TRANSCRIPTOMIC RESPONSE OF *Salmonella enterica* Subspecies *enterica* serovar Typhi TO ANTIBIOTIC TREATMENTS DURING BIOFILM FORMATION

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by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

ATCC	American Type Culture Collection
CCD	Central Composition Design
CDC	Centers for Disease Control
CFU	Colony-forming unit
cDNA	Complementary DNA
℃	Degree celsius
DEPC	Diethylpyrocarbonate
ELISA	Enzyme-linked immunosorbent assay
EPS	Exopolysaccharide
FDA	Food and Drug Administration
g	Gram
>	Greater than
LPS	Lipopolysaccharide
mRNA	Messenger RNA
MRVP	Methyl Red Vogas-Proskauer
µg	Microgram
µg/ml	Microgram per milliliter
µl	Microliter
mg	Milligram
ml	Milliliter
MBEC	Minimum Biofilm Eradication Concentration
AHL	N-acylated-L-homoserine lactones
-	Negative
NGS	Next Generation Sequencing

OD	Optical density
%	Percentage
+	Positive
PBS	Phosphate-buffered saline
QS	Quorum Sensing
qPCR	Quantitative real-time polymerase chain reaction
RSM	Response Surface Methodology
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RIN	RNA integrity Number
SPI1	*Salmonella* Pathogenecity Island 1
<	Smaller than
SIM	Sulphate Indole Motility
TSI	Triple Sugar Iron
v/v	Volume over volume
λ	Wave-length
w/v	Weight over volume
WHO	World Health Organization
XLD	Xylose Lysine Deoxycholate

RESPON TRANSKRIPTOMIK *Salmonella enterica* Subspecies *enterica* serovar Typhi TERHADAP RAWATAN ANTIBIOTIK SEMASA PEMBENTUKAN BIOFILEM

ABSTRAK

Salmonella Typhi (*S.* Typhi) ialah patogen khusus manusia yang menyebabkan demam tifoid. Satu faktor utama yang menyumbang kepada tifoid yang berkekalan adalah dengan kewujudan pembawa tifoid yang tidak bersimptom. Pembentukan biofilem dalam pundi hempedu manusia didakwa berkait dengan pembangunan pembawa. Tujuan kajian ini adalah untuk membangunkan assai pembentukan biofilem *S.* Typhi secara *in vitro* yang menyerupai persekitaraan pundi hempedu. Enam kunci pembolehubah yang terlibat dalam pembentukan biofilem *S.* Typhi telah dioptimumkan menggunakan Metodologi Respon Permukaan (RSM), dan assai yang dihasilkan menjadi asas untuk pengkajian penghasilan biofilem, dalam kehadiran antibiotik kanamisin dan kloramfenikol. Kedua-dua sel planktonik (bebas renang) dan perantara-biofilem telah dikaji dalam kajian ini bagi menentukan corak ekspresi gen yang mempengaruhi komuniti biofilem *S.* Typhi. Keputusan menunjukkan bahawa *S.* Typhi tidak boleh bermandiri dengan rawatan antibiotik tanpa kehadiran biofilem. Bakteria didapati membina biofilem dengan mudah dalam kehadiran kanamisin berbanding kloramfenikol. Untuk memperjelaskan mekanisma penyesuaian ini, RNA berkualiti tinggi telah diekstrak daripada sampel sel *S.* Typhi planktonik and sel perantara-biofilem yang tidak terawat dan terawat antibiotik. Ekspresi gen sel *S.* Typhi dikelaskan kepada empat kategori: 1) pengawalwaturan biofilem, 2) Penghasilan eksopolisakarida dan liposakarida, 3) kerintangan antibiotik, dan 4) laluan metabolik. Empat gen yang terlibat dalam biofilem telah dipilih dan disahkan menggunakan PCR masa sebenar kualitatif (qPCR). Telah dibuktikan

bahawa gen kerintangan pelbagai-stress telah dikawalatur naik dalam sel perantara-biofilem dengan rawatan kloramfenikol dan kanamisin. Juga, gen *tvi*D, yang diperlukan untuk sintesis antigen Vi telah ditemui dikawalatur turun secara signifikan dalam sel planktonik-kanamisin, tetapi tidak pada sel perantara-kanamisin. Gen *yjf*O, yang mengekod untuk stress biofilem dan protein pergerakan telah disahkan dikawalatur naik dalam sel perantara-biofilem-kanamisin, tetapi dikawalatur turun secara signifikannya dalam sel planktonic-kanamisin. Gen protein pengawalatur pembentukan biofilem, *bss*S telah dikawalatur naik dalam sel perantara-biofilem-kanamisin dan dikawalatur turun dalam sel planktonik-kanamisin. Diambil bersama, penemuan ini mencadangkan bahawa proses penyesuaian biofilem melibatkan peningkatan sintesis eksopolisakarida dan liposakarida untuk pembinaan benteng biofilem. Penurunan dalam enzim yang berkaitan dengan aktiviti metabolik adalah konsisten dengan penampilan senesen bakteria, membolehkan bakteria mengelak daripada sistem imun hos. Kajian ini menyediakan asas untuk pemahaman yang lebih baik akan penyesuaian *S.* Typhi dalam pembentukan biofilem, yang membantu bakteria bermandiri dalam stres rawatan antibiotik. Diharapakan, kajian ini akan dapat mempelopori dalam pembangunan langkah-langkah terapeutik di masa hadapan yang akan dapat menguruskan pembawa dan akhirnya membasmi demam tifoid.

TRANSCRIPTOMIC RESPONSE OF *Salmonella enterica* Subspecies *enterica* serovar Typhi TO ANTIBIOTIC TREATMENTS DURING BIOFILM FORMATION

