A STUDY OF SMALL NON-CODING RNAs OF *SALMONELLA ENTERICA* SUBSPECIES *ENTERICA* SEROVAR TYPHI IN BIOFILM DEVELOPMENT

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A STUDY OF SMALL NON-CODING RNAs OF *SALMONELLA ENTERICA* SUBSPECIES *ENTERICA* SEROVAR TYPHI IN BIOFILM DEVELOPMENT

by

KOGAAN ANBALAGAN

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for the degree of
Master of Science**

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

-	Negative or minus
%	Percentage
~	Approximately
<	Less than
>	More than
µg	Microgram
µL	Microliter
10X	Ten times
1X	1 time
bp	Base pair
cm	centimetre
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
E. coli	Escherichia coli
g	gram
IgG	Immunoglobulin G
L	Liter
LA	Luria agar
LB	Luria broth
M	Molar
MDR	Multidrug resistant
mg	Milligram
mL	Milliliter
mM	Millimolar
ncRNA	Non-coding RNA
ng	Nanogram
ºC	Degree Celcius
OD	Optical Density
OMP	Outer membrane protein
PBS	Phosphate-buffer saline
pH	Power of hydrogen
psi	Pounds per square inch
RNA	Ribonucleic acid
S. Typhi	*Salmonella* Typhi
SEM	Scanning Electron Microscope
SPI	*Salmonella* Pathogenicity Island
v/v	Volume per volume
w/v	Weight per volume

KAJIAN RNA KECIL BUKAN PENGEKODAN DARIPADA *SALMONELLA ENTERICA* SUBSPECIES *ENTERICA* SEROVAR TYPHI DALAM PERTUMBUHAN BIOFILEM

ABSTRAK

Salmonella Typhi adalah Gram-negatif bakteria dan patogen khusus bagi manusia yang menyebabkan demam kepialu. Ia merupakan masalah kesihatan yang serius di negara-negara yang kurang membangun dan sedang membangun termasuk Malaysia. Salah satu faktor utama yang menyumbang kepada penyebaran demam kepialu ialah kewujudan pembawa kepialu asimptomatik. Pembentukan biofilm dalam pundi hempedu manusia dikaitkan rapat dengan perkembangan pembawa. Kajian terkini menunjukkan, 97 novel RNA kecil bukan pengekodan (ncRNAs) yang dikenal pasti dari *S*. Typhi sel melalui eksperimen makmal. Daripada 97 ncRNAs, sepuluh daripadanya dikategorikan dalam '*Salmonella* Pathogenicity Island' (SPI). RNA kecil bukan pengekodan adalah molekul RNA yang tidak mengekod protein tetapi terlibat dalam modulasi proses kawal selia sel, termasuk patogenesis bakteria. Oleh itu, tujuan utama kajian ini adalah untuk mengenalpasti peranan sepuluh ncRNAs dari SPI dalam pembentukan biofilem. Untuk menghasilkan jumlah sel biofilem yang banyak, satu model biofilem baru direka dengan menggunakan slaid kaca mikroskop yang bersalut kolesterol diletakkan dalam 6-lubang kultur tisu plat. Tujuh kaedah pengekstrakan RNA telah dinilai untuk mengenal pasti kaedah pengekstrakan RNA yang berkesan daripada sel biofilem. Ianya terdiri daripada empat kit komersial: 1) RNeasy Mini Kit (Qiagen, Germany), 2) NucleoSpin RNA II (Macherey-Nagel, Germany), 3) Phenol-Free Total RNA Purification Kit (Amresco, USA), and 4) Total RNA Purification Kit (Norgen, Canada), dan tiga kaedah pengekstrakan yang berbeza iaitu 1) pengekstrakan

yang menggunakan enzim lysis, 2) sonikasi, dan 3) pengeskstrakan yang menggunakan pemukul manik. Hasilnya, kaedah pemukul manik dengan TRIzol lebih baik dan mampu untuk mengeskstrakan kuantiti RNA yang tinggi dan berkualiti dari biofilem *S*. Typhi berbanding dengan kaedah lain. Degradasi 23S RNA juga dikenalpasti apabila sel *S*. Typhi memasuki fasa kebuluran. Jumlah RNA dari sel plankton, pertengahan dan biofilem dianalisi dengan mengunakan kaedah blot northern. Pelabelan menggunakan digoxigenin bukan radioaktif (DIG) telah digunakan dalam blot Northern untuk mengesan sepuluh ncRNA yang diperolehi dalam SPI. Daripada sepuluh ncRNAs ini, lima ncRNAs ekspresi, iaitu Styr-327, Styr-9, Styr-143, Styr-161, dan Styr-381, telah dikesan. Styr-161 menunjukkan kuantiti ekspresi yang serata dalam semua 3 peringkat sel *S*. Typhi, mencadangkan ia boleh dikategorikan gen juzuk dalam sel *S*. Typhi. Styr-9 dan Styr-381 menunjukkan ekspresi yang rendah dalam sel-sel biofilem berbanding dengan sel-sel plankton. Manakala, Styr-143 dan Styr-327 jelas menunjukkan ekspresi yang tinggi dari sel-sel pertengahan dan biofilem berbanding dengan sel-sel plankton. Oleh itu, ncRNA ini mungkin mempunyai peranan yang penting dalam penyesuian biofilm. Menariknya, Styr-143 hanya diekspres semasa sel-sel pertengahan dan sel-sel biofilem, menunjukkan peranan penting dalam pembentukan biofilem. Sasaran mRNA diramalkan bagi ncRNAs yang dikenalpasti dengan menggunakan pelayar laman web yang dikenali sebagai 'RNAPredator' dan fungsi kemungkinan ncRNAs ini ke arah sasaran mRNA diandaikan dalam kajian ini. Akhir sekali, ncRNAs yang dikaitkan dengan biofilem yang telah dikenal pasti dalam kajian ini boleh berfungsi sebagai bakal calon penanda bio untuk mengenal pasti pembawa Kepialu dan memberikan petunjuk untuk lebih memahami fungsi kawal selia dalam pembentukan biofilem.

