IDENTIFICATION OF PUTATIVE VIRULENCE FACTORS OF *LEPTOSPIRA INTERROGANS* USING A YEAST MODEL

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IDENTIFICATION OF PUTATIVE VIRULENCE FACTORS OF *LEPTOSPIRA INTERROGANS* USING A YEAST MODEL

by

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LIST OF SYMBOLS AND ABBREVIATIONS

µl	Microliter
µg	Microgram
µm	Micrometre
µM	Micromolar
°C	Degree Celsius
%	Percent
Amp	Ampicillin
BSA	Bovine serum albumin
bp	Base pair
Cm	Chloramphenicol
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
H	Hour(s)
HPLC	High-performance liquid chromatography
HT	High-throughput
kDa	Kilodalton
K_d	Dissociation constant
LB	Luria Broth
Leu	Leucine
M	Molar
min	Minutes
ml	Millilitre
mM	Millimolar

mOsM	Milliosmole
Ni-NTA	Nickel- Nitrilotriacetic acid
nm	Nanometer
Na^+	Sodium ion
K^+	Potassium ion
OD	Optical Density
PCR	Polymerase Chain Reaction
Sec	Seconds
SC medium	Synthetic Complete medium
SDS	Sodium Dodecyl Sulfate
Ura	Uracil
VF	Virulence factor
VFs	Virulence factors
YPD medium	Yeast extract-Peptone-Dextrose medium

PENGENALPASTIAN FAKTOR VIRULENS PUTATIF *LEPTOSPIRA INTERROGANS* MENGGUNAKAN MODEL YIS

ABSTRAK

Internalisasi dan percambahan intraselular patogen bakteria *Leptospira interrogans* (*L. interrogans*) di dalam perumah adalah disebabkan oleh protein patogen yang dikenali sebagai faktor virulens (VF) yang menyasarkan mekanisme molekular, proses selular dan struktur eukariot yang terpelihara antara eukariot. Yis *Saccharomyces cerevisiae* (*S. cerevisiae*) merupakan model eukariot yang popular dan digunakan untuk pengenalpastian faktor virulens bakteria. Matlamat kajian ini adalah untuk mengenalpasti VF putatif *L. interrogans* dengan mengekspresikannya di dalam yis dan mengkaji kesan VF putatif pada pertumbuhan yis. Sejumlah 288 gen virulens putatif *L. interrogans* dipilih melalui kaedah bioinfomatik dan kajian penerbitan. Daripada 288 VF putatif yang dipilih, sebanyak 226 VF putatif berjaya diklon dalam plasmid ekspresi yis dan diekspresikan di dalam yis dengan menggunakan format plat 96-telaga. Kemandirian yis yang mengekspresi VF putatif disahkan dengan menggunakan keadah medium cair dan asai bintik pencairan bersiri yis. Sebanyak 11 VF putatif didapati merencatkan pertumbahan yis dan dikaji untuk penyetempatan protein di dalam yis dan interaksi VF dengan protein yis. Protein fluoresen hijau (GFP) ditambah pada VF putatif di terminal-C untuk menyiasat penyetempatan VF putatif di dalam yis dengan menggunakan mikroskop fluoresen. Kesimpulannya, penggunaan model yis adalah kaedah yang ringkas dan efektif untuk mengenal pasti VF putatif yang menyasarkan mekanisme molekular dan proses selular yang terpelihara dalam yis dan sel manusia. Kajian ini telah memberikan maklumat asas untuk kajian mekanisme jangkitan dan pertahanan *L. interrogans*.

IDENTIFICATION OF PUTATIVE VIRULENCE FACTORS OF *LEPTOSPIRA INTERROGANS* USING A YEAST MODEL

ABSTRACT

Internalization and intracellular proliferation of pathogenic bacteria *Leptospira interrogans* (*L. interrogans*) in their host is induced by pathogenic proteins known as virulence factors (VFs) which target molecular mechanisms, cellular processes and eukaryotic structures that are conserved among eukaryotes. Yeast, *Saccharomyces cerevisiae* (*S. cerevisiae*), is a popular eukaryotic model that is used to identify bacterial VFs. The goal of this study was to identify *L.* interrogans putative virulence factors by expressing them in yeast and examining the putative VFs impact on yeast growth. A total of 288 *L. interrogans* putative virulence genes were selected through a bioinformatic approach and literature review. Of the 288 selected putative virulence genes, 226 putative virulence genes were successfully cloned in the yeast expression plasmid and expressed in yeast using a 96-well plate format. The cell viability of yeast expressing putative virulence factors was verified using a liquid medium method and yeast serial dilution spotting assay. Eleven *L. interrogans* putative VFs were found inhibiting yeast growth and they were investigated for their localization in yeast and VF-yeast protein interaction. A green fluorescent protein (GFP) was fused to the C-terminal of the VFs to investigate the localization of putative VFs in yeast with the use of a fluorescent microscope. In conclusion, the yeast model is a simple and effective model to identify bacterial VFs that target conserved molecular mechanisms and cellular processes among yeast and human cells. This study has provided some preliminary information for the future study on *L. interrogans* invasion and evasion mechanisms.

CHAPTER 1

INTRODUCTION

Leptospira spp. is a Gram-negative, spirochete. It can be grouped into pathogenic, intermediate and saprophytic forms and categorized into 21 species which can be further classified into more than 300 serovars. However, only pathogenic *Leptospira* spp. has acquired the ability to cause disease in human and animals (Adler *et al.*, 2011).

Leptospirosis is a worldwide zoonotic disease that is caused by *Leptospira* spp. Approximately over 1 million leptospirosis cases and 60,000 deaths are reported annually (Costa *et al.*, 2015). Leptospirosis has been considered a geographical disease due to many occurrences in tropical climate countries such as South East Asia, Oceania, and Latin America countries (Costa *et al.*, 2015). Those who work outdoor, in agricultural or exposed to animal pasture (veterinarians and farmers), sewage workers, and mine workers are at high risk of the disease (Mwachui *et al.*, 2015). The symptoms of leptospirosis depend on the severity of infection which ranges from moderate to severe. Leptospirosis causes moderate symptoms such as high fever, muscle ache, headache, vomiting, diarrhoea, and rash which are similar to other diseases, cause misdiagnosis and may cause mistreatment (Plank & Dean, 2000). It was reported that 10-15% of leptospirosis patients who do not receive proper treatment might develop potentially fatal severe conditions such as meningitis and multiple organ failure (Weil's disease), it was reported that the mortality rate due to Weil's disease is at 5-15% (Maroun *et al.*, 2011).

