UNIVERSITI SAINS MALAYSIA
GERAN PENYELIDIKAN UNIVERSITI PENYELIDIKAN
LAPORAN AKHIR

DEVELOPMENT OF NOVEL APPROACH RAPID MULTIPLEX REAL-TIME LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (M-QLAMP) ASSAY FOR DETECTION OF MELIOIDOSIS AGENT BURKHALDERIA PSEUDOMALLEI

PENYELIDIK

PROFESOR MADYA DR. CHAN YEAN YEAN

2017

END OF PROJECT REPORT FOR SCIENCEFUND

A. Description of the Project

1. **Project number :**
2. **Project title :** Development of Novel Approach Rapid Multiplex Real-time Loop-mediated Isothermal Amplification (M-qLAMP) Assay for detection of Melioidosis agent Burkholderia pseudomallei
3. **Project leader :**Chan Yean Yean
4. **Project Team (Please provide an assessment of how the project team performed and highlight any significant departures from plan in either structure or actual man-days utilised)**

   NIL
5. **Industrial Partnership :** (Please describe the nature of collaborations with relevant industry)

   NIL
6. **National/International Collaboration** (please identify research organisations and describe the nature of collaboration)

   AIMST University
7. **Project Duration :** 24.0 months.

   Start Date : April(month)2015(Year)

   End Date : March(month)2017(Year)
8. **Total Budget Approved :**

B. Objectives of the project

1. Socio-economic Objectives (SEO)

Which socio-economic objectives are addressed by the project? (Please identify the Research Priority Area, SEO Category and SEO Group under which the project falls. Refer to the Malaysian R&D Classification System, 4th Edition.)

Research Cluster	BIOTECHNOLOGY
SEO Category	: Natural Sciences, Technologies and Engineering
SEO Group	: Biotechnology
SEO Area	: Biotechnology

2. Fields of Research (FOR)

Which are the two main FOR Categories, FOR Groups, and FOR Areas of your project? (Please refer to the Malaysian R&D Classification System, 4th Edition)

a.Primary Field of Research

• FOR Category	: Biotechnology
• FOR Group	: Medical Biotechnology
• FOR Area	: Diagnostics

b. Secondary Field of Research (if applicable)

• FOR Category	: Applied sciences and technologies
• FOR Group	: Nanotechnology
• FOR Area	: Medical devices

C. Objectives achievement

* **Original project objectives** (Please state the specific project objectives as described in Section II of the Application Form)

Phase 1: Development of a Multiplex Loop-mediated Isothermal Amplification (M-LAMP) Assay 1.To design specific primers and optimize a M-LAMP which is capable of simultaneously detecting the presence of B. pseudomallei and internal control genes. Phase 2: Development of a Multiplex Real-time Loop-mediated Isothermal Amplification (M-qLAMP) Assay 2.To design qLAMP-probes for Real-time LAMP's special application of sequence-specific target and internal control genes detection. 3.To optimize the incorporation of qLAMP-probes by anneal on LAMP primers. 4.To optimize a M-qLAMP which is capable of real-time simultaneously detecting the presence of B. pseudomallei and internal control genes. Phase 3: Determination of analysis system for Multiplex Real-time Loopmediated Isothermal Amplification (M-qLAMP) Assay 5.To determine an analysis system for interpretation of M-qLAMP assay. 6.To generate a multiple target interpretation system for the simultaneous detection of multiple genes. Phase 4: Evaluation of the Multiplex Real-time Loop-mediated Isothermal Amplification (M-qLAMP) Assay 7.To perform analytical evaluation of the M-qLAMP assay 8.To evaluate the performance of the M-qLAMP assay using clinical samples.