A STUDY OF SMALL NON-CODING RNAs OF *SALMONELLA ENTERICA* SUBSPECIES *ENTERICA* SEROVAR TYPHI IN BIOFILM DEVELOPMENT

ABSTRACT

Salmonella Typhi is a Gram-negative bacteria and human-specific pathogen which causes Typhoid fever. It is a major health problem in under-developed and developing countries including Malaysia. One of the major factors contributing to Typhoid persistence is due to the existence of asymptomatic Typhoid carriers. Biofilm formation in the human gallbladder has been postulated to be associated with the development of carriers. A recent study found 97 novel non-coding RNAs (ncRNAs) in *S*. Typhi and ten of them were mapped to the *Salmonella* Pathogenicity Island (SPI) of the bacteria's genome. Non-coding RNAs are RNA molecules that do not encode for proteins but are involved in modulating cell regulatory processes, including bacterial pathogenesis. Thus, the main aim of this study was to explore the role of the ten SPI-derived ncRNAs in biofilm formation. To produce a higher amount of biofilm cells, a new biofilm culture model was developed on a cholesterol-coated microscope glass slide in a 6-well tissue culture plate. Seven RNA extraction methods consisting of 4 different commercially available RNA extraction kits and 3 in-house methods were studied in order to identify effective RNA extraction methods from biofilm cells. The commercial kits were; 1) RNeasy Mini Kit (Qiagen, Germany), 2) NucleoSpin RNA II (Macherey-Nagel, Germany), 3) Phenol-Free Total RNA Purification Kit (Amresco, USA), and 4) Total RNA Purification Kit (Norgen, Canada). The 3 different in-house extraction methods were; 1) enzymatic lysis, 2) sonication, and 3) bead-beater with TRIzol. The results showed that the bead beater with TRIzol extraction method was better and could produce high quantity and quality of RNA from *S*. Typhi

biofilm compared to the other methods. Kinetic studies of *S*. Typhi cultures showed degradation of 23S RNA when the *S*. Typhi cell entered into starvation phase. The extracted total RNA from planktonic, intermediate and biofilm cells were then subjected to northern blot analysis using non-radioactive digoxigenin (DIG) method to the detect ten SPI-derived ncRNAs. Out of these ten ncRNAs, five ncRNAs, i.e. StyR-327, StyR-9, StyR-143, StyR-161, and StyR-381 were detected. StyR-161 was equally expressed in all three stages of *S*. Typhi development, suggesting a house-keeping function in *S*. Typhi cells. StyR-9 and StyR-381 were marginally down-regulated in the biofilm cells compared to the planktonic cells, whereas StyR-143 and StyR-327 were clearly up-regulated in the intermediate and biofilm cells compared to the planktonic cells, indicating a possible role of these ncRNAs in biofilm adaptation. Interestingly, StyR-143 was found to be expressed only in intermediate cells and biofilm cells, indicating a specific role in biofilm formation. The possible mRNA targets of these ncRNAs was predicted using an online webserver known as ‘RNAPredator’, and the possible functions of these ncRNAs towards their mRNA target were postulated. Finally, the biofilm-associated ncRNAs that were identified in this study can serve as biomarker candidates for Typhoid carrier identification and provide clues to better understand the regulatory functions in biofilm.

CHAPTER ONE

INTRODUCTION

Typhoid fever is a global infectious disease with an estimated incidence of 26.9 million cases and 269,000 deaths per annum (Buckle *et al*., 2012). *Salmonella enterica* subspecies *enterica* serovar Typhi (*S*. Typhi) is the pathogen responsible for Typhoid fever. *Salmonella* is a Gram-negative bacterium that belongs to the Enterobacteriaceae family. This pathogen is rod-shaped and generally motile. It is a human-specific pathogen and humans are the only known reservoir or carriers of the disease. According to the World Health Organization, the disease occurs mainly in Asia and Africa (Figure 1.1), where the annual incidence of Typhoid fever is about 1-100 cases per 100,000 population (Crump & Mintz, 2010; Crump *et al*., 2004)

Typhoid fever is a systemic disease that is transmitted through faecal-oral route via ingestion of faecal-contaminated water or food. Although it is an ancient disease, Typhoid fever still continues to be a global health problem especially in developing and under-developed countries, where poor sanitation and inadequate access to clean water facilitate the spread of the disease (Crump & Mintz, 2010). In the year 2005, the Ministry of Health, Malaysia reported 1,072 cases of Typhoid fever in the country (Figure 1.2).

The disease is characterized by high fever, malaise, nausea, vomiting, and diarrhea. Following ingestion, *S*. Typhi penetrate intestinal epithelium and invades macrophages. The infected macrophages carry the organism to the systemic circulation infecting the liver, pancreas, and spleen, causing acute fever (Gonzalez-escobedo *et*