The pathogenicity level of *Leptospira* spp. is determined by its virulence factors (VFs). The infection pathway starts with host cell infection which would lead

to interruptions of self-defence mechanism and host cell biological activities and eventually the death of the host cell. Identifying and analyzing *Leptospira's* VFs, by studying its structure and its effect on the host cell, secretory pathway and regulation of VFs expression during the bacterium exposure to host immune defence mechanisms and other unfavourable conditions for example, environmental stress and drug effects will help in the understanding of these VFs (Jain *et al.*, 2010).

To date, the exact cellular mechanisms of the pathogenesis of leptospirosis remain unclear since most genes of *L. interrogans* have not been unidentified and their functions remain to be discovered. According to the review of Adler and team members (2011), a comparison of the genome of pathogenic *L. interrogans*, intermediate pathogenic *L. borgpetersenii* and non-pathogenic *L. biflexa* identified approximately 655 proteins unique to *L. interrogans* which have potentially virulence-associated proteins. However, of the 655 unique proteins of *L. interrogans*, approximately three-quarter have no defined function (Adler *et al.*, 2011). Current leptospiral research is mainly focused on the identification of leptospiral cell surface protein including outer membrane proteins (OMPs), lipopolysaccharide (which is a general virulence factor of Gram-negative bacteria), and adhesion molecules which have been identified that might contribute to the pathogenesis of *Leptospira* infection and disease (Evangelista & Coburn, 2010; Ghazaei, 2018; King *et al.*, 2014; Matsunaga *et al.*, 2003).

In this study, a yeast-based assay was developed to identify putative *L. interrogans* VFs that can cause inhibition on yeast growth. Yeast was used as a model eukaryote in this study due to its similarity to the human cell where it shares a large portion of genes and eukaryotic structures as well as cellular mechanisms (Smith & Snyder, 2006). For over a decade, the utility of yeast to discover new bacterial

virulence proteins have increased, for instance, *Pseudomonas aeruginosa* Pec1, Pec2, and Pec3, when expressed in yeast resulted in yeast growth inhibition, moreover, a protein localization pattern of these three proteins in yeast were also observed in human epithelial type-2 cell (HEp-2) (Zrieq *et al.*, 2015). Therefore, results from a yeast-based assay can provide hints on the impacts of *L. interrogans*' VFs in the human cell.

Firstly, putative *L. interrogans*' VFs were selected through a literature review and analysis of information obtained from VFs databases. Then, a high-throughput cloning protocol was developed for insertion of putative virulence genes into a yeast expression plasmid. Next, a yeast growth inhibition assay was carried out to identify putative virulence factors that inhibited yeast growth. The growth-inhibiting proteins were characterized by performing protein-protein interaction studies and protein localization determination through microscopic observation. Figure 1.1 shows the outline and the flow of this study.

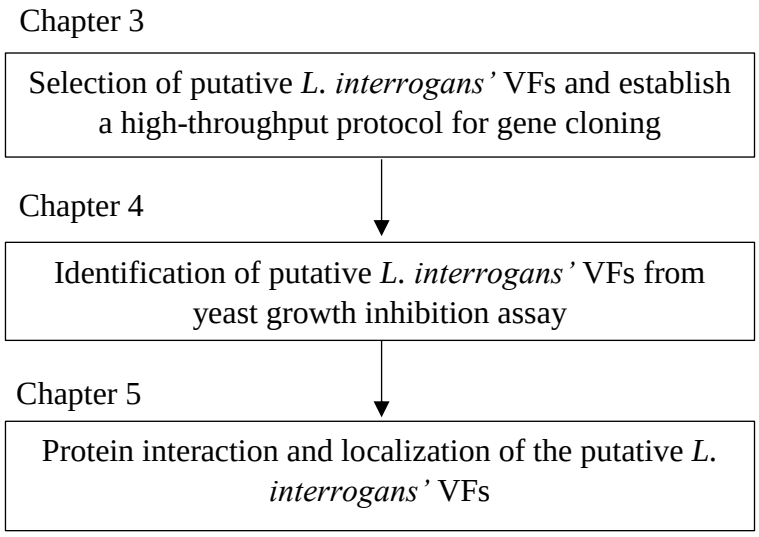

Figure 1.1 Outline and flow of thesis

1.1 Objective

The pathogenicity of *L. interrogans* is determined by its virulence factor. However, not all *L. interrogans* genes encoding VFs are identified. Many *L. interrogans* protein functions remain to be defined and among those proteins with unknown function, some might contribute to the pathogenicity of *L. interrogans*. Therefore, the discovery of proteins that possess virulence function will provide a better insight into the molecular mechanisms of *L. interrogans* pathogenicity.

Animal models have been used to discover genes that control the pathogenicity of *L. interrogans* by observing the host pathophysiological characteristics, immunological responses and survivability. However, this is a relatively laborious method. Meanwhile, yeast is another option that is suitable for the preliminary study to identify bacterial virulence factors as it is less laborious, faster, and can be used in high-throughput assays experimentally. The objective of this study was to identify putative *L. interrogans* VFs by monitoring the yeast survivability upon the expression of putative *L. interrogans* VF genes.

The specific objectives of this study were:

a) To select putative *L. interrogans* VFs through bioinformatics analysis and literature review.
b) To establish a high-throughput protocol for gene cloning and protein expression in yeast.
c) To identify putative *L. interrogans* VFs in a yeast growth inhibition assay.
d) To study the protein interaction and localization of the putative *L. interrogans* VFs.

CHAPTER 2

LITERATURE REVIEW

2.1 *Leptospira*

2.1.1 Introduction of *Leptospira* spp.

Leptospira spp. is a Gram-negative, spirochete that causes a zoonotic disease known as leptospirosis. Leptospirosis has a wide range of symptoms from mild fever, muscle ache, chills and abdominal pain to a severe form condition which is known as Weil's symptoms that involves multiple organs failure such as meningitis, liver and kidney failure which can cause death. Global estimation of reported leptospirosis incidents reached approximately 1 million cases with more than 60,000 deaths per year. However, the number of leptospirosis cases could be underestimated due to the limitation of the investigation system and diagnosis method (Picardeau, 2015). Although leptospirosis is a health burden worldwide, leptospirosis issues have been neglected by the medical community due to inadequate diagnosis and reporting. The high complexity of leptospirosis is due to variation in transmission modes, hundreds of pathogenic serovars, followed by numerous animal reservoirs, nonspecific symptoms and also poor diagnosis method (Mwachui *et al.*, 2015).

