DEVELOPMENT OF APTAMER-BASED MAGNETIC SEPARATION WITH PCR FOR *Salmonella* spp. DETECTION AND EVALUATION OF NANOCARBON APTASENSOR

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DEVLOPMENT OF APTAMER-BASED MAGNETIC SEPARATION WITH PCR FOR *Salmonella* spp. DETECTION AND EVALUATON OF NANOCARBON APTASENSOR

ABSTRACT

Foodborne illness is a major concern worldwide due to its impacts towards health, economics and society. One of the major foodborne diseases is salmonellosis that is caused by the members of the species *Salmonella enterica.* Even though culture method is the gold standard for pathogen detection, this method is too time-consuming and laborious. Therefore, many alternative methods have been developed to increase sensitivity, specificity and speed of detection. Foodborne pathogens detection involves various aspects which are sample preparation, isolation and detection. The objectives of the study were to improve the food sample preparation by using DNA aptamer magnetic separation (AMS) and evaluate the sensitivity and specificity of an aptamer-based biosensor (aptasensor) for *Salmonella* detection. In the first part of this study, biotinylated aptamer was coupled with the streptavidin magnetic beads and then used to isolate whole *Salmonella* cells, followed by detection using PCR targeting the *invA* gene. The limit of the detection of the AMS-PCR was 100 CFU/ml which was 10 times more sensitive than using PCR (10^3 CFU/ml) alone. The DNA aptamer could differentiate ten different *Salmonella* serovars without any cross-reactivity with other non-*Salmonella* spp. This AMS was also evaluated in naturally contaminated food samples (n = 14). The results showed that the use of AMS could reduce the detection time of *Salmonella* to 6 to 7 hours as compared to the conventional methods (pre-enrichment, enrichment, selective plating steps) which took 2 to 3 days. In the second part of this study, the sensitivity and specificity of the amino-modified aptasensor were determined. The aptasensor was previously developed by using carbon nanotube (CNT)

deposited ITO substrate. The sensor conductivity and behaviour were determined by using cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) using the AUTOLAB electrochemical instrument. When the aptasensor was exposed to *Salmonella* cells, the resistance increased, indicating the binding between *Salmonella* and the aptamer. The linear relationship between the peak of the current and different scan rates indicated the stability of the aptasensor. For sensitivity test, the aptasensor was exposed to different concentrations (5.5×10^1 to 10^6 CFU/ml) of *Salmonella* and showed its limit of detection at 55 CFU/ml. When the aptasensor was exposed to non-*Salmonella* cells (*Staphylococcus aureus*, *Vibrio parahaemolyticus* and *E. coli*), it did not show any cross-reactivity. Compared with culture method, this aptasensor could rapidly detect the *Salmonella* cells within one hour. In conclusion, the use of AMS with PCR could concentrate the bacterial cells in the initial food preparation and helped to reduce the total detection time of *Salmonella* in food samples. The aptasensor was shown to be rapid, specific and sensitive and could be further developed for *Salmonella* detection.

Keywords: foodborne pathogens detection; aptamer; *Salmonella*; PCR; aptasensor.

PEMBANGUNAN PEMISAHAN MAGNET BERASASKAN APTAMER DENGAN PCR UNTUK PENGESANAN *Salmonella* spp. DAN PENILAIAN NANOKARBON APTASENSOR

ABSTRAK

Penyakit bawaan makanan menjadi kebimbangan di serata dunia di sebabkan kesannya terhadap kesihatan, ekonomi dan masyarakat. Salmonellosis yang berpunca daripada salah satu spesis *Salmonella enterica* antara penyebab berlakunya penyakit bawaan makanan ini. Meskipun kaedah kultur merupakan kaedah utama pengesanan pathogen, kaedah ini mengambil masa yang terlalu lama dan sukar dikendalikan. Oleh itu, terdapat banyak kaedah pengesanan alternatif yang dicipta bagi meningkatkan tahap sensitif, spesifik and kepantasan pengesanan. Pengesanan pathogen bawaan makanan merangkumi beberapa aspek iaitu penyediaan sampel, pengasingan dan pengesanan. Objektif kajian ini adalah meningkatkan keupayaan tahap penyediaan sampel makanan menggunakan pemisahan magnet berasaskan aptamer DNA (DNA aptamer magnetic separation, AMS) dan menilai tahap sensitif dan spesifik biosensor aptamer (aptasensor) bagi pengesanan *Salmonella*. Pada bahagian pertama kajian ini, 'biotinylated-aptamer' digabungkan bersama ketulan magnet streptavidin dan kemudiannya digunakan untuk mengasingkan seluruh sel *Salmonella*, diikuti oleh pengesanan menggunakan PCR melalui gen *invA*. Had pengesanan AMS- PCR ialah 100 CFU/ml iaitu 10 kali lebih sensitif daripada menggunakan PCR sahaja (10^3 CFU/ ml). DNA aptamer ini mampu membezakan sepuluh jenis serovar *Salmonella* yang berlainan tanpa reaktiviti silang antara spesis bukan *Salmonella*. AMS ini turut diuji dengan sampel makanan yang tercemar secara semula jadi (n = 14). Keputusan kajian menunjukkan AMS mampu mengurangkan masa pengesanan *Salmonella* kepada 6 ke 7 jam berbanding dengan kaedah konvensional '(pre-enrichment, enrichment, selective plating steps)' yang mengambil masa 2 ke 3 hari. Pada bahagian kedua kajian ini, tahap sensitif dan spesifik

‘amino-modified aptasensor’ telah ditentukan. Aptasensor ini telah dibangunkan sebelumnya menggunakan nanotube karbon (CNT) berdeposit substrat ITO. Tahap konduktiviti dan dan kelakuannya telah ditentukan berdasarkan voltammetry kitaran (CV) dan spektroskopi impedens elektrokimia (EIS) menggunakan alat elektrokimikal AUTOLAB. Apabila aptasensor didedahkan kepada sel *Salmonella*, tahap rintangan telah menaik, menunjukkan pengikatan antara *Salmonella* dan aptamer. Hubungan linier antara puncak arus dan kadar imbasan yang berbeza menunjukkan kestabilan aptasensor ini. Bagi ujian tahap sensitif, aptasensor telah didedahkan kepada tahap kepekatan *Salmonella* yang berbeza (5.5×10^1 sehingga 10^6 CFU/ml) dan menunjukkan had pengesanan pada 55 CFU/ml. Apabila aptasensor didedahkan kepada sel bukan *Salmonella* (*Staphylococcus aureus*, *Vibrio parahaemolyticus* dan *E. coli*), tiada reaktiviti silang ditemui. Berbanding dengan kaedah kultur, aptasensor mampu mengesan *Salmonella* dengan pantas dalam masa satu jam. Kesimpulannya, penggunaan AMS-PCR mampu menumpukan sel bakteria pada permulaan penyediaan makanan dan membantu untuk mengurangkan jumlah masa pengesanan bagi *Salmonella* di dalam sample makanan. Aptasensor pula telah menunjukkan kepantasan, spesifik dan sensitif serta boleh terus dibangunkan untuk pengesanan *Salmonella*.

Kata kunci: pengesanan patogen bawaan makanan; aptamer; *Salmonella*; PCR; aptasensor.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

AMS	:	Aptamer magnetic separation
AP	:	Alkaline phosphate
BLAST	:	Basic Local Alignment Search Tool
BPW	:	Buffer peptone water
BS	:	Bismuth sulfite agar
C	:	Capacitance
CDC	:	Centers for Disease Control and Prevention
CFU	:	Colony forming unit
CNT	:	Carbon nanotube
CV	:	Cyclic voltammetry
EIS	:	Electrochemical impedance spectroscopy
ELISA	:	Enzyme Linked Immunosorbent Assay
FDA	:	Food and Drug Administration
FSIS	:	Food Safety & Inspection Service
HE	:	Hekteon enteric
HRP	:	Horseradish peroxidase
IMS	:	Immuno-magnetic separation
ISO	:	Organization for standardization
ITO	:	Indium tin oxide
LAMP	:	Loop-mediated Isothermal Amplification
LB	:	Luria-bertani
LIA	:	Lysine iron agar
LOD	:	Limit of detection
mPCR	:	Multiplex polymerase chain reaction

MWCNT	:	Multi-walled carbon nanotube
NCBI	:	National Centre for Biotechnology Information
OMP	:	Outer Membrane Protein
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
Pt	:	Platinum
R	:	Resistance
R_{CT}	:	Charge transfer resistance
R_S	:	Solution resistance
RV	:	Rappaport- Vassiliadis
RVS	:	Rappaport- Vassiliadis soya
SAM	:	Self-assemble monolayers
SC	:	Selenite cysteine
SCE	:	Saturated calomel electrode
SELEX	:	Systemic Evolution Ligand by Exponential Enrichment
SEM	:	Scanning electron microscopy
ssDNA	:	Single-stranded DNA
TBE	:	Tris-borated EDTA
™	:	Trademark
TSI	:	Triple sugar iron agar
TT	:	Tetrathionate broth
W	:	Warburg
WHO	:	World Health Organization
XLD	:	Xylose lysine deoxycholate agar

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 General introduction

Foodborne illness has remained as a public health concern due to its impacts towards health, economy and society. The cause of this illness is associated with the bacteria, fungi, viruses and chemical. According to World Health Organization (WHO), in the year 2010 the occurrence of foodborne illness has caused 600 million people ill and 420,000 deaths around the globe (WHO, 2015). Other than that, 550 million people have suffered diarrheal disease, the most common disease for foodborne illness which causes 230,000 deaths every year. This outbreak is mainly due to the foodborne pathogens that results in the toxin secretion of the microorganism to the intestinal tract of the infected person. *Salmonella enterica* is one of the main foodborne pathogens that typically present in the dairy products, chicken, beef, eggs, fresh vegetables and fruits. Inefficiency in the detection and treatment can cause epidemic in the general population thus impedes the socioeconomic development and increases economic burden due to the workforce inefficiency. Therefore, rapid and accurate detection of the foodborne pathogens in foods is crucial as the first step of prevention in food safety.

There are two main parts of food detection, which are upstream food sample preparation and downstream foodborne pathogens detection (Brehm-stecher et al., 2009). The purpose of upstream food sample preparation is to recover maximum number of targeted bacteria, eliminate non-target microbiota, purify against extraneous components and exclude the inhibitory substances in food samples environment; thus, increasing the efficacy of detection.

On the other hand, downstream foodborne pathogen detection is used to determine the presence of the targeted bacteria in food samples. In foodborne pathogens detection, cultural method has remained as a gold standard for detection even though area such as

molecular and biosensor detection are continually advancing in diagnostics. Nevertheless, this conventional method is too time-consuming, laborious and lacks standardisation. The advancement of the new detection tools technology has been limited by the relatively high detection limits, necessitating the use of time-consuming enrichment prior to detection. At the same time, the upstream sample preparation is often neglected which may contribute to the interference of the downstream detection method. Therefore, it is necessary to develop highly sensitive and specific upstream sample preparation to separate, purify and concentrate the targeted bacteria from a complex food matrix along with the rapid and accurate detection tools. Even though immuno-magnetic separation (IMS) has been developed to reduce this problem (Jenïkovâ et al., 2000), the use of antibody as recognition element is relatively not stable, difficult to produce as it needs an animal host and has batch to batch variations.

Thus, in recent years, aptamers have become a great research tool. Aptamers are single-stranded oligonucleotides that can specifically bind to target molecules with high affinity by forming specific three-dimensional structures (Jayasena, 1999). It has unique folding patterns depending on its sequences. Aptamers have been widely used as biological recognition element because of its thermal stability, low cost, can be produced in vitro, less batch-to-batch variation process and can be used in various applications (Khati, 2010). Therefore, an aptamer has been used as biological recognition elements in biosensor (aptasensor) application as an alternative to the single-stranded DNA and antibody.

This thesis is divided into two main sections. First, development aptamer magnetic separation (AMS) for upstream sample preparation for food sample followed by PCR detection. Second, evaluation of aptasensor as one of the detection tools by using the similar aptamer sequence in AMS.

1.2 Research questions

This study was conducted to answer these questions;

1. Is aptamer magnetic separation (AMS) a good alternative to increase the efficiency of upstream sample preparation to ensure maximum recovery of *Salmonella* before downstream detection method is conducted especially in food sample?

2. Can aptamer-based nanocarbon biosensor (aptasensor) be an alternative platform for high specificity detection of low amount of *Salmonella* within a short period of time?

1.3 Objectives

The overall goal was to develop and evaluate AMS-PCR and aptasensor for *Salmonella* detection by using DNA aptamer as a biological recognition element in both platforms.

1. To develop and evaluate an Aptamer Magnetic Separation (AMS) with Polymerase Chain Reaction (PCR) for the *Salmonella* detection in food samples. Specifically, the objective was to determine the sensitivity, specificity and detection time of AMS in bacteria cell suspension followed by its application for *Salmonella* detection in various food samples.
2. To evaluate the sensitivity, specificity and detection time of an aptasensor for *Salmonella* spp. detection.

CHAPTER 2: LITERATURE REVIEW

2.1 Foodborne illnesses

Food safety has become a huge concern worldwide due to its impacts towards public health, economy, and food industry. Each year, the Centers for Disease Control and Prevention, USA (CDC) estimates about 48 million people in the United States acquired foodborne illness and in 2013, 818 foodborne outbreaks had been reported resulting 16 deaths (CDC, 2014). This imposes over USD15.5 billion in economic burdens relative to these illnesses per annum in the USA (Hoffmann et al., 2015). According to the World Health Organization (WHO), in South East Asia, foodborne illness has become the second highest burden per population with 150 million cases and 175 000 deaths (WHO, 2016). In Malaysia, the reported foodborne illness in 2015 was low, about 48 cases in 100,000 populations (Ministry of Health, 2016). However, this number is highly underestimated as many food poisoning cases are unreported. Most foodborne illnesses are caused by norovirus, non-typhoidal *Salmonella* spp., *Clostridium perfringens* and *Campylobacter* spp. and others (Scallan et al., 2011). Among these pathogens, non-typhoidal *Salmonella enterica* is the leading cause of hospitalisations and deaths (Scallan et al., 2015).

Consumption of food contaminated with *Salmonella* can result in risk of the salmonellosis. Many factors can contribute to the food contamination especially during food preparation and handling for example poor hygiene practices, biological cross-contamination, lack of water system and inadequate preservation and storage for the fresh products (Ab-Karim et al., 2017; Nidaullah et al., 2017; Rusul et al., 1996). *Salmonella* can also develop into biofilm on a food contact surface for instance plastic cutting board (Chmielewski & Frank, 2003). Attachment of this biofilm would lead to the cross-contamination as it is protected from sanitizer thus increase the possibility of *Salmonella* to enter the food chain at any point from livestock, food processing,

retailing, catering as well as food preparation at home. Study conducted in poultry processing environment in wet markets in Penang and Perlis, Malaysia had shown consistent contamination of *Salmonella* throughout the processing line with 100% prevalence in the whole chicken carcass and chicken cuts including plants and equipment use for product processing as well as water source (Nidaullah et al, 2017). In 2014, 169 cases related to the salmonellosis outbreak reported in Terengganu, Malaysia had shown poor food handling, unhygienic food handlers, poor sanitation and substandard kitchen infrastructure were the main factors of the outbreak (Ab-Karim et al., 2017).

2.2 *Salmonella enterica*

Salmonella is a Gram-negative bacterium which is a member of the Enterobacteriaceae family. According to the Kauffman-White classification and latest subtyping method, 2,500 different serotypes belong to *Salmonella* genus (D'Aoust & Maurer, 2007; Pui et al., 2011). In United States, about 1 million people suffer salmonellosis every year with 23,000 hospitalisations and 450 annual deaths (Scallan et al., 2011) due to *Salmonella* infection. This situation gives impacts towards hospitalisation cost, job loss during recovery and multiple economic burdens as a whole.

Salmonella has two main species which are *Salmonella enterica* and *Salmonella bongori*. The total genus of *Salmonella* is 2,579 in which more than 99.5% of the isolated *Salmonella* are subspecies *enterica* serovars. *Salmonella enterica* consist of six subspecies namely *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae* and *S. enterica* subsp. *indica* (Grimont & Weill, 2007). To avoid possible confusion, the nomenclature of the genus *Salmonella* was standardized. The subspecies name (subsp. *enterica*) does not need to be indicated as only *Salmonella* from this species bear a

name. Serovar (or serotype) names for example *Salmonella enterica* serovar Typhimurium should not be italicized with the first letter is capitalized. After the first use, serovar may be used without a species name, e.g. *S.* Typhimurium (Grimont & Weill, 2007).

Salmonella is typically zoonotic in origin and widely distributed in nature. It is originated from gastrointestinal tract of animals and capable to growth within the wide temperature from 6^0C to 46^0C (Odumeru & León-Velarde, 2012). The infection caused by *Salmonella enterica* is usually from ingestion of animal products contaminated with *Salmonella* species for example, chickens, eggs, beef, turkey, pork and dairy products. Other than that, it can be transmitted via non-animal products like fresh vegetables, fruits and water as well as direct person-to-person transmission (Hara-Kudo & Takatori, 2011; Odumeru & León-Velarde, 2012; WHO, 2016).

