IDENTIFICATION OF POTENTIAL PROTEIN BIOMARKERS FROM *Salmonella enterica* subspecies *enterica* serovar Typhi BIOFILM

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IDENTIFICATION OF POTENTIAL PROTEIN BIOMARKERS FROM *Salmonella enterica* subspecies *enterica* serovar Typhi BIOFILM

by

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TABLE OF CONTENTS

CHAPTER 3.0 RESULTS

APPENDICES

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

Symbols and Abbreviations	Descriptions
-	Negative
%	Percentage
+	Positive
<	Smaller than
>	Greater than
µg	Microgram
µg/ml	Microgram per milliliter
µl	Microliter
∞	Infinity
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
CDC	Centers for Disease Control
CID	Collision-Induced Dissociation
ELISA	Enzyme-linked immunosorbent assay
EPS	Extracellular polymeric substances
g	Gram
H_2S	Hydrogen sulphide
IEF	Isoelectric focusing
IgA	Immunoglobin A
IgG	Immunoglobin G
IPG	Immobilized pH gradient
LB	Luria-Bertani
LPS	Lipopolysaccharide
LSD	Least Significant Difference
M	Molarity
m/z	Mass over charge
mA	Milliampere
mA/gel	Milliampere per gel
MALDI	Matrix Assisted Laser Desorption Ionization
mg	Milligram
ml	Milliliter
mM	Millimolar
MRVP	Methyl Red Vogas-Proskauer
MS	Mass spectrometry
º	Degree
ºC	Degree celsius
OD	Optical density
PI	Isoelectric point
PMS	Protein Mass Fingerprint
ppm	Parts per million
Psi	Pressure per square inch
rpm	Revolution per minute
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIM	Sulphate Indole Motility

TOF	Time of Flight
TSI	Triple Sugar Iron
V	Volt
V/HR	Volt per hour
v/v	Volume over volume
w/v	Weight over volume
WHO	World Health Organization
xg	Gravity
XLD	Xylose Lysine Deoxycholate
λ	wave length
2D	Two-dimension
2D-PAGE	Two-dimension polyacrylamide gel electrophoresis

IDENTIFIKASI BIOMARKER PROTEIN YANG BERPOTENSI DARIPADA BIOFILEM *Salmonella enterica* subspecies *enterica* serovar Typhi

ABSTRAK

Salmonella Typhi adalah patogen demam kepialu yang hanya menjangkiti manusia. Ia merupakan masalah kesihatan yang serius di negara-negara yang sedang membangun termasuk Malaysia. Walaupun langkah pencegahan dan rawatan perubatan telah dijalankan, tetapi wabak demam kepialu masih berlaku. Salah satu faktor yang menyumbang kepada keterusan deman kepialu adalah kewujudan pembawa *(carrier)* deman kepialu yang tiada tanda-tanda klinikal. Wujudnya pembawa deman kepialu adalah dijangka melibatkan biofilem yang terbentuk di dalam pudi hempedu pesakit. Pembentukan biofilem membolehkan bakteria tersebut mengelak serangan sistem imun mangsanya dan ubat antibiotik. Dalam usaha untuk memahami pembentukan biofilem, satu kaedah untuk kultur *S.* Typhi biofilem *in vitro* telah dicipta untuk memudahcarakan kajian ekpresi protein dan kekebalan biofilem terhadap antibiotik.

Dalam kajian ini, *S.* Typhi telah dikultur secara *in vitro* dengan menyamai persekitaran dalam pundi hempedu manusia dengan menggunakan media kultur yang mengandungi sup nutrien, glukosa, hempedu dan garam. Protein biofilem yang dihasilkan dianalisis secara perbandingan dengan pasangan planktoniknya menggunakan kaedah 2D-PAGE. Daripada analisis tersebut, telah didapati lima-belas titik-titik protein yang unik kepada biofilem. Titik-titik protein yang unik telah diekstrakkan dan identitinya dikenalpasti dengan mengunakan kaedah MALDI-TOF.

Salah satu daripada titik-titik protein unik tersebut merupakan protein TolC, iaitu salah satu faktor yang menyumbang kepada kebisaan dan kerintangan bakteria tersebut. Selain daripada itu, ia juga didapati meningkatkan tahap ekpresinya (TolC) secara empirikal apabila terdedah kepada hempedu dalam media kultur biofilem. Tahap kerintangan biofilem juga dinilai dengan kajian cabaran antibiotik *(antibiotic sensitivity test)* termasuk ampicillin, chloramphenicol, kanamycin dan tetracycline. Penilaian kekebalan menunjukkan bahawa biofilem *S.* Typhi memang lebih kebal terhadap kebanyakan antibiotik seperti chloramphenicol, kanamycin, dan tetracycline, tetapi masih sensitif kepada ampicillin.

Kaedah pengkulturan *S.* Typhi biofilem yang dikenal pasti dalam kajian ini boleh digunakan sebagai model kajian *in vitro* pembawa deman kepialu dan juga satu kaedah untuk mengkaji kuantiti antibiotik yang diperlukan untuk menghapuskan biofilem yang berada di dalam pesakit kronik. Protein biofilem yang dikenalpasti dalam kajian ini merupakan calon-calon biomarker yang berpotensi untuk dijadikan ujian diagnostik bagi mengenal-pasti pembawa deman kepialu. Selain daripada itu, *S.* Typhi biofilem merupakan bidang penyelidikan yang baru, dan ilmu pengetahuan yang telah dijana daripada kajian ini boleh menambahkan nilai yang lebih ketara kepada pemahaman mekanisma pembentukan biofilem.

IDENTIFICATION OF POTENTIAL PROTEIN BIOMARKERS FROM *Salmonella enterica* subspecies *enterica* serovar Typhi BIOFILM

ABSTRACT

Salmonella Typhi is a human specific pathogen which causes typhoid fever. It is a major health problem in developing countries including Malaysia. Although prevention and medical treatment are available, typhoid still persists. One of the major factors 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 carriers. Formation of biofilm enables the bacteria to evade the host immune system and resist antibiotics. In order to understand the formation of biofilm, a *S.* Typhi biofilm culture method was developed to facilitate the biofilm protein expression and antibiotic resistance studies.