2.1.2 Morphology and structure of *Leptospira* spp.

Leptospira is a Gram-negative, long, thin and spiral-shaped bacterium with 0.10 µm diameter, length range of 6 to 20 µm and 0.10-0.15 µm of helical amplitude. Different from other spirochetes, *Leptospira* has hooks at one or both ends, these structures are found in both pathogenic and non-pathogenic *Leptospira* spp. However, there are slightly different structures between pathogenic *Leptospira* spp. and non-pathogenic *Leptospira* spp. (Cameron, 2015). A morphological comparison between pathogenic (e.g. *L. interrogans*) and non-pathogenic *Leptospira* spp. (e.g. *L. biflexa*)

(on their leptospiral cell envelop, novel filaments in the periplasm, flagellar motor structure, morphology and distribution of chemoreceptor arrays, spherical cytoplasmic bodies and DNA location) using Cryo-Electron Tomography reported that two major structural differences were observed. One of the differences is the *L. interrogans* cell envelop is thicker by having a thicker lipopolysaccharide layer. The envelop of both *L. interrogans* and *L. biflexa* composed of an inner membrane, outer membrane, peptidoglycan, and lipopolysaccharide. The second structural difference is the size of spherical cytoplasmic bodies within *Leptospira* spp. cell body. *L. biflexa* contain larger spherical cytoplasmic bodies (~100 nm) than *L. interrogans* (~30 nm). (Raddi *et al.*, 2012).

2.1.3 *Leptospira* serotypes and serovars

Leptospira spp. comprises 21 species which are categorized into three clusters: pathogenic, intermediate pathogenic and non-pathogenic. There are nine species which have been found to be pathogenic *Leptospira* species which included *L. interrogans*, *L. kirshneri*, *L. borgpetersenii*, *L. santorosai*, *L. noguchii*, *L. weilli*, *L. alexanderi*, *L. kmetyi*, and *L. alstonii*. While five species are classified as intermediate pathogenic *Leptospira*. They are *L. inadai*, *L. broomii*, *L. fainei*, *L. wolfii* and *L. licerasiae*. And seven species are non-pathagenic *Leptospira* namely *L. biflexa*, *L. wobachii*, *L. meyeri*, *L. vanthielii*, *L. terpstrae*, *L. idonii* and *L. yanagawae* (Voronina et al., 2014). These 21 species of *Leptospira* have been further divided into around 300 serovars, while among these 300 serovars, more than 200 serovars are pathogenic (Bourhy *et al.*, 2013). A phylogenetic tree that exhibits the relatedness of the 21 leptospiral species is shown in Figure 2.1.

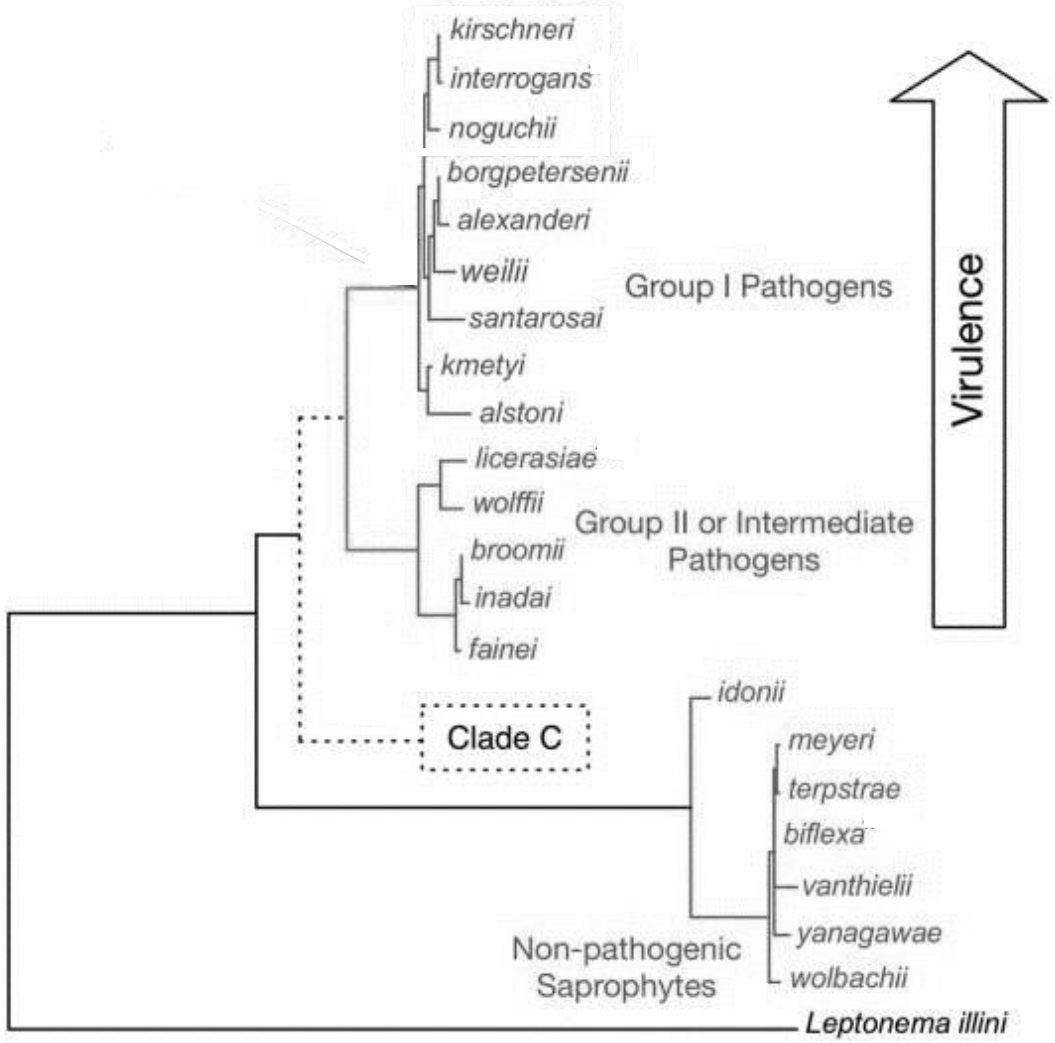

Figure 2.1 The phylogenetic tree of the 21 leptospiral species (Lehmann *et al.*, 2014)