Pathogenicity of the *Salmonella* can be devided into two main stages including invasion and proliferation of the *Salmonella* in the cell (Figure 2.1). There are two main strategies of *Salmonella* invasion which are known as Trigger and Zipper mechanisms (Boumart et al., 2014). Both of these mechanisms invade nonphygocytic cells by modulationg the actin cytoskeleton.

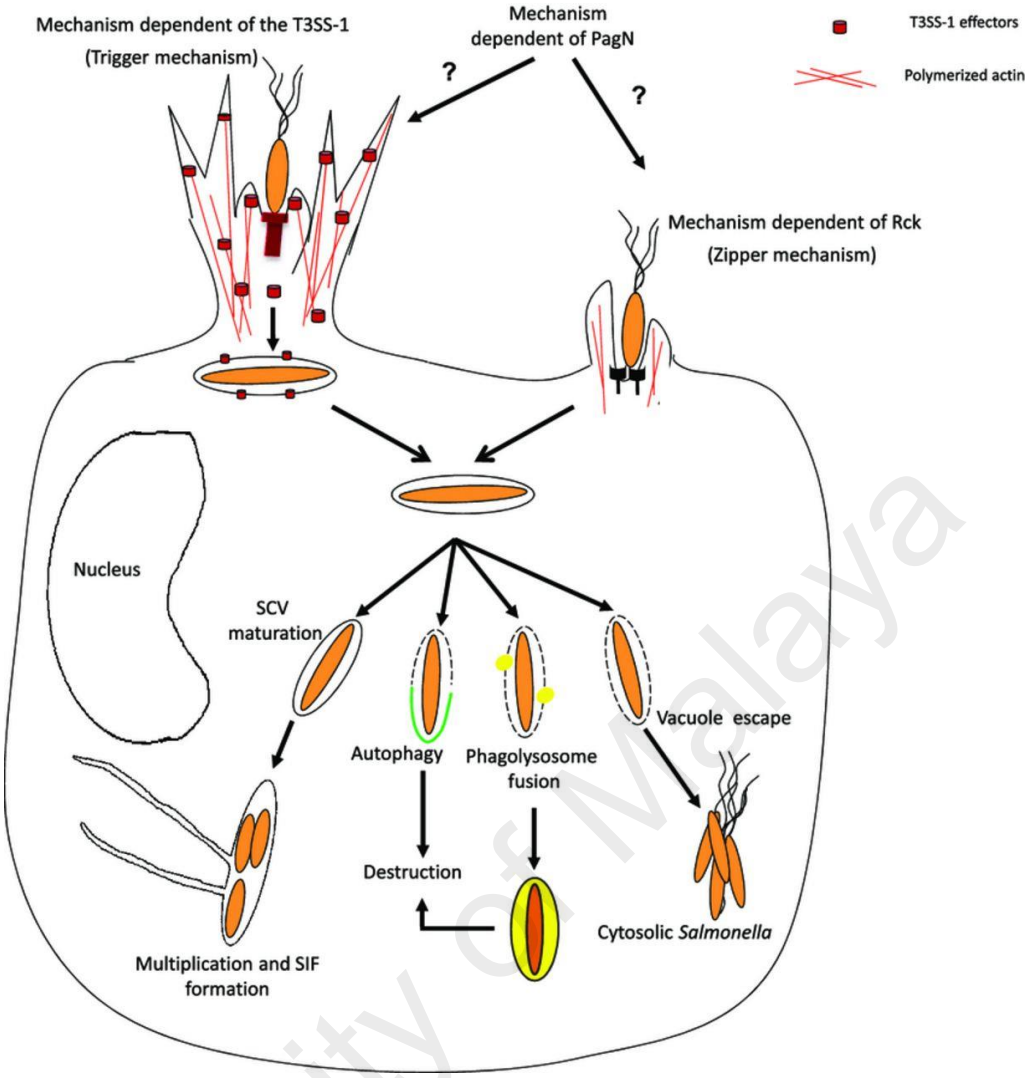


Figure 2.1: The invasion and proliferation strategies of the *Salmonella* in eukaryotic cell**.** SCV, *Salmonella*- containing vacuole; SIF, *Salmonella*-induced filament; T3SS-1, type 3 secretion system 1 (Boumart et al., 2014).

Trigger mechanism involves Type 3 Secretion System-1 (T3SS-1) receptor and bacteria effector proteins for bacteria attachement to enter the host cells (Schroeder & Hilbi, 2008). The interaction of the bacteria surface to the host receptor leads to the activation of host signalling pathway and promotes the actin polymerization and bacteria upatake (Cossart, 2004). Other that that, lastest dicovery of Zipper mechanism involved Rck invasion protein that encoded for *rck* gene located on the large virulence plasmid (Rosselin et al., 2010). Interaction of the *Salmonella* to the host cell receptor induces the signalling cascade and local accumulation of the actin thus promotes minor cytoskeleton action rearrangements and tight membrane extension (Cossart, 2004;

Rosselin et al., 2010). Another invasion strategy namely PagN dependent mechanism had been discovered but its precise mode of action is not well studied (Lambert & Smith, 2008; Boumart et al., 2014).

In term of intracellular proliferation, *Salmonella* internalizes within the cell membrane- compartment as vacoular pathogen (Bakowski et al., 2008). *Salmonella* resides and replicates in the *Salmonella* containing vacoule (SCV) that undergoes different maturation stages. As the SCV matures and surrounded by the actin, it will form *Salmonella* induced filaments (SIFs) that project from SCV and extend throughout the cell. SIFs facilitate the delivery of nutrients in the SCV thus help in bacterial replication (Knolder & Steele-Mortimer, 2003; Salcedo & Holden, 2003). Other than that, portion of the *Salmonella* would escape from the SCV and multiply efficiently in the cytosol of epithelial cells (Malik-Kale et al., 2012). Destruction of the *Salmonella* could be due to the autophagy or repsonse of SCV- lysosome fusion (Viboud & Bliska, 2001; Boumart et al., 2014).

Symptoms of salmonellosis include watery diarrhea, abdominal pain, nausea, fever, headache and occasional constipation (Odumeru & León-Velarde, 2012). In a normal individual, salmonellosis can result in self-limiting diarrhea and will typically recover in a week. Nevertheless, in immunocompromised person for instance HIV and sickle cell anemia patient or infant, it can give a severe extra-intestinal salmonellosis that can spread and cause bacteremia and possibly lead to life-threatening septicemia (D'Aoust & Maurer, 2007; Ricke et al., 2015).

Therefore, development of sensitive and specific diagnostic tools for pathogens detection is important to prevent the outbreak of foodborne diseases and strategise remedial actions. Besides being sensitive and specific, these food forensic tools need to be rapid, easy to use, low cost and portable. In the detection of *Salmonella* particularly,

many diagnostic tools have been developed which can be categorised into two common groups; conventional and molecular diagnostics.

2.3 Diagnostic tools

2.3.1 Conventional methods

For conventional methods, there are several approaches for *Salmonella* recovery from food provided by several agencies for example International Organization for Standardization (ISO), Food and Drug Administration (FDA) and Food Safety and Inspection Services (FSIS) of USDA. Generally, isolation of *Salmonella* from food sample requires pre-enrichment, selective enrichment and selective differential plating followed by the tedious and time consuming biochemical and serological tests (Figure 2.1). Pre-enrichment is used to revive the sub-lethally injured bacteria from the sample. This step is important to ensure any bacteria that are likely to revive will be captured during the detection procedure. Usually, pre-enrichment media are Buffer Peptone Water (BPW) and lactose broth. After that, the incubated pre-enrichment media are inoculated into selective media to suppress the propagation of other bacteria and other inhibitory factors. This step is called enrichment. In *Salmonella* detection, tetrathionate (TT), Rappaport-Vassiliadis (RV) and selenite cystine (SC) medium are used. Although conventional isolation method is considered as the gold standard, this method is time-consuming, lack of inter-laboratory consistency, labour-intensive, tedious and impractical for real-time applications especially in response to outbreak and bioterrorism events (Logue & Nolan, 2012).

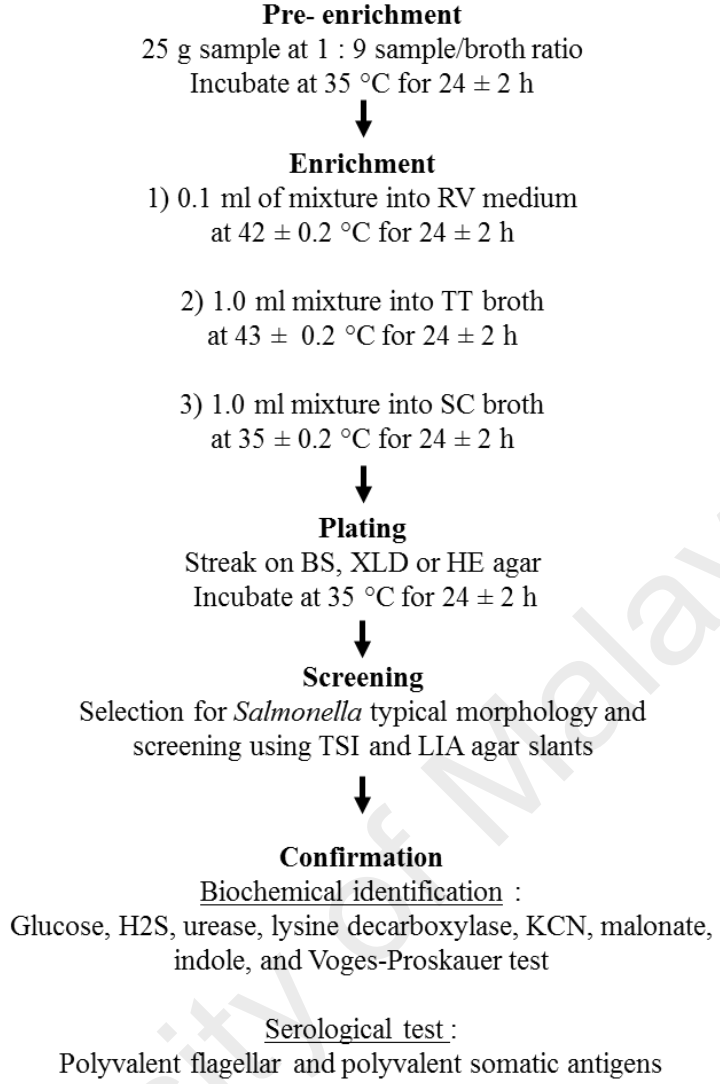


Figure 2.2: A summary for *Salmonella* detection by conventional cultural method according to Food and Drug Administration (FDA) (2007). RV, Rapport-Vassiliadis medium; TT, tetrathionate broth; SC, Selenite cystine broth; BS, bismuth sulfite agar; XLD, xylose lysine deoxycholate agar; HE, Hektoen enteric agar; TSI, Triple sugar iron agar; LIA, Lysine iron agar.

Therefore, advancement in the new selective media has improved the isolation of the target bacteria in the agar for example chromogenic media. Chromogenic media has been formulated with selective antimicrobial reagents and special substrate to grow specific bacteria. However, the results can only be produced within 24 to 48 hours. Other than that, as bacteria species can only be distinguished based on the colour

intensity of the colony, the optical observation may be subjective and limited (Hernandez et al., 2016).

In order to improve the sensitivity, specificity, detection time and at the same time reduce the cost and labor, many researches have been conducted to improve the food diagnostic area. These diagnostic tools are known as rapid diagnostic tools that take lesser time than the conventional culture method. Besides, these diagnostic tools need to be highly sensitive to ensure a very low number of targeted bacteria are detectable. They also need to have high degree of specificity to eliminate false positive results or cross-reactivity that lead to unnecessary additional time which do not represent the concern of the public health.

2.3.2 Polymerase Chain Reaction (PCR)

PCR is the most commonly used molecular method. It is able to detect the low concentration of the targeted DNA in a defined sample; therefore, it shortens the time for enrichment to reach minimum amount of bacteria concentration (Lee et al., 2015). Consecutively, the PCR involves specific primers that can amplify specific region of DNA to identify the presence or absence of the targeted bacteria. However, this method needs post-protocol step such as gel electrophoresis and probe hybridisation before the results analysis, takes 4 to 5 hours to get the result and needs sophisticated instrument to maintain different temperature in different duration. Other than that, multiplex PCR has been developed to target a few foodborne pathogens simultaneously.

Multiplex PCR (mPCR) involves more than a pair of primers in one reaction tube to target multiple number of targets. It can amplify a number of DNA of bacteria present at one time; thus, reducing the time taken for the detection. Designing primes is the key for the mPCR. The process of primers designing might be challenging because not only the primers need to be specific, they must not interact with each other too.

Furthermore, the annealing temperature needs to be adjusted until it achieves similar optimum temperature and produces distinguishable bands to differentiate different targets. Recently, mPCR has been developed to detect the presence of the diarrheagenic bacteria for example *Salmonella* spp., *Vibrio* spp., *E. coli*, *Campylobacter* spp. and *Aeromonas* (Sjöling et al., 2015). In addition, mPCR for detection of five foodborne pathogens was developed and tested in the pork samples and it was reported that this method was reliable, useful and cost effective (Chen et al., 2012) even though time-consuming, pre-enrichment and enrichment steps were still needed.

2.3.3 Real-time PCR

In recent years, real-time PCR or quantitative PCR has been widely used in food safety including detection for foodborne pathogens. It can amplify and quantify number of the targeted DNA presented. The labelled probes or intercalating dyes are used to detect the products based on the intensity of fluorescence. The examples of fluorescent dyes are SYBR green, TaqMan probes and molecular beacons. Each of them has different applications; for example, SYBR green can intercalate non-specific double stranded DNA of the amplicon, whereas TaqMan and molecular beacon are sequence specific DNA probes that are complementary to the targeted DNA and can only permit detection when it is hybridised to the targeted DNA (Dwivedi & Jaykus, 2011).

In addition, multiplex real-time PCRs have also been developed to detect and quantify multiple targets at the same time. For example, multiplex real-time PCR was developed to detect *Salmonella* spp., *Vibrio parahaemolyticus* and *Listeria monocytogens* simultaneously in shrimps (Zhang et al., 2015). After conventional pre-enrichment and DNA extraction, the targeted pathogens were detected within 50 minutes with sensitivity of about 10^2 CFU/ml. In other study, Syto-9 and probes were compared in real-time PCR application to detect four different bacteria namely

Salmonella spp., *Yersinia enterocolitica*, *Listeria monocytogenes* and *Campylobacter* spp. (Skerniskyte et al., 2016). That study reported that specific amplification of the multiplex PCR was more superior using probes compared to Syto-6 dye. Both Syto-6-based reaction and probes-based reaction showed comparable sensitivity with no cross-reactivity with other non-targeted pathogens. Therefore, real-time PCR has become an attractive tool due to its high sensitivity and rapidity. Unlike PCR, it does not need any post-PCR processing such as gel electrophoresis. Using real-time PCR, the amplicons can be quantified; thus, making it suitable for high throughput analysis.

2.3.4 Loop-mediated Isothermal Amplification (LAMP)

In 2000, Notomi et al. developed a novel DNA amplification called Loop-mediated Isothermal Amplification (LAMP). It has high specificity, sensitivity and rapidity which can amplify DNA in isothermal condition. This method involves *Bst* DNA polymerase and a set of four to six primers to recognise a single target gene. The targeted gene can be amplified within one hour with a single temperature, usually 65°C. The result can be observed either by using turbidity meter that measures the turbidity of the magnesium pyrophosphates which is the by-product of the DNA synthesis or by utilising the optical detection using fluorescent chelation reagent.

LAMP possesses a few advantages that include a single chamber for amplification as it involves just one optimum and specific temperature and does not need specific equipment. This single chamber equipment includes water bath or heat block which is commonly and easily available in the laboratory. LAMP also offers a simple detection with high sensitivity and specificity. LAMP kit is commercially available (Mangal et al., 2016) and further development for foodborne pathogens detection includes for example detection of *Salmonella* (Kokkinos et al., 2014; Wang et

al., 2008), *Shigella* (Liew et al., 2014), E. *coli* (Stratakos et al., 2017; Wang et al., 2014) and *Listeria monocytogenes* (Cho et al., 2014; Liu et al., 2015).

2.3.5 Enzyme Linked Immunosorbent Assay (ELISA)

The basic principle of the ELISA is a specific antibody which binds to an antigen for detection. In direct ELISA, the antigen-antibody conjugate is detected directly by the substance attached on the primary antibody; whereas for indirect assay, the primary antibody is captured by the secondary antibody with substance attached on it (Law et al., 2014). In sandwich assay, usually the plate is coated with the antibody which causes the analyte to be captured by capture antibody before being detected by primary antibody, explaining the term 'sandwich'. Then, the secondary antibody conjugated with the substrate is added and the result is observed after substrate interaction. A wide range of the substance is used depending on the application and equipment available to measure the signal. Examples of the substances are peptides, antibodies, hormone, protein and enzyme. Commonly, horseradish peroxidase (HRP), beta-galactosidase and alkaline phosphatase (AP) are used (Yeni et al., 2014). These enzyme substrates are conjugated to the antibody and emit the signal to represent the intensity of the bound antigen/analyte. The result is viewed either by spectrophotometer or visually based on colour changes. Compared to conventional method, ELISA is a faster method and has been widely used to detect pathogenic bacteria and bacteria toxin in food. However, the limit of detection of traditional ELISA is 10^4 CFU/ml (Shan et al., 2016). To increase the sensitivity of the system, double antibody sandwich (ds-ELISA) and indirectly competitive ELISA (ic-ELISA) are developed to detect *E .coli* in milk sample by establishing cascade signal amplification system (Shan et al., 2016). In this method, β-lactamase increases as the amount of *E. coli* increases followed by the hydrolysation of the β-lactamase by the penicillin. This method is able to increase the sensitivity of the

ELISA by 1,000-fold. In addition, ELISA kit is commercially available for example Invitrogen ™ coated ELISA kit by Thermo Fischer Scientific.