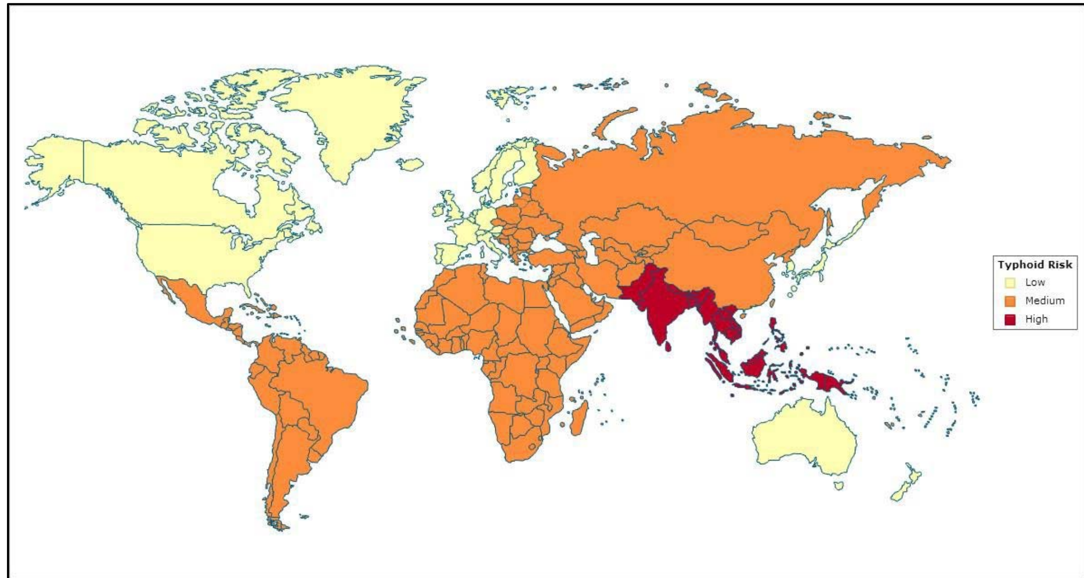
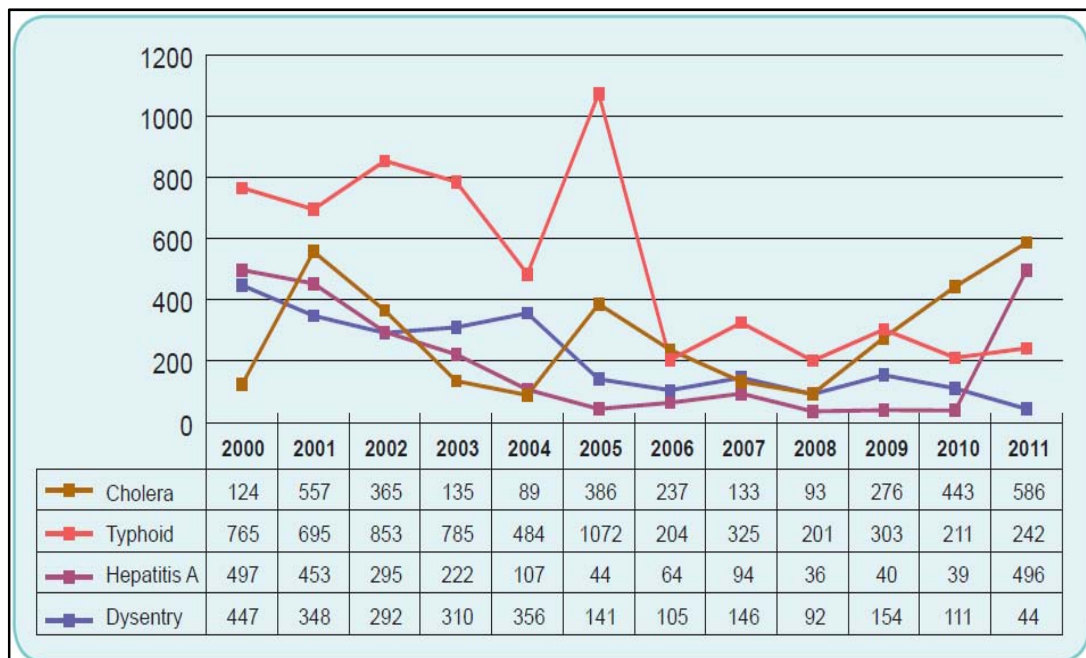


Figure 1.1: Global burden of typhoid fever. (Adapted from Crump *et al.*, 2004)



	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011
Cholera	124	557	365	135	89	386	237	133	93	276	443	586
Typhoid	765	695	853	785	484	1072	204	325	201	303	211	242
Hepatitis A	497	453	295	222	107	44	64	94	36	40	39	496
Dysentry	447	348	292	310	356	141	105	146	92	154	111	44

Figure 1.2: Number of Typhoid fever cases in Malaysia from year 2000-2011. (Data adapted from Disease Control Division, Ministry of Health, Malaysia, 2011)

al., 2011). From the liver, the bacteria migrates to the gallbladder, where either an active infection (cholecystitis) or a chronic infection (carrier state) may develop. The carrier state occurs in about 3 to 5% of people infected with acute Typhoid fever, and is frequently associated with gallbladder abnormalities and cancer (Gunn *et al*., 2014; Prouty *et al*., 2002).

Chronic infection with *S*. Typhi is associated with long-term localization of the bacteria in the gallbladder and continual excretion of the bacilli in the faeces which can result in outbreaks of the disease in the community. The ability of *S*. Typhi to survive in the gallbladder is due to its ability to form biofilm on the surface of human cholesterol gallstones. Biofilms are generally defined as a community of bacteria adherent on a surface and protected by a layer of exopolysaccharide matrix (Gunn *et al*., 2014). Biofilm formation is believed to be a protective mechanism of the bacteria in response to harsh conditions, such as environmental stresses, antibiotics, disinfectants and the host immune system (Pamp *et al*., 2007). As such, it is considered a pathogenic feature of *S*. Typhi. However, the mechanisms associated with biofilm genesis is poorly understood.

Recent studies have shown that the expression of non-coding RNAs plays a critical role in the growth of this pathogen. Ninety-seven unique sRNAs have been found in *S*. Typhi in which ten were mapped to the *Salmonella* Pathogenesis Island (SPI) genes, suggesting their importance in the pathogenesis of the organism (Chinni *et al*., 2010). Since biofilm formation is associated with the bacterial virulence, it is hypothesised that some of these non-coding RNAs are involved in the biofilm formation. Therefore, this project was aimed at investigating the expression of the ten unique non-coding

RNAs in *S*. Typhi in order to understand their role in the formation of biofilm in *S*. Typhi.

CHAPTER TWO

LITERATURE REVIEW

2.1 The Genus *Salmonella*

Salmonella are Gram-negative bacteria that belong to the Enterobactericeae family. These pathogens are rod-shaped and generally motile with peritrichous flagella (flagella that are all around the cell body) except for *Salmonella* gallinarum (Andino & Hanning, 2015). The genus *Salmonella* is classified into two different species; *Salmonella* enterica and *Salmonella* bongori, which can be further subdivided into subspecies and serotypes (serovar). So far, more than 2,500 serotypes have been identified (Jones *et al*., 2008).

2.2 S*almonella enterica* subspecies *enterica* serovar Typhi

Among 2,500 serotypes, *Salmonella enterica* subspecies *enterica* serovar Typhi (*S*. Typhi), is the only pathogen responsible for Typhoid fever. It is a human-specific pathogen and human are the sole reservoir for *S*. Typhi. Typhoid fever is still a global infectious disease with an estimated count of 26.9 million cases and 269,000 deaths per annum (Buckle *et al*., 2012). This systemic disease is transmitted through faecal-oral route via ingestion of contaminated water or food mainly by faeces or urine. The infected patients usually show a few common symptoms, such as abdominal pain, dizziness, high fever, and in some severe cases it can be life threatening

2.3 Typhoid Fever

Worldwide, Typhoid fever continues to be a threat to global health problem especially in under-developed and developing countries where poor sanitation and inadequate