2.1.4 Epidemiology of *Leptospira* spp.

Leptospirosis occurs worldwide. However, the distribution of leptospirosis is affected by three factors, which are geography, human behavior, and poverty (Costa *et al.*, 2015). Leptospirosis mainly occurs in tropical countries such as Southern America, Caribbean, South Asia, and South-East Asia and Oceania where the conditions are humid and warm. Tropical countries had the highest reported leptospirosis disease incidence due to the climate being suitable for *Leptospira* survival and favour transmission of leptospirosis especially during the monsoon season (Costa *et al.*, 2015). Not surprisingly, the outbreak of leptospirosis in South Asia and South-East Asia, especially in developing countries such as India, Bangladesh, Timor East, Thailand, Laos, Nepal, and Cambodia are commonly found to be associated with poor environmental sanitation, farming activities, and natural disasters, for instance, floods during raining season. While in Japan, Korea, Australia, and New Zealand, leptospirosis is considered an occupational disease. Most leptospirosis cases happened among livestock workers, agricultural workers and farmers in the sub-urban area (Victoriano *et al.*, 2009). However, disease burden is also likely to occur in some non-tropical developing countries like in East Sub-Sahara African. Figure 2.2 shows the geographical distribution of leptospirosis cases in the world.

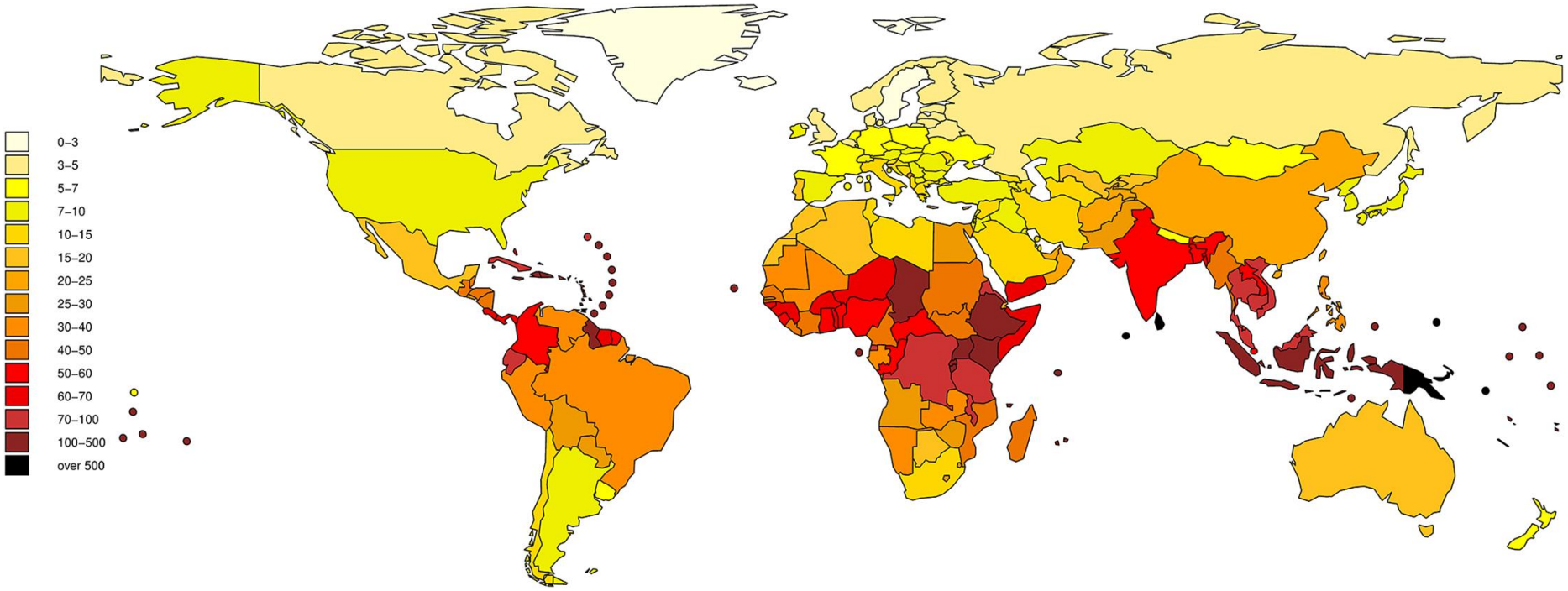

Figure 2.2 Geographical distribution of leptospirosis cases (Torgerson *et al.*, 2015).

Despite geographical and climatic factors, the Leptospira epidemiology pattern can be divided into rural and urban. In rural areas, Leptospira transmission is related to livestock holding and farming activities, where a wet, warm and humid condition of farmlands and animal husbandry facilitated survival of Leptospira (Sehgal, 2006). Transmission of leptospirosis in overpopulated cities especially cities in developing countries is rodent-borne and associated with overcrowding, poor personal hygiene, and poor sanitation (de Vries et al., 2014).

Leptospirosis is also considered an occupational disease. Workers in sewer maintenance, farming and animal husbandry has a higher chance to be infected by Leptospira due to contact directly or indirectly with contaminated water and infected animals (Wynwood *et al.*, 2014). According to Levett (2001), only mammalian animals can be the reservoir host for Leptospira. Hence, Leptospira is commonly detected in animals such as rodents, cows, pigs, and dogs. However, the cat is an exception. Interestingly, different animals may be the reservoir to different *Leptospira* spp. For instance, brown rats (*Rattus norvegicus*) are commonly known as the reservoir host for *Leptospira* serovar Icterohaemorrhagiae and Copenhageni, while mice only harbor the serovar Ballum. Cows harbor serovar Hardjo, Pomona and Grippotyphosa; dogs are the reservoir host for serovar Conicola and sheep harbor serovar Hardjo and Pomona (Levett, 2001). In addition, the outbreak of leptospirosis is also related to recreational activities especially outdoor water sports such as swimming in the river or lake, kayaking, and rafting. This is due to the survivability of pathogenic *Leptospira* spp. in water and moist environment for weeks to months. An experimental result showed that pathogenic *L. interrogans* exhibited 100% cell viability after cultivation in water for 48 h, while intermediate pathogenic *L. borgpetersenii* lost more than 90% of its viability (Bulach *et al.*, 2006). Weather

conditions also contribute to leptospirosis outbreaks, the upsurge of cases usually happen during floods and monsoon season in tropical countries (Barragan *et al.*, 2016).