In this study, *S.* Typhi biofilm was cultured *in vitro* by mimicking the human gallbladder environment using nutrient broth containing bile, glucose, and salts. Biofilm proteins were harvested and compared with the planktonic counterpart using 2D-PAGE. Fifteen unique biofilm protein spots were identified and were excised for further analysis using MALDI-TOF. One of the biofilm proteins identified was TolC, a virulence and persistence factor of the bacteria. It was also found to be empirically upregulated in response to bile concentration in the biofilm culture medium. Biofilm antibiotic resistance was assessed by challenging the biofilm bacteria cells with ampicillin, chloramphenicol, kanamycin, and tetracycline. The results showed that *S.* Typhi biofilm was generally more resistant to certain antibiotics such as

chloramphenicol, kanamycin, and tetracycline, but remained susceptible to ampicillin.

The *S.* Typhi biofilm culture method developed in this study could be used as a model for the typhoid carrier stage and also to screen antibiotics required for elimination of *S.* Typhi biofilm in chronic patients. Biofilm proteins identified in this study are potential biomarker candidates for typhoid carrier identification. Furthermore, since *S.* Typhi biofilm is a new research field, knowledge generated in this study added significant value to the knowledge pool of biofilm and provide clues for better understanding of the mechanism of biofilm formation.

CHAPTER 1.0

INTRODUCTION

1.1 *Salmonella enterica* subspecies *enterica* serovar Typhi

Salmonella enterica subspecies *enterica* serovar Typhi (*S.* Typhi) is the pathogen responsible for typhoid fever in humans. It is a human-specific pathogen which preys specifically on humans, which also serve as their reservoir. It is a Gram negative bacteria which belongs to the genus *Salmonella*, a pathogen which is not present in the normal gut of humans. The size of the bacteria is approximately 2-3 µm in length and 0.4-0.6 µm in diameter (WHO, 2003). In the laboratory, *S.* Typhi exists as motile cells with peritrichous flagella. Like most bacteria, *S.* Typhi is a chemoorganotroph, i.e. they obtain energy and carbon molecules needed for cellular functions from organic compounds (Clark & Barrett, 1987).

1.1.1 Nomenclature of *Salmonella*

The nomenclature of *Salmonella* is a very complex system which consists of over two thousand serovars and the numbers are still growing with newly identified serovars every year (Su & Chiu, 2007). *Salmonella* is named after its founder Daniel Elmer Salmon, an American bacteriologist who first isolated *Salmonella* from porcine intestine in 1884. The isolated *Salmonella* was called *Salmonella* Choleraesuis (Schultz, 2008). *Salmonella* is classified based on serological typing using specific antisera recommended by Kauffmann in the year 1966. *Salmonella* identified after 1966 were designated based on serotyping results. However, there are a few clinically important serovars, such as *S.* Typhi and *S.* London which were identified before 1966 and given special names based on the location the bacteria

was first isolated instead of following the antigenic formula system (Su & Chiu, 2007). To avoid confusion and to accommodate all the *Salmonella* strains, the nomenclature system underwent years of reformation. *Salmonella* nomenclature remains a discussion topic till this day.

Presently, according to the ruling of the Judicial Commission of the International Committee on Systemic Bacteriology, the genus *Salmonella* consist of three species, *Salmonella bongori*, *Salmonella enterica* and *Salmonella subterranean*. *Salmonella enterica* was further divided into 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).

Although *Salmonella* species *subterranean* was officially recognized in 2005, it is yet to be incorporated into the Centers for Disease Control and Prevention of United States of America (CDC) system (Su & Chiu, 2007). The *Salmonella* classification system used at the CDC is based on recommendations by the World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France, which is responsible for updating the scheme (Su & Chiu, 2007).

Table 1.1: Nomenclature of *Salmonella* (adapted from Su & Chiu, 2007)

Taxonomic position (writing format) and nomenclature				No. of serotypes in each species or subspecies
Genus (capitalized, italic)	Species (italic)	Subspecies (italic)	Serotypes (or serovars) (Capitalized, not italic)*	
Salmonella	*enterica*	*enterica* (or subspecies I)	Choleraesuis, Enteritidis, Paratyphi, Typhi	1504
		salamae (or subspecies II)	9, 46:z:z39	502
		arizonae (or subspecies IIIa)	43:z29:-	95
		diarizonae (or subspecies IIIb)	6,7:1, v:1,5,7	333
		houtenae (or subspecies VI)	21 :m, t:-	72
		indica (or subspecies VI)	59:z36:-	13
	bongori	subspecies V	13,22:z39:-	22
	subterranea			

*some selected serotypes (serovars) are listed as examples

1.1.2 Laboratory Identification of *S*. Typhi

Currently there are two major methods for identification of *S*. Typhi in a laboratory setting; the conventional bacteria culture method and the polymerase chain reaction (PCR) "serotyping" method. The bacteria culture method requires less skill but is more time consuming compared to the PCR "serotyping" method.

In the conventional method, the bacteria sample is cultured in a selective medium such as Xylose-Lysine-Desoxycholate (XLD) medium to determine whether the bacteria belong to the genus *Salmonella*. Transparent colonies of bacteria with a black dot in the center are an indication of the genus *Salmonella*. The *Salmonella* bacteria will be subjected to a series of biochemical tests where their biochemical properties will be used to determine their species (Table 1.2). Subsequently, serological tests using specific antisera targeting the somatic (O), flagellar (H) and capsular envelop (Vi) antigens, were carried out to serotype the organism (Table 1.3) (WHO, 2003).

In PCR "serotyping" method, specific primers are used to amplify specific antigen-coding genes, such as *prt* and *tyv* which code for the somatic O antigens; *flicC* codes for flagellar H antigen, and *viaB* gene which codes for Vi antigen in *S*. Typhi (Wattiau *et al*., 2011). PCR "serotyping" method provides more reliable results compared to conventional serotyping as the Vi antigen might not be expressed in certain laboratory conditions (Wain *et al*., 2005).