ABSTRACT

Salmonella Typhi (*S.* Typhi) is a human-specific pathogen which causes typhoid fever. One major factor contributing to typhoid persistence is the existence of asymptomatic typhoid carriers. Biofilm formation in the human gallbladder is postulated to be associated with development of the carrier-state. The aim of this study was to develop an optimized assay for *S.* Typhi biofilm formation *in vitro* that mimics the environment of the gallbladder. Six key variables involved in *S.* Typhi biofilm formation were optimized using Response Surface Methodology (RSM), and the resulting assay formed the basis for transcriptomic investigation of biofilm production, in the presence of the antibiotics kanamycin and chloramphenicol. Both planktonic (free-swimming) and intermediate-biofilm cells were investigated in this study, to determine the patterns of gene expression which influence the *S.* Typhi biofilm community. The results showed that *S.* Typhi could not survive antibiotic treatment in the absence of biofilm. The bacteria were found to construct biofilm more readily in the presence of kanamycin than chloramphenicol. To clarify the mechanism of this adaptation, high quality RNA was extracted from untreated and antibiotic-treated samples of *S.* Typhi planktonic and intermediate-biofilm cells. Transcriptomic analysis was carried out to identify genes responsible for the adaptation. Gene expression of *S.* Typhi cells were grouped into four categories: 1) biofilm regulation, 2) exopolysaccharide and lipopolysaccharide production, 3) antibiotic resistance, and 4) metabolic pathways. Four genes involved in biofilm formation were selected and confirmed using qualitative real-time PCR (qPCR). It

was established that the Multiple-stress-resistance gene *bhs*A was up-regulated in the intermediate-biofilm cells with chloramphenicol and kanamycin treatments. Also, the *tvi*D gene, which is required for synthesis of Vi antigen, was found to be significantly down-regulated in the planktonic-kanamycin cells, but not in intermediate-kanamycin cells. Gene *yjf*O, which encodes for biofilm stress and motility protein, was confirmed to be up-regulated in the intermediate-biofilm-kanamycin cells, but significantly down-regulated in planktonic-kanamycin cells. Biofilm formation regulatory protein gene, *bss*S was up-regulated in intermediate-biofilm-kanamycin cells and down-regulated in planktonic-kanamycin cells. Taken together, these findings may suggest that the biofilm adaptation process involves increased synthesis of exopolysaccharides and lipopolysaccharides for the construction of the biofilm barrier. The decrease in enzymes associated with metabolic activity is consistent with the appearance of bacterial senescence, enabling the bacteria to evade the host's immune system. This study provides a basis for better understanding of the adaptability of *S.* Typhi by means of biofilm formation, which enables the bacteria to survive the stress of antibiotic treatment. It is hoped that this will lead to the future development of therapeutic measures that will permit management of the carriers and final eradication of typhoid fever.

CHAPTER 1.0

INTRODUCTION

1.1 Background

Salmonella enterica subspecies *enterica* serovar Typhi (*S.* Typhi) is a pathogenic bacterium that causes typhoid fever. Despite major treatment and prevention efforts, millions of new typhoid fever infections and thousands of deaths occur worldwide each year (Gonzalez-Escobedo *et al.,* 2011). Over the past decade, the frequency of antibiotic resistance among *S.* Typhi isolates has increased substantially in endemic regions such as Southeast Asia, Western Asia, Latin America, and South and East Africa (Gonzalez-Escobedo *et al.,* 2011; Wong *et al.,* 2015). *S.* Typhi is transferred into the human gastrointestinal system by consumption of unhygienic food or water. It crosses through the intestinal epithelial layer and invades macrophages (specialized cells that combat infections, including *Salmonella*). Macrophages then carry the *S.* Typhi organisms to the liver, pancreas and spleen. There, it is believed that the bacterial cells replicate in both types of cells, whether phagocytic or non-phagocytic (McDougald *et al.,* 2012). About 3-5% of victims may develop chronic infection and become carriers after the bacteria are shed into their gallbladder, where acute infection results. The bacteria interact with bile that is produced by the liver in the gallbladder. A moderate acidic environment (pH 5–6), the toxic effect of bile and the hydrophobicity of gallstones, which are prone to develop in hypercholesterolemia patients, provides the perfect harsh conditions for the bacteria to form biofilms in the gallbladder.

A study by Kariuki *et al.,* (2010) estimated that almost all *S.* Typhi isolated from acute typhoid patients were resistant to at least one front-line antibiotic.

Further, some 60% were resistant to a range of different antibiotics. It is well-known that bile stored in the gallbladder is composed of bile acids, cholesterol, phospholipids and bilirubin, which have antimicrobial effects. However, *Salmonella* is able to survive the bactericidal effects of bile (Prouty *et al.*, 2002). Hence, it has been proposed that sequestration of *S.* Typhi in the gallbladder results in persistent infection and the typhoid carrier state. The pathogen adapts to sub-lethal concentrations of bile by changing its gene expression, leading to biofilm formation in the gallbladder and chronic infection of the host (Hernández *et al.*, 2012). Biofilms protect bacteria from environmental stresses, including antibiotics and disinfectants. These films also provide a barrier that isolates the bacteria from the host's immune system (Castelijn *et al.*, 2012; Robijns *et al.*, 2014) with the result that *S.* Typhi are extremely difficult to eliminate. Around 3-5% of patients who suffer from acute *S.* Typhi infection become carriers; and those with the carrier state frequently have gallbladder abnormalities; these may include carcinoma and/or gallstones. Approximately 90% of chronically infected carriers have gallstones (Gonzalez-Escobedo *et al.*, 2011), and 11.1% have been found to have gallbladder cancer with a 50% mortality rate (Kuba *et al.*, 1998).

Salmonella biofilms are formed on many types of surfaces—whether living cells (biotic) or non-living surfaces (abiotic). Stepanovic *et al.*, (2004) studied biofilm formation in 122 *Salmonella* species taken from humans, as well as animals and food. They cultured these on polystyrene plates, and found that all strains could form biofilm successfully on the plates. Later studies showed that the amount of biofilm produced by *Salmonella* was dependent on the surface and culture conditions (Stepanovic *et al.*, 2004; Steenackers *et al.*, 2012). In line with these studies, it was

found that cholesterol-coated polypropylene tubes were good surfaces for biofilm formation (Crawford *et al.,* 2008). It was also found that bile was essential for the growth of efficient biofilms on the surface of gallstones (Gonzalez-Escobedo *et al.,* 2011). It is well-established that antibiotic treatments are often not effective against *S.* Typhi in carriers who have gallstones. And Prouty *et al.,* (2002) observed that patients who have gallstones are more likely to become typhoid carriers. Since *S.* Typhi is a human-specific pathogen, there is no animal model available. Hence, an *in vitro* biofilm microtiter plate assay may be deemed a useful model for screening of biofilm against antibiotics and for understanding the persistence of *S.* Typhi in typhoid carriers.

