IMMUNOPROTEOMIC ANALYSIS AND SERODIAGNOSIS OF *Salmonella enteretica* Typhi AND INVASIVE NON-TYPHOIDAL *Salmonella* INFECTIONS USING DIFFERENTIALLY EXTRACTED WHOLE CELL BACTERIAL PROTEIN

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2023

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by

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Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

April 2023

ACKNOWLEDGEMENTS

BISMILLAHIRAHMANIRAHIM.

First and foremost, praises and thanks to Allah SWT for giving me the strength, knowledge, and opportunity to undertake this study and complete it satisfactorily. I would like to express my deepest gratitude and appreciation to my research supervisor, Associate Prof. Dr Zainoodin bin Sheik Abdul Kader, for giving me the opportunity to do research and imparting a great deal of knowledge throughout this research. I am thankful for his support and endless guidance throughout the whole project. I am glad to have learnt and experiences a lot of new things throughout this project. I also thanks my co-supervisor, Dr Tuan Noorkorina binti Tuan Kub, for her assistance extended to me throughout my study period. I would like to thank the postgraduate students; Mr. Naim, Kak Feeda, and Kak Ai'shah for their kindness in helping me in materials preparation and handling instruments for the past three years. Special thanks to my labmates and friends; Kak Ida, Kak Auni and Syasya for their encouragement and concerns for me in completing my research project. Finally, I am very thankful to my parents; Abdul Lateef Khan and Ismai Meera, for their unwavering moral support and valuable prayers.

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LIST OF ABBREVIATIONS

AUFI	Acute undifferentiated febrile illnesses
AP	Alkaline phosphatase
BMA	Bone Marrow Aspirate
CBB	Coomassie brilliant blue
CSP	cell surface protein
dH	flagellar antigens
HIV	Human immunodeficiency virus
IEF	Iso-electric focusing
IFN-y	gamma interferon
IgM	Immunoglobulin M
IgG	Immunoglobulin G
IgA	Immunoglobulin A
iNTS	invasive non-typhoidal *Salmonella*
kDa	kilodalton
MDR	multi-drug resistance
NC	Nitrocellulose membrane
NO	nitric oxide
NPV	negative predictive value
O9	somatic antigens
***S*.Typhi**	*Salmonella* Typhi
***S*.spp**	*Salmonella* species
PCR	Polymerase chain reaction
PHS	Pool healthy sera
PHPS	Pool healthy positive sera
PTS	Pool typhoid sera
PVS	Pool Vaccinated sera
POS	Pool other disease sera
pI	Isoelectric point
PPV	positive predictive value
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sdWCP	surface depleted whole cell protein

SPB	sample preparation buffer
SPIs	*Salmonella* pathogenicity islands
TNF-a	tumor necrosis factor alpha
TB	Tuberculosis
TBS	Tris-buffered saline
WCP	whole cell protein
WB	Western blot
XDR	Extremely drug resistant

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ANALISIS IMMUNOPROTEOMIK DAN SERODIAGNOSIS JANGKITAN *Salmonella enteretica* Typhi DAN INVASIF S*almonella* BUKAN TIFOID MENGGUNAKAN KESULURUHAN SEL PROTEIN DENGAN KAEDAH PENGEKSTRAKAN BERBEZA

ABSTRAK

Demam tifoid ialah penyakit berjangkit yang disebabkan oleh bakteria, *Salmonella* Typhi dan boleh membawa maut jika tidak dirawat. Penyakit ini endemik di negara-negara membangun tetapi bilangan kes yang membimbangkan yang dikaitkan dengan sumber domestik telah dilaporkan di negara-negara perindustrian. Manifestasi klinikal demam tifoid tidak spesifik dan mirip dengan penyakit demam akut yang tidak dapat dibezakan (AUFI). Oleh itu, pengenalpastian pencilan yang tepat diperlukan untuk intervensi epidemiologi yang berkesan. Penghasilan ujian diagnostik yang cepat dan kos efektif juga diperlukan untuk memberikan rawatan awal bagi menyelamatkan nyawa. Oleh itu, analisis perbandingan dilakukan untuk pencirian profil protein dua serovars *Salmonella* yang diekstrak secara berbeza dengan kaedah SDS-PAGE. Pengekstrakan protein kepada tiga ekstrak yang berbeza mendedahkan profil protein yang dapat membezakan antara kedua-dua strain dan boleh digunakan untuk mengenal pasti serotip *Salmonella*. Antigen yang diekstrak secara berbeza dari *S*.Typhi dan *S*.spp telah digunakan untuk menghasilkan ujian enzim immuno titik (dot EIA) untuk diagnosis demam tifoid. Prestasi ujian adalah menggalakkan dengan sensitiviti dan spesifisiti yang tinggi. Berikutan kejayaan penghasilan ujian TYPHOIDYNE μSpot M-Ag Arrays EIA, antigen yang diekstrak secara berbeza dicirikan lagi dengan kaedah 'Western blot' (WB). Pencirian dengan kaedah WB mendedahkan pengecaman corak dinamik antara antigen yang diekstrak secara berbeza dan berjaya membezakan pesakit tifoid daripada subjek yang berkemungkinan