Table 1.2: Biochemical tests for identification of *S.* Typhi (adapted from WHO, 2003)

Organism	Triple Sugar Iron Test				Motility, Indol, Urea Tests			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
E. coli	Alkaline	Acid	-	+	+	+	-	-
Klebsiella spp.	Acid	Acid	-	+	-	V	+	+
Citrobacter spp.	V	Acid	+	+	+	V	-	+
Proteus spp.	Alkaline	Acid	+	+	+		+	V

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

Table 1.3: Serological identification of *S.* Typhi (adapted from WHO, 2003)

Serotype	O antigen	H antigen	Serogroup Phase 1:2
S. Typhi	9, 12 (Vi)	d:	Group D1
S. paratyphi A	1, 2, 12	a: (1, 5)	Group A
S. paratyphi B	1, 4, (5), 12	b: 1, 2	Group B
S. paratyphi C	6, 7, (Vi)	c : 1, 5	Group C1

1.2 Typhoid Fever

Typhoid fever is a systemic infectious disease caused by the bacteria *S.* Typhi (Maskalyk, 2003; WHO, 2003). Typhoid transmission is mostly by means of the fecal-oral route, where consumption of contaminated water or food may lead to typhoid (Luxemburger & Dutta, 2005). Typhoid requires an incubation period of 7-14 days after ingestion of the bacteria. The length of the incubation period is dependent on a few factors, such as the quantity of inoculums, host factors, and medications. Typhoid requires a minimum inoculum size of 10^5 organisms. However, in immunocompromised patients, inoculum size of 10^2 organisms might be sufficient to cause clinical illness. Subjects on medications, such as antacids which reduces the gastric acid in the stomach also show higher susceptibility towards typhoid fever (WHO, 2003).

1.2.1 Epidemiology of Typhoid Fever

Worldwide, typhoid continues to be a burden to global health especially in under developed countries (Figure 1.1). Twenty-two million cases of typhoid fever with 216,510 deaths annually were reported in the year 2000 during an exercise to estimate the global morbidity and mortality of typhoid fever (Crump *et al.,* 2004). However, the number reported may be underestimated as the clinical picture of typhoid fever is often masked and confused with other infections. Moreover, modern diagnostic tests are not readily available in less developed countries such as Pakistan and Bangladesh where typhoid is endemic (WHO, 2003; Crump, *et al.,* 2004; Khosla, 2008). Most of the patients are mainly infants, children and adolescents ranging from newborns to 20-year old adults (Crump *et al.,* 2004). Older adults are presumably less susceptible due to frequent boosting of their immune system. Peak

of transmission often occurs after onset of the raining season or disasters such as earthquakes and tsunamis, where proper sanitation is unavailable (Sutiono *et al.,* 2010).

Malaysia is considered as one of the Southeast Asian countries endemic for typhoid fever (Figure 1.1). Two hundred and ten cases of typhoid fever was reported by the Ministry of Health in year the 2010 (Figure 1.2). Among all the states in Malaysia, Kelantan has the highest incidence rate in the year 2010 (Figure 1.3). Kelantan is a typhoid endemic state located in the North-East coast of Peninsular Malaysia. The incidence rate in Kelantan could rise up as high as 56.7 per 100,000 population as observed in the year 2005 (Figure 1.4). As a result of continuous intervention programs by the local health authority, the incidence rate decreased to 3.0 per 100,000 population in 2010 (Kementerian Kesihatan Malaysia, 2011).

1.2.2 Manifestation of Typhoid Fever

During the first week of infection, signs and symptoms of typhoid gradually develop. Patients will experience gradually increasing fever, loss of appetite, and fatigue. Some individuals may also develop frontal headache, dry cough and delirium which increases the malaise (WHO, 2003).

In the second week of infection, the bacteria will emboli on the dermis layer resulting in red rose spots on the body (Khosla, 2008). As the bacteria proliferates, the patient will develop high fever (38-40°C), which has a stepwise pattern (WHO, 2003; Ogoina, 2011).

Figure 1.1: Global distribution of typhoid fever in the year 2000 (Adapted from Crump *et al.*, 2004).

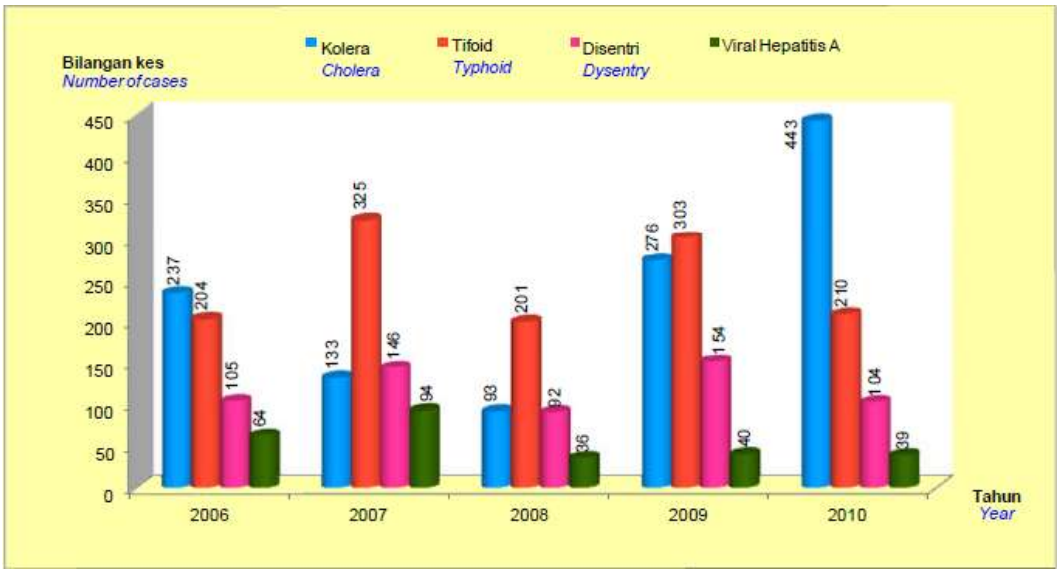

Figure 1.2: Number of Typhoid fever cases in Malaysia from year 2006-2010 (Kementerian Kesihatan Malaysia, 2011).

