesia (Petersen, 2005). Among these age 15-19 years old, about 26% were reported to have healthy periodontal tissue and 5% in those aged 35-44 (Oral Health Divison, 2004).

In 2000, about 9.8% of dentate subjects had healthy gingival, 5.2% had deep pockets, while 57.5% had calculus. However, in 2010, 3.2% of dentate subjects had healthy gingival, 18.2% had deep pockets and 41.4% had calculus (Table 1.1). The data has clearly shown that the percentages of periodontal cases have increased between the year 2000 to 2010. Highest prevalence of deep pockets was found in those age 35-44 in 2000 while high prevalence in the age 65-44 during 2010 (National Oral Health Survey

for Adult, 2010). Based on Oral Health Divison, MOH (2012), severe periodontitis was found in 18.2% of the populations.

Table 1.1: The prevalence of periodontal disease in Malaysia (adapted from National Oral Health Survey for Adult, 2010)

Age group	Year	Participants examined (dentate)	Periodontal status (%)					
			CPI 0 Healthy	CPI 1 Bleeding	CPI 2 Calculus	CPI 3 Shallow pockets	CPI 4 Deep pockets	Excluded sextant
15-19	*1990	1,928	16.9	10.4	68.5	3.9	0.3	-
	2000	1,639	25.8	11.2	60.0	2.9	0.1	0.0
	2010	1,235	9.6	14.1	56.5	16.8	3.0	0.0
35-44	*1990	2,452	4.6	2.6	60.6	23.4	8.5	-
	2000	2,258	5.0	2.8	54.9	28.5	7.2	1.7
	2010	1,629	1.8	1.7	36.1	34.2	25.3	0.9
65-74	*1990	354	4.2	0.6	54.5	24.3	16.4	-
	2000	392	2.6	1.5	40.7	27.1	9.2	19.0
	2010	363	2.0	1.4	26.7	28.1	26.7	15.1
ALL	*1990	12,305	7.2	4.6	65.1	17.0	6	-
	2000	9,932	9.8	4.5	57.5	20	5.2	3.0
	2010	8,332	3.2	4.1	41.4	30.3	18.2	2.7

1.3 *Porphyromonas gingivalis*

P. gingivalis is a gram negative bacteria, non-motile, anaerobic and asaccharolytic rod which usually showed coccal to short rod morphologies (Haffajee and Socransky, 1994). On blood agar media, this bacterium showed black-pigmented, small colonies that resulted from accumulation of iron protoporphyrin IX, oxidized form of haem (Smalley *et al.*, 1998). Haem is a growth requirement that serves as the source of iron and protoporphyrin IX (Lewis *et al.*, 1999). This organism has different colony morphologies in fresh clinical isolates, ranging from smooth to rough colony morphotypes (Reynolds *et al.*, 1989).

P. gingivalis produces many cell components such as lipopolysaccharides, capsule, outer membrane, fimbrae and proteinase that function as virulence factors. The presences of anti-phagocytic capsule protect *P. gingivalis* from host defense. Fimbriae are responsible for binding to host tissue and saliva-coated hydroxyapatite. Proteinase is hydrolytic, proteolytic and lipolytic enzymes which function in periodontal disease progression. *P. gingivalis* also synthesize low endotoxicity lipopolysaccharides which enables bacteria grow and colonize in host cell (Holt *et al.*, 1999).

1.3.1 *PepO* gene

PepO involved in metalloendopeptidase that has a significant homology with human endothelin-converting enzyme 1 which responsible for the conversion of big edothelin-1 to endothelin-1 (Awano *et al.*, 1999). The conversion increases the endothelin-1 level which may lead to heart failure and atherosclerosis (Beck *et al.*, 1996). *PepO* is involved in initial step of invasion or lysis of mammalian cell membrane where deficient in invasion of HeLa cells was reported by using *PepO* mutant (Ansai *et al.*, 2003). Other study also reported that, gene encoding *PepO* is required for optimal

entry of *P. gingivalis* into gingival epithelial cells (Park *et al.*, 2004). Besides that, *pepO* gene has been used in multilocus sequence analysis in order to investigate the genetic relationship of *P. gingivalis* isolates from periodontitis patients (Koehler *et al.*, 2003).

