DEVELOPMENT OF THERMOSTABLE MULTIPLEX qPCR FOR SIMULTANEOUS DETECTION OF PATHOGENS ASSOCIATED WITH FEBRILE ILLNESSES

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by

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-	Negative or subtraction
+	Positive or addition
±	Plus-minus
≤	Less than or equal to
≥	Greater than or equal to
×	Times or multiplication
=	Equal to
~	Approximately
/	Division or 'or'
°C	Degree Celcius
µ	Micro
%	Percentage
5-FU	5- Fluorouracil
a	Atto
A	Adenine
A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
AAT	Accelerated ageing temperature
AT	Ambient temperature
BCCM	Belgian Coordinated Collections of Microorganisms
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
C	Cytosine

cm	Centimetre
D	Duration
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double-stranded DNA
EDTA	Ethylenediaminetetraacetic acid
EMJH	Ellinghausen-McCullough-Johnson-Harris
e.g.	Exempli gratia (for example)
et al.	Et alia (and others)
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
f	Femto
F	Forward primer
g	Gram
g	Relative centrifugal force
G	Guanine
GC	Guanine-cytosine
HCl	Hydrochloric acid
HUSM	Hospital Universiti Sains Malaysia
HSNZ	Hospital Sultanah Nur Zahirah
IAC	Internal amplification control
IDT	Integrated DNA Technologies
i.e.	Id est (In other words)
IMR	Institute for Medical Research

k	Kilo
KKM	Kementerian Kesihatan Malaysia
L	Litre
LAMP	LooP-mediated isothermal amplification
Ltd.	Limited
LSHTM	London School of Hygiene & Tropical Medicine
m	Meter
M	Molar
MAT	Microscopic agglutination test
MKA	Makmal Kesihatan Awam
ml	Miller
MIQE	Minimum information for publication of quantitative real-time PCR experiments
mm	Millimetre
n	Nano
n	Number of samples
nm	Nanometer
no.	Number
NPV	Negative predictive value
MW	Molecular weight
OD	Optical density
p	Pico
p	Sensitivity or specificity expressed as proportion
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PPV	Positive predictive value
psi	Pounds per square inch

Q_{10}	Rate of chemical reaction
R	Degenerative nucleotide for A or G substitution
RNA	Ribonucleic acid
RPA	Recombinase polymerase amplification
rpm	Revolutions per minute
rRNA	Ribosomal RNA
SD	Standard deviation
sp.	species (singular)
spp.	species (plural)
T (or U)	Thymine (or Uracil)
Ta	Annealing temperature
TBE	Tris-borate-EDTA
Tm	Melting temperature
Tris	Tris(hydroxymethyl)aminomethane
Tris-HCl	Tris Hydrochloride
U	Unit
UK	United Kingdom
UNAM	National Autonomous University of Mexico
UPM	Universiti Putra Malaysia
USA	United States of America
USM	Universiti Sains Malaysia
UV	Ultraviolet
V	Volt
v/v	Volume/volume
w/v	Weight/volume
WHO	World Health Organisation
Y	Degenerative nucleotide for C or T substitution

PEMBANGUNAN ASAI TINDAK BALAS RANTAI POLYMERASE TAHAN HABA DENGAN PELBAGAI SASARAN BAGI MENGESAN PATOGEN YANG MENYEBABKAN DEMAM

ABSTRAK

Sehingga kini, terdapat beberapa ujian molekular untuk mengesan penyakit kencing tikus, melioidosis, salmonellosis invasif dan malaria. Ujian-ujian ini membolehkan pengesanan awal asid nukleik patogen, seterusnya berpotensi menjadi alternatif kepada ujian-ujian sedia ada seperti kaedah kultur (pembiakan patogen dalam media tiruan), serologi dan pemeriksaan mikroskopik. Walaubagaimanapun, sebilangan besar ujian-ujian molekular ini tidak mempunyai kawalan kualiti amplifikasi dalaman (IAC), tidak mematuhi garis panduan MIQE dan biasanya hanya dapat mengesan satu jenis patogen sahaja. Untuk menangani kekangan-kekangan tersebut, kajian ini menyasarkan untuk membangunkan satu asai (ujian) molekular menggunakan pentas 'probe hidrolis TaqMan' bagi tujuan pengesanan asid deoksiribonukleik dari empat jenis patogen (iaitu *Leptospira*, *B. pseudomallei*, *Salmonella* and *Plasmodium*) berserta IAC dalam satu tiub. Untuk mencapai tujuan tersebut, empat pasang primer pencetus dan empat '*probe* hidrolis TaqMan' telah direka untuk mengesan patogen *Leptospira*, *B. pseudomallei*, *Salmonella* and *Plasmodium*. Primer-primer pencetus ini mengandungi penyesuai (UN-*adapter*) dan primer UN untuk menambahkan lagi kadar sensitiviti asai ini. Selain itu, sepasang primer pencetus dan *probe* tambahan turut direka untuk tujuan kawalan kualiti amplifikasi dalaman (IAC). IAC yang digunakan adalah gabungan antara gen *rpoB* daripada bakteria batuk kering dan juga gen *HLY5mc1* daripada patogen *Entamoeba*

histolytica. Selepas itu, prestasi asai ini divalidasi selaras dengan garis panduan MIQE. Kemampuan analitikal asai ini diuji dengan asid deoksiribonukleik dari empat jenis patogen utama yang dilarutkan sebanyak 10 kali ganda, dalam beberapa siri. Selain itu juga, kekhususan asai ini diuji terhadap 357 patogen, antaranya 131 patogen *Leptospira*, 105 patogen *B. pseudomallei*, 44 patogen *Salmonella*, 31 patogen *Plasmodium* dan 46 lain-lain patogen. Tambahan lagi, kestabilan asai ini pada beberapa suhu dan prestasinya terhadap 518 sampel pesakit turut dinilai. Dalam kajian ini, adalah didapati bahawa asai yang dibangunkan berjaya mengenalpasti patogen *Leptospira, B. pseudomallei, Salmonella* dan *Plasmodium* pada kadar serendah 5.61 salinan, 8.24 salinan, 19.3 salinan dan 18.1 salinan DNA bagi setiap ujian. Tiada signal meragukan dikesan pada patogen-patogen yang lain. Selain itu, penilaian klinikal menggunakan 518 sampel pesakit menunjukkan bahawa asai ini mempunyai kadar sensitiviti yang sempurna (100%) dan kadar spesifisiti yang tinggi (98.8% – 100%). Tiada tanda-tanda perencatan PCR ditemui dalam sampel yang digunakan. Tambahan lagi, ujian kestabilan asai kering dengan kehadiran 5% gula trehalos menunjukan bahawa asai ini masih lagi stabil selepas 84.7 hari, pada suhu bilik. Kesimpulannya, kajian ini berjaya membangunkan asai molekular yang sensitif dan khusus bagi mengesan patogen penyebab deman kencing tikus, melioidosis, invasif salmonellosis dan malaria.

DEVELOPMENT OF THERMOSTABLE MULTIPLEX qPCR FOR SIMULTANEOUS DETECTION OF PATHOGENS ASSOCIATED WITH FEBRILE ILLNESSES

ABSTRACT

To date, several molecular assays are available for the diagnosis of leptospirosis, melioidosis, invasive salmonellosis and malaria. These reported assays allow early detection of pathogens nucleic acids; hence serve as potential alternatives to the conventional methods including culture, serology and microscopic examination. However, majority of the reported molecular assays lack of internal amplification control (IAC), do not adhere to the MIQE guidelines and usually detect a single organism. To address these major limitations of current molecular assays, this study is aimed to develop a multiplex TaqMan hydrolysis probe-based qPCR that can detect nucleic acids of four different organisms (*Leptospira, B. pseudomallei*, *Salmonella* and *Plasmodium*) and an IAC within a single reaction. To achieve these objectives, four pairs of primers and four TaqMan hydrolysis probes were designed against *Leptospira, B. pseudomallei, Salmonella* and *Plasmodium* genomes. These primer pairs were conjugated with a universal adapter (UN-adapter) and combined with UN primers in order to increase the performance of the assay. In addition, another pair of primers and probe against *Mycobacterium tuberculosis rpoB* gene and *Entamoeba histolytica HLY5mc1* gene was also developed as IAC tool. Validation of the assay was performed according to the MIQE guidelines. First, analytical sensitivity of the multiplex qPCR assay was evaluated on 10-fold serial dilutions of genomic DNA from each microbial target, as well as its specificity on

357 microbial isolates, made up of 131 *Leptospira* isolates, 105 *B. pseudomallei* isolates, 44 *Salmonella* isolates, 31 *Plasmodium* strains and 46 other organisms. Following stability testing at different temperatures, the clinical performance of the qPCR assay was evaluated on 518 retrospective specimens from suspected patients. In this study, it was found that the developed multiplex qPCR assay correctly amplified and differentiated all *Leptospira*, *B. pseudomallei*, *Salmonella* and *Plasmodium* isolates, with limit of detections (LODs) of 5.61 copies, 8.24 copies, 19.3 copies and 18.1 copies per reaction, respectively. No undesired amplification was observed in other tested organisms. Similarly, the clinical evaluation showed that the qPCR assays had clinical sensitivities of 100% and clinical specificities of between 98.8% and 100%. No evidence of PCR inhibition was observed. In terms of stability, in the presence of 5% trehalose, the lyophilised qPCR mix had an estimated shelf-life of 84.7 days, at ambient temperature. Overall, this study successfully developed a multiplex qPCR assay for early detection of four common febrile causing infections, in particular leptospirosis, melioidosis, invasive salmonellosis and malaria, at high sensitivity and specificity, both analytically and clinically.

CHAPTER 1

INTRODUCTION

1.1 *Leptospira* and leptospirosis

Leptospira, a causative organism of leptospirosis was first observed in 1907 by A.M. Stimson from a kidney specimen of a deceased patient (Stimson, 1907). The organism was then called *Spirocheta interrogans*. In July 1913, a Japanese group led by Inada successfully isolated *Leptospira* from guinea-pigs injected with the blood of suspected patients. The organism was named *Spirochaeta icterohaemorrhagiae* (Inada *et al*., 1916). Approximately a year after the Inada's discovery, another two independent German groups successfully propagated *Leptospira* from the same animal host and named the organism as *Spirochaeta nodosa* and *Spirochaeta icterogenes*, respectively (Sellards, 1940).

1.1.1 Biology of *Leptospira* and cultivation

Leptospira spirochetes are thin and helical in shape, with a diameter of 0.1 to 0.3 µm and a length of 6 – 20 µm. The spirochetes usually have pointed ends that resemble a distinctive hook, at either or both poles. At both poles of the bacteria is a pair of endoflagella (also known as periplasmic flagella) that gives motility to this organism (Johnson, 2018).

This bacterium is cultivatable aerobically in artificial media enriched with several essential supplements. Among the important nutritional requirements is the fatty acid that acts as carbon and energy source to *Leptospira*. Unlike *L. biflexa*, the pathogenic *L. interrogans* can only metabolise long chain (>C15) unsaturated fatty acids (Cameron, 2015). At high concentration, this essential carbon-energy source,

however, can be toxic to *Leptospira*. Therefore, the artificial growth medium must also contain detoxicants such as bovine serum albumin (BSA) or sorbitol-fatty acid complexes that promote slow release of fatty acid or serves as a non-toxic form of fatty acid source (Mythri, 2016). Other important supplements include ammonium ions, thiamin, cobalamin, biotin, iron and some salts. In addition, *Leptospira* grows optimally at slightly alkaline pH of 7.2–7.6 (Cameron, 2015).

The most commonly used medium is Ellinghausen-McCullough-Johnson-Harris (EMJH) medium. The liquid or semisolid EMJH (contain additional agar) medium is incubated at a temperature ranging between 28 and 30 °C (Samir and Wasfy, 2013). Unlike the saprophytic group, the growth of most pathogenic strains of *Leptospira* is inhibited at lower temperature, 11–13 °C. The growth of leptospires in the artificial medium can be monitored under the dark field microscope or predicted by the presence of Dinger's ring, located close to the bottom of the medium tube (Nally *et al*., 2018). In terms of doubling time, the growth rate of *Leptospira* depends on the passage and strains. Generally, *Leptospira* growth rate on primary isolation is often slower than a pure subculture. Therefore, during clinical diagnostic, the culture medium is incubated for up to 13 weeks before being discarded (Sayyed Mousavi *et al*., 2017).

In addition, saprophytic *Leptospira* grow faster than the pathogenic counterparts, with maximal growth after 2 – 3 days versus 4 – 7 days, respectively. In terms of generation time, the pathogenic group grows slower (~20 hours) in contrast to the saprophytic group that replicates more rapidly, approximately 5 hours (WHO, 2013). This observation may due to the ability of the saprophytic group to synthesise many essential metabolites such as purines and pyrimidines, as well as its resistance in the environment (Cameron, 2015). Based on these unique cellular

metabolisms, both pathogenic (*L. interrogans* sensu lato) and saprophytic (*L. biflexa* sensu lato) groups can be differentiated based on the nutritional requirement. For instance, in the presence of 8-azaguanine, a purine analogue, the growth of pathogenic group is inhibited, unlike the latter group that is resistant to the supplement (Musso *et al*., 2013).

1.1.2 Classification and taxonomy

The current genus name was proposed by Noguchi, in 1918, to discriminate *Leptospira* from other spirochetes such as *Treponema pallidum* and *Spironema recurrentis*. This genus belongs to Spirochaetes phylum, Spirochaetales order and family of Leptospiraceae. Historically, species name was given based on each new serovar isolated, such as *Leptospira pomona* and *Leptospira canicola*. However, in 1982, this genus was separated into two species, *Leptospira interrogans* sensu lato and *L. biflexa* sensu lato based on its pathogenicity (M. Picardeau, 2012).

With the availability of DNA–DNA hybridization and genome sequencing, modern *Leptospira* genus are speciated according to their DNA relatedness (Levett, 2015). Extensive molecular and phylogenetic analyses revealed that there is a total of 35 distinctive species belong to the *Leptospira* genus, comprised of 13 pathogenic species, 11 intermediate species and remaining 11 saprophytic species (Bourhy *et al*., 2014; Brenner *et al*., 1999; Faine and Stallman, 1982; Matthias *et al.*, 2008; Puche *et al.*, 2017; L. Smythe *et al.*, 2013; Yasuda *et al.*, 1987). In 2018 alone, 13 new leptospiral species were described (Thibeaux *et al.*, 2018). Details of the latest *Leptospira* species are listed in Table 1.1.

In addition, *Leptospira* is also concurrently classified into different serovars, based on their antigenic relatedness, using cross agglutination absorption test (CAAT). This test is cumbersome and laborious due to the stringent requirement of rabbit immune sera that need to be prepared and maintained (Mgode *et al.*, 2015). Each serovar contains different carbohydrate component of the outer membrane lipopolysaccharide (LPS). To date, more than 250 serovars have been described. Related serovars are clustered into serogroups and identified using the microagglutination test (MAT). In the clinical application, serological typing of *Leptospira* poses limited usefulness for early detection of clinical leptospirosis (Sayyed Mousavi *et al.*, 2017). However, MAT is significant in epidemiological research and disease surveillance (Jorge *et al.*, 2017).

Yet, these molecular and serology classification systems do not correlate well with each another. It is because a given serogroup or serovar may present across several *Leptospira* species (David A Haake and Levett, 2015). For example, 26 described serovars of the Tarassovi serogroup are found in *L. alexanderi* (*n* = 1), *L. borgpetersenii* (*n* = 7), *L. kmetyi* (*n* = 1), *L. santarosai* (n = 11), *L. weilii* (*n* = 4), *L. inadai* (*n* = 1) and one unknown species. Similarly, 20 serovars within Icterohaemorrhagiae serogroup are found in *L. interrogans* (*n* = 10), *L. borgpetersenii* (*n* = 1), *L. kirschneri* (*n* = 6), *L. terpstrae* (*n* = 1) and two unknown species. The poor relationship between molecular and serotyping classification may due to potential horizontal gene transfer between the leptospiral species (Cerqueira and Picardeau, 2009).

Remarkably, approximately 90% of the described serovars belong to the pathogenic group of *Leptospira*, which is significant to human infections. Of this pathogenic group, 150 serovars belong to *L. interrogans, Leptospira santarosai and*

L. borgpetersenii. Meanwhile, the remaining 27 serovars belong to the intermediate and saprophytic groups of *Leptospira* which rarely isolated from human cases (Levett, 2015). Table 1.2 showed the distribution of serovars across the *Leptospira* genus.

Table 1.1 Latest species classification of *Leptospira* and their pathogenicity group. Adapted from Thibeaux *et al*. (2018)

No	Organism	Group	Reference
1	*Leptospira adleri*		Thibeaux *et al.* (2018)
2	*Leptospira alexanderi*		Brenner *et al.* (1999)
3	*Leptospira alstonii*		Smythe *et al.* (2013)
4	*Leptospira barantonii*		Thibeaux *et al.* (2018)
5	*Leptospira borgpetersenii*		Yasuda *et al.* (1987)
6	*Leptospira ellisii*		Thibeaux *et al.* (2018)
7	*Leptospira interrogans*	Pathogenic	Faine and Stallman (1982)
8	*Leptospira kirschneri*		Levett *et al.* (2005)
9	*Leptospira kmetyi*		Slack *et al.* (2009)
10	*Leptospira mayottensis*		Bourhy *et al.* (2014)
11	*Leptospira noguchii*		Yasuda *et al.* (1987)
12	*Leptospira santarosai*		Yasuda *et al.* (1987)
13	*Leptospira weilii*		Yasuda *et al.* (1987)
14	*Leptospira broomii*		Levett *et al.* (2005)
15	*Leptospira fainei*		Levett *et al.* (2005)
16	*Leptospira haakeii*		Thibeaux *et al.* (2018)
17	*Leptospira hartskeerlii*		Thibeaux *et al.* (2018)
18	*Leptospira inadai*		Yasuda *et al.* (1987)
19	*Leptospira licerasiae*	Intermediate	Matthias *et al.* (2008)
20	*Leptospira neocaledonica*		Thibeaux *et al.* (2018)
21	*Leptospira perolatii*		Thibeaux *et al.* (2018)
22	*Leptospira saintgironsiae*		Thibeaux *et al.* (2018)
23	*Leptospira venezuelensis*		Puche et. al. (2017)
24	*Leptospira wolffii*		Slack *et al.* (2009)
25	*Leptospira biflexa*		Faine and Stallman (1982)
26	*Leptospira brenneri*		Thibeaux *et al.* (2018)
27	*Leptospira harrisiae*		Thibeaux *et al.* (2018)
28	*Leptospira idonii*		Saito *et al.* (2013)
29	*Leptospira levettii*		Thibeaux *et al.* (2018)
30	*Leptospira macculloughii*	Saprophytic	Thibeaux *et al.* (2018)
31	*Leptospira meyeri*		Yasuda *et al.* (1987)
32	*Leptospira terpstrae*		Smythe *et al.* (2013)
33	*Leptospira vanthielii*		Smythe *et al.* (2013)
34	*Leptospira wolbachii*		Yasuda *et al.* (1987)
35	*Leptospira yanagawae*		Smythe *et al.* (2013)

Table 1.2 Distribution of *Leptospira* serovars and species. The respective species within the pathogenic group is shaded in red, intermediate group in blue and saprophytic group in green. Adapted from Levett (2015).

Serogroup	**# serovars**	***Leptospira* species**			
Andaman	1	*L. biflexa*			
Australis	14	*L. borgpetersenii*	*L. interrogans*	*L. kirschneri*	*L. noguchii*
Autumnalis	16	*L. borgpetersenii*	*L. interrogans*	*L. kirschneri*	*L. noguchii*
		L. santarosai			
Ballum	7	*L. borgpetersenii*	*L. mayottensis*	*L. santarosai*	
Bataviae	11	*L. interrogans*	*L. kirschneri*	*L. noguchii*	*L. santarosai*
Canicola	13	*L. interrogans*	*L. kirschneri*		
Celledoni	5	*L. borgpetersenii*	*L. weilii*		
Codice	1	*L. wolbachii*			
Cynopteri	2	*L. kirschneri*	*L. santarosai*		
Djasiman	6	*L. interrogans*	*L. kirschneri*	*L. noguchii*	
Grippotyphosa	9	*L. interrogans*	*L. kirschneri*	*L. santarosai*	*Undetermined*
Hebdomadis	13	*L. alexanderi*	*L. borgpetersenii*	*L. interrogans*	*L. kirschneri*
		L. santarosai	*L. idonii*		
Holland	1	*L. vanthielii*			
Hurstbridge	2	*L. broomii*	*L. fainei*		
Icterohaemorrhagiae	20	*L. borgpetersenii*	*L. interrogans*	*L. kirschneri*	*L. terpstrae*
		Undetermined			
Iquitos	1	*L. licerasiae*			
Javanica	16	*L. alexanderi*	*L. borgpetersenii*	*L. santarosai*	*L. weilii*
		L. meyeri			
Louisiana	3	*L. interrogans*	*L. noguchii*		
Manhao	5	*L. alexanderi*	*L. weilii*	*L. inadai*	
Mini	10	*L. alexanderi*	*L. borgpetersenii*	*L. interrogans*	*L. mayottensis*
		L. santarosai	*L. weilii*		
Panama	3	*L. noguchii*	*L. inadai*		
Pomona	8	*L. borgpetersenii*	*L. interrogans*	*L. kirschneri*	*L. noguchii*
		L. santarosai			
Pyrogenes	17	*L. borgpetersenii*	*L. interrogans*	*L. noguchii*	*L. santarosai*
		L. weilii			
Ranarum	3	*L. alstonii*	*L. interrogans*	*L. weilii*	
Sarmin	6	*L. interrogans*	*L. santarosai*	*L. weilii*	*Undetermined*
Sehgali	1	*L. interrogans*			
Sejroe	22	*L. borgpetersenii*	*L. interrogans*	*L. santarosai*	*Undetermined*
Semaranga	4	*L. biflexa*	*L. meyeri*	*L. yanagawae*	
Shermani	5	*L. noguchii*	*L. santarosai*	*L. inadai*	
Tarassovi	26	*L. alexanderi*	*L. borgpetersenii*	*L. kmetyi*	*L. santarosai*
		L. weilii	*L. inadai*	*Undetermined*	
Undesignated	3	*L. alstonii*	*L. inadai*	*L. wolffii*	
Total	254				

1.1.3 Sources of leptospirosis infections

Rodents are considered an important animal reservoir that can continuously excrete *Leptospira* throughout their lifespan without any apparent symptoms. Among the most common *Leptospira* serovars isolated from rodents are Ichterohaemorrhagiae, Copenhageni, Pomona and Ballum (Andersen-Ranberg *et al.*, 2016). In dogs, serovar Canicola and Bratislava are more common, in contrast to serovar Hardjo and Sejroe that are more prevalent in cattle (Jorge *et al.*, 2017; Nally *et al.*, 2018). The prevalence of *Leptospira* serovars among these animal hosts, however, may differ by the locations (Ellis, 2015). Apart from rodents, ruminants and canine, *Leptospira* also may present in wildlife mammals such as foxes, racoons and skunks (Shearer *et al.*, 2014).

These animal reservoirs (also known as maintenance host) are the main source of environmental *Leptospira* contamination. In general, rats shed 8×10^8 leptospires cells per mL of urine, the highest concentration as compared to other mammals. This animal host (rodents), as well as dogs play more significant roles in the urban areas, in which the animal density and prevalence of carrier among host will increase higher dissemination of leptospires (Guernier *et al.*, 2017). In terms of absolute quantity, cattle and deer excrete the biggest amount of *Leptospira,* 6.3×10^8 and 6.1×10^8 cells, respectively due to the significantly larger volume of urine (V. Barragan *et al.*, 2017). The sheer volumes of urine from large mammal provide consistent inoculation of *Leptospira* to the environment, even at low density of animal carrier (V. Barragan *et al.*, 2016).

Upon dissemination onto the environment (soils and water), the persistence of leptospires are affected by many physical factors. The moisture content of >20 % and approximately neutral pH (6.2 to 7.2) improve recovery of viable leptospires

contaminated soils (Saito *et al.*, 2013). In freshwater, *Leptospira* can remain pathogenic and viable at low temperature, poor-nutrient and acidic conditions over at least 10 months (Andre-Fontaine *et al.*, 2015). Another factor that affects the viability of *Leptospira* is the presence of environmental microbiota. Potential syntrophic interactions between leptospires and *Sphingomonas* and *Azospirillum brasilensis* improve growth and/or resistance of *Leptospira* to an antibiotic, increased temperature and UV radiation, possibly due to the formation of biofilms (V. A. Barragan *et al.*, 2011; Vinod Kumar *et al.*, 2016).

In addition, extreme weather, such as floods and storms is also associated with leptospirosis outbreaks. The incidence of such water-related catastrophes increases dispersal and persistence of *Leptospira* in the environments (Cann *et al.*, 2013). In urban settings, floods overwhelm sewage systems and disperse environmental leptospires to wider areas. Meanwhile, in other circumstances, floods attract more peridomestic animal to readily limited spaces of dry habitat and lead to a higher number of infected animal hosts. Subsequently, more *Leptospira* is shed into the environments, increasing the risk of human leptospirosis (Gomes-Solecki *et al.*, 2017). Figure 1.1 illustrates the roles of animal reservoirs and environments to human leptospirosis.

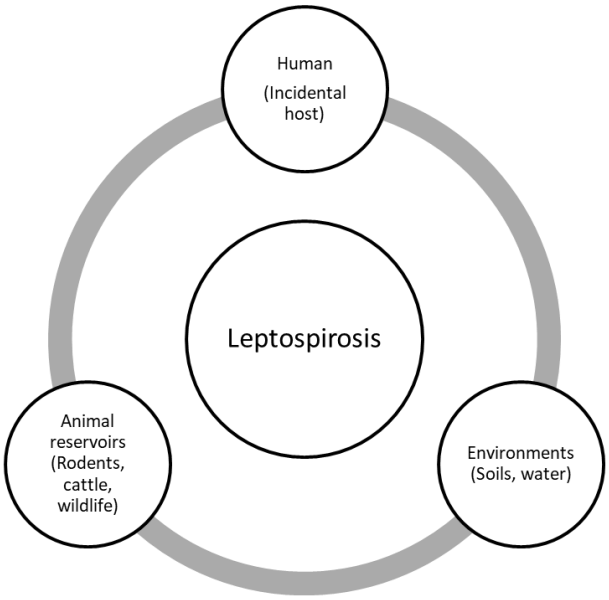


Figure 1.1 Factors that contribute to human leptospirosis and the relationships between the incidental host (humans), natural animal reservoirs and environments. Adapted from Haake and Levett (2015).

1.1.4 Pathogenesis

Currently, the overall pathogenesis mechanism of *Leptospira* remains poorly understood. However, the presence of several putative virulence factors, such as lipopolysaccharides (LPS), hemolysins and outer membrane proteins (OMPs) provide some possible causes of the leptospiral pathogenicity. Noticeably, many of these genes present only in the pathogenic *Leptospira* group (Adler, 2014). Table 1.3 summarises common putative *Leptospira* virulence proteins associated with human leptospirosis.

During entry, *Leptospira* bind to extracellular matrix (ECM) components of the hosts using several outer surface proteins such as *OmpL36*, *OmpL37*, *OmpL47* and *OmpL54*, also known as adhesins. These molecule complexes are capable of binding to the fibroblasts, renal epithelial cells and endothelial cells efficiently (Adler, 2014). Other accessory proteins that probably play roles in facilitating leptospiral adhesion include *LigA*, *LigB* and *LigC*. These *lig*-proteins resemble immunoglobulin-like domains and are partially exposed on the leptospiral surface (Mathieu Picardeau, 2015).

Hemolysin is another virulence factor that plays important roles in pathogenicity of leptospirosis. This protein is able to lyse the cellular membrane of erythrocytes. Among the described hemolysin proteins are Tly, sphingomyelinase C (encoded by *sphA*), SphH and Sph2 (Evangelista and Coburn, 2010). Upon invasion, the bacteria disseminate through the blood before settling in the interstitium and tubular lumen of liver, renal and lung. During leptospiremia, the burden of bacteria can be as high as 10^6 per ml of blood (S. B. Agampodi *et al.*, 2012). As such, a concentration of more than 10^4 per ml of blood (or $\geq 10^4$ per g lung, kidney and

muscle) was associated with a severe form of leptospirosis (Patrick Hochedez *et al.*, 2015; Segura *et al.*, 2005).

Table 1.3 Putative leptospiral proteins and its associated host components. Adopted from Adler (2014) and Evangelista and Coburn (2010).

Leptospiral component	Host component
LigA, LigB	ECM, fibronectin
LigB fragment	Elastin, tropoelastin
LigB fragment	Fibronectin, collagen III
LipL32	Laminin, collagen I, collagen V
LipL32	Fibronectin, collagen IV
Lipl32, LIC12730, LIC10494,	Plasminogen
LMB216 expressed in L. biflexa	Fibronectin
Lp29, Lp49, LipL40, MPL36, LIC12238	Plasminogen
Lp95	Laminin, fibronectin
Lsa20	Laminin, plasminogen
Lsa21	Laminin, collagen IV, fibronectin
Lsa24	Laminin
Lsa25, Lsa33	Laminin
Lsa27	Laminin
Lsa30	Plasminogen
Lsa44, Lsa45	Laminin
Lsa63	Laminin
Lsa66	Fibronectin, laminin, plasminogen
OmpL1	Fibronectin, laminin, plasminogen
OmpL37	Elastin, fibronectin, fibrinogen, laminin
Unidentified 36 kDa protein	Fibronectin
Viable leptospires	L929 fibroblasts
Viable leptospires	Primary renal epithelial cells
Viable leptospires	L929 fibroblast ECM
Viable leptospires	Vero fibroblasts
Viable leptospires	Chondroitin sulfate B
Viable leptospires	Cadherin

1.1.5 Leptospirosis in human

Exposure to contaminated environments and animal reservoirs are the two main sources of human leptospirosis. Usually, *Leptospira* gains entrance into the body via cut or abrasion or via penetration through the mucous membranes of the conjunctivae or respiratory tract or oral cavity during occupational or recreational activities (De Brito *et al.*, 2018). Following infection, the onset of disease appears on average between 7 and 12 days, although it can be as early as 1 day, and up to months (Evangelista and Coburn, 2010; David A Haake and Levett, 2015). Most leptospirosis cases are mild or subclinical, while some may develop into severe form and potentially fatal.

During the early phase of leptospirosis, infected patients typically present nonspecific symptoms, especially a sudden onset of febrile illness, chills and severe headache. The frontal, bitemporal throbbing headache is usually accompanied by light sensitivity and retro-orbital pain (Toyokawa *et al.*, 2011). These symptoms can be easily misidentified as influenza, melioidosis, malaria or dengue fever. Other common symptoms are myalgia (associated with calves and lower back) and conjunctival suffusion, occurred in 54.6% to 78.3% of confirmed cases (Vanasco *et al.*, 2008). Meanwhile, nausea, arthralgia, sore throat, vomiting, diarrhoea and abdominal pain could also be reported.

Meanwhile, severe leptospirosis involves multiple organs failures including liver, renal and brain. Classically, one of the most recognizable severe forms of leptospirosis is the Weil disease, characterised by jaundice and renal dysfunction. Jaundice is caused by acute hemolytic anaemia that elevates the amount of bilirubin in the blood and is more common in patients with glucose-6-phosphate dehydrogenase deficiency (Miyahara *et al.*, 2014). Moreover, infection with certain

Leptospira serogroup i.e. Icterohaemorrhagiae also was associated with the elevated bilirubin and jaundice (Katz *et al.*, 2001). Kidney is another important organ affected by leptospirosis, in which tubule-interstitial nephritis and proteinuria are commonly observed. In the tropical area, a severe renal complication of leptospirosis has become an emerging cause of chronic kidney disease among the survivors (CKD) (C.-W. Yang, 2018). Dry cough has been commonly reported in acute leptospirosis and may be confused with influenza. In 17% – 50% of leptospirosis cases, this non-specific dry cough precedes pathological changes in alveolar septa and intra-alveolar spaces and potentially fatal pulmonary haemorrhage (Gulati and Gulati, 2012). Furthermore, severe leptospirosis manifestation may also include aseptic meningitis, and myocarditis, albeit at less common circumstances but are associated with an increased risk of death (David A Haake and Levett, 2015).

1.1.6 Epidemiology

Early epidemiological studies explored the incidence of leptospirosis in certain occupational groups related to domestic animals especially cattle (Benacer *et al.*, 2016; Goris *et al.*, 2013). With better availability of diagnostic tests and awareness, increasing numbers of institutional-based epidemiology data were reported worldwide. In 1999, the first global effort to determine the leptospirosis incidence was initiated (WHO, 1999). However, the initiative was hampered by limited capacity of the vast majority of countries in the establishment of effective notification systems. As a result, Leptospirosis Burden Epidemiology Reference Group (LERG) was established to develop models for the estimation of human leptospirosis burden via existing data and to identify technical gaps related to the endeavour (WHO, 2010) A year later, the LERG successfully provided the earliest

data on global burden of leptospirosis; a median of 5 cases per 100 000 population (WHO, 2011). Notably, a more recent study estimated the leptospirosis burden was significantly higher in tropical areas, particularly the South East Asia (55.54 cases per 100 000) as compared to other temperate countries (0.65 cases per 100 000) (Costa *et al.*, 2015).

In Malaysia, leptospirosis classified as a mandatory notifiable disease. Following the regulatory enforcement, it was found that the annual leptospirosis incidence rates ranged between 7.94 and 30.2 cases per 100 000 population (Abdul Wahab, 2015; MOH, 2016). Figure 1.2 illustrates incidence and mortality due to leptospirosis in Malaysia, between the year 2001 and 2017. In general, there was a slight increase in leptospirosis incidence, peaked in 2015. Meanwhile, the mortality rate coincided with the incidence, except in the year of 2011 and 2012. Of the 12 states and 3 federal territories, Perak, Selangor, Kelantan and Pahang recorded the highest number of leptospirosis cases. Most of these cases were associated with the monsoon seasons (Benacer *et al.*, 2016).

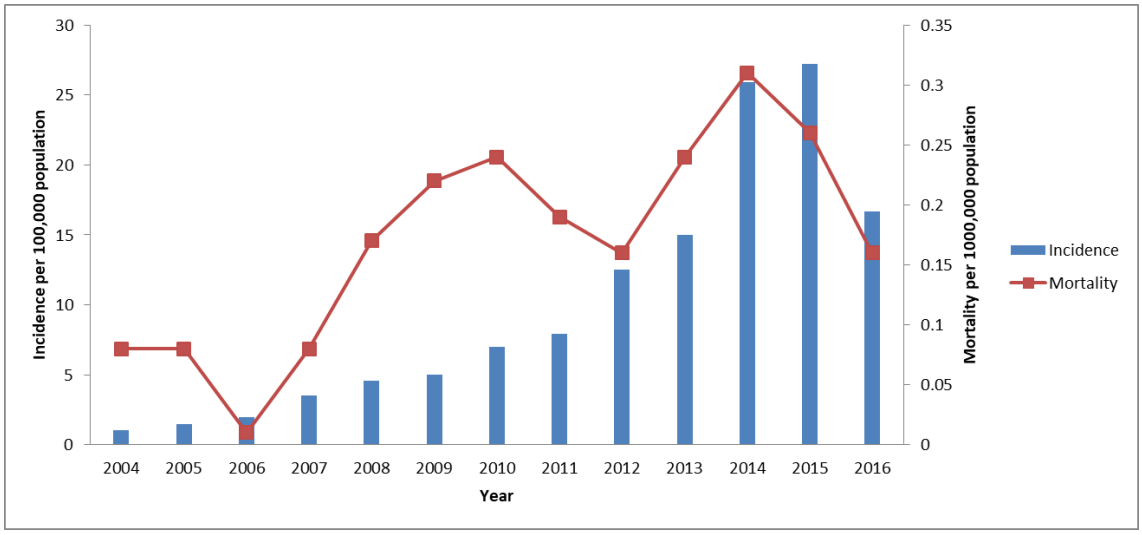


Figure 1.2 Leptospirosis incidence and mortality (per 100,0000 population) in Malaysia between 2004 and 2016 based on MOH annual reports.

1.1.7 Risk factors

Several factors are associated with increased risk of leptospirosis in both tropical and temperate countries. In general, it can be divided into (i) socioeconomic and (ii) environmental factors. Socioeconomic factors include rapid urbanisation, rural area and type of occupations. During urbanisation, the size and density of population increased rapidly within a limited area of living. Consequently, an increased number of people live in poor housing or slum dwellings with poor sanitation and are in close proximity to open sewage (Mgode *et al.*, 2015). Moreover, overcrowded areas usually have garbage management problem that attracts presences of stray animals, especially rodents and stray dogs. In several prevalence studies, between 16% and 82% of captured rodents and stray dogs were positive for *Leptospira* (Agudelo-Flórez *et al.*, 2009; Kimari, 2016; Zaidi *et al.*, 2018).

In addition, certain occupational group also associated with higher risk of leptospirosis. Of all, mining is the earliest sectors that were identified as leptospirosis occupational risk group (Inada *et al.*, 1916). The prevalence of leptospiral antibodies among miners was as high as 65.3%. Those mine workers who exposed to waters, cattle rearing and rat infestation had augmented risk, with odds ratio (OR) of 10.6, 10.4 and 4.6, respectively (Natarajaseenivasan *et al.*, 2016). Farmers also have higher risk of leptospiral infection. Due to this occupational association, leptospirosis was also called as rice-field worker's disease, cane cutter's fever, harvest fever and Japanese (akiyami) autumnal fever (Rovid Spickler and Leedom Larson, 2013). Other occupations include animal farmers, abattoir workers, military, veterinarians and others who have direct contact with animals (Guerra, 2013; A. A. N. Rafizah *et al.*, 2013). Importantly, the magnitude of the risk depends on the degree of exposure,

frequency, prevalence of leptospiral carrier, personal protective equipment availability, knowledge and awareness (David A Haake and Levett, 2015).

Environmental factors also influence the risk of leptospirosis. Usually, a higher number of leptospirosis cases and outbreaks were reported following climatic events such as floods and typhoon. For example, after the 2009 typhoon in Metro Manila, more than 2000 patients were hospitalised due to leptospirosis, of which, 178 died (Amilasan *et al.*, 2012). Apart from occasional typhoons, an upsurge in regular rainfall, as well as increase in humidity were associated with 2% to 4% escalation of leptospirosis cases (Joshi *et al.*, 2017). Hospitalisation usually occurred after 2 weeks of the heavy rainfalls (Matsushita *et al.*, 2018).

Recreational activities such as freshwater swimming, caving, rafting and triathlons have emerged as another important risk factor of leptospirosis. The importance of recreational exposures become more significant in recent years due to the increased popularity of adventurous sports and cheaper cost of travelling to exotic areas. Among the recreational related outbreaks that occurred are; Springfield triathlon (n = 98) (Morgan *et al.*, 2002), Malaysia Borneo Eco-Challenge (n = 80) (Sejvar *et al.*, 2003), Guam triathlon (n = 21) (Haddock *et al.*, 2002), Florida Adventure Race (n = 21) (Stern *et al.*, 2010), Réunion Island triathlon (n = 9) (Pagés *et al.*, 2016), and Martinique canyoning (n = 8) (P Hochedez *et al.*, 2013).

1.1.8 Diagnosis

Clinical diagnosis of leptospirosis is challenging and requires a high index of suspicion. It is because; the clinical presentations are non-specific, often resembling other tropical infections (e.g. melioidosis). Therefore, a complementary laboratory test is always necessary for a definite result. Laboratory diagnosis of leptospirosis

can be either direct demonstration or indirect demonstration of *Leptospira* from clinical specimens.

1.1.8 (a) Microscopic observation

Direct observation of *Leptospira* from body fluid or artificial EMJH culture can be carried out using a dark field microscope. Under the microscope, the leptospires appear as thin, bright and motile spirochetes. In order to be detectable in the microscopic field, the initial *Leptospira* concentration in clinical specimens should be 10^4 cells per mL (Toyokawa *et al.*, 2011). A false negativity may happen when a low number of *Leptospira* present in the sample. In addition, this method is prone to false positivity when artefacts such as fibrin or protein threads and cellular debris are mistakenly identified as leptospires. Differential centrifugation can be performed to concentrate the organism, the technique is only useful for a large volume of specimens (WHO, 2013). Alternatively, some staining procedures, such as Warthin-Starry silver nitrate staining, immunohistochemistry and in situ hybridization to increase sensitivity and specificity. These methods, however, requires highly experienced technicians and laborious, hence are less useful as routine diagnostic tests (Ahmad *et al.*, 2005).

1.1.8 (b) Isolation of *Leptospira*

Demonstration of leptospiral growth in artificial media is a definitive indicator of *Leptospira* presence. Body fluids such as blood, plasma, urine and CSF can be inoculated into a primary culture. To date, several culture media has been described, such as EMJH media, Fletcher's media and *Leptospira* Vanaporn Wuthiekanun (LVW) agar (Philip *et al.*, 2018). Additional additives, including

rabbit's serum (8-15% v/v), fetal bovine serum, sodium pyruvate, Tween-80, superoxide dismutase enzyme and magnesium salts can be included to improve recovery of *Leptospira* (Chideroli *et al.*, 2017). Moreover, 5-fluorouracil, nalidixic acid, rifampicin, polymyxin B, vancomycin can be added to inhibit the growth of contaminating organisms. Yet, the addition of selective antibiotics may compromise the growth of certain leptospiral strains or when low numbers of viable leptospires are available. Culture media for *Leptospira* are incubated at 30 °C, for up to 6 months to accommodate the slow growth of certain serovars, such as Hardjo and Bratislava (Khaki, 2016). Due to this slow growth rate and long incubation time, culture is less useful for routine diagnostic testing (Chideroli *et al.*, 2017; Wagenaar *et al.*, 2006).

1.1.8 (c) Molecular diagnosis

PCR has been used for direct detection of *Leptospira* DNA for more than 20 years (Mérien *et al.*, 1992). Since then, many molecular assays have been developed, utilising conventional PCR, real-time PCR (qPCR), reverse-transcription PCR (rtPCR) and isothermal amplification platforms. These assays are useful for early diagnosis of leptospirosis (first week of infection), in which *Leptospira* usually present in the bloodstream at low concentration. PCR amplify unique sequences of housekeeping genes such as *rrs*, *fliC*, *gyrB* and *secY* or virulence genes associated with pathogenic species of *Leptospira*, including *lipL32*, *lipL21* and *lfb1* (Thaipadungpanit *et al.*, 2011).

Historically, conventional PCR was widely used to detect *Leptospira* DNA from various clinical samples. Among the commonly used primers were targeting 331 bp sequence of *Leptospira* rrs gene and the G1/G2 primers targeting 285 bp of

secY (Gravekamp *et al.,* 1993). Conventional PCR had sensitivity approximately 10 pg *Leptospira* DNA (equivalent to 2×10^3 DNA copies) per reaction (Fonseca *et al.*, 2006; Mérien *et al.,* 1992). Amplicon of conventional PCR subsequently can be used for sequencing and identification of infecting *Leptospira* species (Andrew T Slack *et al.*, 2006).

SYBR and TaqMan hydrolysis qPCR are better alternatives to conventional PCR. qPCR is more sensitive and independent of gel electrophoresis step. To date, several SYBR and TaqMan hydrolysis qPCR have been described. Those assays had limits of detection between 10 to 50 DNA copies per reaction, hence are more sensitive than the conventional PCR (Ahmed *et al*., 2009). Moreover, TaqMan hydrolysis qPCR enable broader application of assay in which multiple targets are possible. Such assays allow detection and differentiation between pathogenic and non-pathogenic groups of *Leptospira* (Bedir *et al.*, 2010). Due to high sensitivity and specificity, qPCR is useful for routine testing but provides limited data on the infecting serovars.

1.1.8 (d) Serological diagnosis

Serological assays are commonly used to screen and to diagnose leptospirosis. The assays are also widely utilised in surveillance and prevalence studies. In these assays, antibodies (IgM or IgG or both) against *Leptospira* are detected to demonstrate the indirect presence of the organism. Antibodies are produced after 5 – 7 days of infection and may persist for months or years (Budihal and Perwez, 2014). Serology-based assays include microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA).

MAT or microscopic agglutination test was first described by Pettit and Martin in 1918 and remains as a reference test for diagnosis of leptospirosis (Martin and Pettit, 1918). In this test, agglutination between patient's sera and viable *Leptospira* cultures is observed under darkfield microscopy. Maintenance of at least 19 *Leptospira* strains of certain serogroups is necessary, yet laborious. Positivity is confirmed when more than 50% of leptospiral cells agglutinate with patient's sera relative to the control well. At this stage, the MAT requires experienced technicians to perform and to analyse the agglutinations (WHO, 2003).

Alternatively, ELISA is less laborious and does not require viable *Leptospira* cells. This method allows detection of IgM against *Leptospira,* using recombinant proteins or cellular extract of circulating strains. Despite its commercial availability and widely used, performances of leptospiral ELISA are inconsistent. For instance, the sensitivity & specificity of *Leptospira* IgM ELISA varies depending on location, i.e. 96.6 % and 93.3% in Brazil (Ooteman *et al.*, 2006), 76.1% and 82.6% in Thailand (Desakorn *et al.*, 2012) and 70% and 78% in Laos (Tanganuchitcharnchai *et al.*, 2012). In other studies, the sensitivity and specificity highly ranged, 32% – 99% and 52% – 100%, respectively (Rosa *et al.*, 2017). As a result, complementary tests, such as MAT and PCR are highly recommended to confirm the diagnosis of leptospirosis.

Other more rapid tests that screen the presence of anti-*Leptospira* antibodies are also available. These tests are aimed for application at resource-limited settings, in which results are ready in as early as 10 minutes (J.-W. Lee *et al.*, 2016). Those tests include latex bead agglutination, indirect immunofluorescence, indirect hemagglutination and immunochromatography tests (Mathieu Picardeau *et al.*, 2014).

1.1.9 Management and treatment

Majority of leptospirosis cases are mild and self-limiting, hence do not require antimicrobial treatment. However, in some circumstances, early initiation of antibiotic prevents a severe form of leptospirosis. According to the WHO, less severe leptospirosis can be treated with oral antibiotics, such as amoxicillin and ampicillin. Other options include doxycycline, erythromycin, ceftriaxone, cefotaxime, and quinolones. Meanwhile, in severe leptospirosis, the WHO recommends high doses of intravenous (IV) penicillin, with some supportive care such as dialysis and mechanical ventilation, when necessary (WHO, 2003). In very rare cases, high dose penicillin cause Jarisch-Herxheimer reaction (JHR), characterised by acute inflammatory response due to the production of large amounts of cytokines (also known hypercytokinemia) and fever (Guerrier and D'Ortenzio, 2013).

In Malaysia, recommended treatment for severe leptospirosis in adults is 2 M unit IV C-penicillin, every 6 hours, for 5 to 7 days. For less severe cases, 2 mg to 100 mg per kg doxycycline (or tetracycline, ampicillin, amoxicillin) can be prescribed orally every 12 hours for 5 – 7 days. Meanwhile, in paediatric patients, 100000 U IV C-penicillin per kg, every 6 hours, for 7 days is preferred. Alternatively, patients of below 8 years old may be prescribed with oral doxycycline 4mg per kg for 12 hours, for 1 week (MOH, 2011).

1.2 *Burkholderia pseudomallei* and melioidosis

B. pseudomallei, a causative organism of melioidosis was first observed in 1911 by Alfred Whitmore and C. S. Krishnaswami from postmortem tissues of morphine addicts in Rangoon, Burma (Whitmore, 1913). Upon inoculation into guinea pigs, the isolated bacilli were similar to *Burkholderia mallei*, a causative agent for glanders, but the organism was motile. Eventually, the organism was called *Bacillus pseudomallei* or *Bacille de Whitmore (or Bacillus whitmorii).* In other occasions of the last decade, the *B. pseudomallei* was also referred to as *Actinobacillus pseudomallei, Lofflerella whitmori, Malleomyces pseudomallei, Pfeifferella pseudomallei, Pfeifferella whitmori, Pseudomonas pseudomallei* (Bart J Currie, 2015). Until 1992, the organism is officially recognised as *Burkholderia pseudomallei*, based on the DNA-DNA homology and 16S rRNA relatedness (Yabuuchi *et al.*, 1992).

During the early days, Whitmore's reported that first 38 fatal cases of this disease in morphine injectors who had abscesses in multiple organs and bacteraemia. Consequently, the disease was called the 'Whitmore's disease' or the 'morphine injector's septicaemia'. The term, melioidosis was given by Stanton and Fletcher in 1932, which detailed similar diseases in patients from Malaya, Ceylon and Indochina. The name was taken from the Greek 'melis' (distemper of asses), 'oid' (like) and 'osis' (condition) which refer to glanders-like infection (Stanton and Fletcher, 1921). Figure 1.3 summarises the important events in regards to melioidosis.

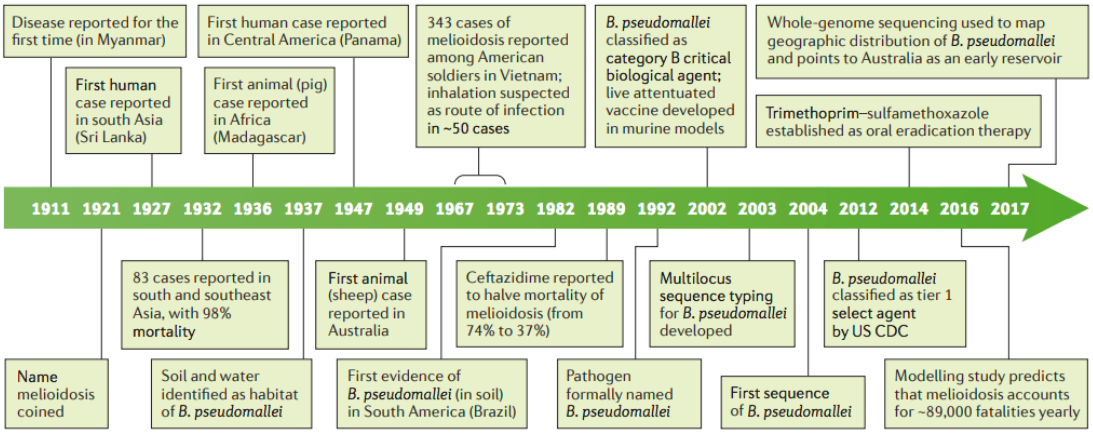


Figure 1.3 Important milestones in the history of melioidosis. Adopted from Wiersinga *et al*. (2018)

1.2.1 Biology of *B. pseudomallei* and cultivation

B. pseudomallei are motile Gram-negative bacilli, with a diameter of 0.4 to 0.8 µm and a length of approximately 1.5 µm. The motility is due to the presence of a cluster of two to four flagella, located at one end of the organism. Microscopically, *B. pseudomallei* exhibit bipolar staining resembles 'safety-pin' appearance (Liu, 2014).

This organism is non-fastidious and metabolically versatile. It can utilise many carbon sources, both aerobically and anaerobically. In anaerobic condition, *B. pseudomallei* require the presence of arginine or nitrate (Vietri and DeShazer, 2007). Despite its ability to grow in various routine media, selective media such as the Ashdown medium and Mac Conkey agar are favourable for the non-sterile specimen in order to prevent potential misdetection due to overgrowth of other organisms. The culture media can be incubated at 24 °C to 32 °C, and up to 42 °C (Hemarajata *et al.*, 2016). On the Ashdown media, overnight *B. pseudomallei* colonies are typically pinpoint in size and will develop into flat, purplish, wrinkled colonies post 48 hours incubation. Meanwhile, on MacConkey agar, overnight colonies are typically colourless, non-lactose-fermenting and will develop into a pinkish rigours colony with metallic sheen after 48 hours. Additional biochemical tests, i.e. oxidase test, L-arabinose and other monosaccharide fermentations and indole test, as well as susceptibility to gentamicin, polymyxin B and amoxicillin/clavulanic acid are necessary to identify *B. pseudomallei* (Wiersinga *et al.*, 2018).