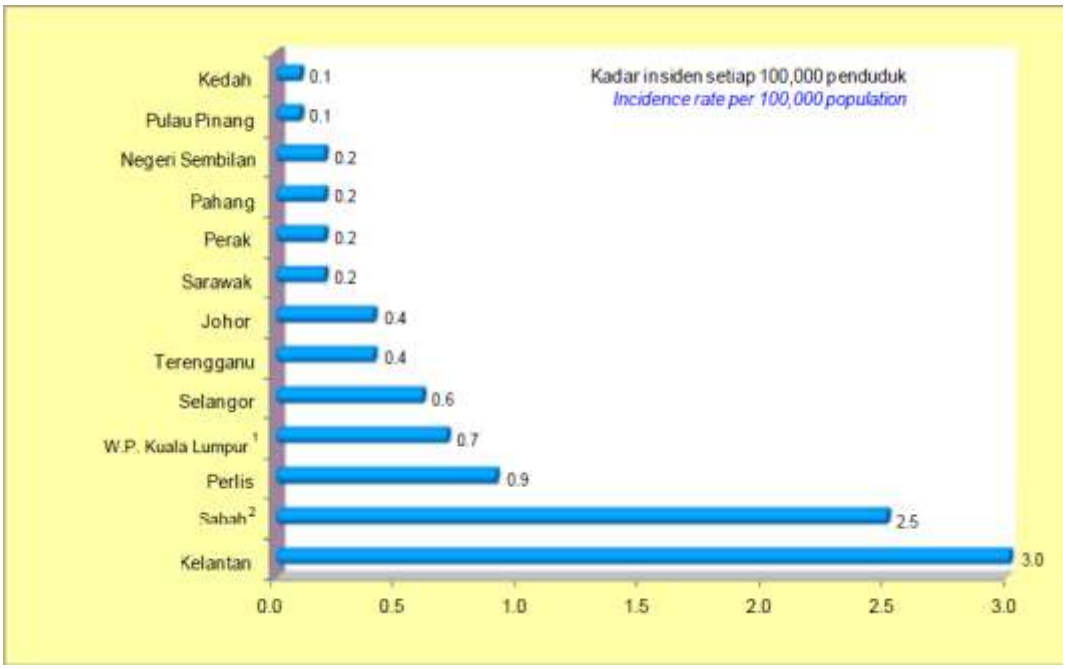

Figure 1.3: Incidence of typhoid fever in all states in Malaysia in the year 2010 (Kementerian Kesihatan Malaysia, 2011).

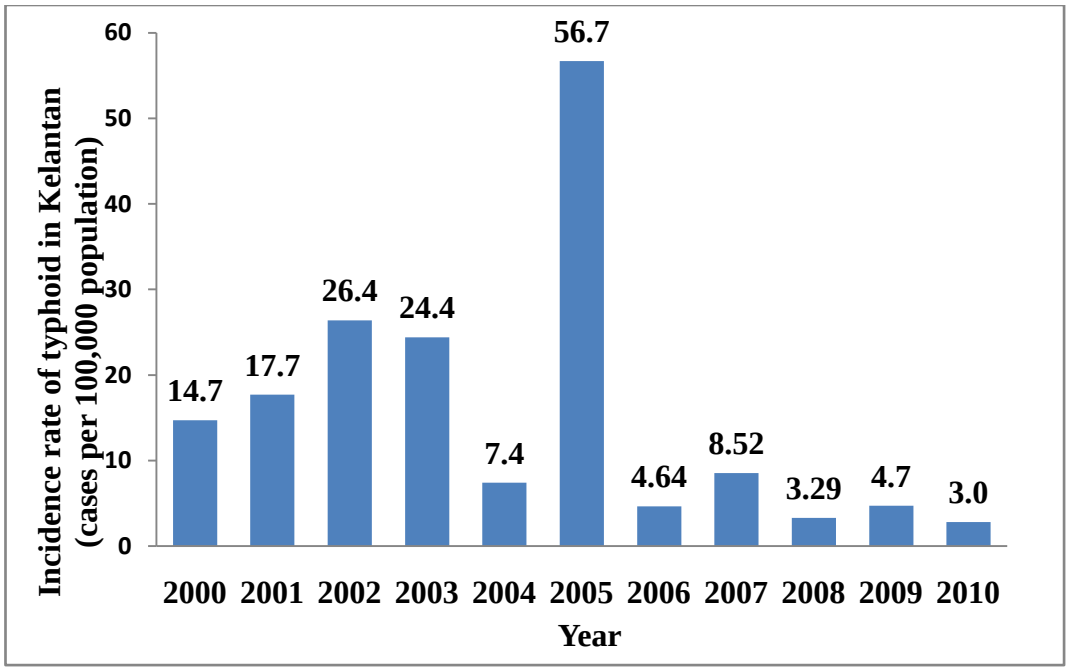

Figure 1.4: Incidence of typhoid fever in Kelantan from year 2000-2010 (Kementerian Kesihatan Malaysia, 2011).

In the third week, febrile typhoid patients will experience severe abdominal distension may occur to some patients causing foul green liquid diarrhea (WHO, 2003). At this stage, the patient is characterized by apathy, confusion and even psychosis. Necrosis of the Peyer's patches may cause intestinal perforation and peritonitis (Everest *et al.,* 2001). At this point, overwhelming toxaemia may cause intestinal hemorrhage resulting in death (Lott *et al.,* 1980; WHO, 2003).

The patient may slowly recover if he survives through the fourth week but will require a few months to regain weight. Although the patient is cured, some may become asymptomatic typhoid carriers, which are capable of transmitting the bacteria unconsciously to other individuals (WHO, 2003; Gonzalez-Escobedo, 2011).

1.2.3 Typhoid Carriers

Prescription of antibiotics is the most common and effective way of treating typhoid fever. However, only 95% of typhoid patients fully recover from the disease; the remaining 5% become asymptomatic carriers (WHO, 2003; Gonzalez-Escobedo, 2011). In general, a typhoid carrier is a person who harbours *S.* Typhi in his body without showing any clinical signs or symptoms. Carriers are mostly former typhoid patients who continue to excrete the bacteria in their stools or urine for longer than one year after the onset of acute typhoid fever (WHO, 2003). Carriers without a history of acute typhoid fever have also been reported in endemic areas (WHO, 2003). Patients with cholelithiasis are predisposed to develop the carrier state (Levine, *et al.,* 1982).