mempunyai sejarah demam tifoid. Seterusnya, protein yang diekstrak secara berbeza daripada *S*.Typhi dan *S*.spp dipecahkan dengan kaedah pemfokusan isoelektrik (IEF) dan dicirikan melalui SDS-PAGE diikuti dengan immunoblot. Analisis proteomik SDS-PAGE pecahan protein tulen diikuti dengan prosedur pewarnaan berganda mendedahkan pelbagai protein yang terdapat dalam jumlah yang sikit dan banyak dengan berat molekul yang berbeza di semua pecahan. Kesemua pecahan dicirikan dengan kaedah dot EIA untuk mendapatkan diagnosis yang tepat dengan sensitiviti yang dipertingkatkan untuk demam tifoid dan *Salmonella* invasif bukan tifoid (iNTS). Corak gerak balas imun dinamik yang membezakan antara demam tifoid dan penyakit iNTS telah diperoleh. Daripada seratus dua puluh pecahan protein, empat belas antigen dikenal pasti sebagai penanda biologi prospektif untuk penghasilan diagnosis pembezaan dan definitif demam tifoid dan penyakit iNTS dalam satu ujian. Empat belas pecahan antigen yang dipilih tertakluk kepada pencirian lanjut dengan kaedah WB. Kajian perbandingan telah dijalankan antara serum pesakit tifoid yang disatukan (PTS) dan serum pesakit iNTS yang dikonjugatkan dengan isotype antibodi IgM, IgG, dan IgA untuk memetakan keantigenan pecahan terpilih. Keputusan WB menunjukkan bahawa protein imunreaktif yang ditemui dapat membantu membezakan antara peringkat akut dan konvalesen demam tifoid. Kesimpulannya, penyelidikan ini amat berguna untuk mewujudkan immunocerakin serologi kedua untuk diagnosis pelbagai penyakit Salmonella dalam satu ujian. Selain itu, ia juga membina asas yang amat berguna untuk mengenal pasti komponen antigenik untuk pembangunan vaksin universal dengan perlindungan terhadap demam tifoid dan penyakit iNTS.

IMMUNOPROTEOMIC ANALYSIS AND SERODIAGNOSIS OF *Salmonella enteretica* Typhi AND INVASIVE NON-TYPHOIDAL *Salmonella* INFECTIONS USING DIFFERENTIALLY EXTRACTED WHOLE CELL BACTERIAL PROTEIN

ABSTRACT

Typhoid fever is a potentially fatal infectious disease caused by *Salmonella* Typhi. The disease is endemic in developing countries, but alarming number of cases linked to domestic sources have been reported in industrialized countries. The clinical presentation of typhoid fever is highly variable and often overlap with other acute undifferentiated febrile illnesses (AUFI). Hence, accurate identification of isolates is important for effective epidemiological intervention. The development of rapid, cost-effective diagnostic test is also crucial for early life saving treatment. Therefore, comparative analysis was performed to characterize protein profiles of two differentially extracted *Salmonella* serovars by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Separation of protein into three different extractions revealed discriminative protein profiles between the two strains which provided an effective adjunct that can be used as a tool for protein serotyping. A standardized protocol of the dot Enzyme Immunoassay (dot EIA) with differentially extracted antigens derived from *S*.Typhi and *S*.spp was established for definitive and differential diagnosis of typhoid fever. The performance of the test was encouraging with high sensitivity and specificity. Following the successful development of 'TYPHOIDYNE μSpot M-Ag Arrays, the antigenic components of the differentially extracted proteins were further characterized by western blot (WB) assay. WB characterization revealed a dynamic pattern of recognition between the differentially extracted antigen that successfully discriminated typhoid patients from healthy

subjects with possible exposure to typhoid fever. Subsequently, the differentially extracted proteins derived from *S*.Typhi and *S*.spp were fractionated by liquid phase preparative isoelectric focusing (IEF) system and characterized by SDS-PAGE followed by immunoblotting. SDS-PAGE proteomic analysis of highly purified protein fractions coupled with double staining procedure revealed a range of low abundant and high abundant protein with varied molecular weight across all fractions. To obtain a differential and definitive diagnosis with enhanced sensitivity for both typhoid fever and invasive non-typhoidal *Salmonella* (iNTS), the fractions were subjected to immunological characterization by dot EIA. A dynamic pattern of immune response discriminating between typhoid fever and iNTS disease was observed. Out of the hundred twenty protein fractions, fourteen antigens were identified as prospective biomarkers for development of definitive and differential diagnosis of typhoid fever and iNTS disease in a single test. The fourteen selected antigens were subjected to further characterization by WB assay. Comparative study was carried out between pooled typhoid sera (PTS) and iNTS serum that were conjugated with IgM, IgG, and IgA antibody isotypes in order to map the antigenicity of the selected fractions. The WB results suggest that the discovered immunoreactive proteins could help differentiate between the acute and convalescent stages of typhoid fever. In summary, this study would clearly be relevant for the development of second serologic immunoassay for detection of multiple *Salmonella* diseases in a single test. Elucidation of the antigenic components for development of universal vaccine with protection against typhoid fever and iNTS disease would be other spin off.

CHAPTER 1

GENERAL INTRODUCTION

1.1 History of typhoid fever

Typhoid is well-known as an ancient re-emerging infectious disease (Vanderslott and Kirchelle, 2019). Back in the days of Hippocrates, in the Greek Mythology, people believed that typhoid disease associated with fatal high fever with death was responsible for the plague during the war of Athens. The word typhi originated from the Greek word 'Typhos' which means smokes, haze, or stupor (Bawa, 2010). In the year 23 BC, a Greek botanist who is also the roman physician for Emperor Augustus became famous as he was honored for curing this illness of the emperor by cold compresses (Bawa, 2010).