However, there are a few disadvantages of ELISA application. As ELISA is based on the antibody, the pH, temperature and shelf-life need to be maintained for the reagents to work optimally. The selection of the antibodies is important to ensure strong affinity to the targeted analyte. The signal of the method could be varied depending on the antibody, antigen, substrate, assay plate as well as signal detection equipment.

2.3.6 Biosensor

Over the past few years, biosensors have become one of the major research endeavours in foodborne pathogens detection due to their promising capabilities to provide high selectivity and improve the limit of detection rapidly (Logue & Nolan, 2012; Umesha & Manukumar, 2016; Zhao et al., 2014). A biosensor is an analytical device that will convert a biological response into an electric signal (Velusamy et al., 2010). It is composed of two important parts which are a biorecognition element and a transducer. A biorecognition element is important to recognise the target analyte, while transducer will convert the target analyte into a measurable signal (Hermann & Patel, 2000). Examples of biorecognition elements in biosensor are nucleic-acid, antibody enzyme, cell, biomimetic and phage; whereas, transducer can be classified into a few groups which are optical, electrochemical, thermometric, micromechanical, magnet or mass-based (Law et al., 2014; Velusamy et al., 2010; Zhao et al., 2014).

When the biorecognition elements capture the target analyte namely bacteria, the corresponding biological response will be converted into a signal by a transducer. The small input from this transducer is delivered into a large output by an amplifier. It is then processed by a signal processor and the results will be stored, displayed and analysed accordingly. The biorecognition element needs to be immobilised on the solid

surface, for example electrode (Amaya-González et al., 2013; Song et al., 2008; Zelada-Guillen et al., 2009). This concept possesses a few advantages such as enhanced structural stability, prolonged the life span and is useful for the real-time detection application (Acquah et al., 2015). Electrochemical biosensor that has been developed for foodborne pathogens by using different nanocomposites for example graphene oxide chitosan nanocomposite for *Salmonella enterica* serovar Typhi detection (Singh et al., 2013), gold nanoparticles modified pencil graphene electrode for *Bacillus cereus* detection (Izadi et al., 2016), single-wall nanocarbon tube for *E. coli* detection (Yamada et al., 2014), graphene oxide with electrodeposited gold nanoparticles for *Salmonella* spp. detection (Ma et al., 2014) and diazonium-based in screen-printed carbon electrode for *Salmonella* detection (Bagheryan et al., 2016). Despite all these biosensor developments, other nanocomposites have been explored to improve the effectiveness of the biosensor for foodborne pathogens in terms of sensitivity, specificity, time of detection, portability, stability and costs.

To develop a robust technology for foodborne pathogens, biological recognition elements are the key for a specificity and sensitivity of the technology. A bioreceptor is a molecular agent that will bind to the target of interest for detection. It can be classified into five different categories including antibody, enzymes, nucleic acid, biomimetic and bacteriophage (Velusamy et al., 2010).

In this study, a DNA aptamer was used as a biorecognition element. Application of aptamer in biosensor is known as aptasensor. Table 2.1 shows the list of aptasensors that have been developed to specifically detect the *Salmonella enterica*.

Table 2.1: List of aptasensor developed to detect *Salmonella* spp.

Aptasensor	**Sensitivity (CFU/ml)**	**Time taken**	**Sample food matrix**
Reduced graphene-based azophloxine nanocomposite aptasensor by potentiometric method (Muniandy et al., 2017)	10	10 min	Chicken meat
Electrochemically-reduced graphene oxide chitosan complex (Dinshaw et al., 2017)	10	Not stated	Chicken meat
Surface-enhanced Raman spectroscopy (SERS) with gold nanoparticles (Zhang et al., 2015).	15	3 hours	Pork sample
Glassy carbon electrode modified with graphene oxide and gold nanoparticles by using thiolated-aptamer (Ma et al., 2014)	3	Not stated	Pork sample
Lateral flow aptasensor (Fang et al., 2014)	10	10 min	Milk
Aptamer-based viability sensors for bacteria (AptaVISens-B) using electrochemical detection (Labib et al., 2012).	600	Not stated	Not tested
Ultrasensitive fluorescence detection of viable *Salmonella* enteritidis by using enzyme-induce cascade two stage teohold strand displacement-driven assembly of the G-quadruplex DNA (Zhang et al., 2016)	60	Not stated	Not tested

2.4 Aptamer: A biological recognition element.

An aptamer is a synthetic single-stranded DNA that can be generated *in vitro* by using a process known as Systemic Evolution of Ligand by EXponential Enrichment (SELEX) (Ellington & Szostak, 1990; Tuerk & Gold, 1990). Even though an aptamer is a single-stranded DNA or RNA, it has a different mechanism of binding to the complementary target. When an aptamer is generated by the SELEX process, it has the ability to form a functional specific 3D structure. Therefore, it can specifically bind to the target through structural recognition (Sun et al., 2014). In the presence of the target, an aptamer will fold into well-defined binding pocket due to hydrogen bonds and will unfold upon dissociation or absence of the target (Hermann & Patel, 2000). For larger molecules, for example proteins, the aptamer interacts with the target via noncovalent bond with a mechanism known as induce-fit. This mechanism shows a flexibility of the aptamer structure which can complementarily fit the target site and result in high specificity and affinity (Hermann & Patel, 2000).

Many aptamers have been developed to target *Salmonella* spp. (Table 2.2) with different properties including their own unique sequences, different dissociation constant (K_D value), structure and specific target, for example outer membrane protein, lipopolysaccharide, flagella or lipoprotein and others. Compared to other biological agents, such as antibody and proteins, aptamers possess a few advantages that make them more favourable for detection of microorganisms. An aptamer can be chemically synthesised *in vitro*. Therefore, it is possible to tailor its physiochemical properties based on the required application (Famulok & Mayer, 2011). This could be achieved as the conditions of the selection during the SELEX procedures can be customised.

Table 2.2: List of aptamers developed for *Salmonella* detection.

***Salmonella* serovars**	**Sequence and structure**	**References**
S. Enteritidis and *S.* Typhimurium	5'CACACCGGAAGGGATGCCACCTAAACCCC-3' 5'CACAGATGACGTCTGGCACATAATTAACAC-3' 5'-CCGATGTCCGTTAGGGCTCCTCCATAGA-3'	(Park et al., 2014)
S. Typhimurium	5'CTGATGTGTGGGTAGGTGTCGTTGATTTCTTCTGGTGGGG-3'	(Dwivedi et al., 2013)
S. Typhimurium	5'AGTAATGCCCGGTAGTTATTCAAAGAGAGTAGGAAAAG A-3'	(Duan et al., 2013)
S. Typhimurium	5′ACGGGCGTGGGGGCAATGCCTGCTTGTAGGCTTCCCCTGTGCGCG-3'	(Moon et al., 2013)
S. Typhimurium	5'TATGGCGGCGTCACCCGACGGGGACTTGACATTATGACAG-3'	(Joshi et al., 2009)

Unlike an antibody, an aptamer does not require host animal for production, therefore, making the cost less expensive and the process less tedious. Due to its properties that have high affinity, specificity and sensitivity to the target molecules, it is able to eliminate the possibility of false-positive and false-negative results; thus, increasing its reliability. Unlike an antibody that will permanently lose its stability at high temperature, an aptamer is a stable compound that is able to transfer in ambient temperature (Famulok & Mayer, 2011; Jayasena, 1999). It can also be chemically modified with a functional group at the end of the aptamer sequence without affecting their ability and stability. This characteristic is very important especially in development and modification of biosensors. Last but not least, it has the ability to detect the whole bacteria cell; thus, eliminating the tedious DNA extraction step and decreasing the time taken for detection. Consequently, many types of researches related to aptamer as molecular recognition elements are growing at an enormous pace.

2.5 Sample preparation and its challenges

Regardless of the many developments in the detection methods, the need for an efficient upstream sample preparation is equally important. It has been suggested that a good pre-analytical process or upstream sample preparation is crucial to increase and optimise the sensitivity and specificity of the downstream detection (Brehm-stecher et al., 2009). This is important to ensure maximum recovery for the bacteria detection as well as to reduce the loss of the targeted bacteria during the pre-analytical sample preparation. As food is a complex environment with the presence of fats, salts, preservatives and diversity of the microbiota background, it may interfere with the detection of the bacteria. One of the biggest dilemmas in sample preparation is to separate, concentrate and purify the targeted pathogens from the food matric background. Therefore, many methods have been introduced to facilitate this purpose including physical, chemical and biological methods (Stevens & Jaykus, 2004; Suh et al., 2013). One of the examples of biological methods used to facilitate this purpose is immunomagnetic separation (IMS) (Skjerve et al., 1990). Usually, these methods are combined with the current diagnostic tools to enhance the rapid, sensitive and specific detection especially in the complex food sample.

IMS was developed over two decades ago in which the magnetic beads are coated with the specific antibody that complement the antigen of the targeted bacteria. The bacteria are separated from the complex food matrix by using a magnet. IMS is commercially available for instance Dynabeads ® Anti-*Salmonella* by the Life Technologies Corporation and has been widely used for this purpose. IMS can be detected automatically or manually. Usually, IMS is coupled with Polymerase Chain Reaction (PCR) (Jenïkovâ et al., 2000; Wang et al., 2013), real-time PCR (Notzon et al., 2006; Wang et al., 2014) or loop-mediated isothermal amplification method as a subsequent detection tool (Song, 2008). Antibody is broadly used due to its high

specificity to the targeted antigen. However, antibody is not stable and sensitive to the room temperature which can lead to irreversible denaturation. The production of antibody involves animal suffering and has batch to batch variation. It is important to check the physiological environment in the cells that may give stress impact to the antibody which may reduce it performances (Zhao et al., 2014).

Therefore, in this study, an aptamer was used as the biological recognition element for two different purposes; firstly, to separate *Salmonella* from a complex food matrix and secondly, as a detection element to recognize *Salmonella* by using nanocarbon biosensor as a platform. The objectives of this study were to develop and evaluate the DNA aptamer as a biological recognition element for *Salmonella* detection by using magnetic beads follow by PCR as a downstream detection method; and to evaluate aptamer-based nanocarbon biosensor (aptasensor) for *Salmonella* detection.

CHAPTER 3: METHODOLOGY

3.1 PART A: Aptamer conjugated magnetic separation (AMS) followed by PCR

3.1.1 Materials

3.1.1.1 Media, buffers and chemical preparation

All media, buffers and chemical preparation steps are listed in Appendix A.

3.1.1.2 Bacteria isolate collection

Ten different serovars of *Salmonella enterica* were used, namely *Salmonella enterica* Typhimurium (*S.* Typhimurium), *Salmonella enterica* Enteritidis (*S.* Enteritidis), *Salmonella enterica* Typhi (*S.* Typhi), *Salmonella enterica* Albany (*S.* Albany), *Salmonella enterica* Braenderup (*S.* Braenderup), *Salmonella enterica* Corvallis (*S.* Corvallis), *Salmonella enterica* Indiana (*S.* Indiana), *Salmonella enterica* Pullorum (*S.* Pullorum), *Salmonella enterica* Paratyphi A (*S.* Paratyphi A) and *Salmonella enterica* Paratyphi B (*S.* Paratyphi B). Non-*Salmonella* enterica species were also used which included *Escherichia coli*, *Vibrio parahaemolyticus*, *Shigella flexneri* and *Staphylococcus aureus*. These strains were from the culture collection from Laboratory of Biomedical Science which have been previously confirmed and identified. The details of the strains are shown in Appendix B.

3.1.1.3 Aptamer sequence

The aptamer sequence (5'T ATG GCG GCG TCA CCC GAC GGG GAC TTG ACA TTA TGA CAG-3') was as previously reported by Joshi et al. (2009). The 5' ends of the ss DNA aptamer was modified with biotin that would be further captured by streptavidin coated paramagnetic beads. The sequence was synthesised by Integrated DNA Technologies (IDT, United States).

3.1.1.4 Primers

The *Salmonella* primers were designed in-house and the sequence were 5' ATC CCT TTG CGA ATA ACA TCC T and 5' GGG CGC CAA GAG AAA AAG A. These invA primers specifically for *Salmonella* were used and checked by BLAST algorithm on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to minimise the likelihood of unspecific amplification for non-target loci. Then, the primers were tested through *in silico* uniplex assay with the PCR amplification program via http://insilico.ehu.es/PCR/. The other in-house primers (sequences not shown) specific for *Staphylococcus aureus*, *Shigella*, *E. coli* and *Vibrio* for specificity test were also used.

3.1.2 Methods

3.1.2.1 Revival of the bacterial strains and their confirmation by PCR.

Pure isolates of *Salmonella* were inoculated into 1 ml of Luria Bertani (LB) medium and incubated overnight at 37^0C with shaking. Then, a loopful of the culture was streaked on the LB agar and incubated overnight at 37^0C to get a well-isolated single colony. The identity of the strains was further re-confirmed by conventional PCR reaction by using *invA* gene as a targeted locus (described in Section 3.1.2.5). The representative PCR product was sent for purification and sequencing (First BASE Laboratories Sdn. Bhd., Malaysia). The confirmed cultures were cultivated in LB medium and preserved at -80^{o}C in 50% (v/v) glycerol for future use.

3.1.2.2 Preparation of the bacteria culture

Pure isolates of *Salmonella* were inoculated into 1 ml of Luria Bertani (LB) broth and incubated overnight at 37^0C with shaking. After that, 1:10 dilution of the overnight culture was inoculated in the LB broth and incubated for 3 hours at 37^0C with shaking. Then the cell culture was centrifuged at 5000 rpm for 10 minutes, the supernatant was discarded and the cell pellet was washed with 1 ml of 1 × Phosphate

Buffered Saline (PBS) (0.1M, pH 7.4). The washing was repeated twice. The cell pellet was resuspended in 1 ml of 1 × PBS. By using DEN-1 densitometer (Biosan, Latvia), the concentration was adjusted to 0.5 McFarland at 565 ±15 nm. The bacterial suspension was serially diluted ten folds and the corresponding colony forming unit (CFU) was determined by plate counting method.

3.1.2.3 Preparation of Aptamer Magnetic Beads Separation (AMS)

Streptavidin Magnesphere® Paramagnetic Particles (Promega, Madison, WA) or magnetic beads were washed with 1 × PBS and prepared as per manufacturer's instruction. Briefly, 1 vial of the magnetic beads was washed three times with 1 ml of 1 × PBS. After magnetic separation by using Polyattract ® System 100 Magnetic Separation Stand (Promega, Madison, WA), the magnetic beads were resuspended in 1 ml of 1 × PBS. A total of 4 µl of 0.4 nmol of the synthesised biotinylated aptamer was coupled with 200 µl of magnetic beads in 1 × PBS at ambient room temperature. The aptamer-conjugated magnetic beads were used within 30 minutes.

3.1.2.4 Sensitivity test

To determine the sensitivity, 1 ml of each serially diluted *Salmonella* suspension (10^0 CFU/ml to 10^7 CFU/ml) was mixed with aptamer-conjugated paramagnetic beads and incubated for 30 minutes at room temperature with continuous shaking. Then, the bound-bacteria-aptamer-conjugated magnetic beads were recovered by using magnetic stand and the supernatant was discarded. Bound bacteria-magnetic aptamer particles were washed four times with 1 × PBS- 5% Tween 20 buffer and finally dissolved in 200 µl of 1 × PBS. The bacteria cells were eluted by using nuclease-free water.

The DNA was extracted from direct boiled cell lysate. Briefly, the eluted bacteria were heated at 99°C for 5 minutes and snapped cooled on ice. After a brief centrifugation at 13,400 rpm for 5 minutes, the supernatant was transferred into another

sterile microfuge tube. In comparison, DNA was also extracted from the same serially diluted bacteria cells that were not subjected to AMS step.

3.1.2.5 Polymerase Chain Reaction (PCR)

Primers were commercially synthesised by Integrated DNA Technologies (IDT, United States). Each 25 µl PCR mixture contained 1 × PCR buffer, 1.8 mM of $MgCl_2$, 0.12 mM of dNTPs, 0.8 µM for each forward and reverse *invA* primers, 0.06 U of GoTaq Flexi DNA polymerase (Promega, Madison, WI, U.S.) and 5 µl of DNA were used as templates. PCR conditions consisted of initial denaturation at 95 °C for 5 minutes; 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 45 s; and final extension at 72 °C for 10 minutes. The PCR products were electrophoresed on 2% agarose gel in 0.5 × Tris-borated EDTA (TBE) buffer. The gel was stained in GelRed™ (Biotium) and visualised by GelDoc™XR imaging system.