The human body's susceptibility to infection has been the focus of most studies to date that have considered the effects of antibiotics on biofilm. Utilization of streptomycin and vancomycin has increased susceptibility of *S.* Typhimurium infection toward antibiotic treatments (Sekirov *et al.,* 2008). Antibiotics eliminate metabolic activities of gut commensals which affect sugars release and bile acids as a potential mechanism for antibiotic resistance (Ng *et al.,* 2013; Ferreyra *et al.,* 2014). Multiple studies have shown that treatment with high concentrations of antibiotics reduces most of the products of bacterial metabolism. These include such things as: secondary bile acids, and short-chain fatty acids (Theriot *et al.,* 2014; Jump *et al.,* 2014). Conversely, bacterial precursors increase when subject to high-dose antibiotics. These include primary bile acids, oligosaccharides, and sugar alcohols (Yap *et al.,* 2008; Romick-Rosendale *et al.,* 2009; Zhao *et al.,* 2013).

Each antibiotic that is used in treating bacteria triggers a particular reaction, as can be seen by an analysis of responses of the global bacterial transcriptome (Goh *et al*., 2002; Ng *et al*., 2003; Lin *et al*., 2005; Yim *et al*., 2006). In some cases, the physiology of the bacteria reveals that a reflection response has been initiated toward the antibiotic, which inhibits bacterial growth. In other cases, however, an adaptive response is evident in the bacteria - it becomes resistant, or adaptive, to the antibiotic. Ultimately, the use of antibiotics increases the rate of mutation (Alonso *et al*., 1999; Henderson-Begg *et al*., 2006), as well as the recombination and the horizontal transfer of genes (Ubeda *et al*., 2005; Lopez *et al*., 2007), and mechanisms by which resistance to antibiotics is expressed. In short, a global adaptive response is triggered as a result of the stress engendered by antibiotic use.

In this study, chloramphenicol and kanamycin have been used as antibiotic treatments for *S.* Typhi. Chloramphenicol increases the expression of genes involved in tRNA biosynthesis. It magnifies the expression of gene translation and vitamin biosynthesis, as well as phosphate transport, response to stressors, proton motive force, and antibiotic resistance (Maurice *et al*., 2013). Based on a report by Tezel *et al*., (2016), 76.9% resistance has been observed among *Salmonella* Infantis strains against kanamycin. This study aims to investigate parameters and subsequent patterns of gene expression which influence the biofilm community and also, to determine the functional mechanisms of chloramphenicol and kanamycin in *S*. Typhi biofilm.

1.2 Rationale of the Study

As new diseases are continually discovered, some diseases which have been a problem in the past re-emerge to threaten the human population. This is especially dangerous to those who have not previously been exposed to the diseases. One such classical example is *Salmonella* infections. The frequency of antibiotic resistance among *Salmonella* isolates has increased profoundly in regions where the bacterium is naturally located. One of the factors that contribute to high salmonellosis prevalence is the formation of biofilm in infected patients, making them chronic carriers of the *Salmonella* bacteria which propagate the disease in the population.

Biofilm formation is an important step in bacterial pathogenicity. In biofilm, the resistance of the bacteria towards antibiotics increases exponentially. Antibiotics will kill the bacteria while it is still in the free-swimming planktonic stage. The problem is that the use of antibiotics to treat biofilm in the human body would require a cocktail of high dosage antibiotics in the hope of penetrating the exopolysaccharide (EPS). Moreover, toxicity due to drug-to-drug interaction during the antibiotic treatment is a cause of many deaths, rather than death from the bacterial infections. In addition, if antibiotics continue to be used incorrectly, drug-resistant bacteria strains will continue to proliferate. Hence, the aim of this study was to develop an optimized assay for *Salmonella* Typhi biofilm that mimics the environment of the gallbladder as an experimental model for chronic typhoid fever.

It is hypothesized that the typhoid carrier state is associated with *S.* Typhi biofilm formation in the gallbladder. Studies have been conducted regarding the significance of antibiotics triggering biofilm formation (Linares *et al.*, 2006, Yim *et*

al., 2007). To date, there has been little conclusive information published that provides detail regarding the molecular ecology or the evolutionary dynamic of biofilm formation within the human gallbladder. In order to lessen the recurrence of future outbreaks of this infection disease, it is vital to gain a greater understanding of environmental factors that are involved in its life-cycle, including the persistence, growth, and transmission of *Salmonella* pathogens and the host's response to environmental changes.

In this study, chloramphenicol and kanamycin were chosen as antibiotic treatments for their different responses towards *S.* Typhi biofilm. Chloramphenicol effects protein translation through inhibition of the elongation step in the 50S ribosome subunit, while kanamycin effects protein translation by tRNA mismatching (mistranslation) in the 30S ribosome subunit (Kohanski *et al*., 2010). Hence, bacteria responded differently when chloramphenicol and kanamycin antibiotics were used.

RNA-Seq-based transcriptomic analysis of antibiotic-resistant bacteria has been investigated using the Illumina sequencing system. Next-generation sequencing method triggered a huge amount of mRNA reads. In transcriptome, the distribution associated with reads was mapped randomly through the comparative position along the genome for each sample. The two completely sequenced *S.* Typhi strains which are usually used as reference genomes were Ty2 and CT18. Ty2 is commonly used in experimentation and vaccine development (Deng *et al*., 2003). CT18 is a multi-drug resistant isolate from Vietnam (Parkhill *et al*., 2001). Hence, this study used *S.* Typhi CT18 as reference genome and aimed to investigate parameters and subsequent

patterns of gene expression which influence biofilm community and the functional mechanisms of chloramphenicol and kanamycin on *S.* Typhi biofilm.

1.3 General Objective

To evaluate gene expression of *S.* Typhi planktonic and intermediate-biofilm cells with chloramphenicol and kanamycin treatments.

1.4 Specific Objectives

1. To develop an *in vitro S.* Typhi biofilm culture using polypropylene microtitre plates and Response Surface Methodology.
2. To determine the growth-curve of *S.* Typhi cells and antibiotic susceptibility for planktonic and intermediate-biofilm cells.
3. To investigate the effect of chloramphenicol and kanamycin treatments on gene expression of *S.* Typhi cells using Transcriptome.
4. To evaluate and confirm gene expression of *S.* Typhi with antibiotic treatments using qPCR.