2.1.5 Pathogenesis of *Leptospira* spp.

Theoretically, only the pathogenic *Leptospira* spp. acquired the ability to cause disease. However, intermediate pathogenic *Leptospira spp.* was also detected from leptospirosis patients. Intermediate pathogenic *L. fainei* has been found in cattle and in human in Australia while intermediate pathogenic *L. wolffii* was isolated from a suspected leptospirosis patient in Thailand (Levett *et al.*, 2006; Slack *et al.*, 2008). Surprisingly, Chiriboga and the team members found that as many as 96% of leptospiral DNA detected from human showed identity with intermediate pathogenic *Leptospira* spp. while only 4% showed identity with pathogenic *Leptospira* spp. (Chiriboga *et al.*, 2015). This finding suggests that intermediate pathogenic *Leptospira* spp. can cause human leptospirosis, but this needs to be validated.

During leptospiral infection, *Leptospira* spp. enter the human body through the wound and oral cavity. According to the review paper of Gomes-Solecki, Santecchia, and Werts (2017), almost 90% of leptospirosis cases are mild and treatable before they progress to a more severe condition. At the initial stage of leptospirosis, the infected patient shows mild symptoms for about one week characterized by diarrhoea, fever, chills and muscle ache. In this phase, *Leptospira* spp. entered human blood capillaries (leptospiraemia) or it can be found in the cerebrospinal fluid. Because febrile symptoms of *Leptospira* infections is similar to other bacterial infections, therefore, misdiagnoses will cause a delay of treatment, whereby hematogenous dissemination of Leptospira results in the harbouring of Leptospira in patient organs and this has worsened the infected patient situation and developing severe symptoms, also called Weil's disease. However, less than 10% of

Leptospira infected patients progress to Weil's disease where the failure of multisystem organs take place like meningitis, kidney and liver failure (Gomes-Solecki *et al.*, 2017). Figure 2.3 shows the overview of the Leptospira infection.

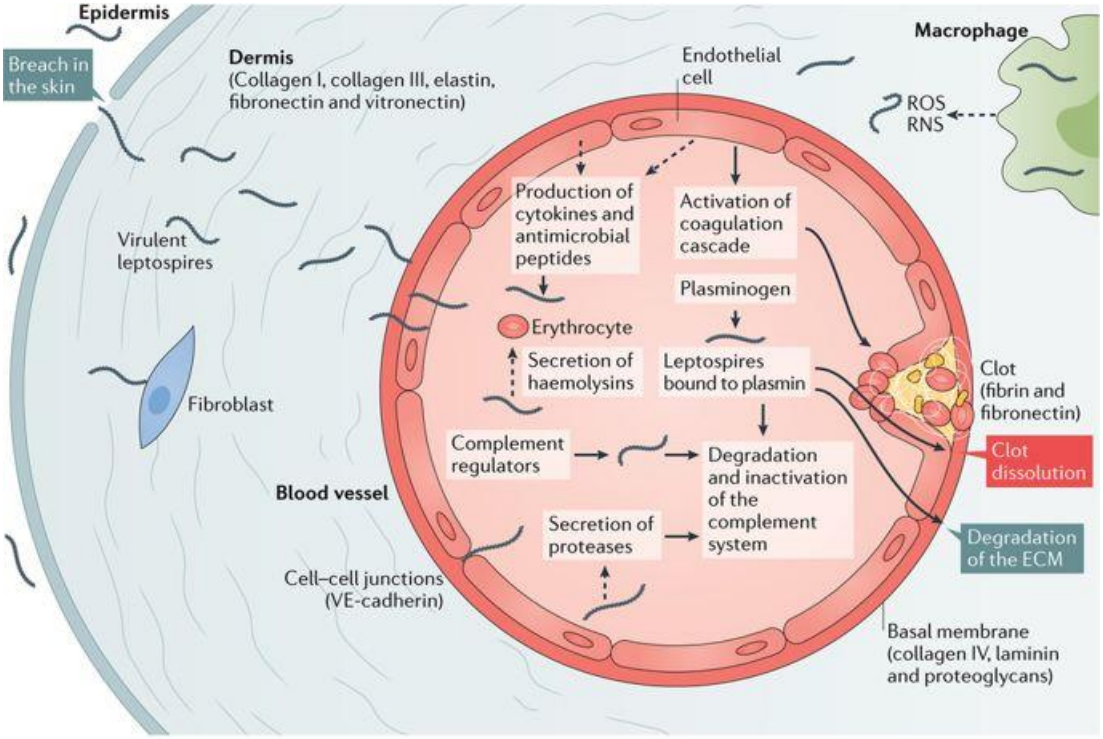

Figure 2.3 An overview of *Leptospira* infection (Picardeau, 2017).

2.2 Virulence factors

2.2.1 Introduction of virulence factors

The human body is harboured by various bacteria where they localized on the skin, mucus membrane and gastrointestinal tract. While these bacteria and human is in a mutualism relationship, and provided protection and benefits, for instance, intestinal bacteria contribute in mucosal cell turnover, muscle wall thickness, digestive enzyme activity and cell-mediated immunity (Canny & McCormick, 2008).

However, some opportunistic bacteria enter the host through wound, nasal cavity and respiratory system. After entering the host, these invader bacteria evade host immune system, cross host barriers and harbour in targeted organs for multiplication and lead to disease. To get through all the host barrier and eventually propagate, bacteria express a variety of macromolecules called virulence factors to assist them in every stage from invasion, evasion to proliferation in the host (Ribet & Cossart, 2015).

The pathogenicity level of bacteria depends on its ability in causing disease. The expression level of virulence factor is related to bacteria pathogenicity which is associated with chronic or persistence infection whereas more expressed virulence factor which has greater invasion ability and strongly resists to host immune system (Hu & Ehrlich, 2008).

2.2.2 *Leptospira interrogans* virulence factors

Comparison of the genome between pathogenic *L. interrogans*, intermediate pathogenic *L. borgpetersenii*, and non-pathogenic *L. biflexa* reflected that genes that are absent in intermediate pathogenic and non-pathogenic *Leptospira* spp. but present in pathogenic *Leptospira* spp are believed to encode proteins that contribute in *Leptospira* pathogenicity and are important during the host invasion and dissemination in the host.