3.1.2.6 Specificity test

To test the specificity of the aptamer, the same bacteria concentration of *Salmonella* spp., *S.* Typhi, *S.* Albany, *S.* Braenderup, *S.* Corvallis, *S.* Paratyphi A, *S.* Paratyphi B, *S.* Enteritidis. *S.* Pullorum, *S.* Typhimurium and *S.* Indiana were used. Then, the bacteria were tested with AMS followed by PCR as described earlier.

For other non-*Salmonella* species, the same concentration of *Vibrio parahaemolyticus*, *Escherichia coli*, *Staphylococcus aureus* and *Shigella flexneri* including *S.* Typhimurium were mixed to form a bacterial cocktail before they were tested by AMS. To ensure the aptamer did not target any non-*Salmonella* bacteria cells, the eluted bacteria were tested by using specific in-house primers (sequences not shown).

3.1.2.7 Detection of *Salmonella* in foods.

Fourteen food samples including chicken (n = 4), vegetables (n = 8) and beef (n = 2) were purchased from different retail markets around Klang Valley and analysed on the same day. The overall protocols for *Salmonella* detection in foods by using AMS-PCR is shown in Figure 3.1 The details of the food samples are shown in Appendix C.

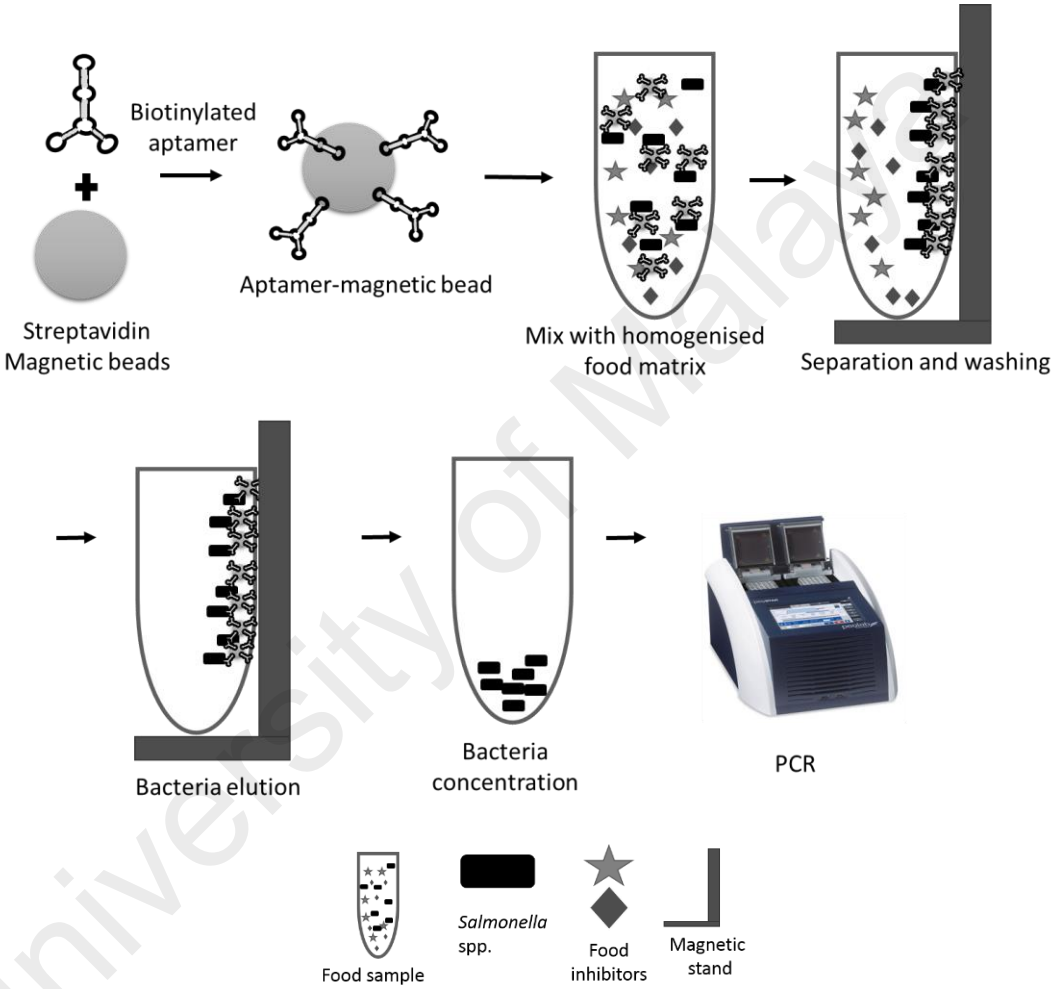


Figure 3.1: The illustration of AMS-PCR method for detection of *Salmonella* in food samples.

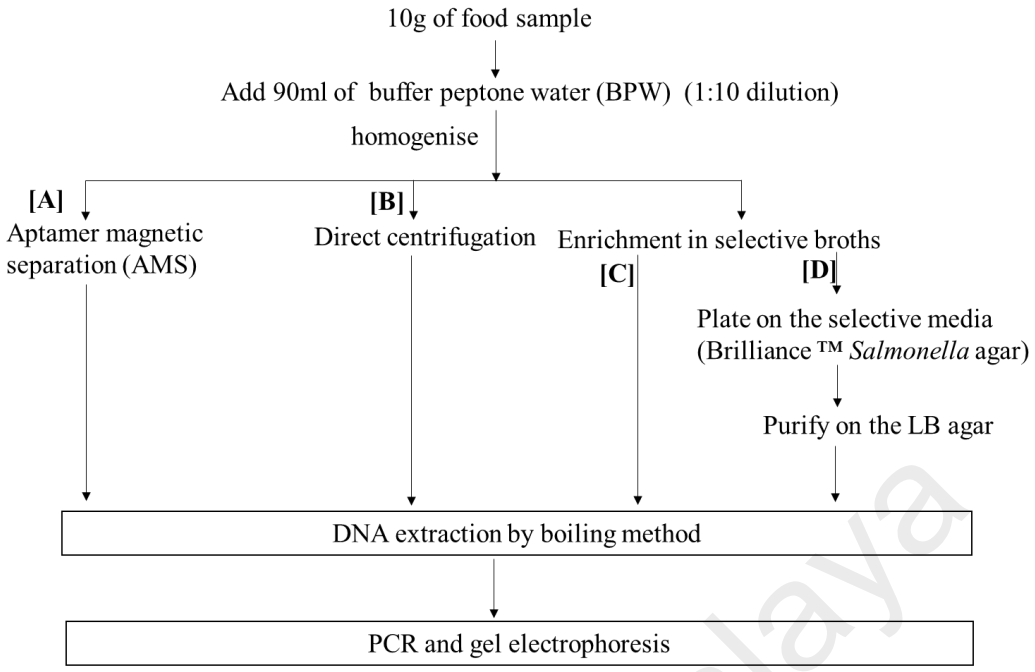


Figure 3.2: The workflow for AMS-PCR detection for *Salmonella* detection by comparing four different procedures namely [A], [B], [C] and [D]. All of these procedures were conducted on each food sample.

The scheme for evaluation of food testing is illustrated in Figure 3.2. After homogenisation and incubation of the sample for three hours in Buffer Peptone Water (BPW), an aliquot of 1 ml of the incubated pre-enrichment was mixed with the aptamer-conjugated magnetic beads [A], while another 1 ml was directly processed for DNA extraction without AMS step [B]. In [C] which is the conventional culture approach, 1 ml of the incubated pre-enrichment was transferred and incubated in the selective enrichment broths which included Rappaport-Vassiliadis Soya (RVS) Peptone Broth (Oxoid, UK) for 24 hours at 37 °C or Selenite Cystine (SC) Broth (Oxoid) for 12 hours at 42 °C. An aliquot of these selective enrichment broths was processed for DNA extraction. In [D], aliquots of the selective broth overnight cultures (RVS/SC) were streaked onto selected media (Brilliance ™ *Salmonella* Agar) for *Salmonella* isolation. Presumptive *Salmonella* colonies (purple colour) were picked and purified on LB agar. DNA extraction from A, B, C and D were carried by direct boiled cells lysate as

described earlier. The DNAs were then further tested with PCR for confirmation of *Salmonella*.

3.2 PART B: Aptamer-based nanocarbon biosensor

The preparation and characterisation of the aptasensor were conducted by Dr. Rakibul Hassan from Nanotechnology & Catalysis Research Centre (NANOCAT), University Malaya (Parts 3.2.2.1, 3.2.2.2. and 3.2.2.3).

3.2.1 Materials

Multi-walled carbon nanotubes (MWCNTs) were supplied by Bayer Material Science AG (Germany). Prior to the electrochemistry studies, the crude carbon nanotubes (CNTs) were refluxed in nitric acid, followed by hydrochloric acid at ambient temperature. The electrochemical deposition was carried out using a DC power supply. Indium tin oxide (ITO) coated glass substrate was used for CNTs platform for sensor probe preparation. A powersonic 420 ultrasonic cleaner was used to disperse CNTs and clean electrodes.

The *Salmonella* aptamer sequence 5'-T ATG GCG GCG TCA CCC GAC GGG GAC TTG ACA TTA TGA CAG 3' (Joshi et al., 2009) with amino modification at 5' end was purchased from Integrated DNA Technologies (IDT). The aptamer was diluted with nuclease free water and it was stored at −20 °C when not in use.

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were performed in the Metrohm silicate glass cell using a PGSTAT302N electrochemical workstation (Autolab Metrohm, Netherlands). A conventional three-electrode system having: a platinum (Pt) plate of area 1 cm^2 as a counter electrode; a saturated calomel electrode (SCE) as a reference electrode and the sensor probe with an area of 1 cm^2 as a working electrode.

3.2.1.1 Media, buffer and chemical preparation

All media, buffer and chemical preparation steps are listed in Appendix A.

3.2.2 Methods

3.2.2.1 Purification of carbon nanotubes

The summary of aptasensor development is shown in Figure 3.3. Briefly, CNTs were treated before performing electro-deposition on indium tin oxide (ITO) glass substrate. In this study, the CNTs samples were first heated at 480 $^{\circ}$C in the presence of oxygen followed by 12 hours reflux using 3 M HNO_3/HCl. Generally, raw CNTs consisted of impurities such as fullerenes, amorphous carbon and catalyst metal nanoparticles (nickel, iron, cobalt). In order to minimise the electrochemical interference of these particles with respect to possible electro-catalytic effects, these impurities must be removed to ensure the purity of CNTs (Yang et al., 2010).

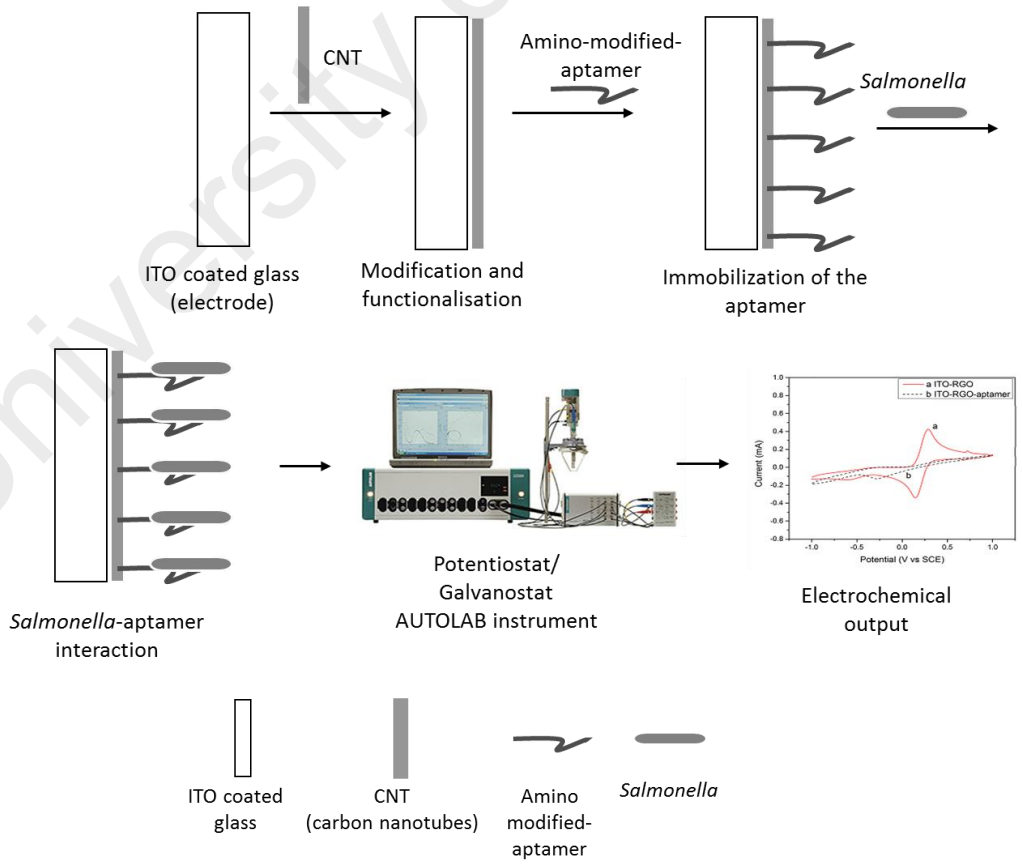


Figure 3.3: The illustration of aptasensor development for *Salmonella* detection.

3.2.2.2 Fabrication of aptasensor

A total of 1 mg/ml solutions were prepared before the start of CNTs electro-deposition. 30 V was applied for 1 minute for one-step deposition technique. After gentle washing of the CNT/ITO substrate with ultrapure water, the immobilisation of carboxyl groups onto the CNT/ITO substrate was performed *via* EDC-NHS reaction for few hours. Then, 25 µl of 5 µM amino-modified *Salmonella* aptamer was dropped onto the activated electrode for one hour at 25 $^{\circ}$C. Aptamer/CNTs/ITO bioelectrode was thoroughly washed to removed unbound DNA. This sensing platform was stored at 4 $^{\circ}$C until further use.

3.2.2.3 Characterisation of aptasensor and electrochemical detection of *Salmonella*.

The structure and surface morphology of the CNTs were characterised by Raman microscope (Renishaw inVia, UK) and Scanning Electron Microscopy (SEM - Hitachi, Japan), respectively. Specifically, a disassembled ITO glass substrate was uniformly covered with purified CNTs. Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were carried out to characterise the linearity, sensitivity and specificity values for *Salmonella* detection. The electrolyte used in the experiment was 5 mmol.l^{-1} $[Fe(CN)_6]^{3-/4-}$ solution containing KCl solution at various scan rates. The CV measurements were performed in a potential range of -0.2 V to +0.8 V (*vs.* SCE) with a scan rate of 50 mV s^{-1}. EIS measurements were carried out at a frequency range between 10^{-1} Hz to 10^{5} Hz, with amplitude of 5 mV around the open circuit potential (OCP). NOVA 1.11 software interfaced with the potentiostat was used to analyse the impedance data.

Charge transfer resistance (R_{CT}) and the D-value resistance (ΔR_{CT}) were calculated using the following equation (Jia et al., 2016; Wu et al., 2012).

$$\Delta R_{CT} = R_{CT}\,(\text{aptamer} - \text{bacteria}) - R_{CT}\,(\text{aptamer})$$

Where, R_{CT} (aptamer) is the resistance value of aptamer modified ITO substrate and R_{CT} (aptamer – bacteria) is the resistance after aptamer conjugation while exposed to *Salmonella* samples.

3.2.2.4 Reviving and bacteria preparation for aptasensor detection.

Well-isolated *Salmonella enterica* colonies were transferred into a 50 ml Falcon tube containing 2 ml of LB broth and incubated overnight at 37 ºC with consistent shaking. The overnight cell culture was centrifuged at 5000 rpm for 10 minutes, then the supernatant was discarded and the cell pellet was washed with 1 ml of 1 × PBS (0.1 M, pH 7.4). The washing was repeated twice. The cell pellet was resuspended in 1 ml of 0.85% (w/v) saline and then serially diluted 10 folds from 10^{-1} to 10^{-8}. For each dilution, the optimal density at 600 nm and the corresponding colony forming unit (CFU) was determined.

3.2.2.5 Sensitivity and specificity tests

For sensitivity test, different *Salmonella* cell concentrations were tested ranging from 10^1 to 10^6 CFU/ml. The modified electrode was incubated with different *Salmonella* cell concentration at ambient temperature. Then the electrode was washed with ultrapure water to remove unbound *Salmonella* to the aptamer. After that, the EIS measurement were conducted by immersing the electrode in the electrolyte 5 mmol.l^{-1} $[Fe(CN)_6]^{3-/4-}$ solution containing KCl solution.

Specificity experiments were conducted by using different *Salmonella* serovars namely *S*. Paratyphi A, *S*. Paratyphi B and *S.* Typhi and non-*Salmonella* bacteria such as

Staphylococcus aureus, *Escherichia coli*, and *Vibrio parahaemolyticus*. The fresh electrode was used for each type of bacterial sample. All the specificity experiments were conducted using same bacterial cell density of 10^3 CFU/ml.