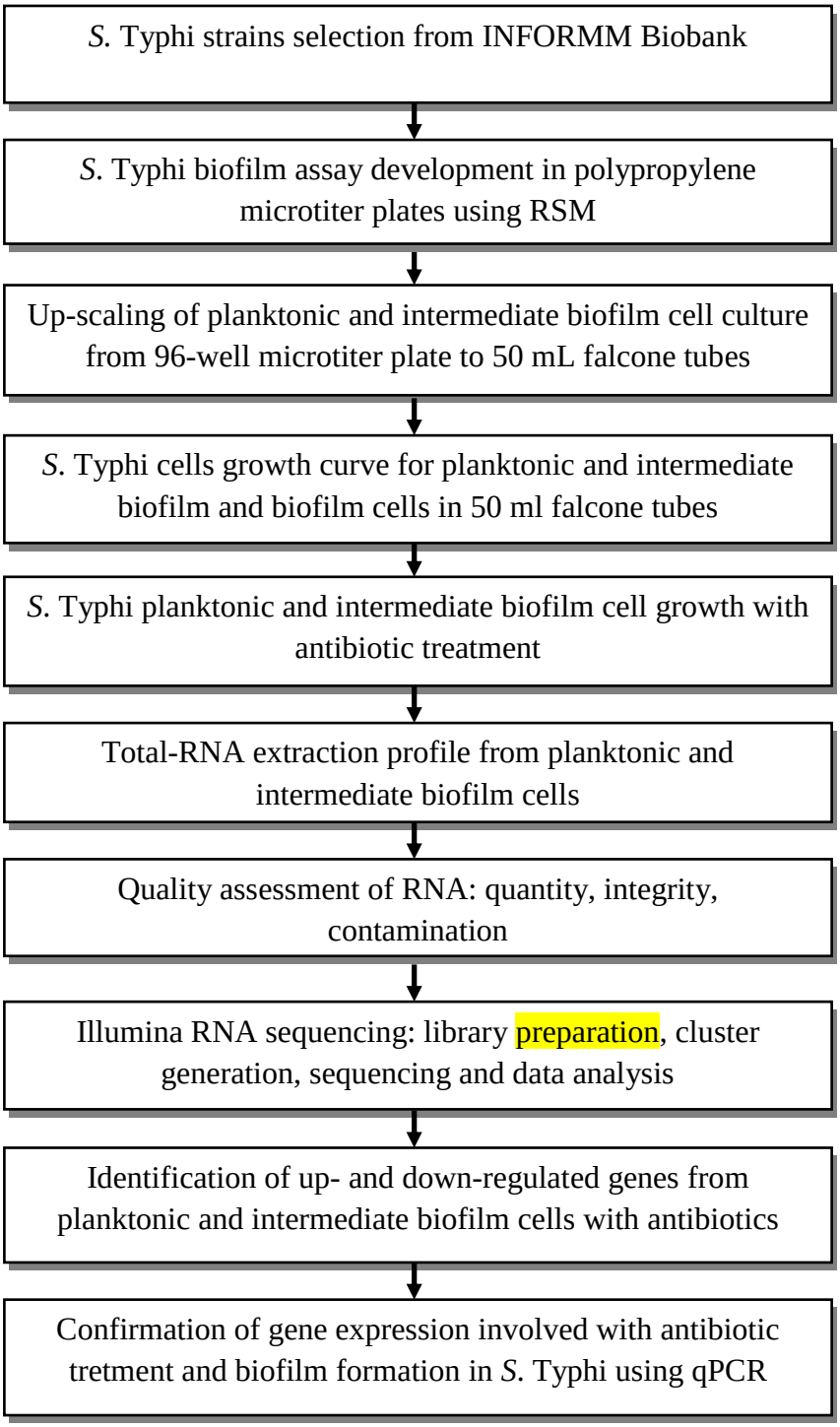

Figure 1.1: Flow chart of research experimental overview

CHAPTER 2.0

Literature Review

2.1 *Salmonella enterica* Subspecies *enterica* serovar Typhi

The *Salmonella* serovars that can cause enteric fever in humans include *Salmonella enterica* subspecies *enterica* serovar Typhi (*S.* Typhi). This subspecies is the agent that causes typhoid fever. Lignieres named *Salmonella* in 1900, in recognition of Daniel Elmer Salmon. He was the bacteriologist who, in 1885, isolated *Salmonella Choleraesuis* (*S. Choleraesuis*) in pigs (Threlfall and Frost, 1990). The genus *Salmonella* is clustered within the family *Enterobacteriaceae* (Chart, 2003). It has well-established identifying characteristics incuding ubiquity, Gram-negativity, and general motility with peritrichous flagella. In addition, *Salmonella* is known for its straight-rod shape and being non-encapsulated. *Salmonella* is facultative and non-spore forming (Gray and Fedorka-Cray, 2002; Molbak *et al.*, 2006). The bacterium ranges in width from 0.7 to 1.5 μm, and in length from 2.0 to 5.0 μm (Holt *et al*., 1994). It can grow successfully in a range of temperatures from 8°C up to 45 °C (Hanes, 2003), and the most favourable pH for growth is within 6.5 to 7.5 (Garcia-Del Portillo, 1999).

2.1.1 Nomenclature of *Salmonella*

The nomenclature of *Salmonella* is a complicated process which contains more than two thousand serovars and the numbers of identified serovars inceasing annually. The *Salmonella* classification system is based on recommendations by CDC (Centers for Disease Control and Prevention) and WHO (World Health Organization) for Reference and Research on *Salmonella*, located at the Pasteur Institute, Paris, in France (Su and Chiu, 2007). According to the Judicial

Commission of the International Committee on Systemic Bacteriology, the genus *Salmonella* consists of three species, *Salmonella bongori*, *Salmonella enterica* and *Salmonella subterranean*. In addition, *Salmonella enterica* was divided further, with the identification of six subspecies: 1) *S. enterica* subspecies *enterica*; 2) *S. enterica* subspecies *salamae*; 3) *S. enterica* subspecies *arizonae*; 4) *S. enterica* subspecies *diarizonae*; 5) *S. enterica* subspecies *houtenae* and 6) *S. enterica* subspecies *indica*. Serovar strains in subspecies I are mostly clinical pathogen identified before serotyping was introduced which include *S.* Typhi, *S.* Typhimurium, and *S.* Enteritidis etc. (Table 1.1).