After ingestion of *S.* Typhi, the bacteria passes through the digestive track and reaches the small intestine. They rapidly penetrate into the mucosal epithelial cells and emerge in the lamina propria when they elicit an influx of macrophages. These macrophages ingest them but do not generally kill them (Monack *et al.,* 2004). They remain hidden within the macrophages and are circulated throughout the body by means of the lymphatic and blood circulatory systems (WHO, 2003). It is believed that during the acute stage of infection, the bacteria also invades many other organs, such as the liver, lymphatic system, bone marrow, nerve neuron system and gallbladder. Among these organs, the gallbladder is the preferred niche area for infection (Gonzalez-Escobedo *et al.,* 2011).

Typhoid carriers are capable of shredding *S.* Typhi into the environment and infecting the community. The situation is more critical when the carriers work as food-handlers, such as road-side hawkers or housewives (WHO, 2003). Due to the asymptomatic nature of these individuals, identification of typhoid carriers is difficult. Typhoid carriers are not aware of their condition until an outbreak occurs and the source of infection is traced back to them by the public health authority (WHO, 2003).

Typhoid carriers are usually treated immediately upon diagnosis with high dosages of antibiotics. According to WHO recommendations, ampicillin (100 mg per kg per day) plus probenecid (1 g orally or 23 mg per kg body weight for children) or trimethoprim-sulfamethoxazole (1600 to 800 mg twice daily) for 6 weeks, will be sufficient to treat most carrier cases (WHO, 2003). Cholecystectomy is usually

recommended for carriers with abnormal gallbladder problems, such as gallstones in order to cure the disease completely (Levine *et al.,* 1982; WHO, 2003).

1.3 Biofilm

Biofilms are generally defined as a community of bacteria adherent on a surface and forming a protective layer of matrix (Prakash, *et al.*, 2003). Biofilm formation is a protective mechanism of the bacteria in response to harsh conditions, such as environmental stresses, antibiotics, disinfectants and the host immune response (Flemming & Wingender, 2010).

1.3.1 Life Cycle of Biofilm Cells

Biofilm formation commence with a few free swimming planktonic cells attaching themselves onto a desired surface (Figure 1.5). The attached surface could be a biotic surface, such as animal cells, plant cells, or abiotic surface such as gallstone and polystyrene (Steenackers *et al*., 2012). The attachment is believed to be irreversible as most of the attached cells lose their motility capability such as flagella (Monroe, 2007). The attached cells will eventually form a matrix of extracellular polymeric substances (EPS). 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 level, planktonic cells or a portion of the biofilm may disperse into the environment seeking better habitats (Prakash *et al*., 2003).

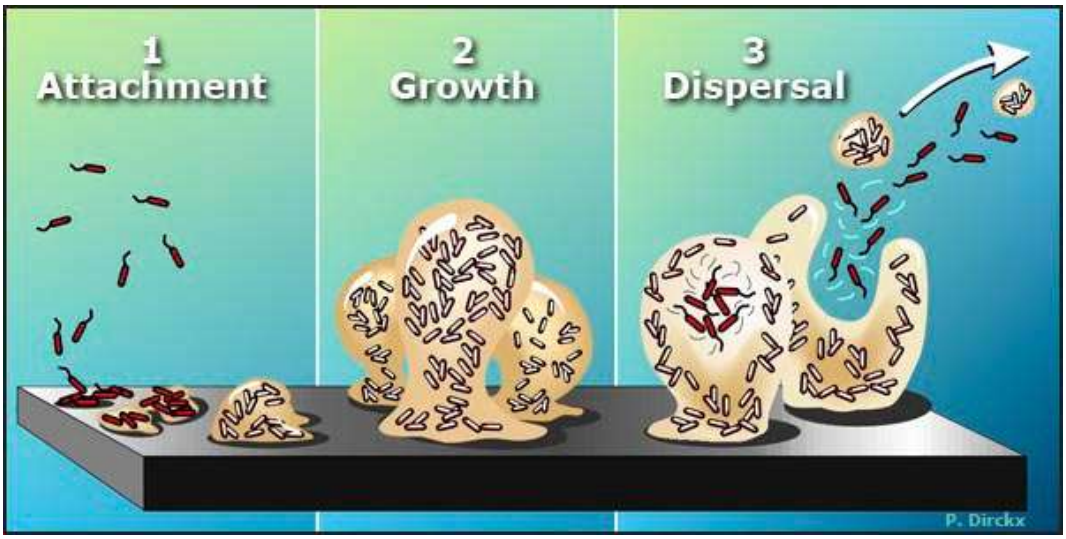

Figure 1.5: **Life cycle of biofilm cells. Stage 1, the planktonic cells attach onto a surface. Stage 2, the surface-attached cells form a layer of protective matrix which later becomes the biofilm community. Stage 3, the biofilm becomes mature and disperse into the environment (adopted from Sauer, 2003).**

1.3.2 Biofilm and Chronic Diseases

Biofilm plays an important role in persistence of chronic diseases. According to the National Institution of Health, more than 60% of persistent bacterial infections are caused by biofilms (Lewis, 2001). Manifestation of bacterial biofilm-associated diseases ranges from mild dental plague to chronic diseases such as cystic fibrosis (Parsek & Singh, 2003). Distinguishing biofilm infection from acute infection is a tedious task as biofilm infection may coexist with acute infection. Furthermore, there is no known biomarker for the biofilm state. However, biofilm infection could still be identified based on a few fundamental characteristics of the biofilm such as; a) the infecting bacteria are adherent onto a surface; b) examination of the infected tissues show signs of bacteria clustering, or microcolonies enclosed in a matrix; c) the infection is usually confined to a particular area; d) the infection requires higher dosage or is unable to be eliminated with antibiotics (Parsek & Singh, 2003). Cystic fibrosis is one of the chronic diseases caused by *Pseudomonas aeruginosa* biofilm (Moreau-Marquis *et al*., 2008). This infection can persist in the patient for years or even decades. Chronic cystic fibrosis infection causes inflammation in the patient's lungs. As the lung's function decline, the patient suffers from respiratory failure.