CHAPTER 4: RESULTS

4.1 PART A: Aptamer Magnetic Separation (AMS) followed by PCR

4.1.1 Evaluation on invA primer sequences through BLAST and in silico PCR amplification tool

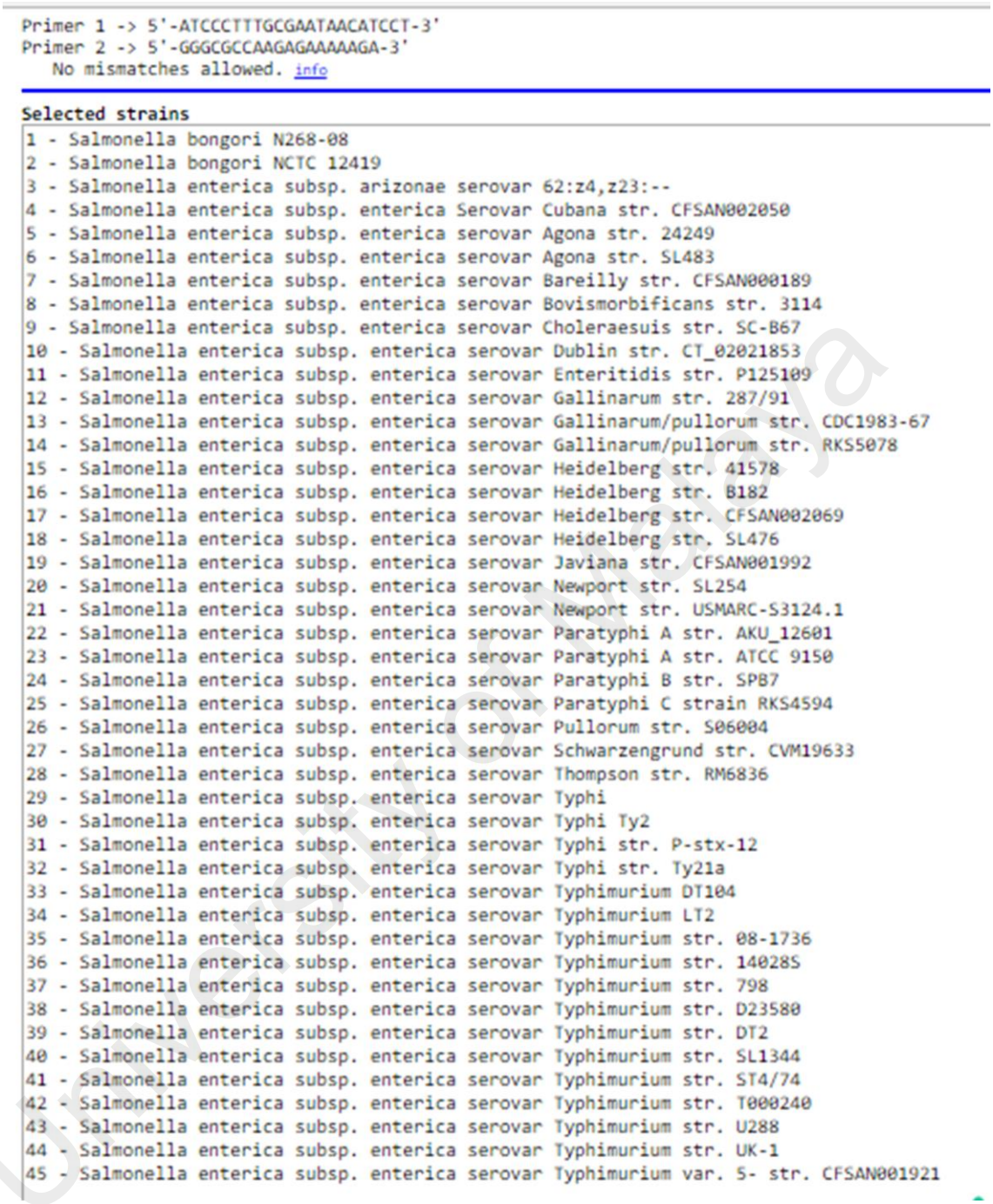
The invA primers were checked by using online BLAST tool in NCBI website. Based on Table 4.1, the results show that the primes were specific to the *Salmonella enterica* spp..

Table 4.1: Evaluation of primers by using BLAST in NCBI website

Primer	Target gene	Similarity identity
invA	*inv A*	100% to *Salmonella enterica* subsp. enterica strain RM11060 chromosome, complete genome (CP022658.1) 100% to *Salmonella enterica* subsp. enterica strain 08-00436, complete genome (CP020492.1) 100% to *Salmonella enterica* strain SA20084699, complete genome (CP022497.1)

Then, the primers were further evaluated by *in silico* uniplex assay with the online PCR amplification program. The result of in silico is shown in Figure 4.1. Both BLAST and in silico results show that invA primers were specific to *Salmonella* spp..

a) ***In silico* PCR Amplification**



Primer 1 -> 5'-ATCCCTTTGCGAATAACATCCT-3'
Primer 2 -> 5'-GGGCGCCAAGAGAAAAAGA-3'
No mismatches allowed. info

Selected strains

1 - Salmonella bongori N268-08
2 - Salmonella bongori NCTC 12419
3 - Salmonella enterica subsp. arizonae serovar 62:z4,z23:--
4 - Salmonella enterica subsp. enterica Serovar Cubana str. CFSAN002050
5 - Salmonella enterica subsp. enterica serovar Agona str. 24249
6 - Salmonella enterica subsp. enterica serovar Agona str. SL483
7 - Salmonella enterica subsp. enterica serovar Bareilly str. CFSAN000189
8 - Salmonella enterica subsp. enterica serovar Bovismorbificans str. 3114
9 - Salmonella enterica subsp. enterica serovar Choleraesuis str. SC-B67
10 - Salmonella enterica subsp. enterica serovar Dublin str. CT_02021853
11 - Salmonella enterica subsp. enterica serovar Enteritidis str. P125109
12 - Salmonella enterica subsp. enterica serovar Gallinarum str. 287/91
13 - Salmonella enterica subsp. enterica serovar Gallinarum/pullorum str. CDC1983-67
14 - Salmonella enterica subsp. enterica serovar Gallinarum/pullorum str. RKS5078
15 - Salmonella enterica subsp. enterica serovar Heidelberg str. 41578
16 - Salmonella enterica subsp. enterica serovar Heidelberg str. B182
17 - Salmonella enterica subsp. enterica serovar Heidelberg str. CFSAN002069
18 - Salmonella enterica subsp. enterica serovar Heidelberg str. SL476
19 - Salmonella enterica subsp. enterica serovar Javiana str. CFSAN001992
20 - Salmonella enterica subsp. enterica serovar Newport str. SL254
21 - Salmonella enterica subsp. enterica serovar Newport str. USMARC-S3124.1
22 - Salmonella enterica subsp. enterica serovar Paratyphi A str. AKU_12601
23 - Salmonella enterica subsp. enterica serovar Paratyphi A str. ATCC 9150
24 - Salmonella enterica subsp. enterica serovar Paratyphi B str. SPB7
25 - Salmonella enterica subsp. enterica serovar Paratyphi C strain RKS4594
26 - Salmonella enterica subsp. enterica serovar Pullorum str. S06004
27 - Salmonella enterica subsp. enterica serovar Schwarzengrund str. CVM19633
28 - Salmonella enterica subsp. enterica serovar Thompson str. RM6836
29 - Salmonella enterica subsp. enterica serovar Typhi
30 - Salmonella enterica subsp. enterica serovar Typhi Ty2
31 - Salmonella enterica subsp. enterica serovar Typhi str. P-stx-12
32 - Salmonella enterica subsp. enterica serovar Typhi str. Ty21a
33 - Salmonella enterica subsp. enterica serovar Typhimurium DT104
34 - Salmonella enterica subsp. enterica serovar Typhimurium LT2
35 - Salmonella enterica subsp. enterica serovar Typhimurium str. 08-1736
36 - Salmonella enterica subsp. enterica serovar Typhimurium str. 14028S
37 - Salmonella enterica subsp. enterica serovar Typhimurium str. 798
38 - Salmonella enterica subsp. enterica serovar Typhimurium str. D23580
39 - Salmonella enterica subsp. enterica serovar Typhimurium str. DT2
40 - Salmonella enterica subsp. enterica serovar Typhimurium str. SL1344
41 - Salmonella enterica subsp. enterica serovar Typhimurium str. ST4/74
42 - Salmonella enterica subsp. enterica serovar Typhimurium str. T000240
43 - Salmonella enterica subsp. enterica serovar Typhimurium str. U288
44 - Salmonella enterica subsp. enterica serovar Typhimurium str. UK-1
45 - Salmonella enterica subsp. enterica serovar Typhimurium var. 5- str. CFSAN001921

Figure 4.1: The specificity results of *in silico* PCR of invA primers (screenshot). (a) The results showed invA primers were highly specific to *Salmonella* species.

b)

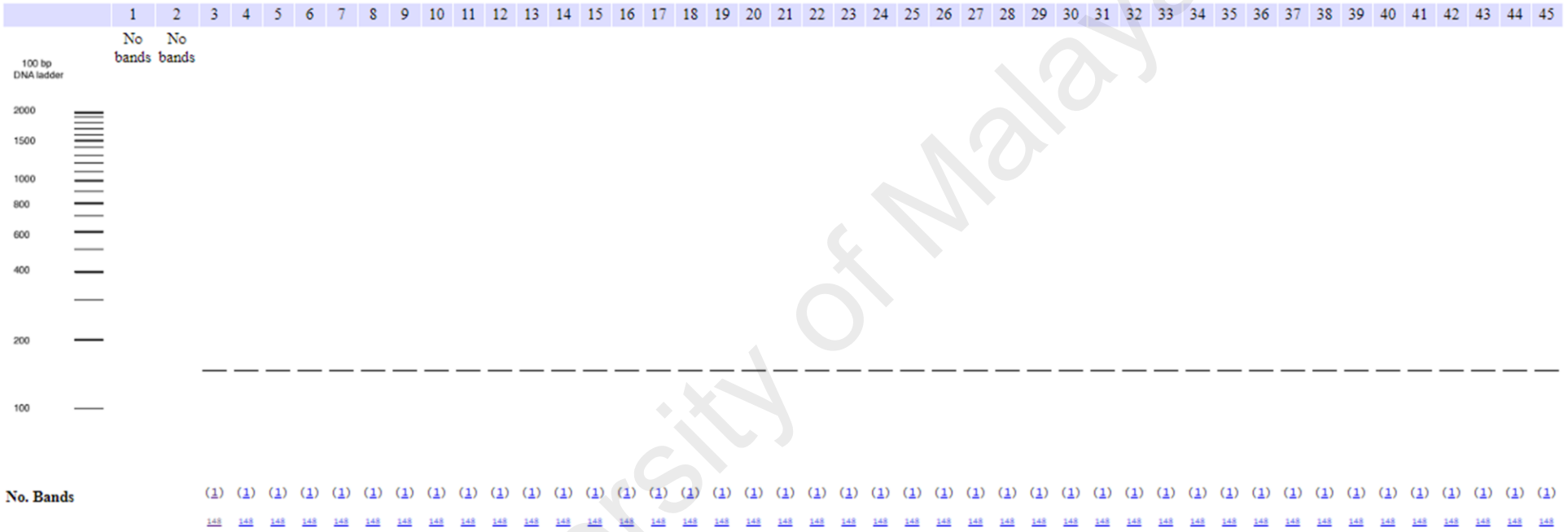


Figure 4.1, continued. The specificity results of *in silico* PCR of invA primers (screenshot). (b) No 1 to 45 referring to *Salmonella* species in (a). The analysis showed the size of the amplicons was approximately 149 bp.

4.1.2 The sensitivity test with pure *Salmonella* cultures.

The sensitivity of the AMS-PCR and PCR were determined by detecting the lowest concentration of cells using colony forming unit (CFU) counting. The enumeration of CFU of *Salmonella* on pure culture is shown in Table 4.2.

Table 4.2: Enumeration of colony forming unit (CFU) of *Salmonella* on pure culture at 0.5 McFarland. Data for each dilution factors was based on triplicate experiments. The test was replicated twice.

Test	**Dilution factors**	**No of colonies**				**Calculation, CFU/ml** $\frac{\text{no of colonies}}{\text{amount plated (ml)}} \times \text{dilution factor}$
		Plate 1	Plate 2	Plate 3	Average	
1	10^4	TNTC	TNTC	TNTC	N/A	$\frac{87.3}{0.1} \times 10^5 = 8.7 \times 10^7$
	10^5	100	64	98	87.3 ± 20.2	
	10^6	5	11	10	8.7 ± 3.2	
2	10^4	TNTC	TNTC	TNTC	N/A	$\frac{73.3}{0.1} \times 10^5 = 7.3 \times 10^7$
	10^5	66	68	86	73.3 ± 11.0	
	10^6	7	12	10	9.7 ± 2.5	
					Average	**$8 \times 10^7 \pm 1.0$**

TNTC= Too Numerous To Count

N/A = Not Available.

The plate counting method showed that at 0.5 McFarland, the concentration of bacteria was $8 \times 10^7 \pm 1.0$ CFU/ml. Therefore, before bacteria dilution was conducted, the initial reading of bacteria suspension was adjusted and standardised to 0.5 McFarland.

The sensitivity of the AMS-PCR was determined by detecting the lowest concentration of cells that showed 149 bp band. Based on Figure 4.2a, the lowest detection limit of PCR-AMS was 100 CFU/ml. Compared to PCR alone (Figure 4.2b), AMS-PCR showed 10 times higher sensitivity. Typically, the infective dosage of

Salmonella can be as low as 100 organisms (D'Aoust, 1985) or ≥ 10^5 organisms (Kothary & Babu, 2001; Hara-Kudo & Takatori, 2011) even though it can be varied based on the exposed population, for instance age, immunity, illness and characteristics of the pathogens, i.e. pathogenicity (Hara-Kudo & Takatori, 2011).

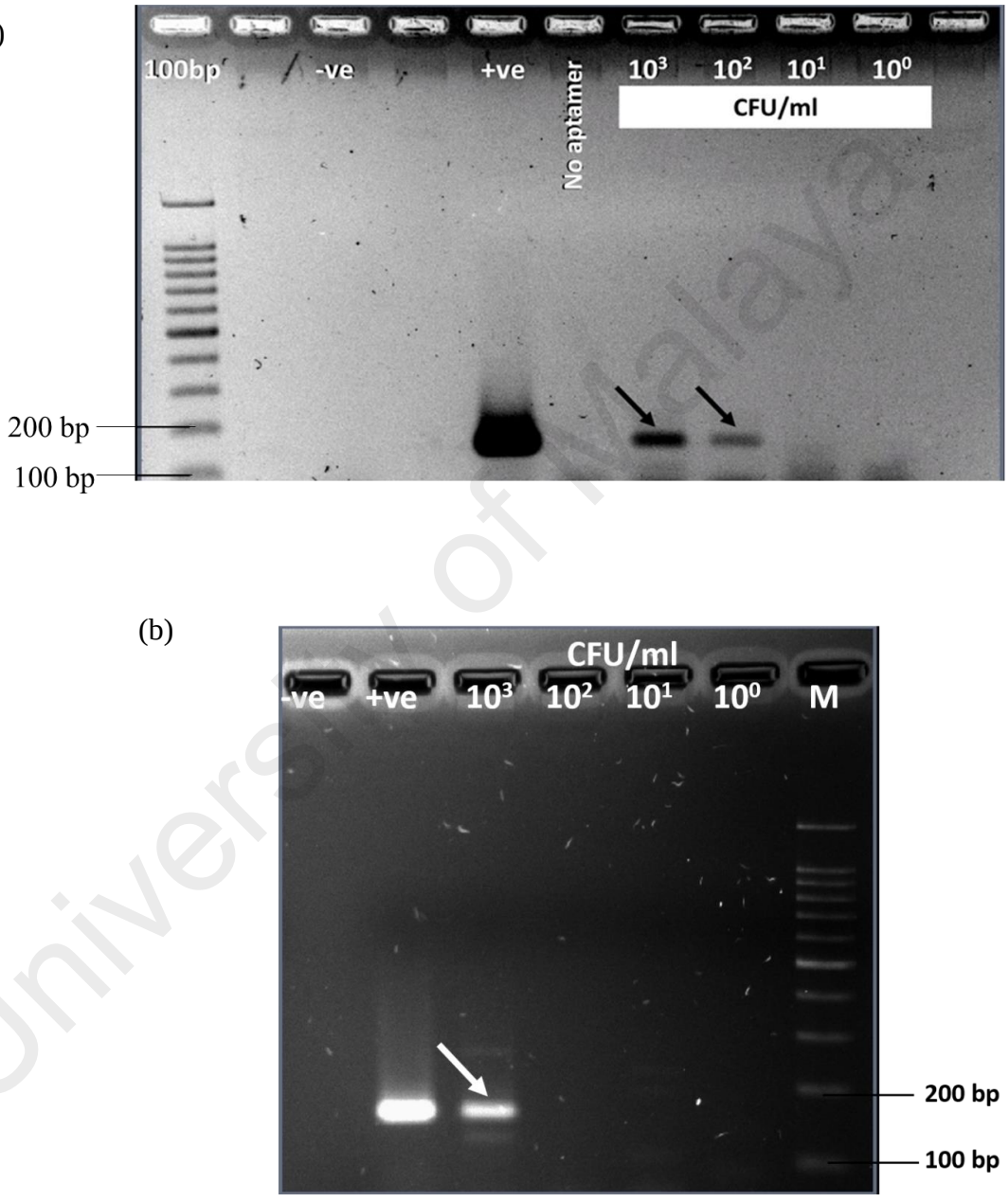


Figure 4.2: The comparison of sensitivity test results when tested with AMS – PCR and without AMS in *Salmonella* detection. (a) The limit of detection of AMS – PCR was 100 CFU/ml. (b) PCR results without AMS application showed the limit of detection was 10^3 CFU/ml. Arrow indicated the size of the amplicon (~149 bp).