* **Objectives Achieved** (Please state the extent to which the project objectives were achieved)

1. Development of a Loop-mediated Isothermal Amplification (LAMP) Assay 1.1 Completion of primers design and standardization of LAMP for detection of B. pseudomallei target on specific region (TTS1-orf2) 2.Development of a Multiplex Loop-mediated Isothermal Amplification (M-LAMP) Assay 2.1 Completion of primers design of internal control. 2.2 Incorporation of internal control to the assay for the assay validation purpose. 2.3 Optimization of the M-LAMP assay with known strains. 3. Development of a Multiplex Real-time Loop-mediated Isothermal Amplification (M-qLAMP) Assay 3.1 Dual-function oligonucleotides were developed for multiplex real-time loop-mediated isothermal amplification assay usage. 3.2 Incorporation of the newly design dual-function oligonucleotides to the M-LAMP assay. 3.3 Completion of the optimization steps for the M-qLAMP assy which is real-time and simultaneously detecting the presence of B. pseudomallei and internal control genes. 4. Determination and evaluation of analysis system for Multiplex Real-time Loop-mediated Isothermal Amplification (M-qLAMP) Assay 4.1 Analytical evaluation of the M-qLAMP assay was performed using known bacteria strains. 4.2 The performance of the M-qLAMP assay was determined using blood spiked samples.

* **Objectives not achieved** (Please state the extent to which the project objectives were achieved)

NIL

D. Technology Transfer / Commercialisation Approach, if any. (Please describe the approach planned to transfer / commercialise the results of the project)

This study has successfully developed a novel strategy for detection of melioidosis agent with newly designed dual-functions oligonucleotide. The platform can be adapted and used in developing similar assay for others infectious diseases. The use of dual-functions oligonucleotide accelerate the assay and monitoring the signal amplification simultaneously in real-time help us to promote the use of isothermal amplification technology in diagnostic microbiology laboratory to detect infectious disease rapidly. The outcome of this project include high-impact publications (Analyst, Q1, IF: 4.1), international and national conference presentations and patent filed and pending at the intellectual property corporation of Malaysia (MyIPO). (PI2016702747)

E. Assessment of Research Approach (Please highlight the main steps actually performed and indicate any major departure from the planned approach or any major difficulty encountered)

1. Design of specific LAMP primers for detection of Burkholderia pseudomallei and internal control. 2. Optimization of M-LAMP for simultaneous detection of target and internal control 3. Design of dual-functions LAMP primers and incorporate with usual LAMP primers for formation of M-qLAMP system 4. Optimization of M-qLAMP for real-time detection of multiple genes 5. Determination of analytical sensitivity and specificity of the M-qLAMP assay 6. Evaluation of the M-qLAMP assay with clinical isolates blood spiked samples 7. Determination of diagnostic sensitivity and specificity of the assay

F. Assessment of the Project Schedule (Please make any relevant comment regarding the actual duration of the project and highlight any significant variation from plan)

Project was conducted in line with milestones stated which took two years to developed and evaluate the novel multiplex real-time loop-mediated isothermal amplification assay.

G. Assessment of Project Costs (Please comment on the appropriateness of the original budget and highlight any major departure from the planned budget)

Funds were well spent as planned with the original budget.

H. Additional Project Fundling Obtained (In case of involvement of other funding sources,please indicate the source and total funding provided)

NIL.

I. Benefits of the Project (Please identify the actual benefits arising from the project as defined in Section III of the Application Form. For examples research direct outputs, organisational outcomes and sectoral/national impacts, please refer to Section III of the Guidelines for the Application of R&D Funding under ScienceFund)

1. Direct Outputs of the Project (please describe as specifically as possible the outputs achieved and provide an assessment of their significant to users)

i. Technical contribution of the project

a. What was the achieved direct output of the project:

Basic Research Project **Description:**

- [ ] Algorithm
- [ ] Structure
- [X] Data
- [ ] Other, please specify :

Basic Research Project **Description:**

- [X] Method/technique
- [X] Demonstrator/prototype

[ ] Product/component

[ ] Process

[ ] Software

[ ] Other, please specify :

b. How would you characterise the quality of this output?

[ ] Significant breakthrough

[X] Major improvement

[ ] Minor improvement

ii. Contribution of the project to knowledge

a. How has the output of the project been documented?