Typhoid fever was first clinically described by Thomas Willis in 1659. In 1782, Huxham gave the first clinical differentiation of the disease when he described putrid typhus (febris putrida) and slow nervous fever (febris nervosa lenta) which later known as typhoid. In 1829, Pierre Louis identified successfully pioneered the term 'typhoid fever' when he identified the lesions on the abdominal lymph nodes of patients who died from "gastric fever". In 1869, William Bud specified the disease as a water-borne disease (Bawa, 2010). German and English bacteriologists, which include Robert Koch also confirmed and verified Eberth's findings. Despite the contribution by many scientists to the quest, the genus "*Salmonella*" was named after an American veterinary pathologist, Daniel Elmer Salmon who leaded the USDA research program (Marineli et al., 2013). A bacillus suspected to cause cholera was identified by Salmon and Smith in 1885. This bacillus was then identified to belong in the typhoid group and hence designated as *Salmonella* Typhi. In 1896, the first vaccine was made by German scientist, Richard Pfeiffer and Wilhelm Kolle where they demonstrated on

how immunity against typhoid fever was discovered when inoculated with killed typhoid bacteria. On the same year, George Fernand Isidore Widal and Siccard coined out a diagnostic method for the disease by agglutination reaction. Therefore, the serological test known as Widal's agglutination test was pioneered. The Vi antigen associated with virulence of the bacteria was also described by Felix and Pitt. In 1903, three methods were pointed out by Robert Koch for controlling typhoid disease which were improving handling of sewage, disinfect excrete at its source and quarantine or isolate infected patients until they are tested negative for the presents of this bacillus. Despite that, there was still high increase in case fatality until the year 1948 when Theodore Woodward and his partners reported the use of chloromycetin to sterilize blood cultures in typhoid patient. This marked as the first era on the use of antibiotics for treatment of typhoid fever (Qadri and Ayub, 1989).

Typhoid fever became a known pandemic in the 1900s when about 3000 New Yorkers were infected through a well-known first documented healthy carrier, Mary Mallon who worked as a cook. She became the main source of infection, and condemned as "Typhoid Mary" when many people around her started to be infected with typhoid fever, even causing death to some (Marineli et al., 2013). Another asymptomatic carrier that caused a pandemic was a cowman or a milker known as Mr. N. This carrier alone had caused an outbreak of 205 cases in southeast England from the year 1899 to 1909.

In 1902, German scientist, Robert Koch introduced the concept of asymptomatic carrier of infectious disease. Koch also expressed in his typhoid research papers on how his discovery from the study of malaria epidemic in New Guinea proofed the concept of carriers triggering an epidemic also applies on typhoid cases as could be seen in the case of Mary Mallon (Gradmann et al., 2019).

1.2 Global epidemiology of typhoid fever

Low income and under-developed countries with poor hygienic condition and polluted food or water have higher chances of typhoidal infection. Whereas, in higher income country, typhoid infection is usually due to travellers coming from endemic countries. In the year 2000, *S.*Typhi has caused up to 216,000 deaths and up to 21 million illnesses worldwide (Ajibola et al., 2018). Based on the surveillance report until the year 2000, typhoid fever has been observed as the major infectious disease-causing death in Pakistan, sub-Saharan regions of Africa and Indian subcontinent and Southeast Asia (Crump et al., 2004).

In Sub-Saharan Africa, fever is one of the most frequently occurring symptom that led people to seek for healthcare services. The resembling clinical presentations of acute undifferentiated febrile illness (AUFI) such as Malaria in Sub-Saharan Africa with typhoid fever create a diagnosis dilemma for this disease. Although typhoid fever can be ruled out by bacteria isolation, limited resources in most areas of the country has caused a major challenge in development of rapid and low-cost diagnosis for typhoid fever (Crump, 2012).

Certain factors can increase the risk of typhoid fever, such as residing in larger households, living near water bodies, lower economic status, and lower literacy rates. In India, prevention and control measures are crucial in reducing the incidence of typhoid fever and diagnosing cases at an early stage. However, the country faces challenges due to limitations in resources and the unavailability of detecting typhoid carriers (Kanungo et al., 2008). To mitigate the risk of typhoid fever, it is important to prioritize efforts in providing safe drinking water, improving sanitation and hygiene practices, and increasing vaccination coverage. Early diagnosis and treatment are also crucial in preventing the spread of the disease.

1.3 Epidemiology in Malaysia

Incidence of typhoid fever in Malaysia has been reported since the 1900s. In 1988, there were 10.2 -17.9 typhoid cases out of 100,000 population per year from 1978 to 1980 (Yap and Puthucheary, 1998). Due to the highest number of typhoid cases reported between 1998 to 1999 especially among children, Kelantan has become the center of typhoid epidemiology research in Malaysia (Malik and Malik, 2001). From a population study in 2013, it has been reported that most of the typhoid cases were from the area of Kota Bharu which is the capital of the state. This could be due to high population in the capital compared to other area (Penido et al., 2013). The Ministry of Health also reported that the number of cases from 2001 to 2007 were 1.89 to 4.10 per 100,000 population which showed tremendous improvement from the 1900s.