The PCR amplicons were validated by using DNA sequencing. The sequence result was analysed by using BLAST program compared to the sequences in the NCBI GenBank and the similarity was found to be 99% to the *Salmonella* spp. (Figure 4.3).

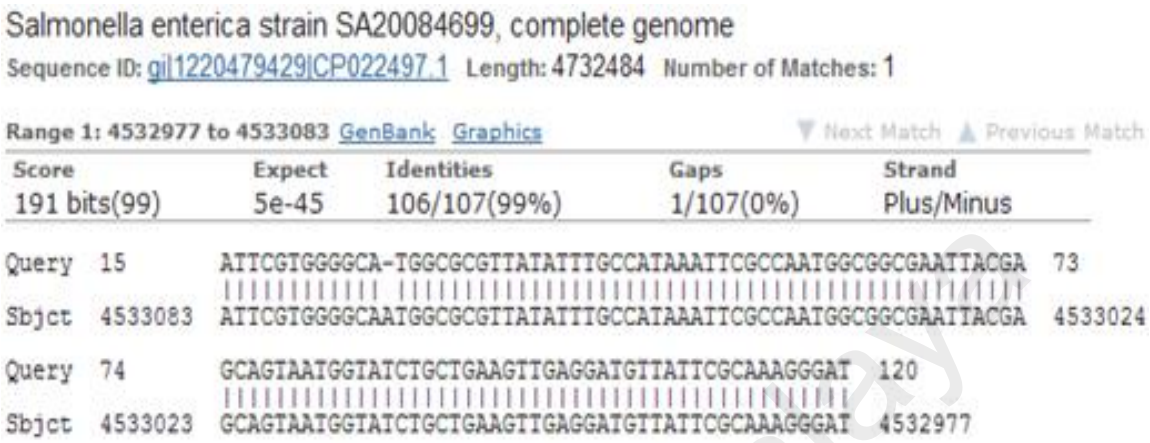


Figure 4.3: The sequence analysis of the PCR amplicon of *invA* gene from *Salmonella*. The analysis shows that the amplicon was from *Salmonella enterica*.

4.1.3 The specificity results

Ten different *Salmonella* serovars and four non-*Salmonella* species namely *E. coli*, *S. aureus*, *Shigella flexneri* and *Vibrio parahaemolyticus* were tested to determine the specificity of the aptamer. Based on Figures 4.4 and 4.5, the aptamer was highly specific to the *Salmonella* serovars with no cross-reactivity with non-*Salmonella* cells. In order to ensure the magnetic beads did not affect the binding of *Salmonella* to the aptamer, magnetic beads without aptamer were tested in bacteria suspension. Based on Figure 4.5, magnetic beads did not show any unspecific binding with the *Salmonella* and consistent with the result in Figure 4.2.

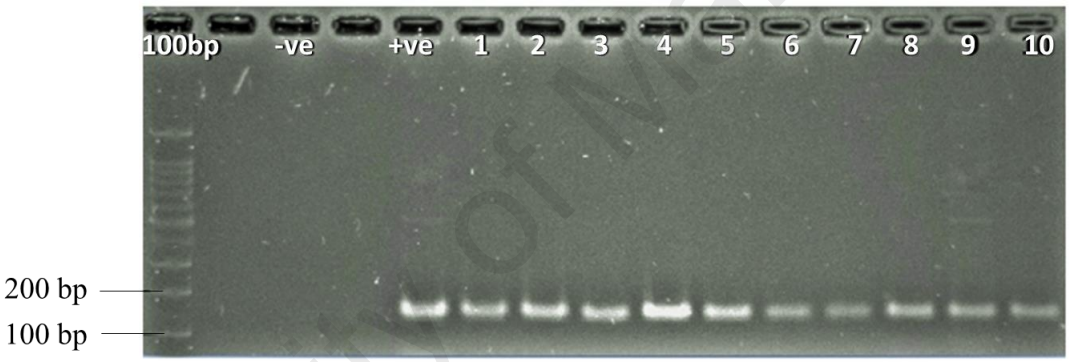


Figure 4.4: Specificity results for different *Salmonella* serovars**.** Lanes 1- *S.* Typhi, 2- *S.* Albany, 3- *S.* Braenderup, 4- *S.* Corvallis, 5- *S.* Paratyphi A, 6- *S.* Paratyphi B, 7- *S.* Enteritidis. 8-*S.* Pullorum, 9- *S.* Typhimurium and 10- *S.* Indiana. +ve - positive control; -ve - non-template control.

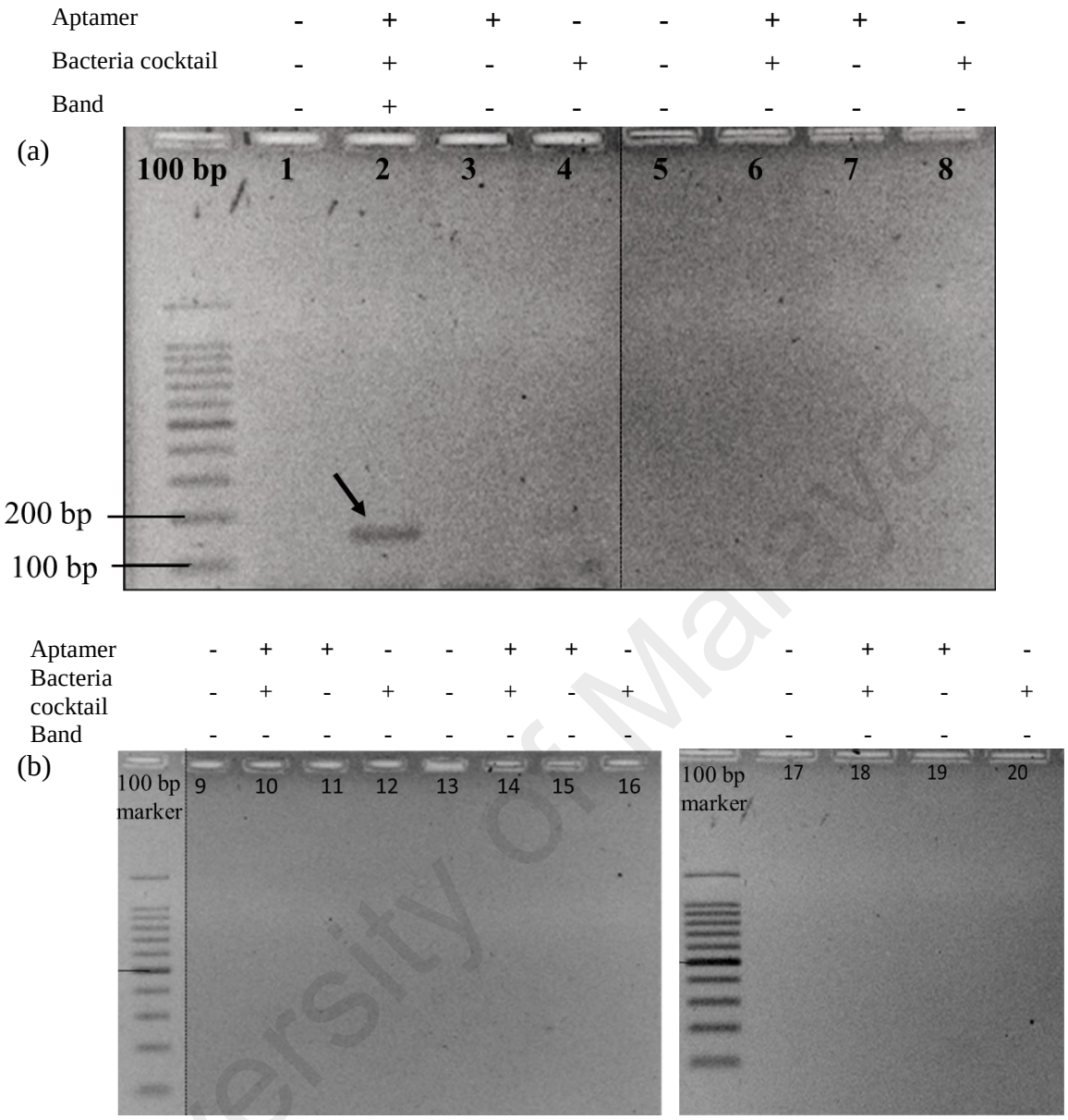


Figure 4.5: The specificity results for AMS - PCR when tested with the bacteria cocktail of *Salmonella*, *E. coli*, *S. aureus*, *Shigella flexneri* and *Vibrio parahaemolyticus*. The PCR was conducted using the same master mix except the primers which were specific for each species. The sign "+" indicates presence and "-" indicates absence of aptamer in magnetic beads, bacterial cocktail as DNA templates and band of DNA amplicon accordingly. (a) Lanes 1-4: used inv A primers for *Salmonella* detection. Lanes 5-8: used primers for *S. aureus*. (b) Lanes 9-12: used primers for *E. coli*. Lanes 13-16: used primers for *Shigella*. Lanes 17-20: used primers for *Vibrio*.

4.1.4 Naturally contaminated food samples

A total of fourteen raw foods purchased from the market including chicken (n = 4), vegetables (n = 8) and beef (n = 2) were analysed with four different approaches (Table 4.3). In approach A, AMS-PCR was used. This method was compared with PCR results of DNA extracted from pre-enriched broth-culture (approach B) and selective broth culture (approach C).

Table 4.3: Summary of the AMS-PCR results of *Salmonella* detection in naturally contaminated food samples by four approaches: [A], [B], [C] and [D**]** (see footnote).

Types of food	Sample number	PCR results (149 bp)				Interpretation
		[A]	[B]	[C]	[D]	
Chicken	1	-	-	+	+	False negative
	2	+	-	+	+	True positive
	3	+	-	+	+	True positive
	4	+	-	+	+	True positive
Vegetables	5	-	-	-	-	True negative
	6	-	-	-	-	True negative
	7	+	-	+	+	True positive
	8	+	+	+	+	True positive
	9	-	-	-	-	True negative
	10	-	-	-	-	True negative
	11	-	-	-	-	True negative
	12	-	-	-	-	True negative
Beef	13	-	-	+	+	False negative
	14	-	-	-	-	True negative

"+" or "-" indicates the presence or absence of *Salmonella* DNA amplicon
[A]: An aliquot of food homogenate → mixed AMS → elute → extract DNA → PCR.
[B]: An aliquot of food homogenate → centrifuged and wash → extract DNA→PCR.
[C]: An aliquot of food homogenate →selective enrichment broth →aliquot for DNA extraction →PCR.
[D]: An aliquot of food homogenate →selective enrichment broth →streaked on selective medium →picked presumptive colonies, purify on the LB→DNA extraction →PCR.

Out of the 14 food samples tested with the AMS-PCR, five were tested as true positives, seven as true negatives and two as false negative. The false negative results were shown in beef and chicken samples respectively. All vegetable samples however,

showed true positives results, indicating that the nature of the food may influence the activity of AMS-PCR.

Based on these result, it shows that AMS method was comparable with the culture conventional method in detecting *Salmonella* in the contaminated food samples. Even though approach B without AMS initial step could reduce the time of detection to one hour, it decreased the sensitivity of the detection. The AMS-PCR (approach A) was able to reduce the time of detection from 2 days (approach C) to 3 days (approach D) into 6 to 7 hours. In approach D, the purple colonies on the Brilliance ™ *Salmonella* Agar (Figure 4.6) were purified and further tested with PCR. The results of approach D which is the conventional and gold standard method for *Salmonella* detection were consistent with approach C even though it took extra one day for detection.

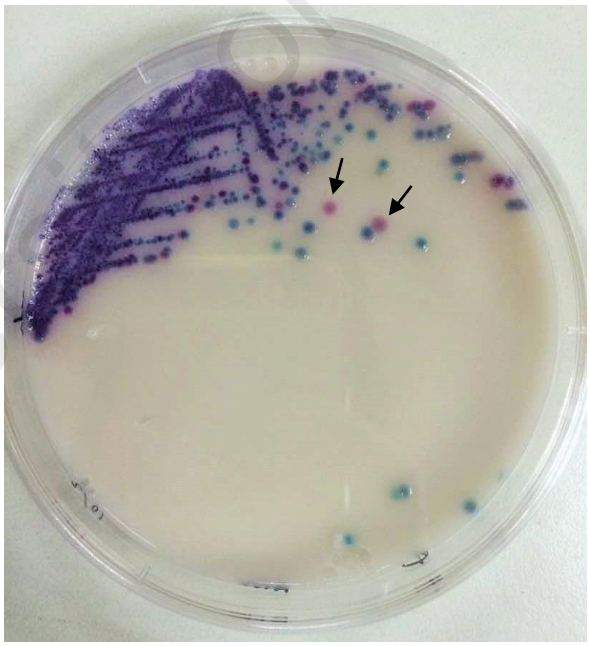


Figure 4.6: Representative image of *Salmonella* purple colonies (arrow) that were observed on the Brilliance ™ *Salmonella* agar. These single colonies would be purified on the LB agar for further DNA extraction and PCR detection.

4.2 PART B: Nanocarbon aptasensor

In the second part of the study, the same aptamer sequence was used as a biological recognition element for biosensor. However, in order to functionalise the aptamer to the electrode platform, the aptamer was modified with amino group at 5' end.

4.2.1 Characterisation of the carbon nanotube aptasensor

Nano-like tubes structure was observed for raw CNTs (Figure 4.7a) The SEM images of the aptasensor with raw CNTs before (Figure 4.7b) and after exposure (Figure 4.7c) to *Salmonella* were captured to understand its morphology. A stable and regular dispersion after electrodeposition of CNT on the surface of ITO electrode was observed. The CNTs coating was not damaged after exposure to *Salmonella* cells.

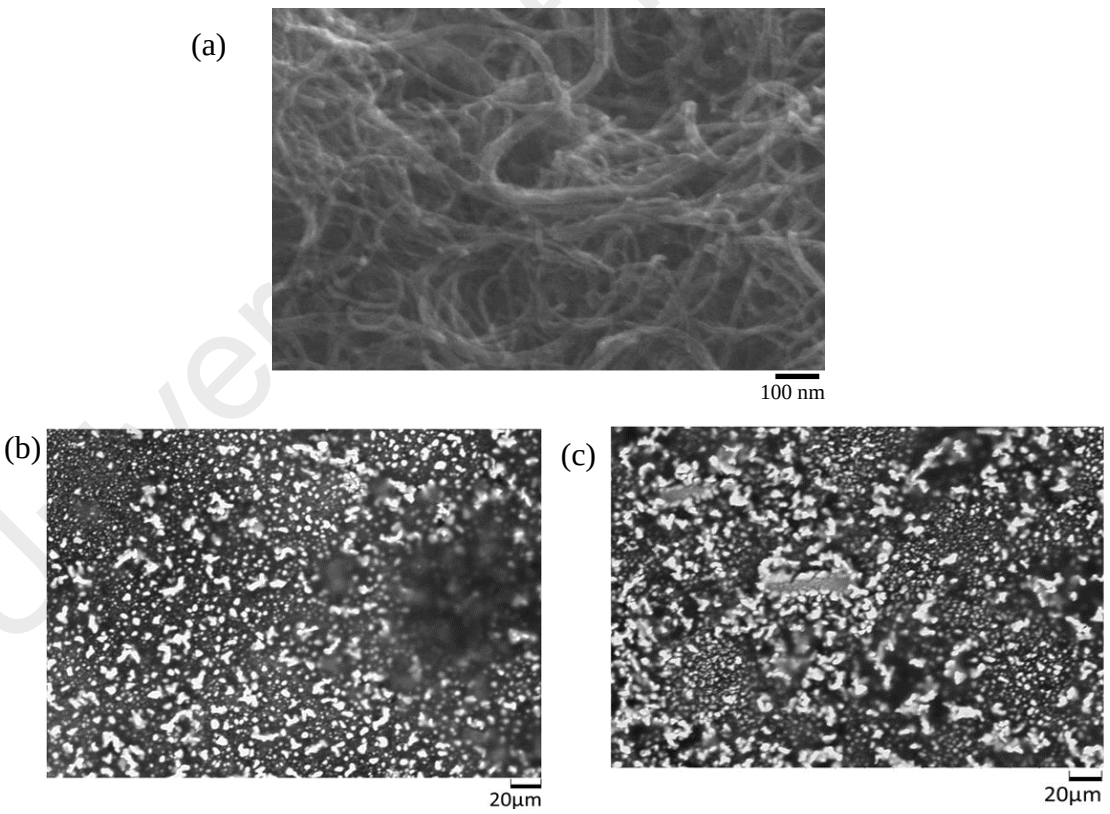


Figure 4.7: Morphology of the aptasensor surface based on SEM image. (a) Raw CNTs. (b) Before and (c) after exposure to *Salmonella* cells.