Comparison between the genome of *L. interrogans* genome and *L. borgpetersenii* showed that the *L. borgpetersenii* genome is smaller than *L. interrogans* genome by around 16%, moreover, up to 12% of *L. borgpetersenii* coding regions encode pseudogenes and transposases while there are less than 4% in *L. interrogans (Bulach et al., 2006)*. This indicates that *L. borgpetersenii* might have undergone a genome reduction process that was prompted by an insertion-sequence element (IS element) (Bulach *et al.*, 2006). Genes that are absent in *L. borgpetersenii* mostly are genes encoding proteins with the functions of metabolisms, sensory transduction, and defense mechanisms and this has weakened the adaptation and survivability of L. *borgpetersenii* in diverse environments (Bulach *et al.*, 2006). Fewer metabolisms pathways will limit *L. borgpetersenii* in obtaining enough nutrients from diverse environments. Therefore, it is possible *L. borgpetersenii* is unable to survive in an environment that lacked nutrients such as soil and water. *L. borgpetersenii* is unable to survive beyond their host either in the cattle or a human due to the absence of genes that functions in adaptation and survival in environments external to the host. However, *L. borgpetersenii* is still able to cause acute infections in human and this proved that the loss of genes in *L. borgpetersenii* may not affect its invasiveness into the host (Bulach *et al.*, 2006).

2.2.2 (a) Leptospiral outer membrane proteins

Variation in pathogenicity of *Leptospira* spp. might be affected by the variance of the leptospiral surface proteins which are located at the outer membrane. While lipoprotein is one of the components that made up the outer membrane and which also believed that it contributes to the pathogenicity of *Leptospira* spp. There are 164 predicted lipoproteins in *L. biflexa*, and 184 and 130 predicted lipoproteins in *L. interrogans* and *L. borgpetersenii*, respectively (Picardeau *et al.*, 2008). However, 89 *L. biflexa* lipoprotein genes have no orthologs in the *L. interrogans* and *L. borgpetersenii* and at least 90 lipoproteins genes from pathogenic *Leptospira* spp. have no orthologs in *L. biflexa* (Picardeau et al., 2008). This means even *L. biflexa* has more predicted lipoprotein than *L. borgpetersenni*, but most of the lipoproteins in *L. biflexa* might not be pathogenic. Importantly, orthologs of known pathogenic lipoprotein such as Lig surface proteins, LfhA (factor H binding protein), Outer membrane proteins (LipL32, LipL36, LipL41, and few LipL45 related proteins) are not found in *L. biflexa* as well (Picardeau *et al.*, 2008).

Many pathogenic bacteria produce Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM) which are important proteins that are involved in colonization to the host cell during bacterial infection (Foster *et al.*, 2014). Leptospiral cell membrane proteins included the Len family proteins, LipL32, LipL53, leptospiral surface adhesin proteins (Lsa20, Lsa21, Lsa25, Lsa33, Lsa63, Lsa66), OmpL1, OmpL37, and leptospiral immunoglobulin-like (Lig) family proteins were found as extracellular matrix binding proteins which acquired the ability to adhere to lamina and fibronectin during infection (Fernandes *et al.*, 2012; Oliveira *et al.*, 2010; Pinne *et al.*, 2010). However, not all membrane proteins found in leptospira play roles in adhesion, for example, LfhA, instead of adhering to the

extracellular matrix, binds to the host factor H (FH). FH is a prominent regulator protein in the alternative complement pathway which downregulates the pathway by degrading the C3b to avoid the inadvertent activation of the complement system on host cell surfaces (Verma *et al.*, 2006). Therefore, FH regulatory activity is inhibited by binding with LfhA and cause complement-mediated damages to host tissues. In addition, *Leptospira* spp. might express distinct lipoprotein at different stages of infection including adhesion and proliferation in the host (Pinne *et al.*, 2010). For example, an experimental result showed that the LipL36 is expressed in vitro cultivation at 30°C, but it was downregulated when *L. interrogans* infected the mammalian host. This suggests LipL36 might play a role in adapting to the extracellular environment for leptospiral survival instead of contributing to invasion (Haake *et al.*, 1998). In contrast, other important lipoproteins such as LipL32, OmpL1, and LipL41 are not affected by these stage changes and are found expressed in the infected hamster kidney tissues (Haake *et al.*, 1998).

In *L. interrogans*, LigA and LigB are recognized as MSCRAMM and these two proteins are important for *L. interrogans* invasion and proliferation inside the host (Choy *et al.*, 2007). The absence of LigA and LigB proteins is correlated with the loss of virulence of the pathogenic Leptospira (Matsunaga *et al.*, 2003). Regulation of LigA and LigB have been shown to correlate with environment signal, both proteins were found up-regulated by increase of osmotic conditions from 60 mOsM to 300 mOsM and temperature from 30°C to 37°C in which the osmolarity and temperature of the cultivation conditions mimic human ones (Matsunaga *et al.*, 2007).

Multiple roles of LigA and LigB are involved in *L. interrogans* infection included leptospiral adhesion, invasion, and immune evasion. LigA and LigB bind to extracellular matrix proteins such as fibronectin, laminin, and collagen I and collagen

IV during the leptospiral adhesion (Choy *et al.*, 2007). Among these extracellular matrix proteins, LigA and LigB both possess a high binding affinity on fibronectin which is composed of several domains including a N-terminal domain (NTD) and a gelatine-binding domain (GBD), a cell-binding domain (CBD), a fibrin-binding domain II and a heparin binding domain II (hep-2) (Henderson *et al.*, 2011; Lin *et al.*, 2010). Although both LigA and LigB were speculated to possess the role in host cell colonization, however, only LigB is found in three pathogenic *Leptospira* strains (*L. interrogans* serovar Copenhageni, and *L. interrogans* serovar Pomona, *L. interrogans* serovar Lai) and one intermediate pathogenic *Leptospira* (*L. borgpetersenii* serovar Hardjo), and LigA is absence in *L. interrogans* serovar Lai and *L. borgpetersenii* serovar Hardjo (Nascimento *et al.*, 2004). This discovery indicates that LigB might be more crucial for leptospiral adhesion to the host cell and it was studied in detail. The LigB comprises three domains which are Lig conserved region (LigCon; amino acid 1-630), LigB Central variable region (LigBCen; amino acid 631-1417) and C-terminal variable region of LigB (LigBCtv; amino acid 1418-1889) (Lin & Chang, 2008). The amino acid sequence of LigBCen is identical with the conserved region of LigA, however, the conserved region of both LigA and LigB does not acquire ability in fibronectin adhesion, while LigBCen and LigCtv were recognized as fibronectin binding domains in the previous study (Forster *et al.*, 2013; Lin & Chang, 2008). In addition, fibronectin-binding affinity of LigBCen is higher due to LigBCen has lower dissociation constants (K_d = 0.01 µM) compared with LigCtv (K_d = 8.55 µM) (Lin & Chang, 2008). In a detailed study, LigBCen2 (amino acid 1014-1165) was discovered to strongly bind with the fibronectin compared with LigBCen1 (amino acid 631-1013), and LigBCen3 (amino acid 1166-1417) however, the association of LigBCen2 with

the NTD of fibronectin is stronger than other domains of fibronectin (Lin & Chang, 2007).