Flood risk areas with contaminated water source were identified to have higher rate of typhoid incidence (Shah et al., 2012). Kelantan together with Sabah have been reported to be the major contributor to the statistic of typhoid cases in Malaysia (Kementerian Kesihatan Malaysia, 2015). Data from Ministry of Health Malaysia (2015) also showed a tremendous increment of cases especially in Sarawak and Johor. Based on the investigation done, the source of infection was food contamination. Some of the food handlers in the area showed positive reaction in IgA antibody isotype when tested with Typhidot C, indicating that the outbreak could be attributed by possible healthy carriers (Nizal et al., 2018). In 2019, Sabah declared typhoid outbreak in the district following report of 5 new cases (The Star, 2019).

1.4 General characteristics of *Salmonella*

Typhoid fever is an infection caused by *Salmonella* enteretica serotype Typhi which is also known as typhoidal *Salmonella*. Humans are the only host for *Salmonella* Typhi (*S*.Typhi), and the disease is usually transmitted by consumption of contaminated food or water. The mode of transmission is through faecal-oral route (WHO, 2018).

S.Typhi is a gram-negative bacterium from the Enterobacteriaceae family. It is a non-sporing, actively motile bacterium with peritrichous flagella. It is facultative anaerobe and aerobe bacteria that grows well on MacConkey agar and Blood sheep agar (Kementerian Kesihatan Malaysia et al., 2019; Qadri and Ayub, 1989). The size of bacterium is about 2- 4 um x 0.6 um.

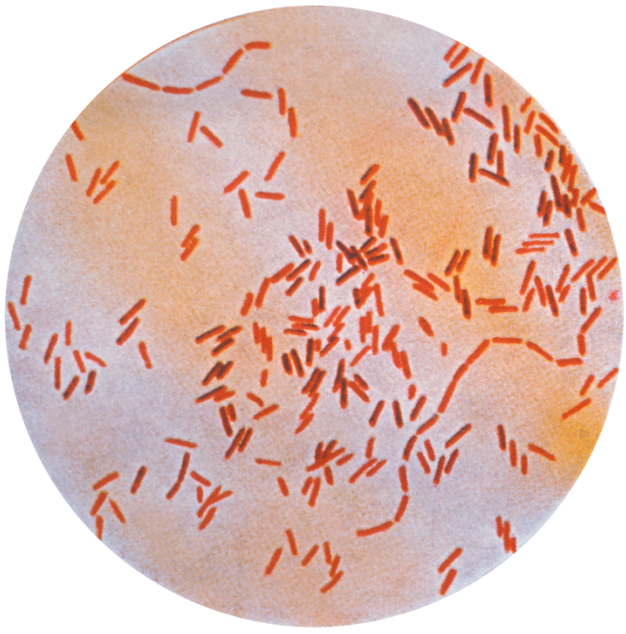

Figure 1.1 The illustration shows appearance of *S*.Typhi using a Gram staining procedure. Adapted from (CDC, 1954)

Salmonella has a complex lipopolysaccharide (LPS) structure on its cell wall. The components of the LPS structure consist of O-polysaccharide chain, inner

oligosaccharide (R core) core and lipid A (Zenk et al., 2009). The three moieties of LPS plays various important role in virulence, inflammatory response and humoral immunity. First, the O-polysaccharide chain is an important virulence factor of the organism. *Salmonella* can become less virulent or avirulent without the complete sugar sequence of the O-polysaccharide chain. *Salmonella* with O-chains are termed "smooth" (S) strains because of the appearance of their colonies. *Salmonella* lacking the LPS O-chains are usually less virulent or avirulent and defined as "rough" (R) strains. Second, many Gram-negative bacteria share the common R core structure of LPS. As a result, the humoral immune response produced against the R core can provide universal protection against infection by various Gram-negative bacteria. Third, the lipid A also known as endotoxin structure of the LPS is responsible for initiating potent systemic inflammatory response and can lead to septic shock infection (Garrett and Onderdonk, 2014).