1.4 Detection of *P. gingivalis*

1.4.1 Loop Mediated Isothermal Amplification (LAMP)

Notomi *et al.* (2001) introduced (LAMP), a molecular method to amplify specific segment of DNA under isothermal conditions. LAMP is sensitive, rapid and cost effective. This DNA amplification technique has been applied for the detection of pathogenic viruses (Hagiwara *et al.*, 2007; Yang *et al.*, 2011), bacteria (Neonakis *et al.*, 2011; Yamazaki *et al.*, 2011) and fungus (Uemura *et al.*, 2008; Sun *et al.*, 2010). Unlike the conventional polymerase chain reaction (PCR) based detection method, the strand displacement amplification require special equipment such as thermal cycler (Notomi *et al.*, 2000; Nagamine *et al.*, 2002). LAMP does not require special equipment such as thermal cycler. It can amplify DNA rapidly under isothermal conditions by using DNA polymerase with strand-displacements activity instead of *Taq* polymerase (Fu *et al.*, 2011). The final amplified products of positive samples are separated by agarose gel, giving a ladder like pattern (Hagiwara *et al.*, 2007).

LAMP is characterized by the recognisable eight distinct regions on the target gene with the use of six different primers which are forward inner primer (FIP), backward inner primer (BIP), two outer primers (F3 and B3) and two loop primers (FLP and BLP). The outer primers play their role in the strand displacement, while the internal primers help in the formation of an artificial stem-loop since they have both sense and antisense sequence. The eight distinct regions of the target gene are F1c, F2c,

F3c and FLP region at the 3' end and the B1, B2, B3 and BLP regions at the 5' end (Figure 1.2).

There are two steps of LAMP amplification which are non-cyclic and cyclic steps. In non-cyclic steps, there is formation of DNA with stem-loops at each ends that serve as the starting structure for the amplification and in LAMP cycling steps, one internal primer hybridizes to the loop on the product and initiates displacement DNA synthesis, producing the original stem-loop DNA and new stem-loop DNA with stem twice as long (Parida *et al.,* 2008). The final products of LAMP amplification are stem-loop DNAs with several inverted repeats of the target and cauliflower-like structure with multiple loops which formed by annealing between inverted repeats of the target in same strand (Parida *et al.,* 2008; Fu *et al.,* 2011).

The LAMP assay has become a powerful tool to facilitate genetic testing for the rapid diagnosis of infectious diseases (Endo *et al.*, 2004). Studies have reported the detection of *P. gingivalis* in subgingival plaque using LAMP assays (Maeda *et al.*, 2005; Yoshida *et al.*, 2005; Miyagawa *et al.*, 2008). Previous studies have shown that the addition of loop primers will accelerate the LAMP reaction and increase the sensitivity of LAMP assays (Nagamine *et al.,* 2002; Maeda *et al.*, 2005). LAMP assay has been demonstrated to have almost identical results to conventional real-time PCR with advantages of rapidity in amplification which indicates potential usefulness of LAMP assay in detecting and quantifying *P. gingivalis* in plaque samples (Maeda *et al.*, 2005; Yoshida *et al.*, 2005). The addition of heat-treatment step to LAMP assay has been shown to increase the sensitivity of LAMP assay (Ihira *et al.*, 2007).