Table 2.1: Nomenclature of *Salmonella* (adapted from Su and Chiu, 2007)

Genus (italic)	Species (italic)	Subspecies (italic)	Serovars (not italic)	No of Serovars
Salmonella	*enterica*	*enterica* (subspecies I)	Choleraesuis, Enteritidis, Paratyphi, Typhi	1504
		salamae (subspecies II)	9, 46:z:z39	502
		arizonae (subspecies IIIa)	43:z29:-	95
		diarizonae (subspecies IIIb)	6,7:1, v:1,5,7	333
		houtenae (subspecies VI)	21 :m, t:-	72
		indica (subspecies VI)	59:z36:-	13
Salmonella	*bongori*	(subspecies V)	13,22:z39:-	22
Salmonella	*subterranean*	(subspecies VII)		

2.1.2 Laboratory Identification of *Salmonella* Typhi

Presently there are two strategies being practised in the laboratory for identification of *S.* Typhi; the conventional bacterial culture technique and the serotyping method. The bacterial culture technique involves less skill but requires more steps and is more time-consuming than the serotyping techniques. In order to identfy *Salmonella* accurately the bacterium must be isolated, followed by biochemical characterization and serotyping. Isolating *Salmonella* from clinical samples such as stool requires the use of non-selective media for general culture, followed by specific culturing in selective media. Alternatively, selective and differential media can be used from the outset. The selective differential media that are most commonly used to isolate *Salmonella* are Xylose Lysine Deoxycholate agar (XLD) and MacConkey agar (Perilla *et al*., 2003). Transparent colonies of bacteria with a black dot in the center are an indication of the genus *Salmonella* using XLD while colorless colonies of *Salmonella* grow on MacConkey agar.

Most *Salmonella* strains are motile, and achieve their effect with the fermentation of glucose and the production of acid and gas. This assists in the biochemical characterization of the bacterium. Biochemical tests that are used to differentiate bacteria are based on the metabolic activities of the organism, together with minor changes in growth conditions. These conditions include such things as nutrient concentration, temperature, and pH. Biochemical properties of the *Salmonella* were subjected to the following five tests: Triple Sugar Iron (TSI) Medium, Urea Agar Base, Simmons Citrate Agar, Sulphide Indole Motility (SIM) Medium, and Methyl Red-Voges Proskauer (MRVP) Medium (Table 1.2).

Triple Sugar Iron (TSI) medium is sometimes used by the Food and Drug Administration (FDA) as a key to begin the process of identifying enterobacteria. The medium consists of three sugars: glucose, lactose and sucrose and it differentiates among various types of enteric Gram-negative bacteria based on the fermentation of carbohydrate and production of H_2S (Vanderzant and Splittstoesser, 1992). Urea agar is commonly used as an aid to identify enteric Gram-negative bacilli based on urea hydrolysis. In a positive test the colour will change due to ammonia production, while no colour change will be observed in *S*. Typhi if urease is negative. Another test is performed with Simmons citrate agar, which is used to diagnose enteric Gram-negative bacilli. Findings are based on the utilization of inorganic ammonium salt as a source of nitrogen, and sodium citrate as a source of carbon (Simmons, 1926).

SIM medium combines three tests in a single tube: Indole and H_2S production and motility (Perilla *et al*., 2003). A further test can be performed by adding drops of Ehrlich's or Kovac's reagent to a growth medium. If a purple-red coloration occurs this indicates the presence of indole (Harrigan and McCance, 1966), while an acid reaction (yellow colour) at the bottom of the medium indicates lack of indole; this latter reaction is caused by the fermentation of glucose (Vandeppitte *et al*., 2003). MR-VP Medium is a test that can be used to assist in the differentiation of enteric Gram-negative bacilli. MR-VP is based on methyl red and acetylmethylcarbinol (Voges-Proskauer) reactions, and determines the ability of the bacterium to convert glucose into acidic products including lactate, acetate and formate (Clark and Lubs, 1915).

Table 2.2: Biochemical tests for identification of *S.* Typhi (adapted from WHO,2003)

Organism	Triple Sugar Iron Test				Motility, Indol, Urea Test			Citrate Test
	Slant	Butt	H_2S	Gas	Motility	Indol	Urea	
S. Typhi	Alkaline	Acid	Wk+	-	+	-	-	-
S. Paratyphi A	Alkaline	Acid	-	+	+	-	-	-
Other *Salmonella spp*.	Alkaline	Acid	V	V	+	-	-	V

'+' = Positive; '-' = Negative; Wk+ = Weak positive; V = Variable result
H_2S = Hydrogen sulphide

Serotyping is based on a distinctive antigen-specific (antibody) response that is created by an organism which invades a mammalian host. Proteins and polysaccharides are among the bacterial antigens which promote the antibody response that is used for serologic classification (Riley, 2009). Serotyping is only valid on strains that have already been confirmed biochemically to be *Salmonella,* because the counteragent may cross-react with other types of bacteria (Ellermeier and Slauch, 2006). The serotyping method provides more a reliable result compared to conventional serotyping as the Vi antigen may not be expressed in certain laboratory conditions (Wain *et al*., 2005).

2.2 Overview of Typhoid Fever

Typhoid fever is a severe disease that can have a major effect on the entire human body. In particular, it involves the reticuloendothelial system and gall bladder. It is normally indicated by a lengthy fever, abdominal discomfort, malaise, headache, constipation, rose-coloured spots on the chest, hepatomegaly and splenomegaly. It is caused by *Salmonella enterica* serovar Typhi. It is usually spread by ingestion of water or food that is contaminated and has not been properly treated

(Luxemburger and Dutta, 2005). Children and elderly (i.e., those with compromised immune systems) are disproportionally affected by the disease, especially among populations in parts of Asia (South Central, Southeast), Africa (Southern) and Latin America. One Southeast Asian country endemic for typhoid fever is Malaysia. According to Ministry of Health records, in 2010 there were two hundred and ten reported cases of typhoid fever (Kementerian Kesihatan Malaysia, 2011).

Symptoms of the disease manifest 10 to 14 days post-innoculation and vary with geography. The latest WHO report estimated that typhoid fever caused over 21 million illnesses with 217,000 deaths in 2000 (Crump *et al.,* 2010). Moreover, The CDC estimated that at least 22 million illnesses and 600,000 deaths worldwide were attributed to typhoid fever and person-to-person spread of a low, infectious dose of *S.* Typhi (CDC, 2007). Blood antibody titers are highest during the first week, but delayed onset or absence altogether of overt symptoms in many patients makes the clinical diagnosis of typhoid fever challenging (Parry *et al.,* 2002). In addition, *S.* Typhi is transmitted via the fecal-oral route, yet stool cultures are positive in only 30% of symptomatic typhoid patients (Parry *et al.,* 2002). A regimen of fluoroquinolones such as ciprofloxacin and bed rest were used to treat most cases of enteric fever, but the introduction of multi-drug resistant strains in endemic areas is making management of the disease particularly problematic.