1.2.2 Classification and taxonomy

Historically, *Burkholderia* was placed under the rRNA group II of the *Pseudomonas* genus. Following taxonomic advancement through rRNA–DNA hybridization technology, *Burkholderia* spp. were transferred into its own genus, which was first described by Walter H. Burkholder in 1942 (Hemarajata *et al.*, 2016). The genus belongs to class Betaproteobacteria, order Burkholderiales and family Burkholderiaceae and consist of more than 50 species (listed at http://www.bacterio.net/burkholderia.html) (Wisplinghoff, 2017).

Genetically, *Burkholderia* species are divided into three main clusters; (i) *B. pseudomallei*-complex (Bpc) group, (ii) *B. cepacia*-complex (Bcc) group and (iii) *Paraburkholderia* group, as illustrated in Figure 1.4. In the last two years, the latter group eventually was proposed to be placed into a new genus due to its unique genetic composition, phenotypic similarities and non-pathogenicity on humans, animals and plants (Anatoly and Mansour, 2016). The *B. pseudomallei*-complex contains *B. pseudomallei* (melioidosis), *B. mallei* (glanders) and *B. thailandensis* (avirulent). Meanwhile, remaining species are clustered in the Bcc group, that may become opportunistic pathogens in cystic fibrosis (CF) and immunocompromised patients (Lowe *et al.*, 2016).

The whole genome of *B. pseudomallei* was completely sequenced in 2004. The chromosome of this organism present in two copies, Chromosome 1 (~4.07 Mb) that encodes genes related to metabolisms, biosynthesis of cellular components, DNA replication and transcription and the smaller Chromosome 2 (~3.17 Mb) that contains genes involving host-environmental adaptation functions (Holden *et al.*, 2004). There are 16 ‘genomic islands’ (GI) within the *B. pseudomallei* genome, potentially acquired through horizontal gene transfer and are responsible for

various metabolic and virulence advantage. Among the encoded virulence genes are adhesins, fimbriae, capsular exopolysaccharides, LPS and Type III secretion systems (TTSS), responsible for the invasion and pathogenesis of *B. pseudomallei* (White, 2013).

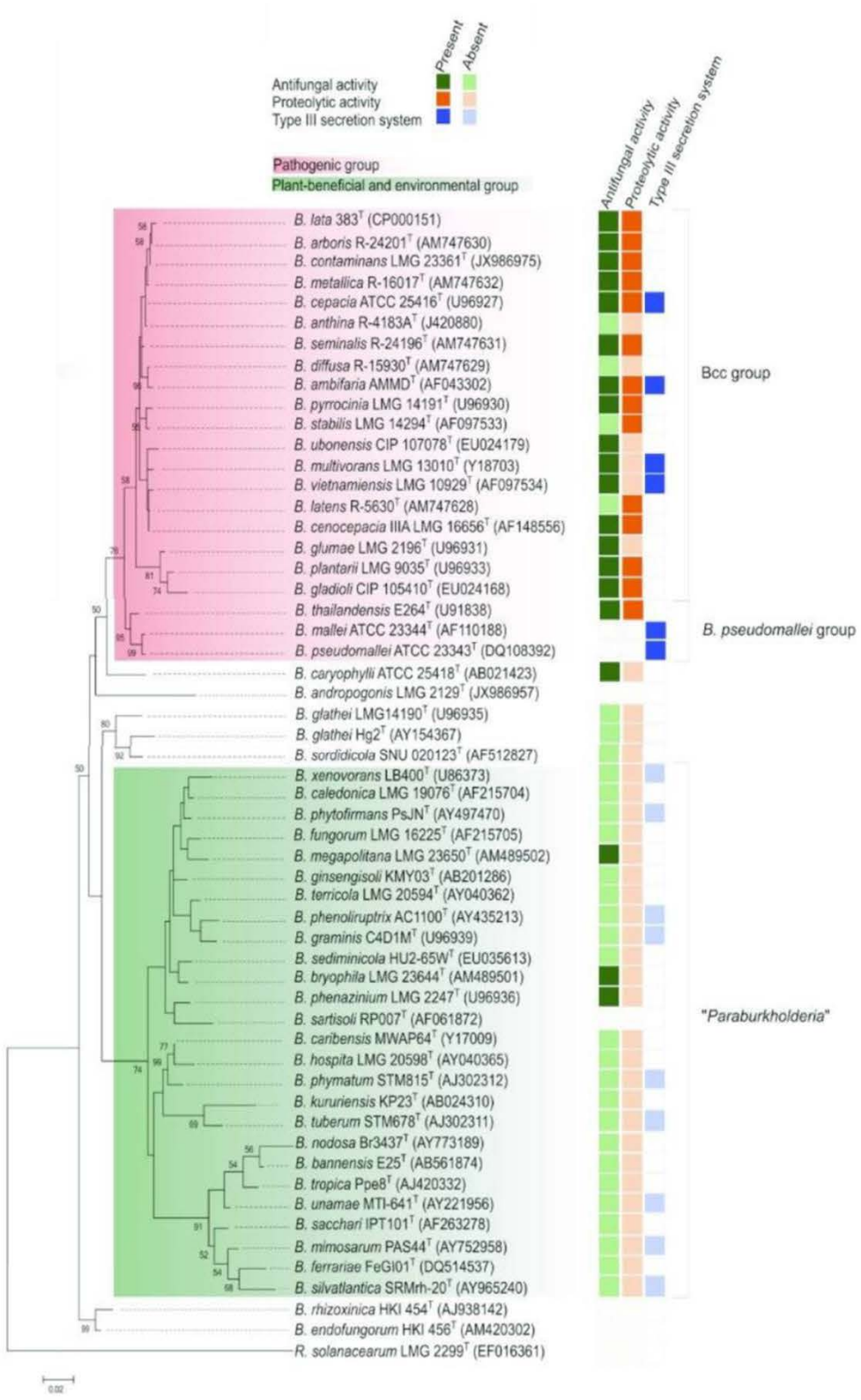


Figure 1.4 Phylogenetic tree of *Burkholderia* species based on 16S rRNA gene sequences. Adopted from Eberl and Vandamme (2016).

1.2.3 Pathogenesis

B. pseudomallei usually infect humans who are regularly in contact with water and soils. The common routes of infection are percutaneous inoculation, inhalation, and ingestion of contaminated soil or water or aerosolised organisms (Nathan *et al.*, 2018). This initial route of infection was found to be associated with the clinical manifestation of melioidosis. For instance, infections through inhalation were associated with pneumonia, while infections through ingestion were associated with septicaemia (C. Lim *et al.*, 2016). In terms of the animal host, melioidosis has affected various animals including cattle, camels and kangaroos. However, there is very limited data on zoonotic melioidosis and its associated animal reservoir (Choy *et al.*, 2000).

During entry into the human body, *B. pseudomallei* attaches to epithelial cells of broken skin or the mucosal surface, mediated by the type IV pili (such as PilA protein), polysaccharides capsule and BoaA and BoaB adhesin proteins. Subsequently, the bacteria enter the cell through endocytosis and can remain in the endocytic vesicles. Prior to lysozyme activation, type III secretion system (TTSS) transports certain effector proteins, such as ecotin (a periplasmic serine protease inhibitor), BopA (BPSS1524), BopB (BPSS1514), BopC (BPSS1516), BopE (BPSS1525) and CHBP (BPSS1385). These proteins are responsible for bacterial endocytic escape (J. K. Stone *et al.*, 2014). *B. pseudomallei* can replicate in various cells including neutrophils, monocytes and macrophages and some epithelial cell lines (J. K. Stone *et al.*, 2014).

Apart of important virulence proteins that are responsible for invasion, endocytic escape and intracellular survival, pathogenicity of *B. pseudomallei* is also caused by the production of BLF1 (*Burkholderia* lethal factor 1) toxin that

inactivates host protein synthesis (Hautbergue and Wilson, 2012). Other virulence proteins and their associated function are listed in Table 1.4. Due to the high pathogenicity, *B. pseudomallei* are regarded as CDC Tier 1 select agent and a Category B Priority Pathogen by the National Institute of Allergy and Infectious Diseases (NIAID).

Table 1.4 Selected *B. pseudomallei* virulent proteins. Adapted from Wiersinga *et al*. (2018)

Gene	Antigen	Function
ahpC	Alkyl hydroperoxide reductase	Resistance to oxidative stress
bimA	T5SS autotransporter	Escape from phagosome and actin tail formation
bipB	Translocator protein	TTSS components
bipC	Effector protein	Involved in endocytic vesicle survival, escape and cell invasion
bipD	Translocator protein	Involved in endocytic vesicle survival, escape and cell invasion
boaA	Adhesins	T5SS autotransporters
boaB	Adhesins	Role in cell attachment and possibly intracellular replication
bopA	Effector protein	• TTSS component • Involved in endocytic vesicle membrane disruption and avoidance of autophagy
bopE	Guanine nucleotide exchange	TTSS effector
bpaC	Adhesin	• T5SS trimeric autotransporter adhesin • Protects from complement killing • Involved in cell attachment
bsaQ	TTSS structural component	Involved in endocytic vesicle escape, cell invasion and plaque formation
bsaU	TTSS structural component	Involved in endocytic vesicle escape and early onset activation of the caspase 1
bsaZ	TTSS structural component	Implicated in endocytic vesicle escape and intracellular replication
CHBP	ATP and GTP binding protein	TTSS structural component
dpsA	DNA starvation and stationary	Resistance to oxidative stress
fliC	Flagellin structural component	• Required for flagellar assembly • Involved in cell adherence
hcp1	Hcp1 family T6SS effector	Role in cell fusion and macrophage cytotoxicity
irlR	Transcriptional activator protein IrlR	Mutants displayed reduced invasion
katG	Catalase-peroxidase	Resistance to oxidative stress
pilA	Type IV pilin subunit protein PilA	• Temperature-dependent adherence and formation of microcolonies • Intracellular motility
purM	Phosphoribosylformylglycinamidine	Purine biosynthetic pathway
rpoE	RNA polymerase σ-factor RpoE	Biofilm formation, heat stress response via RNA polymerase σ-factor
rpoS	RNA polymerase σ-factor RpoS	Suppresses iNOS activity by upregulating SOCS3 and CIS cytokines
sodC	Superoxide dismutase	Resistance to oxidative stress
virAG	Two-component regulatory system	Regulates T6SS transcription

1.2.4 Melioidosis in human

Exposure to contaminated environments is an exclusive source of human melioidosis. *B. pseudomallei* may enter the body via percutaneous cut or abrasion, inhalation or ingestion during occupational or recreational activities. Upon entry, the disease progression is influenced by many aspects such as mode of infection, host risk factors, pathogenicity of the infecting strains and its inoculum (C. Lim *et al.*, 2016).

On average, the onset of melioidosis appears after 9 days (range: 1 – 21 days) (Bart J Currie, 2015). In other instances, the symptoms appeared within 24 hours of presumed infection, i.e. after severe climatic events or near-drowning incidence (Chierakul *et al.*, 2005; Bart J. Currie and Jacups, 2003; Hinjoy *et al.*, 2018). Meanwhile, the longest reported incubation period was 62 years, in an American veteran who was previously imprisoned during World War II (Ngauy *et al.*, 2005).

The most common presenting symptom of melioidosis is fever and cough. During hospital admission, between 78.5% – 100% melioidosis patients had reported fever (Afroze *et al.*, 2017; Churuangsuk *et al.*, 2016; Deris *et al.*, 2010). Other common clinical presentation includes pneumonia (36% – 58%) and soft tissue or skin abscess (17% – 36%) (Kingsley *et al.*, 2016; Nathan *et al.*, 2018; Zueter *et al.*, 2016). Meanwhile, less common clinical presentations are liver abscess, splenic abscess, prostate abscess, parotid abscess, genitourinary, neurologic abscess and osteomyelitis/septic arthritis (Tipre *et al.*, 2018). In an unusual case, a 56-year-old melioidosis patient presented with jaundice (Tyagi *et al.*, 2014). On the other hand, majority of exposure to *B. pseudomallei* are subclinical and do not cause active infection (Limmathurotsakul and Peacock, 2011). Figure 1.5 summarises association between mode of *B. pseudomallei* infection and disease progression of melioidosis.

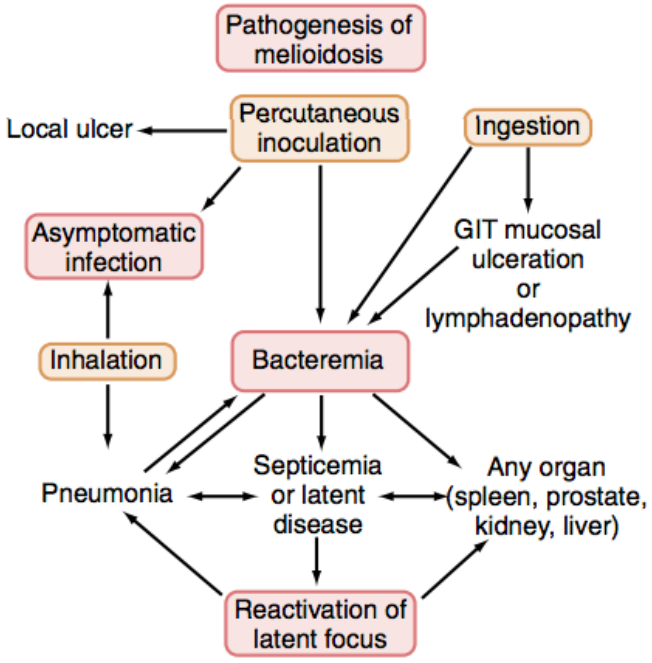


Figure 1.5 Association between mode of *B. pseudomallei* entry and progression of melioidosis. Adopted from Currie (2015).

1.2.5 Epidemiology

Melioidosis is endemic to the tropic regions, located between 20 °N and 20 °S of the equator. In the early 1900s, limited data were available on melioidosis cases, in which, most of the reported cases were in travellers. In more recent years, emerging data suggested that the wider geographic areas are affected by melioidosis, such as the Hong Kong, Taiwan and the Indian subcontinent (Bart J. Currie *et al.*, 2008). Moreover, sporadic cases have been reported in the Middle East, Africa and the Americas, especially after heavy rainfalls. Overall, it is estimated that more than 3 billion people are at risk of melioidosis, with 5.0 cases per 100,000 people. Of these, South Asia and East Asia & Pacific are the most affected regions with 73,000 and 65,000 cases, annually (Limmathurotsakul *et al.*, 2016).

Thailand and Northern Territory of Australia are two classical melioidosis endemic countries. In both regions, as high as 24.63% of community-acquired septicemic cases are caused by *B. pseudomallei* (Churuangsuk *et al.*, 2016). In Malaysia, melioidosis was first described in 1913 by Stanton and Fletcher, from the Institute of Medical Research (Jayaram, 2005). To date, this disease is not compulsorily notifiable in Malaysia. Noticeably, melioidosis incidences vary between states. For example, in Pahang and Kedah, the 4.3 cases per 100,000 population and 16.35 cases per 100,000 population were reported, respectively (How *et al.*, 2009; Nathan *et al.*, 2018). Few melioidosis outbreaks have been reported, associated with environmental exposure during public evacuation and among military personnel (Sapian *et al.*, 2012; Thin *et al.*, 1970). Figure 1.6 illustrates melioidosis cases and death in Malaysia, between 1975 and 2015.

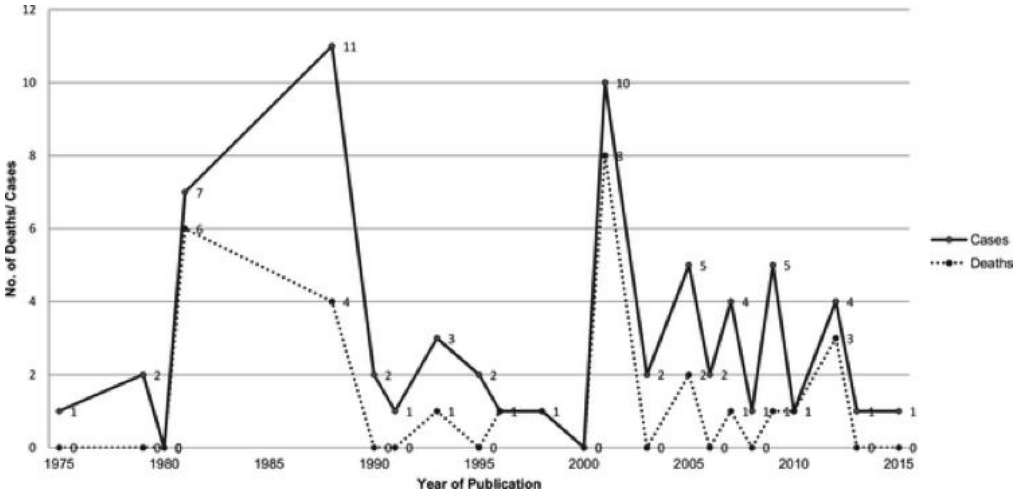


Figure 1.6 Reported melioidosis cases and deaths in Malaysia, between 1975 and 2015. Adopted from Kingsley *et al*. (2016).

1.2.6 Risk factors

Diabetes mellitus is the most common risk factor associated with melioidosis. This predisposing condition present in more than 50% melioidosis patients worldwide (Wiersinga *et al.*, 2018). In a certain region, as high as 60.9% of affected patients also had this diabetes mellitus (Suputtamongkol *et al.*, 1999). It is shown that patients with diabetes mellitus have impaired immune response to *B. pseudomallei*, in particular, reduced capacity of macrophages phagocytic and bacteriolysis activity, that eventually lead to increased susceptibility to this infection (Dunachie *et al.*, 2017). In addition to diabetes mellitus, other comorbidities associated with increased risk of melioidosis are; renal impairment (10% – 27%), pulmonary disease (27%), thalassaemia (7% – 8%, due to excessive iron and subsequent neutrophil dysfunction), congestive heart failure, corticosteroid therapy, malignancy and immunosuppression (Wiersinga *et al.*, 2018).

Certain occupational classes are also known risk factors to melioidosis. For instance, paddy rice worker or farmer accounted for 78% – 84.9% of melioidosis patients in Thailand and Sri Lanka (Corea *et al.*, 2016; Suputtamongkol *et al.*, 1999). Other high-risk occupation groups include military, fishing and veterinary. Interestingly, recreational/non-occupational activities have become an emerging source of infection. For instance, a retrospective study in Darwin, Australia revealed that 75% (n = 407) patients were infected during recreational activities, in contrast to 18% (n = 96) who had direct exposure during occupational activities (Currie *et al.*, 2010). Similarly, only 18.6% of melioidosis patients in Kedah, Malaysia worked in the farming, forestry, fishing (Hassan *et al.*, 2010).

Occupational status is also related to gender and age group. Overall, male were frequently associated with melioidosis, with the percentage of between 73.4% and 84%. Similarly, those who were of mean age of between 44 – 50 were also associated with increased risk of melioidosis (L. Pang *et al.*, 2017; Zueter *et al.*, 2016). In a certain region, specific race, ie. people of Malay ethnicity in Malaysia were predominate melioidosis patients (71% – 89%) (Nathan *et al.*, 2018). A similar incidence was observed in Singapore, in which on average, 2.4 Malays per 100,000 population were reported to have melioidosis, as compared to the Chinese and Indian (L. Pang *et al.*, 2017). In another study, the aboriginality and alcohol consumption were shown to have relative risk of 2.1 and 8.1, respectively, in contrast to the other counterparts (Cheng and Currie, 2005).

1.2.7 Diagnosis

Early diagnosis enables the initiation of appropriate treatment and improves prognosis. Clinically, acute melioidosis features are non-specific and mimicking other diseases, as described previously (Meumann *et al.*, 2012). Therefore, complementary laboratory tests are important for confirmation of melioidosis. To date, several laboratory tests have been described to assert the presence of *B. pseudomallei* in clinical specimens. These laboratory tests are categorised into; (i) culture methods, (ii) serology method and (iii) molecular assays.

1.2.7 (a) Culture method

Culture method, paired with additional biochemical tests (for identification) is considered the gold standard for direct demonstration of viable *B. pseudomallei*. This ubiquitous non-fastidious organism may grow on various culture media, such as

blood agar, Mac Conkey agar and Ashdown media. On non-selective agar, *B. pseudomallei* growth may be mistakenly identified as contaminating microorganism. It is because, following 18 – 24 hours incubation, the colonies are colourless and non-lactose fermenting resemble oxidase-positive *Pseudomonas* (on Mac Conkey) or are small, smooth, cream-coloured on blood agar. The wrinkled metallic sheen or pinkish, rugose appearance only manifest after prolonged incubation (usually >48 hours or up to 5 days) (Hemarajata *et al.*, 2016). The slow growth of *B. pseudomallei* on the suboptimal media may be out-competed by other normal commensals. As a result, when melioidosis is highly suspected, more selective agar, such as Ashdown agar should be considered. On this agar, colonies are typically pinpoint in size after 18 – 24 hours incubation and will become flat, purple in colour and wrinkled at 48 hours. Other clues for suspicion of *B. pseudomallei* is the bipolar staining of Gram-negative bacilli, earthy or musty smell and intrinsic resistant to polymyxins and gentamicin (albeit not all strains) (Podin *et al.*, 2014).

In addition, routine biochemical test such as oxidase, indole, L-arabinose test or commercial tests i.e. API 20NE, Remel RapID NF, Phoenix, Vitek 2 and matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry can be used for identification of *B. pseudomallei*. However, the performance of the assays varied among laboratory hence validation on local strains may be warranted. For instance, the commercial API 20NE identified 99.0% of 800 isolates and 37% of 71 isolates respectively in two independent evaluation studies, from Thailand and Australia (Amornchai *et al.*, 2007; Inglis *et al.*, 2005).

1.2.7 (b) Serological diagnosis

Different serological assays have been developed for detecting anti-*B. pseudomallei* antibodies using whole cell lysates or poorly characterised antigens. Most of these tests are 'in-house' and not validated extensively. Among others, the indirect hemagglutination assay (IHA) is commonly used to detect antibodies produced against *B. pseudomallei.* In this assay, sheep red blood cells (RBCs) are sensitised with crude antigen, prepared from local clinical strains of *B. pseudomallei*. The sensitized red blood cells are then mixed with serially diluted inactivated patient serum. Titre is denoted by the highest dilution of patient serum that distinctively agglutinated with the sensitised RBCs (Alexander *et al.,* 1970). Although IHA is relatively simple, the reported sensitivity of this test was between 56% and 73% (Wiersinga *et al.*, 2018). Moreover, in another study, 68% of patients who were initially negative on admission subsequently exhibited seroconversion (Cheng *et al.*, 2006). Due to these inconsistent sensitivities and delayed seroconversion, serology-based assays are not routinely used as a sole method for diagnosis of melioidosis.

In other laboratories, ELISA is available for detection of melioidosis specific IgM and/or IgG. These tests are rapid and omit the bias of IHA observer. Whole bacterial cells, exopolysaccharide (EPS) and lipopolysaccharide (LPS) are commonly used antigens. However, utilisation of unpurified antigens leads to lack of standardization and reproducibility. For instance, dual evaluations of an *in-house* ELISA within the same institute revealed that the sensitivity of the developed IgM ELISA reduced from 82.2% to 79.8%. Similarly, the specificity reduced from 94.9% to 89.7% (Noor *et al.*, 2015; Yi *et al.*, 2017).

1.2.7 (c) Molecular diagnosis

A number of molecular assays for the detection of *B. pseudomallei* nuclei acid have been described in the literature including conventional and real-time PCR. Some of the described PCR assays exclusively detect *B. pseudomallei*, while the remaining were designed to identify and differentiate *B. pseudomallei* from its close genetic relatives, such as *B. thailandensis* or *B. mallei* (Lowe *et al.*, 2016).

In general, real-time PCR is more sensitive and more rapid than the conventional PCR. For instance, the PPM2 conventional PCR could detect 1 ng DNA per reaction, while the TaqMan qPCR may detect 0.000076 ng DNA (Lew and Desmarchelier, 1994; Novak *et al.*, 2006). Even though qPCR is more sensitive, only a few of the reported assays were validated clinically. Evaluation of qPCR on clinical specimens is important to confirm its usability and practicality. In an instance, two developed TaqMan qPCR (designated 8653 and 9438) had analytical specificity of 100%, both. However, upon validation on confirmed specimens, the actual clinical specificity were 82% and 88 %, and clinical sensitivity of 71% and 54%, respectively (Supaprom *et al.*, 2007). Relative to culture and serology method, direct detection of *B. pseudomallei* DNA is more sensitive and specific, hence potentially enable early diagnosis of melioidosis.

1.2.8 Management and treatment

Timely initiation of appropriate antibiotic treatment reduces the mortality rate by approximately 50% (Dance, 2014). Usually, melioidosis treatment is divided into two phases: (i) induction phase, in which intravenous antibiotics are prescribed for at least 10 days, to prevent overwhelming sepsis and potential death, and (ii) maintenance phase, in which oral antibiotics are prescribed, usually for 20 weeks,

with the aim to prevent relapse episode of melioidosis. Table 1.5 summarises recommended antibiotic treatment for melioidosis.

Apart of antibiotic treatment, management of melioidosis includes, correction of electrolytes, fluids and acid-base imbalances, initiation of insulin therapy (for diabetic patients), respiratory support (when necessary) and drainage of abscess (if present).

Table 1.5 Recommended antibiotic regimes and melioidosis phase. Adapted from Dance (2014) and MOH (2011).

Phase	**Antibiotics**	**Duration**
Induction	Ceftazidime 50 mg/kg/dose (up to 2 g) every 6–8 h	10-14 days or ≥4 weeks for deep-seated infection
	Meropenem 25 mg/kg/dose (up to 1 g) every 8 h	
Maintenance	Trimethoprim/sulfamethoxazole • 8/40 mg/kg/dose orally BD* • >60 kg : 2 × 160/800 mg (960 mg) tablets BD • 40–60 kg : 3 × 80/400 mg (480 mg) tablets BD • <40 kg : 1 × 160/800 mg (960 mg) or : 2 × 80/400 mg (480 mg) tablets BD	3–6 months, depending on clinical response to therapy
	With or without doxycycline 2.5 mg/kg/dose (up to 100 mg) orally BD	
Life threatening melioidosis	• IV Meropenem (25mg/kg/dose; usual dose for adult: 1 gm TDS), or • IV Imipenem (50mg/kg/day; usual adult dose 1gm TDS)	at least 2 weeks

1.3 *Salmonella* and invasive salmonellosis

Salmonella are ubiquitous Gram-negative facultative anaerobic, Gram-negative bacteria belong to Enterobacteriaceae family. This organism was first isolated from infected bovine in 1855 by Theobald Smith and named after the research team leader, Dr Daniel Elmer Salmon (Schultz, 2008). *Salmonella* genus is made up of two species, *Salmonella enterica* and *Salmonella bongori*. The earlier species is commonly isolated, and further divided into six subspecies; *enterica, salamae, arizonae, diarizonae, houtenae, indica.*

Apart from classification based on the genetic relatedness, *Salmonella* serovars have been described based on their antigenic determinants and designation by the White-Kauffmann-Le Minor scheme (Issenhuth-Jeanjean *et al.*, 2014). Most of these serovars are adapted to humans and animal host. In clinical settings, invasive human salmonellosis is caused by the serovars Typhi or Paratyphi A, B, C, or invasive non-typhoidal *Salmonella* (iNTS) serovars.

1.3.1 Biology of *Salmonella* and cultivation

Salmonellae are motile non-spore forming Gram-negative bacilli with a dimension of approximately 1 μm (in diameter) × 2 – 5 μm (in length) (Kawamoto *et al.*, 2013). The motility of this organism is due to the presence of peritrichous flagella, located all over of the bacterial cell (Andino and Hanning, 2015). Microscopically, *Salmonella* are indistinguishable to other Gram-negative bacilli and do not display unique staining pattern (Malt *et al.*, 2015).

Salmonella ferment glucose, sucrose, mannitol and citrate as a carbon source (Steven and David, 2014). These organisms are non-fastidious facultative anaerobes and can be cultivated in the microbiology laboratory using various basic agars, such

as blood agar and nutrient agar. Besides, selective (and/or differential) agar can be used including Mac Conkey agar, Hektoen (HE) agar and selenite broth with brilliant green (Steven and David, 2014). These organisms are oxidase-negative, predominantly non-lactose fermenting and can produce hydrogen sulphide (H_2S). Emerging number of *Salmonella* (particularly non-typhoidal serovars) are either H_2S negative (7% – 40%) or lactose fermenter (<1%) (Latif *et al.*, 2014; Lin *et al.*, 2014; Wu *et al.*, 2016). These atypical phenotypic characteristics of *Salmonella* may cause misidentification of the organism, like *E. coli* or other Enterobacteriaceae.

1.3.2 Classification and taxonomy

During its discovery, *Salmonella* were formerly named as *Bacillus choleraesuis.* In 1900, the name was changed to *Salmonella choleraesuis* (Ryan *et al.*, 2017). Since then the nomenclature remains complex and evolving. In 2005, the Judicial Commission of the International Committee on Systematics of Prokaryotes has decided that the *Salmonella* genus consists of two species; *Salmonella enterica* and *Salmonella bongori* (Tindall *et al.*, 2005). The earlier species is further divided into six subspecies; designated with respectively Roman numeral (not italicised), *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV), and *S. enterica* subsp. *indica* (VI) (Lamas *et al.*, 2018). Among all subspecies, *S. enterica* subsp. *enterica* (I) are commonly isolated and accounted for 99% of salmonellosis in human and mammals. Meanwhile, the remaining subspecies II – VI and *S. bongori* (V) rarely infect human and predominantly isolated from cold-blooded animal and the environment (Fookes *et al.*, 2011; Heredia and García, 2018).

Furthermore, the species or subspecies are further classified into different serovars (or serotypes). This classification is based on the surface antigenic compositions of the bacterium. The antigens include somatic (O), capsular (K) and flagellar (H) determinants. In all antigens present in the *S. enterica* subsp. *enterica* (I), the serovar can be named after the location where it was isolated. Otherwise, serovar with partial antigenic determinants will be named according to the antigenic formula, similar to the other five subspecies (II, IIIa, IIIb, IV and V) (Ryan *et al.*, 2017).

To date, 2659 different serovars have been described. Some serovars, such Typhi and Paratyphi A are exclusive to the human host. Meanwhile, serovar Rissen, Derby and Lexington are more prevalent in animals (Vo *et al.*, 2006). For both human and animals, serovar Enteritidis, Typhimurium and Weltevreden are commonly encountered (Fardsanei *et al.*, 2016; Vo *et al.*, 2006). Details of reported serovars are listed in Table 1.6 and Figure 1.7.

Table 1.6 Numbers of serovars of *S. enterica* subspecies *and S. bongori* determined by the White-Kauffmann-Le Minor scheme. Adapted from Fardsanei *et al*. (2016).

Salmonella species/subspecies	Number	# serovar
S. enterica		
subsp. *enterica*	I	1586
subsp. *salamae*	II	522
subsp. *arizonae*	IIIa	102
subsp. *diarizonae*	IIIb	338
subsp. *houtenae*	IV	76
subsp. *indica*	VI	13
S. bongori	V	22
Total		2659

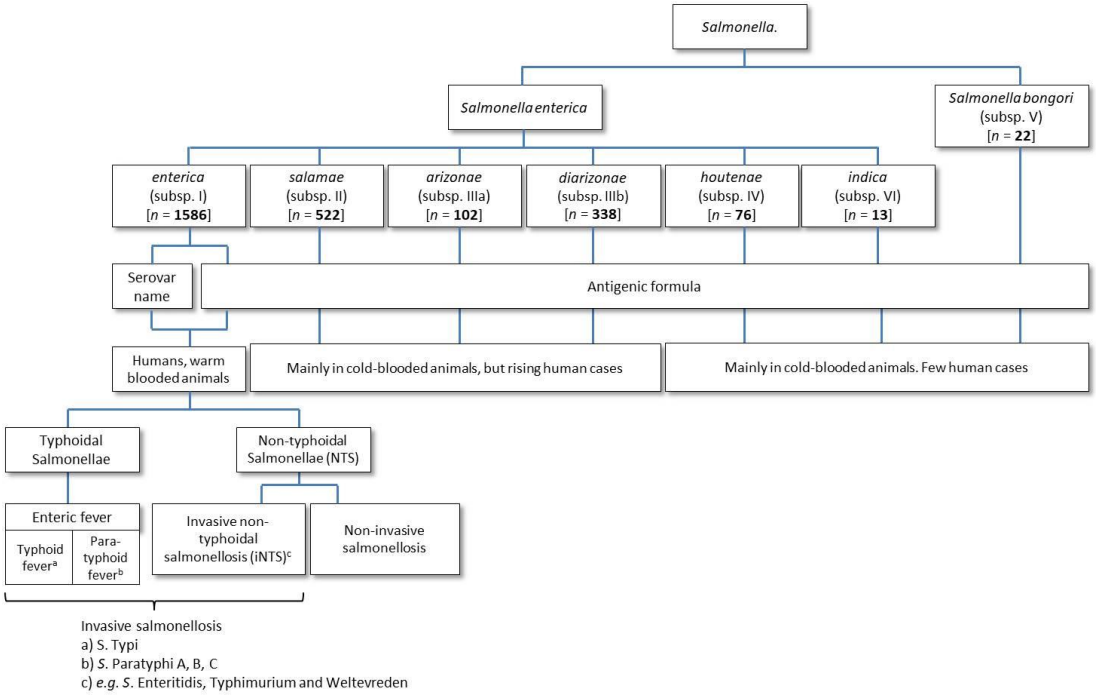


Figure 1.7 Summary of *Salmonella* nomenclature, their respective host and clinical involvement.

1.3.3 Pathogenesis

Following ingestion, *Salmonella* attach to the non-phagocytic epithelial M cells of the Peyer patches using fimbriae or pili. Subsequently, the bacteria invade the cells by exploiting the cellular antigen uptake and transport function (Andino and Hanning, 2015). Upon entry, *Salmonella* disseminate into macrophages, neutrophils and several other cells. Once reside in the host cells, *Salmonella* survive and replicate within a specialised compartment called *Salmonella*-containing vacuoles (SCV). Intracellular survival of *Salmonella* is due to many pathogenic factors, including effector proteins associated with the Type III secretion system (TTSS), encoded by the *Salmonella* pathogenicity island 2 (SPI-2). The effector proteins include *GogB, SodC* and *SspHI* (Fookes *et al.*, 2011). Apart from intracellular replication, certain serovars, especial non-typhoidal, replicates vastly in the gastrointestinal lumen (Dougan and Baker, 2014). Figure 1.8 illustrates invasion and replication of typhoidal and non-typhoidal *Salmonella*.

The ability of *Salmonella* to survive in the host cells is paramount for its pathogenicity. For instance, the complement-mediated lysis and antibody-mediated opsonisation are effectively blocked by the Vi antigen (Ilyas *et al.*, 2017). Besides, by exploiting the mononuclear cell migration, *Salmonella* spread through the system, especially the liver, spleen and bone marrow. In few cases, gallbladder became the main location of *S.* Typhi (or Paratyphi), in which long-term excretion have been reported (Gunn *et al.*, 2014).

Moreover, the presence of typhoid toxin, encoded by *CdtB, PltA, and PltB* further improve the virulence of serovar Typhi (Chong *et al.*, 2017). In non-typhoidal serovars, another homologue, Javiana toxin has been described (Rodriguez-Rivera *et al.*, 2015). These toxins protein contains an N-terminal Sec signal peptide, which

mediates the transportation from the cytoplasm to the periplasm. Upon release, the toxins bind to Neu5Ac-terminated glycans, which are exclusively expressed in human cells (Gao *et al.*, 2017). At high concentration, typhoid toxin is capable of inducing the death of host cells (Chong *et al.*, 2017). In addition to these proteins, other putative pathogenic genes and their associated functions in salmonellosis are listed in Table 1.7.

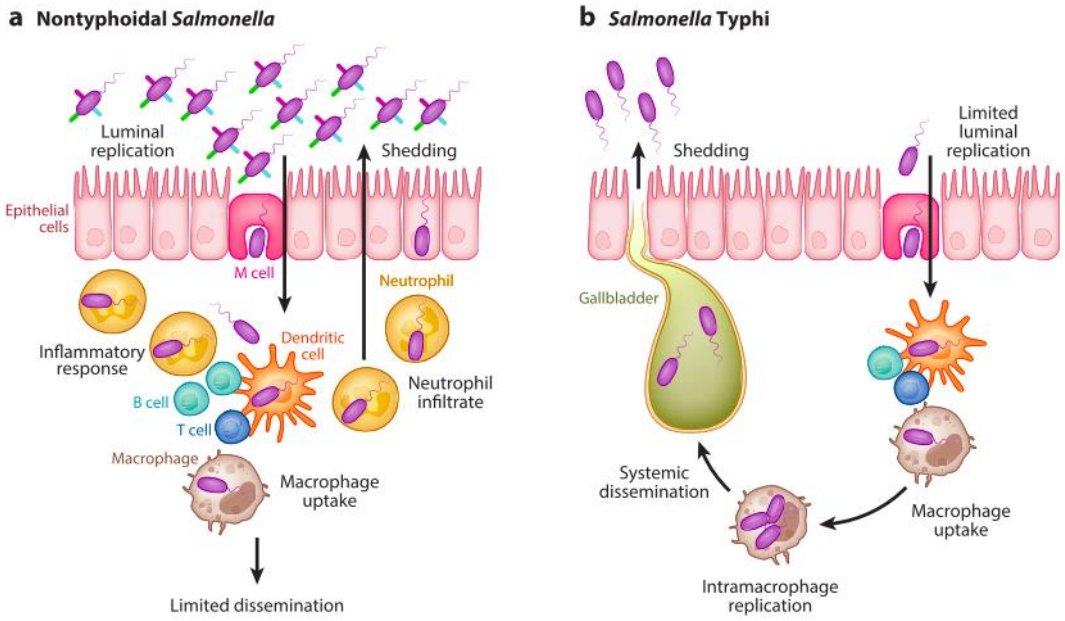


Figure 1.8 Invasion and replication of typhoidal and non-typhoidal *Salmonella*. Adopted from Dougan and Baker (2014).

Table 1.7 Selected *Salmonella* virulence genes and putative roles in infection. Adapted from Ilyas, Tsai and Coombes (2017)

Gene	**Gene location**	**Role in infection**
GogB	Gifsy-1	Inhibition of pro-inflammatory response, survival within macrophages
SodC	Gifsy-2	Resistance to oxidative stress, survival within SCV
SspH1	Gifsy-3	Immune suppression, survival within SCV
SopE	SopEΦ	iNOS activation, luminal colonization
SipA, SipB	SPI-1	Membrane ruffling, invasion
SseF, SseG	SPI-2	SCV localization, intracellular survival
MisL	SPI-3	Attachment, long term colonization
SiiE	SPI-4	Attachment to intestinal epithelium, aids in invasion
SopB	SPI-5	Membrane ruffling, invasion of epithelial cells, activation of pro-inflammatory response
SciG, SciS	SPI-6	Intracellular survival, systemic infection
Tae4	SPI-6	Overcoming colonization resistance, luminal colonization
PagC, PagD, EnvE, EnvF	SPI-11	Resistance to antimicrobial peptides, survival within macrophages
SspH2	SPI-12	Immune evasion, intracellular survival
STM3118, STM3119	SPI-13	Immune evasion, survival within macrophages
LoiA	SPI-14	Regulation of SPI-1 genes, important for invasion
STM0557	SPI-16	Serotype conversion, long term colonization

1.3.4 Salmonellosis in human

Contaminated food is the major cause of *Salmonella* outbreaks in humans. Among reported sources of foodborne outbreaks were peanut butter, snacks, dog food, cereal and pies. This association was supported by close genetic relatedness found between *Salmonella* isolates from humans and foods (Fardsanei *et al.*, 2016). Besides, human to human infections, post-climatic events outbreaks are also possible but are less frequent.

To infect humans, at least 1000 *Salmonella* are necessary. To reach that number, it is possible that the bacterium multiplies in food sample until reaching the infective dose (Fangtham and Wilde, 2008). In human infections, the clinical manifestations occur after 7 to 14 days, and are divided into (i) enteric fever, (ii) gastroenteritis, (iii) bacteraemia and other extra-intestinal complications (invasive salmonellosis) and (iv) chronic carrier state (Crump *et al.*, 2015).

Enteric fever is caused by typhoidal *Salmonella* serovars, i.e. *S.* Typhi (caused typhoid fever) and *S.* Paratyphi A, B and C (caused paratyphoid fever). These serovars exclusively infect humans and transmitted via contact with water or food, contaminated with stools of the chronic carrier or infected patients. Following infection, the incubation period ranges between 7 – 14 days. Enteric fever is characterised by a myriad of non-specific symptoms including headache, coated tongue, rose-spots on chest (not always), abdominal pain, bradycardia, diarrhoea (or constipation) and high fever. In addition, infected patients may also develop hepatomegaly, splenomegaly, pancreatitis, cholecystitis and gastrointestinal haemorrhage (Eng *et al.*, 2015).

Gastroenteritis (or stomach flu) is caused by serovars other than Typi and Paratyphi, also known as non-typhoidal *Salmonella* (NTS). Medically important NTS include the *S.* Enteritidis, *S.* Typhimurium and *S.* Weltevreden. Infection by NTS has a shorter incubation period (6 – 12 hours) with common symptoms of headache, vomiting, nausea, non-bloody diarrhoea, abdominal cramps and myalgia. These symptoms usually are self-limiting and last for approximately 10 days. A certain group, such as immunocompromised patients, infants, young children and elderly people (> 50 years) have higher risks of NTS infection and may develop severe invasive salmonellosis (Asmar and Abdel-Haq, 2015; Dhanoa and Fatt, 2009).

Invasive salmonellosis is a spectrum of extra-intestinal diseases which include enteric fever (i.e., typhoid and paratyphoid fever) and invasive nontyphoidal *Salmonella* (iNTS) infection (Khan *et al.*, 2016). Bacteraemia is an example of major extra-intestinal complications, in which *Salmonella* disseminate into the bloodstream after invading the intestinal barrier. Like enteric fever, bacteraemia is characterised by high-grade fever (≥ 38°C), persists for more than 12 hours (Gibani *et al.*, 2015). In certain circumstances, bacteraemia develops into septic shock, due to dysregulation of immune response (Thompson Bastin *et al.*, 2016). It is estimated that 5% – 9% NTS bacteraemia patients developed septic shock, commonly with pulmonary involvement (Dhanoa and Fatt, 2009; Eng *et al.*, 2015). Other extra-intestinal sequalae include cellulitis, urinary tract infections (UTI), meningitis and endocarditis (Tapia *et al.*, 2015).

In addition, approximately 2% – 5% of enteric fever patients may become chronic carriers, in contrast to 0.15% occurrence rate in patients with NTS (Gunn *et al.*, 2014). Chronic carriage incidence is associated with existing gallstone (90% prevalence), whereby its presence foster synthesis of *Salmonella* biofilm (Crawford

et al., 2010; Gunn *et al.*, 2014). These asymptomatic carriers are believed to have important roles in the maintenance of *Salmonella* within human populations since the first report of the Typhoid Mary in 1939 (Soper, 1939).

1.3.5 Epidemiology

Enteric fever and iNTS disease are emerging major public health concerns, especially in developing countries. The incidences are associated with behavioural, environmental and infrastructural factors, especially in areas with inadequate sanitation facilities and poor water supply systems (Khan *et al.*, 2016; Phu Huong Lan *et al.*, 2016). In many situations, epidemiological data for invasive salmonellosis are separated into (i) enteric fever; consist of typhoid fever & paratyphoid fever, and (ii) iNTS infections, based on the etiologic agents for each clinical manifestations.

Worldwide, it was estimated that between 6.9 and 48.4 million cases of enteric fever were reported annually (Saad *et al.*, 2018). While *S.* Typhi accounted for 217,000 deaths, *S.* Paratyphi A, B and C were estimated to cause 0.5% death of the total incidence (Buckle *et al.*, 2012). The vast majority (~80%) of this burden occurred in Africa and Asia, particularly India, Bangladesh, Nepal, Pakistan, China, Laos, Vietnam and Indonesia (Chau *et al.*, 2007; Kothari *et al.*, 2008).

Meanwhile, 2.1 – 6.5 million iNTS cases were reported worldwide, every year. Of these iNTS cases, approximately 20% were fatal, which were equivalent to 681,316 (range 415,165 – 1,301,520) deaths, annually (Ao *et al.*, 2015). In other studies, case fatality rates (CFR) were estimated between 3% (62,275 – 195,228 death) and 47% (1,037,912–3,253,800 death). More than 50% of the global cases occurred in sub-Saharan African countries, especially among the children (0 – 4 years) and young adults (30 – 40 years) (Ao *et al.*, 2015). In Malaysia, typhoid and

paratyphoid fever are listed as mandatorily notifiable diseases, in which potential outbreaks and incidence are monitored continuously. According to the Ministry of Health Malaysia, the prevalence of typhoid fever is 0.57 cases per 100,000 population with 0.01 death per 100,000 population (MOH, 2016).

1.3.6 Risk factors

Host genetic factors play critical roles in the epidemiology of salmonellosis. For instance, individuals with HLA-DRB1*0405 gene within the major histocompatibility complex (MHC) region, have 5-fold lower risk of developing enteric fever (Dunstan *et al.*, 2014). This resistance may due to the differences in the antigen presentation and subsequent immune response. Meanwhile, patients with impaired expression of IL-12Rβ1 chain of the interleukin-12 receptor (IL-12R) were highly susceptible to *Salmonella* infections. Deficiency in IL-12R expression impairs pro-inflammatory interferon-γ (IFN-γ) production and type 1 helper T cell (T_H1) responses, which are important in overcoming intracellular infections (de Jong *et al.*, 1998). In other areas, patients (especially children) with malnutrition, sickle cell disease and malarial anaemia were associated with higher incidences of salmonellosis (S. C. Wen *et al.*, 2017). In a Malaysian study, 65.5% of iNTS patients had at least one underlying immunosuppression, particularly malignancy, AIDS or Systemic Lupus Erythematosus (SLE) (Dhanoa and Fatt, 2009).

Besides, household-related factors, such as unhygienic preparation of food, poor hand washing behaviour, and recent *Salmonella* infections within the family members were also associated with salmonellosis (Fangtham and Wilde, 2008). Similarly, increased odd-ratios (ORs) were observed in individuals with low score of

hand-washing practice (i.e. before eating, after defecating), washing food produce before eating and eating *lolo* (squeeze coconut) (Prasad, 2015).

In addition to household aspects, increased salmonellosis incidences were also associated with environmental factors. For instance, increased rainfall and close distance to river increased the risk of salmonellosis (de Alwis *et al.*, 2018). In the more temperate area, increase in temperature is associated with higher number of cases (Akil *et al.*, 2014). For example, one unit increase in temperature (1 Fahrenheit) in Maryland, USA was associated with 4% increased risk of salmonellosis. At coastal areas of the same state, such an increase in temperature and increased rainfall resulted in a higher salmonellosis rate (7.1%) (Jiang *et al.*, 2015). Moreover, a high number of salmonellosis incidences were reported following major climatic incidences, as seen in 20.5% survivors who reported gastroenteritis and diagnosed as salmonellosis, 2 weeks after the catastrophic 2004 Acheh Tsunami (Sutiono *et al.*, 2010).

1.3.7 Diagnosis

Diagnosis of salmonellosis is important for initiation of appropriate treatment and control of the disease. To date, the laboratory tests for diagnosis of salmonellosis are categorised into; (i) culture methods, (ii) serology method and (iii) molecular assays.

1.3.7 (a) Culture method

Isolation of *Salmonella* colonies from clinical specimens, such as blood, bone marrow and stool is a definitive diagnosis of salmonellosis. This method is most useful for detection of bloodstream infection during the first two weeks of the disease onset. Without prior antibiotic treatment, the sensitivity of *Salmonella* culture is as high as 80%. However, the sensitivity drops to as low as 40% in treated patients (Andrews and Ryan, 2015).

Several culture media can be used for isolation of these non-fastidious Gram-negative bacilli. For inoculation of non-sterile specimens such as stool, selective differential media such as Hektoen (HE) agar and selenite broth with brilliant green (Steven and David, 2014). This medium contains (i) bile salts that inhibit certain growth of coliforms, (ii) acid fuchsin & bromothymol blue to indicate carbohydrate dissimilation and (iii) ferric iron to allow the formation of H_2S from thiosulfate. On HE agar, *Salmonella* colonies are blue-green colonies with or without production of blackish H_2S (Corry, 2003). Meanwhile, for the cultivation of *Salmonella* from sterile specimens, such as blood that contains a low number of the organism (<10 colonies per ml), enriched media such as blood culture fluid (BD BACTEC and BacT/Alert vials) can be used. Following isolation, *Salmonella* can be screened using routine biochemical tests such as Triple Sugar Iron (TSI) agar, Simmons Citrate Agar, urea agar and Motility-Indole-Ornithine (MIO) agar (Mikoleit *et al.*, 2015). For definitive identification, commercial API 20E, Remel RapID ONE, Phoenix, Vitek 2 and MALDI-TOF mass spectrometry can be used (Wisplinghoff, 2017). Complementary serotyping based on the H, O and Vi antigen of the Kauffman-White scheme are necessary to confirm the serovars (Mikoleit *et al.*, 2015).

1.3.7 (b) Serological diagnosis

In addition to bacterial culture, the indirect presence of *Salmonella* can be demonstrated by the detection of specific antibodies. In certain regions, the Widal test is used to measure agglutination between antibodies (from patient sera) and *Salmonella* antigens, (H and O). Despite its simplicity and inexpensiveness, the performance of this test is limited. In an evaluation study, the clinical sensitivity and specificity were found between 72% – 80 % and between 51% – 58%, respectively (Adhikari *et al.*, 2015). Lower sensitivity (36%) and specificity (58%) were observed in a recent study in India (Rao, 2018). Meanwhile, the clinical performance of other serological assays, including rapid diagnostic tests (RDTs) and ELISA are listed in Table 1.8.

Table 1.8 Clinical sensitivity & specificity of selected rapid diagnostic tests (RDTs) and ELISA for salmonellosis diagnosis

Kit	**Sensitivity (%)**	**Specificity (%)**	**References**
Cromotest® O : semiquantitative	95	4	(Keddy *et al.*, 2011)
Cromotest® H : semiquantitative	80	50	
Cromotest® O: single tube	87	7	
Cromotest® H: single tube	95	14	
Typhidot® IgM	75	61	
Typhidot® IgG	69	70	
MyBioSource ELISA IgM	95	94	(Adhikari *et al.*, 2015)
MyBioSource ELISA IgG	96	95	
Test-It Typhoid	69	90	(Wijedoru *et al.*, 2017)
TUBEX	78	87	

1.3.7 (c) Molecular diagnosis

Nucleic acid amplification testing for direct detection of *Salmonella* DNA has been explored since the early 1990s (Song *et al.*, 1993). In the early days, conventional PCR was developed against common genes encoding for flagellin or more specific genes such as *invA* and *spvC* with a sensitivity of approximately 200 – 250 organisms per reaction. The assays may detect a particular serovar (i.e. *S.* Typhi or *S.* Typhimurium), a combination of serovars (i.e. *S.* Typhi & *S.* Paratyphi A) or a particular species (i.e. *S. enterica*) (Chiu and Ou, 1996; Goay *et al.*, 2016; G. G. Stone *et al.*, 1994).