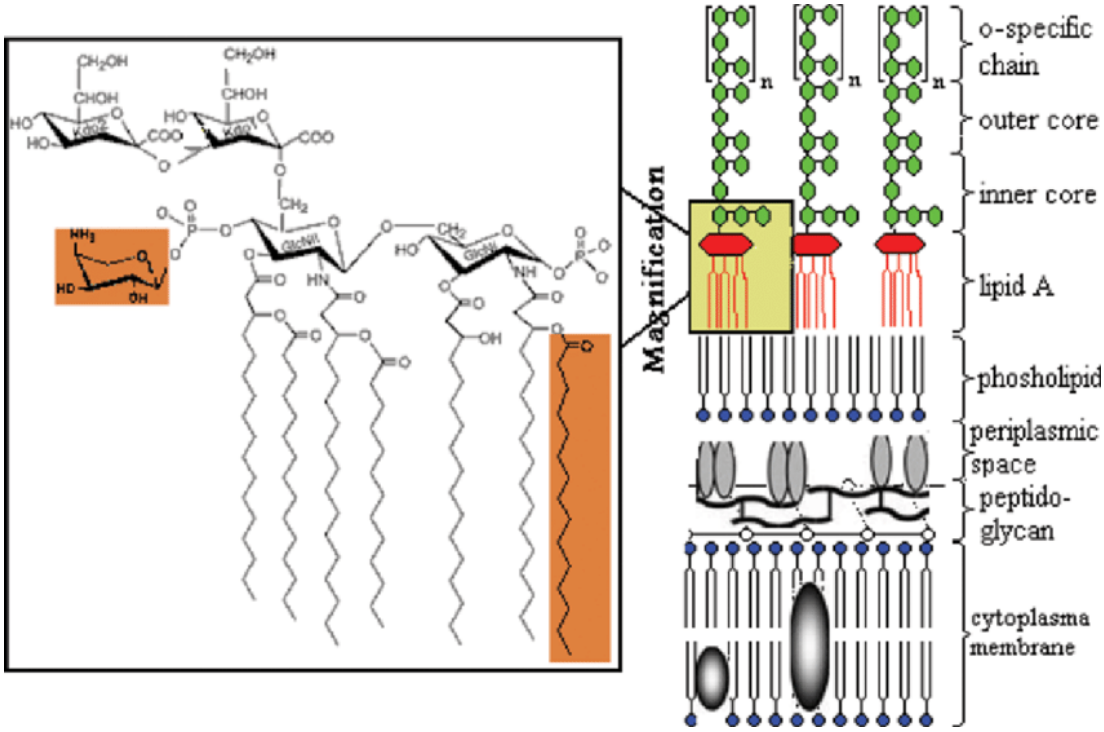

Figure 1.2 The illustration shows lipopolysaccharide structure of *Salmonella*. Adapted from picture of LPS (Redwan, 2012).

On the other hand, this bacterium has three major types of antigens which are the polysaccharide (Vi) envelop antigens, flagellar (H) antigens and somatic (O) antigens.

1.4.1 Flagellar (H) antigens

Between these three antigens, the flagellar H antigen is the most immunogenic. There are approximately 114 identified H antigens. The H antigens are the slender threadlike part of the flagella of bacteria that are composed of heat-labile protein (Oludairo et al., 2022; Udayangani, 2020). This antigen produced the highest titer of antibody upon infection or immunization (Bawa, 2010). The H antigen can be expressed in both phase 1 and phase 2 or transition from one phase to the other. *Salmonella* strains that exhibit phase variation are called diphasic strains (Fujita et al., 1973).

1.4.2 Polysaccharide (Vi) antigens

The Vi antigens is a capsular polysaccharide possessed by a few numbers of *Salmonella* serovars that causes severe infection in humans such as *S*.Typhi and *S*.Paratyphi C (Giannella, 1996; Virlogeux-Payant and Popoff, 1996). The Vi antigen backbone is composed of poly—(14)-linked N-acetylgalactosaminuronic acid that has been modified with O-acetyl residues needed for effective vaccine development (Wear et al., 2022). Although the Vi antigen has low immune response upon infection, but the presence of this antigen is sustained for a longer period of time. Therefore, this antigen has potential to be used for diagnosis of carrier (Bawa, 2010).

1.4.3 Somatic (O) antigens

The O antigens are located on the outer membrane's surface of the bacteria and are defined by its unique sugar chain sequences on the surface of the cell (Giannella, 1996). The O antigens are heat stable since they are composed of polysaccharides (Udayangani, 2020). There are approximately 60 O antigens in *Salmonella*, and these antigens are labelled in numerals forms (Oludairo et al., 2022; Xiong et al., 2018) . The O antigens are generally less immunogenic when compared to the H antigens. This antigen also produces lower titer of O antibody response to infection or immunization (Bawa, 2010).

1.5 Identification and Classification of *Salmonella*

The genus of *Salmonella* is divided into two main species which includes *Salmonella* enteretica and *Salmonella* bongori. *Salmonella* enteretica is divided into six subspecies: *Salmonella* enterica subsp enterica, *Salmonella* enterica subsp arizonae, *Salmonella* enterica subsp diarizonae, *Salmonella* enterica subsp houtenae, *Salmonella* enterica subsp indica, and *Salmonella* enterica subsp salamae. Serotypes in the serogroup of *Salmonella* enteretica subsp enteretica are linked to more than 99% of *Salmonella* diseases (Chen et al., 2013).

The commonly used culture media for isolation and characterization of *Salmonella* from clinical specimens are Bismuth Sulfide agar, MacConkey agar, Blood agar, Xylose Lysine Deoxycholate agar (XLD agar), Deoxycholate Citrate agar (DCA agar) and Brilliant Green agar (BGA) (Public Health England, 2021; Todar, 2015). *Salmonella* can be identified and distinguished by their surface antigens and biochemical testing (Smith et al., 2016).

1.5.1 Biochemical test

Traditionally, biochemical tests are performed to identify and distinguish bacterial strains. Some of the vital biochemical test for identification of *Salmonella* are indole test, oxidase test, urease test, Triple sugar iron (TPI) agar test, catalase test and citrate test (Parija, 2012; Rapid Microbiology, 2022). These conventional biochemical tests are laborious and time consuming (Aryal, 2022). However, there is a commercially available identification system called the AP1-20E bacterial identification kit (BioM´erieux, Marcy-l'Etoile, France), which includes 21 standardized biochemical tests and an integrated database. This system is user-friendly and provides accurate identifications based on the extensive API database. This system can identify and classify bacterial strains from the Enterobacteriaceae family (BioMérieux, 2019; Martinez-Urtaza et al., 2006).

1.5.2 Kauffman-White Classification

The Kauffman-White classification scheme introduced in 1934 has been the cornerstone in classification of *Salmonella* according to their serotypes. In the Kauffman-White classification scheme, each *Salmonella* serovars were classified according to their surface antigens which are the somatic (O) antigen, polysaccharide (Vi) antigen and flagella (H) antigens. (Chattaway et al., 2021; Grimont and Weill, 2007; Smith et al., 2016).