Over the past decade, high-dose antibiotic therapy has resolved fewer and fewer chronic infections, to the point that less than two-thirds of chronic infections can be treated, today. The incidence of antibiotic resistance in endemic regions has increased dramatically. Currently, resistance to multi-drug treatment is evident in up

to 60% of all known strains (Gonzalez-Escobedo *et al.*, 2011). Victims of the disease who subsequently become carriers after their recovery from acute illness may be as high as 3 to 5 percent of all infections (Gupta *et al.*, 2006). Whether or not a patient becomes a carrier, initial infection can be fatal in the immunocompromised, in young children, and in the elderly.

2.3 Typhoid Chronic Carriage

Comprehensive studies during chronic carriage regulatory processes facilitate the understanding of what is happening during biofilm development. *Salmonella enterica* subspecies *enterica* serovar Typhi (*S.* Typhi) can linger in the human carrier's gallbladder, suggesting that there is a connection between the bacteria and individuals with gallbladder abnormalities (Prouty and Gunn, 2003). When *S.* Typhi crosses the epithelial layer of the intestine, it invades macrophages and thence is transported to the liver, pancreas and spleen. There, it is thought to replicate, regardless of whether the cells are both phagocytic and non-phagocytic (McDougald *et al*., 2012). Bacteria in the liver are sloughed off and move to the gallbladder, shed into the intestine and interact with bile. Bile, in turn, emulsifies lipids which are shed in faeces. These contain the bacteria carried by the bile. *Salmonella* forms biofilm to protect itself from the high concentration of bile, which consists of bile acids, cholesterol, phospholipids and bilirubin (Prouty *et al.*, 2002).

The carrier state in typhoid patients is frequently connected with those who have gallbladder abnormalities, for example gallstones. Approximately, 90% of chronically infected carriers have gallstones (Gonzalez-Escobedo *et al.*, 2011). Antibiotics prescription is a common and effective method to treat typhoid fever.

Only 90% of typhoid patients fully recover from the disease. However, 10% will continue to shed the bacteria for up to 3 months, and other 5% become long-term carriers without showing any clinical symptoms; yet they will continue shedding bacteria in their stools for over a year (Parry *et al*., 2002; WHO, 2005). The chronic carrier stage is characterized by long-term colonization of the bacilli in the hepatobiliary system (Gupta *et al*., 2006). It has been suggested that *S.* Typhi can form biofilms on the gallstones, providing a more permanent habitat (Prouty *et al*., 2002) from acute and chronic infection.

Antibiotic treatments are not effective in eliminating bacteria (Figure 2.1) in the gallbladder. The infection can seldom be eradicated if the substrate, to which the bacterial cells are attached, is not removed. Hence, the only option to get rid of the biofilm is by surgically removing the gallstones (Prouty *et al.*, 2002). In many cases, removing a biofilm-colonized gallbladder in the case of chronic typhoid infection is the only means to address the persistence of typhoid disease (Gonzalez-Escobedo *et al*., 2011). Furthermore, the typical community of bacteria in the biofilm (Figure 2.1) is sessile and bound in a matrix. From that biofilm community, intermediate-biofilm (free individual) cells fall. In humans, this may cause further infection or lead to the release of the bacterium into the environment through faeces or urine (Selander, 1991). Thus, carriers contribute to transmission of the pathogen in the wider human community (Pradier *et al*., 2000; Olsen, 2001; Esteves *et al*., 2005). In addition, it has been shown that chronic carriers of *S.* Typhi are seriously in danger of developing hepatobiliary carcinomas. In fact, they have an 8.47-fold higher risk of developing cancer of the gallbladder (Davey and O'Toole, 2000).

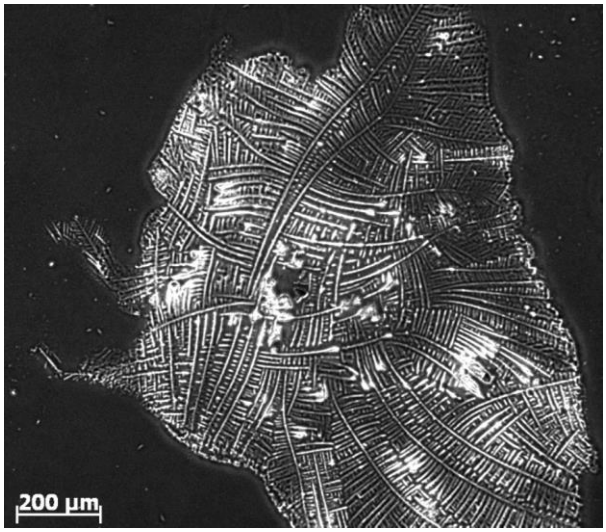

Figure 2.1: *S.* Typhi biofilm community under fluorescence microscope

2.4 Life Cycle of Biofilm Cells

To an ever-increasing extent during the past twenty years, bacterial biofilms have been implicated as a global danger to public safety. Biofilms can be defined as disparate communities of microorganisms that cling to one another and also to biotic or abiotic substrates (Steenackers *et al.,* 2012). *S.* Typhi biofilm is grown through a complex process that requires transporting microbial cells as well as organic and inorganic molecules, to the surface.

In the 1980s, biofilm formation was described in three stages: (1) absorption, (2) attachment, and (3) colonisation (Fletcher, 1980). But in the 1990s, biofilm formation was outlined as an eight-step process. The steps include, among others, the early-stage formation of a layer that provides conditioning, the reversible and irreversible adherence of bacteria to the surface (biotic or abiotic), and the eventual release of cells from the mature biofilm; these subsequently re-gather to colonize afresh and start another attachment (Characklis and Marshal, 1990). In the first decade of the 21st century, biofilm formation was discribed in four different stages:

(1) preliminary attachment to a surface, (2) microcolony formation, (3) maturation of biofilm, and (4) diffusion and cell death (Russo *et al.*, 2006).

Figure 2.2 shows a schematic representation of *S.* Typhi biofilm formation according to Russo's description. Before the attachment takes place, *S.* Typhi approaches a solid-liquid interface closely. The motility of *S.* Typhi tends to slow down and establishes a temporary association with other cells on the surface (Prakash *et al*., 2003). Once *S.* Typhi reaches the surface, flagellated appendages such as fimbriae and pilli take over the attachment role. Fimbriae are vital in cell-to-surface hydrophobicity binding while pilli are important for host-cell colonization (Prakash *et al*., 2003).