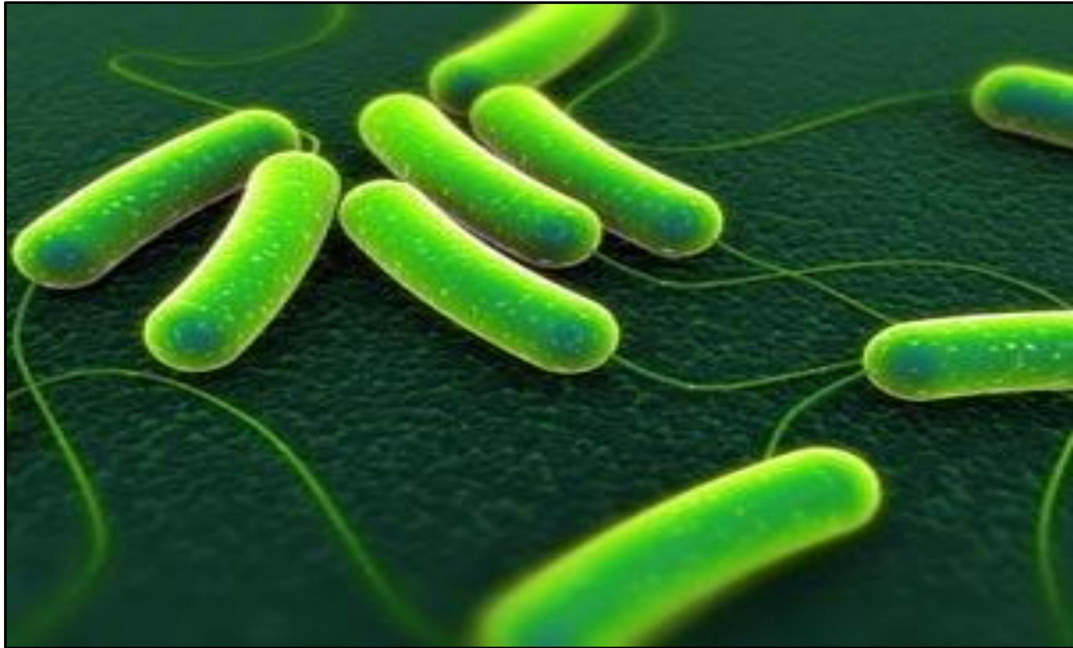


Figure 2.1: ***S*****. Typhi is rod-shaped, possesses long and peritrichous flagella. (Image adapted from http://salmonellatyphi.org/)**

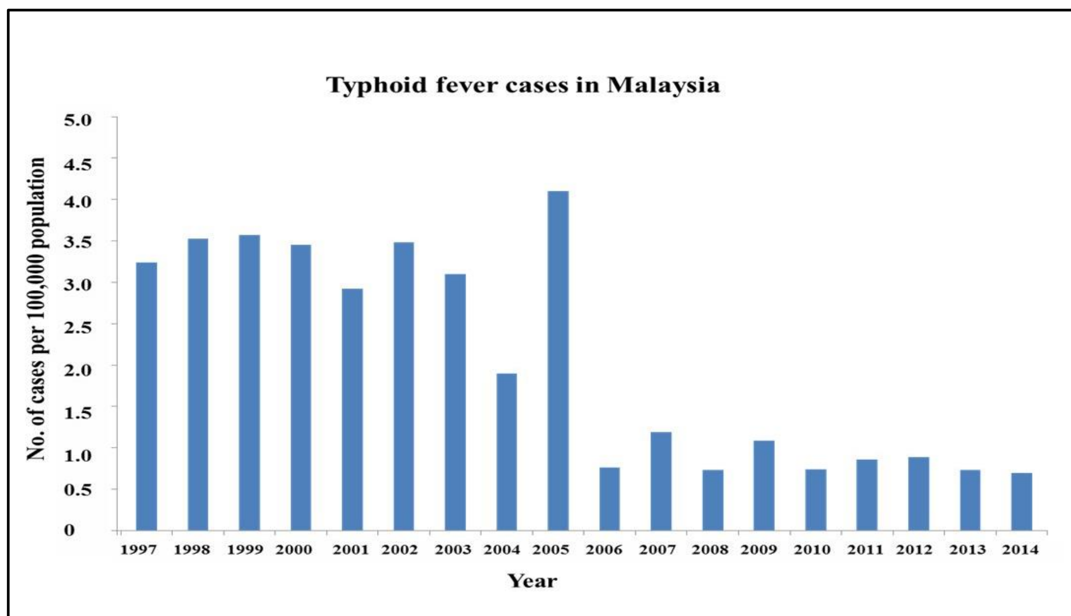


Figure 2.2: Trend of typhoid fever cases in Malaysia from year 1997 till 2014. (Data adapted from the Disease Control Division, Ministry of Health, Malaysia, 2015)

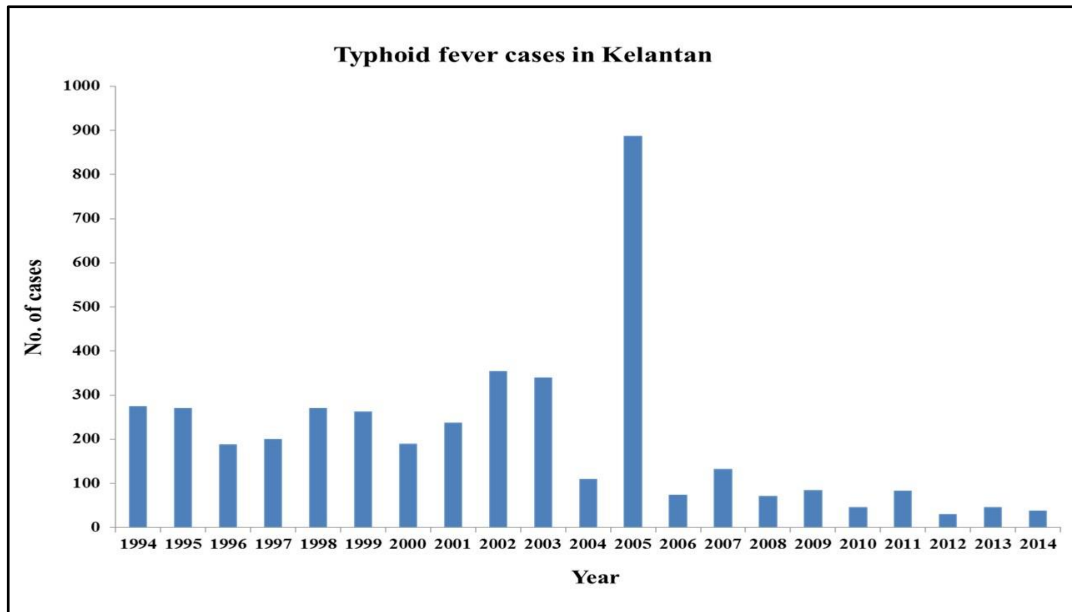


Figure 2.3: Trend of typhoid fever cases in Kelantan from year 1994 till 2014. (Data from Kelantan State Public Health Department, 2015)

access to clean water facilitated the spread of the disease (Crump & Mintz, 2010).