The *Salmonella* nomenclature published by Kauffman and White has been the gold standard classification for *Salmonella*. However, this traditional phenotypic approach can be time-consuming and costly. Additionally, the characterization of more than 2,500 *Salmonella* serovars requires skilled experts and more than 250 good quality reagents. Many primary and secondary healthcare institutes depend on a restricted number of commercially available antisera that cover only a handful of

serotypes and therefore hampered the progress in epidemiological investigation and intervention (Bee and Kwai, 2009; Franklin et al., 2011).

1.5.3 Genome based Identification

Another alternative method of identification of *Salmonella* is the genetic sequence construction of each antigen encoding gene based on the complete Kauffmann-White scheme. This method of identification is known as genoserotyping. Programmes such as Multi Locus Sequence Typing (MLST), Metric-Oriented Sequence Typer (MOST), SeqSero and *Salmonella*TypeFinder have been developed for genome-based serotyping of *Salmonella*. MLST is a serotyping method based on housekeeping genes of known function and chromosome position (Dahiya et al., 2013). MOST is a system established and used by Public Health England for deducing a sequence type from short reads and identifying related serotypes using a local database. SeqSero is an in silico serotyping tool that identifies the presence of O and H antigen from next generation sequencing (NGS) data and correlate with antigens used in conventional serotyping (Banerji et al., 2020). *Salmonella*TypeFinder is a pipeline used for determination of the serotype using combination of results from SeqSero and MLST tool (Underwood, 2016). Identification of *Salmonella* by genoserotyping is a rapid and cost-effective method that does not require use of antisera reagents. However, some of the challenges in the application of this method in routine identification includes the unknown genetics of antigen production, failure to correlate with gold standard and the presence of novel sequence type which have not been formally validated. The limitation led to lower sensitivity, specificity, and accuracy. Therefore, the performance of this method is not equivalent to the performance of classic serotyping (Chattaway et al., 2021; Yang et al., 2021).

1.5.4 MALDI-TOF based Identification

In recent years, Matrix Assisted Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) has been introduced as an alternative promising analytical technology for identification and definitive diagnosis of pathogens. The MALDI-TOF identification system is fast, accurate, and cost-effective (Singhal et al., 2015). Recent study showed the excellent and satisfactory performance of MALDI-TOF-MS identification system in for bacterial pathogens (Justesen et al., 2011; La Scola et al., 2011; Y. Li et al., 2019) . Figure 1.3 illustrated the application of MALDI-TOF in identification of bacteria grown on agar plate, blood cultures or urine samples.

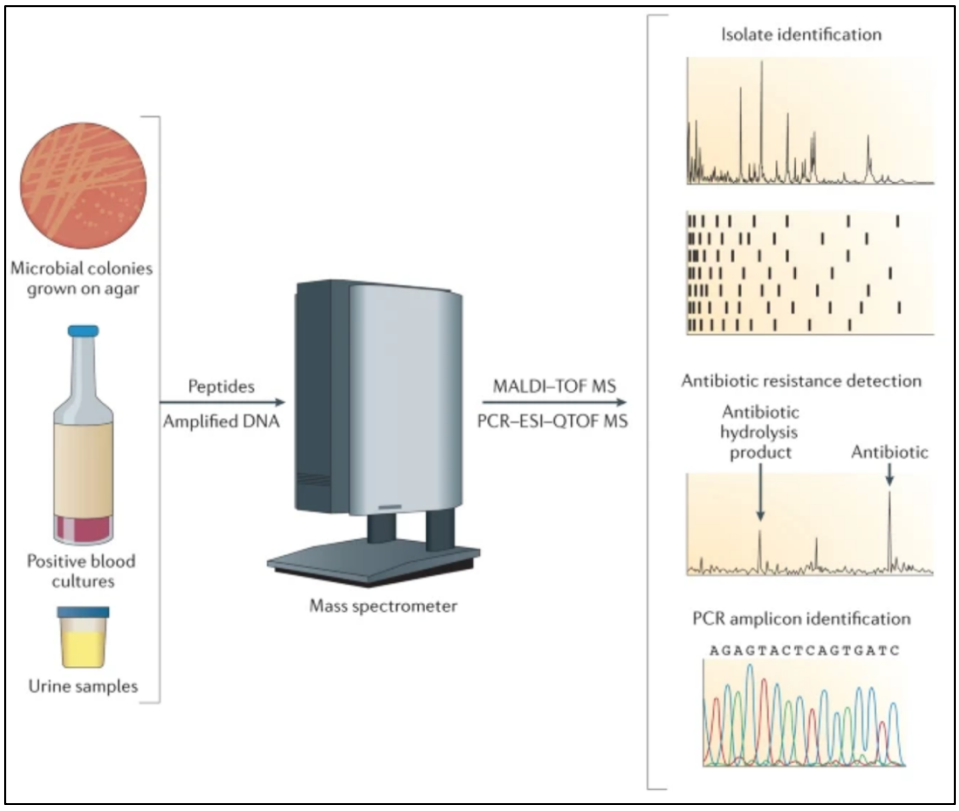

Figure 1.3 The application of MALDI-TOF mass spectrometry in bacterial identification. The figure was adopted from (Fournier et al., 2013).

1.6 Transmission and Pathogenesis

S.Typhi is predominantly an intestinal parasite with human as its only host. *S*.Typhi has an incubation period of approximately 2 weeks, which is marked by fever and illness (Crump, 2015). *S*.Typhi binds to the mucosa cells in small intestine and multiples within macrophages of lymphoid follicles, liver and spleen. Based on reported study conducted in human volunteers, the infectious dose for *S*.Typhi ranges from as low as 1000 to a million bacteria and it can cause local and systemic immune response in human.