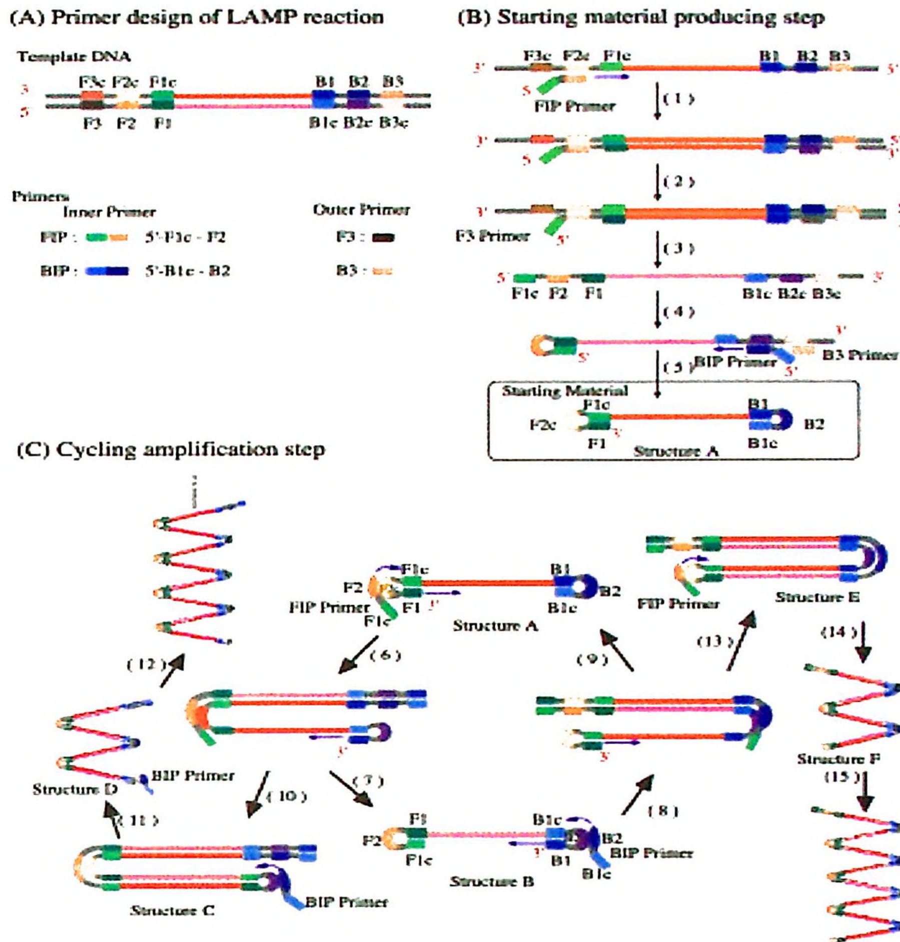


Figure 1.2: LAMP amplification step (Adapted from Parida *et al.*, 2008)

(A) Schematic representation of primer design for LAMP assay showing of the six types of primers based on the following eight distinct regions of the target gene: the F1c, F2c, F3c and FLP regions at the 3' end and the B1, B2, B3 and BLP regions at the 5' end. (B) Principles of LAMP amplification. Non-cyclic step (1-5): generation of stem loop DNA with dumbell-shaped structure at both ends that is ready to enter into cylic amplification step. (C) Principles of LAMP amplification. Cyclic amplification step (9-11): exponential amplification of original dumbbell- shaped stem-loop DNA employing internal primers. The product consisting of alternately inverted repeats of the target seqeunce on the same strand, giving a cauliflower-like structure.

1.4.2 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) assay has been used for direct detection of periodontal pathogens such as *B. forsythus*, *A. actinomycetemcomitans* and *F. nucleatum* in subgingival samples (Avila-Campos *et al.,* 1999). It also has been used in detection of *P. gingivalis* (Boutaga *et al.*, 2003). Other study has reported the use of PCR in detection of *P. gingivalis* using saliva sample which showed that *P. gingivalis* was more frequently detected in saliva than in subgingival plaque samples (Sakamoto *et al.*, 2001). Beside conventional PCR, real-time PCR showed good sensitivity and specificity in detection of *P. gingivalis* (Morillo *et al.*, 2003). Previous studies have reported rapid and accurate detection of *P. gingivalis* using simple real-time PCR method (Boutaga *et al.*, 2007; Braga *et al.*, 2010). Detection of *P. gingivalis* was also twice more often by real-time when compared to cultivation method (Jervøe-Storm *et al.*, 2005). Based on previous study, both PCR methods (conventional and real-time PCR) have been shown to be more sensitive, rapid and accurate than culture method in detection of *P. gingivalis* in saliva samples (Sakamoto *et al.*, 2001). Other study has reported the usefulness of multiplex PCR in detection and evaluation of the prevalence of *P. gingivalis* (Estrela *et al.,* 2010).

1.4.3 Others detection methods

Culture technique has been used for a long period of time for the detection of bacteria (Eick and Pfister, 2002). The cultivation method was considered as a gold standard, although it has many limitations such as inability of some species to grow on selective agar, detection of non-viable bacteria and high costs (Loomer, 2004). Other alternative methods that have been developed for the detection of *P. gingivalis* are immunoassay and DNA probe assay (Boutaga *et al.*, 2003; Liu *et al.*, 2003).