On the other hand, qPCR (SYBR and TaqMan) are better alternatives, in terms of sensitivity and specificity. Example of SYBR qPCR includes the Combinatory SYBR®Green qPCR System (CoSYPS) that enables the detection and differentiation of *Salmonella* genus, *S. enterica* and S. *enterica* subp. *enterica*. Despite its good analytically sensitivity and specificity, this assay is not validated clinically (Barbau-Piednoir *et al.*, 2013). Another instance is *clyA*-TaqMan qPCR, developed for detection of *S.* Typhi and *S.* Paratyphi A. However, upon validation on blood specimens collected from suspected patients, this assay had clinical sensitivity and specificity of 28.6% and 94.7%, respectively. Moreover, this assay also cross-reacted with diarrheagenic *E. coli* (Tennant *et al.*, 2015).

1.3.8 Management and treatment

Salmonella gastroenteritis is usually self-limiting and does not require antimicrobial treatment. However, antibiotic treatment for this infectious diarrhoea remains controversial as more than one organism (i.e. EPEC, *Vibrio cholerae*) may present during the illness. On the other hand, several studies have shown that antibiotic treatment in non-typhoidal salmonellosis prolonged NTS shedding in faeces and increased risk of relapse (Gillies *et al.*, 2015; Nelson *et al.*, 1980). Therefore, antibiotic treatments are recommended for invasive salmonellosis (enteric fever and iNTS), as well as for patients who are at high risk of developing invasive disease. In the absence of effective therapy, the case-fatality rate is between 10% – 30%. The fatality is reduced to 1% – 4% when appropriate antibiotic treatment is administered (Buckle *et al.*, 2012).

Both inpatient and outpatients with enteric fever or invasive salmonellosis should be monitored for complication. Supportive measures such as electrolyte replacement and rehydration should be considered when indicated. While most patients can be treated at home, about 10% – 22% may require hospitalisation and parenteral antimicrobial treatment (Chen *et al.*, 2013). Table 1.9 summarises WHO recommendation for treatment of enteric fever in non-severe and severe cases.

Meanwhile, the Ministry of Health Malaysia (MOH) further specifies treatment based on age groups. Among non-severe cases, ampicillin or ciprofloxacin is recommended for adult patients. Meanwhile, for non-severe paediatric cases, third generation cephalosporin, ceftriaxone is suggested. Alternatively, chloramphenicol can also be considered. Meanwhile, for severe cases, higher doses of respective antibiotics should be administered. Details of antibiotic treatment per recommendation of MOH are listed in Table 1.10.

Table 1.9 WHO recommendations of antibiotic treatment in (A) non-severe and (B) severe enteric fever

Optimal therapy				Alternative therapy		
(A) Susceptibility	Antibiotic	Daily dose mg/kg	Days	Antibiotic	Daily dose mg/kg	Days
Fully sensitive	Ciprofloxacin Ofloxacin	15	5-7	Chloramphenicol	50-75	14-21
				Amoxicillin	75-100	14
				TMP-SMX	8-40	14
Multidrug resistance	Ciprofloxacin	15	5-7	Azithromycn Cefixime	8-10 15-20	7 7-14
	Ofloxacin					
	Cefixime	15-20	15-20			
Quinolone resistant	Azithromycin Ceftriaxone	8-10 75	7 10-14	Cefixime	20	7-14

Optimal therapy				Alternative therapy		
(B) Susceptibility	Antibiotic	Daily dose mg/kg	Days	Antibiotic	Daily dose mg/kg	Days
Fully sensitive	Oxofloxacin	15	10-14	Chloramphenicol	100	14-21
				Amoxicillin	100	14
				TMP-SMX	8-40	14
Multidrug resistance	Ciprofloxacin	15	10-14	Ceftriaxone or	60	10-14
	Ofloxacin			cefotaxime	80	
Quinolone resistant	Ceftriaxone or	60	10-14	Ciprofloxacin	20	7-14
	cefotaxime	80		Ofloxacin		

Table 1.10 MOH recommendations of antibiotic treatment of non-severe and severe enteric fever cases

Cases	**Antibiotic**	**Dose (mg per kg)**	**Days**
Adult (Uncomplicated)	Ampicillin	500	14
	Ciprofloxacin	750	5-7
	Chloramphenicol	500	14
Paediatric (Uncomplicated)	Ceftriaxone	60-100	5-7
Adult (Complicated)	Ceftriaxone	30	5-6
	Ciprofloxacin	200	5-7
Paediatric (Complicated)	Ceftriaxone	60-100	5-7
Resistant	Depend on sensitivity		

1.4 *Plasmodium* and malaria

Malaria is an infection caused by *Plasmodium* protozoan parasites. The name of the disease originated from ‘mala’ and ‘aria’ in Italian which mean ‘bad air’. The discovery of this disease began in 1880, by Alphonse Laveran, who observed black-pigmented crescentic bodies of the parasites in the blood specimens of malarial patients. The organism was named *Oscillaria malariae* (CDC, 2015). Seventeen years later, William MacCallum and Eugene Opie, of the Johns Hopkins University discovered sexual stages of *Plasmodium*. During this period of time, the important roles of mosquito vectors; the female *Anopheles*, in the transmission of *Plasmodium*, were discovered by Sir Ronald Ross. Finally, the complete cycle of this parasite was concluded in 1982, when Wojciech Krotoski successfully demonstrated the dormant stage of *Plasmodium* in the liver (Phillips *et al.*, 2017).

1.4.1 Biology of *Plasmodium* and cultivation

Plasmodium are eukaryotic protozoa that have complex life cycles, within two different hosts. Like other eukaryotes, these malarial pathogens have linear genomes and organelles such as ribosomes, mitochondria, enclosed by a plasma membrane and pellicular cisterna (Phillips *et al.*, 2017).

Among all, the *Plasmodium* merozoites are well characterised. Merozoites are oval in shape and are approximately 1.5 µm (in length) and 1 µm (in width). The apical end of merozoites contains polar rings and some secretory vesicles that are important for cellular invasions. Once released in the blood, merozoites are short-lived and invade a new erythrocyte for survival. The poor extracellular survival of malaria parasite hampered research studies on *Plasmodium* and dependent of

constant need of an animal host for a continuous supply of parasites (Bannister *et al.*, 2000).

In vitro cultivation of *Plasmodium* was pioneered by William Trager and J.B. Jensen in 1976 who described artificial media that could support malarial growth (Trager and Jensen, 1976). Artificial media for *Plasmodium* usually consists of a minimal tissue culture media i.e. Roswell Park Memorial Institute (RPMI)1640 medium or MEM medium, seeded with serum and erythrocytes. Culture plates are incubated at 38 °C with CO_2 (2% – 5%), O_2 (3% – 18%) and N_2 gases (Childs *et al.*, 2013). The condition of the culture is closely monitored and synchronised to mimic original condition in mammalian hosts.

1.4.2 Classification and taxonomy

Plasmodium are unicellular protozoa which belong to the family *Plasmodiidae* and genus *Plasmodia*. These obligate intracellular eukaryotes made up more than 100 species, of which, five species are capable of infecting humans and cause malaria. The clinically important species include *Plasmodium falciparum, P.malariae, P.ovale, P.vivax* and *P. knowlesi* (zoonotic malaria) (Sherman, 2012).

Traditionally, *Plasmodium* are characterised based on its morphology, depending on its cycles and species. However, in more recent years, molecular tools have been available to explore relatedness of *Plasmodium* genus. Among the commonly used nucleic acid sequences is the mitochondrial genome. Mitochondrial genomes have been shown to be unique, different structure, size and organisations. In *Plasmodium*, the mitochondrial genome is linear and consist of distinctive tandem repeats (Fuehrer and Noedl, 2014). With the availability of whole-genome

sequencing techniques such as next-generation sequencing (NGS) enables acceleration of genomic research.

To date, twenty complete *Plasmodium* genomes have been described, including 5 species that can infect humans. Based on the genomic analysis, malarial *Plasmodium* has three genomes that include (i) a nuclear genome with 14 linear chromosomes, (ii) a linear mitochondrial genome and (iii) a vestigial circular plastid genome homologous to red-alga's chloroplasts. Summary of genomic characteristic of five medically important *Plasmodium* species is listed in Table 1.11.

Table 1.11 Characteristic of malaria genomes according to each medically important *Plasmodium* species. Adapted from Antinori *et al.* (2012).

Malaria species	Overall genome size (Mb)	Number of genes	Overall A+T content (%)	Overall G+C content (%)
P. falciparum	23.3	5,403	80.6	19.4
P. knowlesi	23.5	5,188	62.5	37.5
P. malariae	31.9	5,930	75.3	24.7
P. ovalae	35.7	8,421	71.1	28.9
P. vivax	26.8	5,433	57.7	42.3

1.4.3 Pathogenesis

The life cycles of *Plasmodium* consist of (i) exogenous sexual phase (named sporogony), which take place in *Anopheles* mosquitoes and (ii) endogenous asexual phase (named schizogony), which happen in humans. The latter phase is further divided into (a) exo-erythrocytic cycle and (b) erythrocytic cycle. Figure 1.9 illustrates complete life cycle of *Plasmodium* parasite. In general, during a blood meal, the female *Anopheles* carrier inoculated sporozoites into humans, marked as the start of the exo-erythrocytic cycles (CDC, 2018). Sporozoites remain in the parenchyma cells, mature into schizonts and ruptured as merozoites (Phillips *et al.*, 2017).

The release of merozoites into bloodstream is the beginning of the erythrocytic cycle. Merozoites invade erythrocytes and developed into trophozoites and schizonts, which eventually ruptured and release more merozoites. Meanwhile, some trophozoites develop into gametocytes. Gametocytes are made of microgametocytes (male) and macrogametocytes (female) and ingested by *Anopheles* mosquitos during a blood meal (Cowman *et al.*, 2016).

Within the vector host, sexual sporogonic cycle begins in which microgametes penetrates macrogametes and become ookinetes. Ookinetes develop into oocysts in the mosquito midgut wall (Antinori *et al.*, 2012). Eventually, oocysts will rupture, release sporozoites that travel to the salivary gland of the mosquitoes, ready for inoculation into a new human host (Phillips *et al.*, 2017). Characteristics of malaria infections based on respective species are summarised in Table 1.12.

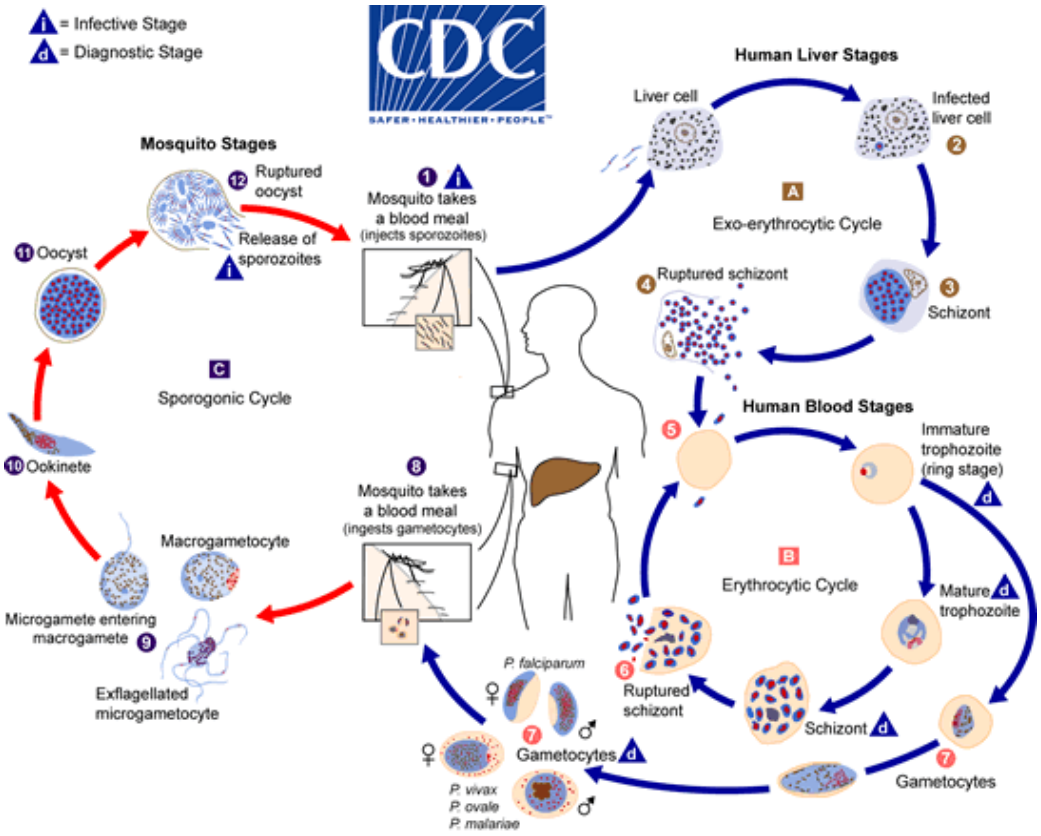


Figure 1.9 Life cycle of malaria parasites, in mosquito vectors and human hosts. Adopted from CDC (2018).

Table 1.12 Characteristic of malaria infection according to each medically important *Plasmodium* species. Adapted from Antinori *et al*. (2012).

Characteristics	*P. falciparum*	*P.vivax*	*P.malariae*	*P.ovale*	*P.knowlesi*
Exo-erythrocytic stage (days)	5 – 7	6 – 8	14 – 16	9	8 – 9
Pre-patent period* (days)	9 – 10	11 – 13	15 – 16	10 – 14	9 – 12
Erythrocytic cycle (hours)	48	48	72	50	24
Red cells affected	All	Reticulocytes	Mature erythrocytes	Reticulocytes	All
Av. parasitaemia (per µl)	20,000 – 500,000	20000	6000	9000	600 – 10,000
Max. parasitaemia (per µl)	2000000	100000	20000	30000	236000
Febrile paroxysm (hours)	16 – 36 or longer	8 – 12	8 – 10	8 – 12	8 – 12
Severe malaria	✓	✓	X	X	✓
Relapses from liver forms	X	✓	X	✓	X
Recurrences	✓ (treatment failure)	✓ (treatment failure)	✓	X	✓

* Interval period between sporozoites inoculation (during mosquito blood meal) and trophozoites detection in blood

1.4.4 Malaria in human

Upon inoculation of malarial parasites by *Anopheles* into a human host, the parasites are detectable in the blood between the first two weeks. This time interval is also known as the pre-patent period. During this stage, initial presentation are non-specific, which include fever (92.5% – 100%), headache (89%), myalgia (58%), diarrhoea (20.6% – 72%) and cough (28.8%) (Arévalo-Herrera *et al.*, 2015; Bouyou-Akotet *et al.*, 2014; Braga *et al.*, 2015; Mutanda *et al.*, 2014). In young children, loss of appetite (64%) and vomiting (31%) are also common (Mutanda *et al.*, 2014). During uncomplicated malaria, patients may have parasitaemia level between 1,000 – 50,000 parasites per µl of blood (Phillips *et al.*, 2017).

Untreated or partially treated patients may develop severe malaria. According to the WHO, severe malaria is characterised by impaired consciousness (Glasgow coma score <11), generalised weakness, more than two convulsion within 24 hours, acidosis, hypoglycaemia (<2.2 mmol/L), severe anaemia, renal impairment, jaundice, pulmonary oedema, haemorrhage, compensated shock and hyperparasitaemia (WHO, 2015). In terms of frequency, approximately 10% of malaria cases developed into these severe form (Arévalo-Herrera *et al.*, 2015; Kurth *et al.*, 2017). However, the severe rate is significantly higher among children (41.1%) and pregnant women (47.7%) (Geleta and Ketema, 2017; Maltha *et al.*, 2014).

1.4.5 Epidemiology

Malaria is a fatal infection caused by *Plasmodium* parasites that are transmitted by infected *Anopheles* mosquito vectors. According to the WHO, between 196 and 263 million people were infected in 2016. Despite a slight decrease in death, the mortality number remains steady at 225,000 cases, globally. Remarkably, more than 90% of cases and death occurred in Africa, almost entirely caused by *P. falciparum*. Outside Africa, *P. vivax* is a common cause of malaria in Americas (63%), Mediterranean (40%) and South-East Asian (>30%) (WHO, 2017b).

In Malaysia, malaria cases must be notified and actively monitored. As shown in Figure 1.10, the overall trend of malaria is steadily reducing, from over 7,000 cases in 2008 to less than 2,500 cases in 2016. Similarly, indigenous cases and imported cases of malaria remain low, since the past 6 years (WHO, 2017a).

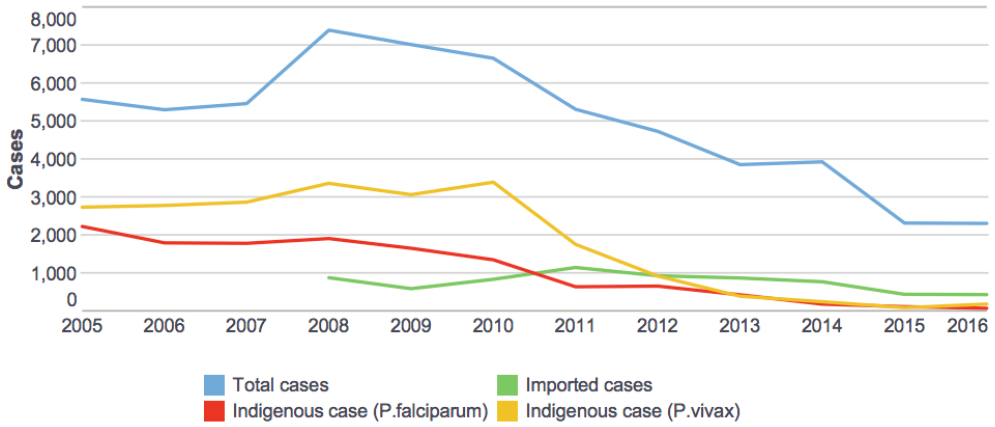


Figure 1.10 Malaria cases in Malaysia, between the year of 2005 and 2016. Adapted from WHO (2017a)

1.4.6 Risk factors

Transmissions of malaria are attributed to several aspects that can be clustered into (i) human-related factors, (ii) environmental factors and (iii) biological factor. In endemic regions, the age of below 40 years was associated with an increased risk of malaria (Schwartz *et al.*, 2001). Specifically, WHO estimated that 285,000 children under 5 years old died due to the severe complication of malaria, which includes severe anaemia and cerebral malaria, every year (WHO, 2018). Of this young age group, children aged between 6 months and 5 years were most susceptible as they have lost maternal immunity, at the same time, specific immunity against malaria have not been fully developed (Schumacher and Spinelli, 2012).

Pregnancy is another risk factor associated with malaria. In Africa alone, approximately 200,000 stillbirth cases were due to *P. falciparum* during pregnancy (K. A. Moore *et al.*, 2017). Higher risk of malaria infection during pregnancy may due to the hormonal changes, the attractiveness of mosquitoes towards expecting mother, as well as the presence of specific receptors such as chondroitin sulphate A (CSA) and sequester in the placenta (Takem and D'Alessandro, 2013).

In addition to these factors, the density of *Anopheles* (>1.5 *Anopheles* per house), late outdoor activities, time spent outdoors and lack of preventative measures such as indoor residual spraying (IRS) and bed net were also associated with increase of malaria cases (Chirebvu *et al.*, 2014; Protopopoff *et al.*, 2009; Roberts and Matthews, 2016). Other factors that influence the risk of malaria are illustrated in Figure 1.11.

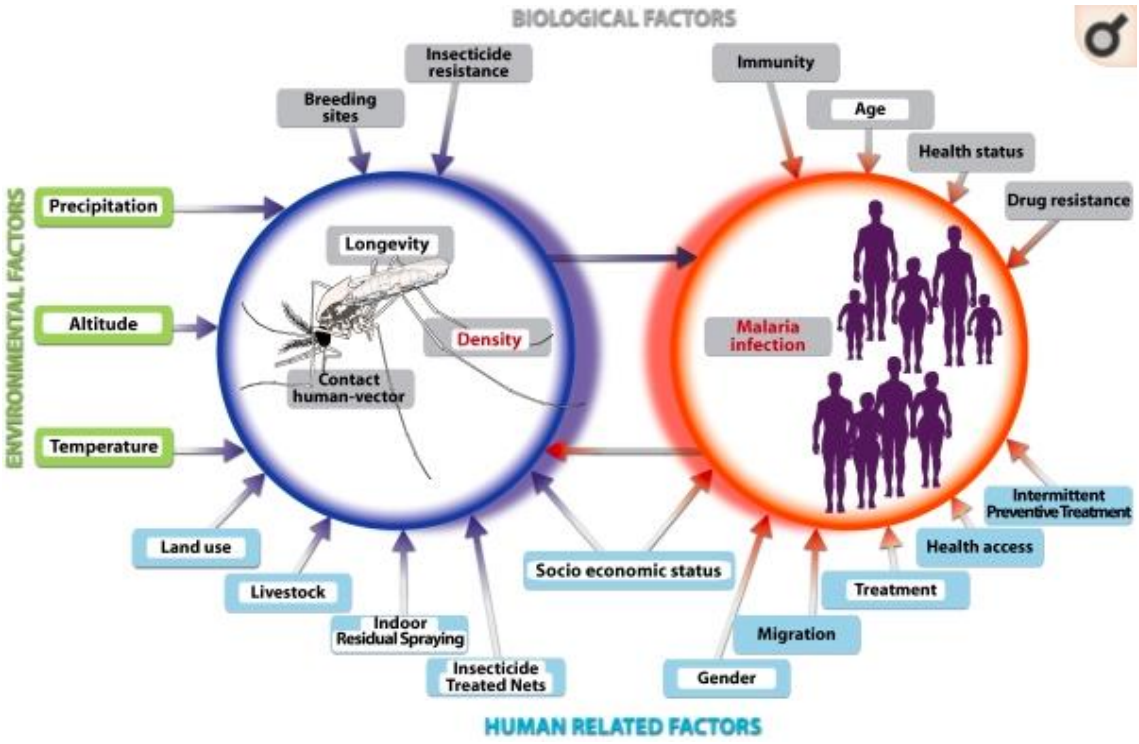


Figure 1.11 Factors associated with malaria infections. Adopted from Protopopoff *et al*. (2009)

1.4.7 Diagnosis

Accurate and prompt diagnosis of malaria significantly reduces mortality and morbidity. Similar to leptospirosis, melioidosis and invasive salmonellosis, presumptive diagnosis for malaria is challenging due to the lack of pathognomonic clinical signs or symptoms. As a result, complementary laboratory diagnosis is necessary to confirm the infection.

1.4.7 (a) Microscopic observation

Microscopic examination of blood smear with Giemsa stain is reference standard for diagnosis of malaria. This method allows identification of infecting *Plasmodium* species and quantification of parasitaemia. However, the sensitivity of microscopy method is inconsistent (10 – 1000 parasites per µl blood), depending on the experience of the laboratory technicians, quality of smear and stains, and concentration of parasites (Kakkilaya, 2015; Mukry *et al.*, 2017). Microscopy is less reliable at low parasitaemia (<50 parasites per µl) i.e. during pregnancy malaria where the parasites sequester at placenta (Fried *et al.*, 2012; Lo *et al.*, 2015).

Quantitative Buffy Coat (QBC) is another method that is useful for screening malaria. This assay utilises staining of the *Plasmodium* DNA with acridine orange on centrifuged blood samples. Unlike Giemsa staining, this assay requires a fluorescence microscope, and less convenient for differentiation of infecting malarial species (CDC, 2018).

1.4.7 (b) Rapid diagnostic test (RDT)

Several antigen-based tests are available to confirm the presence of *Plasmodium*. Among antigens of interest include histidine-rich protein 2 (HRP2) and *Plasmodium* lactate dehydrogenase (pLDH), utilising immunochromatographic lateral flow technology. An FDA-validated test that detects both HRP2 antigen and antibodies; BinaxNOW Malaria has sensitivity and specificity of 84% and 99%, respectively for non-*P. falciparum* malaria (Farcas *et al.*, 2003). However, the assay was only able to correctly detect 29% of *P. knowlesi* infections (D. Foster *et al.*, 2014).

1.4.7 (c) Molecular diagnosis

Molecular assays generally are superior in terms of sensitivity (and specificity). To date, several molecular assays have been described. Such assays detect 18S ribosomal RNA (rRNAs), cytochrome C oxidase I gene (*cox1*) for pan-detection of *Plasmodium* genus and/or differentiation of *Plasmodium* species. In the analytical evaluation, the assays had a limit of detections of between 5 – 100 copies per reaction (Gruenberg *et al.*, 2018; Kamau *et al.*, 2013).

Upon evaluation on clinical specimens, certain qPCR assays suffered from reduced sensitivity, as low as 62.5%, with apparent evidence of non-specific amplicons (Echeverry *et al.*, 2016; Ojurongbe *et al.*, 2013). It is implied that differences in the distribution of infecting malarial species negatively affected the clinical performance of molecular assays (Gruenberg *et al.*, 2018).

1.4.8 Management and treatment

Prompt diagnosis and appropriate treatment are important for better prognosis, as well as reducing the risk of malaria transmission. Currently, artemisinin-based combination therapies (ACTs) remain as a core treatment for malaria. In uncomplicated *P. falciparum* malaria, 5 ACTs combination are recommended, which include either (i) artemether + lumefantrine, (ii) artesunate + amodiaquine, (iii) artesunate + mefloquine, (iv) dihydroartemisinin + piperaquine, or (v) artesunate + sulfadoxine-pyrimethamine. Meanwhile, in uncomplicated *P. vivax, P. malariae, P. ovale* or *P. knowlesi* malaria, treatment with chloroquine or standard regime of ACT is recommended. The latter is advocated in areas with prevalent chloroquine-resistance (WHO, 2015). In Malaysia, artemether + lumefantrine is the first line treatment for uncomplicated *P. falciparum* malaria (MOH, 2013).

In severe malaria (i.e. patients with impaired consciousness, acidosis, jaundice), intramuscular or intravenous artesunate is recommended for at least 24 hours. Once patients tolerate orally, treatment is continued with an ACT (WHO, 2015).

1.5 Laboratory diagnosis of infectious diseases

For the past two decades, clinical microbiology laboratories have been carrying significant roles in determining a plethora aetiologies of infections that are paramount to patients' well-being and treating physicians (Isenberg, 2003). Since then, clinical microbiology laboratories have evolved correspondingly to the clinical needs, as well of the technology advancements. The current state-of-the-art, modern clinical microbiology laboratories employ a variety of diagnostic platforms, ranging from basic staining methods to molecular assays.

1.5.1 Conventional method

Conventional microbiology testing includes microscopy, culture and serology. The utilisation of microscopy to demonstrate the direct presence of microorganisms remains relevant and are considered as gold-standard in many instances, such as stool and malarial parasites (Isenberg, 2003). Moreover, culture also remains important as certain organisms are cultivatable in artificial media and serve as another method for laboratory diagnosis of bacterial infection. Despite their relative simplicity, microscopy and culture are less sensitive when the pathogens exist in low number. Moreover, culture is prone to false negativity when organisms fail to grow (i.e. due to antibiotic treatment) and require a long incubation period of time (Limmathurotsakul *et al.*, 2010).

Meanwhile, serological assays utilised detection of specific antibodies against infecting microorganism, typically produced two weeks after initial exposure. As a result, serological tests pose limited usefulness during the acute phase of infections (Musso *et al.*, 2013). Alternatively, molecular methods, such PCR and qPCR are perceived as powerful choices (in terms of sensitivity & specificity), that can potentially be applied for early detection of infection (Laupland and Valiquette, 2013).

1.5.2 Molecular method

"Molecular revolution" significantly impacts clinical microbiology laboratories and gradually replacing conventional methods in many regions (Smith and Osborn, 2009). Molecular assays, such as (conventional) PCR utilise *Taq* polymerase for amplification of desired nucleic acid targets, in the presence of at least a pair of primers, buffers, salts and dNTPs. In all occasion, results (positivity or

negativity) are recognised by the presence (or absence) of PCR products (or amplicons) with the correct sizes, achieved after ~ 60 minutes of gel electrophoresis. In many diagnostic laboratory settings, conventional PCR successfully replaced enzyme immunoassay (EIA) as a reference method i.e. for chlamydia detection (Laupland and Valiquette, 2013). However, the compulsory post-PCR electrophoresis is laborious and increases the risk of PCR product contamination through aerosolisation.

Simultaneous quantification of amplified PCR products was pioneered by Higushi *et. al*. in 1992. The team included ethidium bromide in the PCR reaction which enabled detection of fluorescence increment, proportionately to the amount of PCR product, during each cycle. By plotting the fluorescent signals and cycle number, a complete graph with real-time detection of the amplification product can be produced. This technology omits requirement of post-amplification gel electrophoresis (Patrinos and Ansorge, 2005).

Since then, the real-time PCR (qPCR) technology evolves rapidly, producing many derivative applications with different types of fluorescent dyes. These real-time molecular assays are grouped based on the fluorescent agents used; (i) intercalating dyes and (ii) fluorophores attached to oligonucleotides. The latter group can be further divided into (i) primer-probe (probe functions as a primer), (ii) hydrolysis probe and (iii) nucleic acid analogues. Figure 1.12 summarises types of qPCR assays based on their respective fluorescent agents.

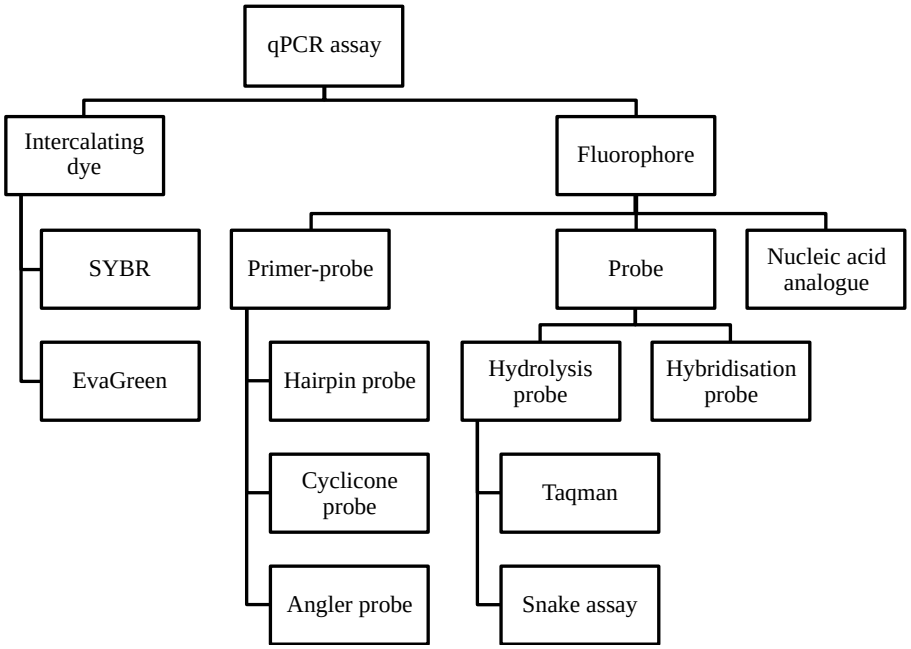


Figure 1.12 Types of qPCR assays based on the fluorescent agent used.

SYBR® Green I is commonly used intercalating dye. The structure is based on cyanine dye (2-[N-(3-dimethylaminopropyl)-N-propylamino]-4-[2,3- dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene]-1-phenyl-quinolinium). Under thermal cycling condition, the dye is positively charged (2+) which will bind to the minor groove of dsDNA with strong affinity. This dsDNA-SYBR complex absorbs and emits lights (λ_{max}) at 497 nm and 520 nm, respectively (Arya *et al.*, 2005). In addition to SYBR, more recent intercalating dyes include EvaGreen, a more stable dye, useful for high-resolution melt (HRM) analysis. Despite the possibility to analyse the melting temperature of resulting PCR products, the risk of false positivity due to primer-dimerisation cannot be fully excluded (Sánchez *et al.*, 2017). Moreover, multiplexing in SYBR/EvaGreen (to detect more than one target) is challenging, rely solely on the difference in the melting peaks (Navarro *et al.*, 2015).

Another qPCR group employs fluorophores (also called as dye), that are attached to oligonucleotides. Fluorophores are small fluorescent molecules, made up of reporter and quencher, which are attached to oligonucleotides function as probes. During absorbance, 'ground state' reporter fluorophore absorbs energy from light and become to an 'excited state'. To return to the 'ground state', reporter fluorophore will emit energy (emission) as fluorescence. The emitted light from a donor has lower energy, lower frequency and longer wavelength than the absorbed light. Therefore, if another quencher fluorophore presents in close distance (10 to 100 Å), it can absorb the emitted light from the reporter fluorophore. This transfer is expressed as Fluorescence Resonance Energy Transfer (FRET) (Crisalli and Kool, 2011).

Primer-probe is the first group of qPCR platform that utilises fluorophores. Primer-probe is a fragment of oligonucleotides that acts as both primer and probe. These oligonucleotides have conjugated fluorophores. Fluorescence is measured when primer-probe oligonucleotides are linearised during PCR amplification (Navarro *et al.*, 2015). Primer-probes can be further divided into (i) hairpins primer-probes (i.e. Scorpion, Amplifluor®, LUX™), (ii) Cyclicon primer-probe and (iii) Angler® primer-probes. Details of primer-probes are illustrated in Figure 1.13.

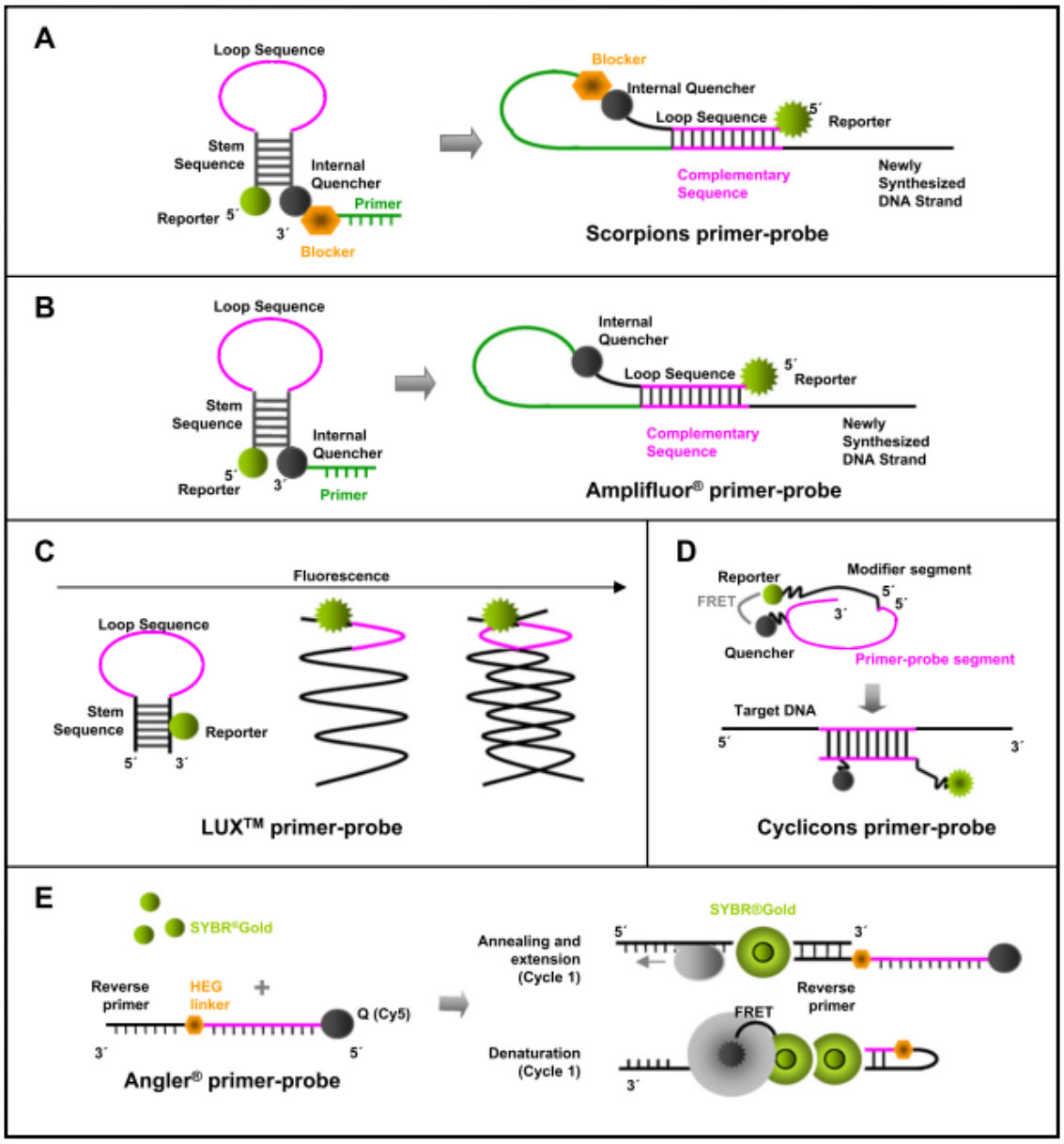


Figure 1.13 Action mechanism of primer-probes. Adopted from Navarro *et al.* (2015).

Probe is the second group of qPCR platform that utilises fluorophores. Unlike primer-probe that serves a dual function, the probe is a single-stranded oligonucleotide with a reporter fluorophores and/or a quencher. The probe is further categorised into (i) hydrolysis probe and (ii) hybridisation probe. Hydrolysis probe utilises 5' – 3' exonuclease activity of Taq polymerase enzyme (Holland *et al.*, 1991). It can be further divided into (a) TaqMan probe and (b) Snake assay. Mechanism of TaqMan probe is detailed in section 1.6. Meanwhile, the mechanism of snake assay is similar to TaqMan probe except that the complementary primer contains special sequence (snake primer). Following amplification, this special sequence will self-hybridise to form a small loop. The probe will bind next to this loop and get hydrolysed. Details of the snake assay are illustrated in Figure 1.14.

Hybridisation probe also utilises hydrolysis probe and quencher. The differences are the location of fluorophores (either on the same oligonucleotide or different oligonucleotides), the location of reporter dye (at 5'-end, 3'-end or in the middle), the presence of loop, as well as the presence of a 3′-end blocker (3′-octanediol or phosphate). Hybridisation probe includes Hybprobes/FRET probe, Molecular Beacon probe, HyBeacons™ probe, MGB-probes, ResonSense® probe and Yin-Yang probe (Kong *et al.*, 2007).

The last category of qPCR utilises nucleic acid analogue, which resembles DNA, but contains alteration at their phosphate DNA backbone and pentose sugar substitution, as shown in Figure 1.15. Once paired with a fluorophore, nucleic acid analogue can serve as probes. This nucleic acid analogues are more stable at a low salt concentration of PCR buffer, resistant to nucleases and bind strongly to complementary DNA templates Examples of nucleic acid analogue include peptide nucleic acids (PNA), locked nucleic acids (LNA) and zip nucleic acids (ZNA) (Bala

et al., 2016). Figure 1.16 illustrates the mechanism of ZNA. In this ZNA assay, a probe conjugated with cationic spermine unit is attracted to the negatively charged DNAs. Hybridisation happens once the probe binds to its complementary sequence located on the DNA target (Navarro *et al*., 2015).

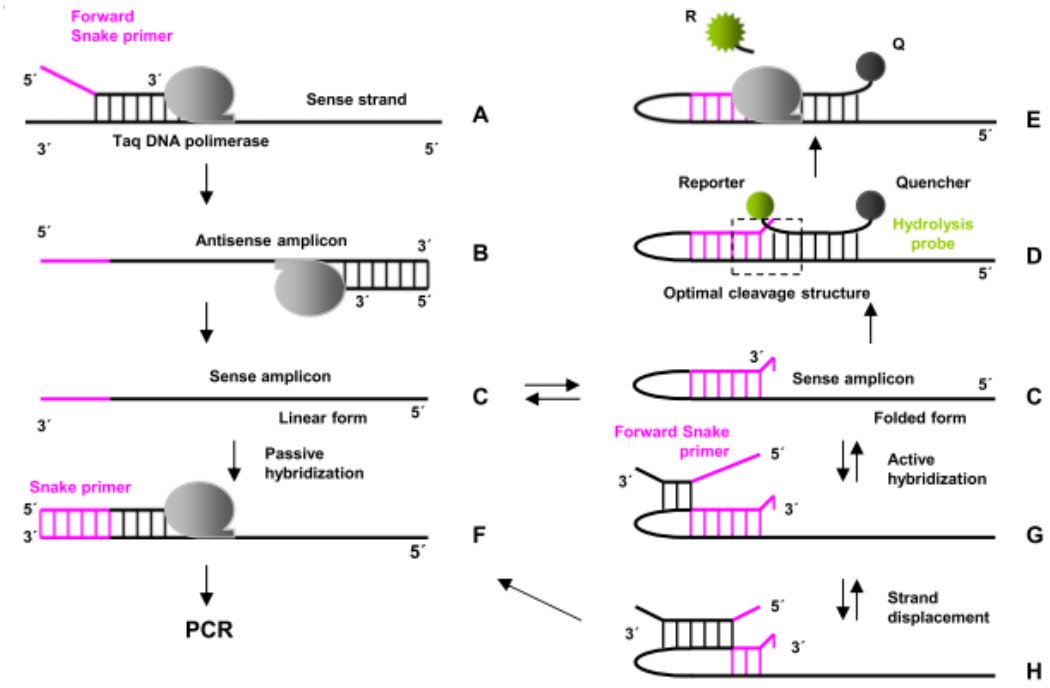


Figure 1.14 Mechanism of snake assay that utilises hydrolysis probe. Adopted from Navarro *et al*. (2015).

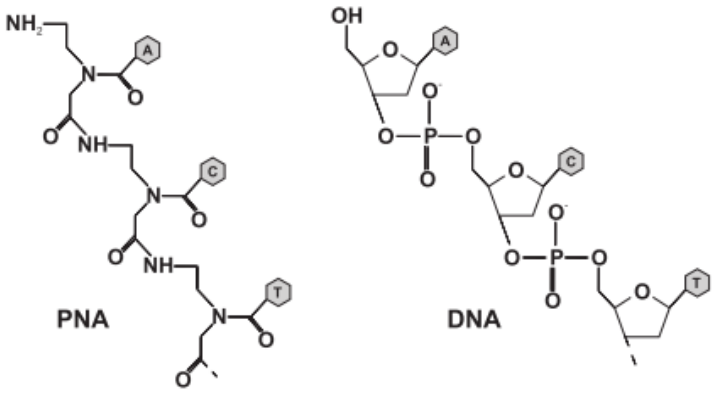


Figure 1.15 Comparison of chemical structures between a nucleic acid analogue, PNA and DNA. Adopted from Navarro *et al*. (2015).

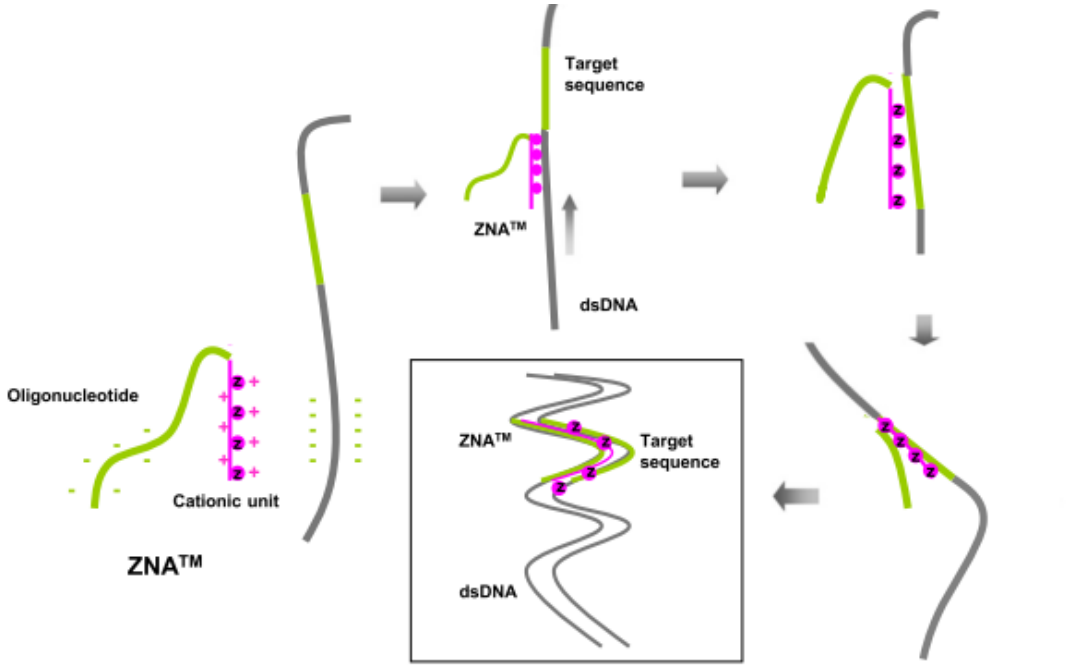


Figure 1.16 Binding of a nucleic acid analogue, ZNA to DNA template during PCR amplification. Adopted from Navarro *et al*. (2015).

In addition to conventional PCR and qPCR, isothermal amplification has been used to amplify nucleic acid from pathogens at a single temperature, with or without additional digestive enzyme such as helicase (Zhao *et al.*, 2015). To date, at least, 15 different isothermal amplifications have been described and can be classified according to the reaction kinetics; (i) exponential amplification, (ii) linear amplification and (iii) cascade amplification.

The exponential amplification group consists of nucleic acid sequence-based amplification (NASBA), exponential strand displacement amplification (E-SDA), exponential rolling circle amplification (E-RCA), loop-mediated isothermal amplification (LAMP), Helicase-Dependent Amplification (HDA) and recombinase polymerase amplification (RPA). Moreover, exponential isothermal amplification also includes exponential amplification reaction (EXPAR), whole genome amplification (WGA) and emerging exponential isothermal amplification, such as smart amplification process version 2 (SMAP 2) and Beacon-assisted detection amplification. These methods rely on exponential DNA replication hence pose high amplification efficiency and good sensitivity (Asiello and Baeumner, 2011). However, some assays suffer from non-specific amplification (production of multiple bands) and challenging in the design.

Alternatively, linear isothermal amplifications are simpler and fast. This method includes linear SDA, linear RCA, transcription-based amplification and signal amplification strategies (Zheng *et al.*, 2016). Yet, due to linear amplification strategy, some assays may have a weak signal and false negativity when the initial amount of template is low. To overcome these limitations, cascade amplifications have been introduced. In cascade amplification, enhancers such as DNAzyme, nucleases and NEase are included to improve the sensitivity of the assays (Zhu *et al.*,

2017). Examples of commonly used isothermal amplification and their characteristics are listed in Table 1.13.

Table 1.13 Characteristic of commonly used isothermal amplification. Adapted from Navarro *et al*. (2015).

Method	Enzyme (required)	Primer #	Temp. (°C)	Incubation (h)	Template	Amplicon
E-SDA	DNA polymerase and NEase	2 or 4	37	2	DNA	dsDNA
EXPAR	DNA polymerase and NEase	0	~60	< 0.5	short DNA/RNA	DNA
HDA	DNA polymerase and helicase	2	37−65	0.5−2	DNA	DNA
HRCA	DNA polymerase and ligase	2	60	1.5	DNA/RNA	DNA
LAMP	DNA polymerase	4	60−65	< 1	DNA	DNA
MDA	DNA polymerase	random primers	30−37	> 8	DNA	DNA
NASBA	Reverse transcriptase & RNA polymerase	2	~41	1.5−2	RNA/DNA	RNA, DNA
PG-RCA	DNA polymerase and NEase	0	60	1−3	DNA/RNA	DNA
pWGA	DNA polymerase and T7 gp4 primase	0	37	0.5−2	DNA	DNA
RPA	DNA polymerase and recombinase	2	37−42	0.5−1.5	DNA	DNA

1.6 Hydrolysis probe-based qPCR

The construction of hydrolysis TaqMan probe in 1988 and the discovery of 5' exonuclease activity of *Taq* DNA polymerase that was capable to degrade fluorophores' of a probe in 1991 marked as important milestones in molecular research (Cardullo *et al.*, 1988; Holland *et al.*, 1991). By combining these fundamental findings together, two related research teams from Genetech, USA successfully developed "real-time" quantitative PCR methods that were able to measure accurate and reproducible expressions of human *β-actin* gene and cystic fibrosis transmembrane transductance regulator (CFTR) mRNA (Gibson *et al.*, 1996; Heid *et al.*, 1996).

The principle of hydrolysis probe-based qPCR (or TaqMan qPCR) relies on hydrolysis probe (also known as a TaqMan probe) and 5' exonuclease activity of Taq polymerase, or any other polymerase with equivalent activity, such as *Tth* and *Tfl* polymerase (Ishino and Ishino, 2014).

Structurally, a hydrolysis probe is a short fragment of sequence-specific oligonucleotides of (25 – 30 bp), double labelled with a reporter dye at 5' end and another quencher at the 3' end. Some probe manufacturers offer additional internal quencher, located in the middle of the probe to further reduce the effect of unnecessary 'noise'. In its intact form, probe does not emit detectable signal because the emitted energy from reporter dye is absorbed by the quencher, in a reaction called FRET (Crisalli and Kool, 2011). Figure 1.17 illustrates typical structure of a TaqMan hydrolysis probe.

Availability of various reporter dyes with distinctive emission and absorbance ranges enable multiplexing in TaqMan qPCR. Numbers of PCR target that can be detected by a TaqMan qPCR depends on the quantity of channel/detectors of the

real-time thermal cycler. To date, almost a dozen companies have developed real-time thermal cyclers. The companies are (according to seniority in the market) Applied Biosystems, Roche, Qiagen, BioGene, Bioneer, Bio-Rad, Cepheid, Corbett Research, Idaho Technology, MJ Research and Stratagene (Tajadini *et al.*, 2014). For instance, Bio-rad CFX96 Touch™ Real-Time PCR Detection System enables simultaneous detection of five different targets and do not require any reference dye, such as ROX (for calibration purpose) (Jordan and Kurtz, 2010). List of reporter dyes and quenchers available in the market, as well as their characteristics are included in Table 1.14.

In order to utilise all channel available within a real-time thermal cycle, reporter dyes and quenchers must be carefully selected. Such reporter dye ideally should have distinctive emission and absorbance range to avoid cross-talk. Crosstalk is an undesired phenomenon where fluorescence signal from a reporter dye is detected by more than 1 channel and results in false-positivity (Weidmann *et al.*, 2008).

During amplification, probe and two other primers hybridise to the complementary DNA targets. Elongation of amplicon will happen from the 5'end to the 3' end, of each primer. Once the new strand reaching the probe, the 5' exonuclease activity of *Taq* polymerase will cleave the reporter dye, releasing them from the probe. When free and no longer in close proximity with a quencher, the emitted energy of the reporter dye is detectable. This process happens in every amplification cycle. As the PCR products accumulate exponentially, stronger signals will be detected by the real-time PCR machine. Accumulation of 'free' reporter dye does not inhibit PCR amplification (Navarro *et al.*, 2015). Figure 1.18 illustrates amplification of DNA targets in a TaqMan qPCR assay. To date, several multiplex

TaqMan qPCR assays have been reported, for the detection of periodontal pathogens such as *Fusobacterium nucleatum, Porphyromonas gingivalis* and *Treponema denticola* and foodborne pathogens such as *Listeria monocytogenes, Giardia lamblia, Yersinia enterocolitica* and Enterovirus (Coffey *et al.*, 2016; Morrison *et al.*, 2015).