1.3.3 Biofilm Protein Studies

Biofilm-associated diseases are usually chronic and difficult to eradicate; hence it poses a threat to human society from the point of medical healthcare (Lewis, 2001). To eradicate the biofilm, one must understand the nature of the biofilm. Biofilm arises from planktonic cells attached onto a surface. By logic the genome of planktonic and biofilm cells should be the same, yet the morphology and the behaviours of planktonic cells and biofilm cells are totally different (Donlan, 2002).

A simple explanation for this phenomenon is that the bacteria alters its protein expression dramatically in the planktonic state in order to become biofilm cells (Sanchez *et al*., 2011). Studies on various bacteria biofilms showed that there are significant differences in protein expression of biofilm cells and planktonic cells. Apart from the up regulation and down regulation of common proteins, novel biofilm proteins have also been found in a comparative proteomic study of *Streptococcus suis*. In addition, some biofilm proteins were found to be immunogenic which have potential as diagnostic markers (Wang *et al*., 2012).

1.4 Protein Analysis and Identification

1.4.1 Two-Dimensional Polyacrylamide Gel-Electrophoresis (2D-PAGE)

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is a powerful technique used to separate proteins based on their isoelectric points and molecular masses (Person *et al.,* 2006; Posch, 2008). Single dimension SDS-PAGE method separates proteins based on their molecular weight. However, this method is not ideal when the samples contain more than one protein that have the same molecular weight. These proteins will appear as one band on the gel but consist of two or more proteins. In order to distinguish them, another stage of separation is needed (Liebler, 2001).

Proteins are a polymer chain containing various amino acids with different chemical properties. Combination of these amino acids give the protein polymer different physical and chemical properties and functions. Amphoteric molecules carry charges either positive or negative depending on the surrounding pH. When an electric field is applied to these molecules, they will migrate toward a certain pH zone where they

could become neutral; the point where a certain amphoteric molecule becomes neutral is defined as the isoelectric point (PI) (Liebler, 2001; Geiser *et al.*, 2011).

2D-PAGE separates proteins based on isoelectric focusing (IEF) in the first stage and molecular mass in the second stage. In the first stage, the protein sample is absorbed onto a pH gradient dried gel strip and the gel is hydrated. When an electric current is applied to the gel, acidic proteins at the alkaline side will be ionized and become negatively charged and migrate towards lower pH side (acidic) on the gel, whereas the alkaline protein at the acid side will become positively charged and migrate toward higher pH (alkaline) side on the gel. These proteins will eventually reach a point (isoelectric point) where they become neutral by losing their charge and stop moving. In the second stage, the proteins are separated by SDS-PAGE based on the molecular mass of the proteins (Garfin, *et al.*, 2003). Thus, as a result the 2D gel will have spots of proteins separated by means of isoelectric focusing and molecular mass.

1.4.2 Mass Spectrometry

Mass Spectrometry (MS) is a powerful analysis tool used for rapid and accurate measurement of molecular mass of a sample. It is widely used in various fields, such as protein analysis in biotechnology, drug discovery in pharmaceuticals, drug testing in clinical settings and pesticide quantitation in the environmental. MS is highly accurate and reproducible. It is able to determine the molecular mass of organic compounds up to an accuracy of 5 parts per million (ppm) (Ashcroft, 1997). Due to the high accuracy, identification of protein peptides can be done by confirming the peptide mass against a protein database.

In general, MS can be divided into three fundamental components, the ionization source, analyzer and the detector. The ionization source provides energy for the molecules to be vaporized into ionic form (Ashcroft, 1997). The analyzer separates these ions base on their mass to charge ratio (m/z) and the detector detects these ions as electronic signals at the end of the detector and analyze them based on their relative abundance quantity and molecular weight in the form of the m/z spectrum (Ashcroft, 1997).

Technology advances as time passes. Currently there are many varieties of mass spectrometry methods, the most widely used in biochemical studies is the Matrix Assisted Laser Desorption Ionization (MALDI). MALDI is compatible with thermo labile, non-volatile, and high molecular mass organic compounds. Proteins, glycoproteins, oligosaccharides are highly recommended (Lim, *et al.*, 2003). Bacteria protein analysis has also achieved a great deal of successes using MALDI (Murray, 2010). MALDI uses a laser bombardment-based ionization technique. The samples are pre-mixed with a matrix which have a high energy absorbent component such as Alpha-cyano-4-hydroxycinnamic acid (ACHA) (Hernandez-Guillamon *et al.*, 2010). The matrix absorbs and transfers the excitation energy to the samples which lead to sputtering of ions from the surface to the analyzing chamber. The excited ions are analyzed based on their Time of Flight (TOF) signals, as the ions travel through the analyzing chamber, molecules with lower m/z ratio (lighter molecules) will travel faster while molecules with higher m/z ratio (heavier molecules) will travel slower towards the detector (Ashcroft, 1997; Downard, 2004).

There are two modes available in mass spectrometry data analysis; 1) MS or Protein Mass Fingerprint (PMF), and 2) MS/MS (tandem MS). MS data is the mass of the total peptides, and MS/MS data is the mass of each peptide further fragmented by gas collision. For protein identification, the MS data is normally sufficient to provide identity of the protein. However with the MS/MS data, the results can be presented with more confidence (Webster & Oxley, 2012).

1.5 Rational of the Study

Today, sanitation and clean water supply have greatly decreased the number of cases and outbreaks of typhoid fever. However, the threat still remains. Persistence of typhoid carriers is the main reason why typhoid still exist today. To reduce the frequency of typhoid outbreaks, carriers must be identified and treated as soon as possible. Currently, typhoid carriers are identified using stool culture method (Wain *et al.,* 1998). Stool samples are collected from treated patients one year after the onset of acute typhoid fever and cultured for the presence of *S.* Typhi (WHO, 2003). However, this method has low sensitivity since only 1-5% of carriers are detected by stool or rectal swab culture methods (Wain, *et al.,* 1998; WHO, 2003). Also, stool sample collection and bacteria cell culture are tedious procedures. Serological methods which depend on detection of elevated IgA and/or IgG antibodies against *S.* Typhi antigens require paired sera which are not easily obtainable.