Asia was reported to have the highest incidence rate of typhoid fever, especially in South-central and South-eastern Asia, including Malaysia, India, Pakistan, and Bangladesh with more than 100 cases per 100,000 population in the year 2000 (Crump *et al*., 2004). However, according to the data collected by the Ministry of Health, Malaysia was classified as a low endemic region for typhoid fever with an annual incidence rate of less than 10 cases per 100,000 population from year 1997 to 2014 (Figure 2.2). Among all the states in Malaysia, Kelantan has the highest incidence rate of typhoid fever and classified as a medium endemic region (10-100 per 100,000) (Shah *et al*., 2012). The annual typhoid incidence estimated in year 2001 for Kelantan was 37 per 100,000 (Wan Mansor *et al*., 2005), which declined to 24.4 per 100,000 in 2003, 10 per 100,000 in 2008, 3.29 per 100,000 in 2009, and 2.8 per 100,000 in year 2010. This was attributed to the introduction of proper monitoring and surveillance systems by the Kelantan State Public Health Department (Wan Mansor *et al*., 2011). As shown in Figure 2.3, a highest incidence of typhoid fever occurred in the year 2005 due to a major flood in the state. It was reported that wells were contaminated with sewage overflow, and rural communities which depended on wells for water supply were infected (Shah *et al*., 2012).

2.4 Pathogenesis and Clinical Manifestations

The incubation period of *S*. Typhi is generally 7 to 14 days, even though it may be as short as 3 and as long as 60 days depending upon the size of the inoculum and the status of the hosts immune system (Crump & Mintz, 2010; Crump *et al*., 2004; Maskalyk, 2003). In the first week of infection, gastrointestinal manifestations, such as abdominal pain and diarrhea are the first symptoms that happen to most people.

Constipation may also occur as the Peyer's Patches undergo inflammation and narrowing of the bowel lumen (Gunn *et al.*, 2014). Some individuals may also develop headache, dry cough and delirium, which leads to malaise (WHO, 2003).

After ingestion, *S.* Typhi is able to survive the acidic barrier in the stomach and colonize the small intestine (Gunn *et al.*, 2014). The bacterium crosses the intestinal epithelium via three routes: (i) active invasion of enterocytes; (ii) passive invasion of M cells in the Peyer's Patches (lymphoid associated tissue of the distal ileum); and (iii) direct uptake by CD18+ cells (macrophage's or dendritic cells) that intercalate epithelial cells by extending protrusions into the gut lumen (Álvarez-Ordóñez *et al.*, 2011; Crump *et al.*, 2004; Gonzalez-escobedo *et al.*, 2011).

The invading bacteria from the first two routes are internalized by macrophages and dendritic cells which reside in the lamina propria. *S.* Typhi infected phagocytes migrate to the intestinal lymphoid follicles and the draining mesenteric lymph nodes and spread systemically via lymph and blood (Gonzalez-escobedo *et al.*, 2011). The most common sites of secondary infection are the liver, spleen, bone marrow and the gallbladder. The bacteria reaches the gallbladder via the vasculature or through the ducts emanating from the liver (Gonzalez-escobedo *et al.*, 2011). Bacteria that are excreted in the bile can then reinvade the intestinal wall. The gallbladder is the main reservoir during a chronic infection with *S.* Typhi as the bacteria are able to overcome the toxic effect of bile (Gonzalez-escobedo *et al.*, 2011; Gunn *et al.*, 2014).

2.5 Typhoid Carriers

Most Typhoid fever patients recover from the acute phase of the disease after adequate treatment; however, between 3 to 5% of individuals infected with *S*. Typhi develop a chronic infection in the gallbladder regardless of treatment (Maskalyk, 2003). Chronic carriers are defined as individuals shedding *S*. Typhi in their feces more than 1 year after the acute infection (Gonzalez-escobedo *et al*., 2011). As *S*. Typhi is a human-restricted pathogen, chronic carriers serve as a critical reservoir for further spread of the disease through persistent bacterial shedding in feces and urine (Gunn *et al*., 2014; Prouty *et al*., 2002). Chronic *S*. Typhi infections can persist for decades and although highly contagious, they are typically asymptomatic, making identification of carriers within a population difficult (Gonzalez-escobedo *et al*., 2011).

Besides that, chronic carriage also presents a high possibility of developing gallbladder cancer (Gonzalez-escobedo *et al*., 2011). The uniqueness of *S*. Typhi is its ability to interact with bile, a highly complex, lipid-rich and protein-poor digestive secretion produced by the liver. The components of bile are include bile acids, cholesterol, bilirubin and phospholipids and functions as a potent antimicrobial substance in the gastrointestinal tract (Gonzalez-escobedo *et al*., 2011). However, the primary site of *S*. Typhi carriage is in the bile rich gallbladder, which proposes a dilemma. How does *S*. Typhi develop resistance mechanisms which are utilised by the bacteria to develop resistance and persistence in this hostile environment (Gonzalez-escobedo *et al*., 2011). Therefore, the survival of the bacteria in the gallbladder is a key to understand bacteria persistence and biofilm formation must be a critical method.

2.6 Bacterial Biofilm

A bacterial biofilm can be defined as a community of bacteria adherent to a surface and each other by an extracellular matrix secreted by the bacterial cells (Sutherland, 2001). Bacterial preference to survive in groups attached to surfaces and biofilms were seen as simply "chunks" of randomly positioned bacteria within a matrix material. However, it has been discovered that biofilms are not stationary cells or inactive groups of cells but in fact they were complex, diverse community of cells with an ordered and efficient three dimensional structure (Gerstel & Römling, 2003; Sutherland, 2001). A biofilm community has a large benefit to an individual bacterium, protection from environmental stress where the bacteria can be shredded via an extracellular matrix. Hence, at the same time bacterial cells receiving all of the nutrients needed to survive from the extracellular matrix (McDougald *et al*., 2011).