Table 1.14 Types of primer-probe used in qPCR. Adopted from http://www.genelink.com/oligo_modifications_reference/

Quencher & Dye Selction Guide

Dye Name	Excitation Max, nm	Emission Max, nm	Color	Quencher
Alexa 350	346	442	Blue	Dabcyl λ (max) = 453 nm Range = 380-530 nm
Pacific Blue	416	451		
Marina Blue	362	459	Blue-Green	
Acridine	362	462		
Edans	336	468	Green	
Coumarin	432	472		
BODIPY 493/503	493	503		
Cy2	489	506		
BODIPY FL-X	504	510	Yellow-Green	
DANSYL	335	518		
Alexa 488	495	519		BHQ-1 λ (max) = 534 nm Range = 480-580 nm
FAM	495	520		
Oregon Green	500	520		
Rhodamine Green-X	503	528		
NBD-X	466	535		
TET	521	536		
Alexa 430	434	541		
BODIPY R6G-X	529	547	Yellow	
JOE	520	548		
Yakima Yellow	531	549		
Alexa 532	532	554		
VIC	538	554		
HEX	535	556		
R6G	524	557		
Alexa 555	555	565	Yellow-Orange	BHQ-2 λ (max) = 579 nm Range = 550-650 nm
BODIPY 564/570	563	569		
BODIPY TMR-X	544	570		
Cy3	550	570		
Alexa 546	556	573		
TAMRA	555	576		
Rhodamine Red-X	560	580		
BODIPY 581/591	581	591		
Redmond Red	579	595		
Cy3.5	581	596		
ROX	575	602	Orange	
Alexa 568	578	603		
Cal Red	583	603		
BODIPY TR-X	588	616	Orange-Red	
Alexa 594	590	617		
BODIPY 630/650-X	625	640	Red	BBQ-650 λ (max) = 650 nm Range = 550-750 nm
LC Red 640	625	640		
Alexa 633	632	647		
BODIPY 650/665-X	646	660		
Alexa 647	650	665		
Cy5	649	670		
Alexa 660	663	690	Far Red	
Cy5.5	675	694		
Alexa 680	679	702		
LC Red 705	689	705		
Alexa 700	702	723		
Alexa 750	749	775		

Figure 1.17 Typical structure of TaqMan hydrolysis probe. Reporter dye (red circle) and quencher (black circle) located in close proximity within an oligonucleotide (blue line).

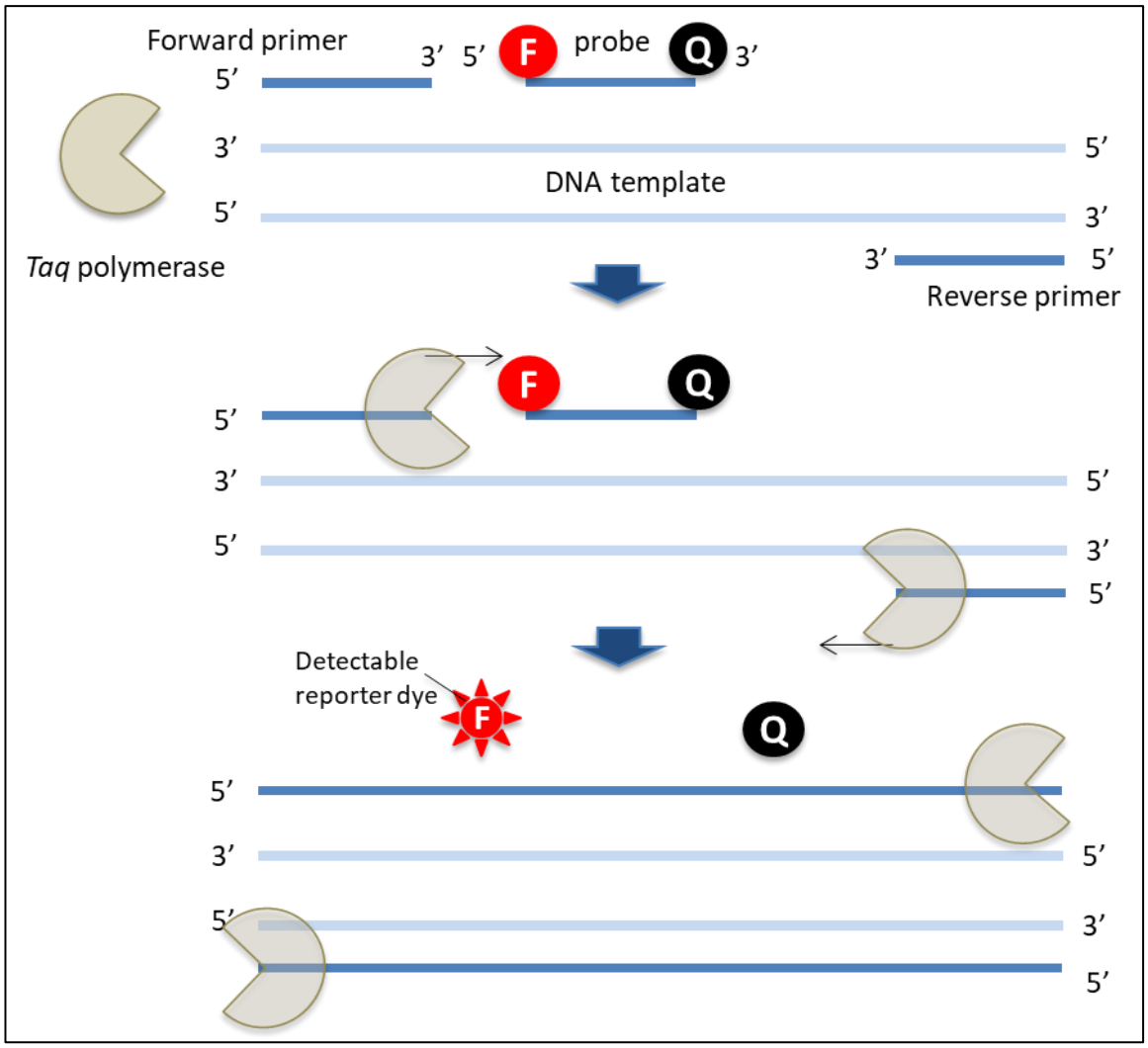


Figure 1.18 Amplification of DNA targets in TaqMan hydrolysis probe-based qPCR.

1.7 Rationale of the study

Leptospirosis, melioidosis, invasive salmonellosis and malaria are important causes of morbidity and mortality, worldwide, especially among those of the tropical and subtropical regions (WHO, 2014). Altogether, these communicable diseases accounted for 243.7 million cases and more than 1.4 million deaths, annually (Costa *et al.*, 2015; Crump *et al.*, 2015; Greenwood, 2017; Limmathurotsakul *et al.*, 2016).

Early detection enables prompt diagnosis and initiation of appropriate treatment. Subsequently, it increases patients' prognosis, reduces the risk of death, the burden of diseases and potential economic loss (Colzani *et al.*, 2017; Nii-Trebi, 2017). However, during the early phase of infection, patients develop febrile illnesses, together with other non-specific clinical features such as malaise, cough and diarrhoea, that perplexes clinical diagnosis (Beresford and Gosbell, 2016). As a result, complementary laboratory tests are required in order to confirm the diagnosis.

Currently, each of these infectious diseases is detected by a different conventional laboratory test. In leptospirosis case, the existing gold standard, MAT has inconsistent sensitivity & specificity and less useful during the early phase of infection (Limmathurotsakul *et al.*, 2012). Meanwhile, conventional culture for *B. pseudomallei* and *Salmonella* are laborious and time-consuming, requires more than 24 hours incubation (Hemarajata *et al.*, 2016). Microscopy examination for malarial parasites is specific, but less sensitive during low-level parasitaemia and entails experienced technicians (Lo *et al.*, 2015). In undesired circumstances, potential misdiagnosis happens when either of the laboratory tests is requested or available. On top of that, there is no laboratory test that can simultaneously detect *Leptospira*, *B. pseudomallei*, *Salmonella* and *Plasmodium* in a single reaction.

To address the urgent needs of a reliable diagnostic assay for early diagnosis of leptospirosis, melioidosis, invasive salmonellosis and malaria, this study is designed to exploit the flexible platform of hydrolysis probe-based qPCR for the detection of pathogens' DNA from clinical specimens. This assay will detect nucleic acids of pathogens, which usually exist during the first two weeks of infection, at high sensitivity and specificity. In addition to these four pathogenic targets, an extra built-in internal amplification control (IAC) is also included to exclude potential false negativity due to PCR inhibitors and others. This important IAC function is often omitted and unavailable in many reported molecular assays.

1.8 Objective of the study

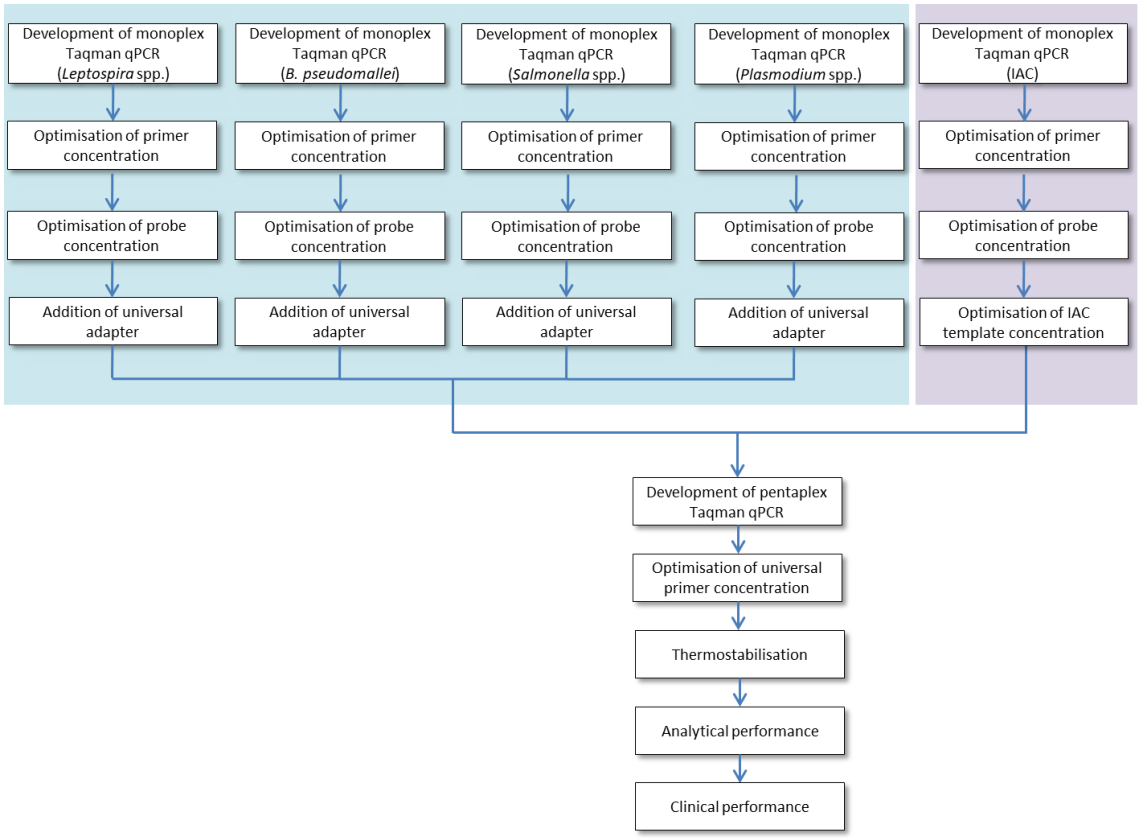
General objective:

To develop a sensitive and specific multiplex qPCR for the detection of pathogens associated with four common febrile infections (leptospirosis, melioidosis, invasive salmonellosis and malaria) simultaneously.

Specific objectives:

1. To design specific probes and primers, and optimize the developed multiplex qPCR.
2. To optimize the incorporation of internal amplification control (IAC) in the developed assay.
3. To perform the analytical evaluation (sensitivity & specificity) of the developed multiplex qPCR.
4. To evaluate the stability and clinical performance of the developed multiplex qPCR.

1.9 Overview of the study



CHAPTER 2

MATERIALS AND METHODS

2.1 General materials and equipment

2.1.1 Chemical and reagents

Details of chemicals and reagents used in the study are listed in Table 2.1.

2.1.2 Consumables and kits

Details of consumables and commercial kits used in the study are listed in Table 2.2 and Table 2.3.

2.1.3 Equipment

Details of the equipment used in the study are listed in Table 2.4.

Table 2.1 List of chemicals and reagents used in this study

Name	**Source**
5-fluorouracil	Merck, Germany
Absolute ethanol	Merck, Germany
Agarose powder	First Base Laboratories, Malaysia
Ampicillin	Sigma Aldrich, USA
Bacteriological agar	Oxoid, UK
Benzyl alcohol	Sigma Aldrich, USA
Deoxycholate Citrate Agar (DCA)	Oxoid, UK
Deoxyribonucleoside triphosphate (dNTPs)	Fermentas, Lithuania
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich, USA
GeneRuler™ 1 kb DNA Ladder	Thermo Scientific, USA
GeneRuler™ 100bp Plus DNA	Thermo Scientific, USA
Glycerol	Merck, Germany
Hydrochloric acid (HCL)	Merck, Germany
Leptospira Enrichment EMJH	Difco, USA
Leptospira Medium Base EMJH	Difco, USA
MacConkey No.3 powder	Oxoid, UK
Magnesium chloride ($MgCl_2$)	Sigma Aldrich, USA
Magnesium sulphate ($MgSO_4$)	Sigma Aldrich, USA
Mineral oil	Sigma Aldrich, USA
Mueller Hinton (MH) agar powder	Oxoid, UK
Mueller Hinton (MH) broth powder	Oxoid, UK
Oligonucleotides	Integrated DNA Technologies, Singapore
Orange G	Sigma Aldrich, USA
PCR grade water	Sigma Aldrich, USA
Proteinase K	Merck, Germany
Raffinose	BDH, UK
RedSafe™ Nucleic Acid Staining Solution	iNtRON Biotechnology, Korea
Sodium bicarbonate ($NaHCO_3$)	Sigma Aldrich, USA
Sodium carbonate (Na_2CO_3)	Sigma Aldrich, USA
Sodium chloride (NaCl)	Merck, Germany
Sodium citrate ($C_6H_5O_7Na_3$)	Merck, Germany
Sodium dihydrogen phosphate (NaH_2PO_4)	Sigma Aldrich, USA
Sodium hydroxide (NaOH)	Merck, Germany
Taq DNA polymerase	Fermentas, Lithuania
Trehalose	Merck, Germany
Tris-base	Merck, Germany
Tris-hydrochloride (Tris-HCl)	Invitrogen, USA

Table 2.2 List of kits used in this study

Name	Source
NucleoSpin® Blood QuickPure	Macherey Nagel, Germany
NucleoSpin® Tissue	Macherey Nagel, Germany
SsoAdvanced™ universal probes supermix	Bio-Rad Laboratories, USA

Table 2.3 List of consumables used in this study

Name	Source
Aluminium foil	Diamond, USA
Conical polypropylene tube	Corning, USA
CryoBeads™	Hardy Diagnostics, USA
Disposable Petri dish	Corning, USA
Disposable surgical face mask	Medicos, Malaysia
Filtered pipette tips	Axygen, USA & GoldenGate Bioscience, USA
Gloves	Iron Skin, Malaysia
Microcentrifuge tube	Axygen, USA
N95 Mask	3M, Korea
UV/Vis cuvette	Eppendorf, Germany

Table 2.4 List of equipment used in this study

Name	Source
Autoclave (Tomy ES-315)	Tomy Seiko, Japan
Balance (CPA225D)	Sartorius, Germany
Biological safety cabinet (E-series)	Esco Technologies, USA
Microcentrifuge (5424)	Eppendorf, Germany
Refrigerated centrifuge (5920)	Kubota, Japan
Microcentrifuge (Force 7)	Denver Instruments, USA
Centrifuge (Hettich Mikro 22R)	Hettich, Germany
Centrifuge (Micro 1730R)	LaboGene, Denmark
Chiller (Chill-1050)	Tech-Lab Scientific, Malaysia
Darkfield microscope (BX51)	Olympus, Japan
Dry cabinet (TD-100)	Eureka, Taiwan
Freezer -20 °C (LY350LT)	Snow, Malaysia
Freezer -20 °C (MDF-436)	Sanyo, Japan
Freezer -80 °C (VIP® Series)	Sanyo, Japan
Gel imaging system (Chemilmager 5500)	Alpha Innotech, USA
Heating block (IC-20)	Cole-Parmer, USA
Gel electrophoresis (Owl Separation Systems)	Thermo Scientific, USA
Ice maker (AF100)	Scotsman, USA
Incubator (IN260)	Memmert, Germany
Incubator Shakers (Innova 40)	New Brunswick, USA
Light microscope (CX31)	Olympus, Japan
Lyophiliser (Heto Lyolab 3000)	Thermo Scientific, USA
pH meter (CyberScan pH 510)	Thermo Scientific, USA
Pipettes (Pipetman)	Gilson, USA
Spectrophotometer (UV-Vis 1201)	Shimadzu, Japan
Spectrophotometer (BioPhotometer)	Eppendorf, Germany
Thermocycler – qPCR (CFX96)	Bio-Rad Laboratories, USA
Thermocycler – Conventional PCR (Mastercycler)	Eppendorf, Germany
Vacuum centrifugal concentrator (Heto)	Thermo Scientific, USA
Vacuum pump (RZ 6 rotary vane pump)	VacuuBrand, Germany
Vortex mixer (IKA® MS 3)	Mason Technology, Ireland
Water bath (TE-10A thermoreulator)	Techne, USA
Water distiller (W4L)	Favorit, UK

2.2 Preparation of chemical stocks, buffers and media

2.2.1 Preparation of buffer and stock solutions

2.2.1 (a) 5-Fluorouracil (5-FU) solution, 10 mg/ml

This stock solution was prepared by dissolving 1 g 5-FU antibiotic powder in 100 ml 0.02 M phosphate buffer, pH 7.4, followed by sterilisation using 0.2 μm syringe filter. The solution was stored at room temperature until further use.

2.2.1 (b) Hydrochloric acid (HCl) solution, 1 M

This solution was prepared by adding 41.7 ml 36% HCl into 300 ml distilled water. Following gentle mixture, solution mixture was adjusted to 500 ml using distilled water.

2.2.1 (c) Orange G, 0.2%

One gram Orange G powder was dissolved in 500 ml distilled water and sterilised using 0.2 μm syringe filter. The solution was stored at 4 – 8 °C until further use.

2.2.1 (d) Sodium hydrochloride (NaOH) solution, 1 N

This solution was prepared by adding 20 g NaOH into 500 mL distilled water, mixed well until complete dissolve and stored at room temperature.

2.2.1 (e) Tris-HCl solution, 0.1 M, pH 7.4

To prepare this solution, 7.88 g Tris-HCl was dissolved in 500 ml distilled water. The pH was adjusted to 7.4 using HCl. The solution was sterilised using 0.2 μm syringe filter and stored at room temperature until further use.

2.2.1 (f) Glycerol stock, 15% (v/v)

Fifteen ml glycerol was dissolved in 85 ml distilled water, mixed and sterilised at 121 °C for 15 minutes. The solution was stored at 4 – 8 °C until further use.

2.2.2 Preparation of culture media

2.2.2 (a) Liquid Ellinghausen-McCullough-Johnson-Harris (EMJH)

This leptospiral growth media was prepared by dissolving 2.3 g *Leptospira* medium base in 900 ml distilled water, with frequent stirring. Upon complete dissolution, the solution was autoclaved and allowed to cool. Next, 100 ml *Leptospira* enrichment EMJH and 200 μg/ml 5-FU were added, mixed thoroughly and dispensed into 15 ml polypropylene conical tubes, at a volume of approximately 8 ml. The aliquots were stored at 4 – 8 °C until further use.

2.2.2 (b) Semisolid EMJH

The steps were similar to the preparation of liquid EMJH, except additional 0.5 g bacteriological agar was added, together with 2.3 g *Leptospira* medium base in 900 ml distilled water.

2.2.2 (c) MH agar

Nineteen gram MH agar powder was dissolved in 500 ml distilled water and boiled. Following autoclaving at 121 °C for 15 minutes, the sterilised medium was poured into sterile disposable Petri dishes.

2.2.2 (d) MH broth

A mixture of 10.5 g MH broth powder in 500 ml distilled water was prepared and autoclaved at 121 °C for 15 minutes. The medium was allowed to cool down and aliquoted into sterile tubes.

2.2.2 (e) MacConkey No.3 agar

To prepare this agar, 25.75 g MacConkey agar powder was dissolved in 500 ml distilled water, boiled and autoclaved at 121 °C for 15 minutes. The medium was poured into sterile disposable Petri dishes.

2.2.2 (f) Deoxycholate Citrate Agar (DCA)

To prepare this agar, 24.25 g DCA was dissolved in 500 ml distilled water and boiled by frequent agitation using flame. As a precaution, the agar was not autoclaved or heated. The medium was poured into sterile disposable Petri dishes.

2.2.2 (g) Todd-Hewitt broth

Fifteen-gram Todd-Hewitt powder was dissolved in 500 ml distilled water and autoclaved at 121 °C for 15 minutes. Cool sterilised media were then transferred into sterile tubes.

2.3 Development of TaqMan qPCR assay

2.3.1 Designing of synthetic DNA for IAC target

Unlike the other microbial targets (*Leptospira*, *B. pseudomallei*, *Salmonella* and *Plasmodium*) that relied on genomic DNA as templates, synthetic double-stranded DNA (dsDNA) or gBlocks® Gene Fragments was used as the PCR target for IAC. The synthetic DNA was designed based on a fusion of *Mycobacterium tuberculosis rpoB* gene (Accession no. NC_000962.3: 759807-763325) and 83-110 bp region of *Entamoeba histolytica HLY5mc1* gene (Accession no. Z29969.1) (https://sg.idtdna.com/pages/products/genes/gblocks-gene-fragments/).

2.3.2 Designing of oligonucleotides

Primers and probes were designed using the online IDT PrimerQuest tool (https://sg.idtdna.com/Primerquest/Home/Index). A total of sixty-four nucleotide sequences of *Leptospira rrs* genes, *B. pseudomallei orf2* genes, *Salmonella StyR-3* gene, *Plasmodium* 18S ribosomal RNA (18S rRNA) genes and human GAPDH gene were retrieved from the National Center for Biotechnology Information (NCBI) database and used for the oligonucleotides designing.

The sequences' compositions were; 24 *Leptospira rrs* genes (as listed in Table 2.5), 10 *B. pseudomallei orf2* genes (as listed in Table 2.6), 15 *Salmonella StyR-3* gene (as listed in Table 2.7), 14 *Plasmodium* 18S rRNA genes (as listed in Table 2.8) and one human GAPDH gene (NC_000012.12, from 6534405 bp to 6538375 bp). The sequences were aligned using VectorNTI software and visualised using GeneDoc software to determine the conserved region. For the exogenous IAC, the respective oligonucleotides were designed against the synthetic DNA as described in section 2.3.1.

Initial specificities of the designed oligonucleotides were checked using online Basic Local Alignment Search Tool (BLAST) by the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3.3 Bioinformatics characterisation of oligonucleotides

Characteristic of the designed primers and probes were checked using online IDT OligoAnalyzer tool (https://sg.idtdna.com/calc/analyzer) for potential hairpin formation and primer dimerisation. Standard pre-set qPCR parameters were used during the bioinformatics analysis (50 mM Na^{+}, 3 mM Mg^{2+} and 0.8 mM dNTPs).

Table 2.5 List of accession numbers of *Leptospira rrs* gene sequences used for designing of primers and probe

No	Accession number	Location of gene	Species	Serovar	Group
1	AY631880	1 to 1432	*L. alexanderi*	Manhao	Pathogenic
2	AOHD02000026	296 to 1806	*L. alstonii*	Pingchang	Pathogenic
3	AM050581	1 to 1318	*L. borgpetersenii*	Ballum	Pathogenic
4	AY996794	1 to 1431	*L. interrogans*	Australis	Pathogenic
5	AY996791	1 to 1431	*L. interrogans*	Autumnalis	Pathogenic
6	EF536987	1 to 1385	*L. interrogans*	Bataviae	Pathogenic
7	DQ991476	1 to 1319	*L. kirschneri*	Agogo	Pathogenic
8	AHMP02000003	562206 to 563714	*L. kmetyi*	Malaysia	Pathogenic
9	KJ847187	1 to 1313	*L. mayottensis*	N/A	Pathogenic
10	AHOP02000022	21126 to 22634	*L. noguchii*	Autumnalis	Pathogenic
11	AY461889	1 to 1181	*L. santarosai*	N/A	Pathogenic
12	AY631877	1 to 1431	*L. weilii*	Celledoni	Pathogenic
13	AY792329	1 to 1431	*L. broomii*	N/A	Intermediate
14	AY996789	1 to 1464	*L. fainei*	Hurstbridge	Intermediate
15	AY631891	1 to 1431	*L. inadai*	Aguaruna	Intermediate
16	EF612278	1 to 1348	*L. licerasiae*	Varillal	Intermediate
17	EF025496	1 to 1318	*L. wolffii*	Khorat	Intermediate
18	AY631876	1 to 1422	*L. biflexa*	Patoc	Saprophytic
19	AB721966	1 to 1443	*L. idonii*	N/A	Saprophytic
20	AY631889	1 to 1422	*L. meyeri*	Hardjo	Saprophytic
21	AY631888	1 to 1422	*L. terpstrae*	Hualin	Saprophytic
22	AY631897	1 to 1422	*L. vanthielii*	Holland	Saprophytic
23	AY631879	1 to 1422	*L. wolbachii*	Codice	Saprophytic
24	AY631882	1 to 1422	*L. yanagawae*	Saopaulo	Saprophytic

Table 2.6 List of accession numbers of *B. pseudomallei* TTSS-*orf2* gene sequences used for designing of primers and probe

No	Accession number	Location of gene
1	AF074878.2	25391 to 26344
2	CP018404.1	2131427 to 2132380
3	CP018402.1	2693333 to 2694286
4	CP018388.1	2406331 to 2407284
5	LK936443.1	1885696 to 1886649
6	BX571966.1	1919731 to 1920690
7	AY626775.1	1 to 891
8	HM235938.1	1 to 131
9	CP004043.1	1726185 to 1727138
10	CP008891.1	1977666 to 1978619

Table 2.7 List of accession numbers of *Salmonella StyR-3* gene sequences used for designing of primers and probe

No	Accession number	Location of gene	Organism	Serovar	Strain
1	AL513382	622812 to 623305	*S. enterica*	Typhi	CT18
2	CP012151	1761103 to 1761596	*S. enterica*	Typhi	B/SF/13/03/195
3	LT906560	4137882 to 4138375	*S. enterica*	Typhi	OVG_041
4	LT904870	1639553 to 1640046	*S. enterica*	Typhi	SGB90
5	LT904881	3025972 to 3026465	*S. enterica*	Typhi	UI2120
6	CP009049	2860086 to 2860579	*S. enterica*	Paratyphi A	CMCC 50973
7	CP023508	2376776 to 2377269	*S. enterica*	Paratyphi A	FDAARGOS_368
8	CP000886	2486471 to 2486964	*S. enterica*	Paratyphi B	SPB7
9	CP000857	630078 to 630571	*S. enterica*	Paratyphi C	RKS4594
10	CP019177	3323035 to 3323528	*S. enterica*	Albany	ATCC 51960
11	CP007639	677299 to 677792	*S. enterica*	Choleraesuis	C500
12	CP018657	3841800 to 3841951	*S. enterica*	Enteritidis	92-0392
13	CP015924	3453164 to 3453315	*S. enterica*	Newport	Levine 15
14	CP022168	3417343 to 3417494	*S. enterica*	Typhimurium	WW012
15	CP014996	3538585 to 3539078	*S. enterica*	Weltevreden	1655

Table 2.8 List of accession numbers of *Plasmodium* 18S rRNA gene sequences used for designing of primers and probe

No	Accession number	Location of gene	Species
1	NC_004325.1	474888 to 477036	*P. falciparum*
2	XR_002273095.1	1 to 2149	*P. falciparum*
3	AL844501.2	473739 to 475887	*P. falciparum*
4	CP016991.1	474581 to 476729	*P. falciparum*
5	KF018657.1	1 to 2096	*P. ovale*
6	KF219559.1	1 to 2094	*P. ovale*
7	AB182493.1	1 to 2094	*P. ovale*
8	LT727657.1	74467 to 75768	*P. knowlesi*
9	LT727650.1	964364 to 965664	*P. knowlesi*
10	M54897.1	999 to 2319	*P. malariae*
11	LT594624.1	1104390 to 1105716	*P. malariae*
12	LT615240.1	136671 to 137951	*P. vivax*
13	LT615257.1	166254 to 167528	*P. vivax*
14	U07367.1	789 to 2063	*P. vivax*

2.3.4 Reconstitution of oligonucleotides stock solutions

Primers and probes were synthesised by Integrated DNA Technologies (IDT), Singapore and purified using standard desalting procedure by the same manufacturer. Upon arrival, the lyophilised primers and probes were centrifuged at 10, 000 × g for 3 minutes. One hundred µM oligonucleotides stocks were prepared by adding an appropriate amount of water (or TE buffer, for probes) to the lyophilised nucleotides. The reconstituted oligonucleotides were vortexed for 5 – 10 minutes, briefly centrifuged and stored at -20 °C until further use.

2.3.5 Preparation of oligonucleotides working solutions

Primers working stocks and probes working stocks were prepared in 20 µM concentration in water (or TE buffer, for probes). An appropriate amount of water of TE buffer was added to reach the desired concentration based on the $m_1v_1 = m_2v_2$ equation. The diluted oligonucleotides were briefly vortexed, centrifuged and stored at -20 °C until further use.

2.3.6 TaqMan qPCR assay

To determine the functionality of the designed oligonucleotides, TaqMan qPCR mixtures were prepared. Reactions consisting 1 × SsoAdvanced Universal Probes Supermix, 50 – 600 nM primers, 50 – 200 nM probes, 2 – 8 µl DNA template and water, adjusted to 20 µl, was prepared. The reactions were subjected to thermal cycling program as listed in Table 2.9.

Data from the assay were analysed using Biorad CFX Manager software. Baseline threshold was set at 25 RFU for FAM signal and 50 RFU for Texas Red, HEX, Cy5 and Cy5.5 signals. Any Cq ≤40 is considered positive (Bustin *et al.*, 2009).

Table 2.9 TaqMan qPCR cycling condition

Mode	**Temperature (°C)**	**Time**	**Cycle**
Initial denaturation	95	3 min	1
Denaturation	95	15 sec	50
Annealing & elongation	61.3	30 sec	

2.3.7 Optimisation of PCR parameters

2.3.7 (a) Optimisation of oligonucleotides' concentrations

To determine the optimal concentration of primers that result in the lowest Cq, PCR reactions were prepared as described in section 2.3.6. The primers concentrations were adjusted to 100 nM, 200 nM, 300 nM, 400 nM, 500 nM and 600 nM. The reactions were subjected to thermal cycling program as listed in Table 2.9.

Once optimal primers concentrations were determined, additional PCR reactions were prepared to define optimal probes concentration. Probes concentrations were adjusted to 50 nM, 100 nM, 150 nM, 200 nM. The reactions were subjected to thermal cycling program as listed in Table 2.9.

2.3.7 (b) Optimisation of unconjugated universal primer concentration

Presence of unconjugated universal primer may improve PCR efficiency (Yuan *et al*., 2009). To determine the optimal concentration of unconjugated universal primer, PCR reactions were prepared as described in section 2.3.6, using the previously determined optimal conditions. Additional unconjugated universal primer was added, at concentration of 100 nM, 200 nM, 300 nM and 400 nM. The reactions were subjected to thermal cycling program as listed in Table 2.9.

2.4 Analytical evaluation of the developed TaqMan qPCR assay

2.4.1 Reference strains

Microorganisms used for this study were divided into two categories; clinical isolates and environmental isolates. Of these isolates, *L. interrogans* serovar Canicola strain UPM, *B. pseudomallei* local strain (USM BUPS 15/07/A), *S. enterica* serovar Typhi (ATCC 7251) and *P. falciparum* (ATCC PRA¬405D) were used as reference strains.

2.4.2 Clinical isolates and ATCC strains

A total of 253 clinical isolates and ATCC strains, consisted of 27 *Leptospira*, 105 *B. pseudomallei*, 44 *Salmonella*, 31 *Plasmodium* and 46 other organisms were used in this study. The strains were obtained from the Department of Medical Microbiology & Parasitology, School of Medical Sciences, Universiti Sains Malaysia, Hospital Raja Perempuan Zainab II (HRPZII), Kota Bharu, Malaysia, Institute for Medical Research (IMR), Malaysia, Malaysia, Hospital Sultanah Nur Zahirah (HSNZ), Kuala Terengganu, Malaysia, Department of Veterinary Medicine, Universiti Putra Malaysia (UPM), Malaysia, Belgian Coordinated Collections of Microorganisms (BCCM), Belgium, London School of Hygiene & Tropical Medicine (LSHTM), England, American Type Culture Collection (ATCC), National Collection of Type Cultures (NCTC), London, United Kingdom and National Autonomous University of Mexico (UNAM), Mexico. Details of clinical and ATCC strains used in this study are listed in Table 2.10.

2.4.3 Environmental *Leptospira* isolates

A total of 104 environmental *Leptospira* isolates were obtained from Department of Medical Microbiology & Parasitology, School of Medical Sciences, Universiti Sains Malaysia and from peridomicilar area of leptospirosis patients residences (Azali *et al.*, 2016; M. R. M. R. Mohd Ali, Mohamad Safiee, Yusof, *et al.*, 2017). Details of environmental *Leptospira* isolates used in this study are listed in Table 2.11.

Table 2.10 List of clinical isolates and ATCC strains used in this study

Organism (*n* = 253)	**Source**	**No. of strains**
Acinetobacter baumanii	USM, Malaysia	1
Acinetobacter baumanii (ATCC 19606)	ATCC, USA	1
Aeromonas hydrophilia	USM, Malaysia	1
Aspergillus fumigatus	USM, Malaysia	1
Bacillus subtilis	USM, Malaysia	1
Burkholderia cepacia	USM, Malaysia	6
Burkholderia pseudomallei	USM, Malaysia	95
Burkholderia pseudomallei	HSNZ, Malaysia	10
Burkholderia thailandensis	USM, Malaysia	1
Campylobacter jejuni	USM, Malaysia	1
Candida albicans	USM, Malaysia	1
Citrobacter freundii	USM, Malaysia	1
Entamoeba dispar	UNAM, Mexico	1
Entamoeba histolytica	UNAM, Mexico	1
Entamoeba moshkovskii	LSHTM, England	1
Enterococcus avium (LMG 10744)	BCCM, Belgium	1
Enterococcus faecalis	USM, Malaysia	1
Enterococcus faecalis (LMG 16216)	BCCM, Belgium	1
Enterococcus faecium	USM, Malaysia	1
Enterococcus faecium (LMG 16200)	BCCM, Belgium	1
Enterococcus gallinarum	USM, Malaysia	1
Enterococcus hirae (LMG 6399)	BCCM, Belgium	1
Enterohemorrhagic *Escherichia coli* (EHEC)	IMR, Malaysia	1
Enteroinvasive *Escherichia coli* (EIEC)	IMR, Malaysia	1
Enteropathogenic *Escherichia coli* (EPEC)	IMR, Malaysia	1
Enterotoxigenic *Escherichia coli* (ETEC)	IMR, Malaysia	1
Haemophilus influenzae	USM, Malaysia	1
Klebsiella oxytoca	USM, Malaysia	1
Klebsiella ozaenae	USM, Malaysia	1
Klebsiella pneumoniae	USM, Malaysia	1
Leptospira biflexa serovar Patoc	IMR, Malaysia	1
Leptospira biflexa serovar Patoc	UPM, Malaysia	1
Leptospira borgpetersenii Celledoni	IMR, Malaysia	1
Leptospira borgpetersenii serovar Ballum	UPM, Malaysia	1
Leptospira fainei serovar Hurtsbridge	IMR, Malaysia	1
Leptospira fainei serovar Hurtsbridge	UPM, Malaysia	1
Leptospira interrogans serovar Australis	UPM, Malaysia	1
Leptospira interrogans serovar Autumnalis	IMR, Malaysia	1
Leptospira interrogans serovar Bataviae	IMR, Malaysia	1
Leptospira interrogans serovar Bataviae	UPM, Malaysia	1
Leptospira interrogans serovar Canicola	UPM, Malaysia	1

Organism (*n* = 253)	**Source**	**No. of strains**
Leptospira interrogans serovar Copenhageni	IMR, Malaysia	1
Leptospira interrogans serovar Djasiman	IMR, Malaysia	1
Leptospira interrogans serovar Hardjoprajitno	IMR, Malaysia	1
Leptospira interrogans serovar Hebdomadis	UPM, Malaysia	1
Leptospira interrogans serovar Icterohaemorrhagiae RGA	UPM, Malaysia	1
Leptospira interrogans serovar Javanica	IMR, Malaysia	1
Leptospira interrogans serovar Pomona	IMR, Malaysia	1
Leptospira interrogans serovar Pomona	UPM, Malaysia	1
Leptospira interrogans serovar Pyrogenes	IMR, Malaysia	1
Leptospira interrogans serovar Pyrogenes	UPM, Malaysia	1
Leptospira interrogans serovar Tarassovi	IMR, Malaysia	1
Leptospira licerasiae serovar Varillal	IMR, Malaysia	2
Leptospira meyeri serovar Semaranga	IMR, Malaysia	2
Leptospira wolffii	IMR, Malaysia	1
Listeria monocytogenes	UPM, Malaysia	1
Plasmodium falciparum	MKA Kota Bharu, Malaysia	10
Plasmodium falciparum (ATCC PRA405D)	ATCC, USA	1
Plasmodium knowlesi	MKA Kota Bharu, Malaysia	10
Plasmodium vivax	MKA Kota Bharu, Malaysia	10
Proteus vulgaris	USM, Malaysia	1
Proteus mirabilis	USM, Malaysia	1
Providencia stuartii	IMR, Malaysia	1
Salmonella Agona (ATCC 51957)	ATCC, USA	1
Salmonella Albany	USM, Malaysia	1
Salmonella Bordeaux	USM, Malaysia	1
Salmonella Braenderup (ATCC BAA-664)	ATCC, USA	1
Salmonella Emek	USM, Malaysia	1
Salmonella Enteritidis (ATCC 13076)	ATCC, USA	1
Salmonella Hadar	USM, Malaysia	1
Salmonella Heidelberg (ATCC 8326)	ATCC, USA	1
Salmonella Java	USM, Malaysia	1
Salmonella Kibi	USM, Malaysia	1
Salmonella Kissi	USM, Malaysia	1
Salmonella Newport	USM, Malaysia	1
Salmonella Paratyphi A (ATCC 9150)	ATCC, USA	1
Salmonella Paratyphi B (ATCC BAA 1250)	ATCC, USA	1
Salmonella Paratyphi C (ATCC 9068)	ATCC, USA	1
Salmonella Poona	USM, Malaysia	1
Salmonella Regent	USM, Malaysia	1
Salmonella Richmond	USM, Malaysia	1

Organism (*n* = 253)	**Source**	**No. of strains**
Salmonella Tshiongwe	USM, Malaysia	1
Salmonella Typhi	ATCC, USA	10
Salmonella Typhi (ATCC 7251)	USM, Malaysia	1
Salmonella Typhimurium (ATCC 14028)	ATCC, USA	1
Salmonella Uppsala	USM, Malaysia	1
Salmonella Virchow	USM, Malaysia	1
Salmonella Weltevreden (NCTC 6534)	NCTC, London	1
Salmonella spp.	USM, Malaysia	10
Shigella boydii	IMR, Malaysia	1
Shigella dysenteriae	IMR, Malaysia	1
Shigella flexneri	IMR, Malaysia	1
Shigella sonnei	IMR, Malaysia	1
Staphylococcus aureus	USM, Malaysia	1
Staphylococcus epidermidis	USM, Malaysia	1
Staphylococcus saprophyticus	USM, Malaysia	1
Vibrio cholerae	IMR, Malaysia	1
Vibrio parahaemolyticus	IMR, Malaysia	1

Table 2.11 List of environmental *Leptospira* isolates used in this study

Organism (*n* = 104)	**Isolation location**	**No. of isolates**
Pathogenic group		
L. alstonii	Pasar Wakaf Che Yeh	1
L. kmetyi	Jeram Linang	13
L. kmetyi	Kelantan & Terengganu	7
Intermediate group		
L. fainei	Jeram Linang	2
L. fainei	Kelantan or Terengganu (Unspecified)	1
L. inadai	Jeram Linang	2
L. inadai	Kelantan or Terengganu (Unspecified)	1
L. licerasiae	Jeram Linang	3
L. licerasiae	Kelantan & Terengganu	2
L. wolffii	Jeram Linang	26
L. wolffii	Pasar Wakaf Che Yeh	3
L. wolffii	Pasar Siti Khadijah	2
L. wolffii	Kelantan & Terengganu	3
Saprophytic group		
L. idonii	Jeram Linang	1
L. meyeri	Pasar Wakaf Che Yeh	18
L. meyeri	Jeram Linang	13
L. meyeri	Pasar Siti Khadijah	2
L. meyeri	Kelantan & Terengganu	4

2.4.4 Cultivation and maintenance of organisms

Liquid and semi-solid EMJH media were prepared for cultivation and short-term maintenance of *Leptospira*, respectively. For non-leptospiral bacteria, strains were cultured in MH broth. More fastidious bacteria, such as *Streptococcus* and *Haemophilus* were cultivated on enriched growth media such as Todd-Hewitt broth and commercial chocolate blood agar (CBA). Selective media such as MacConkey agar and DCA were also used for Enterobacteraciae. CryoBeads™ and/or 15% (v/v) glycerol stocks were used for long-term preservation of bacteria, stored at -80°C. Procedures for preparation of culture media were described in section 2.2.2.

Bacteria were revived by inoculating the stock into EMJH media (for *Leptospira*) or MH broth (for non-fastidious organism) or enriched growth media (for *Streptococcus* and *Haemophilus*) or other selective media. Inoculated EMJH media were incubated at 30°C, for 7 days on a shaker at 40 rpm and examined under dark field microscope for their presumptive hooked-ends, helical coils and motility. Other media were incubated at 37°C for 24 – 48 hours. Fresh cultures were used for subsequent genomic DNA extraction.

2.4.5 Genomic DNA extraction

Genomic DNA from clinical isolates were used in specificity testing. The extractions of the genomic DNA were performed using NucleoSpin® Tissue kit DNA from fresh bacterial cultures. Briefly, one millilitre of fresh bacterial culture was centrifuged at 8, 000 × g for 5 minutes. The supernatant was discarded and the pellet was re-suspended in 180 µl buffer T1, mixed, added with 25 µl Proteinase K, briefly centrifuged and incubated at 56 °C until complete lysis (at least 1 – 3 hours,

or overnight). Sample was vortexed, added with 200 µl buffer B3 and incubated at 70 °C for 10 minutes.

Following the addition of 210 µl absolute ethanol and vigorous vortex, sample was placed into column and collection tube and centrifuged at 11, 000 × g for 1 minute. Column was placed into a new collection tube, added with 500 µl buffer BW and centrifuged at 11, 000 × g for 1 minute. Column was placed into a new collection tube, added with 600 µl buffer B5 and centrifuged at 11, 000 × g for 1 minute. Column was placed into a new collection tube and centrifuged at 11, 000 × g for 1 minute. Next, the column was placed in a 1.5 ml microcentrifuge tube, applied with 100 µl of pre-warmed buffer BE (70 °C). Following incubation at room temperature for 10 minutes, DNA was eluted by centrifugation at 11, 000 × g for 1 minute. Dilution of DNA was carried out when necessary and stored at -20 °C.

2.4.6 Quantification and quality analysis of DNA

2.4.6 (a) DNA quantitation

DNA was quantitated using dsDNA program of Biophotometer at 260 nm wavelength. Fifty microliter elution buffer was used as a blank. Before sample reading, 2 µl DNA was added to 48 µl elution buffer. The purity of the diluted samples was also checked at the wavelengths of 230 nm, 280 nm and 320 nm.

2.4.6 (b) Agarose gel electrophoresis

Agarose gel electrophoresis was performed to determine the intactness and quality of the extracted genomic DNA or PCR products, when applicable. Briefly, 0.8% – 2% (w/v) agarose powder was dissolved in 0.5 × TE buffer, by heating the mixture in a microwave for 2 – 3 minutes. After the mixture was cooled to the

temperature of approximately 50 °C, 1 × RedSafe™ Nucleic Acid Staining Solution was homogeneously added. The gel was cast and allowed to solidify. Then, the gel was placed in the tank, emerged with 0.5 × TE buffer. Five microliter of DNA or PCR product was mixed with 1 µl 6 × Orange G loading dye and loaded to respective well of the gel. GeneRuler 100 bp Plus DNA Ladder was included to enable size estimation of the DNA or PCR product. The bands were separated by subjecting the gel to 150 V for 5 minutes and subsequent 100 V for 45 minutes. The bands were visualised using Alpha Innotech Chemi Imager 5500.

2.4.7 Analytical sensitivity

A set of 10-fold dilution of DNA templates from the reference strains were prepared, ranging from 10 ng μl^{-1} to 1 fg μl^{-1}. PCR reactions were prepared as described in section 2.3.6. Two microliter of each dilution was used, in triplicates. Standard curves were constructed based on the mean of Cq values and $\log_{10}$ (DNA copies). Copy numbers were calculated using an online tool by the URI Genomics & Sequencing Center (http://cels.uri.edu/gsc/cndna.html). The genome sizes for *Leptospira, B. pseudomallei, Salmonella and Plasmodium* are 4,627,366 bp (Nascimento *et al.*, 2004), 7,247,547 Mbp (Holden *et al.*, 2004), 4,809,037 bp (Parkhill *et al.*, 2001) and 22,853,764 bp (Gardner *et al.*, 2002), respectively. When specified, a 500 bp synthetic DNA was used to substitute *Plasmodium* DNA. Linearity and efficiency are calculated from logarithmic standard curves using Microsoft Excel.

Analytical sensitivity is defined by the lowest amount of DNA detectable by at least one of the replicates. Meanwhile, a more stringent definition was used for LOD, exemplified as the lowest concentration of DNA template detectable at 95%

likelihood (Bustin *et al.*, 2009). Twenty four replicates were performed in three occasions, using bacterial genomic DNA concentration of 80 fg, 40 fg, 20 fg, 5 fg and 2 fg per reaction and 20 ag, 10 ag, 5 ag, 2 ag and 0.2 ag of *Plasmodium* synthetic DNA per reaction.

2.4.8 Analytical specificity

Analytical specificity testing was carried out on reference strains, clinical isolates and environmental isolates as listed in Table 2.10 and Table 2.11. Twenty-nanogram DNA of each isolate was used as templates, unless specified otherwise. PCR reactions were prepared as described in section 2.3.6.

2.4.9 Assay precisions

2.4.9 (a) Repeatability (Intra-assay evaluation)

Repeatability is defined by the variation of replicates within the same run. PCR assays were prepared, as described in section 2.3.6, in triplicates. Standard deviation (SD) of the Cqs was calculated using Microsoft Excel. Two different template concentrations, 200 pg and 200 fg were used.

2.4.9 (b) Reproducibility (Inter-assay evaluation)

Reproducibility is defined by the variation of replicates between different runs. PCR assays were prepared, as described in section 2.3.6, in triplicates, for two different days. Standard deviation (SD) of the Cqs was calculated using Microsoft Excel. Two different template concentrations, 200 pg and 200 fg were used.

2.4.10 Stability of TaqMan qPCR assay

Lyophilisation of the multiplex assay was performed using Heto LyoLab 3000 freeze-dryer connected to a vacuum concentrator. Master mix solutions containing optimised amount oligonucleotides, 10% (w/v) trehalose and water were prepared, aliquoted into 0.5 ml microcentrifuge tubes, and lyophilised at 8×10^{-2} mBar for 2 hours. The tubes containing dried pellets were capped, sealed and kept in designated temperatures (4 °C, 25 °C, 37 °C and 45 °C) at different days until subsequent use. Appropriate controls such as pre-lyophilised master mix solutions were also included.

To test the lyophilised oligonucleotides, pellets were dissolved in water, added with 1 × SsoAdvanced Universal Probes Supermix and 2 – 8 µl DNA template. The assays were then subjected to thermal cycling condition as listed in Table 2.9.

The shelf-life was estimated using equations by (Clark, 1991); $\text{Estimated shelf life} = \left(\text{D} \times {Q_{10}}^{0.1(\text{AAT}-\text{AT})}\right) + \text{Actual age}$, where

D = duration at elevated temperature

Q_{10} = rate of chemical reaction

AAT = accelerated ageing temperature

AT = ambient temperature

2.5 Clinical evaluation of the developed TaqMan qPCR assay

Performance of the developed multiplex assays was evaluated on retrospective and prospective clinical samples. Retrospective samples were previously confirmed by gold standard methods or reference methods; leptospirosis – MAT/PCR/culture, melioidosis – culture, invasive salmonellosis – culture and malaria – BFMP/PCR. Meanwhile, prospective samples were collected from febrile patients presented to the Department of Emergency Medicine, Hospital Universiti Sains Malaysia.

2.5.1 Ethical approval

Prior to recruitments of patients, ethical approvals were obtained from appropriate ethical committees. This study protocol has been approved by the Human Ethics Committee of Universiti Sains Malaysia (Protocol code: USM/JEPeM/16080260) and the Medical Research and Ethics Committee (MREC) of the Ministry of Health Malaysia (Protocol code: NMRR-16-2117-33181) (Appendix A and B).

2.5.2 Sample size calculation

Sample sizes were calculated using public spreadsheet developed by Dr Wan Nor Arifin, from Universiti Sains Malaysia (Wan Nor Arifin, 2017). Sample size for retrospective specimens was estimated based on sensitivity & specificity formula by Buderer study (1996). Prevalence of leptospirosis, melioidosis, salmonellosis and malaria in the febrile patients were assumed to be 8.4% (Rafizah *et al*., 2013), 5% (Berger, 2018), 7.4% (Brown *et al*., 1984) and 6.2% (Berger, 2018), respectively. Expected sensitivity & specificity was set to 98% (Liesenfeld *et al*., 2014). Precision

was set at 10% with an alpha error of 0.05. Additional 10% drop-out was also included.

Meanwhile, the sample size for prospective specimens was calculated using the prevalence formula; $n = \frac{Z^2 P(1-P)}{d^2}$ by (Naing, 2003). Where n = sample size, Z = Z statistic corresponding to 95% confidence (1.96), P = expected prevalence (8.4%), and d = precision (0.05).

Details of sample sizes calculation (for both retrospective and prospective specimens) were listed in Appendix C.

2.5.3 Clinical samples collection

Retrospective clinical samples were collected based on the estimated sample sizes. EDTA blood samples from confirmed leptospirosis and malaria status were collected from the Makmal Kesihatan Awam Kota Bharu, Kelantan. The samples were previously tested for leptospiral DNA using GenoAmp® Real-Time PCR Leptospirosis kit or for malarial DNA using abTES™ Malaria qPCR I Kit. Besides, EDTA blood samples from confirmed melioidosis and invasive salmonellosis status were collected from the Department of Haematology and Department of Medical Microbiology & Parasitology, School of Medical Sciences, USM. The collected EDTA blood samples had parallel samples tested for viable organisms (i.e. *B. pseudomallei* isolated, *S.* Typhi isolated, no growth) through blood culture method.