False negative diagnose of typhoid carriers might lead to outbreaks of the disease as these carrier subjects are usually unaware that they are still shredding the bacteria into the environment that might infect other people. The situation may become crucial if the carrier is a food-handler, such as a chef or roadside hawker, and cause

outbreaks of the disease. In order to detect typhoid carriers efficiently, a simple but sensitive test based on proteins specific to *S.* Typhi in the carrier state should be made available. Hence, this has become the thrust of this research.

In this study, we hypothesized that the typhoid carrier state is associated with biofilm formation by *S.* Typhi sequestered in the gallbladder of its victims. Biofilm formation protects *S.* Typhi from bile and other environmental stress in the gallbladder. Since biofilm is associated with bacterial persistence, it is proposed that biofilm cells represent the carrier state, whereas the planktonic (free swimming) cells represent the acute state of the disease. However, since *S.* Typhi is a human-specific pathogen, no animal model system is available to culture the organism and produce biofilm for laboratory analysis. Hence, an important aim of this study was to develop an *in vitro* method for production of *S.* Typhi biofilm cells by culturing the pathogen under conditions that mimic the gallbladder environment. Apart from that, by comparing the protein expression profiles of the planktonic and biofilm cells using 2D gel electrophoresis and gel image processing technology, biofilm proteins may be uncovered that may have potential to be used as laboratory diagnostic biomarkers for detection of typhoid carriers.

1.6 Objectives of the Study

The goal of this study was to identify potential proteins that are important for development of *S.* Typhi biofilm under *in vitro* gallbladder environment. The experimental overview of this study is shown in Figure 1.6. This study was divided into 4 phases, which include:

Phase I Development of an *in vitro S.* Typhi biofilm culture method by mimicking the gallbladder environment

- To determine the ingredients needed for of *S.* Typhi biofilm culture
- To optimize the factors affecting *S.* Typhi biofilm production

Phase II Elucidation of proteins expressed in *S.* Typhi biofilm

- To grow and extract both planktonic and biofilm proteins
- To analyze planktonic and biofilm proteins using 2D-PAGE
- To identifly novel biofilm proteins spot using MALDI-TOF

Phase III Investigation of the effect of bile on TolC protein expression

- To establish optimal concentration of bile needed for TolC production
- To analyze the TolC protein profile using 2D-PAGE

Phase IV Antibiotic resistance study of *S.* Typhi biofilm

- To grow biofilm in different concentrations of various antibiotics
- To elucidate the effect of various antibiotics on biofilm growth

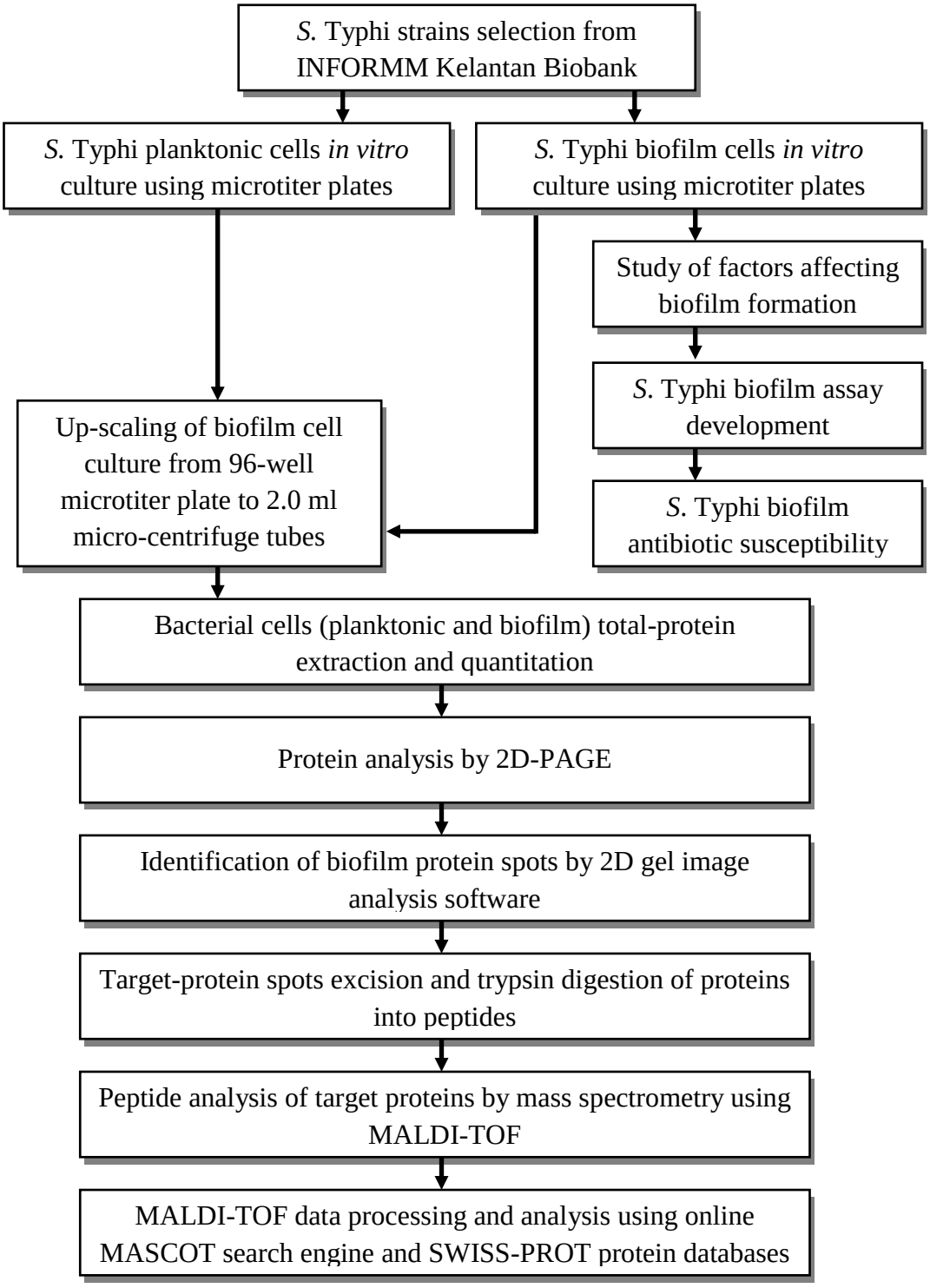

Figure 1.6: Flow chart of research experimental overview.