In the process to the development of microcolonies, cell-surface interaction is not sufficient. A stable cell-cell interaction is also required to hold the microcolony (Stanley and Lazazzera, 2004). At this stage, signal transduction is needed for the formation of surface motility structures, for example the pilli, and the production of exopolysaccharide (EPS), which mediates the stabilization of cell-cell interaction. Quorum sensing (QS) is helpful at this stage. *S.* Typhi secretes the signal molecules and when signal molecules reach a threshold, the genes that control EPS production will be stimulated (Prakash *et al*., 2003). These signals are important in adaptation to the environment. The maturation of biofilm is regulated by several signals such as QS, catabolism repression, and starvation. Studies showed that at this stage, biofilm develop properties such as increased antibiotic tolerance, development of EPS, increased ability to withstand UV light, and elevated production of secondary metabolites. It has been proposed that at this stage pilli within water channels are

formed in order to permit nutrients to enter and waste to leave (Danese *et al*., 2000; Stanley and Lazazzera, 2004).

At the dispersion stage, the cells in the biofilm will migrate to become planktonic cells. This condition occurs especially when the environment is not favourable. The detachment could be triggered by internal factors, for example QS or external factors such as the shear forces or nutrient depletion (Donlan, 2002). As yet, there is still considerable research to be done to understand the mechanism, the function, and the regulation of detachment from the *S.* Typhi biofilm.

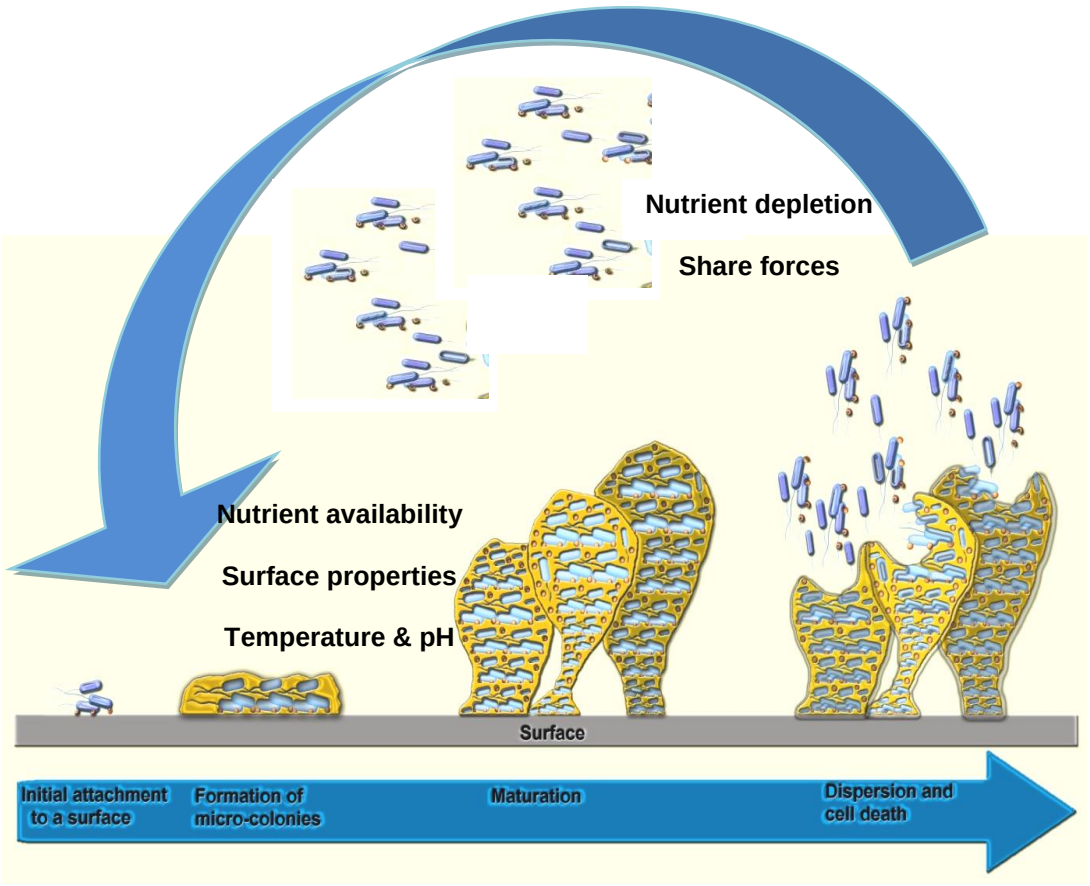

Figure 2.2: Life cycle of biofilm cell. The intermediate-biofilm cells are attached on a favourable surface and form a microcolony which leads to biofilm maturation. Exopolysaccharide is formed surrounding the bacteria during biofilm maturation. Dispersion of the cells occurs when there are unfavourable conditions involved

2.5 Structural Components of Biofilm

Microbial species operate in various ways, including the mechanism by which they manage to come in contact with a surface, attach them to it, and promote cell-to-cell communication. Their growth as complex structures also demonstrates multiple facets (Wang *et al*., 2004). Typically microbes find it easier to adhere to rough surfaces, as well as those that exhibit hydrophobic properties, or have conditioning films on their surface (Chae *et al*., 2006). There are several properties of the cell's surface that are important for biofilm formation and development: notably EPS production, inter-cell communication, and the existence of extracellular appendages (Davies *et al*., 2003). Other structural components that are important during *S.* Typhi biofilm formation include flagella, curli and other fimbriae, BapA, and anionic O-antigen capsule. Cellulose, colonic acid and fatty acids are also implicated (Steenackers *et al.*, 2012).

2.5.1 Exopolysaccharide

Hori and Matumoto (2010) have demonstrated that roughly 50-90% of the total organic carbon of biofilms is attributable to EPS. EPS consists of an elaborate admixture of biopolymers; this includes polysaccharides, proteins, humic substances, nucleic acids, and lipids. Further, EPS facilitates the introductory attachment of cells to various strata, and it protects the cells from dehydration and from environmental stress (Vu *et al.*, 2009). Depending on its structure, EPS may be either hydrophilic (water-loving) or hydrophobic (repelled by water) (Donlan, 2002), and yet, EPS itself contains in the vicinity of 97% water (Sutherland, 2001; Karatan and Watnick, 2009). This means that it is highly hydrated. Polysaccharaides are an important

element of biofilm matrices. Its chemical and physical properties could vary based on the monomer units and also its glycoside linkages (Pamp *et al*., 2007).