1.5 Problem statement

P. gingivalis is an obligate anaerobic periodontopathogenic bacterium. Due to this characteristic, these bacteria are difficult to maintain during sample collection and transportation. In addition, the culture methods require experienced personnel, labour intensive, expensive, time consuming and need a prolonged period before results can be obtained (Boutaga *et al.*, 2003). Because of these drawbacks, the quantitative culture of putative periodontopathogenic bacteria is not a routine procedure in most of the clinical microbiological laboratories. Several techniques such as biochemical tests, immunoassays and hybridization using nucleotide probes have been developed for microbiological diagnosis of periodontal diseases (Harper-Owen *et al.*, 1999; Maeda *et al.*, 2005). However, all of these methods are laborious and time consuming for routine clinical examinations (Maeda *et al.*, 2005).

Molecular biological techniques such as PCR based assays have enabled the development of culture-independent methods (Slots *et al.*, 1995; Harper-Owen *et al.*, 1999; Maeda *et al.*, 2005). However, these methods have several drawbacks such as time-consuming, potentially leading to laboratory contamination and the requirement of special apparatus (Parida *et al.*, 2008).

1.6 Rationale of study

To the best of our knowledge, very few studies have reported the use of LAMP for the detection of *P. gingivalis* and there is no reported on the detection of *P. gingivalis* using heat-treated saliva. Therefore, this study aims to evaluate the applicability of this novel nucleic acid amplification method for rapid detection of *P. gingivalis* using heat-treated saliva in comparison with extracted DNA using commercial kit. The isothermal amplification assay results obtained were compared with those results obtained by PCR assay.

For application of the LAMP method to clinical diagnostic, we have attempted to simplify the sample preparation. Previous study has suggested the possibility of direct use of plaque sample for LAMP examination without the DNA extraction step (Miyagawa *et al.,* 2008). Therefore, in this study heat-treated saliva were used as the source of DNA template besides the extracted DNA using the commercial kit to examine the effect of heat-treated saliva on LAMP sensitivity.

1.7 Objectives of study

1.7.1 General objective:

- ❖ To evaluate the applicability of (LAMP) using heat-treated saliva for rapid detection of *P. gingivalis* in comparison to conventional PCR.

1.7. 2 Specific objectives:

a) To compare the performance of LAMP using heat-treated saliva and extracted DNA prepared from commercial kit.

b) To compare the performance of PCR using heat-treated saliva and extracted DNA prepared from commercial kit.

c) To compare the performance of LAMP and PCR assays for the detection of *P. gingivalis* in periodontitis patients and healthy subjects.