[X] Detailed project report

[X] Product/process specification documents

[ ] Other, please specify :

b. Did the project create an intellectual property stock?

[ ] Patent obtained

[X] Patent pending

[ ] Patent application will be filed

[ ] Copyright

c. What publications are available?

		National	International
[X]	Articles (s) in scientific publications	How many :	1
[X]	Paper(s) delivered at conferences/seminars	How many : 1	1
[ ]	Book	How many :	
[ ]	Other, please specify : GenBank		

d. How significant are citations of the results?

- [ ] Citations in national publications — How many :
- [ ] Citations in international publications — How many :
- [X] Not yet
- [ ] Not known

2. Organisational Outcomes of the Project (Please describe as specifically as possible the organisational benefits arising from the project and provide an assessment of their significance)

i. Contribution of the project to expertise development

a. How did the project contribute to expertise?

Area of Specialisation:

[X]	PhD degrees	How many : 1
[ ]	MSc degrees	How many :
[X]	Research staff with new specialty	How many : 2
[ ]	Other, please specify :	

b. How significant is this expertise?

[X]	One of the key areas of priority for Malaysia
[ ]	An important area, but not a priority one

ii. Economic contribution of the project?

a. How has the economic contribution of the project materialised?

		Description (if applicable)
[ ]	Sales of manufactured product/equipment	
[ ]	Royalties from licensing	
[X]	Cost savings	
[X]	Time savings	
[ ]	Other, please specify :	

b. How important is this economic contribution?

[ ]	High economic contribution	Value : RM
[X]	Medium economic contribution	Value : RM 0.00
[ ]	Low economic contribution	Value : RM

c. When has this economic contribution materialised?

- [ ] Already materialised
- [ ] Within months of project completion
- [ ] Within three years of project completion
- [X] Expected in three years or more
- [ ] Unknown :

iii. Infrastructural contribution of the project

a. What infrastructural contribution has the project had?

☐ New equipment	Type :	Value : RM 0.00
☐ New/improved facility	Investment :	Value : RM 0.00
☐ New information networks		
☐ Other, please specify :		

b. How significant is this infrastructural contribution for the organisation?

- ☐ Not significant/does not leverage other projects
- ☐ Moderately significant
- ☒ Very significant/significantly leverages other projects

iv. Contribution of the project to the organisationâ€™s reputation

a. How has the project contributed to increasing the reputation of the Organisation

- ☐ Recognition as a Center of Excellence
- ☐ National award (Type and category)
- ☐ International award (Type and category)
- ☐ Demand for advisory services
- ☐ Invitations to give speeches on conferences
- ☐ Visits from other organisations
- ☐ Other, please specify :

b. How important is the projectâ€™s contribution to the organisationâ€™s reputation?

- ☐ Not significant
- ☒ Moderately significant
- ☐ Very significant

3. **National Impacts of the Project** (If known at this point in time, please describe as specifically as possible the potential sectoral/national benefits arising from the project and provide an assessment of their significance)

i. Contribution of the project to organisational linkages

a. Which kinds of linkages did the project create?

- [X] Domestic industry linkages
- [ ] International industry linkages
- [X] Linkages with domestic research institutions, universities
- [ ] Linkages with international research institutions, universities

b. What is the nature of the linkages?

- [ ] Staff exchanges
- [X] Inter-organisational project team
- [ ] Research contract with a commercial client
- [X] Informal consultation
- [ ] Other, please specify :

i. Social-economic contribution of the project

a. Who are the direct customer/beneficiaries of the project output?

Customers/beneficiaries :	Number :

b. How has/will the socio-economic contribution of the project materialised?

- [X] Improvements in health
- [ ] Improvements in safety
- [ ] Improvements in the environment
- [ ] Improvements in energy consumption/supply
- [ ] Improvements in international relations
- [ ] Other, please specify :

c. How important is this socio-economic contribution?

- [X] High social contribution
- [ ] Medium social contribution
- [ ] Low social contribution

d. When has/will this social contribution materialised?