Colonization of the host cell is the first step of leptospiral infection, in the following step, leptospiral invasion, LigA, and LigB are involved in causing degradation of fibrin also known as fibrinolysis (Castiblanco-Valencia *et al.*, 2016). Fibrinogen is a matrix protein that is important in clot formation, inflammation and wound healing. During tissue injury, fibrinogen converts into fibrin with the presence of thrombin to form the fibrin-based blood clot (Mosesson, 2000). Fibrinolysis is a natural enzymatic process in the human body that breaks down the fibrin-based blood clot by plasmin (PLA) when the wound is healed (Kolev & Longstaff, 2016). During the leptospiral invasion, LigA and LigB are associated with plasminogen (PLG) which is converted into plasmin (PLA) in the presence of the urokinase-type activator (uPA) (Castiblanco-Valencia *et al.*, 2016). Excess of plasmin resulting hyperfibrinolysis which exhibits the inhibitory effect on blood clotting and wound healing, this activity has allowed more *L. interrogans* to enter into the host and host circulation for dissemination (Choy, 2012).

Other than causing fibrinolysis, plasmin can also proteolyze another two key component molecules known as C3b and C5 which are the proteins that play roles in the complement system which is a part of the innate immune system that improves the capability of the antibodies and opsonisation during pathogens clearance (Castiblanco-Valencia *et al.*, 2016). C3b protein has the most significant role in the opsonising activity, while C5, a complement protein that composes C5a and C5b in which C5b initiates the formation of membrane attack complex (MAC) which causes lysis of the pathogens, while C5a triggers inflammation (Horiuchi & Tsukamoto, 2016). Other than plasmin, complement regulatory proteins such as Factor H (FH) and C4b-binding

protein (C4BP) are also involved in degrading C3b and C4b, respectively (da Silva *et al.*, 2015). Degradation of C3b, C4b, and C5 complement proteins will down-regulate the activation of the complement system.

Vieira and team members have discovered the *L. interrogans* that bound with plasminogen or plasmin have improved its penetration activity through human endothelial cell monolayers compared with the *L. interrogans* without plasminogen or plasmin in their study (Vieira *et al.*, 2013). On the other hand, matrix metalloprotease 9 (MMP-9) might also play an important part in leptospiral infection. The up-regulation of MMP-9 in human endothelial cell was detected in the culture that human endothelial cell incubated with plasmin associated *L. interrogans* compared with the human endothelial cell without *L. interrogans*, hence, the attachment of plasmin on the *L. interrogans* might induce the expression of MMP-9 in the human endothelial cell directly or indirectly (Vieira *et al.*, 2013). MMP-9 is one of the matrix metalloprotease family (MMPs) involved in the extracellular matrix degradation and providing an important character in cell growth, cell differentiation, apoptosis and response to bacterial infection when MMPs are precisely regulated but excessive amounts of MMPs may cause immunopathology to the host and facilitate the dissemination of *L. interrogans* in the intracellular space during infection (Elkington *et al.*, 2005; Xue *et al.*, 2013). The roles of LigA and LigB in leptospiral infection is showed in Figure 2.4.

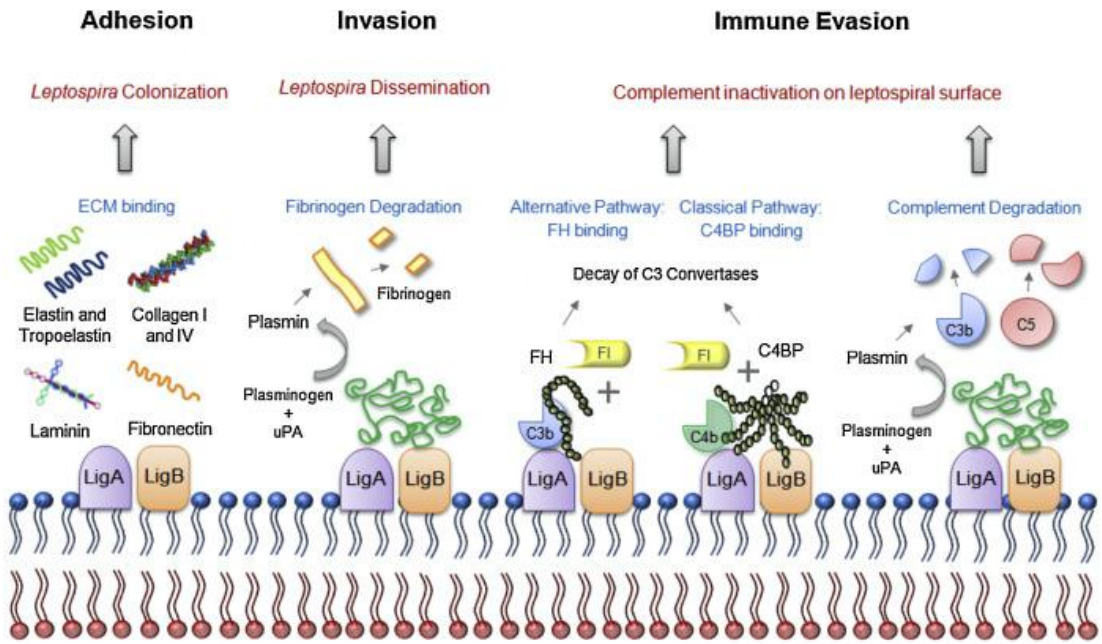

Figure 2.4 The roles of LigA and LigB in leptospiral infection (Castiblanco-Valencia *et al.*, 2016)**.**

Sphingomyelinases-like proteins (Smase) are a type of enzyme found in several serovars of pathogenic *L. interrogans*. *L. Interrogans* serovar Lai, serovar Copenhageni and serovar Pomona consist of genes that encode Smase – Sph1, Sph2, Sph3 Sph4, and SphH. However, intermediate pathogenic *L. borgpetersenni* only encoded proteins SphA, SphB, and Sph4, and all *sph* genes are absent in non-pathogenic *L. biflexa* (Narayanavari *et al.*, 2012a). The catalytic active site of Smase was found to be important for haemolytic and sphingomyelinases activity that happens on erythrocyte and endothelial cells. The catalytic active site is a protein structure that formed by residues Glu-53, His-151, Asp-195, Asn-197, Asp-295 and His-296 which is a consensus site that found in the Smase of *Bacillus cereus* (Bc-Smase), *Lesteria ivanovii*, (SmcL) and *Staphylococcus aureus* (Sa-Smase) (Narayanavari *et al.*, 2012b). The Smase of these bacterial were confirmed in performing catalysis on the cell membrane of erythrocyte and endothelial cell.