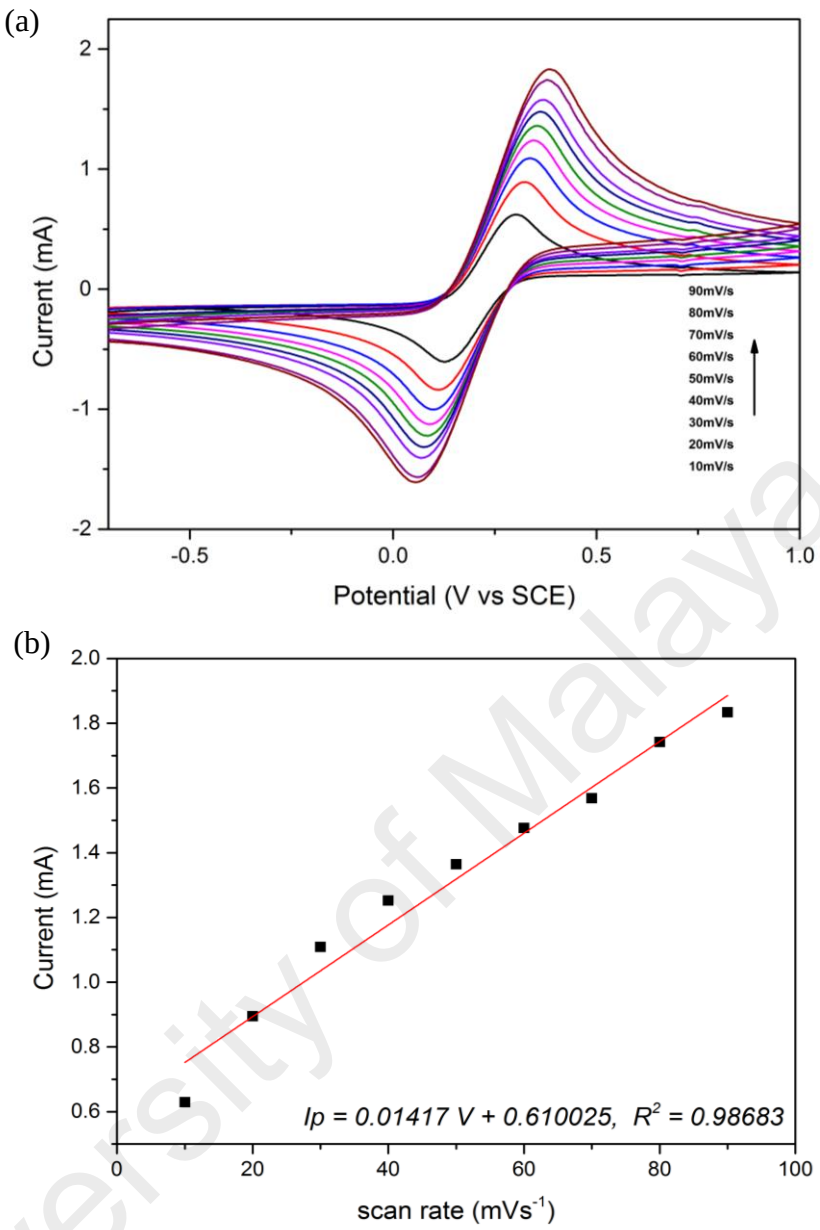


Figure 4.8: The electrochemical characterisation of aptasensor. (a) CV curves for different scan rates ranging from 10 $mV.s^{-1}$ to 90 $mV{\cdot}s^{-1}$. (b) A linear relationship of peak current vs scan rate of aptasensor in 0.1 $mol{\cdot}l^{-1}$ KCl (5 $mmol{\cdot}l^{-1}$ $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$) solutions.

Figure 4.8a shows the CVs of the aptamer/CNTs/ITO electrodes (aptasensor) at different scan rates ranging from 10 $mV{\cdot}s^{-1}$ to 90 $mV{\cdot}s^{-1}$. As the scan rates increased, the peak of the current increased accordingly showing the increment of the electron transfer across the electrolytes. Figure 4.8b indicates straight-line behavior, i.e. linear relationship between peak currents of the electrodes at different scan rates with I_P= 0. 0.01417V + 0.610025 with R^2=0.98683).

4.2.2 The sensitivity tests

To determine the sensitivity of the aptasensor, six different concentrations of *Salmonella* cell suspension were tested ranging from 5.5 $\times 10^1$ to 10^6 CFU/ml. Based on Figure 4.9a, there was a significant change of the peak of the current as the concentrations of *Salmonella* increased.

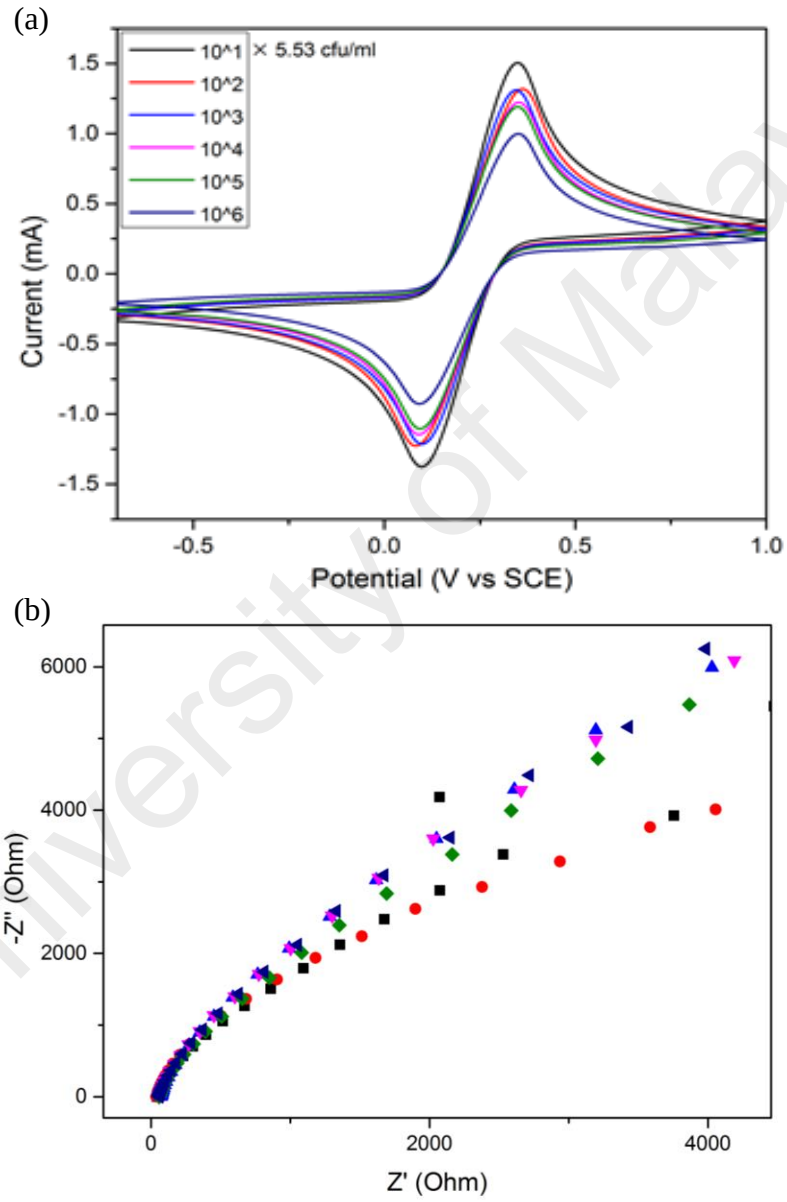


Figure 4.9: The sensitivity test of *Salmonella* by using aptasensor. (a) CV and (b) Nyquist plot of the aptasensor corresponded to different *Salmonella* concentrations (10^1 to 10^6 CFU/ml). As the concentrations increased, impedance increased.

The electrochemical impedance of the aptasensor was also measured by using potentiometry as shown in Figure 4.9b. The results suggest that as the cell concentration increased, there was corresponding increase in the impedance and decrease in the peak of the current. This is shown in the Nyquist plot when semi-arc diameter increased with the increase in the cell concentrations.

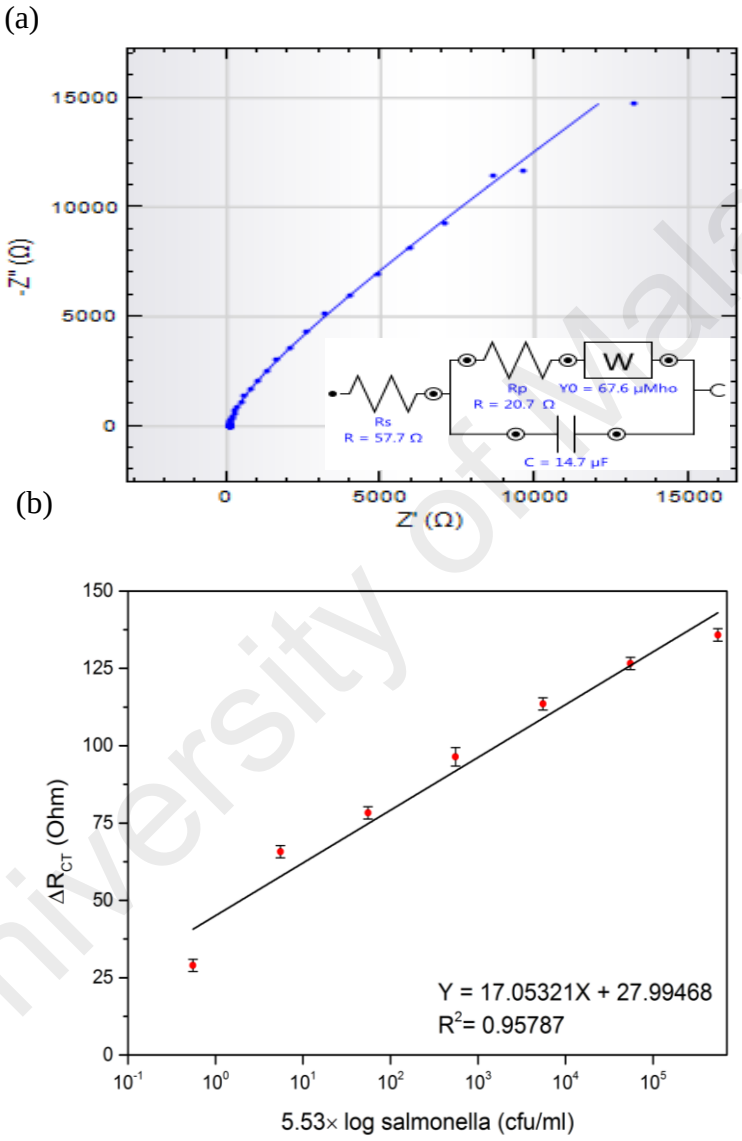


Figure 4.10: Electrochemical impedance spectroscopy of the developed aptasensor**.** (a) Circuit fitting of the Nyquist plot of the aptasensor aptamer/CNT/ITO electrode with the Randles equivalent model (inset) for *Salmonella* at concentration 10^3 CFU/ml in 0.1 mol·l^{-1} KCl (0.1 mol·l^{-1} KCl (5 mmol·l^{-1} $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$) solutions. (b) The linear relationship of the D-value resistance (ΔR_{CT}) to different concentrations of the *Salmonella*.

In order to determine the resistance value of each parallel determination, Randles equivalent circuit model through fitting analysis was used as shown in Figure 4.10a. R_S is the uncompensated solution resistance, R_{CT} (or R_P) is the charge transfer resistance at the aptasensor-electrolyte interface and W is the Warburg impendence. A linear relationship between ΔR_{CT} and the logarithm of the complementary bacteria concentrations ranging from 5.5×10^1 to 5.5×10^6 CFU/ml was plotted as shown in Figure 4.10b. The data demonstrated that the limit of detection of the *Salmonella* was 55 CFU/ml This can be expressed in regression equation, $\Delta R_{CT} = 17.05321 \log C + 27.99468$ where correlation factor = 0.95787. The

4.2.3 The specificity tests

Four different *Salmonella* serovars namely, *S.* Typhimurium, *S.* Paratyphi B, *S.* Typhi and *S.* Paratyphi A and three non-*Salmonella* cells (*S. aureus*, *V. parahaemolyticus* and *E. coli*) were tested for specificity test. Based on Figure 4.11, the current for non-*Salmonella* was higher than *Salmonella* spp.. These results indicate that the aptasensor had higher specificity for *Salmonella* spp. compared to non- *Salmonella* spp..

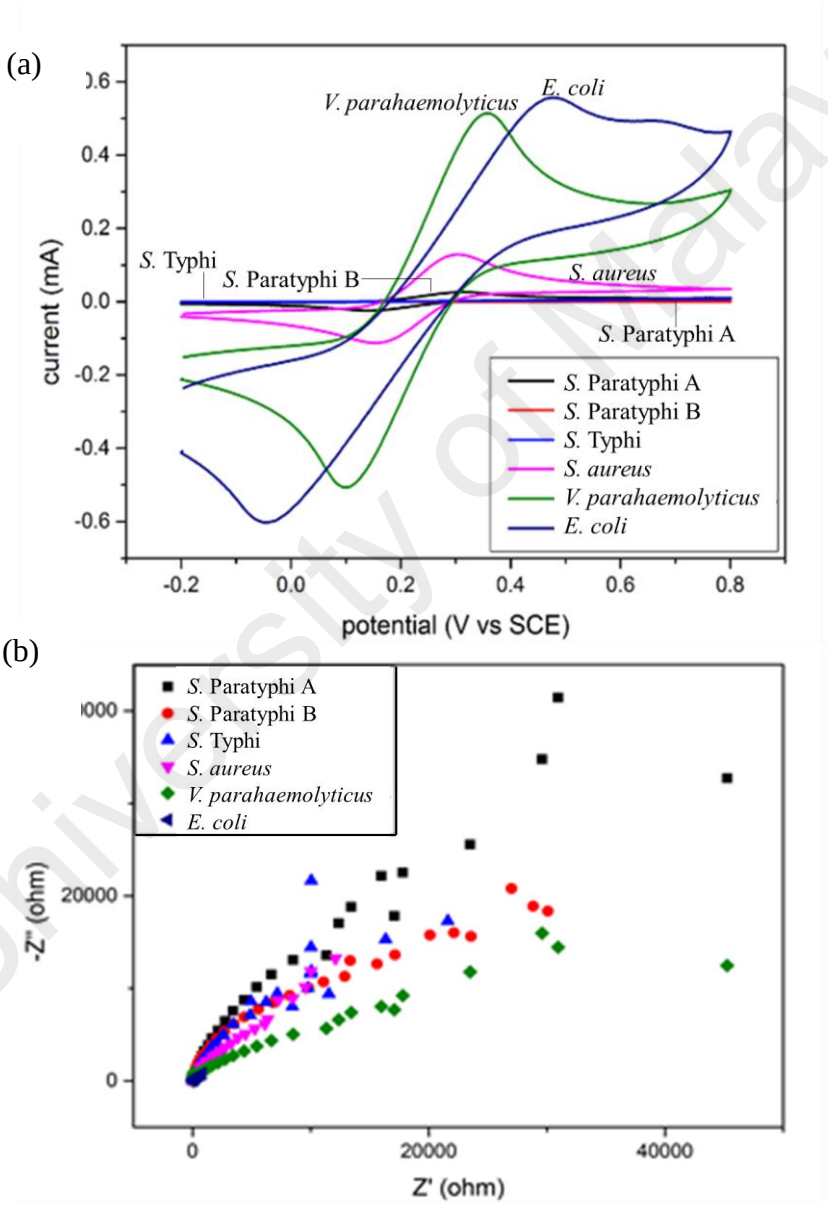


Figure 4.11: The specificity test of the aptasensor binding to *Salmonella* and non-*Salmonella* cells. (a) CV and (b) Nyquist plot of the CNT-aptamer modified electrode corresponded to the same concentration (10^3 CFU/ml) of different bacteria.

CHAPTER 5: DISCUSSION

5.1 PART A: Potential use of aptamer-magnetic separation (AMS) - PCR as an alternative for *Salmonella* detection in food.

Various methods have been developed to increase the specificity, sensitivity and rapidity of foodborne pathogens detection. However, it has been suggested that the upstream preparation is equally important to separate, concentrate and purify the bacteria from a complex food matrix (Suh et al., 2013). The conventional food analysis for instance pre-enrichment and enrichment of bacteria are usually time-consuming. Previously, immunomagnetic separation (IMS) was used to reduce the overall analysis time. However, as IMS is based on the antibody-antigen principle, it is relatively not stable, has limited shelf-life and is expensive (Dong et al., 2014).

In this study, the potential use of the DNA aptamer was evaluated as an alternative for upstream preparation in food testing specifically to separate and concentrate the *Salmonella* from different food matrix. DNA aptamer has been chosen as the biological recognition element in this study due to its high specificity to detect the target organism by forming secondary or tertiary structure upon binding (Fang & Tan, 2010). As the aptamer binds to the whole-cell organism (Joshi et al., 2009), it becomes a great advantage for upstream sample separation because the targeted organism can directly bind to the aptamer without initial DNA extraction step. By using cell-SELEX (Systematic Evolution of Ligands by EXponential enrichment) process, this aptamer will specifically target the outer membrane protein (OMP) of the live *Salmonella* which are expressed on the surface of cells. Unlike ssDNA that only binds to its complementary strand without discriminating the live or dead cells, aptamer can specifically bind to live cells (Fang & Tan, 2010) that are critical for its biological function which can cause salmonellosis. Other than that, this method also eliminates

laborious enrichment steps that are usually necessary for foodborne pathogen detection in foods. An aptamer is not only highly specific like antibody-antigen interaction but it is more stable and binds tightly to the targeted cell epitope due to stem-loop structure formation that is responsible for the biological activity (Park et al., 2014).