CHAPTER 2.0

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals, Media and Reagents

Chemicals, media and reagents used in this study are listed in Table 2.1.

2.1.2 Research Kits and Consumables

Research kits and consumables used in this study are listed in Table 2.2.

2.1.3 Software

Software used in this study is listed in Table 2.3.

2.1.4 Instruments

Instruments used in this study are listed in Table 2.4.

2.1.5 Bacteria Isolates

Bacteria isolates used in this study are listed in Table 2.5.

2.1.6 Enzymes and Antibiotics

Enzymes and antibiotics used in this study are listed in Table 2.6.

Table 2.1: List of chemicals, media and reagents

No.	Name	Source
1.	3-dimethylammonio-2-hydroxy-1-propanesulfonate (CHAPS)	Amresco, USA
2.	Acetic acid glacial	Merck, Germany
3.	Acetonitrile (ACN)	Sigma-Aldrich, USA
4.	Acrylamide / bis solution 37.5:1 30%	Bio-Rad, USA
5.	Alpha-cyano-4-hydroxycinnamic acid (ACHA)	Sigma-Aldrich, USA
6.	Amidosulfobetaine-14 (ASB-14)	CALBIOCHEM, UK
7.	Ammonium bicarbonate (ABC)	Sigma-Aldrich, USA
8.	Ammonium persulfate (APS)	Bio-Rad, USA
9.	Ampholytes 3-10	Bio-Rad, USA
10.	Bile purified	Fluka, USA
11.	Bromophenol blue	Bio-Rad, USA
12.	Complete EDTA-free protease inhibitor cocktail	Roche, Germany
13.	Coomassie blue R250	Bio-Rad, USA
14.	Crystal Violet dye	Riedel-de Haën, USA
15.	Dithiothreitol (DTT)	Bio-Rad, USA
16.	Ethanol	HmbG chemical, Germany
17.	Glucose (D+)	Sigma-Aldrich, USA
18.	Glycerol	Merck, Germany
19.	Iodoacetamide (IAA)	Bio-Rad, USA
20.	Kovac reagents	Remel, UK
21.	Methanol	Merck, Germany
22.	Methyl red solution	Remel, UK
23.	Methyl red Vogas-Proskauer (MRVP) medium	Oxoid Ltd, UK
24.	Mineral oil	Bio-Rad, USA
25.	Nutrient broth powder	Oxoid Ltd, UK
26.	Potassium chloride (KCl)	Merck, Germany
27.	Simmons citrate Agar	Oxoid Ltd, UK
28.	Sodium chloride (NaCl)	Merck, Germany
29.	Sodium dodecyl sulphate (SDS)	Bio-Rad, USA
30.	β-mercaptoethanol	Bio-Rad, USA
31.	Sulfobetaine (SB 4-10)	CALBIOCHEM, UK
32.	Sulphate Indole Motility (SIM) medium	Oxoid Ltd, UK
33.	Thiourea	Sigma-Aldrich, USA
34.	Trifluoroacetic acid (TFA)	Sigma-Aldrich, USA
35.	Triple Sugar Iron (TSI) agar	Oxoid Ltd, UK
36.	Tris (hydroxymethyl) aminomethane (TRIS)	Bio-Rad, USA
37.	Urea	Amresco, USA
38.	Urea agar base	Oxoid Ltd, UK
39.	Xylose Lysine Deoxycholate (XLD) agar	Oxoid Ltd, UK

Table 2.2: List of research kits and consumables

No.	Name	Source
1.	96-well not coated cell culture microtiter plates	Nunc, USA
2.	Antisera poly O, poly H, H-d, Vi and 09	Remel, USA
3.	Bovine serum albumin (BSA) solution	Bio-Rad, USA
4.	Immobilized pH gradient (IPG) strip	Bio-Rad, USA
5.	Micro-centrifuge tubes 2.0 ml, 1.5 ml, 0.6 ml, 0.2 ml	Axygen, USA
6.	Pipette tips 5.0 ml, 1.0 ml, 200 µl, 10 µl	Axygen, USA
7.	RCDC protein concentration assay	Bio-Rad, USA
8.	Zip Tip	Milipore, USA

Table 2.3: List of software

No.	Name	Purpose	Source
1.	Soft-Max Pro Series 5	Optical density analysis	Molecular device , USA
2.	PDQuest	2D-PAGE gel analysis	Bio-Rad, USA
3.	MASCOT Search	Peptides identification	Matrix Science, USA
4.	Unistat	Statistic analysis	Unistat Ltd, UK

Table 2.4: List of instruments

No.	Name	Source
1.	Autoclave machine	Hirayama, Japan
2.	Bath sonicator	Illinois, USA
3.	Centrifuge machine	Eppendorf, USA
4.	Chiller (4^{o}C)	Hitachi, Japan
5.	Deep freezer (-70^{o}C)	Thermo scientific, USA
6.	Freezer (-20^{o}C)	Sanyo, Japan
7.	IEF system	Bio-Rad, USA
8.	Incubator	Memmert, Germany
9.	Incubator shaker	Thermo scientific, USA
10.	Microscope	Olympus, Japan
11.	Mini Protean II	Bio-Rad, USA
12.	Orbital shaker	Finemould precission, Korea
13.	pH meter	Villerbanne Cedey, France
14.	Refrigerator centrifuge	Eppendorf, USA
15.	ELISA spectrophotometer	Molecular device, USA
16.	Ultrasonicator	Misonix Incoperated, USA
17.	Vortex	ERLA, USA
18.	Weighing Machine	A&D company limited, Japan
19.	Densitometer	Bio-Rad, USA
20.	Vacuum concentrator	Eppendorf, USA
21.	MALDI-TOF/TOF 5800	AB Sciex, USA