Meanwhile, prospective EDTA blood samples from febrile patients with unknown status of leptospirosis, melioidosis, salmonellosis and malaria were collected from the Emergency Department, HUSM.

The inclusion criteria for prospective patients were:

- Aged more than 18 years old and
- Has fever with temperature more than 38 °C and/or
- Has suspected or proven severe infection or sepsis

Meanwhile, prospective patients with these conditions were excluded;

- Has suspected or proven healthcare-associated pneumonia (HAP/HCAP) and/or ventilator-associated pneumonia (VAP) and/or
- Has proven disease/s other than leptospirosis, melioidosis, invasive salmonellosis and malaria.

Clinical evaluation was carried out by preparing multiplex TaqMan qPCR as described in section 2.3.6. For each assay, eight microliter DNA template was used. Reactions were subjected to the cycling condition as described previously.

2.5.4 Genomic DNA extraction from clinical samples

Genomic DNA from clinical specimens was used for clinical evaluation. Total genomic DNA from blood specimens was extracted using NucleoSpin® Blood QuickPure kit. Briefly, 200 µl blood, 200 µl lysis buffer BQ1 and 25 µl Proteinase K were mixed and incubated at 70 °C for 10 – 15 minutes. Following the addition of 200 µl absolute ethanol, the sample was mixed, vortexed, applied onto the column and centrifuged at 11, 000 × g for 1 minute. Then 350 µl buffer BQ3 was added to the column and centrifuged at 11, 000 × g for 3 minutes. Another 200 µl buffer BQ3

was added to the column, followed by centrifugation at 11, 000 × g for 1 minute. The column was placed in a 1.5 ml microcentrifuge tube, applied with 50 µl of pre-warmed buffer BE (70 °C). Following incubation at room temperature for 10 minutes, DNA was eluted by centrifugation at 11, 000 × g for 1 minute. Eluted DNA samples were stored at -20 °C until further use.

Meanwhile, other clinical specimens, DNA were extracted using NucleoSpin® Tissue kit. Depending on the sample type, pre-treatment was necessary and performed according to the manufacturer's recommendations. For urine sample, 1 ml of the sample was centrifuged at 13, 000 × g for 30 minutes, followed by removal of the supernatant. This step was repeated three times. Then, the pellet was re-suspended in 180 µl buffer T1 and 25 µl Proteinase K. The subsequent steps were similar to the section 2.4.5.

In addition, blood culture fluid (BCF) samples were treated according to the M5 method of a previous study (Villumsen *et al.*, 2010). A hundred microliter of BCF was added to 100 µl lysis buffer (5 M guanidine hydrochloride in 100mM Tris-HCl, pH 8.0) and 10 µl proteinase K (20 mg/ml). Following incubation at room temperature for 10 minutes, 600 µl ultrapure water and 800 µl >99% benzyl alcohol were added to the mixture. Samples were mixed and centrifuged at 20, 000 × g for 5 minutes at room temperature. Two hundred microliter of the supernatant were collected and added to 200 µl of BQ1 buffer and 200 µl absolute ethanol, and were processed according to procedures for the blood specimens, using NucleoSpin® Blood QuickPure kit, as described previously.

2.5.5 Sequencing of PCR products

Any retrospective sample with a discordant result (negative by the reference method, positive by multiplex qPCR) was confirmed by sequencing. Briefly, another reaction tube containing 0.75 unit of *Taq* Polymerase, 1× PCR buffer, 0.16 mM dNTP, 2.5 mM $MgCl_2$, 200 nM of respective forward and reverse primers and five microliter PCR products. The reaction was adjusted to 20 µl using water. The mixture was then subjected to a primary denaturation at 95 °C for 3 minutes, followed by 40 cycles of; 94 °C for 30 seconds, 60 °C for 30 seconds, 72 °C for 30 seconds; and a final elongation at 72°C for 5 minutes. Subsequently, PCR products purification and sequencing were outsourced to Integrated DNA Technologies, Singapore.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Preparation of genomic DNA templates

L. interrogans serovar Canicola strain UPM, *B. pseudomallei* local strain (USM BUPS 15/07/A), *S. enterica* serovar Typhi (ATCC 7251) and *P. falciparum* (ATCC PRA¬405D) were used as reference strains. The remaining clinical isolates and environmental isolates were used in the subsequent analytical specificity analysis. Genomic DNA templates were extracted following cultivation of respective microorganisms using commercial extraction kits.

Quantity and quality of the extracted genomic DNA from reference strains were determined using UV spectroscopy. Ratios of absorbance at the wavelength of A_{260}/A_{280} and A_{260}/A_{230} were used as indicator of purity. Ideally, the ratio at A_{260}/A_{280} should lie between 1.8 and 2, which is accepted indicator for pure DNA. Ratio outside this range may indicate protein or RNA contamination. Meanwhile, ratio at A_{260}/A_{230} should ideally lies within 1.8 and 2.2 and is useful to indicate contamination by residual phenol, salt, protein, polysaccharide or guanidine, usually originating from the extraction reagent (Olson and Morrow, 2012).

Residual contamination may affect the spectrophotometric measurements. To further confirm the quality of the DNA, extracted samples were analysed using gel electrophoresis (Didelot *et al.*, 2013). This method provides an important indication of DNA intactness, as well as further estimation of the DNA quantity and sizes. A single high molecular band without smear indicated intact extracted genomic DNA, as well as two smaller bands at ~500 bp and ~250 bp which represented the

Plasmodium synthetic DNA and IAC synthetic DNA. (Figure 3.1 and Figure 3.2). Meanwhile, the presence of low molecular band may indicate RNA contamination.

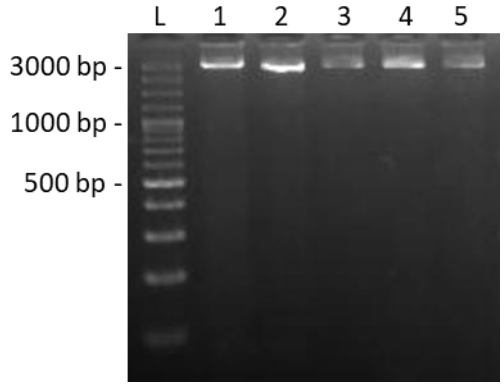


Figure 3.1 Gel electrophoresis of purified genomic DNA from *L. interrogans* (Lane 1), *B. pseudomallei* (Lane 2) and *S.* Typhi (Lane 3) and 2 other organisms (Lane 4 and 5). Lane L contained GeneRuler 100 bp Plus DNA Ladder.

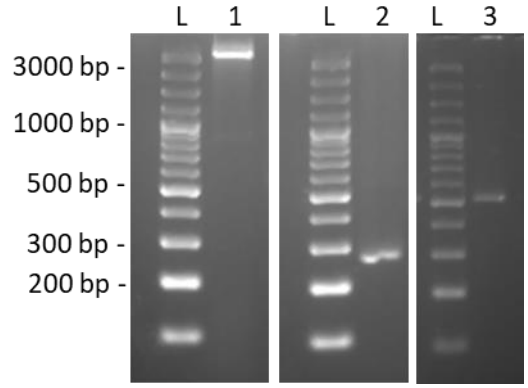


Figure 3.2 Gel electrophoresis of purified *P. falciparum* genomic DNA (Lane 1), IAC synthetic DNA (Lane 2) and *Plasmodium* synthetic DNA (Lane 3). Lane L contained GeneRuler 100 bp Plus DNA Ladder.

3.2 Preparation of synthetic DNA fragment as IAC template and *Plasmodium* DNA substitute

IAC was designed synthetically against a fusion of *M. tuberculosis rpoB* gene and 83-110 bp region of *E. histolytica HLY5mc1* gene (later will be denoted as IAC synthetic DNA or IAC target). Full sequence of the IAC synthetic DNA is listed in Figure 3.3. The quality of synthetic DNA fragment, also known as gBlocks® Gene Fragment (*rpoB*-EH synthetic DNA) was measured using UV spectrophotometer, as well as gel electrophoresis. A single molecular band at ~250 bp indicated intact synthetic DNA fragment (Figure 3.2).

In certain circumstances, *Plasmodium* synthetic DNA was used to substitute commercially purified *P. falciparum* strain 3D7 ATCC® PRA-405D. The 495-bp *Plasmodium* synthetic DNA was designed based on partial sequence of *P. falciparum* 18S rRNA gene. Full sequence of *Plasmodium* synthetic DNA is listed in Figure 3.4. Quality of the synthetic DNA was measured using UV spectrophotometer and gel electrophoresis. Figure 3.2 illustrated size and intactness of *Plasmodium* synthetic DNA, at a molecular band size of approximately 500 bp.

Figure 3.3 Sequence of IAC synthetic DNA used in this study. The locations of forward (green), reverse (red) primers and probe (brown) are highlighted in respective colours.

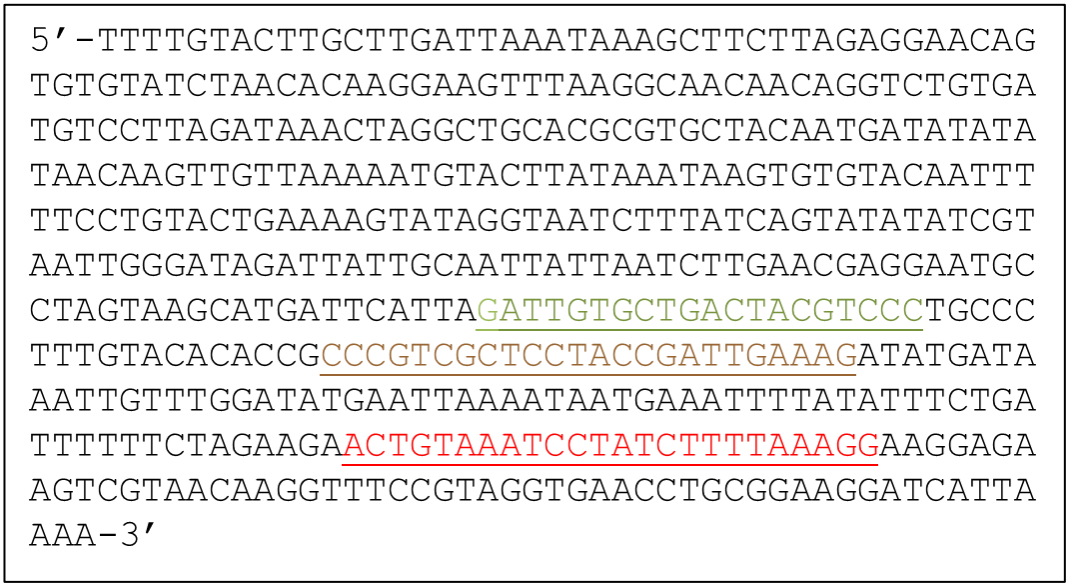


Figure 3.4 Sequence of *Plasmodium* synthetic DNA used in this study. The locations of forward (green), reverse (red) primers and probe (brown) are highlighted in respective colours.

3.3 Oligonucleotides design

3.3.1 Selection of target genes and sequence alignments

Selection of the target gene is an important initial step for designing and validating PCR assays. A target gene can be a specific gene for a particular microbial species or a functional gene coding for a protein or non-protein coding (NPC) sequences from a similar genus (Hanna *et al.*, 2005). Sometimes, a target gene can be selected from a polymorphic area of a common housekeeping gene (Adékambi *et al.*, 2009; Bondoso *et al.*, 2013; J. T. Foster *et al.*, 2008). The nature of the target gene is exploited in order to be used for broad detection of a particular microbial species or targeting a particular species or strain, depending on the study interest (Kumar and Chordia, 2015).

Several genes have been described and used for the detection of *Leptospira* spp.. Among the widely used genes is *lipL32* that encodes for a 32 kDa outer membrane lipoprotein, highly conserved among pathogenic leptospiral species (D A Haake *et al.*, 2000; Nurul Najian *et al.*, 2016). As increasing number of studies have reported the detection of intermediate and saprophytic *Leptospira* from human patients (Arzouni *et al.*, 2002; Chiani *et al.*, 2016; Krogfelt *et al.*, 2001), this study utilised *rrs* gene as PCR target for *Leptospira* genus. Twenty four *rrs* gene sequences from 22 *Leptospira* species have been retrieved from the NCBI database and aligned. *L. interrogans* serovar Bataviae (Accession no. EF536987) was selected as the reference sequence. From the alignment, a region between nucleotides 246 and 424 was selected due to high similarity among the leptospiral species (Figure 3.5).

Meanwhile, the *B. pseudomallei* type III secretion system (TTSS) genes cluster has been extensively studied and associated with the pathogenicity of the bacteria. This gene cluster encodes for more than 30 proteins, and several open reading frames (ORFs) (Gong *et al.*, 2015). Among the ORFs, *orf2* was selected as the target gene due to its uniqueness to the *B. pseudomallei*. A sequence from the AF074878.2 was used as the reference. Alignment of ten *orf2* genes revealed a region between nucleotides 554 and 651 that is conserved among the sequences, as shown in Figure 3.6.

Next, *StyR-3* gene was used as the *Salmonella* target. This non-protein coding (npc) gene is unique to the *Salmonella* genus (Chinni *et al.*, 2010). Fifteen *StyR-3* gene sequences from 10 serovars were retrieved and aligned. Sequence from AL513382 was set as the reference. A conserved region between nucleotide 161 and 312 was determined and used for oligonucleotide designing (Figure 3.7).

Another potential target, the 18S rRNA gene is a common house-keeping in eukaryotes, including *Plasmodium*. Fourteen 18S rRNA gene sequences were retrieved and aligned to determine specific malarial region. Sequence from NC_004325.1, of *P. falciparum* was used as the reference. A conserved region, specific to *Plasmodium* were found, located at nucleotide 1945 and 2097, as shown in Figure 3.8.

In the fifth target; IAC, a fusion of *M. tuberculosis rpoB* gene and 83-110 bp region of *E. histolytica HLY5mc1* gene was designed as PCR template. The template was ordered as a synthetic DNA fragment as illustrated in Figure 3.3.

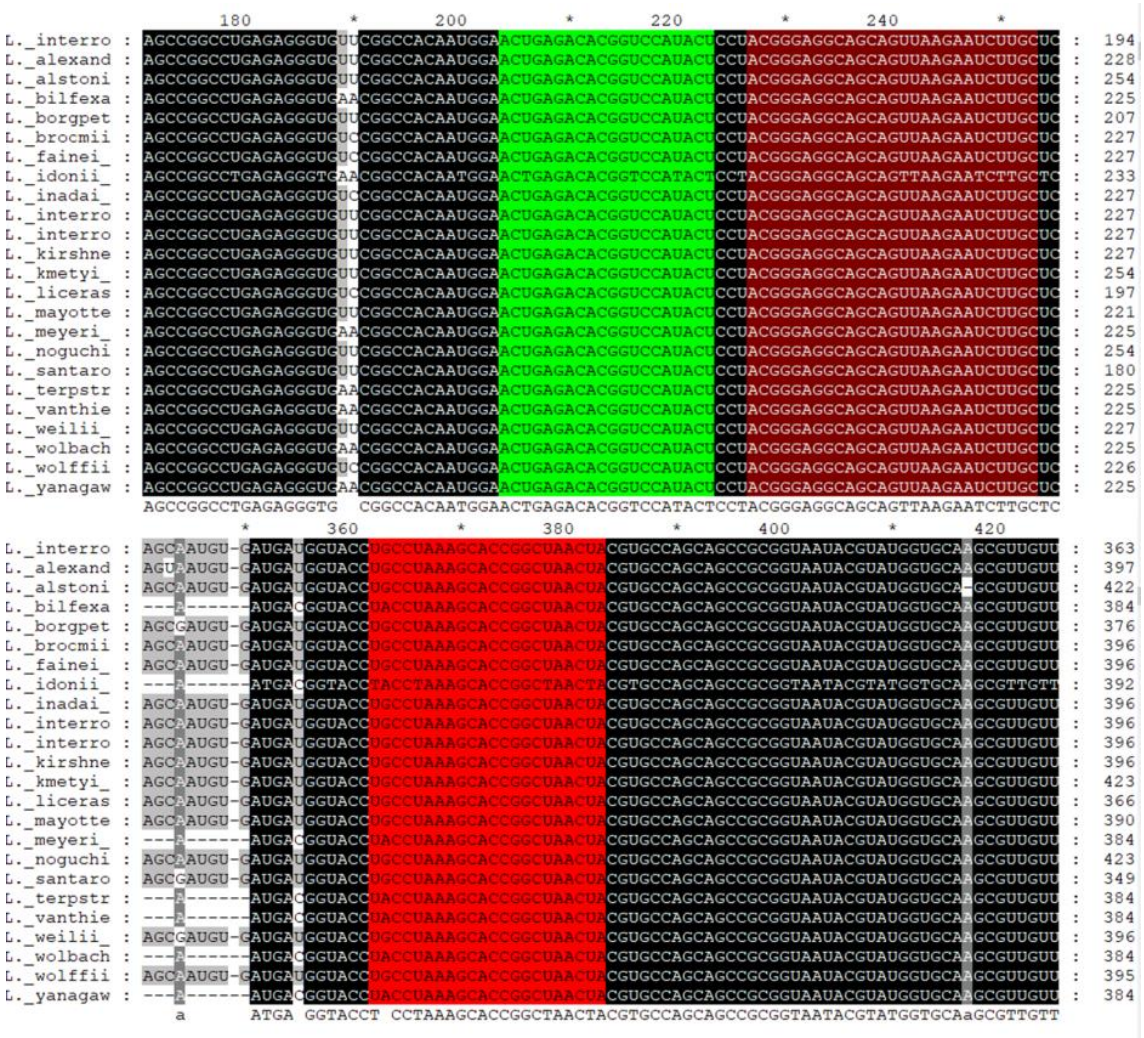


Figure 3.5 Alignment of selected *Leptospira rrs* gene sequences. Shaded areas represent forward (green), reverse (red) primers and probe (brown).

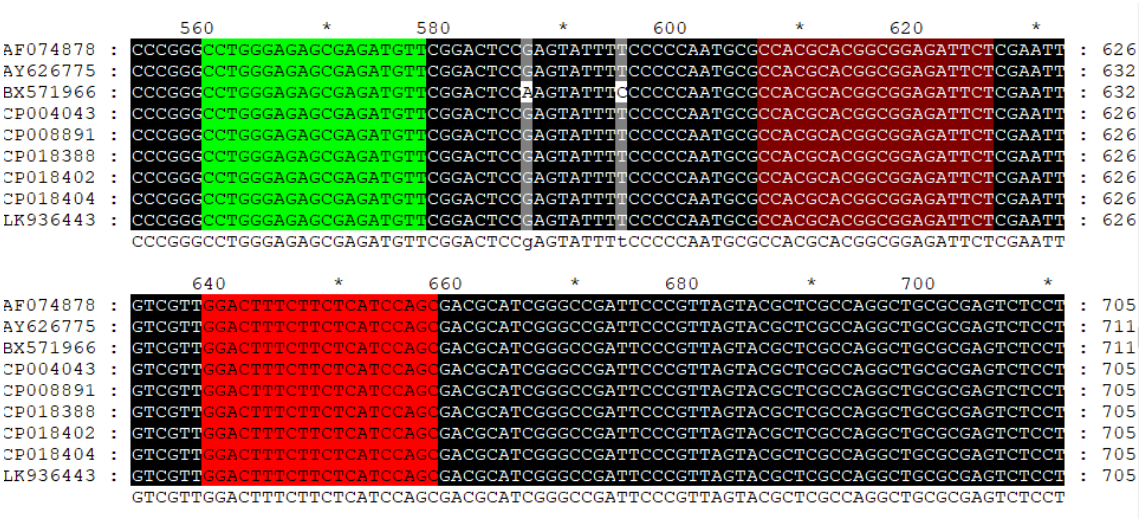


Figure 3.6 Alignment of selected *B. pseudomallei orf2* sequences. Shaded areas represent forward (green), reverse (red) primers and probe (brown).

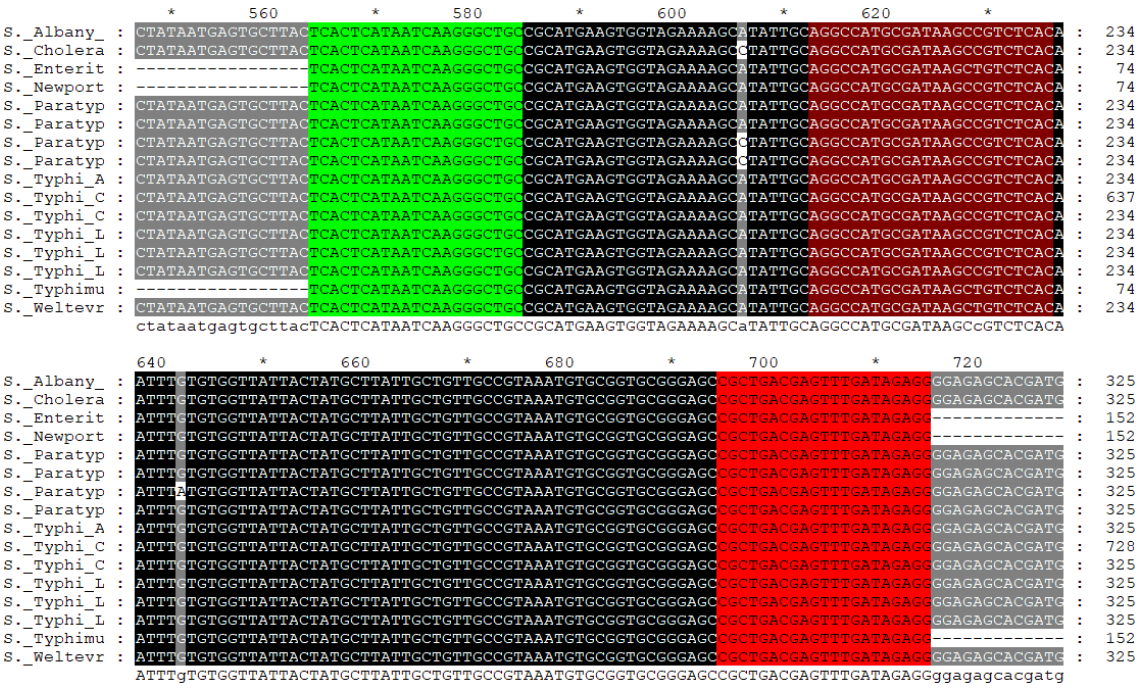


Figure 3.7 Alignment of selected *Salmonella StyR-3* sequences. Shaded areas represent forward (green), reverse (red) primers and probe (brown).

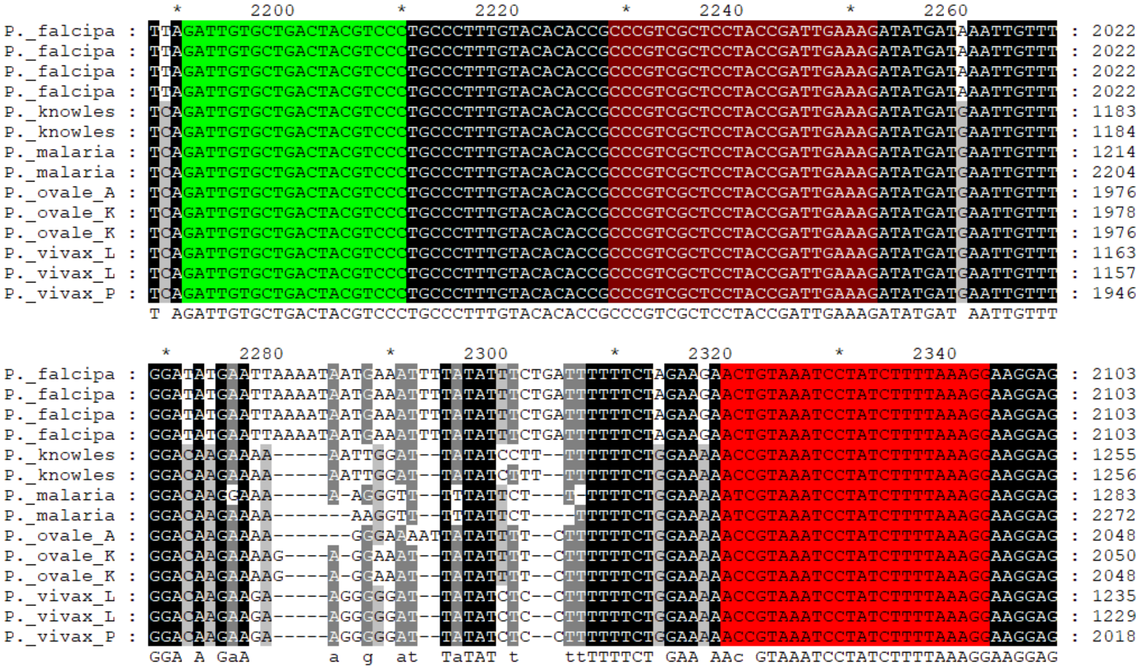


Figure 3.8 Alignment of selected *Plasmodium* 18S rRNA sequences. Shaded areas represent forward (green), reverse (red) primers and probe (brown).

3.3.2 Primers designs and characteristics

Following the identification of target region of the selected genes, primers were designed using IDT PrimerQuest tool. The sequences of the developed primers were listed in Table 3.1. Locations of primers on the respective gene alignments are shown in Figure 3.5 - Figure 3.8. Degenerative nucleotides were used when area of polymorphism occurred in the target.

In addition, a CT-rich universal adapter was added to each primer (except IAC), as a previous study had shown that modification at 5' end of primers may increase PCR efficiency (Yuan *et al.*, 2009). The sequences of modified primers (also denoted as primer with universal adapter or UN-primers) are listed in Table 3.2. Several parameters have been reported as optimal oligonucleotides characteristics. In terms of length, desirable primers should contain 18 – 35 bp (Basu, 2015). Some authors suggested slightly shorter ones, between 16 – 24 bp (J. Lim *et al.*, 2011). Without the addition of a universal adapter, the non-modified primers' lengths were still within the recommended range, 19 – 24 bp.

Conversely, following the addition of universal adapter, the length increased by 17 bp. Though this modification caused the primers' length to fall outside the optimal range, during the initial annealing and elongation, only the microbial-specific sequence will bind to the complementary target, leaving the adapter hanging. Full sequence of modified primers would play their roles in the subsequent cycles (Arif and Ochoa-Corona, 2013). Furthermore, previous studies have shown that this strategy may improve PCR efficiency (Afonina *et al.*, 2007; Yuan *et al.*, 2009). Therefore, *in vitro* validation is important in order to verify this hypothesis.

Another important primer characteristic is the GC content. Prior to the addition of universal adapter, all primers had GC% between 31.2% and 57.9%, except the IAC reverse primer that contained 64.7% (Table 3.1). This value is slightly higher than the GC% recommendation; 40 – 60% (Basu, 2015). Some guidelines tolerate much higher upper limit, up to 80% (Rodríguez *et al.*, 2015). Following addition of the universal adapter, the GC% increased to 46.4% and 62.2% (Table 3.2). Careful caution should be taken into consideration because high GC% and lengthy primer will increase melting temperature, which subsequently reduce PCR amplification efficiency and denaturation (J. Lim *et al.*, 2011). In the modified primer, the melting temperature range was 73.6 °C to 77.6 °C. It is anticipated that the melting temperature will increase proportionately to the oligonucleotide lengths (Arif and Ochoa-Corona, 2013).

Potential formation of secondary hairpin structure of primers was investigated using online OligoAnalyzer 3.1 software using default qPCR parameter setting. Ideally, hairpin structure should be avoided. However, its presence may be acceptable when the structure is readily denatured (or linearised) at low temperature. Table 3.3 summarises the potential hairpin formation of the developed primers and their melting temperatures. Without modification at 5' end, all primers have no potential formation of hairpin at ≥60 °C.

Meanwhile, additions of universal adapter to the primer affect hairpin structure slightly differently, as listed in Table 3.4, in which, the additional universal adapter to the forward primer of *B. pseudomallei* may result in hairpin formation, possible melts at 61.3 °C. Meanwhile, the other modified primers did not form hairpin structure at ≥60 °C.

Table 3.1 List of primers used in this study and respective characteristics

Organism	Primer type	Sequences (5' → 3' end)	Length (bp)	GC (%)	T_m (°C)
Leptospira	F	ACTGAGACACGGTCCATACT	20	50.0	62.3
	R	TAGTTAGCYGGTGCTTTAGGYA	22	45.5	63.2
B. pseudomallei	F	CCTGGGAGAGCGAGATGTT	19	57.9	63.1
	R	GCTGGATGAGAAGAAAGTCC	20	50.0	60.1
Salmonella	F	TCACTCATAATCAAGGGCTGC	21	47.6	62.3
	R	CCTCTATCAAACTCGTCAGCG	21	52.4	62.1
Plasmodium	F	GATTGTGCTGACTACGTCCC	20	55.0	62.2
	R	CCTTTAAAAGATAGGATTTACRGT	24	31.2	59.7
IAC	F	AAGGAGTTCTTCGGCACCA	19	52.6	63.5
	R	GGCGCTTGTGGGTCAAC	17	64.7	63.3

Table 3.2 List of primers with 5' end modification (UN-primers) used in this study and respective characteristics. Letters in brown represent universal sequences used as an adapter or an universal primer.

Organism	Primer type	Sequences (5' → 3' end)	Length (bp)	GC (%)	T_m (°C)
Leptospira	UN-F	CCTTCCTTCCTTCCCCCC-ACTGAGACACGGTCCATACT	38	57.9	76.1
	UN-R	CCTTCCTTCCTTCCCCCC-TAGTTAGCYGGTGCTTTAGGYA	40	55.0	75.9
B. pseudomallei	UN-F	CCTTCCTTCCTTCCCCCC-CCTGGGAGAGCGAGATGTT	37	62.2	77.6
	UN-R	CCTTCCTTCCTTCCCCCC-GCTGGATGAGAAGAAAGTCC	38	57.9	75.6
Salmonella	UN-F	CCTTCCTTCCTTCCCCCC-TCACTCATAATCAAGGGCTGC	39	56.4	75.6
	UN-R	CCTTCCTTCCTTCCCCCC-CCTCTATCAAACTCGTCAGCG	39	59.0	76.0
Plasmodium	UN-F	CCTTCCTTCCTTCCCCCC-GATTGTGCTGACTACGTCCC	38	60.5	76.4
	UN-R	CCTTCCTTCCTTCCCCCC-CCTTTAAAAGATAGGATTTACRGT	42	46.4	73.6
Universal	UN	CCTTCCTTCCTTCCCCCC	18	66.7	63.3

Table 3.3 Summary of hairpin structure presence among primers at 60 °C and other temperatures

Organism	**Primer type**	**Presence of hairpin**	
		at ≥ 60 °C	**at < 60 °C**
Leptospira	Forward	No	Yes, 32 °C
	Reverse	No	Yes, 44.7 °C
B. pseudomallei	Forward	No	Yes, 13.4 °C
	Reverse	No	Yes, 31.2 °C
Salmonella	Forward	No	Yes, 16.5 °C
	Reverse	No	Yes, 35.4 °C
Plasmodium	Forward	No	Yes, 24.2 °C
	Reverse	No	Yes, 31.9 °C
IAC	Forward	No	Yes, 29.4 °C
	Reverse	No	Yes, 32 °C
Universal	UN	No	No

Table 3.4 Summary of hairpin structure among UN-primers at 60 °C and other temperatures

Organism	**Primer type**	**Presence of hairpin**	
		at ≥ 60 °C	**at < 60 °C**
Leptospira	UN-Forward	No	Yes, 32.0 °C
	UN-Reverse	No	Yes, 43.3 °C
B. pseudomallei	UN-Forward	Yes, 61.3 °C	Yes, 54.3 °C
	UN-Reverse	No	Yes, 39.8 °C
Salmonella	UN-Forward	No	Yes, 51.8 °C
	UN-Reverse	No	Yes, 35.4 °C
Plasmodium	UN-Forward	No	Yes, 32.7 °C
	UN-Reverse	No	Yes, 39.7 °C

3.3.3 Probes designs and characteristic

Probes were designed using IDT PrimerQuest tool, located between the developed forward and reverse primers. The sequences of the developed probes were listed in Table 3.5. Ideally, the melting temperature should be 6 – 10 °C higher than the primers. In comparison to the unmodified primers, the T_m differences were in parallel to the recommendation. However, following the addition of universal adapter, the modified primers had higher T_m than the probes. This less desired scenario might affect PCR because there is possible tendency where the modified primers will hybridise earlier to the target DNA than the probe. As a result, amplification might happen without probes which subsequently reduce the PCR performance. Careful optimisation and *in vitro* validation should be carried out to rule out these undesirable possibilities.

Similarly, potential formation of secondary hairpin structure of probes was investigated using online OligoAnalyzer 3.1 software using default qPCR parameter setting. Even though hairpin structure may compromise PCR, its existence may be acceptable when the structure is readily denatured (or linearised) at low temperature. Table 3.6 summarises the potential hairpin formation of the developed probes and their melting temperature. At the temperature of 60°C and higher, the probes did not form any hairpin.

In addition, autolysis of probe may happen in the event where the 5’ of the probe contain guanine. In the designed probes, no guanine base is located at the mentioned location.

Table 3.5 List of probes used in this study and respective characteristics

Organism	**Sequences (5' → 3' end)**	**Size (bp)**	**GC (%)**	**T_m (°C)**
Leptospira	FAM-ACGGGAGGCAGC-ZEN-AGTTAAGAATCTTGC-IBFQ	27	51.9	69.9
B. pseudomallei	TexRed-CCACGCACGGCGGAGATTCT-IBRQ	20	65.0	68.6
Salmonella	HEX-AGGCCATGCGAT-ZEN-AAGCTGTCTCAC-IBFQ	24	54.2	68.1
Plasmodium	Cy5-CCCGTCGCTCCT-TAO-ACCGATTGAAAG-IBRQ	24	58.3	67.9
IAC	Cy5.5-TTCCTGCTATTCTCATTCGCATCCATGT-IBRQ	28	42.9	67.9

Table 3.6 Summary of hairpin structure presence among probes at 60 °C and other temperatures

Organism	**Presence of hairpin**	
	at ≥ 60 °C	**at < 60 °C**
Leptospira	No	Yes, 36.4 °C
B. pseudomallei	No	Yes, 56.5 °C
Salmonella	No	Yes, 46.4 °C
Plasmodium	No	Yes, 31.2 °C
IAC	No	Yes, 31.0 °C

3.3.4 Self- and hetero-dimerisation activities between oligonucleotides

Dimerisation of primers also may affect PCR amplification. Generally, ΔG value of ≤9 kcal mole^{-1} should be avoided and are deemed to favour production of dimerisation product. To determine the possible dimerisation, the sequences of oligonucleotides were analysed using IDT OligoAnalyzer using default qPCR parameters. The ΔG values of each primer pairs were listed in Table 3.7. Noticeably, three primer pairs had ΔG values of between 9.61 and -12.3 kcal mole^{-1}.

Meanwhile, the ΔG values of each probe versus primer or probe were listed in Table 3.8 and Table 3.9, respectively. Four probe-primer and four probe-probe pairs had ΔG value of less than -9 kcal mole^{-1}. Analytical performance should be performed in order to validate the functionality of these primers and probes in the multiplex reaction. In contrast to primers, probes are less susceptible to dimerisation due to the presence of reporter dyes and quenchers in close proximity that may affect its structure (Johansson, 2006).

Furthermore, the effects of additional universal adapter to each primer were also investigated. Summary of potential primer dimerisation was listed in Table 3.10. Following addition of universal adapter to primers, three more primer pairs had ΔG values of less than -9 kcal mole^{-1}. Similarly, extra four pairs of probe versus UN-primers had ΔG values of more than -9 kcal mole^{-1} (Table 3.11). It is anticipated that addition of universal adapter will affect the ΔG values (Arif and Ochoa-Corona, 2013).

Table 3.7 Potential dimerisations between primers used in this study. The ΔG values (kcal mole^{-1}) were coloured in gradient, from green (the highest value) to red (the lowest value).

Primers interaction (Primer vs. primer)		*Leptospira*		*B. pseudomallei*		*Salmonella*		*Plasmodium*		IAC	
		F	R	F	R	F	R	F	R	F	R
Leptospira	F	-3.61									
	R	-6.68	-8.19								
B. pseudomallei	F	-5.02	-5.47	-3.61							
	R	-6.6	-5.49	-4.74	-4.64						
Salmonella	F	-5.13	-7.07	-4.74	-6.6	-3.14					
	R	-5.13	-8.26	-6.78	-6.69	-6.69	-3.61				
Plasmodium	F	-4.95	-4.74	-7.71	-4.64	-6.14	-9.61	-6.3			
	R	-4.89	-5.49	-4.67	-5.49	-6.61	-5.61	-4.95	-8.74		
IAC	F	-4.64	-12.3	-5.19	-8.64	-5.09	-6.46	-6.44	-6.61	-3.61	
	R	-5.02	-5.47	-8.35	-3.54	-5.5	-8.35	-4.61	-4.12	-5.02	-9.89

Table 3.8 Potential dimerisations between primers and probes used in this study. The ΔG values (kcal mole^{-1}) were coloured in gradient, from green (the highest value) to red (the lowest value).

Oligonucleotides interaction (Primer vs. probe)		*Leptospira*	*B. pseudomallei*	*Salmonella*	*Plasmodium*	IAC
		Probe	Probe	Probe	Probe	Probe
Leptospira	F	-4.89	-4.64	-10	-8.02	-6.7
	R	-6.69	-8.26	-7.07	-7.19	-5.85
B. pseudomallei	F	-4.67	-6.75	-5.02	-11.5	-8.33
	R	-6.69	-5.12	-6.7	-4.64	-10.1
Salmonella	F	-9.83	-5.09	-6.21	-6.14	-5.09
	R	-6.24	-6.75	-6.69	-8.35	-6.75
Plasmodium	F	-8.65	-6.44	-4.74	-4.95	-5.09
	R	-6.59	-3.61	-4.89	-8.02	-6.24
IAC	F	-7.06	-3.61	-6.21	-8.26	-6.24
	R	-4.74	-6.75	-6.75	-6.75	-6.75

Table 3.9 Potential dimerisations between probes used in this study. The ΔG values (kcal mole^{-1}) were coloured in gradient, from green (the highest value) to red (the lowest value).

Oligonucleotides interaction (Probe vs. probe)		*Leptospira*	*B. pseudomallei*	*Salmonella*	*Plasmodium*	IAC
		Probe	Probe	Probe	Probe	Probe
Leptospira	Probe	-5.12				
B. pseudomallei	Probe	-9.77	-6.75			
Salmonella	Probe	-6.69	-8.7	-9.28		
Plasmodium	Probe	-11.1	-8.02	-8.33	-5.19	
IAC	Probe	-8.29	-6.75	-11.8	-5.19	-5.38

Table 3.10 Potential dimerisations between modified primers used in this study. The ΔG values (kcal mole^{-1}) were coloured in gradient, from green (the highest value) to red (the lowest value).

Oligonucleotides interaction (Between UN-primers)		*Leptospira*		*B. pseudomallei*		*Salmonella*		*Plasmodium*		IAC		Universal primer
		UN-F	UN-R	UN-F	UN-R	UN-F	UN-R	UN-F	UN-R	F	R	UN
Leptospira	UN-F	-3.61										
	UN-R	-6.68	-8.19									
B. pseudomallei	UN-F	-8.09	-7.71	-7.71								
	UN-R	-6.68	-5.85	-8.35	-5.12							
Salmonella	UN-F	-6.61	-7.74	-7.74	-6.61	-7.74						
	UN-R	-5.13	-8.26	-7.71	-10.3	-7.74	-3.61					
Plasmodium	UN-F	-6.68	-5.85	-7.71	-5.12	-6.61	-9.61	-6.3				
	UN-R	-6.24	-6.24	-7.71	-6.68	-9.68	-6.24	-6.68	-8.74			
IAC	F	-8.19	-12.3	-8.19	-8.64	-8.19	-8.19	-8.26	-8.19	-3.61		
	R	-9.43	-6.14	-8.35	-6.75	-6.14	-8.35	-6.14	-6.14	-5.02	-9.89	
Universal primer	UN	-3.07	-4.67	-7.71	-5.12	-6.61	-1.94	-1.57	-6.24	-8.19	-6.14	0

Table 3.11 Potential dimerisations between modified primers and probes used in this study. The ΔG values (kcal mole^{-1}) were coloured in gradient, from green (the highest value) to red (the lowest value).

Oligonucleotides interaction (Between UN-primers and probes)		*Leptospira*	*B. pseudomallei*	*Salmonella*	*Plasmodium*	IAC
		Probe	Probe	Probe	Probe	Probe
Leptospira	UN-F	-7.71	-4.64	-10	-8.02	-6.7
	UN-R	-7.71	-9.82	-6.68	-8.02	-5.7
B. pseudomallei	UN-F	-7.71	-6.75	-5.02	-11.5	-8.33
	UN-R	-9.75	-9.82	-6.75	-4.64	-10.1
Salmonella	UN-F	-9.83	-5.09	-6.21	-6.14	-5.09
	UN-R	-7.71	-6.75	-6.69	-8.35	-6.75
Plasmodium	UN-F	-9.75	-6.68	-4.74	-5.19	-5.19
	UN-R	-7.71	-4.64	-4.89	-8.02	-6.24
IAC	F	-7.06	-3.61	-6.21	-8.26	-6.24
	R	-4.74	-6.75	-6.75	-6.75	-6.75
Universal primer	UN	-7.71	-4.64	-4.67	-3.54	0

3.3.5 Amplicon sizes and characteristics

PCR product or amplicon is also another important factor for successful PCR. Ideally, amplicon size should be between 50 and 150 bp. Even though shorter amplicon is desired, ones with less than 400 bp can be amplified efficiently (Rodríguez *et al.*, 2015). In addition, CG content should remain moderate because high GC content (>80%) can potentially reduce or cause unsuccessful PCR amplification (Naz and Fatima, 2013). In this assay, the amplicon sizes are less than 250 bp (107 – 215 bp) and contain between 40.7% and 58.5% GC (Table 3.12). Theoretically, at 95 °C, all amplicons are readily denatured.

Table 3.12 Amplicon sizes and respective characteristics

Organism	**Target gene**	**Length (bp)**	**GC content (%)**	**T_m (°C)**
Leptospira	16S rRNA	215	58.5	76.1
B. pseudomallei	TTSS-*orf2*	135	53.0	79.4
Salmonella	*StyR*-3	188	51.6	78.0
Plasmodium	18S rRNA	189	40.7	79.9
IAC	*rpoB-HLY5mc1* fusion	107	55.1	81.0

3.4 Development of monoplex TaqMan qPCR for the detection of *Leptospira* spp., *B. pseudomallei*, *Salmonella* spp. and *Plasmodium* spp. DNA

3.4.1 Functionality of each monoplex TaqMan qPCR

Following *in silico* validation of respective primers and probes, the developed oligonucleotides were tested *in vitro* to confirm their functionality. PCR reactions consisted 1 × SsoAdvanced Universal Probes Supermix, 200 nM primers of each primer pair, 100 nM probe, 2 µl DNA template (10 – 20 ng μl^{-1}) and water were prepared and subjected to thermal cycling condition as described in Table 2.9.

3.4.1 (a) Functionality of monoplex assay for detection of *Leptospira* spp. DNA

Functionality of primers pair and probe for the detection of *Leptospira* on real-time PCR platform was investigated. Selected strains from pathogenic, intermediate and saprophytic *Leptospira* group were used as PCR template.

Amplification of desired products was observed at first channel of the CFX machine, which captured signal of 450 - 730 nm wavelengths. For the leptospiral probe, FAM probe was used (Wavelength: 450 – 490 nm). As shown in Figure 3.9, the developed monoplex assay was able to amplify all of the tested leptospiral strains which included the *L. interrogans* serovar Canicola strain UPM strains and five other *Leptospira* spp. with Cq values ranged from 18 to 20. Next, the preliminary specificity of the developed assay on *B. pseudomallei*, *Salmonella sp.*, *Plasmodium sp.* and healthy human DNA was investigated.

3.4.1 (b) Functionality of monoplex assay for detection of *B. pseudomallei* DNA

Functionality of primers pair and probe for the detection of *B. pseudomallei* on real-time PCR platform was investigated. Several *B. pseudomallei* isolates were used as PCR template. Amplification of PCR products was expected at wavelength of 560 – 590 nm, through the Texas Red channel. As shown in Figure 3.10, the developed monoplex assay was able to amplify *B. pseudomallei* reference strain (USM BUPS 15/07/A) and six other *B. pseudomallei* isolates with Cq values between 26 and 28. Next, the preliminary specificity of the developed assay on *L. interrogans, Salmonella sp., Plasmodium sp.* and healthy human DNA was investigated.

3.4.1 (c) Functionality of monoplex assay for detection of *Salmonella* spp. DNA

Functionality of the developed primers and probe for detection of *Salmonella* in monoplex assay were tested against selected *Salmonella* strains. Specific HEX-labelled probe, detectable at wavelength of 515 – 535 nm was selected.

As shown in Figure 3.11, the developed assay was able to amplify the tested *Salmonella* Typhi reference strains and other *Salmonella* isolates with Cq values ranging from 25 to 27. Next, the preliminary specificity of the developed assay on *L. interrogans, B. pseudomallei, Plasmodium sp.* and healthy human DNA was investigated.

3.4.1 (d) Functionality of monoplex assay for detection of *Plasmodium* spp. DNA

Functionality of the designed oligonucleotides for amplification of malarial nucleic acid was determined using *P. falciparum* reference strain (ATCC PRA¬405D), synthetic *Plasmodium* DNA and three other *Plasmodium* strains. A Cy5-labelled probe, detectable at wavelength 620 – 650 nm was used. As shown in Figure 3.12, the designed primers and probe was able to amplify the entire tested malarial DNA. Next, the preliminary specificity of the developed assay on *L. interrogans, B. pseudomallei, Salmonella sp.* and healthy human DNA was investigated.

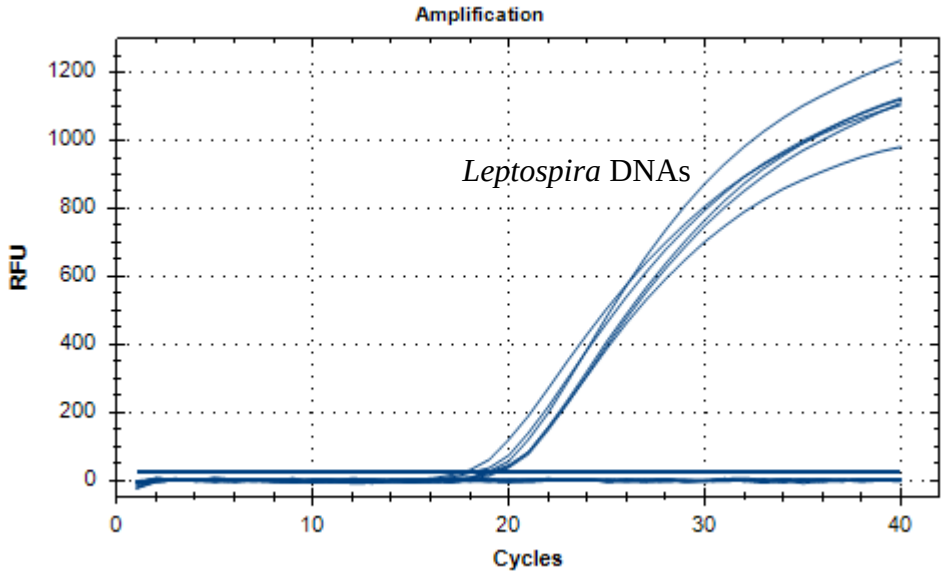


Figure 3.9 Amplification of *Leptospira* spp. *rrs* genes in the monoplex assay

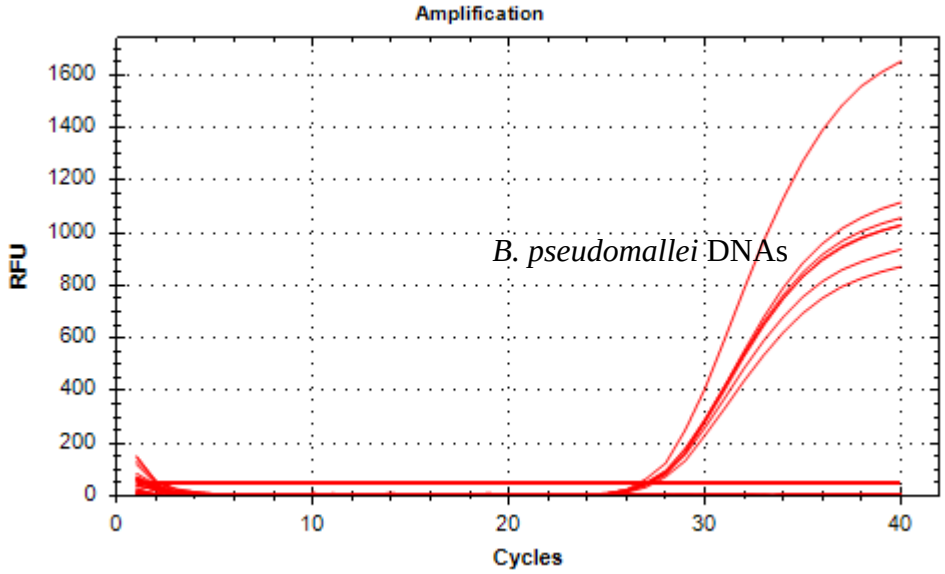


Figure 3.10 Amplification of *B. pseudomallei* TTSS-*orf2* genes in the monoplex assay

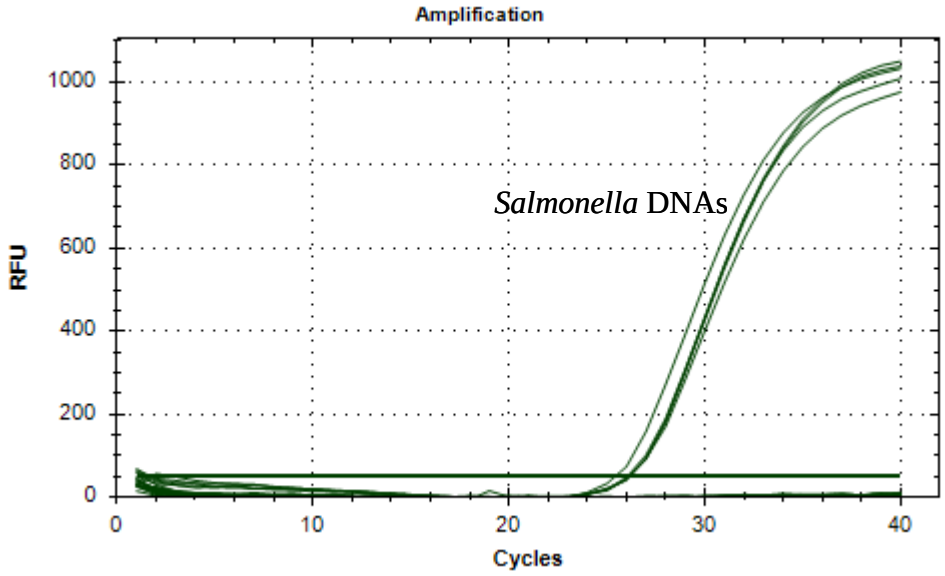


Figure 3.11 Amplification of *Salmonella StyR-3* genes in the monoplex assay

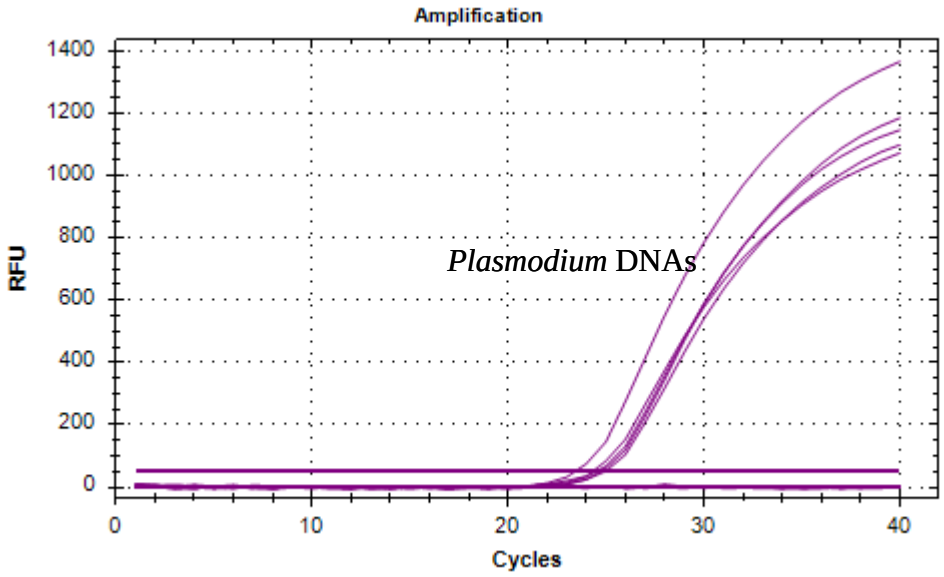


Figure 3.12 Amplification of *Plasmodium* 18S rRNA genes in the monoplex assay

3.4.2 Preliminary specificity testing of the developed monoplex TaqMan qPCR

Prior to optimisation of assays, potential cross-amplification of the developed monoplex assay was tested on *Leptospira* spp., *B. pseudomallei*, *S.* Typhi, *P. falciparum* and healthy human DNA. As shown in Figure 3.13 - Figure 3.16, the monoplex assays exclusively amplified their desired target, respectively. No cross amplification was observed on other organisms. This preliminary specificity results suggest that the primers are specific for individual detection of *Leptospira, B. pseudomallei, Salmonella* and *Plasmodium* respectively, hence can be potentially used in real-time PCR platform.