Prior to this study, preliminary investigation has been conducted to determine cell count (CFU/ml) by using plate counting experiment. In this experiment, the *Salmonella* cells suspension were adjusted to 0.5 McFarland. The number of colonies for 10^{-5} dilution was in the suitable colony counting range (30 - 300 colonies). Using standard bacteria counting method, it was determined that the concentration of *Salmonella* at 0.5 McFarland was equivalent to the 10^7 CFU/ml. Therefore, 0.5 McFarland was used as initial concentration before the cell suspensions were diluted accordingly.

In this study, the aptamer was modified with biotin at 5' end to complement the streptavidin adapter of magnetic beads via non-covalent bonds. When *Salmonella* was introduced into the biotinylated-aptamer, specific 3D structure of the aptamer would form and bind to the outer membrane protein (Joshi et al., 2009) of the live *Salmonella*. By using magnetic stand, cells/aptamer/magnetic beads complex would be attracted to it and the unbound particles would be eluted. The washing process involved 1 × PBS – 5% Tween 20 buffer that helped to disrupt the hydrophobic and electrostatic interaction between bacteria and the food surface (Goulter et al., 2003; Ukuku & Fett, 2002). A study by Dickson et al. (1989) showed that the presence of the negative charges on the surface of the *Salmonella* cells was related to the attachment and interaction to the muscle and fat cells of the meat samples. Another study by Calicioglu et al. (2002) demonstrated that the effectiveness of using 5% Tween 20 in pre-spraying would reduce the number of the *E. coli* O157:H7 attached to the surface. This is because Tween 20 would interfere by lowering the surface tension properties thereby influenced its

hydrophobicity and attachment of the bacteria in the complex food environment. Therefore, the use of Tween 20 helped to detach the bacteria cells from complex food matrix; thus, concentrating and facilitating bacterial elution process. This is known as separation process.

The subsequent detection was conducted by using conventional PCR because PCR is known as the rapid detection tool that can amplify small amount of targeted DNA with high throughput. InvA primers were confirmed to amplify the *invA* gene using sequencing technology. Even though invA primers have been used in other studies (Salehi et al., 2005), the sequence and fragment used to amplify were different. *InvA* is coded invasion protein of *Salmonella* that is essential for invasion to the epithelial cells (Ginocchio & Galan, 1995; Salehi et al., 2005). The important feature of pathogenic *Salmonella* is it invades the cells that are nonphagocytic. This gene is present in most, if not all, of the pathogenic *Salmonella* and absence of this gene seems to be rare (Malorny et al., 2003). However, this method could be improved by using more sensitive downstream detection for example real-time PCR.

Thus, this study showed a proof-of-concept of this technique by eliminating the time-consuming step of conventional enrichment method and introduced a rapid method by combining AMS for upstream sample preparation followed by PCR for detection of *Salmonella* spp..

Typically, the infectious dosage of *Salmonella* in human is within 100 organisms (D'Aoust, 1985) to 10^5 organisms even though it can vary based on the infected population for example environment, immunity, age and illness as well as pathogenicity of the bacteria (Hara-Kudo & Takatori, 2011). When bacterial suspension was subjected to AMS, the sensitivity of the PCR detection increased (100 CFU/ml) unlike PCR without initial AMS step (10^3 CFU/ml). It showed that AMS-PCR was able to detect the

Salmonella within pathogenicity dosage with high sensitivity as compared to without AMS step. This is because the high affinity of the aptamer was able to separate and concentrate the targeted *Salmonella* cells which contributed to the higher sensitivity of detection and decreased the loss of the bacteria in the sample. These upstream separation and concentration steps are important to ensure maximum number of targeted bacteria is recovered for final detection especially in application of real food samples. In recent years, many researches have been conducted to improve the upstream pre-treatment step which is critical to replace laborious and conventional pre-enrichment and enrichment steps. For example, the development of the bacteriophage coupled with PCR to separate *E. coli* from the other bacterial cocktail had shown the same sensitivity as AMS-PCR method (10^2 CFU/ml) (Wang et al., 2016). This method was able to detect viable *E. coli* within 3 hours. Other than that, IMS has been used for a similar application to detect *Alicyclobacillus* strains in apple juice (Wang et al., 2013; 2014) with different downstream detections, for example real-time PCR and ELISA. These studies showed that real-time PCR had limit of detection (LOD) 10 CFU/ml, while LOD for ELISA was 10^5 CFU/ml. Even though the similar IMS procedures were used for upstream separation and concentration, the downstream detection is equally important to increase the sensitivity of the detection.

In order to determine the specificity of the aptamer, 10 different *Salmonella enterica* serovars and non-*Salmonella* cells were tested. Based on the results, this aptamer had high specificity as it was able to detect a wide range of *Salmonella* serovars and it did not bind to non-*Salmonella* such as *E. coli, S. aureus, Shigella* and *Vibrio* which are commonly present in food. This would be a great advantage to detect broad range of *Salmonella* serovars thus contributing to early and fast detection in food safety management. Similar specificity results were observed in other studies by using the same aptamer sequence but with different modification and platform (Ma et al., 2014;

Yuan et al., 2014). Our results also showed that bare magnetic beads alone did not influence detection of *Salmonella*. This indicates the important role and specificity of the aptamer for detection by using AMS.

One of the main concerns of foodborne pathogens detection is the ability to eliminate the food inhibitors in complex food matrix which may influence the sensitivity and ability of the detection assay (Jenïkovâ et al., 2000). Besides that, the targeted foodborne pathogens need to be separated and concentrated from complex mixtures. Therefore, the ability of the AMS-PCR to detect the *Salmonella* spp. in the naturally contaminated food samples was tested. Using AMS, targeted bacteria were expected to be separated from the complex environment of food including non-target microbiota and food ingredients for example, fats, protein, divalent cations and phenolic compounds that may act as inhibitors (Brehm-stecher et al., 2009). This will decrease the time of detection from days to hours which is important in foodborne outbreak.

Different types of naturally contaminated foods were tested including chicken, vegetables and beef. AMS-PCR showed comparable results with the conventional culture method as a gold standard, thus demonstrating its reliability to specifically detect *Salmonella*. As AMS helped to concentrate the targeted bacteria in the initial separation process prior to detection, it helped to reduce the time of detection from 2 to 3 days of conventional method into 6 to 7 hours only, which is the key advantage of this method. While conventional method is too laborious and involves multiple steps, AMS-PCR offers simple detection with high selectivity.

Nevertheless, the discrepant result for two samples (false negatives) in chicken and beef respectively could be attributed to the nature of the food complex itself. For instance, high fat content in the beef caused the separation process of the bacteria to the sample food matrix to be difficult. A high amount of the food particles observed in the

both chicken and beef samples was stuck to the magnetic beads and caused the failure of the magnetic beads recovery. Unlike beef and chicken samples, in vegetable samples, bacteria were removed from the vegetable surface without producing too much debris, thus did not interrupt the interaction of aptamer-magnetic beads to the targeted bacteria during separation process. Besides, the carry-over particles in chicken and beef may affect the efficacy of the PCR amplification due to the competitive environment between bacteria and complex environment of the food especially food inhibitors (Stevens & Jaykus, 2004). Overall, the AMS is applicable for upstream sample preparation before detection method is conducted and suitable as an alternative to conventional cultural method.

5.2 Part B: The evaluation of aptamer-based nanocarbon biosensor

Since the tested aptamer showed its high potential to be used for bacteria detection, it was further explored and incorporated in a biosensor as a biological recognition element. Recently, DNA aptamers have been widely used in biosensor applications including foodborne detection (Liu & Zhang, 2015) and biomedical applications (Zhou et al., 2014). Compared to other detection methods, aptamer-based biosensor is considered as rapid and sensitive detection. The development of this biosensor needs an integrated knowledge of biology, nanotechnology and chemistry. So far, many aptamers have been developed against foodborne pathogen for example *Salmonella* spp. (Duan et al., 2013; Dwivedi et al., 2013; Joshi et al., 2009), *Campylobacter jejuni* (Dwivedi et al., 2010), *Listeria monocytogens* (Duan et al., 2013a; Suh & Jaykus, 2013) *Shigella dysenteriae* (Duan et al., 2013b), *Vibrio parahemolyticus* (Duan et al., 2012), *E. coli* (Kim et al., 2013) and *Staphylococcus aureus* (Cao et al., 2009; DeGrasse, 2012). Other than highly specific aptamer, nanomaterials that acts as sensor surface in biosensor is also one of the important components to provide highly specific, fast response, sensitive and low cost biosensor.

In this study, the electrochemical detection of the *Salmonella* spp. was explored. Carbon nanotubes (CNTs) were used as the transducer layer of this biosensor. They were deposited on the surface of ITO glass as an electrode. The detection was based on the recognition and specific binding between two biorecognitions, such as the *Salmonella* aptamer and targeted *Salmonella*. Therefore, ITO/CNT electrode was incubated with the *Salmonella*-specific aptamer for immobilisation, namely apatsensor. This aptasensor was immersed in the different concentration of *Salmonella* cells suspension. The electrochemical signal of the bioaffinity electrochemical sensor was measured by electrochemical impedance spectroscopy (EIS) measurement.

CNT which has seamless cylindrical shape (Figure 4.7) was chosen due to its high sensitivity, speedy response, simple and easy operation as well as favourable portability (Jacobs et al., 2010). Most importantly, CNTs has high conductivity, chemically stable and mechanically strong for biosensor application. In addition, CNTs can enhance and promote electron transfer between the biorecognition element due to its small size, specific surface area and extraordinary electrochemical properties (Pandey et al., 2008). In this study, as CNT has a wide surface area, it promotes the immobilisation of a vast number of binding sites for the aptamer. During the electrode fabrication process, all the impurities for example catalyst nanoparticles (nickel, carbon, iron), amorphous and defective carbon that might contribute to the electro-catalytic effects (Yang et al., 2010) were successfully removed (results not shown).

After the purification process, the CNT was deposited onto the surface of the ITO followed by aptamer immobilisation. Covalent bonding of surface activation method with EDC/NHS was applied by using physical absorption immobilisation technique. This aptamer which was bound to the carboxyl-rich CNT/ITO substrates formed strong π- π interactions between aptamer and hexagonal carbon structures. It also formed

amide bonds via amine group in 5' aptamer and carboxylic acid group of the CNT. The study of fabricated electrode (Figure 4.8) showed that as scan rates increased, the peak of the currents increased. The linear relationship between these two variables shows the surface-confined process of the fabricated electrode of ITO/CNTs/ aptamer as suggested by Jia et al. (2016). Other than that, the formation of self-assemble monolayers (SAM) of the amino-modified aptamer onto the surface of the ITO/CNT electrode had reduced its current, indicating the successful binding of the aptamer to the surface of the electrode. Other study had used the similar method for aptamer immobilisation with the use of SAM for example, *Salmonella* Typhimurium detection-based poly[pyrrole-co-3-carboxyl-pyrrole] copolymer aptasensor (Sheikhzadeh et al., 2016). Other than using carbodiimide covalent binding to the active surface via EDC/NHS mixture, immobilisation of the aptamer includes attachment of the self-assemble of organised monolayers of thiol-modified aptamer to the gold transducer. This method has been previously described in the detection of *Staphylococcus aureus* (Jia et al., 2014) and *Salmonella* (Ma et al., 2014). Biotin-avidin coupling method is another way to link the biotinylated aptamer to the avidin coated surface as described by Bonel et al. (2011) in which paramagnetic microparticles beads (MBs) were functionalised with biotinylated aptamer prior to their localisation onto the surface of the screen-printed carbon electrodes (SPCEs). This aptasensor targeted the ochratoxin contaminants in wheat samples. As aptamer can be chemically synthesised; thus, maintaining product quality, it can also be modified and labelled based on its platform and purpose within the sequence.

To test the sensitivity of the ITO/CNTs/aptamer aptasensor, different concentrations of *Salmonella* were incubated. As the concentration increased, the impedance increased. This is because, the *Salmonella* cells were specifically attached to the aptamer causing the current flow to decrease. The negatively charged bacteria of the *Salmonella* caused

the repulsion to the redox probe anion, $[Fe(CN)_6]^{3-/4-}$ and hindered the transfer of the electron from the electron mediator solution, $[Fe(CN)_6]^{3-/4-}$ to the electrode. Impedimetric is correlated to the binding of the analyte to the biosensor surface (Ahmed et al., 2014). It is recorded by using resistance (R) and capacitance (C) in order to characterise the biological system.

As the concentration of *Salmonella* increased, it retarded the electron transfer to the surface of the electrode and generated higher resistance that was reflected in larger arc on the Nyquist plots as shown in Figure 4.9. When impedance (R_{CT}) was plotted against the bacteria cell concentration, linear relationship was observed with regression equation, $y = 17.05321x + 27.99468$, where y is R_{CT} and x is concentration of *Salmonella*. The resistance value of each parallel determination was obtained through fitting procedure. Through fitting analysis, ΔR_{CT} exhibited a linear range in bacteria cell concentration ranging from 5.5×10^1 to 5.5×10^6 CFU/ml. R_{CT} was used to monitor the binding event of the aptasensor. The limit of detection (LOD) of this aptasensor was determined by the lowest CFU of the bacteria that could be detected and therefore, the LOD was 55 CFU/ml.

To ensure the aptasensor is specific to *Salmonella enterica*. only, it was compared with different *Salmonella enterica* serovars and different bacteria. The EIS results in Figure 4.11 show the aptasensor was specific to *Salmonella entrica* only and did not interact with the non-*Salmonella* species. It means, the aptasensor had no cross-reactivity with other bacteria which is crucial to ensure only targeted bacteria were detected. This result was confirmed and consistent with the results in AMS in Part A.

Other than being highly sensitive, specific and stable, fast detection is one of the requirements for ideal bacteria detection for biosensor (Ahmed et al., 2014). In this study, *Salmonella* could be detected within one hour which is faster than other

conventional culture method (2 to 3 days) and molecular-based method, for example PCR (3 to 4 hours). Aptasensor detection does not require additional post detection steps; thus, reducing the time taken for detection. This rapid detection allows early diagnostic and more effective detection during an emerging outbreak; thereby, preventing full-blown infection that may lead to mortality.

In the future, this aptasensor should be tested with real food samples for field application. Besides, combination of the AMS and the aptasensor may be evaluated to speed up the detection process which will eliminate hours of enrichment steps during upstream sample preparation as well as speed up the downstream foodborne pathogens detection. The combination of these methods not only allows fast detection, it will also increase the specificity of the detection and eliminate the inhibitors that may affect the sensitivity and reaction of the aptasensor.

CHAPTER 6: CONCLUSION

To conclude, in the first part of the study, a highly specific, sensitive and rapid aptamer magnetic separation (AMS) for upstream sample preparation followed by final detection of PCR was successfully developed. AMS helped to separate, purify and concentrate the *Salmonella* cells in the food matrix. This method was able to detect as low as 100 CFU/ml of *Salmonella enterica* in various food samples including vegetables, chickens and beefs. It also showed high specificity for pan-*Salmonella* detection with no cross-reactivity with other common foodborne pathogens. It could reduce detection time from 2 to 3 days by using standard gold of conventional cultural method to 6 to 7 hours. This method has been proven to facilitate the upstream sample preparation in foodborne pathogen specifically in *Salmonella*.

In second part of this study, by using the similar aptamer as biological recognition element, an aptamer-based nanocarbon biosensor, aptasensor was evaluated. Due to the CNT properties and specificity of the DNA aptamer, it allowed the aptasensor to detect *Salmonella* with a limit of detection 55 CFU/ml within one hour. It also showed high specificity towards *Salmonella enterica* with no cross-reactivity with other foodborne pathogens. This finding was consistent with first part experiment of AMS-PCR detection. This study also showed a specific, rapid and effective aptasensor could be developed from CNT with amino-modified DNA aptamer by using electrochemical approach.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publication;

1. Hassan, M.D.. Pulingam, T., Appaturi, J. N., **Zifruddin, A. N**., The, S. J., Lim, T. W., … Thong, K. L. (2018). Carbon nanotube-based aptasensor for sensitive electrochemical detection of whole-cell *Salmonella*. *Analytical Biochemistry, 554*, 34-43.

Conferences and presentations:

1. **Zifruddin, A.N.** & Thong, KL. (2016, Disember). Development of aptamer-based nanocarbon biosensor for *Salmonella* spp. detection. Presented in Biological Sciences Graduate Congress (BSGC), University Malaya, Kuala Lumpur, Malaysia.

2. Thong, K.L. **Zifruddin, A. N**. Hassan M. R. & Abd- Hamid, S.B. (2016, October) Potential used of aptamer-based nanocarbon biosensor for *Salmonella* detection. Presented in 4th Asia Pacific International Food Safety Conference and 7th Asian Conference on Food and Nutrition Safety 2016, Pulau Pinang, Malaysia.