2.7 Life Cycle of Biofilm

Biofilm formation begins with a few free swimming planktonic cells attaching themselves onto a desired surface. The attached surface could be a biotic surface, such as animal cells, plant cells, or abiotic surface, such as gallstones or polystyrene materials (Sutherland, 2001). There are four distinct stages in the formation of biofilm for most bacteria species: surface attachment, development of microcolonies, mature biofilm, and finally dispersion (Figure 2.4). The attachment stage is believed to be irreversible as most of the attached cells lose their flagella and motility (Monroe, 2007). The attached cells eventually form an extracellular matrix known as exopolysaccharide (EPS) (McDougald *et al*., 2011). The EPS matrix provides structural support and protection for the cells within it. As the biofilm matures, the biofilm structure becomes more complex. When the biofilm reaches a certain maturity

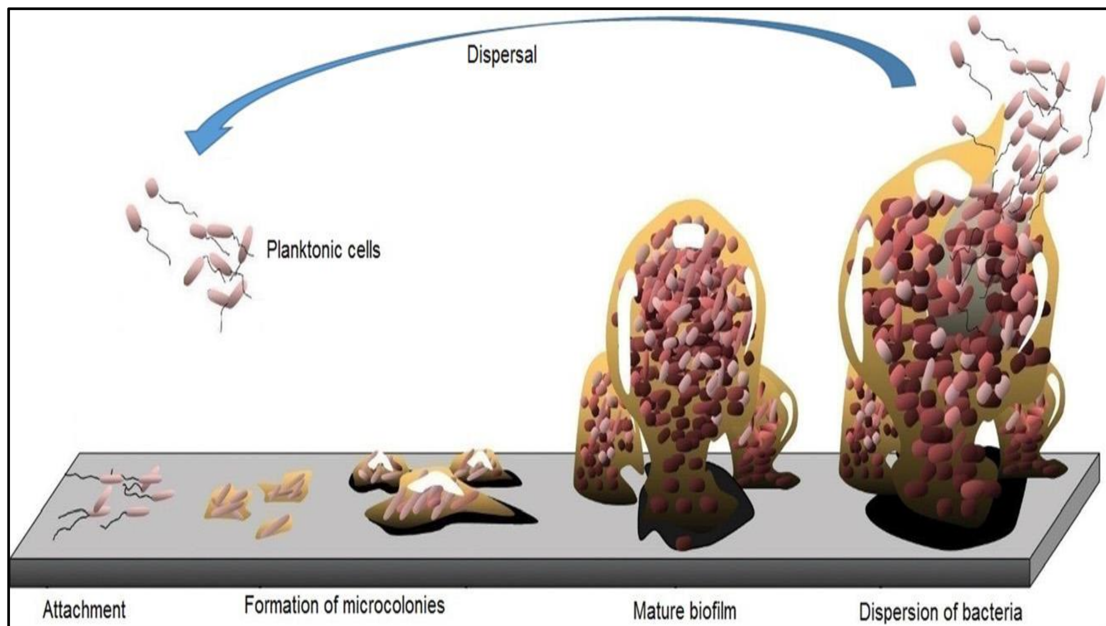


Figure 2.4: Life cycle of biofilm. Stage 1, the planktonic cells attach onto a surface. Stage 2, the surface-attached cells form a layer of protective matrix which later becomes the biofilm community. Stage 3, the biofilm becomes mature. Stage 4, dispersion of the bacteria into the environment. (Adapted from Chung & Toh, 2014)

level, planktonic cells or a portion of the biofilm may disperse into the environment looking for another habitat (Pamp *et al*., 2007).

2.8 *Salmonella* Biofilm Matrix

In the biofilm, bacteria cell are embedded in EPS the matrix. The EPS matrix is usually produced by the bacteria itself and can be hydrophilic or hydrophobic. It is composed approximately 97% of water (Pamp *et al*., 2007; Sutherland, 2001). Thus, with hydrated matrix, desiccation of the biofilm can be prevented. Beside water, the EPS matrix also contains cellulose, curli, fimbriae, colanic acid, biofilm related proteins and nucleic acids (Götz, 2002). The EPS matrix components of *Salmonella* biofilms vary considerably with the environmental conditions and the type of surface (Prouty *et al*., 2002). The mixed components in the bacterial biofilms provide rigidity to the biofilm and protection for the bacterial cells embedded within the biofilm.

Flagella has been shown to promote surface binding during the initial stages of biofilm development in various microorganisms (Sutherland, 2001). The expression of *S*. Typhimurium flagella was to be up-regulated in biofilm formation on polystyrene wells and glass surfaces (Prouty *et al*., 2002), and directly facilitates the biofilm development on cholesterol coated surfaces, suggesting its contribution of this surface appendage to biofilm formation varies with binding substrates (Crawford *et al*., 2008). It has been reported that, the conserved *S*. Typhimurium flagellar regulon gene *flhE*, involved in the flagellar type III secretion specificity switch, is not required for flagella production or swimming, but appeared to play a role in swarming and biofilm formation (Davies *et al*., 1993).

In addition, fimbriae has been shown to facilitate adherence during biofilm initiation (attachment stage) and cell-cell interactions during growth of *Salmonella* biofilm (Müsken *et al*., 2010). Moreover, curli fimbriae (thin aggregative fimbriae) are amyloid-like cell-surface proteins and it is important in host-colonization, persistence, invasion and host-immune evasion (Bordeau & Felden, 2014; Steenackers *et al*., 2012). Colanic acid and cellulose are capsular extracellular polysaccharide that plays a role in *Salmonella* biofilm formation, specifically to create extensive three-dimensional structures (Crawford *et al*., 2008).

The O-antigen that is conserved amongst *Salmonella* species consisting of more than 2,300 repeating tetrasaccharide units. Structurally, the O-antigen capsule is similar to Lipopolysaccharides (LPS) O-antigen of *S*. Enteritidis, and have similar repeating sugar units. The LPS O-antigen differs in size, charge, substitution patterns, and immunoreactivity (Pamp *et al*., 2007). The O-antigen capsule is postulated to play an important role in environmental persistence (Gibson *et al*., 2006). Furthermore, production of O-antigen capsule in *S*. Typhimurium was shown to be related to murine infection activity, suggesting involvement in systemic infection and the cause of Typhoid fever outbreaks (Barak *et al*., 2007). The O-antigen capsule was also shown to be a crucial determinant of biofilm culture on gallstones and cholesterol-coated surfaces (Crawford *et al*., 2008).