- [ ] Already materialised
- [ ] Within three years of project completion
- [X] Expected in three years or more
- [ ] Unknown

Date : 4/5/2017 Signature :

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SCIENCEFUND
END OF PROJECT & BENEFITS REPORT

se fill in the relevant details in the space provided.
se tick (/) where appropriate and provide further description, if required.

PROJECT DESCRIPTION

Project Number		
Project Title	Development of Novel Approach Rapid Multiplex Real-time Loop-mediated Isothermal Amplification (M-qLAMP) Assay for detection of Melioidosis agent *Burkholderia pseudomallei*	
Project Leader's Details	Name: DR. Chan Yean Yean	
	Position: Associate Prof	Address: Department of Microbiology & Parasitology, School of Medical Science, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan.
Project Funding		
Project Time Frame (According to agreement)	Start Date : :___April /2015; Completion Date : :_March /2017 Duration :24 months	
Project Extension (approval from MOSTI; if applicable;)	1st Extension : NA months; Revised completion date :___months /___year 2nd Extension : NA months; Revised completion date :___months / ___year	

2.0 OBJECTIVES ACHIEVEMENT

Problem Statement

Melioidosis is an emerging infectious disease caused by the Gram-negative bacterium *Burkholderia pseudomal* (Dance, 2000, Josephson, 2001, Raja et al., 2005, Valade et al., 2009). This environmental (soil) organism disseminated across Southeast Asia and northern Australia. This disease presentations range from asymptomatic, localized skin infection or pneumonia, to disseminated disease with abscesses in multiple organs, and resulting fulminant sepsis with mortality rates of 50% (Dance, 2000, Josephson, 2001, Raja et al., 2005, Valade et al., 2009). TH increase in numbers of cases globally has reflecting in both improved diagnostics (to detect the disease) and an increa in cases in those living in or traveling from melioidosis endemic region (Mirjam et al., 2012).

However, culture method still remains as a "gold standard" for diagnosis of melioidosis but it gives drawback to th sensitivity, because of the unidentifiable or unfamiliarity with *B. pseudomallei* in laboratories, and poor specificity c biochemical tests (Cooper et al., Wajanarogana et al., Kunakorn et al., 1991, Desakorn et al., 1994, Khrapova et al 1995, Smith et al., 1995, Dharakul et al., 1996, Alex et al., 2006, Altukhova et al., 2007, Kaestli et al., 2007, Mirjam et al 2012). The rapid diagnosis is very important for early detection and treatment, and delay in diagnosis can result in life threatening.

Other diagnostic techniques for *B. pseudomallei* detection include antigen detection by immunofluorescence microscopy latex agglutination and matrix-assisted laser desorption ionization - time of flight (MALDI-TOF) mass spectrometry (Mirjan et al., 2012). However, these methods caused reduced in sensitivity and it is depend on an initial culture step, whicl delaying in time to diagnosis (Mirjam et al., 2012). In addition, the serological diagnosis is unreliable due to backgrounc antibody levels in endemic areas and gave a low sensitivity and specificity assay (Mirjam et al., 2012).

Although various other molecular platforms, such like DNA microarrays and gene sequencing have been developed fo rapid detection of melioidosis but with its high-throughput technologies, yet are not feasible for routine diagnostics (Mirjan et al., 2012). Besides that, the isothermal DNA amplification (LAMP) and real-time PCR assays targeting specific region of the *B. pseudomallei* genome have been developed for rapid detection of melioidosis, but the nature of the LAMF technology itself, constrained to single target detection (monoplex), which made the assay validity is in query and limiting the utility of this technique. Subsequently, the real-time PCR assay offering sensitive and real-time result, but with expensive reagent and multi-thermal amplification causes longer amplification time (3 hours). In addition, both techniques require well trained molecular diagnostic staff to perform the assay because of its multiple micropippetting steps, whicl will furthermore causes contamination to the reaction. Although those conventional LAMP and real-time PCR assays fo detection of Melioidosis agent *Burkholderia pseudomallei* were reported in previous research (Dong et al., Fang et al. Hsu et al., Kasahara et al., Liang et al., Luo et al., Mahony et al., Shao et al., Wang et al., Zerilli et al., Iseki et al., 2007 Narisara et al., 2008, Nathan et al., 2012), the limitations of these methods to be used as diagnostic assay or to be commercialize is still restricted.