CHAPTER 2.0

MATERIALS AND METHODS

2.1 Workflow of study

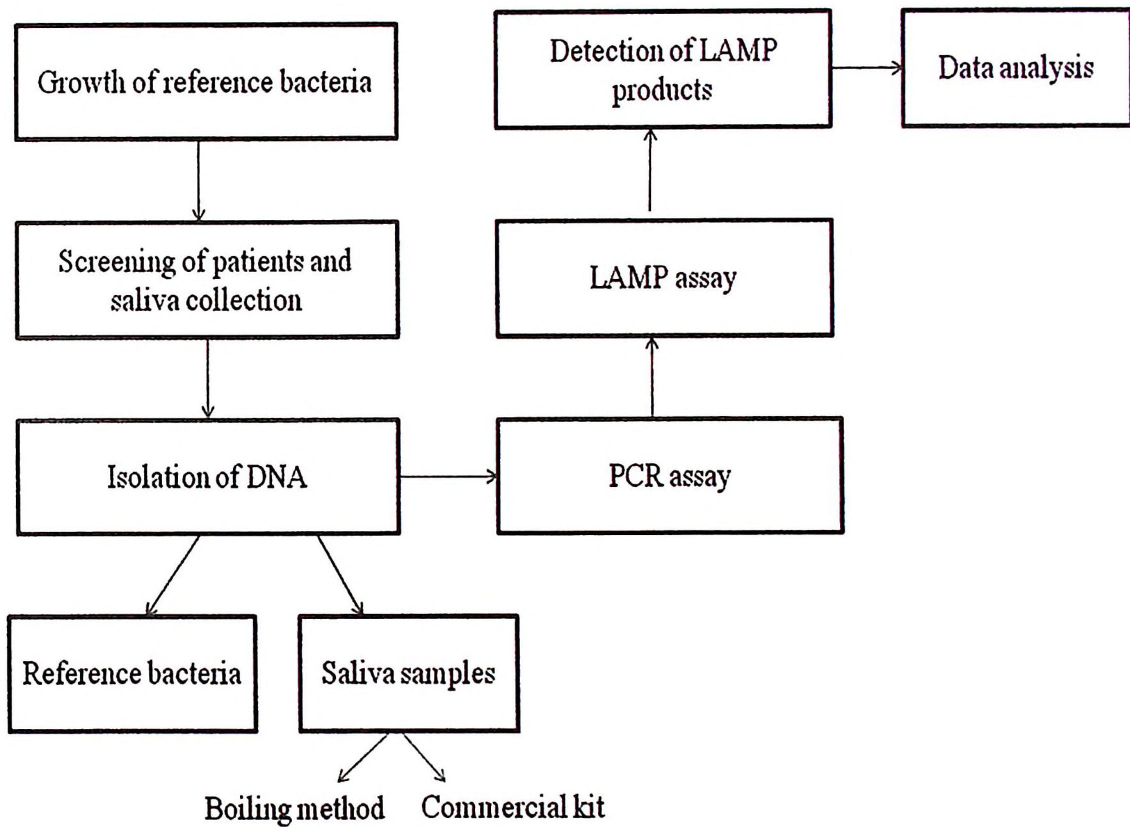


Figure 2.1: Workflow of study

2.2 Study design

This is a pilot study which involved five periodontis patients and five healthy subjects.

2.3 Materials

2.3.1 Chemicals and reagents

All general chemicals and reagents used in this study are listed in Table 2.1.

Table 2.1: List of general chemicals and reagents

Name	Manufacturer
100 bp DNA ladder	Invitrogen, USA
Ethanol	Merck, Germany
10X Blue Juice Loading dye	Invitrogen, USA
Agarose powder	Invitrogen, USA
LB Buffer	Faster Better Media, LLC
Syber Safe DNA stain dye	Invitrogen, USA
Diethyl pyrocarbonate (DEPC)	Sigma - Aldrich, Co., USA
SYBR Green I	Molecular Probes, Inc., Eugene

2.3.2 Kits and consumables

All laboratory kits and consumables used in this study are listed in Table 2.2

Table 2.2: List of kits and consumables

Kits and Consumable	Manufacturer
DNeasy Blood & Tissue Kit	Qiagen, USA
LoopampDNA amplification kit	Eiken Chemical Co. Ltd., Tokyo, Japan
QIAamp® DNA blood mini kit	Qiagen, USA
PCR tubes	Axygen Scientific, USA
Filter pipette tips	Axygen Scientific, USA
1.5 mL microcentrifuge tubes	Eppendorf, Germany

2.3.3 General buffer and stock solution

2.3.3.1 2% Agarose gel (w/v)

To prepare a 2% agarose gel (w/v), 0.5 g of agarose powder was added to25 mL of 1X LB buffer. Then, 2.5 μL of Syber Safe DNA stain was added into the mixture. The mixture was completely dissolved by heating the solution in microwave oven for 30 seconds. Then, the agar solution was poured into a gel casting and a gel comb was then placed into the gel casting tray. The gel was left to harden for 30 minutes. After the gel hardened completely, gel cast consisting of gel was placed into electrophoresis tank containing 1X LB buffer. Three μL of DNA sample was then loaded onto the gel and electrophoreses was run at 80 V for 45 minutes. The gel was visualized under Gel Doc.

2.3.3.2 1X Lithium Borate (LB) Buffer

LB buffer was prepared by using sterilized 0.0001% (1mL/ 10 000 mL) DEPC-treated water (1mL of DEPC added into 10 000 mL distilled water and incubated overnight). A 1X LB buffer was prepared by diluting 100 mL of 10X LB buffer stock solution into 900 mL of DEPC treated water.