However, the catalytic active site was only found in Sph2 (Smase of L. interrogans) and SphA (Smase of L. borgpetersenii) (Narayanavari *et al.*, 2012b). Therefore, these two Smase exhibiting strong haemolytic activity on the Sph2/SphA treated sheep erythrocytes. However, in an experimental test that was carried out by Wang and team member, Sph1 and Sph3 exhibited haemolytic activity as well although their degrees of haemolytic activity was relatively weaker than Sph2. This may be due to Sph1 and Sph3 are conserved in only three out of five essential residues for the formation of the catalytic active site (Wang *et al.*, 2012).

On the other hand, SphH was identified as a pore-forming protein (Lee *et al.*, 2002). Instead of breaking down the sheep erythrocyte, pore formation was detected on the SphH-treated sheep erythrocyte membrane, moreover, SphH acquired function in mediating sublytic damage on mammalian cells membrane (Lee *et al.*, 2002). This

discovery suggests that SphH is more likely a membrane-disrupting cytolysin protein than a haemolysin. Some predictions about the Smase functions had been made. Iron acquisition from the host might be one of the functions since iron is essential for bacterial as it is involved in various metabolic processes which guarantee bacterial survival in different environments (Narayanavari *et al.*, 2012b). Haemolytic activity and pore formation on erythrocyte cell membrane might be an important approach for bacteria to uptake enough iron for dissemination inside the host.

In another case, pore-forming proteins might contribute to resisting host immune system. *L. interrogans* serovar Manila was found to have acquired the ability to survive within murine macrophages and escaped from it to return into the extracellular environment without inducing apoptosis in macrophages which may require the sphingomyelinase activity and pore-forming activities that carried out by Sph2 and SphH, respectively (Toma *et al.*, 2011). However, several studies have shown that *L. interrogans* serovar Lai and *L. interrogans* serovar Copenhageni induced cell death of murine and human macrophages, therefore, the responses of the macrophages infected with different strains of *Leptospira* spp. are unclear (Toma *et al.*, 2011).

2.2.2 (b) Leptospiral virulence factor for stress response

During invasion into the host, *Leptospira* spp. will face stresses from the changes of the environment. These stresses include oxidative stress, temperature changes, and osmolarity stress. These stresses could damage the *L. interrogans* DNA and cause the death of the *L. interrogans* thus, preventing DNA mutation and stabilizing DNA replication is crucial to allow the synthesis of the native stress resistance proteins. DNA damage can trigger the SOS response which is a complex system for DNA repair. SOS response induces DNA repair pathway including homologous recombination, nucleotide excision repair and translesion synthesis (TLS). Nucleotide excision repair (NER) system which repairs the double-stranded breaks (DSB) involves several NER genes – *uvrA*, *uvrB*, *uvrC* and *uvrD* (Baharoglu & Mazel, 2014). UvrA acts as a damaged DNA recognition protein and facilitates the interaction between UvrB with the damaged DNA site, while UvrB binds with UvrC to form a UvrB-UvrC complex which makes incisions at the DNA damaged site (Hsu *et al.*, 1995). UvrD, also as known as DNA helicase II, unwinds the DNA damaged site to allow the DNA polymerase I complete the repair process (Kang & Blaser, 2006). Surprisingly, UV irradiation did not affect the transcription of the *L. interrogans* NER genes, meanwhile, the expression levels of other SOS response genes such as *recN*, *recA*, and *dinP* were promoted (Fonseca *et al.*, 2013).

Homologous recombination DNA repair system is performed differently by recombinases (Rec) and DNA polymerase IV (DinP) and allows the repair of the single-stranded breaks (SSB) and double-stranded breaks (DSB). In the SSB, RecJ exonuclease dissociates nucleotides from DNA nick in 5'- 3' direction and allow the binding of RecFOR exonuclease/helicase complex. While RecBCD exonuclease/helicase complex that only recognizes DSB also removes nucleotides

from DNA nick in 5'- 3' direction to form an overhang (Baharoglu & Mazel, 2014). Both exonuclease/helicase complexes recruit the RecA to the overhangs and form a nucleofilament that degrades the repressor LexA which represses the SOS response (Schons-Fonseca *et al.*, 2016). After the formation of nucleofilament, RecA initiates the homologous recombination through strand invasion with the sister chromosome which carries out by resolvases (RuvABC complex) (Schons-Fonseca *et al.*, 2016). Interestingly, if RecBCD pathway is inactivated due to mutation of SbcB (suppressor of *recBC*) and SbcD (co-suppressor of *recBC*), the RecFOR pathway will be activated (Bentchikou *et al.*, 2010). The experimental results showed that the deletion of these SOS response genes have attenuated the DNA repairing ability of the bacteria and the cell survival was decreased drastically (Dillingham & Kowalczykowski, 2008; Handa & Kowalczykowski, 2007; Ivancic-Bace *et al.*, 2005; Lage *et al.*, 2010; Le Masson *et al.*, 2008).

Heat stress induces expression of leptospiral chaperone proteins such as GroEL, DnaK, HtpG and ClpB which are essential in stabilizing the structure of the host cell, retaining the functions of the cell organelles, prevent protein aggregation and reform misfolded proteins (Lo *et al.*, 2009). On the other hand, reactive oxygen species (ROS) is a by-product of the metabolism of oxygen in mammalian mitochondrion during generating of adenosine triphosphate (ATP) through electron transport system and ROS includes superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) (Das & Roychoudhury, 2014). An experimental result showed that appropriate concentration of ROS supports the growth and survival of the mammalian tissues by regulating prominent cellular mechanisms such as proliferation, cell apoptosis, and differentiation (Carrasco *et al.*, 2016; Clement & Pervaiz, 2001).