a.	**Original Project Objective** (Based on MOSTI approval)	The proposed study is divided into four phases: **Phase 1: Development of a Multiplex Loop-mediated Isothermal Amplification (M-LAMP) Assay** 1. To design specific primers and optimize a M-LAMP which is capable of simultaneously detecting the presence of *B. pseudomallei* and internal control genes. **Phase 2: Development of a Multiplex Real-time Loop-mediated Isothermal Amplification (M-qLAMP) Assay** 2. To design qLAMP-probes for Real-time LAMP' s special application of sequence-specific target and internal control genes detection. 3. To optimize the incorporation of qLAMP-probes by anneal on LAMP primers. 4. To optimize a M-qLAMP which is capable of real-time simultaneously detecting the presence of *B. pseudomallei* and internal control genes. **Phase 3: Determination of analysis system for Multiplex Real-time Loopmediated Isothermal Amplification (M-qLAMP) Assay** 5. To determine an analysis system for interpretation of M-qLAMP assay. 6. To generate a multiple target interpretation system for the simultaneous

		detection of multiple genes. **Phase 4: Evaluation of the Multiplex Real-time Loop-mediated Isothermal Amplification (M-qLAMP) Assay** 7. To perform analytical evaluation of the M-qLAMP assay 8. To evaluate the performance of the M-qLAMP assay using clinical samples.
b.	**Original Achieved** (Please state the extent to which the project objectives were achieves)	**1. Development of a Loop-mediated Isothermal Amplification (LAMP) Assay** i. Completion of primers design and standardization of LAMP for detection of *B. pseudomallei* target on specific region (TTS1-*orf2*) **2. Development of a Multiplex Loop-mediated Isothermal Amplification (M-LAMP) Assay** i. Completion of primers design of internal control. ii. Incorporation of internal control to the assay for the assay validation purpose. iii. Optimization of the M-LAMP assay with known strains. 3. **Development of a Multiplex Real-time Loop-mediated Isothermal Amplification (M-qLAMP) Assay** i. Dual-function oligonucleotides were developed for multiplex real-time loop-mediated isothermal amplification assay usage. ii. Incorporation of the newly design dual-function oligonucleotides to the M-LAMP assay. iii. Completion of the optimization steps for the M-qLAMP assy which is real-time and simultaneously detecting the presence of *B. pseudomallei* and internal control genes. **4. Determination and evaluation of analysis system for Multiplex Real-time Loop-mediated Isothermal Amplification (M-qLAMP) Assay** **i.** Analytical evaluation of the M-qLAMP assay was performed using known bacteria strains. **ii.** The performance of the M-qLAMP assay was determined using blood spiked samples.
c.	**Original Not Achieved** (Please state the objectives that were no achieved and provide reason)	**N/A**
d.	**Summary / Conclusion of Research Findings**	In conclusion, the study has successfully developed a novel multiplex real-time loop-mediated isothermal amplification assay detection of *B. pseudomallei* with present of internal control for result verification. Study used the newly developed dual-functions oligonucleotide with optimized M-qLAMP protocol to effectively detect *B. pseudomallei*.The assay was found to be highly sensitive given limit of detection of *B. pseudomallei* at genomic DNA level was 10 fg/ul, 18 CFU/ml at bacterial cell level. The assay was highly specific and able to specifically detect all blood spiked with *B. pseudomallei* without giving any false result interpretaion. Thus, the overall diagnostic performance of the assay in term of diagnostic sensitivity, diagnostic specificity, positive predictive value (PPV), and negative predictive value (NPV) were all 100%. Real-time monitoring the LAMP assay with BioRad CFX96 real-time system and dual-functions oligonucleotide enhance rapidity of assay to obtain positive result as soon as 20-30 min.