There are many genes known to regulate the expression of biofilm extracellular matrix components via CsgD, a transcriptional activator of the curli operons as well as other biofilm related operons, adrA, bapA, and bcsABCD (Gerstel & Römling, 2003) . CsgD is the master curli regulator in *S*. Typhi biofilm and other Gram-negative bacteria. It

was found that in *E. coli* biofilm, CsgD also altered the cell physiology to enable production of curli (Beloin *et al*., 2004). Biosynthesis of cellulose at the inner bacterial membrane is also positively regulated by CsgD via direct binding and subsequent transcriptional stimulation of AdrA in *S*. Typhimurium biofilm (He & Ahn, 2011; Wang *et al*., 2015). Therefore, the CsgD can be seen as the biofilm control point by regulating the expression of all major *Salmonella* biofilm constituents and controlling the transition between planktonic cells to biofilm cells (Gerstel & Römling, 2003; Wong, 2011). Moreover, the *csgD* expression is also influenced by a large number of proteins, non-coding RNA and signalling molecules (Gerstel & Römling, 2003; Wang et al., 2015). This complex regulation allows *csgD* expression to respond to a number of different extracellular inducements, such as pH, temperature, salt concentration, and nutrient supply amongst others (Gerstel & Römling, 2003; Wang *et al*., 2015; Zou *et al*., 2012). Recent studies also show that non-coding RNAs (ncRNAs) are involved in bacteria cellular network which, include biofilm regulation, bacterial virulence, and also response to environmental stress (Chambers & Sauer, 2013; Finnegan & Matzke, 2003; Gripenland *et al*., 2010).

2.9 Non-Coding RNA

Two major classes of RNA types have been identified in cells derived from all organisms: 1) Protein-coding RNAs or mRNAs serve as templates for protein synthesis; 2) non-coding RNAs (ncRNAs) that are not translated into protein but act as functional RNA (Finnegan & Matzke, 2003; Michaux *et al*., 2014). As the name suggests, ncRNAs do not encode for proteins as they do not contain open reading frames (ORFs). However, this does not mean that they do not contain information nor have function. These regulatory RNAs, which are also widely known as small RNAs

(sRNAs), non-protein-coding RNAs (npcRNAs), functional RNAs (fRNAs), untranslated RNAs (utRNAs), and small non-messenger RNAs (snmRNAs) (Finnegan & Matzke, 2003; Gripenland et al., 2010; Storz et al., 2011). The ncRNAs are present in all kingdoms of life forms and have become increasingly recognized as a novel class of gene expression regulators and play many indispensable roles in bacterial cells. The ncRNAs are generally between 50–300 nucleotides in length (Finnegan & Matzke, 2003). They play diverse physiological roles, such as those in the regulation of stress responses and metabolism, control of bacterial envelope composition, RNA processing and modification, mRNA stability and translation, transcriptional regulation, protein stability and secretion (Chowdhury *et al*., 2013; Michaux *et al*., 2014; Romilly *et al*., 2014; Vogel, 2009). In addition, ncRNAs also play vital regulatory roles in bacterial virulence and quorum sensing besides being involved in many aspects of microbial physiology such as, regulation of outer membrane protein synthesis, iron homeostasis and nutrient metabolism (Bejerano-Sagie & Xavier, 2007; Gripenland *et al*., 2010).

Most characterized ncRNAs regulate gene expression by base pairing with the target mRNAs with having extensive potential for base pairing with their target ncRNA or those with more limited complementarity (Storz et al., 2011; Waters & Storz, 2009). The ncRNAs regulation or interaction can be classified into two distinct classes based on their genomic locations: 1) cis-encoded ncRNAs and 2) trans-encoded ncRNAs (Papenfort & Vogel, 2010; Storz et al., 2011; Waters & Storz, 2009).

2.10 Cis-encoded base pairing Non-Coding RNAs

In bacteria, *cis*-encoded base pairing occurs when the ncRNA binds with extensive complementarity to their target mRNAs, and they are located on the DNA strand

opposite to the mRNA targets. Even though the two transcripts are encoded in the same region of DNA, the able to transcribe from opposite strands as discrete RNA type, and mostly it functions as a trans as diffusible structural unit or molecules (Waters & Storz, 2009). The cis-encoded antisense sRNAs mostly exist in plasmids or other Mobile Genetic Elements (MGE) (type of DNA that can move around within the genome). However, these ncRNAs are increasingly being found in chromosome. Most of the cis-encoded antisense sRNAs expressed from plasmids, bacteriophage, and transposons function to maintain the appropriate copy number of the MGE (Brantl, 2009). In addition, cis-encoded antisense sRNAs modulate the riboswitches, which are part of the 5'UTRs of their target mRNA, that undergoes change in their structures upon binding of a regulatory molecule (Figure 2.5). Moreover, the physiological roles of the cis-encoded antisense ncRNAs expressed from bacterial chromosomes promotes degradation or represses translation of mRNAs (De Lay *et al*., 2013; Waters & Storz, 2009).

It has been reported that in *E. coli*, base-pairing between the stationary phase-induced GadY antisense ncRNA and the *gadXW* mRNA leads to cleavage of the duplex between the *gadX* and *gadW* genes (Padalon-Brauch *et al*., 2008). In *cyanobacterium Synechocystis*, the iron stress-repressed IsrR antisense ncRNA base pairs with sequences within the *isiA* coding region of the *isiAB* transcript and leads to decreased levels of an *isiA* transcript (Dühring *et al*., 2006; Waters & Storz, 2009).

2.11 Trans-encoded base pairing Non-Coding RNAs

Another class of base-pairing ncRNAs is the trans-encoded ncRNA, where this type ncRNAs only share limited complementarity with their target mRNAs. They generally