For example, as illustrated in Figure 3.13, the monoplex leptospiral qPCR assay only amplified *Leptospira* spp. DNA. No signal was observed in the other tested organism. Similarly, the monoplex qPCR assays for *B. pseudomallei* (Figure 3.14), *Salmonella* spp. (Figure 3.15) and *Plasmodium* spp, (Figure 3.16) only amplified *B. pseudomallei*, *Salmonella* spp. and *Plasmodium* spp. DNA, respectively. No cross-amplification of non-related organisms was observed.

These results are consistent with the BLAST analysis that found that the designed oligonucleotides have high specificity to their particular targets. Further extended empirical specificity on wider spectrum of microorganism would be carried out, once a multiplex qPCR assay is successfully developed. Subsequently, the optimal primers and probe concentrations for each monoplex qPCR assays were determined, individually.

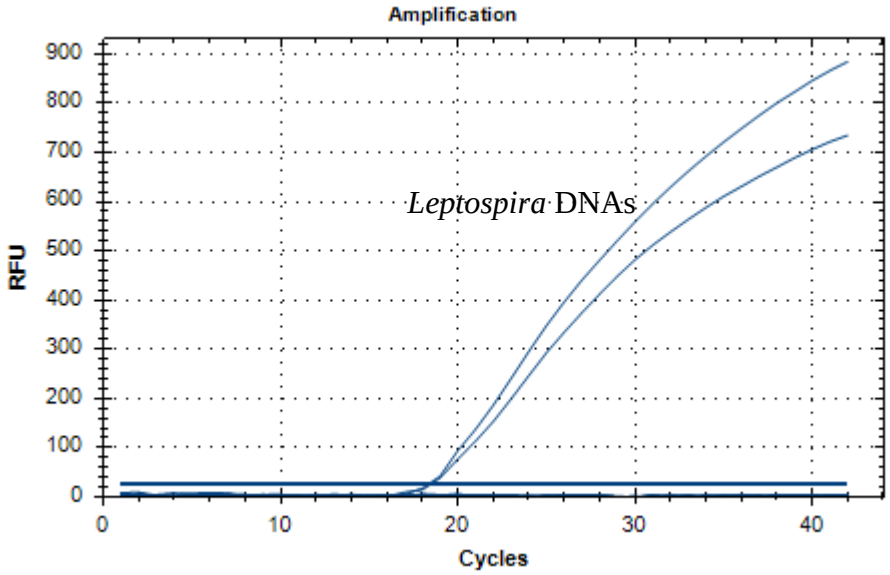


Figure 3.13 Preliminary specificity testing of the monoplex *Leptospira* qPCR

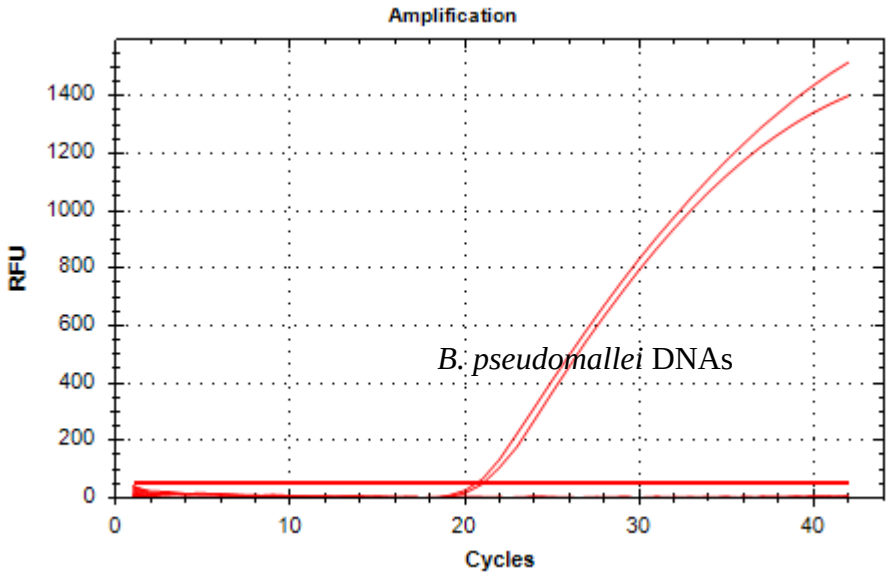


Figure 3.14 Preliminary specificity testing of the monoplex *B. pseudomallei* qPCR

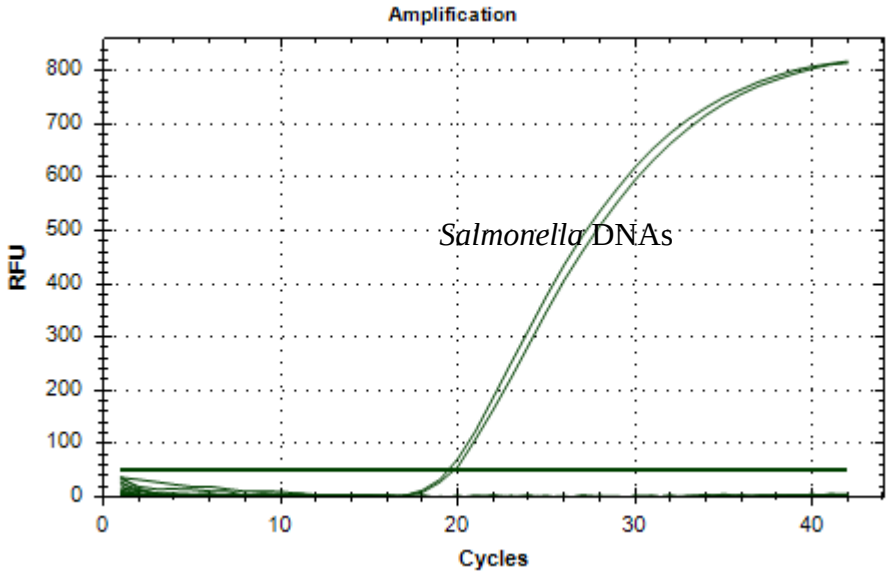


Figure 3.15 Preliminary specificity testing of the monoplex *Salmonella* qPCR

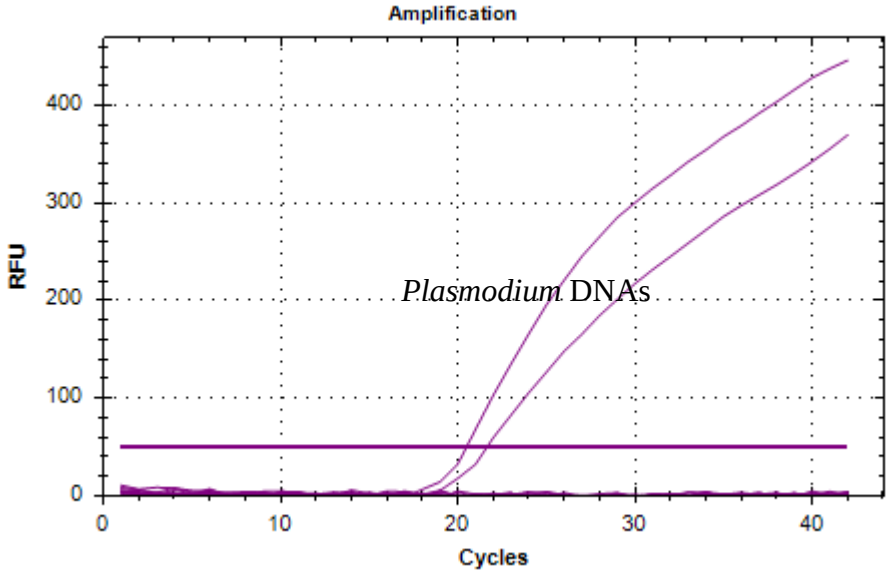


Figure 3.16 Preliminary specificity testing of the monoplex *Plasmodium* qPCR

3.4.3 Optimisations of primer concentrations of the developed monoplex TaqMan qPCR

Following preliminary specificity analysis, optimal primer concentration that resulted in the lowest (earliest) Cq value was determined. Respective monoplex qPCR reactions using different primer concentration were assayed.

3.4.3 (a) Optimisation of primer concentrations for monoplex *Leptospira* qPCR assay

Leptospiral primers at 100 nM, 200 nM, 300 nM, 400 nM, 500 nM and 600 nM were tested. As shown in Table 3.13, the mean of Cq increased proportionately to the increase of primers concentration. Even though the differences were close, especially at concentration of 100 – 300 nM, 200 nM resulted in the lowest Cq.

3.4.3 (b) Optimisation of primer concentrations for monoplex *B. pseudomallei* qPCR assay

Next, the optimal concentration of *B. pseudomallei* primers was determined (Table 3.14). No significant changes in the Cq values were seen across the tested primer concentration (100 nM – 600 nM). The Cq differences between primer concentrations lied between 0.35 and 0.57. Primer concentration of 200 nM was chosen as the optimal concentration.

3.4.3 (c) Optimisation of primer concentrations for monoplex *Salmonella* qPCR assay

Optimal concentration of *Salmonella* primers was determined by testing the concentrations at 100 nM until 600 nM. As shown in Table 3.15, the difference of Cq value ranged closely, between 0.05 to 0.54, relative to the lowest Cq value, achieved at primer concentration of 200 nM.

3.4.3 (d) Optimisation of primer concentrations for monoplex *Plasmodium* qPCR assay

Optimal concentration of primers was determined by testing the assay at different concentration of oligonucleotides, ranging between 100 nM and 600 nM. As shown in Table 3.16, primer concentration of 400 nM resulted in the lowest Cq.

Table 3.13 *Leptospira* primers optimisation

Primers concentration	Mean Cq	Cq difference ($Cq_{lowest} - Cq_{any}$)
100 nM	15.34	+ 0.04
200 nM	15.30	0
300 nM	15.71	+ 0.41
400 nM	16.01	+ 0.71
500 nM	16.05	+ 0.75
600 nM	16.41	+ 1.11

Table 3.14 *B. pseudomallei* primers optimisation

Primers concentration	Mean Cq	Difference ($Cq_{lowest} - Cq_{any}$)
100 nM	19.08	+ 0.35
200 nM	18.73	0
300 nM	19.04	+ 0.31
400 nM	19.23	+ 0.50
500 nM	19.14	+ 0.41
600 nM	19.3	+ 0.57

Table 3.15 *Salmonella* primers optimisation

Primers concentration	Mean Cq	Difference ($Cq_{lowest} - Cq_{any}$)
100 nM	17.73	0.51
200 nM	17.22	0
300 nM	17.47	0.25
400 nM	17.27	0.05
500 nM	17.76	0.54
600 nM	17.67	0.45

Table 3.16 *Plasmodium* primers optimisation

Primers concentration	Mean Cq	Difference ($Cq_{lowest} - Cq_{any}$)
100 nM	22.78	+ 1.91
200 nM	21.27	+ 0.40
300 nM	20.93	+ 0.05
400 nM	20.87	0
500 nM	21.05	+ 0.18
600 nM	20.88	+ 0.01

3.4.4 Optimisations of probe concentrations of the developed monoplex TaqMan qPCR

Leptospira monoplex qPCR assay used FAM-labelled probe, detectable at channel 1, wavelength of 520 nm. Meanwhile, *Salmonella* monoplex qPCR assay used HEX-labelled probe, detectable at channel 2, wavelength of 555 nm. *B. pseudomallei* and *Plasmodium* monoplex qPCR assays utilised Texas Red-labelled probe and Cy5-labelled probe, respectively. The probes are detectable separately at channel 3, wavelength of 617 nm and channel 4, wavelength of 668 nm.

Optimal probe concentration that resulted in the lowest (earliest) Cq value was determined by testing the monoplex qPCR assay at different concentration. Unlike primer, probes are significantly expensive, hence should be utilised judiciously (Tajadini *et al.*, 2014). During this study, TaqMan hydrolysis probes cost between RM 1600 to RM 2300 per tube (data not shown). Therefore, in this optimisation steps, Cq differences of approximately 0.5 is considered less significant. Therefore, lowest possible concentration of probe that resulted ±0.5 Cq difference will be selected.

3.4.4 (a) Optimisation of probe concentrations for monoplex *Leptospira* qPCR assay

Optimal probe concentration was determined, between 50 and 200 nM. As shown in Table 3.17, the Cq decreased as the probe concentration increased. In contrast to selection of primer where the concentration that produces the lowest Cq is selected, differences between the lowest Cq values, as well as their respective probe concentration were carefully considered. In this optimisation, probe at 200 nM resulted in the lowest Cq. However, another probe concentration, the 100 nM was

chosen because the Cq difference is less than 0.5 from the 200 nM, hence will save the cost by approximately 50%.

3.4.4 (b) Optimisation of probe concentrations for monoplex *B. pseudomallei* qPCR assay

Optimal *B. pseudomallei* probe concentration was determined by testing the monoplex assay using different concentration of Texas Red probe (Table 3.18). Higher concentration of probe decreased Cq value of the assay. Probe concentration of 100 nM was selected as the Cq difference was close to 0.5.

3.4.4 (c) Optimisation of probe concentrations for monoplex *Salmonella* qPCR assay

Four different probe concentrations (50 nM, 100 nM, 150 nM and 200 nM) were tested. Generally, increase of probe concentration resulted in lower Cq value (Table 3.19). Probe concentration of 100 nM was used as it produced the Cq within 0.4 Cq difference from the 200 nM.

3.4.4 (d) Optimisation of probe concentrations for monoplex *Plasmodium* qPCR assay

Optimal malarial probe concentration was determined by testing the monoplex assay using different concentration of Cy5-labelled probe. Probe concentration of 200 nM produced the lowest Cq, as compared to other tested concentration. However, probe concentration of 100 nM was chosen in subsequent analysis as the Cq difference was less than 0.5 from the lowest Cq value (Table 3.20).

Table 3.17 Optimisation of *Leptospira* probe

Probe concentration	Mean Cq	Cq difference
50 nM	15.41	0.77
100 nM	15.12	0.48
150 nM	14.98	0.34
200 nM	14.64	0

Table 3.18 Optimisation of *B. pseudomallei* probe

Probe concentration	Mean Cq	Cq difference
50 nM	19.26	1.29
100 nM	18.51	0.54
150 nM	18.11	0.13
200 nM	17.97	0

Table 3.19 Optimisation of *Salmonella* probe

Probe concentration	Mean Cq	Difference
50 nM	18.08	1.03
100 nM	17.45	0.40
150 nM	17.12	0.07
200 nM	17.05	0

Table 3.20 Optimisation of *Plasmodium* probe

Probe concentration	Mean Cq	Cq difference
50 nM	22.71	0.92
100 nM	22.28	0.49
150 nM	21.98	0.19
200 nM	21.79	0

3.4.5 Effect of universal adapter to PCR amplification

Additions of unique nucleotides to the 5' end of primers, also known as adapter or flap, have been reported to improve PCR efficiency. To date, several universal adapters have been reported and utilised in molecular platform (Afonina *et al.*, 2007; Yuan *et al.*, 2009). In this study, the effect of universal adapter/conjugated-primers (UN-primers) on PCR performance was investigated.

3.4.5 (a) Performance of *Leptospira* monoplex qPCR assay using primer with/without universal adapter.

As shown in Table 3.21, the PCR efficiency of primer with the presence of universal adapter was 96.9%, almost 2% better than primer without additional adapter. In terms of analytical sensitivity, the primer with universal adapter was more sensitive, able to amplify as low as 20 fg of leptospiral DNA, as compared to the primer without universal adapter. As the *Leptospira* DNA was diluted, the Cq increased proportionately (Table 3.22). Standard curves were constructed based on the mean Cq and $\log_{10}$ (copies number). The linearity of the assays, denoted by the R^2 values was close to 1.

3.4.5 (b) Performance of *B. pseudomallei* monoplex qPCR assay using primer with/without universal adapter.

Performance of qPCR utilising primer with universal adapter was compared with primer without universal adapter. As shown in Table 3.23, both primer types had comparable PCR efficiency, 93.9% and 93.4%, respectively. In terms of sensitivity (Table 3.24), both assays could detect as low as 20 fg *B. pseudomallei* DNA per reaction. For subsequent optimisation, primer with universal adapter was

chosen to allow standardisation of primers, as well as due to its potential in improving PCR efficiency in the multiplex assay.

3.4.5 (c) Performance of *Salmonella* monoplex qPCR assay using primer with/without universal adapter.

Effect of universal adapter on PCR performance was compared with primer that did not contain universal adapter. As shown in Table 3.25, presence of universal adapter slightly increased the PCR efficiency, by 0.2%. In terms of linearity and sensitivity (Table 3.26), both types of primer had comparable performance, linearity of close to 1, and sensitivity of as low as 20 fg of *Salmonella* DNA.

3.4.5 (d) Performance of *Plasmodium* monoplex qPCR assay using primer with/without universal adapter.

PCR performance of assay utilising standard primers was compared with primers conjugated with universal adapter. As shown in Table 3.27, the presence of conjugated universal primer significantly improved PCR efficiency to 94.2%, as compared to assay without universal adapter which scored 87.2%. In terms of linearity and sensitivity (Table 3.28), both assays had R^2 values of close to 1, and were able to amplify as low as 200 ag *Plasmodium* synthetic DNA.

Table 3.21 Performance of *Leptospira* monoplex qPCR assay using primer without adapter and primer with adapter

Parameter	***Leptospira* qPCR**	
	without UN-adapter	with UN-adapter
Slope	-3.4454	-3.3982
PCR efficiency	95.1%	96.9%
Linearity, R^2	0.9976	0.9942

Table 3.22 Sensitivity of *Leptospira* monoplex qPCR assay using primer without adapter and primer with adapter

Amount of *Leptospira* DNA per reaction (pg)	Mean Cq	
	Primer without UN-adapter	Primer with UN-adapter
20000	17.69	19.72
2000	21.22	23.63
200	25.19	27.65
20	28.44	30.78
2	31.95	34.68
0.2	34.72	37.33
0.02	-	39.96
0.002	-	-

Table 3.23 Performance of *B. pseudomallei* monoplex qPCR assay using primer without adapter and primer with adapter

Parameter	***B. pseudomallei* qPCR**	
	without UN-adapter	with UN-adapter
Slope	-3.475	-3.489
PCR efficiency	93.9%	93.4%
Linearity, R^2	0.9991	0.9993

Table 3.24 Sensitivity of *B. pseudomallei* monoplex qPCR assay using primer without adapter and primer with adapter

Amount of *B. pseudomallei* DNA per reaction (pg)	Mean Cq	
	Primer without UN-adapter	Primer with UN-adapter
20000	18.36	17.96
2000	22.07	21.33
200	25.35	24.53
20	28.75	28.16
2	32.37	31.65
0.2	35.38	35.60
0.02	39.58	38.65
0.002	-	-

Table 3.25 Performance of *Salmonella* monoplex qPCR assay using primer without adapter and primer with adapter

Parameter	***Salmonella* qPCR**	
	without UN-adapter	with UN-adapter
Slope	-3.172	-3.168
PCR efficiency	106.7%	106.9%
Linearity, R^2	0.996	0.994

Table 3.26 Sensitivity of *Salmonella* monoplex qPCR assay using primer without adapter and primer with adapter

Amount of *Salmonella* DNA per reaction (pg)	Mean Cq	
	Primer without UN-adapter	Primer with UN-adapter
20000	17.03	18.35
2000	20.18	21.53
200	23.63	25.13
20	27.14	28.54
2	30.31	32.14
0.2	33.51	34.63
0.02	35.52	36.85
0.002	-	-

Table 3.27 Performance of *Plasmodium* monoplex qPCR assay using primer without adapter and primer with adapter

Parameter	***Plasmodium* qPCR**	
	without UN-adapter	with UN-adapter
Slope	-3.673	-3.469
PCR efficiency	87.2%	94.2%
Linearity, R^2	0.9999	0.9977

Table 3.28 Sensitivity of *Plasmodium* monoplex qPCR assay using primer without adapter and primer with adapter

Amount of *Plasmodium* synthetic DNA per reaction (pg)	Mean Cq	
	Primer without UN-adapter	Primer with UN-adapter
20	17.50	17.97
2	21.26	21.50
0.2	24.94	25.30
0.02	28.44	28.90
0.002	32.15	32.30
0.0002	35.98	35.05
0.00002	-	-

3.5 Development of monoplex TaqMan qPCR for the detection of *IAC*

3.5.1 Functionality of monoplex assay

Following the designing of primers and probe against *rpoB*-EH IAC synthetic DNA, functionality of the oligonucleotides was evaluated. Amplification of desired product was observed at channel Cy5.5 that detect wavelength of 694 nm. As shown in Figure 3.17, the developed IAC monoplex assay was able to amplify *rpoB*-EH IAC synthetic DNA.

3.5.2 Preliminary specificity testing

The preliminary specify of the IAC oligonucleotides was also tested against *L. interrogans, B. pseudomallei, S.* Typhi, *P. falciparum* and human DNA. As shown in Figure 3.18, no undesired cross amplification was observed in the unrelated DNA templates.

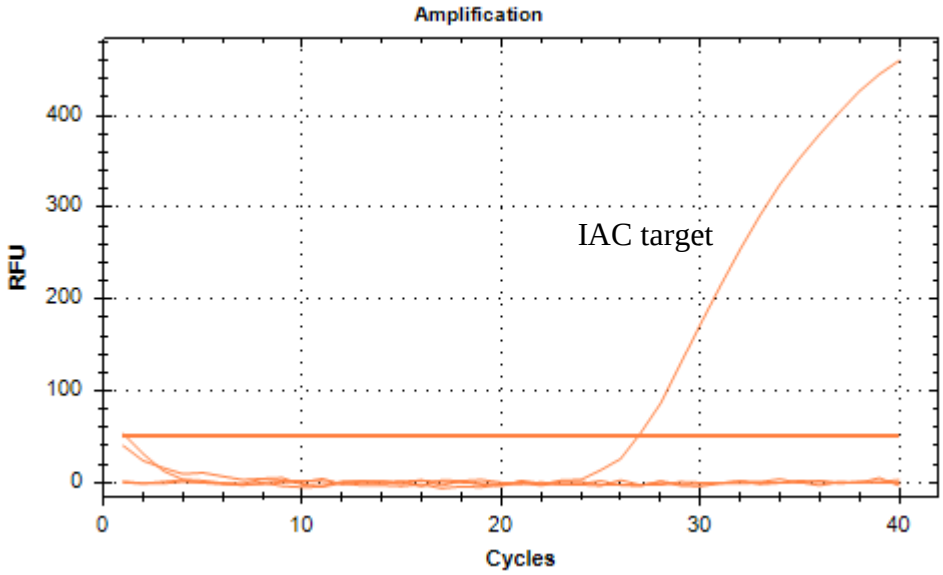


Figure 3.17 Amplification of IAC *rpoB*-EH fusion target in the monoplex assay

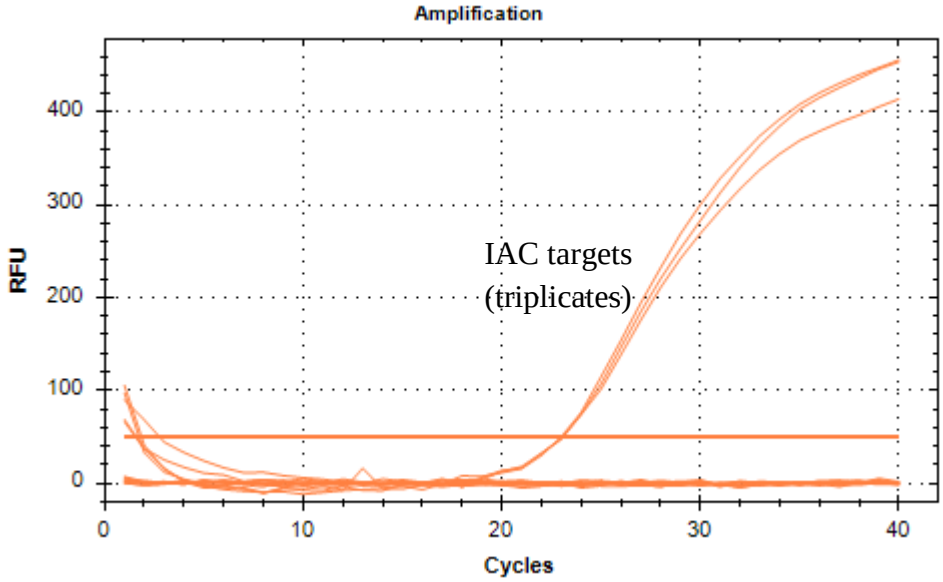


Figure 3.18 Preliminary specificity testing of the IAC monoplex assay

3.5.3 Optimisation of IAC template concentrations

Competitive amplification strategy where all targeted pathogens and IAC were amplified within the same reaction was implemented. This strategy enabled more accurate determination of (true) negative specimen; that may also be caused by the presence of inhibitor, sub-optimal polymerase enzyme activity or imprecise PCR mixture within a single tube, as compared to the non-competitive strategy. Moreover, competitive strategy is more cost-effective as only a single reaction tube is needed. However, careful assay design should be carried out as competitive may in strategy favour to biased amplification, and subsequent false negativity.

Ideally, to reduce the possibility of primers competition between IAC and pathogenic targets, the IAC primers concentration should be lower than the pathogen-specific primers (Hoorfar *et al.*, 2004). Therefore, in this study, the concentration of IAC primers was reduced to 50% - 75% of the pathogen-specific primers, set at 100 nM and 50nM, for forward primer and reverse, respectively. This strategy was implemented to favour amplification of the desired microbial targets. Meanwhile, IAC probe concentration was maintained at 100 nM to maximize the signal strength.

Another important parameter is the IAC target concentration. Too high IAC target may compete with pathogen DNA or may not be able to detect low presence of inhibitor. To determine the optimal concentration of IAC, a set of IAC template (2 pg – 2 ag per reaction) was tested. As shown in Figure 3.19 IAC target concentration of 2 fg was chosen as it gave the Cq value of approximately 32.

3.5.4 Linearity, efficiency and limit of detection (LOD)

Based on the previous data (as illustrated in Figure 3.19), the analytical performance of the IAC qPCR was determined. The PCR efficiency was 76.9% and linearity value of 0.9975 (Table 3.29). Even though the efficiency is lower than the other microbial targets, it is anticipated due to low primer concentrations used. Another study that had similar assay efficiency and linearity reported that such conditions did not affect the assays' reproducibility (Coffey *et al.*, 2016). Besides, the developed assay was able to detect as low as 20 ag of IAC synthetic DNA (Table 3.30). These parameters would be used in the multiplex qPCR assays in order to favour amplification of *Leptospira, B. pseudomallei, Salmonella* and *Plasmodium*.

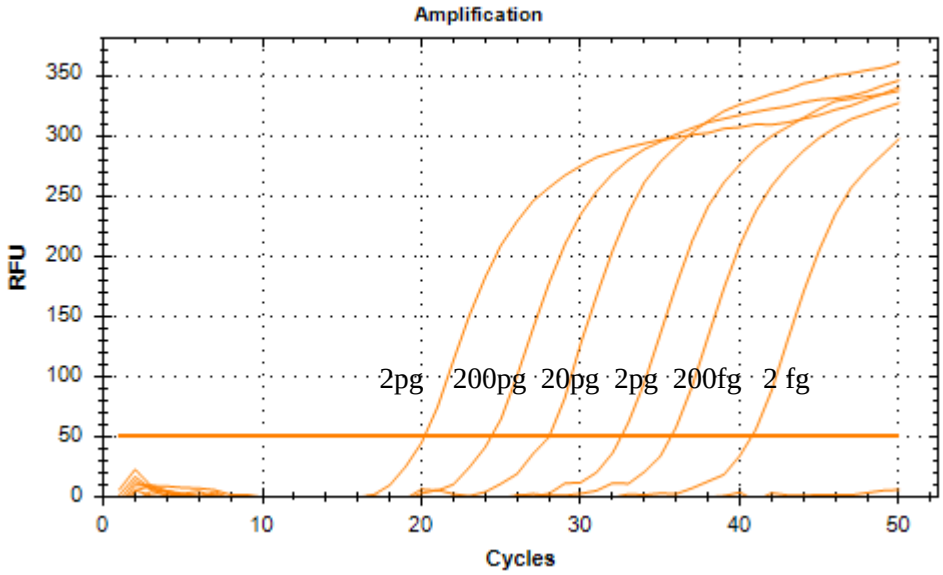


Figure 3.19 Optimisation of IAC template concentrations

Table 3.29 Performance of IAC monoplex qPCR assay

Parameter	***IAC* qPCR**
Slope	-4.035
PCR efficiency	76.9%
Linearity	0.9975

Table 3.30 Sensitivity of IAC monoplex assay

Amount of *IAC* synthetic DNA per reaction (pg)	Mean Cq
2	20.17
0.2	24.37
0.02	28.01
0.002	32.53
0.0002	35.67
0.00002	40.73
0.000002	-

3.6 Development of multiplex TaqMan qPCR for the detection of *Leptospira, B. pseudomallei, Salmonella and Plasmodium* DNA with the incorporation of IAC.

3.6.1 Functionality of the multiplex assay

Following the development of separate monoplex qPCR assays for detection of individual microbial target and IAC, all primer pairs and probes were multiplexed into a single reaction. Preliminary functionality of the multiplex reaction was investigated. As illustrated in Figure 3.20, all of the targets, *Leptospira*, *B. pseudomallei*, *Salmonella*, *Plasmodium* and IAC were successfully amplified.

Next, the concentration of unconjugated universal primer would be optimised, followed by performance comparisons of the multiplex qPCR assay versus monoplex qPCR assays.

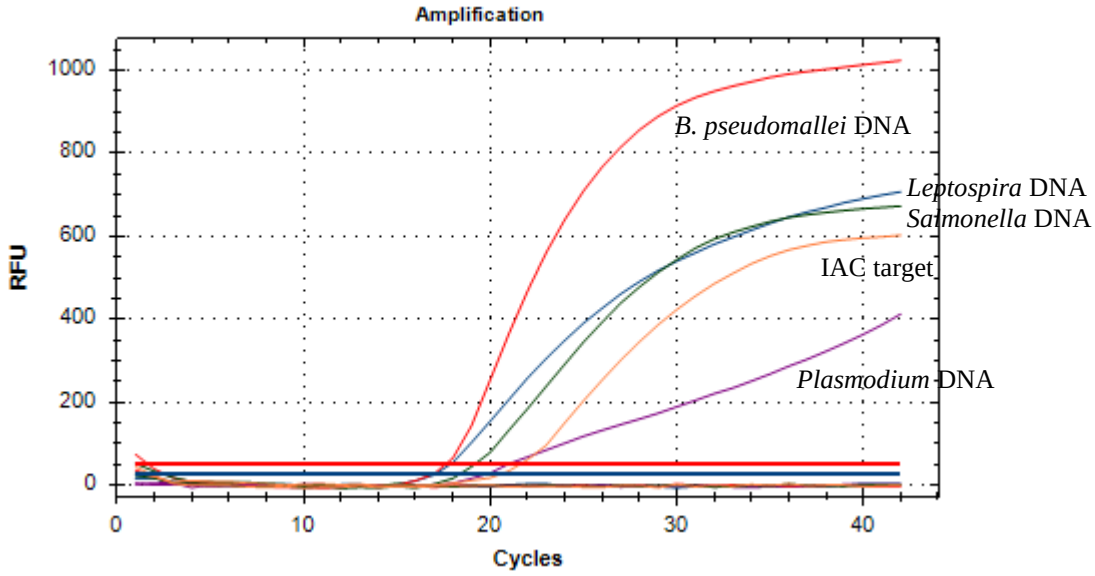


Figure 3.20 Functionality test of the developed multiplex qPCR

3.6.2 Optimisation of un-conjugated universal primers concentration

Effect of additional unconjugated universal primer (UN) to PCR was investigated at concentrations of 100 nM – 400 nM. As shown in Table 3.31, addition of unconjugated universal primer reduced the Cq values by 0.58 and 0.6 for *B. pseudomallei* and *Plasmodium* targets, respectively. For remaining targets, the *Leptospira* and *Salmonella*, additional un-conjugated universal primers resulted in slightly reduced Cq, of approximately 0.2. In the subsequent multiplex qPCR assays, 200 nM unconjugated universal primer will be added to the reactions, as it resulted in the lowest Cq values, in *B. pseudomallei*, *Salmonella* and *Plasmodium* targets. Mechanisms of actions of unconjugated universal primer (UN) and universal adapter are illustrated in Appendix D.

Table 3.31 Optimisation of un-conjugated universal primers concentration

Target	Sample	Mean Cq	Difference
Leptospira	0 nm UN	16.13	0.22
	+ 100 nM UN	15.93	0.02
	+ 200 nM UN	16.03	0.12
	+ 300 nM UN	16.11	0.2
	+ 400 nM UN	15.91	0
B. pseudomallei	0 nm UN	18.78	0.58
	+ 100 nM UN	18.49	0.29
	+ 200 nM UN	18.20	0
	+ 300 nM UN	18.4	0.2
	+ 400 nM UN	18.34	0.14
Salmonella	0 nm UN	18.31	0.16
	+ 100 nM UN	18.17	0.02
	+ 200 nM UN	18.15	0
	+ 300 nM UN	18.19	0.04
	+ 400 nM UN	18.19	0.04
Plasmodium	0 nm UN	17.63	0.6
	+ 100 nM UN	17.33	0.3
	+ 200 nM UN	17.03	0
	+ 300 nM UN	17.26	0.23
	+ 400 nM UN	17.37	0.34

3.6.3 Efficiency and linearity of multiplex versus monoplex qPCR

Standard curves were constructed using amplification data of the developed multiplex qPCR assay, as well as data from the monoplex qPCR assays. As shown in Table 3.32, the PCR efficiency of the multiplex qPCR versus *Leptospira* monoplex qPCR assay was 91.04% versus 95.09%, respectively. It is expected that PCR efficiency can be affected once a monoplex assay is combined into a multiplex assay (Deng *et al.*, 2013; Jin *et al.*, 2017). Some groups reported that PCR efficiency can be reduced as many as 13% in a multiplex platform (Giry *et al.*, 2017). In this study, the 91.04% PCR efficiency of the multiplex assay for the leptospiral target is still within the acceptable range; of 90% efficiency and above (Z. Pang *et al.*, 2014; Svec *et al.*, 2015).

In the next target; the *B. pseudomallei* DNA (Table 3.33), the multiplex qPCR had considerably higher efficiency (103.26%) than the monoplex assay (93.43%). Similarly, for the detection of malarial DNA (Table 3.35), the multiplex qPCR assay had slightly higher efficiency (95.22%) than the monoplex assay (94.22%). Meanwhile, *Salmonella* multiplex and monoplex qPCR assays had comparable efficiency of 105.3% and 106.3%, respectively (Table 3.34). Overall, the multiplex qPCR assays scored more than 90% efficiency for all of the desired microbial targets.

In terms of analytical sensitivity, the multiplex assays had comparable sensitivity with the monoplex assay (Figure 3.21-Figure 3.24). Noticeably, regardless of the lower PCR efficiency of the leptospiral multiplex qPCR assay, the multiplex assay also had similar analytical sensitivity with the leptospiral monoplex assay (Figure 3.21). Similarly, in terms of linearity, all multiplex and monoplex assays had R2 values between 0.9868 and 0.9986; very close to 1.

Table 3.32 Comparison of performance between monoplex and multiplex qPCR assays for detection of *Leptospira* DNA

Parameter	***Leptospira* qPCR**	
	Multiplex	Monoplex
Slope	-3.5571	-3.3111
PCR efficiency	91.04%	100.45%
Linearity	0.9980	0.9957

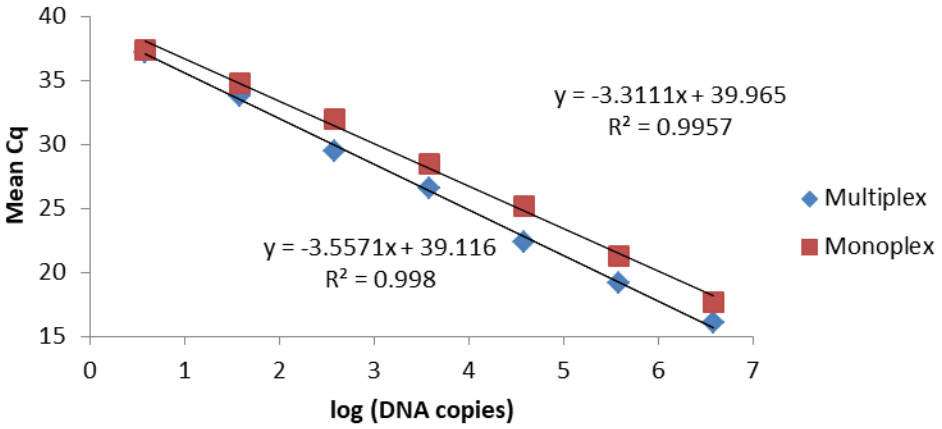


Figure 3.21 Standard curve of the developed qPCR for the detection of *Leptospira* DNA. Multiplex (blue) versus monoplex (red) assays.

Table 3.33 Comparison of performance between monoplex and multiplex qPCR assays for detection of *B. pseudomallei* DNA

Parameter	***B. pseudomallei* qPCR**	
	Multiplex	Monoplex
Slope	-3.2461	-3.4904
PCR efficiency	103.26%	93.43%
Linearity	0.9958	0.9993

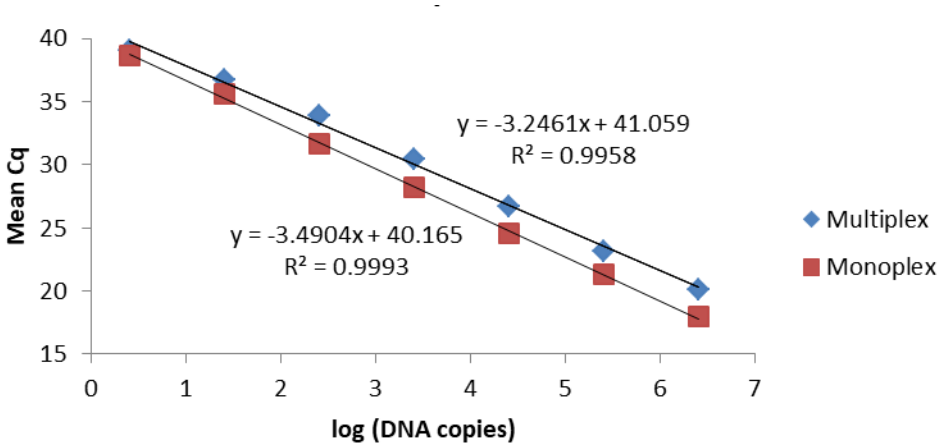


Figure 3.22 Standard curve of the developed qPCR for the detection of *B. pseudomallei DNA*. Multiplex (blue) versus monoplex (red) assays.

Table 3.34 Comparison of performance between monoplex and multiplex qPCR assays for detection of *Salmonella* DNA

Parameter	***Salmonella* qPCR**	
	Multiplex	Monoplex
Slope	-3.2011	-3.1682
PCR efficiency	105.30%	106.85%
Linearity	0.9868	0.9940

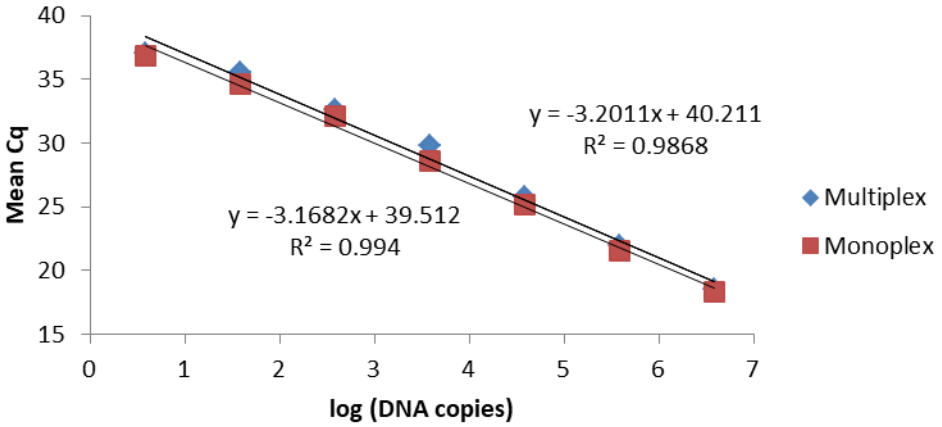


Figure 3.23 Standard curve of the developed qPCR for the detection of *Salmonella* DNA. Multiplex (blue) versus monoplex (red) assays.

Table 3.35 Comparison of performance between monoplex and multiplex qPCR assays for detection of *Plasmodium* DNA

Parameter	***Plasmodium* qPCR**	
	Multiplex	Monoplex
Slope	-3.4421	-3.4686
PCR efficiency	95.22%	94.22%
Linearity	0.9986	0.9977

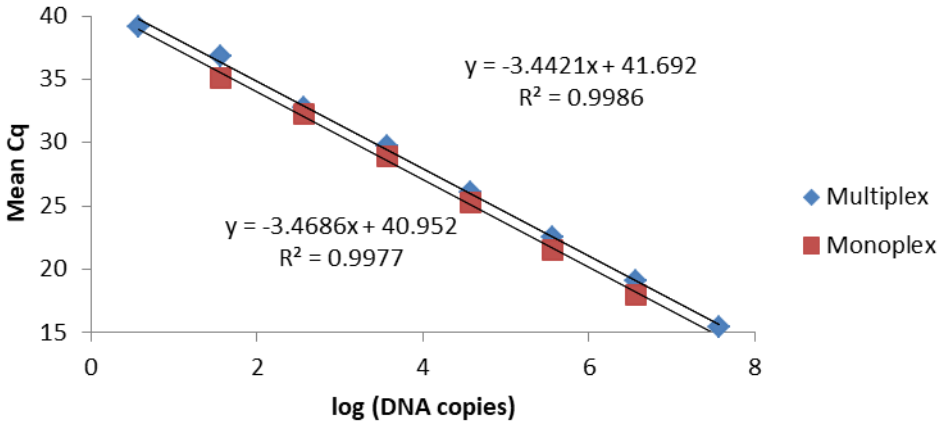


Figure 3.24 Standard curve of the developed qPCR for the detection of *Plasmodium* DNA. Multiplex (blue) versus monoplex (red) assays.

3.6.4 Limits of detection (LOD)

The limits of detection (LOD) of the developed multiplex qPCR assays were determined using Probit nonlinear-regression model, based on data of 24 replicates, targeting 5 different DNA copy numbers and distributed across different days. LOD is defined by the lowest DNA copies at which 95% of the samples were positive. Details of replicates positivity rate and amount of DNA in each reaction were listed in Table 3.36. Respective Probit analysis graphs were constructed from the data.

As shown in Figure 3.25, the LOD of the multiplex qPCR assay for the detection of *Leptospira* DNA was approximately 5.61 copies per reaction. The LOD is comparable to other reported molecular assays which detected 1 – 10 leptospiral DNA copies per reaction (Ahmed *et al.*, 2009; Bedir *et al.*, 2010; Bourhy *et al.*, 2011; Levett *et al.*, 2005; Merien *et al.*, 2005; A. Slack *et al.*, 2007; Thaipadungpanit *et al.*, 2011).

The LOD of the multiplex qPCR assay for the detection of another target, the *B. pseudomallei* DNA was ~ 8.24 DNA copies per reaction (Figure 3.26). Similarly, the LOD is analogous to previously described hydrolysis probe-based molecular assays that can amplify 5 – 10 copies *B. pseudomallei* DNA per reaction (Al-Marzooq and Mustafa, 2011; Kaestli *et al.*, 2012; Novak *et al.*, 2006; B. Zhang *et al.*, 2012).

Meanwhile, the LOD of the multiplex qPCR assay for the *Salmonella* target was around 19.3 DNA copies per reaction (Figure 3.27). In comparison to other studies, the reported LODs were comparable, ranging from 40 to 1000 DNA copies per reaction (González-escalona *et al.*, 2012; Maurischat *et al.*, 2015). In many instances, the reported molecular assays require pre-enrichment prior to the DNA extraction (Reynisson *et al.*, 2006; G. G. Stone *et al.*, 1994).

The fourth target of the multiplex qPCR assay; *Plasmodium;* had LOD of approximately 18.1 copies per reaction (Figure 3.28). As the target gene presents in 5 copies per malarial genome, the calculated LOD could be around 3.7 organisms per reaction (Gonçalves *et al.*, 2012; Mangold *et al.*, 2005). Relative to other studies, the reported LOD was between 40.3 and 250 copies per reaction (Divis *et al.*, 2010; Pholwat *et al.*, 2017). Other assays that targeted 18S rRNA gene had reported LOD of 5 to 50 copies per reaction (Mangold *et al.*, 2005; Rougemont *et al.*, 2004).

Table 3.36 Testing of multiplex qPCR assays on 80 fg - 2 fg bacterial DNA and 20 ag – 0.2 ag *Plasmodium* DNA

Organism	DNA amount	DNA copies	# Positive	# Replicates	Positivity %
Leptospira	80 fg	16	24	24	100%
	40 fg	8	23	24	96%
	20 fg	4	22	24	92%
	5 fg	1	4	24	17%
	2 fg	0.4	0	24	0%
B. pseudomallei	80 fg	10.24	24	24	100%
	40 fg	5.12	21	24	88%
	20 fg	2.56	15	24	63%
	5 fg	0.64	7	24	29%
	2 fg	0.256	1	24	4%
Salmonella	80 fg	15.24	24	24	100%
	40 fg	7.62	18	24	75%
	20 fg	3.81	13	24	54%
	5 fg	0.9525	4	24	17%
	2 fg	0.38	2	24	8%
Plasmodium	20 ag	37.1	24	24	100%
	10 ag	18.55	23	24	96%
	5 ag	9.275	18	24	75%
	2 ag	3.71	9	24	38%
	0.2 ag	0.371	0	24	0%

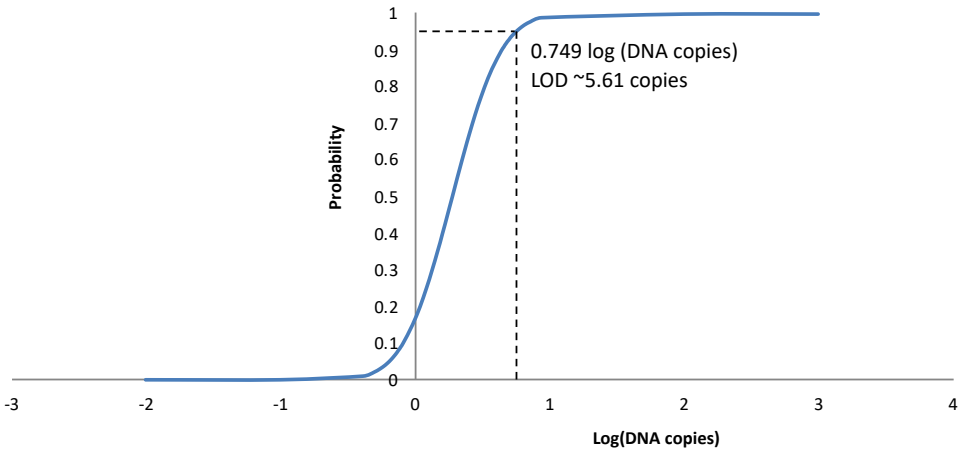


Figure 3.25 Probit nonlinear regression analysis of the developed multiplex qPCR for the detection of *Leptospira* DNA

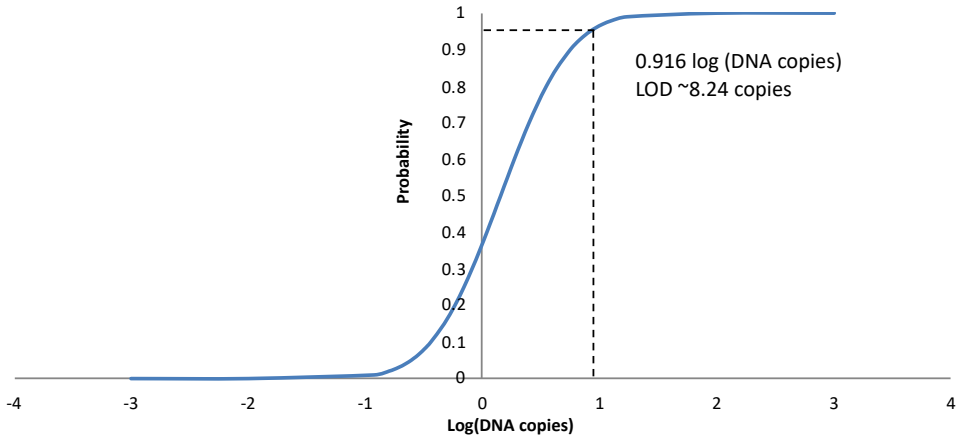


Figure 3.26 Probit nonlinear regression analysis of the developed multiplex qPCR for the detection of *B. pseudomallei* DNA

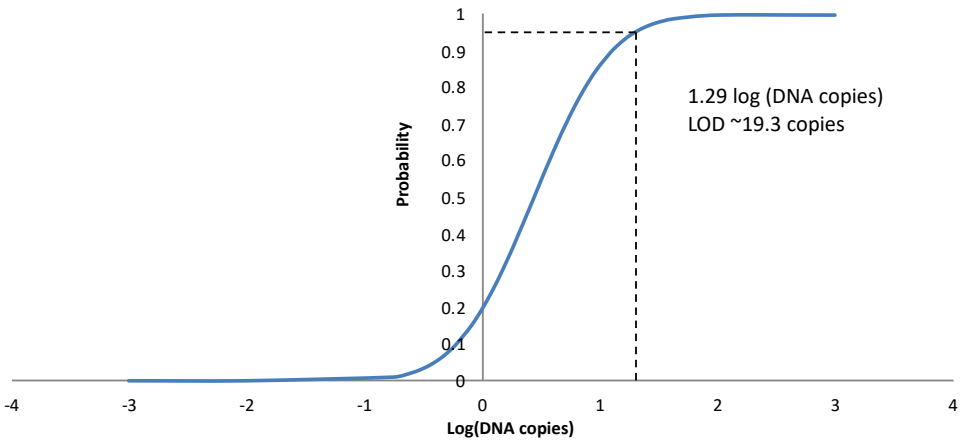


Figure 3.27 Probit nonlinear regression analysis of the developed multiplex qPCR for the detection of *Salmonella* DNA

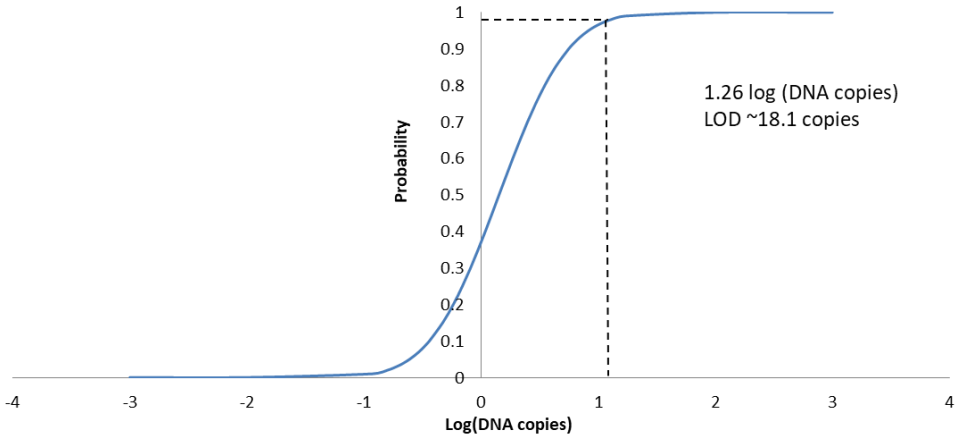


Figure 3.28 Probit nonlinear regression analysis of the developed multiplex qPCR for the detection of *Plasmodium* synthetic DNA.