Cellulose is an example of a polysaccharide which is widely described in *E. coli* and *Salmonella* spp. (Pamp *et al.*, 2007). EPS makes an important contribution toward the morphology and internal structure of biofilm, because is dictates properties of the biofilm, including physical and chemical aspects. Also, EPS matrix acts as a barrier to allow selective transportation. For example, it protects biofilm against adverse conditions such as high concentrations of biocides and antimicrobials. It also contains molecules that are essential for cell-to-cell communication and cellular conduct within the community (Simões *et al.,* 2010). EPS forms locally-charged biofilm matrices that appear in three dimensions, and have a gel-like appearance. Frequently, when EPS is not present in the biofilm, bacteria still attach to the surface but multilayer biofilms are unable to grow (Karatan and Watnick, 2009).

2.5.2 Lipopolysaccharide

Another major constituent of Gram-negative bacteria is lipopolysaccharide (LPS) (Rojas *et al*., 2001). It is an amphiphilic molecule, a lipid- and fat-loving molecule (Gronow *et al*., 2010). LPS is a complex glycolipid which can be divided into three regions; lipid A, core polysaccharide and O-polysaccharide which is known as O-antigen (Sperandeo *et al*., 2009). This lipopolysaccharide structure is known as 'smooth lipopolysaccharide' (S-LPS). O-polysaccharide consists of repeating units (O-unit) of monomers and a single glycosidic link (Consterton, 2009).

In *Salmonella*, the monomers are galactose, rhamnose, mannose, and abequose (Wang *et al.,* 1996). O-polysaccharide forms the surface and forms a protective layer. The long chain of O-polysaccharide becomes a virulent factor in most bacteria (Consterton, 2009). LPS plays a role in helping to stabilize the membrane of Gram-negative bacteria (Gronow *et al.,* 2010). Besides, it assists with the integrity of the outer cell membrane. The characteristics of this component allow the outer membrane to act as a barrier to prevent the entry of toxic molecules. The length of O-polysaccharide is essential to absorbing the nutrients and it is important in resistance to many different detergents and antibiotics (Christie, 2010).

2.6 Planktonic, Intermediate-Biofilm and Biofilm Cells

Planktonic cells are free-swimming in the absence of stress, whereas biofilm cells have adapted in response to bile stress. Biofilms are groups of cells that are embedded in a layer of polysaccharide matrices and adhere to the surfaces. According to Kiedrowsky and Horswill (2011), intermediate-biofilm cells clump together irreversibly and form small aggregates. So far, there is little evidence to define the stage at which intermediate-biofilm forms, but microscopy studies on *in vivo* biofilms have provided images confirming the existence of these aggregated cell clumps (Stoodley *et al*., 2008). Based on a report by Kiedrowski & Horswill (2011), intermediate-biofilm cells are formed during biofilm microcolony formation. However, Chua *et al*., (2014) proposed that intermediate-biofilm cells are formed in the final stage of the biofilm lifecycle (dispersal), and that the process of dispersal confers ‘protection-in-advance mechanisms’ to cope with environmental insults. Chelvam *et al*., (2015) measured variable responses to carbon utilization between planktonic, induced biofilm (intermediate-biofilm) and biofilm cells of *S*. Typhi.

The current study allows for either of these two views: that intermediate-biofilm forms during the microcolony formations stage, or during the biofilm dispersal stage. When growth conditions are optimal, nutrients are available and in the absence of stress planktonic cells are formed. Then, the stress level increases (through nutrient depletion, pH and temperature changes, shear forces and antibiotics) and during this period the microcolony matures into an established biofilm. Therefore, intermediate-biofilm cells are transition cells as they seek to adapt to the new environmental stresses before they fully attach to the surface.

Antibiotics may control disease symptoms by eliminating the planktonic cells, but neglect those bacteria which are in the inner layers of the biofilm. Once the antibiotic treatment stops, the biofilm may act as a platform for revival of the disease. Intermediate-biofilm plays a crucial role in this process, as the dispersion of biofilm cells allows the disease to persist in the presence of environmental antibiotic stimuli. Few studies have considered the effect of antibiotic on intermediate-biofilm. Howden *et al*., (2010) have noted vancomycin resistance at the intermediate-biofilm level due to various regulatory mutations that increase cell wall thickness.

2.7 Antibiotics Responses in *Salmonella*

Excessive dose or low dose of antibiotics can have a deleterious effect on the human host, as it can lead to collateral damage of microflora within the duodenum, or increase the chance of antibiotic resistance in Salmonellosis. Increasing the antibiotic concentrations to 5% of total bacterial culture increases stress response and decreases transcript modulation, which inhibits the growth of the bacteria (Fajardo and Martínez, 2008). In addition, low concentrations of antibiotics

could serve as signalling molecules (Linares *et al*., 2006; Yim and Wang, 2007) activating specific bacterial responses. Fajardo and Martínez, (2008) reported that such antibiotic-mediated signalling may indeed be initiated by the use of antibiotics used therapeutically against the organisms. After they sense the presence of antibiotics, the bacteria exhibit complex responses in resistance to that antibiotic. For example: *Salmonella's* response to antimicrobial mechanisms prompts the PhoP/PhoQ mediator to promote survival within macrophages (Prost *et al*., 2007).

Studies have been conducted to assess the resistance of *Salmonella* biofilms against antibiotics and the repeatability and reproducibility of these assays have been reported to be effective for biofilm treatment (Ghasemmahdi *et al.,* 2015). Antibiotic resistance observed in biofilm may be due to several predictable mechanisms such as efflux pump, low antibiotic doses or slow penetration of antibiotic and pH changes. Randall and Woodward (2002) demonstrated that Sequence alignment analysis showed the gene *opr*J has high similarity to *tol*C which pumps out bile acids, antibiotics, dyes and disinfectants. Moreover, *tol*C is required for efficient bacteria adherence to a surface during biofilm formation (Lee, 2012). *Klebsiella pneumonia* required more than 500 µg/mL of ampicillin to eradicate biofilm (Anderl *et al*., 2000). In addition, slow antibiotic penetration may occur due to ionic attraction between the antibiotic and the exopolysaccharide of the biofilm, hindering its mobility (Prakash *et al*., 2003). As the oxygen concentration reduces in the inner layers of the biofilm, anaerobic acidic niche causes pH changes in the microenvironment and deactivates the antibiotics. This leads to survival of the biofilm cells (König *et al*., 1993). For example: kanamycin is only effective when pH is at the optimum range of 7-8.