Salmonella enters the body orally and invades the digestive tract by penetrating epithelial cell lining of intestinal wall using the type III secretion system. The *Salmonella* pathogenicity islands (SPIs) located at the chromosomal DNA region is responsible for the invasion process to occur. The effectors injected by the bacteria into the host cell's cytoplasm cause changes in the actin cytoskeleton and epithelial cell membrane ruffle, which allows the bacteria to survive in the host cell. The morphology of the invasion resembles phagocytosis process (Eng et al., 2015).

1.7 Non-typhoidal *Salmonella*

Non typhoidal *Salmonella* (NTS) serotypes are frequently associated with localized gastroenteritis infection in human. However, several species of invasive NTS (iNTS) are indicated in life threatening localized and bloodstream infection in young children, elderly and immunocompromised patients (Feasey et al., 2012). The iNTS group include *S*.Enteritidis and *S*.Typhimirium (Phu Huong Lan et al., 2016). To our knowledge, the first report of iNTS was published in literature in 1983, whereby the *S*.Typhimirium was isolated from two immunocompromised African patients in Belgium. Following the initial occurrence of iNTS in the series of patients with AIDS

in the US in 1984, several cases of iNTS among patients with AIDS were also recorded in Africa in the same year (Feasey et al., 2012). As elegantly mentioned by Brent *et al*, Malaria infection are often associated with iNTS disease (Brent et al., 2006). In 1987, children in Africa that were infected with Malaria were reported with iNTS disease. In a previous publication in 1990, iNTS was identified as a common pathogen observed in HIV patients (Gilks et al., 1990).

The economic burden of this disease warrants for a prompt and accurate diagnosis for early life saving treatment. The clinical diagnosis of iNTS is hampered by the overlapping clinical presentations with other febrile diseases including typhoidal fever. In order to confirm the diagnosis of iNTS, culture isolation by blood or stool is required. However, culture isolation is costly, time-consuming and requires laboratory facilities which is limited in areas with low resource settings. To date, no available serological diagnostic tool for accurate and rapid identification of iNTS has been reported (Feasey et al., 2012; Gilchrist and MacLennan, 2019).

Numerous prospective vaccine candidates for iNTS disease are still being developed which includes live-attenuated, recombination antigen-based, and sub-unit-based compounds vaccine (Haselbeck et al., 2017). However, the efficacy of these vaccines against gastroenteritis and invasive disease is uncertain. It is also unclear if these vaccine candidates can provide protection against typhoidal *Salmonella* (Tennant et al., 2016).

1.8 Acute undifferentiated febrile illnesses (AUFI)

Acute undifferentiated febrile illnesses (AUFI) are associated with fever with non-localized signs and symptoms. The clinical study published in literature shows Malaria, dengue fever, typhoid fever, leptospirosis, rickettsiosis, Japanese

encephalitis, and hantavirus are the prevalent aetiologies of AUFI (Jung et al., 2015). Most of the patients with AUFI present with the common symptoms which include myalgia, low-grade fever, rash, unspecific malaise, arthralgia, and general headache (Capeding et al., 2013; Wangdi et al., 2019). Recent finding showed that case of Covid-19 that was initially categorized as AUFI due to the non-specific, overlapping symptoms with other febrile diseases (Nunthavichitra et al., 2020). Therefore, clinical based diagnosis of AUFI cases is difficult and often not always possible to conclude accurate diagnosis. The similar symptoms of the diseases create a setback in obtaining accurate and reliable clinical diagnosis without confirmation with laboratory diagnosis. Hence, cases of AUFI often lead to sub-optimal prophylaxis due to false and delayed diagnosis.

In developing countries particularly during seasonal epidemics, AUFI has significantly increased morbidity, fatality, and financial burden. In a recent cohort study, dengue and enteric fever were highlighted as the two most common AUFI (Choudhary et al., 2019; Wangdi et al., 2019). Therefore, to improve healthcare system, it is essential to establish an inclusive and reliable database of various undifferentiated fever reported around the world.

1.9 Clinical presentations of typhoid fever

Typhoid fever is a serious bacterial infection that presents with a range of symptoms, ranging from mild to severe. The early symptoms of typhoid fever usually begin gradually, appearing 1 to 3 weeks after exposure to the bacteria. These initial symptoms include fever, which starts low but increases throughout the day and can reach as high as 104 degrees Fahrenheit, along with chills, headache, weakness, fatigue, muscle aches, stomach pain, diarrhea or constipation, and sometimes a skin

rash or rose spot. Additionally, individuals may experience cough, loss of appetite, and sweating. As the illness progresses, it can cause intestinal problems, resulting in stomach pain, a very swollen stomach, and in severe cases, sepsis, which is a life-threatening condition caused by gut bacteria spreading throughout the body. In the most severe cases of typhoid fever, individuals may experience alterations in their mental state, such as confusion, disorientation, and impaired cognitive functioning, which may result in an inability to adequately respond to stimuli in their environment. These neurological symptoms can have life-threatening consequences if not promptly addressed by medical professionals. Finally, some people may experience a recurrence of symptoms up to a few weeks after the fever has gone away (Centers for Disease Control and Prevention (CDC), 2018; Habte et al., 2018; Mayo Clinic, 2020).