3.6.5 Amplification of microbial target in the presence of IAC

Simultaneous amplification of IAC and microbial targets (titred close to the LOD) were investigated. As shown in Table 3.37, the means Cq of *Salmonella* target (HEX probe) without and with amplification of IAC targets were comparable, 35.53 and 35.91, respectively. Similarly, the means Cq of *Plasmodium*, in the presence and absence of IAC were analogous, 38.61 and 38.20 each.

Meanwhile, the means Cq of individual *Leptospira* and *B. pseudomallei* target were lower than the *Leptospira* and *B. pseudomallei* with IAC, by more than 1 Cq. This result may due to the competitive amplification between *Leptospira* or *B. pseudomallei* with IAC or potential dimerisation between *Leptospira* and IAC primers, as well as potential dimerisation between *B. pseudomallei* and IAC primers which had ΔG values of -10.3 and -12.3 kcal $mole^{-1}$as shown in section 3.3.4. Remarkably, if the latter factor is significant, sensitivity and amplification efficiency of multiplex qPCR should be lower than the monoplex qPCR. As shown previously in the section 3.6.3, both monoplex and multiplex qPCR assay had similar sensitivity. In terms of amplification efficiency, in contrast to leptospiral target, *B. pseudomallei* multiplex qPCR assay had higher PCR efficiency than the monoplex assay. Other factors such as Poisson distribution effect of DNA targets and random pipetting error might also affect the variations.

Meanwhile, the IAC (Cy5.5 probe) had very stable Cq in all reaction. The recorded IAC Cq values were approximately 32, with and without co-amplification of the microbial target. In this sectional analysis, it is found that the multiplex qPCR assay was able to simultaneously amplify both microbial and IAC target, at low copy numbers albeit increased in the Cq values in certain targets. As there is a possibility that certain sensitivity of the assay may be affected by amplification competition

between IAC and low concentration microbial targets (Flores *et al.*, 2009), further study can evaluate the oligonucleotides competition effects on the assays LOD.

Table 3.37 Amplification of microbial targets in the absence or presence of IAC target

Target		Specific probe (FAM/HEX/TexRed/Cy5)			IAC probe (Cy5.5)		
		Mean Cq	SD	CV%	Mean Cq	SD	CV%
Leptospira		36.57	1.26	3.4	-	-	-
Leptospira	+ IAC	37.62	0.32	0.8	32.46	1.07	3.3
B. pseudomallei		36.24	0.52	1.4	-	-	-
B. pseudomallei	+ IAC	37.74	1.10	2.9	32.38	0.43	1.3
Salmonella		35.53	1.29	3.6	-	-	-
Salmonella	+ IAC	35.91	0.54	1.5	32.09	0.48	1.5
Plasmodium		38.20	0.34	0.9	-	-	-
Plasmodium	+ IAC	38.61	0.80	2.1	32.07	0.38	1.2
IAC		-	-	-	32.36	0.2	0.6

3.6.6 Analytical specificity of the multiplex assay

Extended analytical specificity testing was carried out on 357 microbial isolates, consisted of 131 leptospiral strains, 105 *B. pseudomallei* isolates, 44 *Salmonella* isolates, 31 *Plasmodium* strains and 46 other organisms. As shown in Table 3.38, the developed multiplex qPCR assay was able to detect and differentiate the entire organisms of interest, in particular *Leptospira* (100% detection by the FAM channel), *B. pseudomallei* (100% detection by the Texas Red channel), *Salmonella* spp. (100% detection by the HEX channel) and *Plasmodium* spp. (100% detection by the Cy5 channel). No other organism was detected by any of the mentioned channels.

For the IAC (denoted by the Cy5.5 probe), the signals were detected in other organisms, suggesting successful PCR / absence of PCR inhibitors. In the desired microbial targets, the IAC might not be amplified perhaps due to primers-targets competition, in which the target DNA presents in a proportionally larger amount than the IAC (Hoorfar *et al*., 2004).

Table 3.38 Summary of analytical specificity of the developed multiplex qPCR assay on extended spectrum of microorganisms

Organisms tested	# strains	# positive in Multiplex qPCR					Total
		Leptospira (FAM)	*B. pseudomallei* (Texas Red)	*Salmonella* (HEX)	*Plasmodium* (Cy5)	IAC (Cy5.5)	
Leptospira spp.	131	131	-	-	-	-	131
B. pseudomallei	105	-	105	-	-	-	105
Salmonella spp.	44	-	-	44	-	-	44
Plasmodium spp.	31	-	-	-	31	-	31
Other organisms	46	-	-	-	-	46	46

3.6.7 Intra- and inter-assay variation

Precision of the developed multiplex qPCR assays was determined based on intra-assay variation (repeatability) and inter-assay variation (reproducibility) (Bustin *et al.*, 2009). In this study, two different DNA concentrations; 200 pg and 0.2 pg were used.

Overall, the coefficients of variations (CV) of intra-assays were between 0.1% and 2.8% (Table 3.39). For the leptospiral target, the coefficient of variation (CV) of the first and second runs was 0.1% and 0.7%. Similarly, the *Salmonella, Plasmodium* and IAC targets also had CV% between 0.1% and 0.2%. Slightly higher CV% values were observed in 0.2 pg *B. pseudomallei* targets in which, each run scored 2.8% and 1.8% respectively.

For inter assays variation, as shown in Table 3.39, the CV% of for each microbial targets were less than 4%. Most microbial targets had CV of 2.0% and below, except slightly higher CV% were observed in 0.2 pg *B. pseudomallei* target (3.7%) and 0.2 pg of *Plasmodium* target (2.7%).

Table 3.39 Precision of the developed multiplex qPCR assay represented by the intra- and inter-assay variations

Organism	Amount of DNA (pg)	Run 1			Run 2			Inter-assay CV%
		Mean	SD	CV%	Mean	SD	CV%	
Leptospira	200	22.31	0.02	0.1	23.01	0.07	0.3	1.7
	0.2	33.74	0.15	0.4	34.45	0.25	0.7	1.3
B. pseudomallei	200	26.66	0.16	0.6	26.91	0.22	0.8	0.8
	0.2	36.76	1.03	2.8	37.12	0.66	1.8	3.7
Salmonella	200	25.73	0.09	0.4	26.07	0.01	0.1	0.8
	0.2	35.43	0.43	1.2	36.57	0.27	0.7	2.0
Plasmodium	200	24.61	0.14	0.6	24.94	0.27	1.1	1.0
	0.2	31.39	0.29	0.9	32.92	0.12	0.5	2.7
IAC	0.002	32.36	0.20	0.6	32.38	0.28	0.9	0.7

3.6.8 Preliminary stability testing of lyophilised oligonucleotide mixture

Lyophilisation (also known as freeze-drying or thermo-stabilisation improve molecular assays' stability (Foo *et al.*, 2017; Nagaraj *et al.*, 2018; Nurul Najian *et al.*, 2016). However, during lyophilisation, some PCR component may be negatively affected and resulted in reduced molecular performances. Some lyoprotectants such as trehalose, raffinose and several others have been utilised to preserve PCR components, as well as improving the molecular performance.

In this study, real-time stability of lyophilised multiplex oligonucleotides mixtures was investigated, with and without addition of lyoprotectant. Five per cent trehalose has been chosen in this preliminary testing, as reported by previous assays (Gaertig *et al.*, 2015; Klatser *et al.*, 1998; S. V Lee *et al.*, 2011; Nagaraj *et al.*, 2018). Briefly, wet mixtures and lyophilised mixtures (with and without 5% trehalose) were stored at three different temperatures (-20 °C, 25 °C and 37 °C) and tested every week.

Table 3.40 - Table 3.44 and Figure 3.29 - Figure 3.33 portrayed the mean Cq value and stability trends of the respective mixtures tested on different occasions. In general, no significant Cq differences were observed in the wet oligonucleotides mixtures and lyophilised oligonucleotides mixtures, tested across the stipulated period of 28 days.

However, in terms of CV%, the Texas Red (*B. pseudomallei*) signals in wet oligonucleotides mixtures, stored at 25 °C and 37 °C had CV% of 4.7% and 8.9%, respectively. The other Texas Red parallel thermo-stabilised mixture (with or without trehalose) scored slightly lower CV% values (3.0% - 4.3%) (Table 3.41).

In addition, it is noticeable that the Cy5 (*Plasmodium*) signals in the lyophilised mixture that did not contain additional trehalose had the highest CV% (~6%) as compared to the other mixtures' (0.8% - 1.8%) (Table 3.43). This observation suggests that lyophilisation process possibly had slightly negative effect on the amplification of *Plasmodium* target. García-Ruíz *et al*. (2016) had reported reduced fluorescence signals and delayed Cq following lyophilisation (García-Ruíz *et al.*, 2016). In addition, another study also reported that the Cy5 probe was more susceptible to post-lyophilisation prolonged storage, even in the presence of lyoprotectants, as compared to FAM, HEX and Texas Red probes (Rombach *et al.*, 2014). It is suggested that further sensitivity/LOD analysis using extended dilutions of DNA templates should be carried to confirm this inference, with appropriate controls and settings.

Meanwhile, the FAM (*Leptospira*), HEX (*Salmonella*) and Cy5.5 (IAC) signals, from wet and lyophilised mixtures scored CV% values between 1.0% and 3.8% during the 28-day evaluation time, consistent with a previous report (Rombach *et al.*, 2014). Figure 3.29, Figure 3.31 and Figure 3.33 provide overall trends of probes' stabilities at different form, stabiliser status and temperature storage.

Shelf life of the lyophilised multiplex oligonucleotides mixture was estimated using Q_{10} accelerated ageing technique (Clark, 1991). Based on the calculation, the estimated shelf life of the dried mixture is 84.7 days at ambient temperature. Given that the Cq was still stable after 28 days, further study can investigate the lyophilised mixture stability after 12 months, as recommended the UNICEF (Rombach *et al.*, 2014; UNICEF, 2014).

Calculation of the estimated shelf life of the dried mixture is as follow;

Accelerated study

Q_{10} = 1.8

Storage temperature, T_e = 37 °C

Ambient temperature, T_a = 25 °C

Length of time at elevated temperature = 28 days

$Acceleration\ factor = 1.8^{(0.1 \times (37-25))} = 2.025$

$Accelerated\ age = 28 \times 2.025 = 56.7\ days$

$Estimation\ of\ shelf\ life = 56.7\ days + 28 = 84.7\ days$ at 25 °C

In summary, it is found that the multiplex oligonucleotides mixture was readily stable in wet form, as well as in the lyophilised form. Further study should test its stability at long period of time i.e. 12 months, using different concentration of targets.

Table 3.40 Stability of wet and lyophilised multiplex oligonucleotides mixtures, with and without stabiliser at different temperature and length of times – FAM-labeled probe (*Leptospira*)

Dye	Storage temp.	Lyo-philisation	Amount of stabiliser	Mean Cq					Mean	SD	CV %
				Day 0	Day 7	Day 14	Day 21	Day 28			
FAM	25 °C	-	-	15.63	16.21	16.15	16.23	15.72	15.99	0.29	1.8
		Yes	-	15.18	15.82	16.68	16.46	15.72	15.97	0.60	3.8
		Yes	5%	16.02	16.01	16.3	16.66	15.53	16.10	0.42	2.6
	37 °C	-	-	15.63	16.09	16.04	16.22	15.5	15.90	0.31	2.0
		Yes	-	15.18	16.05	16.05	16.30	15.88	15.89	0.43	2.7
		Yes	5%	16.02	15.97	16.17	15.62	15.62	15.88	0.25	1.6
	- 20 °C	-	-	15.63	16.17	14.70	14.4	15.94	15.37	0.78	5.1

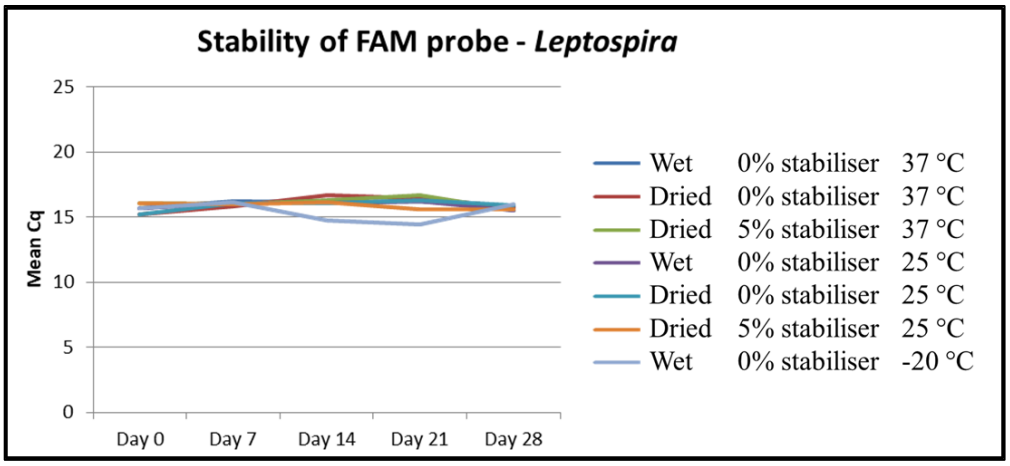


Figure 3.29 Trends of FAM probe stability at different form, stabiliser status and temperature

Table 3.41 Stability of wet and lyophilised multiplex oligonucleotides mixtures, with and without stabiliser at different temperature and length of times – Texas Red-labeled probe (*B. pseudomallei*)

Dye	Storage temp.	Lyo-philisation	Amount of stabiliser	Mean Cq					Mean	SD	CV %
				Day 0	Day 7	Day 14	Day 21	Day 28			
Texas Red	25 °C	-	-	19.73	19.17	20.50	18.06	19.90	19.47	0.92	4.7
		Yes	-	19.59	19.35	20.91	19.87	20.21	19.99	0.61	3.0
		Yes	5%	20.25	19.18	20.83	19.68	19.59	19.91	0.64	3.2
	37 °C	-	-	19.73	19.03	20.90	16.31	19.38	19.07	1.70	8.9
		Yes	-	19.59	19.11	20.85	20.54	20.04	20.03	0.70	3.5
		Yes	5%	20.25	19.14	20.63	18.55	19.88	19.69	0.84	4.3
	- 20 °C	-	-	19.73	19.70	20.66	21.09	20.13	20.26	0.60	3.0

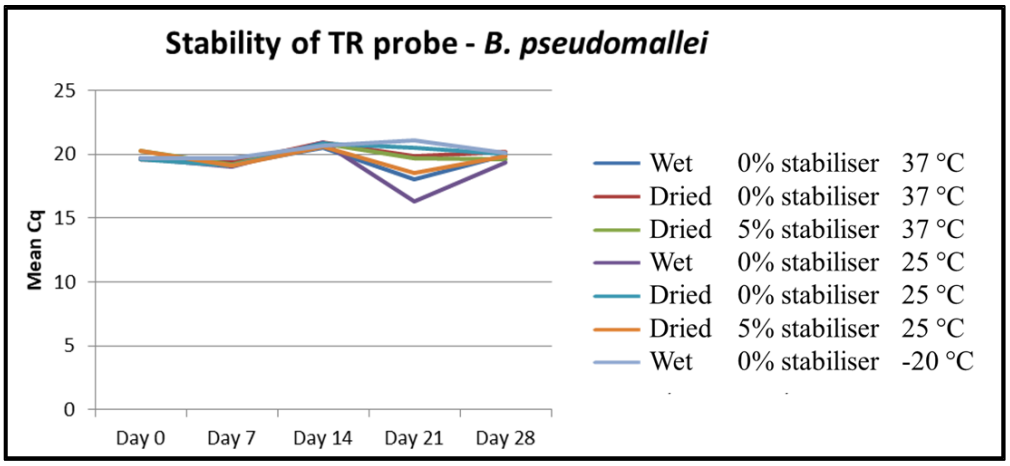


Figure 3.30 Trends of Texas Red probe stability at different form, stabiliser status and temperature

Table 3.42 Stability of wet and lyophilised multiplex oligonucleotides mixtures, with and without stabiliser at different temperature and length of times – HEX-labelled probe (*Salmonella*)

Dye	Storage temp.	Lyo-philisation	Amount of stabiliser	Mean Cq					Mean	SD	CV %
				Day 0	Day 7	Day 14	Day 21	Day 28			
HEX	25 °C	-	-	18.76	18.24	19.00	18.56	18.64	18.64	0.28	1.5
		Yes	-	18.76	18.40	19.08	18.59	18.70	18.71	0.25	1.3
		Yes	5%	19.06	18.27	18.68	18.86	18.49	18.67	0.31	1.7
	37 °C	-	-	18.76	18.4	19.01	18.72	18.32	18.64	0.28	1.5
		Yes	-	18.76	18.49	19.02	18.47	18.65	18.68	0.23	1.2
		Yes	5%	19.06	18.27	18.89	18.75	18.68	18.73	0.30	1.6
	- 20 °C	-	-	18.76	18.74	19.12	18.59	18.71	18.78	0.20	1.1

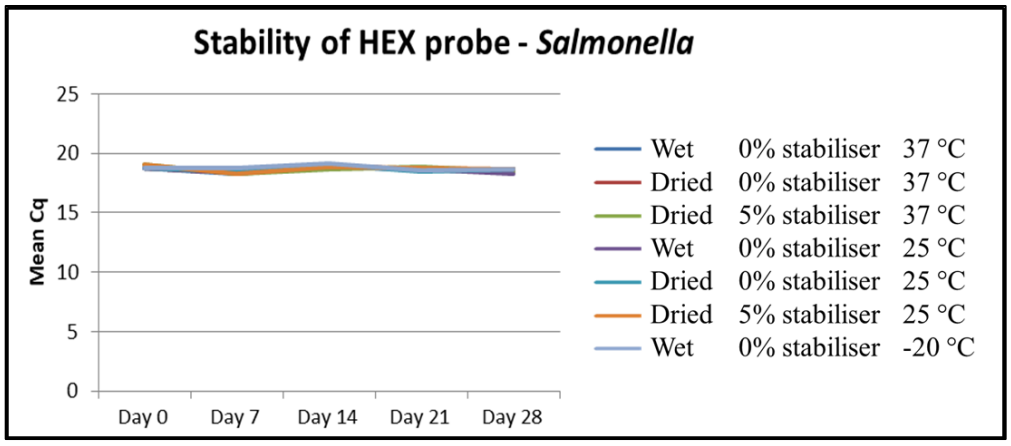


Figure 3.31 Trends of HEX probe stability at different form, stabiliser status and temperature

Table 3.43 Stability of wet and lyophilised multiplex oligonucleotides mixtures, with and without stabiliser at different temperature and length of times – Cy5-labelled probe (*Plasmodium*)

Dye	Storage temp.	Lyo-philisation	Amount of stabiliser	Mean Cq					Mean	SD	CV %
				Day 0	Day 7	Day 14	Day 21	Day 28			
Cy5	25 °C	-	-	18.18	18.1	17.66	17.39	17.85	17.84	0.32	1.8
		Yes	-	20.78	18.22	18.17	18.25	17.79	18.64	1.21	6.5
		Yes	5%	18.13	18.35	18.04	17.96	18.09	18.11	0.15	0.8
	37 °C	-	-	18.18	18.12	18.01	17.44	18.09	17.97	0.30	1.7
		Yes	-	20.78	18.19	18.56	17.86	18.00	18.68	1.20	6.4
		Yes	5%	18.13	18.14	18.01	18.11	17.78	18.03	0.15	0.8
	- 20 °C	-	-	18.18	18.05	18.13	18.16	17.71	18.05	0.19	1.1

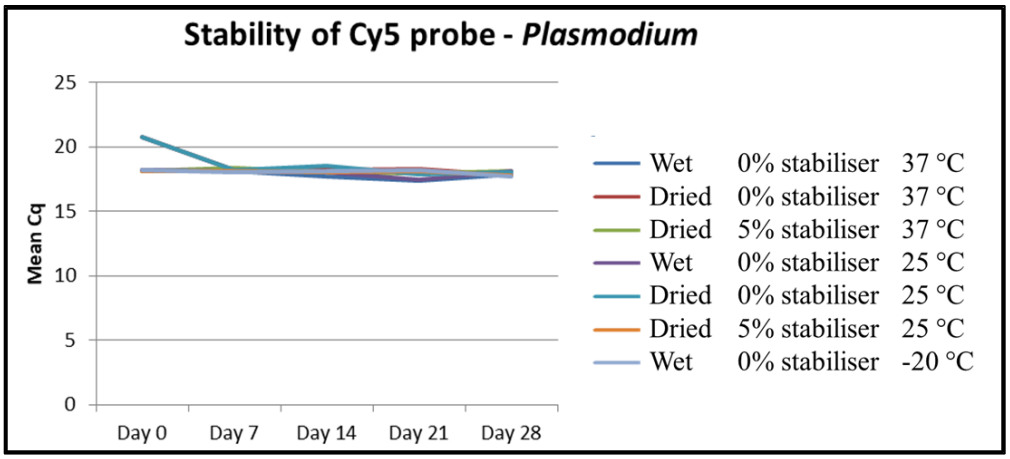


Figure 3.32 Trends of Cy5 probe stability at different form, stabiliser status and temperature

Table 3.44 Stability of wet and lyophilised multiplex oligonucleotides mixtures, with and without stabiliser at different temperature and length of times – Cy5.5-labelled probe (IAC)

Dye	Storage temp.	Lyo-philisation	Amount of stabiliser	Mean Cq					Mean	SD	CV %
				Day 0	Day 7	Day 14	Day 21	Day 28			
Cy5.5	25 °C	-	-	19.87	19.92	19.63	20.35	20	19.95	0.26	1.3
		Yes	-	20.08	20.26	20	20.67	19.93	20.19	0.30	1.5
		Yes	5%	19.65	19.5	19.23	20.31	20.02	19.74	0.43	2.2
	37 °C	-	-	19.87	20.12	20.33	20.31	20.33	20.19	0.20	1.0
		Yes	-	20.08	19.96	21.16	19.67	20.11	20.20	0.57	2.8
		Yes	5%	19.65	19.84	21.05	19.6	20.18	20.06	0.60	3.0
	- 20 °C	-	-	19.87	19.78	20.21	19.7	20.02	19.92	0.20	1.0

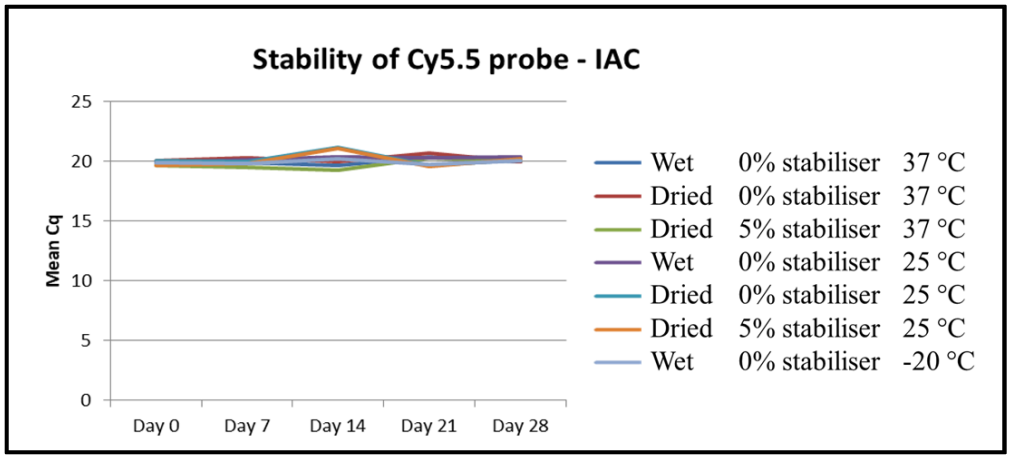


Figure 3.33 Trends of Cy5.5 probe stability at different form, stabiliser status and temperature

3.6.9 Clinical evaluation of the developed multiplex TaqMan qPCR assay

3.6.9 (a) Retrospective samples

Clinical validation of the developed multiplex qPCR assay was carried out using 518 clinical samples that were previously tested with respective gold standard / conventional tests. Of that, 100 leptospirosis-suspected samples were initially confirmed using GenoAmp® Real-Time PCR Leptospirosis kit. Meanwhile, 282 melioidosis- and salmonellosis-suspected clinical samples were cultured beforehand in BD BACTEC™ blood culture system. The remaining 136 clinical samples were previously tested using abTES™ Malaria qPCR I Kit.

As shown in Table 3.45, the multiplex qPCR assay correctly amplified all confirmed specimens. Among the negative specimens, one sample was amplified by the multiplex qPCR assays. While there was no evidence of cross-contamination, the PCR product was sent out for sequencing. BLAST analysis of the sequencing result showed that the sequences resembled those of *L. interrogans* and *L. kirschneri*. This finding had been reported in Molecular & Cellular Probe journal (M. R. Mohd Ali *et al.*, 2018). This discordant result may due to the differences of sensitivity of the qPCR assays, compounded by the nature of the *Leptospira rrs* gene that presents in two copies per genome (Bourhy *et al*., 2013). In addition, any leptospirosis caused by intermediate or saprophytic (rare) *Leptospira* strains, which does not have pathogenic genes may be undetectable by the GenoAmp® Real-Time PCR Leptospirosis kit, as the kit is exclusive for the pathogenic *Leptospira* counterparts. The remaining negative samples had identical results as the developed multiplex qPCR assays.

Clinical performance of the developed multiplex qPCR assay was calculated and summarised in Table 3.46. Overall, the developed assay had 100% clinical sensitivity in detecting samples with positive *Leptospira, B. pseudomallei, Salmonella* and *Plasmodium*. Similarly, high clinical specificity was observed in all targets. PPV and NPV were also very high. In leptospiral target, 95.2% PPV recorded due to a single discordant where leptospiral DNA was amplified by the multiplex qPCR assay from one negative specimen, as mentioned previously.

Table 3.45 Summary of clinical evaluation of the developed multiplex qPCR assay using confirmed clinical specimens

Targets	Conventional assay positive		Conventional assay negative	
	Multiplex qPCR +	Multiplex qPCR -	Multiplex qPCR +	Multiplex qPCR -
Leptospira (*n* = 100)	20	0	1	79
B. pseudomallei (*n* = 168)	20	0	0	148
Salmonella (*n* = 114)	20	0	0	94
Plasmodium (*n* = 136)	50	0	0	86

Table 3.46 Clinical sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of the developed multiplex qPCR assay

Targets	Sensitivity		Specificity		PPV	NPV
	%	95% CI (%)	%	95% CI (%)	%	%
Leptospira	100	83.2 to 100	98.8	93.2 to 99.9	95.2	100
B. pseudomallei	100	83.2 to 100	100	97.5 to 100	100	100
Salmonella	100	83.2 to 100	100	96.2 to 100	100	100
Plasmodium	100	92.9 to 100	100	95.8 to 100	100	100

3.6.9 (b) Prospective prevalence study

Lastly, the developed multiplex qPCR assays were tested prospectively on 155 patient samples with febrile symptom. Of that, 102 were males and 53 were females. The overall age average was 37.7 ± 20.2 years. Based on gender, the average age for males and females were 35.9 ± 18.9 years and 41 ± 22.8 years, respectively.

Of the 155 samples, 16 had detectable *Leptospira* DNA (10.32%), 4 had detectable *B. pseudomallei* DNA (2.58%), one sample was positive for malarial DNA (0.65%) and none for *Salmonella*. In addition, two samples were positive for both *Leptospira* and *B. pseudomallei* DNA (1.29%). However, the findings were not compared with concurrent diagnostic microbiological investigations because most of the samples were not tested for all diseases of interest (leptospirosis, melioidosis, invasive salmonellosis and malaria). Feasibly, a further retrospective study can be carried out to ideally compared the performance of this multiplex qPCR assay with MAT/leptospiral qPCR, culture and BFMP/malarial PCR on patients with unknown febrile illness.

CHAPTER 4

GENERAL DISCUSSION

The difficulties in timely diagnosis of leptospirosis, melioidosis, invasive salmonellosis and malaria remain as perplexing problems, especially to clinicians of the endemic areas. This is attributed to the lack of specific disease characteristics of each particular organism and their infections share a common clinical spectrum of signs and symptoms. Due to these reasons, leptospirosis, melioidosis and malaria are referred as the Great Mimicker (Ghosh and Nicolson, 1977; Izurieta *et al.*, 2008; Meumann *et al.*, 2012). The title can be expanded to invasive salmonellosis, as this infection also share overlapping clinical manifestations (Crump *et al.*, 2015).

During acute phase of leptospirosis, melioidosis, invasive salmonellosis and malaria, the most common presenting symptom is fever. For example, 91% - 99% of leptospirosis patients reported of having elevated temperature within the first week of infection (De Francesco Daher *et al.*, 2017). Similarly, 94.2% of patient with salmonellosis had fever of between 38 °C - 40 °C after seven to fourteen days of infection (Tapia *et al.*, 2015). For malaria and melioidosis, fever was reported in 96% and 96.8% of the cases, respectively (D'Acremont *et al.*, 2002; Vidyalakshmi *et al.*, 2012). Other common signs and symptoms include diarrhoea, myalgia, lethargy and pulmonary involvements, such as cough. While some infections are asymptomatic and do not require hospitalisation (i.e. patients may seek treatments from primary care facilities and been prescribed with general medications), in other cases, the infection may progress to severe forms of potentially fatal diseases.

Several misdiagnoses have been reported worldwide because leptospirosis, melioidosis, invasive salmonellosis and malaria were mistakenly diagnosed as other diseases (Kishimoto *et al.*, 2004; A. N. Rafizah *et al.*, 2012; Y.-M. Wen *et al.*, 2009). For example, only approximately one-third of MAT positive patients were diagnosed as leptospirosis during discharge (A. N. Rafizah *et al.*, 2012). In other circumstances, definitive diagnosis becomes more complicated when more than one organism infect the same patient. To date, several studies have reported leptospirosis-melioidosis co-infection (Hin *et al.*, 2012; Lu and Tseng, 2005; M. R. M. R. Mohd Ali, Mohamad Safiee, Thangarajah, *et al.*, 2017; Sapian *et al.*, 2012), leptospirosis-malaria co-infection (Srinivas *et al.*, 2007; Yong and Koh, 2013) and malaria-*Salmonella* co-infections (Keong and Sulaiman, 2006). Even though co-infections are uncommon, such cases are possible because of the similarity of the organisms geographical distribution. In addition, these infections are potentially under-diagnosed when clinical suspicion is lacking and reliable laboratory tests are limited or not available.

Due to the challenges in the clinical diagnosis, clinicians rely on laboratory test for confirmation. Currently, the gold standard test for diagnosis of leptospirosis is MAT and EMJH culture. The MAT utilises detection of host antibodies against *Leptospira*, which are typically produced after two weeks of infection, hence is less useful during acute leptospirosis (Suneth B. Agampodi *et al.*, 2016). Meanwhile, the fastidious nature of leptospiral growth leads to delay of positive culture, which usually requires several weeks of incubation, sometimes longer for pathogenic strains of *Leptospira* (Cameron, 2015). In addition, both methods are also laborious and suffer from inconsistent sensitivity and specificity.

Similarly, *B. pseudomallei* and *Salmonella* spp. are routinely detected by conventional culture method. Isolation of the bacteria requires more than 24 hours, followed by another 24 – 48 hours for biochemical identification (Andrews and Ryan, 2015; Lau *et al.*, 2015). Potential misidentification of the organism may happen due to inexperienced laboratory technologist or high genome plasticity and diverse phenotypic characteristic of the bacteria (Zong *et al.*, 2012). Based on the Bayesian model, the estimated culture sensitivity for *B. pseudomallei* and *Salmonella* was between 60.2% and 75% (Limmathurotsakul *et al.*, 2010; Parry *et al.*, 2011; Wilkins, 2009). In addition, blood culture requires large amount of samples; approximately 20 – 30 ml blood sample per patient, and is challenging for neonates and elderly (Kaur *et al.*, 2018).

Meanwhile, the current gold standard detection for *Plasmodium* is microscopy. Following Giemsa staining, blood smear is examined under the light microscope. Apart from low sensitivity, potential misdetection may happen due to inexperienced laboratory technologist (Joveen-Neoh *et al.*, 2011; Ting Goh *et al.*, 2013). The microscopic detection limit is between 10 – 50 trophozoites/µl (Trampuz *et al.*, 2003).

Unavailability of useful tool for early detection and differentiation of these important tropical infections lead to risk of misdiagnosis or under-diagnosis. Over the past 20 years, increasing number of real-time molecular assays have been reported worldwide, developed for many applications including pathogen detections (Huggett *et al.*, 2015; Pabinger *et al.*, 2014). Of these, hydrolysis or TaqMan probe is widely used due to its high specificity, simplicity and multiplexing flexibility, owing to the strict requirement of two primers and an additional probe for a positive amplification (Navarro *et al.*, 2015). Following total hybridization of primers and

probe, elongation of PCR product takes place. Upon reaching the probe, the 5' end exonuclease activity of the polymerase will cleave the previously coupled probe-quencher resulting in freeing of the detectable emitting probe (Navarro *et al.*, 2015). Many hydrolysis qPCR assays have been described for individual detection of leptospirosis, melioidosis, salmonellosis and malaria causative agents (Divis *et al.*, 2010; Kaestli *et al.*, 2012; Stoddard *et al.*, 2009; Villumsen *et al.*, 2012; Woods *et al.*, 2008). However, to date, there is no diagnostic assay that can simultaneously detect and differentiate *Leptospira, B. pseudomallei, Salmonella* and *Plasmodium* in a single tube.

This study is aimed to acknowledge the urgent need for a laboratory assay that enables simultaneous diagnosis of leptospirosis, melioidosis, salmonellosis and malaria. The developed multiplex TaqMan qPCR for *Leptospira, B. pseudomallei, Salmonella* and *Plasmodium* DNA was designed according to the MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments". This guideline was introduced to promote standardisation of reporting and consistencies inter laboratories (Bustin *et al.*, 2009). Recommendations from the MIQE guidelines were divided into (i) experimental design, (ii) sample, (iii) nucleic acid extraction, (iv) qPCR target information, (v) qPCR oligonucleotides, (vi) qPCR protocol, (vii) qPCR validation and (viii) qPCR data analysis. This present study adheres to the recommendations, albeit the developed multiplex assay is more qualitative, rather than quantitative.

4.1 Analytical sensitivity

Definitions of analytical sensitivity or LOD also vary between publications. Traditionally, LOD (or analytical sensitivity) is defined as the lowest amount of target that can be detected as positive in all replicates, or as low as one of them (Apfalter *et al.*, 2003; Augustin *et al.*, 2017; Pontiroli *et al.*, 2011). Meanwhile, some studies defined LOD as the minimum amount of analyte that can be statistically differentiated from a blank. This definition, however is more practical for chemical application (Armbruster and Pry, 2008). According to the MIQE, LOD is the lowest copy number of a target that can be determined correctly at a certain degree (Bustin *et al.*, 2009). Although the guideline does not specify the percentage of probability, 95% interval is commonly used. To determine LOD, replicates of diluted DNA templates were used. The larger number of replicates tested, the higher confidence level will be achieved. Some groups used between 6 and 9 replicates to determine LOD (Euler *et al.*, 2013; M. D. Moore and Jaykus, 2017; Muhd Radzi *et al.*, 2015). In this study 24 replicates were tested. In the subsequent steps, Probit regression analysis was used to determine LOD at 95% probability.

In terms of analytical sensitivity, the developed multiplex qPCR assay was able to detect as low as ~5.61 leptospiral DNA copies per reaction at 95% probability. The determined LOD is comparable to other probe-based qPCR assays that amplified between 1 – 10 *Leptospira* DNA copies per reaction (Ahmed *et al.*, 2009; Bourhy *et al.*, 2011; Brenner *et al.*, 1999; Levett *et al.*, 2005; Merien *et al.*, 2005; A. Slack *et al.*, 2007; Thaipadungpanit *et al.*, 2011). During the acute phase of leptospirosis, it is estimated that bacterial load ranges between 10^2 – 10^7 leptospires per ml of blood (Musso *et al.*, 2013). Taking into account the nucleic acid extraction and PCR set-up in this study, the detection of 5.61 DNA copies per reaction equals a

concentration of 175.3 leptospires per ml blood. The developed multiplex qPCR assay is supposed to be able to detect leptospires of this range. In addition, PCR is more useful than EMJH culture for patients who have received prior antibiotic treatment (Boonsilp *et al.*, 2011). Following administration of antibiotic, *Leptospira* is inactivated, leading to a negative culture.

The analytical sensitivity of the multiplex qPCR assay for the detection of *B. pseudomallei* DNA was approximately 8.24 DNA copies per reaction, equivalent to 257.5 CFU per mL of blood. Other reported hydrolysis probe-based molecular assays amplified 5 – 10 DNA copies per reaction (Al-Marzooq and Mustafa, 2011; Kaestli *et al.*, 2012; Novak *et al.*, 2006; B. Zhang *et al.*, 2012). During septicemic melioidosis, the estimated bacterial load was between 1 and 10^3 copies per mL of blood. This number is highly variable, depending on individuals (Wuthiekanun *et al.*, 2008). Even though the multiplex assay's LOD is within this range, a portion of low-level bacteremia may remain undetectable. As compared to culture method, PCR is more sensitive and less susceptible to pre-administration of antibiotic. Besides, culture method is also prone to contamination and requires up to seven days of incubation.

In the *Salmonella* target, the developed multiplex qPCR assay can detect approximately 19.3 DNA copies per reaction, or 603.1 CFU per mL blood. This LOD is relatively higher than the estimated number of *Salmonella* load in blood, 0.3 – 387 CFU per ml (J Wain *et al.*, 1998; Zhou *et al.*, 2016). Even though the other reported studies had analytical sensitivity of 1 – 1000 DNA copies per reaction, most of them were designed against limited serovars, required pre-enrichment steps and not validated for clinical use (González-escalona *et al.*, 2012; Maurischat *et al.*, 2015; Reynisson *et al.*, 2006). Differences of amount of clinical specimen used, as

well as the variation in commercial extraction kit also affected the sensitivity of assay (Tennant *et al.*, 2015).

In the fourth target, the developed multiplex qPCR assay can amplify approximately 18.1 malarial DNA copies per reaction. Since the target gene presents in 5 copies per *Plasmodium* genome, the calculated LOD could be around 3.7 organisms per reaction or 115.6 organisms per ml blood (Gonçalves *et al.*, 2012; Mangold *et al.*, 2005). Other reported studies had LOD is between 5 and 250 copies per reaction (Divis *et al.*, 2010; Mangold *et al.*, 2005; Pholwat *et al.*, 2017; Rougemont *et al.*, 2004).

Noticeably, in one sub-category of the MIQE guideline, the Cq variation at LOD of this assay was not determined because the LOD was determined statistically using Probit regression model. However, partial data of the multiplex qPCR assay performance at 20 fg DNA (for *Leptospira*, *B. pseudomallei* and *Salmonella*) and 2 ag (for *Plasmodium* synthetic DNA) showed that the CV% values were between 2% and 4%. As there is no definite acceptable range of CV% available, a threshold of 5% is favourable (Giry *et al.*, 2017). In this study, the reported variations at LOD and at repeatability and reproducibility testing were relatively small; even though some reports anticipated that a very low (and very high) concentration of DNA template will result in high CV%. (Srisutham *et al.*, 2017; Tennant *et al.*, 2015). To enhance precision of assay that suffers from high CV%, larger volume of each PCR component and minimum of 3 – 4 replicates could be considered (Svec *et al.*, 2015).

Among the most important factor that influence LOD (or sensitivity) is the DNA extraction process, a part of the qPCR itself. Usually, DNA recovery from the clinical samples is ≤30% (Augustin *et al.*, 2017). If this low DNA recovery is ignored, the initial microbial concentration in the clinical samples will be

underestimated. In addition, the DNA recovery efficiency is dependent on the type of specimen used. Therefore, using a set of diluted microorganism spiked into different matrices, such as blood, serum and plasma may provide useful information on the sensitivity of the assay across the potential clinical specimens. In addition, spiking experiment in different matrices also gives important information on potential presence of inhibitors in the tested samples, such as heparin (L. D. Smythe *et al.*, 2002). Remarkably, with the availability of many commercially available nucleic acid extraction kits that utilise silica column, the inhibitory effects are reduced, as compared to conventional methods that rely on organic-phase separation or alcohol precipitation (Khot and Fredricks, 2009). In this study, however, different matrices of specimen were not evaluated.

Some studies proposed modifications to enhance nucleic acid extraction and PCR performance. The modifications include elution of DNA in reduced volume of elution buffer (i.e. 100 µl versus 50 µl versus 25 µl) in order to concentrate the DNA (Dauphin *et al.*, 2010; Desneux and Pourcher, 2014; Tennant *et al.*, 2015), pre-warming of elution buffer (Taskin *et al.*, 2011), using larger amount of initial clinical specimens (200 µl versus 1000 µl) (Psifidi *et al.*, 2015), and implementing pre-enrichment steps (G. G. Stone *et al.*, 1994; Zhou *et al.*, 2016). Some assays also had increased total volume of up to 50 µl reaction to maximise the amount of eluted DNA used (Meumann *et al.*, 2006). The developed multiplex assay is a 20 µl reaction, containing 2 – 8 µl of DNA sample, previously eluted in pre-warmed 50 µl TE buffer.

Type of protease enzymes used in the extraction process also may affect DNA extraction. Its presence helps the degradation of cellular proteins and nucleases. Among the proteases that are available include papain, bromelain and

proteinase K (Mótyán *et al.*, 2013). A study had shown that different proteases were optimal for different samples. For an example, proteinase K was capable of yielding the highest number of DNA from semen and saliva, as compared to other proteases (Eychner *et al.*, 2015). The same protease also was efficient for DNA extraction from blood (Merk *et al.*, 2006). In this study, the Proteinase K was readily supplied by the manufacturer of the DNA extraction kit. Performances of different types of proteases were not evaluated in the current study. Interestingly, one study pointed out that high yield of nucleic acid does not always guarantee better chance of detecting microbial DNA. It is because the total amount of extracted DNA is significantly represented by larger mass of host cells, as compared to the infecting (or spiked) microorganism (Podnecky *et al.*, 2013).

In addition, performance of thermostable polymerase enzyme with 5' exonuclease activity in TaqMan qPCR platform also affect processivity; the capability of a polymerase to continuously replicate DNA without dissociation from DNA target (Holland *et al.*, 1991; Ishino and Ishino, 2014). In this study, a commercial SsoAdvanced Universal Probes super mix containing Sso7d fusion protein was used. The patented 7 kDa fusion protein, originated from *Sulfolobus solfataricus* help to stabilise the *Taq* polymerase binding to the DNA template and subsequently improves PCR efficiency, as compared to unmodified polymerase (Geetha Yadav, 2015; Kalichuk *et al.*, 2016; Wang *et al.*, 2004). Apart of the modification to the polymerase, the PCR mix manufacturer (Biorad, Germany) does not disclose the detail composition of its buffer and associated additives, which could also affect the molecular performances (Śpibida *et al.*, 2017). Further study may compare performance of the developed multiplex qPCR assay using other brands of

PCR mixes containing different types of polymerases, buffers and additives (if any) to evaluate the amplification effects (Śpibida *et al.*, 2017).

Several PCR additives, also known as stabilisers or enhancers can be added to improve amplification of difficult sequences. Some additives also can serve as lyoprotectants that inhibit potential loss during lyophilisation process. For examples, addition of betaine can help to stabilise the polymerase and hinder formation of primers secondary structures (Jensen *et al.*, 2010). Similarly, dimethyl sulfoxide (DMSO) also reduces intermolecular binding especially in GC-rich sequences (Bhagya *et al.*, 2013; Z. Zhang *et al.*, 2010). In this study, trehalose was used as additive during lyophilisation. However, there was no significant difference of assay performances between oligonucleotides mixture with- and without- additional trehalose, stored at different temperatures for 28 days. It is proposed that the effect will be more significant if prolong storage time is tested. Another possibility is that the amplicons produced in this study are 107 – 215 bp in length, with GC contents of between 40.7% and 58.5%, fall within the optimal range of qPCR products. Other studies have reported that addition of trehalose facilitated amplification of GC-rich DNA templates (as high as 80%) by decreasing the melting temperature and stabilising the polymerase (Spiess *et al.*, 2004; Q. Yang *et al.*, 2016; Z. Zhang *et al.*, 2010).

4.2 Analytical specificity

Analytical specificity of the developed multiplex qPCR was evaluated on 357 organisms. Overall, the developed assay was highly specific for the intended targets, *Leptospira, B. pseudomallei, Salmonella* and *Plasmodium*. Properties of target genes are important factor that determine specificity. In this assay, two housekeeping genes (*Leptospira* 16S rRNA gene & *Plasmodium* 18S rRNA genes) and two species-specific/genus-specific genes (*orf2*-TTSS of *B. pseudomallei* & *StyR-3* of *Salmonella*) were used. Major advantage of targeting 16S rRNA and 18S rRNA genes is that the gene present in 2 – 5 copies per *Leptospira* and *Plasmodium* genomes, respectively. However, designing oligonucleotides against housekeeping genes that present across many microorganisms is challenging. Therefore, polymorphic region of the conserved genes that is unique to the organism of interest need to be carefully identified. Sometimes, the regions may contain high CG content that disfavour amplification of the target (Bhagya *et al.*, 2013; Kotłowski *et al.*, 2004). Meanwhile, a novel gene that is distinctive to a particular genus or species serves as a good PCR target because theoretically, there is less chance of sequence similarity with other organisms.

Eventually, the assay should be validated empirically using genomic DNA of other organisms to confirm the specificity. In an instance, a molecular assay targeting *clyA* gene that is conserved in *S.* Typhi and *S.* Paratyphi A showed cross-reaction with two diarrheagenic *E. coli* strains (Tennant *et al.*, 2015). In other circumstances, the unique sequence may be short and do not allow many manipulations during oligonucleotides designing. TaqMan qPCR usually can amplify 70 – 150 bp, and up to 400 bp amplicon efficiently (Rodríguez *et al.*, 2015).

In this study, the 16S rRNA gene used in the developed multiplex qPCR assay was aimed to include the entire *Leptospira* genus, comprising the pathogenic, intermediate and saprophytic clusters, in contrast to majority of reported assays that exclusively targeted the former group. The pathogenic group of *Leptospira*, such as *L. interrogans* and *L. borgpetersenii* are commonly isolated from clinical specimens (Bourhy *et al.*, 2010; Chiani *et al.*, 2016). However, increasing numbers of studies have reported detection of the non-pathogenic leptospiral group from human infections, such as *L. fainei, L. wolffii* and *L. broomii* (Arzouni *et al.*, 2002; Bourhy *et al.*, 2013, 2011; Chiani *et al.*, 2016; Krogfelt *et al.*, 2001). Despite disputation on the pathogenicity of some *Leptospira* serovars (A. T. Slack *et al.*, 2009), detection of the bacterial DNA from sterile specimens should be regarded as important. However, careful experiment design should be implemented to avoid risk of false positivity due to contamination of assay from the laboratory settings (Koizumi *et al.*, 2003). Across the whole *Leptospira* genus, the developed assay was evaluated on 11 leptospiral species available in the laboratory. Further study could empirically validate the specificity of the developed assays on the non-tested *Leptospira* spp.

During the analytical specificity testing of the developed multiplex qPCR assay, six *B. cepacia* and one *B. thailandensis* strains were used. Limited number of *B. pseudomallei* complex and *B. cepacia* complex were tested due to unavailability of the strains in the laboratories. Even though *in silico* BLAST analysis showed that the absence of *orf2* gene from non-*B. pseudomallei*, further study could further validate empirical specificity of the developed assay on *B. pseudomallei* relatives including *B. mallei* and *Ralstonia* spp.

Similarly, the microbial panel for analytical specificity testing of the developed assay also lacks *P. malariae* and *P. ovale* strains. Although both species are uncommon to Malaysia, empirical specificity of the assay should be validated on the strains due to their infectivity to human. Retrospectively, the first imported case of *P. ovale* was reported in Malaysia in 2010 (Y. A. L. Lim *et al.*, 2010). Meanwhile, *P. malariae* might be potentially misidentified as it shares many microscopic features with *P. knowlesi*; an emerging causative agent for malaria in Malaysia, and usually confirmed by PCR (William *et al.*, 2014).

In this study, the multiplex qPCR assay was designed to target both typhoidal and non-typhoidal human salmonellosis. Twenty-four *Salmonella* serovars and 10 *Salmonella* spp. clinical isolates were evaluated in the analytical specificity. Those serovars represent strains that cause typhoid fever (*S.* Typhi), enteric fever (+ *S.* Paratyphi A, B and C) and invasive nontyphoidal *Salmonella* disease (the remaining serovars). The developed multiplex qPCR assay exclusively amplified all *Salmonella* strains tested. Presumably, the assay may also amplify other serovars as demonstrated by the *in vivo* BLAST specificity analysis. Further study could validate the assay on more Typhi, Weltevreden, Enteritidis, Typhimurium and Corvallis that were commonly isolated in Malaysia (Hendriksen *et al.*, 2011).