2.3.4 Laboratory apparatus and equipments

Laboratory apparatus and equipments used in this study are listed in Table 2.3.

Table 2.3: List of laboratory apparatus and equipments

Apparatus/ Equipments	Manufacturer
Autoclave	Hirayama HVE-50, Japan
Centrifuge 5415D	Eppendorf, Germany
C100 Thermal Cycler	Bio-Rad, USA
Grant mini waterbath	Fisher Scientific Sdn. Bhd
Gel Doc	Bio-Rad, USA
Autoclave	Hirayama HVE-50, Japan
Block heater	Stuart
Biophotometer Plus	Eppendorf, Germany
Power Pac HC	Bio-Rad, USA
Vortex mixer	Velp.Sientifica, Europe
Pipette	Eppendorf, Germany
Concentrator Plus	Eppendorf, Germany

2.4 Methods

2.4.1 Reference bacterial strain and growth condition

P. gingivalis ATCC 33277 was used as a reference strain in LAMP and PCR assays. This bacterial strain was obtained from the American Type Culture Collection (ATCC). Reference bacterial strain from lyophilized stock was cultured anaerobically on blood agar plates for 7 days at 37°C.

2.4.2 Screening of patients and saliva collection

Saliva samples from five periodontitis patients and five healthy individuals who attended Dental Clinic at Hospital Universiti Sains Malaysia, Kubang Kerian, Kelantan were collected. Potential patients were screened, examined and selected for participation if they met criteria for periodontal disease such as having at least two sites with probing pocket depth (PD) $\geq$ 4 mm with attachment loss and had not received periodontal therapy or antibiotics for the past three months. Ethical approval was obtained from Research Ethics Committee (Human), Universiti Sains Malaysia (USM). Prior to samples collection, all patients were requested to read and sign the consent form. Then, patients were asked to hold their head slightly forward and expectorate all accumulated saliva into sterile collection tubes. Saliva samples were stored at -80°C until used.

2.4.3 Primers for LAMP and PCR

The lists of the primers are shown in Table 2.4 (Yoshida *et al.*, 2005). The primers were designed from *pepO* gene which encodes *P. gingivalis* endopeptidase. Then, the primers specificity were further confirmed by using BLAST on National Centre for Biotecnology Information. For LAMP assays, six primers were used and two primers (F3 and B3) were used for PCR. Two loop primers (LF, LB) were used to accelerate the LAMP reactions. The locations of the primers in *pepO* gene sequence are shown in Figure 2.2.

Table 2.4: Oligonucleotide primers for LAMP and PCR assays

Periodonthopathic bacteria	Primer sequences[a] (5'-3')	Amplicon size (bp)	Source (strain)
Porphyromonas gingivalis	Pg-F3 GGCAGTAATCGGCGCATT Pg-B3 TCGTGCAGGATGTCGAATG Pg-FIP ACTGAGGTCGATGGCCGGTA GCTGCAATGGCAATAAGGGT Pg-BIP CCGCAGGACGACTTTTATCG CTAGCCGTAGCGACTATAAG CA Pg-LF GCTTCCTGTCAGTATCGTTA GTCTG Pg-LB ACTGCAACGGCAATTGGATG	225	381

[a]Accession number: AB010440 for *P. gingivalis pepO* gene.

1 ATGAACAAGACAATCAAGTTCTTTGCCTTGCGCC**GGCAGTAATCGGCGC**

ATT[1]GATGCTG

61 ACCGGCTGCAATGGCAATAAGGGT[3a]**CAGACTAACGATACTG**

ACAGGAAGC[5]GCGAACCGG**TA**

121 CCGGCCATCGACCTCA[3b]GTGCCATGGATACATCCGTACG**CC**

CGCAGGACGACTTTTATCGC[4a]

181 T[3a]**ACTGCAACGGCAATTGGATG**[6]AAAAACAATCCTCTCAAAC C**TGCTTATAG**

TCGCTACGGC

241 T[4b]CATTCGACATCCTGCACGA[2]CAGCACCCTCGAGCGTGTAC ACCTGATTGT

GGACAACCTT

Note: F3: [1], B3: [2], FIP: [3a] and [3b], BIP: [4a] and [4b], LF: [5], LB: [6]

Figure 2.2: The location of the primers in *P. gingivalis*