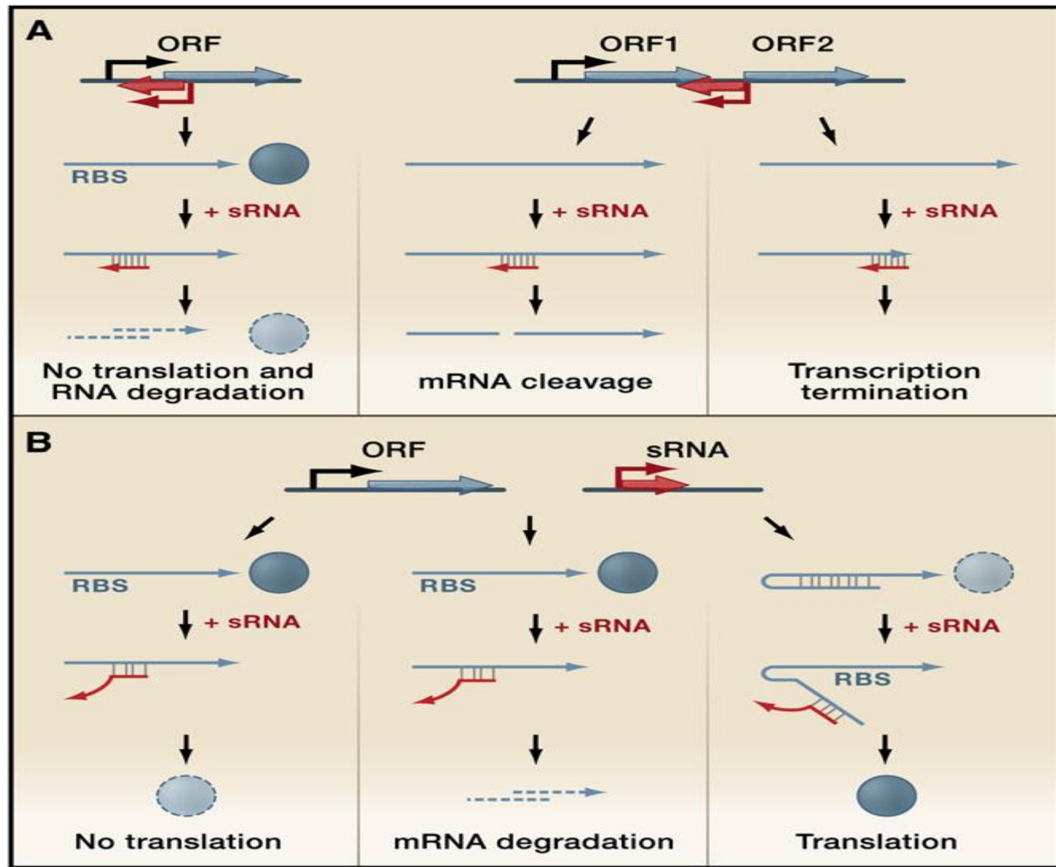


Figure 2.5: Schematic representation of gene regulation mediated by Cis-encoded and trans-encoded base-pairing ncRNAs. (A) Gene encoding cis-encoded antisense sRNAs/ncRNA (red) and their target RNAs (blue), which share extensive complementarity. An sRNA/ncRNA encoded opposite to the 5' UTR of its target mRNA. Base-pairing inhibits ribosome binding and often leads to target mRNA degradation. Base-pairing of the sRNA/ncRNA can target RNases to the region and cause mRNA cleavage, with various regulatory effects, or the sRNA/ncRNA can cause transcriptional termination, leading to reduced levels of downstream genes. (B) Genes encoding trans-encoded antisense sRNAs/ncRNAs (red) are located separately from the genes encoding their target RNAs (blue) and only have limited complementarity. Trans-encoded ncRNA can act negatively by base pairing with the 5' UTR and blocking ribosome binding and/or targeting the ncRNA-mRNA duplex for degradation by RNases (middle panel). Trans-encoded ncRNA can act positively by preventing the formation of an inhibitory structure, which sequesters the ribosome-binding site (RBS). (Adapted from Waters & Storz, 2009)

act by base pairing with target mRNAs with imperfect base-pairing/less complementarity (Storz et al., 2011; Waters & Storz, 2009). The interaction of ncRNAs to the complementarity mRNA are usually short and non-contiguous. Many of these ncRNAs base-pair at or near to the ribosome binding site (RBS) of their targets mRNA and block translation by blocking ribosomes (Figure 2.5) (Waters & Storz, 2009).

In addition, the trans-encoded ncRNAs can complement with their target mRNA at a more distant location and thus interfere with ribosome binding site by preventing the formation of inhibitory secondary structures, or decrease or increase mRNA stability (Storz et al., 2011; Waters & Storz, 2009). Since they usually interact with their targets via imperfect base-pairing, these npcRNAs usually require the aid of RNA chaperone, bacterial RNA binding protein (Hfq). The binding of Hfq is believed to stimulate base pairing interactions by remodelling RNA structures and by increasing local concentrations of mRNA and the ncRNAs (De Lay *et al., 2013).*

2.12 Non-Coding RNA in Biofilm

In *S*. Typhimurium biofilm, it has been reported that biofilm formation phenotype is dependent on the ncRNA called MicA, encoded in the *LuxS* gene. MicA is partially regulated by a quorum sensing system and specifically by the enzyme LuxS (Kint *et al*., 2010). In addition, MicA also involve in the regulation of biofilm formation via RNA chaperone protein (Hfq). The Hfq is a prerequisite for the binding of many ncRNAs to their trans-encoded targets which are involved in the transcription of several ncRNAs, including MicA (Kint *et al*., 2010)

The 93 nucleotide MicF sRNA forms a 20 base-pair imperfect RNA duplex with the translation initiation region of *ompF* mRNA in order to negatively regulate expression of this outer membrane protein (OMP) at the post-transcriptional level (Bak *et al*., 2015) . The Outer membrane (OM) is an important component of Gram-negative bacteria. Together with the peptidoglycan layer and the inner membrane, it forms the bacterial cell envelope in *S.* Typhimurium biofilm. The OM functions as a selective barrier that prevents the entry of many toxic molecules into the biofilm cell, and plays a vital role in bacterial survival in biofilm in diverse environments. So the MicA strongly requires Hfq for its own intracellular stability and for annealing to mRNA (Van Puyvelde *et al*., 2013).

In *E*. coli biofilm, the Csr (carbon storage regulatory) system exerts profound effects on biofilm development. A CsrA ncRNA regulates *E.* coli biofilm formation by post transcriptionally repressing genes required for the production of polysaccharide adhesion of the biofilm (Romeo, 1998). In addition, CsrB and CsrC ncRNAs are part of the *Salmonella* Csr system and antagonize the activity of the RNA-binding protein.

Even though a lot of research on ncRNA related biofilm has been carried out today, there is still no study carried out on *S*. Typhi biofilm. So, this study will focus on ncRNA regulation in *S*. Typhi biofilm.

2. 13 Rational of the Study

Few research has been carried out on *S*. Typhi, the pathogen that causes Typhoid fever in humans. Whereas, *S.* Typhimurium which causes Typhoid-like symptoms in mice, is frequently used as the experimental model to study *Salmonella* virulence, since