4.3 Effect of PCR cycles

Another factor that determines LOD is the PCR cycles. Commonly, a hydrolysis probe qPCR contains 35 – 45 cycles of amplification. In this study, the assay consisted 50 cycles, in parallel with several studies, to increase the intensity of fluorescence signals (Afonina *et al.*, 2007; Tennant *et al.*, 2015; U'Ren *et al.*, 2005; Villumsen *et al.*, 2012). Few assays used up to 60 cycles of amplification (Grúas *et*

al., 2014; Meumann *et al.*, 2006). Those assays that increased PCR cycle beyond 40 usually considered any Cq above the threshold as a positive result (Adamska M, Leonska-Duniec A., Maciejewska A., Sawczuk M. *et al.*, 2010). In this assay, positivity cut-off is set at Cq value of ≤40. Any Cq value arisen after cycle 40 is suggesting of inefficient PCR amplification (Millon *et al.*, 2011; Svec *et al.*, 2015).

4.4 Internal amplification control (IAC)

Internal amplification control (IAC) is an important component of qPCR assays. Incorporation of IAC to a molecular assay enable confirmation of true negative results and avoid false negative results that are usually caused by the presence of inhibitors and unsuccessful/sub-optimal nuclei acid extraction, a part of faulty polymerase and defective PCR composition, such as buffers and dNTPs. Remarkably, majority of the described molecular assays are lacking IAC (Khot and Fredricks, 2009; Price *et al.*, 2012). In practical, IAC target can be an endogenous gene that naturally presents in the sample matrix or an exogenous sequence that artificially introduced into the specimens (Mackay, 2007). Common endogenous gene (also known as housekeeping or reference gene) includes GAPDH, *rnaseP*, *actin* and several others. Using endogenous gene as IAC target can determine the efficiency of DNA extraction and predict presence or absence of PCR inhibitors. However, the concentration of extracted DNA is uncontrollable and sample dependent. Overwhelming amount of host DNA may have detrimental effects on amplification of microbial template that usually presents in low number.

In contrast, exogenous IAC target is unique sequences from non-target organisms (Saunders and Lee, 2013). The exogenous IAC can be added prior to DNA extraction, either to the crude sample or preferably to the lysis buffer. The

latter step is favourable because it minimises DNA degradation and potential loss. However, should the IAC template to be added earlier in the crude samples, a higher concentration of template, i.e. 150 fg IAC template per preparation, or utilisation of protective coated particles can be considered (Mikel *et al.*, 2016; Wei *et al.*, 2016). Advantages of adding IAC template prior to DNA extraction include the ability to determine successful nucleic acid recovery, as well as the presence of inhibitors. In the developed assay, 2 fg IAC template per reaction was included to the master mix to allow homogeneousness and rule-out presence of PCR inhibitors. Using exogenous IAC allow more room for optimisation of molecular assays, such as the primers-probe and IAC concentrations, that can be manipulated to minimise amplification competition with the microbial targets.

Some study recommended that IAC amplicon of less than 150 bp will not affect PCR efficiency and subsequent amplification competition (Hoorfar *et al.*, 2004). Using longer amplicon may not be optimal for relatively short extension time (~ 30 seconds) in qPCR platform (Oikonomou *et al.*, 2008). Moreover, it is also recommended that the PCR efficiency between the desired target and IAC target should be similar, between 90% – 110%. The developed assay utilised IAC amplicon of 107 bp in length. However, its PCR efficiency was 76.9%, due to the lower amount of primers used, purposely designed to minimize parallel competition with the other microbial target. In the scenario which microbial target is abundant, reduced or zero amplification of IAC is anticipated (Oikonomou *et al.*, 2008).

Alternatively, non-competitive IAC strategy can be implemented, in which amplification of microbial target and IAC happen in two different reactions, concurrently. While the non-competitive strategy will not affect the sensitivity of the

assay, the PCR cost is increased due to higher volume of PCR mix and consumables required during the experiments.

4.5 Clinical evaluation

Clinical performance of the developed multiplex qPCR was evaluated on confirmed clinical specimens. The diagnostic sensitivity and specificity were very high, with PPV and NPV of around 100%. Upon implementation in clinical settings, several factors that affect PCR performance should be taken into considerations.

Duration of illness or day of onset has been shown to affect the usefulness of molecular assays. Generally, the onset of fever within two weeks is indicated for molecular testing. For example, the malarial parasitemia is detectable during 5 – 10 days of infection (Trampuz *et al.*, 2003). Likewise, for leptospirosis, PCR was reported to be most sensitive between 5 and 10 days post infection (Musso *et al.*, 2013). However, some cases reported detection of leptospiral DNA after 15 days of stipulated days of onset. In addition, *Salmonella* also presents in the bloodstream during the first one or second week of illness (Crump *et al.*, 2015). However, for molecular detection, it is reported that *Salmonella* DNA is amplifiable within 48 – 72 hours of infection, in one case; during 6 hours post-infection (Darton *et al.*, 2017). Meanwhile, bacteremic melioidosis multiply between day 1 and 21 of infection (B J Currie *et al.*, 2000). In another cases, PCR for *B. pseudomallei* was positive until day 50 of illness (Kim *et al.*, 2015). Theoretically, PCR is most useful during the acute phase of infection. For an example, implementing molecular assay on convalescent / chronic leptospirosis sample may be suboptimal and potentially results in false negativity.

Another important factor is the type of specimens used in the molecular assay. To date, there are limited numbers of studies that determine microbial load from real clinical samples. Alternatively, artificially spiked specimens were used to mimic the infections. In leptospirosis and melioidosis suspected cases, plasma was slightly better than serum for molecular testing (Richardson *et al.*, 2012; Robertson *et al.*, 2015; Stoddard *et al.*, 2009). Meanwhile, serum was reported to be superior to whole blood (S. B. Agampodi *et al.*, 2012). However, another study reported that the differences between plasma, serum and whole blood were not significant (Bourhy *et al.*, 2011). For *Salmonella*, whole blood is considered as the optimal specimens for PCR, most useful during the first 5 days of infection (Andrews and Ryan, 2015; Hatta and Smits, 2007). Alternatively, bone marrow and buffy coats can be used for salmonellosis diagnosis (John Wain *et al.*, 2008).

In the malarial cases, whole blood was 3-fold more sensitive than plasma (Lamikanra *et al.*, 2012). Meanwhile, no significant difference was observed between plasma and serum, used in a malarial PCR assay (Waggoner *et al.*, 2015). In addition, heparin-containing samples should be avoided due to inhibitory reaction (Levett *et al.*, 2005). In this study, the developed assay was validated on EDTA whole bloods, a single clinical matric that can be used consistently across all of the tested infectious diseases.

CHAPTER 5

SUMMARY, LIMITATIONS AND RECOMMENDATIONS FOR FUTURE STUDY

The present study successfully developed a multiplex qPCR assays for simultaneous detection of *Leptospira, B. pseudomallei, Salmonella* and *Plasmodium* DNA, with the incorporation of an IAC. The determined analytical sensitivities were 5.61 copies, 8.24 copies, 19.3 copies and 18.1 copies per reaction, respectively, with 100% specificity. Similarly, the high clinical sensitivities (100%) and specificities (98.8% – 100%) were also observed, upon evaluation on confirmed patient specimens.

Among the limitations of this study is the number of clinical isolates used for determination of the analytical specificity. Noticeably, a small number of *B. pseudomallei* genetic relatives, especially *B. mallei, B. thailandensis, B. oklahomensis* and *Rastolnia* spp. were included in the panel of organisms. Similarly, between 2015 and 2018, the *Leptospira* genus received additional 13 novel leptospiral species, in which 10 species are pathogenic or intermediate. Incorporating more bacteria and other pathogens in the analytical evaluation will provide more information on the assay's specificity, eventually expand its feasibility in environmental and veterinary research.

During the prospective clinical evaluation, the developed assay was used to screen 155 patient samples with febrile symptom. Due to constraints of time and limited availability of resources, the initial qPCR results were not tallied with other microbiological tests, such as MAT, blood culture and BFMP, as well as the final diagnosis upon patients' discharge. By comparing the final diagnosis and other

external factors, such as day of illnesses, severity of diseases, it is believed that more consequential data can be generated. Subsequently, these data will provide insights on factors that contribute positivity (or negativity) of molecular tests for leptospirosis, melioidosis, salmonellosis and malaria. In addition, following molecular detection, conventional methods such as serological typing, or PCR may be required to further speciate *Plasmodium* and *Salmonella.*

Absolute quantification of microorganism in blood is another area to be explored. As reported by other studies, level of bacteraemia and parasitaemia may influence severity and clinical outcomes of infected patients. Moreover, such data is important to be used to monitor treatment effectiveness or failure. Further validation of the developed assay for absolute quantification of *Leptospira*, *B. pseudomallei, Salmonella* and *Plasmodium* DNA in blood samples is favourably anticipated. Moreover, future study may also compare performance of molecular assays on different clinical specimens such as plasma, serum, buffy-coat to determine the most suitable samples for molecular assays. In other circumstances, presence of *Leptospira* in other specimens such as urine (after initial blood sample is PCR positive) can be used to project potential positive growth in artificial culture, which is valuable for epidemiological perspective.

Another limitation lies on the TaqMan hydrolysis probe platform itself. A TaqMan hydrolysis probed-based assays validated in Bio-rad machines may not be used in Applied Biosystems machines or others', and vice versa. To enable implementation of the developed assay on other machines, further studies may be needed to re-optimise the assay (especially the probes compatibility). In addition, ones may also include effects of different PCR master mixes, different types of nucleic acid adapters and other PCR parameters on the performances of the assay.

Nonetheless, in its current form, this assay is relatively sensitive, able to detect between 5 and 20 copies of pathogens per reaction.

Overall, this study successfully developed a multiplex TaqMan hydrolysis probe-based assay for simultaneous detection of *Leptospira, B. pseudomallei, Salmonella* and *Plasmodium* DNA with high sensitivity and specificity. Moreover, subsequent diagnostic evaluation suggested that this assay performed remarkably on the clinical specimens.

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APPENDICES

APPENDIX A: HUMAN ETHICAL APPROVAL (MOH)

JAWATANKUASA ETIKA & PENYELIDIKAN PERUBATAN
(*Medical Research & Ethics Committee*)
KEMENTERIAN KESIHATAN MALAYSIA
d/a Institut Pengurusan Kesihatan
Jalan Rumah Sakit, Bangsar
59000 Kuala Lumpur

Tel.: 03-2287 4032/2282 0491/2282 9085
03-2282 9082/2282 1402/2282 1449
Faks: 03-2282 0015

Ruj.Kami:KKM.NIHSEC. P16-1839 (7)
Tarikh: 02-February-2018

DR Chan Yean Yean
Universiti Sains Malaysia (USM), Health Campus

Mohammad Ridhuan Bin Mohd Ali
Universiti Sains Malaysia (USM), Health Campus

Tuan,

Annual Ethical Renewal for 2018:NMRR-16-2117-33181 (IIR)
FREQUENCY OF LEPTOSPIROSIS AND MELIOIDOSIS AMONG ACUTE FEBRILE PATIENTS AT SELECTED MOH FACILITIES (HOSPITAL RAJA PEREMPUAN ZAINAB II AND MAKMAL KESIHATAN AWAM KOTA BHARU)

With reference to the 'Continuing Review Form' submitted 23-January-2018, we are pleased to inform that the conduct of the above study has been granted approval (via Expedited Review by Chairperson) for a year by the Medical Research & Ethics Comittee, Ministry of Health Malaysia. Please note that the approval is valid until 01-February-2019. To renew the approval, a completed 'Continuing Review Form' has to be submitted to MREC **within 1 month** before the expiry of the approval.

The MREC, Ministry of Health Malaysia operates in accordance to the International Harmonization Good Clinical Practice Guidelines.

Thank you.

"BERKHIDMAT UNTUK NEGARA"

Yours sincerely,

.....
(DR HAJAH SALINA BINTI ABDUL AZIZ)
Chairperson
Medical Research & Ethics Committee
Ministry of Health Malaysia

SMN/Annualrenewal2018/MRECShare

APPENDIX B: HUMAN ETHICAL APPROVAL (USM)

Jawatankuasa Etika Penyelidikan Manusia USM (JEPeM)
Human Research Ethics Committee USM (HREC)

Universiti Sains Malaysia
Kampus Kesihatan,
16150 Kubang Kerian,
Kelantan. Malaysia.
T: 609 - 767 3000 *samb.* 2354/2362
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29th December 2016

Assoc. Prof. Dr. Chan Yean Yean
Department of Medical Microbiology & Parasitology
School of Medical Sciences
Universiti Sains Malaysia
16150 Kubang Kerian, Kelantan.

JEPeM Code : USM/JEPeM/16080260
Protocol Title : Prevalence of Leptospirosis, Melioidosis, Salmonelia Bacteraemia and Malaria among Febrile Patients Presented to the Emergency Department, Hospital USM.

Dear Dr.,

We wish to inform you that your study protocol has been reviewed and is hereby granted approval for implementation by the Jawatankuasa Etika Penyelidikan Manusia Universiti Sains Malaysia (JEPeM-USM). Your study has been assigned study protocol code **USM/JEPeM/16080260**, which should be used for all communication to the JEPeM-USM related to this study. This ethical clearance is valid from **29th December 2016** until **28th December 2017.**

Study Site: Hospital Universiti Sains Malaysia.

The following researchers also involve in this study:

1. Dr. Mohd Hashairi Fauzi
2. Mr. Mohammad Ridhuan Mohd Ali
3. Dr. Nabilah Awang
4. Miss Amira Wahida Mohd Safiee

The following documents have been approved for use in the study.

1. Research Proposal

In addition to the abovementioned documents, the following technical document was included in the review on which this approval was based:

1. Patient Information Sheet and Consent Form (English version)
2. Patient Information Sheet and Consent Form (Malay version)
3. Patient Perfoma Sheet

Attached document is the list of members of JEPeM-USM present during the full board meeting reviewing your protocol.

While the study is in progress, we request you to submit to us the following documents:

1. Application for renewal of ethical approval 60 days before the expiration date of this approval through submission of **JEPeM-USM FORM 3(B) 2015: Continuing Review Application Form.** Subsequently this need to be done yearly as long as the research goes on.
2. Any changes in the protocol, especially those that may adversely affect the safety of the participants during the conduct of the trial including changes in personnel, must be submitted or reported using **JEPeM-USM FORM 3(A) 2015: Study Protocol Amendment Submission Form.**
3. Revisions in the informed consent form using the **JEPeM-USM FORM 3(A) 2015: Study Protocol Amendment Submission Form.**

4. Reports of adverse events including from other study sites (national, international) using the **JEPeM-USM FORM 3(G) 2014: Adverse Events Report.**
5. Notice of early termination of the study and reasons for such using **JEPeM-USM FORM 3(E) 2015.**
6. Any event which may have ethical significance.
7. Any information which is needed by the JEPeM-USM to do ongoing review.
8. Notice of time of completion of the study using **JEPeM-USM FORM 3(C) 2014: Final Report Form.**

Please note that forms may be downloaded from the JEPeM-USM website: **www.jepem.kk.usm.my**

Jawatankuasa Etika Penyelidikan (Manusia), JEPeM-USM is in compliance with the Declaration of Helsinki, International Conference on Harmonization (ICH) Guidelines, Good Clinical Practice (GCP) Standards, Council for International Organizations of Medical Sciences (CIOMS) Guidelines, World Health Organization (WHO) Standards and Operational Guidance for Ethics Review of Health-Related Research and Surveying and Evaluating Ethical Review Practices, EC/IRB Standard Operating Procedures (SOPs), and Local Regulations and Standards in Ethical Review.

Thank you.

"ENSURING A SUSTAINABLE TOMORROW"

Very truly yours,

PROF. DR. HANS AMIN VAN ROSTENBERGHE
Chairperson
Jawatankuasa Etika Penyelidikan (Manusia) JEPeM
Universiti Sains Malaysia

APPENDIX C: SAMPLE SIZE CALCULATIONS

Retrospective sample size calculations (sensitivity test)

According to the Buderer (1996);

$$n_{sensitivity} = Z^2 \times \frac{P_{sn}(1 - P_{sn})}{d^2} \div P$$

Where,

$n_{sensitivity}$	= sample size for sensitivity test	
Z	= Z statistic corresponding to 95% confidence	= 1.96
P_{sn}	= Assay sensitivity or specificity	= 0.98
P	= Prevalence of disease	
$P_{\text{leptospirosis}}$	= 8.4% (Rafizah *et al*., 2013)	
$P_{\text{melioidosis}}$	= 5% (Berger, 2018)	
$P_{\text{salmonellosis}}$	= 7.4% (Brown *et al*., 1984)	
P_{malaria}	= 6.2% (Berger, 2018)	
d	= precision	= 0.10

For leptospirosis samples,

P	= Prevalence of disease (leptospirosis)	= 0.084
$n_{sensitivity}$	$= 1.96^2 \times \frac{0.98\,(1-0.98)}{0.10^2} \div 0.084$	= 89.6 samples

For melioidosis samples,

P	= Prevalence of disease (melioidosis)	= 0.05
$n_{sensitivity}$	$= 1.96^2 \times \frac{0.98\,(1-0.98)}{0.10^2} \div 0.05$	= 150.6 samples

For salmonellosis samples,

P	= Prevalence of disease (melioidosis)	= 0.074
$n_{sensitivity}$	$= 1.96^2 \times \frac{0.98\,(1-0.98)}{0.10^2} \div 0.074$	= 101.8 samples

For malaria samples,

P	= Prevalence of disease (melioidosis)	= 0.062
$n_{sensitivity}$	$= 1.96^2 \times \frac{0.98\,(1-0.98)}{0.10^2} \div 0.062$	= 121.4 samples

Retrospective sample size calculations (specificity test)

According to the Buderer (1996);

$$n_{specificity} = Z^2 \times \frac{P_{sn}(1 - P_{sn})}{d^2} \div (1 - P)$$

Where,

$n_{specificity}$ = sample size for specificity test

Z = Z statistic corresponding to 95% confidence = 1.96

P_{sn} = Assay sensitivity or specificity = 0.98

P = Prevalence of disease

$P_{\text{leptospirosis}}$ = 8.4% (Rafizah *et al*., 2013)

$P_{\text{melioidosis}}$ = 5% (Berger, 2018)

$P_{\text{salmonellosis}}$ = 7.4% (Brown *et al*., 1984)

P_{malaria} = 6.2% (Berger, 2018)

d = precision = 0.10

For leptospirosis samples,

P = Prevalence of disease (leptospirosis) = 0.084

$n_{specificity} = 1.96^2 \times \frac{0.98\,(1-0.98)}{0.10^2} \div (1 - 0.084)$ = 8.2 samples

For melioidosis samples,

P = Prevalence of disease (melioidosis) = 0.05

$n_{specificity} = 1.96^2 \times \frac{0.98\,(1-0.98)}{0.10^2} \div (1 - 0.05)$ = 7.9 samples

For salmonellosis samples,

P = Prevalence of disease (melioidosis) = 0.074

$n_{specificity} = 1.96^2 \times \frac{0.98\,(1-0.98)}{0.10^2} \div (1 - 0.074)$ = 8.1 samples

For malaria samples,

P = Prevalence of disease (melioidosis) = 0.062

$n_{specificity} = 1.96^2 \times \frac{0.98\,(1-0.98)}{0.10^2} \div (1 - 0.062)$ = 8.0 samples

For sensitivity and specificity testing using retrospective clinical samples, the larger number between $n_{sensitivity}$ and $n_{specificity}$ was selected. In general, $n_{sensitivity}$was larger than the $n_{specificity}$, hence was selected as minimum number of samples to be collected. Summary of samples required for retrospective clinical samples evaluation was listed in the following table.

Table A1 Summary of samples required for clinical validation

Cases	$n_{\text{sensitivity}}$	$n_{\text{specificity}}$	n_{required} (including drop-out)
Leptospirosis	90	8	100
Melioidosis	151	8	168
Salmonellosis	102	8	114
Malaria	121	8	136

Prospective sample size calculation

$n = \frac{Z^2 P(1-P)}{d^2}$, where

n = sample size

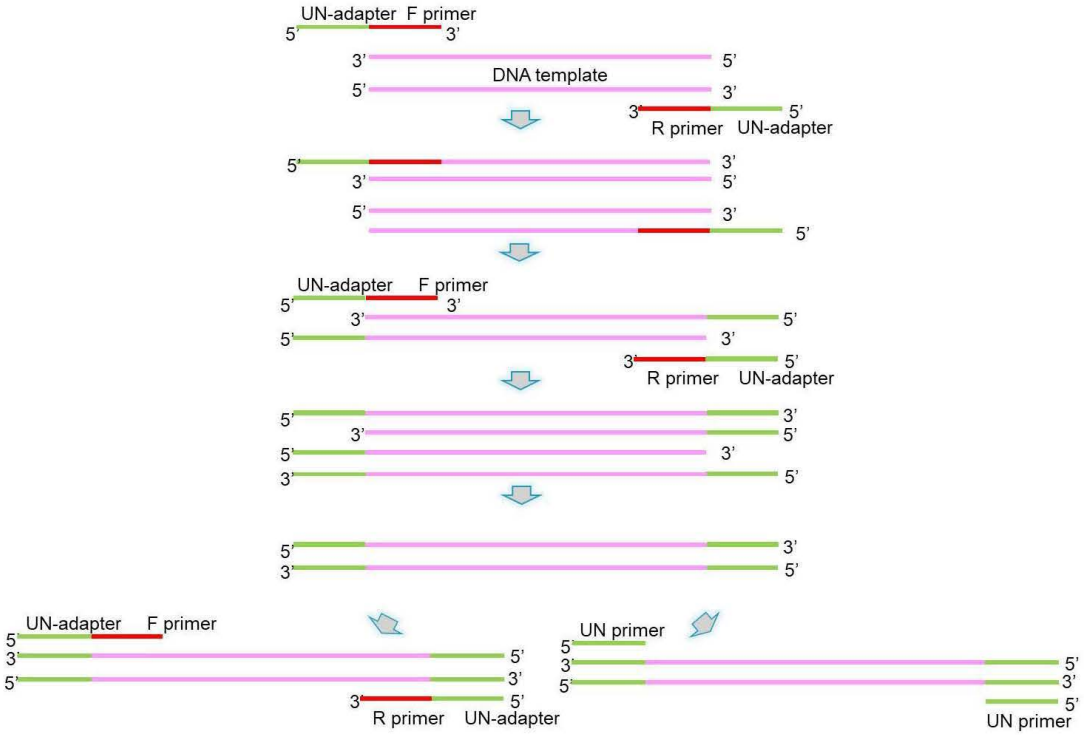
Z = Z statistic corresponding to 95% confidence = 1.96

P = expected prevalence = 0.084 (Rafizah *et al*., 2013)

d = precision = 0.05

∴ No. of samples, $n = \frac{1.96^2 0.084(1-0.084)}{0.05^2}$ = 118 samples

APPENDIX D: ROLES OF UNIVERSAL ADAPTER AND UNIVERSAL PRIMER DURING AMPLIFICATION



LIST OF PATENT AND PUBLICATIONS

Patent

Mohd Ali, M. R, and Yean Yean, C. (2016) Patent title: 'Molecular tool', Patent no: PI2016703845. Filing date:19 October 2016

Accepted publications

Mohd Ali, M. R, Mohamad Safiee, A. W., Thangarajah, P., Fauzi, M. H., Muhd Besari, A., Ismail, N. and Yean Yean, C. (2017) 'Molecular detection of leptospirosis and melioidosis co-infection: A case report', Journal of Infection and Public Health 10(2017), pp. 894–896. doi:10.1016/j.jiph.2017.02.009.

Mohd Ali, M. R., Maning, N. and Yean Yean, C. (2017) 'Detection of *B. pseudomallei* DNA among febrile patients presenting to tertiary hospitals in north eastern Malaysia', Sri Lankan Journal of Infectious Diseases, 7(S29), pp. 38–39. doi: 10.4038/sljid. v7i0.8197.

Mohd Ali, M. R., Mohamad Safiee, A. W., Yusof, N. Y., Fauzi, M. H., Yean Yean, C. and Ismail, N. (2017) 'Isolation of *Leptospira kmetyi* from residential areas of patients with leptospirosis in Kelantan, Malaysia', Journal of Infection and Public Health. 11(2018), pp. 578–580. doi: 10.1016/j.jiph.2017.12.008.

Mohd Ali, M. R., Mohd Safee, A. W., Ismail, N. H., Sapian, R. A., Hussin, H. M., Ismail, N. and Yean Yean, C. (2018) 'Development and validation of pan-*Leptospira* TaqMan qPCR for the detection of *Leptospira* spp. in clinical specimens', Molecular and Cellular Probes. doi: 10.1016/j.mcp.2018.03.001.

Mohd Ali, M. R., Foo, P.C., Hassan, M., Maning, M., Hussin, A., Syed Ahmad Yunus, S. Z., Fauzi, M. H., Muhd Besari, A., Harun, A., M., Ismail, N. and Yean Yean, C. (2018) 'Development and validation of TaqMan real-time PCR for the detection of *Burkholderia pseudomallei* isolates from Malaysia', Tropical Biomedicine (*In press*).

Manuscript in preparation

Mohd Ali, M. R., Harun, A., M., Ismail, N. and Yean Yean, C. (2018) 'Development and clinical validation of a novel multiplex hydrolysis probe-based qPCR for detection of *Leptospira, Burkholderia pseudomallei, Salmonella* and *Plasmodium* with incorporated IAC, *PLoS* Neglected Tropical Diseases (Manuscript in preparation).

Mohd Ali, M. R, and Yean Yean, C. (2016) Patent title: 'Molecular tool', Patent no: PI2016703845. Filing date:19 October 2016

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Pengarah
Innovation and Commercialization Office
Division of Research and Innovation
Chancellory II, Block E42
University Sains Malaysia
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BY COURIER

Attn: Cik Hartini Alias

Dear Sirs

Re: Malaysian Patent Application No: PI 2016703845
Date of Filing: 19 October 2016
Applicant: UNIVERSITI SAINS MALAYSIA
Invention: MOLECULAR TOOL

We refer to the captioned application and have the pleasure of confirming that we have filed the identified Patent application at the Intellectual Property Corporation of Malaysia on **19 October 2016**. Herewith, we enclose the filing details, patent specifications as filed and our invoice (Invoice No.1957) for your records and action.

Now that you have filed a patent application in Malaysia, should you wish to file foreign patent applications for the same invention, you may do so by filing the foreign applications before **19 October 2017**. This deadline is in accordance with the PARIS Convention and The Patent Cooperation Treaty (PCT) if priority is to be claimed. Please note that this date is non extendable. We will be able to assist you in filing patent applications in any country.
The above application(s) will be disclosed to the public by the Malaysian IP office at the 18th month from the date of filing the application. Should you wish to differ disclosure of your application, you may do so by giving us advance notice in writing, at least 2 weeks prior to the scheduled publication date.

The request for substantive examination will be due by **19 April 2018**. Kindly docket this date carefully at your end. Whilst we shall endeavour to send you a reminder, we will not be held responsible if timely instructions are not received from you, at least a week prior to this date, to proceed with the said request.

Thank you for entrusting us with the application.

Yours sincerely,

Sushil Kaur
IP Director
AETAS IP Solutions Sdn.Bhd.

Mohd Ali, M. R, Mohamad Safiee, A. W., Thangarajah, P., Fauzi, M. H., Muhd Besari, A., Ismail, N. and Yean Yean, C. (2017) 'Molecular detection of leptospirosis and melioidosis co-infection: A case report', Journal of Infection and Public Health 10(2017), pp. 894–896. doi:10.1016/j.jiph.2017.02.009.

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Molecular detection of leptospirosis and melioidosis co-infection: A case report

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ABSTRACT

Leptospirosis and melioidosis are important tropical infections caused by *Leptospira* and *Burkholderia pseudomallei*, respectively. As both infections share similar clinical manifestations yet require different managements, complementary laboratory tests are crucial for the diagnosis. We describe a case of *Leptospira* and *B. pseudomallei* co-infection in a diabetic 40-year-old woman with history of visit to a freshwater camping site in northern Malaysia. To our knowledge, this is the first case of such double-infection, simultaneously demonstrated by molecular approach. This case highlights the possibility of leptospirosis and melioidosis co-infections and their underlying challenges in the rapid and accurate detection of the etiologic microorganism.

Introduction

Pathogenic *Leptospira* spp. and *Burkholderia pseudomallei* are emerging tropical diseases that cause leptospirosis and melioidosis, respectively. *Leptospira* infecting more than one million people and cause 58,900 deaths annually [1]. Meanwhile, melioidosis incidence is approximately one tenth of the leptospirosis', but, as high as 89,000 patients succumbed to the disease [2].

Rodents and ruminants are known leptospiral hosts and are presumed to excrete the organism to the environment. Similarly, seroprevalence of *B. pseudomallei* been reported in several animals, including livestock, but contaminated soil remains as one of the main source of melioidosis [3]. Both organism are usually found in moist environment with pH close to 7 [4,5]. Infections may occur by direct inhalation, ingestion, inoculation of the organism or indirectly from contaminated environment [4,6].

Clinically, leptospirosis and melioidosis portray an almost similar wide spectrum of clinical features including fever, headache, myalgia, cough, diarrhoea, vomiting and jaundice [4,7]. As a consequence, physicians rely significantly on the laboratory tests for a confirmatory diagnosis. A misdiagnosis may happen when only either of the tests is considered or available.

Case report

A 40-year-old female school teacher, who has type 2 diabetes mellitus for the past 3 years, poor compliance to medication, was referred to our centre with prolonged high grade fever for 2 weeks, chills and rigor, headache, arthralgia and myalgia, poor appetite, associated with epigastric pain for 2 days duration. There was no history of vomiting, diarrhoea, cough or sputum, rash, seizure, tea coloured urine and contact with tuberculosis. Even though patient resided in dengue endemic area, there was no fogging activity nearby her house at that time. She visited emergency department of another tertiary hospital during her first week of illness, but was discharged with oral antibiotics. There were histories of visiting nearby waterfall one month prior to admission and contact with rats and mice at home.

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http://dx.doi.org/10.1016/j.jiph.2017.02.009

Mohd Ali, M. R., Maning, N. and Yean, C. Y. (2017) 'Detection of *B. pseudomallei* DNA among febrile patients presenting to tertiary hospitals in north eastern Malaysia', Sri Lankan Journal of Infectious Diseases, 7(S29), pp. 38–39. doi: 10.4038/sljid. v7i0.8197.

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DOI: http://doi.org/10.4038/sljid.v7i0.8197

Detection of *B. pseudomallei* DNA among febrile patients presenting to tertiary hospitals in north eastern Malaysia

MRMAli[1,2], NManing[3], CYYean[1,4]

Introduction

Melioidosis, caused by *Burkholderiapseudomallei*, is an important infection, endemic to many tropical regions including South East Asia and northern Australia. In Malaysia, it is estimated that 19% of melioidosis patients died within the first 48 hours of admission, due to septic shock. Early detection plays an essential role in patients prognosis but clinical diagnosis of melioidosis is difficult, as other tropical infections, such as leptospirosis, dengue and scrub typhus also portray similar disease manifestations. A better detection method is therefore urgently needed to allow timely diagnosis and accurate treatment for melioidosis. Our study aimed to evaluate the performance of qPCR for the detection of *B. pseudomallei* among febrile patients who presented to healthcare facilities in north eastern Malaysia.

Methods

A total of 108 blood samples from febrile patients were collected between April 2016 and May 2017 from Hospital Sains Malaysia, Hospital Raja Perempuan Zainab II and Kota Bharu Public Health Laboratory, Malaysia. Genomic DNA were extracted and analysed using real time qPCR to detect the presence of *B. pseudomallei*. Subsequent clinical data, including related microbiological investigation results of patients with positive assay were retrieved to confirm the molecular findings.

Results

Three samples (2.8%, n = 3/108) were found to have detectable *B. pseudomallei*DNA. Parallel laboratory investigations revealed that these 3 patients had positive blood cultures. *B. pseudomallei* were isolated in two samples. Meanwhile, the other patient's blood culture grew multiple organisms; *Klebsiella ozanae* and *K. oxytoca*. Upon retrieval of extensive clinical data, the qPCR-positive, culture-negative patient was reported to have liver abscesses and underlying diabetes mellitus type II. This scenario is possible as conventional culture methods have limited sensitivity (~60%). Therefore, multiple sampling and radiological investigation are recommended in patients with suspected melioidosis. We also found that two of the melioidosis patients also had simultaneous leptospirosis, based on the concurrent leptospiral investigations.

Discussion and Conclusions

This study provides preliminary data in favour of utilisation of qPCR as a sensitive screening tool for melioidosis diagnosis in laboratories of tertiary hospitals in Malaysia, and other endemic regions. Moreover, it also highlights the possibilities of misdiagnosis of

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Mohd Ali, M. R., Mohamad Safiee, A. W., Yusof, N. Y., Fauzi, M. H., Yean Yean, C. and Ismail, N. (2017) 'Isolation of *Leptospira kmetyi* from residential areas of patients with leptospirosis in Kelantan, Malaysia', Journal of Infection and Public Health. 11(2018), pp. 578–580. doi: 10.1016/j.jiph.2017.12.008.

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Isolation of *Leptospira kmetyi* from residential areas of patients with leptospirosis in Kelantan, Malaysia

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ABSTRACT

Background: Environmental sampling provides important information that enhances the understanding of the leptospiral human–environment–animal relationship. Several studies have described the distribution of *Leptospira* in the environment. However, more targeted sites, that is, areas surrounding leptospirosis patients' houses, remain under-explored. Therefore, this study aims to detect the presence of *Leptospira* spp. in the residential areas of patients with leptospirosis.
Methods: Soil and water samples near leptospirosis patients' residences were collected, processed and cultured into EMJH media. Partial 16S rRNA gene sequencing was performed to confirm the identity of *Leptospira*.
Results: EMJH culture and partial 16S rRNA gene sequencing revealed predominant growth of pathogenic *Leptospira kmetyi* (17%, n = 7/42). All tested locations had at least one *Leptospira* sp., mostly from the soil samples.
Conclusion: More than one species of *Leptospira* may be present in a sampling area. The most common environmental isolates were pathogenic *L. kmetyi*.

Introduction

Leptospirosis caused by pathogenic spirochetes *Leptospira* spp. affects more than a million people every year with case fatality rates ranging as high as 20–25% [1–3]. To date, 22 leptospiral species have been described using DNA–DNA hybridization and phylogenetic analysis [4]. Pathogenic *Leptospira* have been isolated in animals such as rodents, cats, dogs, pigs and cattle [5]. The infected animals consistently excrete *Leptospira* into the environment via urination. The bacteria are able to survive and remain virulent for at least 20 months under robust conditions, including low temperature, acidic and nutrient-poor fresh water environments [6,7]. Human are infected through contact with the urine of an animal host or a contaminated environment. In Malaysia, a high number of cases are associated with exposure to the latter factor [8,9]. In order to control leptospirosis, the environmental factor should be taken into consideration in addition to human–animal interfaces [10].

Environmental sampling in Malaysia for *Leptospira* started as early as 1961 [11]. In more recent years, such studies have successfully isolated pathogenic *Leptospira* spp. and several intermediate and saprophytic strains with a positivity rate of around 23% [12,13]. However, the distribution of *Leptospira* in targeted residential areas remains under-explored. Therefore, this study aims to detect the presence of *Leptospira* spp. in the residential areas of patients with leptospirosis.

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Development and validation of pan-*Leptospira* Taqman qPCR for the detection of *Leptospira* spp. in clinical specimens

Mohammad Ridhuan Mohd Ali[a,b], Amira Wahida Mohd Safee[a], Nor Hayati Ismail[c], Roslinda Abu Sapian[b], Hani Mat Hussin[c], Nabilah Ismail[a], Chan Yean Yean[a,d,*]

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ABSTRACT

Background: Early diagnosis of leptospirosis is important for ensuring better clinical management and achieving better outcomes. Currently, serological assays suffer from inconsistent performance and are less useful for early diagnosis of leptospirosis. As an alternative, qPCR is more sensitive, specific and able to detect the presence of leptospiral DNA during the acute phase of the infection. Meanwhile, most molecular assays do not detect the non-pathogenic group of *Leptospira*, even though these groups may also infect humans, although less frequently and less severely.
Methods: A set of primers and probe targeting *rrs* genes of 22 *Leptospira* spp. were designed and evaluated on 31 *Leptospira* isolates, 41 other organisms and 65 clinical samples from suspected patients.
Results: The developed assay was able to detect as low as 20 fg *Leptospira* DNA per reaction (equivalent to approximately 4 copies) and showed high specificity against the tested leptospiral strains. No cross amplification was observed with the other organisms. During the evaluation of the confirmed clinical specimens, the developed assay was able to correctly identify all positive samples ($n = 10/10$). One amplification was observed in a negative sample ($n = 1/55$). The sequencing of the PCR product of the discordant sample revealed that the sequences were similar to those of *L. interrogans* and *L. kirschneri*.
Conclusion: The findings suggest that the developed Taqman qPCR assay is sensitive, specific and has potential to be applied in a larger subsequent study.

Mohd Ali, M. R., Foo, P.C., Hassan, M., Maning, M., Hussin, A., Syed Ahmad Yunus, S. Z., Fauzi, M. H., Muhd Besari, A., Harun, A., M., Ismail, N. and Chan, Y. Y. (2018) 'Development and validation of TaqMan real-time PCR for the detection of *Burkholderia pseudomallei* isolates from Malaysia',
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8th October 2018

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Dear Dr,

The Editorial Team of Tropical Biomedicine is pleased to inform you that your manuscript MS 976/18 "**Development and validation of TaqMan real-time PCR for the detection of *Burkholderia pseudomallei* isolates from Malaysia**" by Mohammad Ridhuan Mohd Ali, Phiaw Chong Foo, Murnihayati Hassan, Nurahan Maning, Azura Hussin, Sharifah Zawani Syed Ahmad Yunus, Mohd Hashairi Fauzi, Alwi Muhd Besari, Azian Harun, Nabilah Ismail and Chan Yean Yean has been accepted for the publication. This manuscript will be published in March Issue Volume 36 No 1, 2019. Thank you for submitting your manuscript in Tropical Biomedicine Journal.

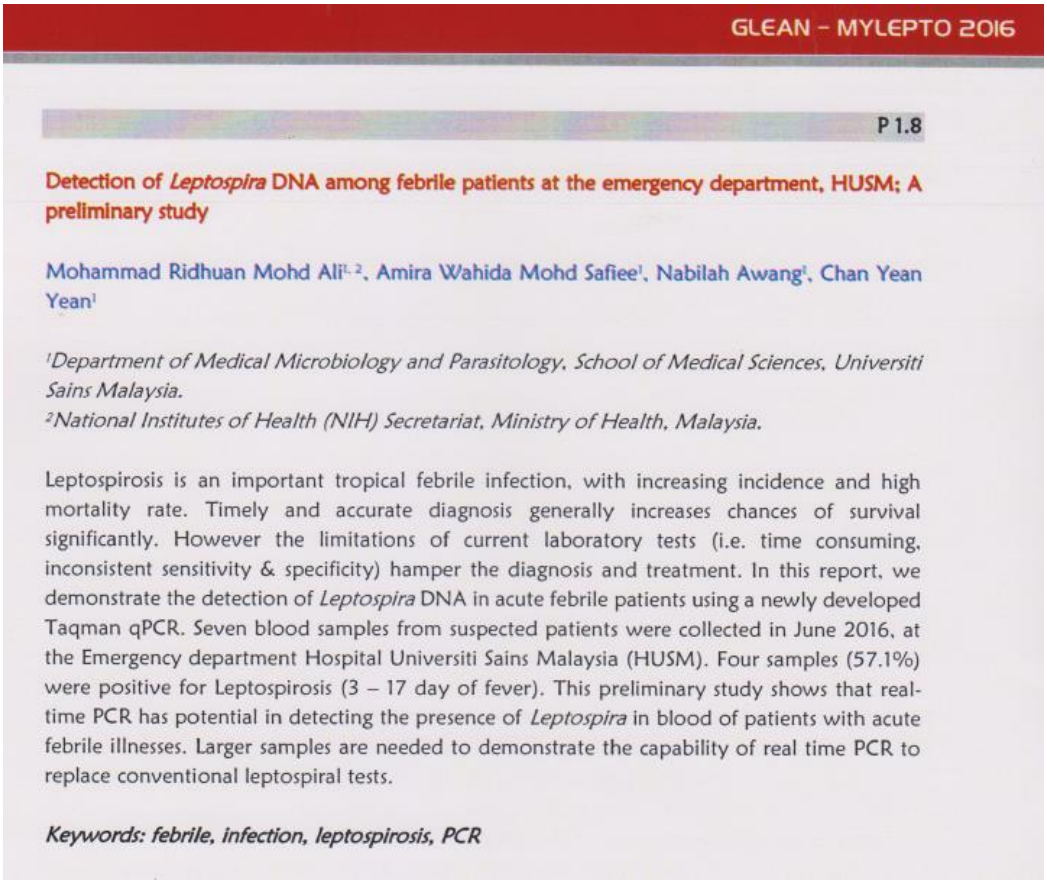
LIST OF PRESENTATIONS

Mohd Ali, M. R, Mohamad Safiee, A. W., Ismail, N. and Yean Yean, C. (2016) Detection of *Leptospira* DNA among febrile patients at the emergency department, HUSM; A preliminary study. *Global Leptospirosis Environmental Action Network (GLEAN) – MyLepto*. *6-8 October 2016*. Universiti Putra Malaysia. (Poster)

Mohd Ali, M. R, Maning, N. and Yean Yean, C. (2017) Detection of *B. pseudomallei* DNA among febrile patients presented to tertiary hospitals in north eastern Malaysian. *2nd South Asian Melioidosis Congress, 29-30 August 2017,* Colombo, Sri Lanka. (Oral & poster – awarded USM Conference Fund)

Mohd Ali, M. R, Ismail, N., Harun, A. and Yean Yean, C. (2018) Novel multiplex real-time PCR for simultaneous detection of *Leptospira, Burkholderia pseudomallei, Salmonella* and *Plasmodium* DNA. *Malaysian Society of Parasitology and Tropical Medicine (MSPTM) Mid-year Seminar in Honour of Prof Mak Joon Wah, 7 July 2018,* International Medical University, Bukit Jalil. (3rd place best poster)

Mohd Ali, M. R, Mohamad Safiee, A. W., Ismail, N. and Yean Yean, C. (2016) Detection of *Leptospira* DNA among febrile patients at the emergency department, HUSM; A preliminary study. *Global Leptospirosis Environmental Action Network (GLEAN) – MyLepto. 6-8 October 2016*. Universiti Putra Malaysia. (Poster)



GLEAN – MYLEPTO 2016

P 1.8

Detection of *Leptospira* DNA among febrile patients at the emergency department, HUSM; A preliminary study

Mohammad Ridhuan Mohd Ali[1, 2], Amira Wahida Mohd Safiee[1], Nabilah Awang[1], Chan Yean Yean[1]

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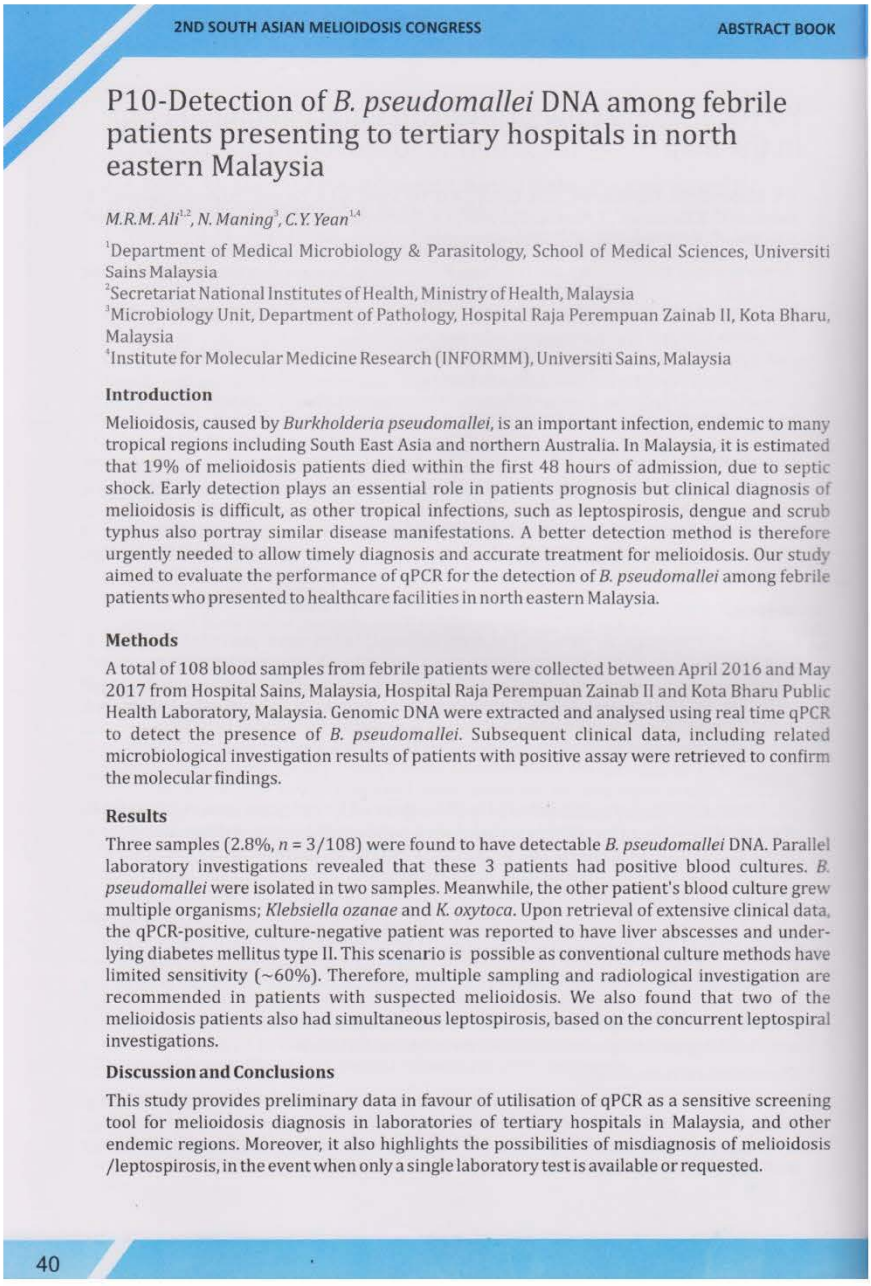
Leptospirosis is an important tropical febrile infection, with increasing incidence and high mortality rate. Timely and accurate diagnosis generally increases chances of survival significantly. However the limitations of current laboratory tests (i.e. time consuming, inconsistent sensitivity & specificity) hamper the diagnosis and treatment. In this report, we demonstrate the detection of *Leptospira* DNA in acute febrile patients using a newly developed Taqman qPCR. Seven blood samples from suspected patients were collected in June 2016, at the Emergency department Hospital Universiti Sains Malaysia (HUSM). Four samples (57.1%) were positive for Leptospirosis (3 – 17 day of fever). This preliminary study shows that real-time PCR has potential in detecting the presence of *Leptospira* in blood of patients with acute febrile illnesses. Larger samples are needed to demonstrate the capability of real time PCR to replace conventional leptospiral tests.

Keywords: febrile, infection, leptospirosis, PCR

Mohd Ali, M. R, Maning, N. and Yean Yean, C. (2017) Detection of *B. pseudomallei* DNA among febrile patients presented to tertiary hospitals in north eastern Malaysian. *2nd South Asian Melioidosis Congress, 29-30 August 2017*, Colombo, Sri Lanka. (Oral & poster – awarded USM Conference Fund)



P10-Detection of *B. pseudomallei* DNA among febrile patients presenting to tertiary hospitals in north eastern Malaysia

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Introduction

Melioidosis, caused by *Burkholderia pseudomallei*, is an important infection, endemic to many tropical regions including South East Asia and northern Australia. In Malaysia, it is estimated that 19% of melioidosis patients died within the first 48 hours of admission, due to septic shock. Early detection plays an essential role in patients prognosis but clinical diagnosis of melioidosis is difficult, as other tropical infections, such as leptospirosis, dengue and scrub typhus also portray similar disease manifestations. A better detection method is therefore urgently needed to allow timely diagnosis and accurate treatment for melioidosis. Our study aimed to evaluate the performance of qPCR for the detection of *B. pseudomallei* among febrile patients who presented to healthcare facilities in north eastern Malaysia.

Methods

A total of 108 blood samples from febrile patients were collected between April 2016 and May 2017 from Hospital Sains, Malaysia, Hospital Raja Perempuan Zainab II and Kota Bharu Public Health Laboratory, Malaysia. Genomic DNA were extracted and analysed using real time qPCR to detect the presence of *B. pseudomallei*. Subsequent clinical data, including related microbiological investigation results of patients with positive assay were retrieved to confirm the molecular findings.

Results

Three samples (2.8%, n = 3/108) were found to have detectable *B. pseudomallei* DNA. Parallel laboratory investigations revealed that these 3 patients had positive blood cultures. *B. pseudomallei* were isolated in two samples. Meanwhile, the other patient's blood culture grew multiple organisms; *Klebsiella ozanae* and *K. oxytoca*. Upon retrieval of extensive clinical data, the qPCR-positive, culture-negative patient was reported to have liver abscesses and underlying diabetes mellitus type II. This scenario is possible as conventional culture methods have limited sensitivity (~60%). Therefore, multiple sampling and radiological investigation are recommended in patients with suspected melioidosis. We also found that two of the melioidosis patients also had simultaneous leptospirosis, based on the concurrent leptospiral investigations.

Discussion and Conclusions

This study provides preliminary data in favour of utilisation of qPCR as a sensitive screening tool for melioidosis diagnosis in laboratories of tertiary hospitals in Malaysia, and other endemic regions. Moreover, it also highlights the possibilities of misdiagnosis of melioidosis /leptospirosis, in the event when only a single laboratory test is available or requested.

Mohd Ali, M. R, Ismail, N., Harun, A. and Yean Yean, C. (2018) Novel multiplex real-time PCR for simultaneous detection of *Leptospira*, *Burkholderia pseudomallei*, *Salmonella* and *Plasmodium* DNA. *Malaysian Society of Parasitology and Tropical Medicine (MSPTM) Mid-year Seminar in Honour of Prof Mak Joon Wah, 7 July 2018*, International Medical University, Bukit Jalil. (3rd place best poster)

P12

NOVEL MULTIPLEX REAL-TIME PCR FOR SIMULTANEOUS DETECTION OF *Leptospira*, *Burkholderia pseudomallei*, *Salmonella* and *Plasmodium* DNA

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Leptospirosis, melioidosis, invasive salmonellosis and malaria portray similar clinical presentations and are difficult to differentiate. To enable timely diagnosis, we developed and evaluated a multiplex Taqman qPCR for the etiologic agents of these infections. Briefly, four primer pairs and probes were designed against *rrs*, *orf2*-TTSS, *StyR-3* and 18S rRNA genes of *Leptospira*, *B. pseudomallei*, *Salmonella* and *Plasmodium* respectively. Analytical specificity of the designed oligonucleotides was tested on 357 microbial isolates including 131 *Leptospira*, 105 *B. pseudomallei*, 44 *Salmonella*, 31 *Plasmodium* and 46 other organisms. The developed assay correctly amplified and differentiated all organisms of interest. No cross amplification was observed in the other non-related isolates. Next, the analytical sensitivity of the assay was evaluated on 10-fold serial dilutions of *Leptospira*, *B. pseudomallei* and *Salmonella* genomic DNA and *Plasmodium* synthetic DNA. It was found that the developed assay can detect between 2.56 – 4 copies of DNA per reaction. These results indicated that the developed multiplex qPCR assay was highly specific and sensitive for molecular detection of *Leptospira*, *B. pseudomallei*, *Salmonella* and *Plasmodium*. Further study can be carried out to evaluate the performance of this assay on clinical samples of suspected patients.