1.10 Laboratory diagnosis for typhoid fever

1.10.1 Culture based diagnosis

The gold standard that has been used for diagnosis of typhoid fever is the isolation of the bacteria from blood of suspected typhoid patient. Blood culture has variable sensitivity upon isolation. The sensitivity is high up to 90% in the first week but falls to lower than 50% on the third week. Although blood culture is still the most used approach for definitive diagnosis of typhoid fever, it has significant drawbacks such as costly, takes 24-72 hours of incubation time with a total of 4 to 5 days for identification and very poor sensitivity, especially when antimicrobial drugs are used extensively before medical screening at hospital. A laboratory with multiple apparatus and material are also needed for bacterial isolation and identification. This will cause a problem to resource limited areas where rapid diagnosis is needed (Bhan et al., 2005; WHO, 2020).

Bone Marrow Aspirate (BMA) method showed higher sensitivity compared to blood culture as the number of microorganisms are higher in bone marrow. Culture from bone marrow also is not affected by antibiotic treatment. However. this method is extremely painful with invasive procedure of taking sample involving the soft tissue inside the bone and this can only be done by a certified medical doctor.

Stool culture has sensitivity lower than 50% and is not recommended for detecting acute typhoid fever. Following *Salmonella* infection, there is usually a period of time of asymptomatic faecal shedding in typhoid patients. Some of these patients will transition to become asymptomatic carrier by continuously shedding the bacteria. Therefore, stool culture can be used for diagnosis and monitoring faecal shedding in chronic and asymptomatic typhoid carriers (WHO, 2020).

Rose spot culture is another alternative for diagnosis of typhoid fever by taking skin snips from the rose spot. This method is usually more beneficial on patients being treated with antibiotics as antibiotics only sterilize the blood stream and not the rose spot (Bawa, 2010). Table 1.2 shows the advantages and disadvantaged between the diagnostic methods for typhoid fever.

1.10.2 Serology diagnosis

Serology based diagnosis is an important alternative approach especially in highly endemic areas. In 1896, Georges Ferdinand Widal developed the classic Widal test to identify the presence of agglutinating antibodies against O and H antigens of *S.*Typhi in suspected typhoid patients. The Widal test is preferred to be used in developing countries as it is cheap and easy to perform (Ajibola et al., 2018). However, the critical drawback in widal test is the misuse and misinterpretation of the results as the antigen is not specific to *S.*Typhi. This test also has low sensitivity and antibody classes (Igm , IgG, IgA) cannot be identified individually (Bhan et al., 2005). Widal

test has also been proven to have poor specificity due to the antibodies that survived or pre-exist in communities in endemic regions and cross reactions with other gram-negative bacteria including non-typhoidal *Salmonella* (T.P1 et al., 2013).

Besides Widal test, newer serological tests that has been developed such as Tubex TF, Typhidot test, TP test and many more as described in Table 1.2. One of the widely used and studied test is the Tubex test which is simple to perform and takes only 2 minutes to obtain result of diagnosis for typhoid fever. However, this test could not diagnose other *Salmonella* serovars responsible for paratyphoid fever and iNTS disease (Bawa, 2010). The efficacy performance of Typhidot assay along with other commercially available serological tests are moderate with variable sensitivity and specificity as shown in Table 1.3. Another problem in the serodiagnosis of typhoid fever is relapse cases that happen 2 to 3 weeks after resolution of fever. Usually, 10% of untreated cases become carriers and 1 to 5% will become chronic carriers. There were also around 25% cases of healthy carriers with no history of typhoid fever (Gal-Mor, 2019). These carriers showed high titer of antibody and will caused false positive results in widal test and other commercially available diagnostic kits. Hence, serological interference has been recognized as a major drawback in the reported diagnostic kits for typhoid fever.

1.10.3 Molecular based diagnosis

The development of molecular diagnostic tests necessitates the use of genetic markers with high sensitivity and specificity in detecting bacterial DNA in typhoid patients. Some of the molecular based diagnostic tests that has been developed include conventional PCR, real time PCR, nested PCR and multiplex PCR. The critical challenge in the development of molecular based diagnosis for typhoid fever is their application in resource limited environment. The other drawback of molecular

approach is the high false positive results due to identification of DNA in non-typhoidal patients (Ajibola et al., 2018; Sattar et al., 2014).

1.11 Antimicrobial treatment

Chloramphenicol has been the standard and longest antibiotic treatment used for typhoid fever since 1948, but it is now limited in several countries due to antibiotic resistance and harmful side effects. Along the line, ampicillin and trimethoprim-sulfamethoxazole has also been the traditional antimicrobial treatment for typhoid fever. However, due to the increasing anti-microbial resistance, fluoroquinolones such as ciprofloxacin and ofloxacin has been used increasingly for treatment of uncomplicated typhoid fever. Carbapenems and tigecycline serves as third line antimicrobial treatment in cases where patients are resistance to first and second-line drugs (Butler, 2011; Crump et al., 2015).

Antibiotic resistance is a growing problem, with multi-drug resistant (MDR) strains of *Salmonella* becoming more common in Africa and Asia. Extensively drug-resistant (XDR) strains have emerged in Pakistan, and their increasing prevalence could make the treatable typhoid become untreatable (Akram et al., 2020; Eng et al., 2015).

1.12 Prevention

Multiple health approach is required for prevention and control of typhoid fever including public health intervention such as sanitation and hygiene education (CDC, 2021; Project, 2016; UNICEF, 2020; WHO, 2022).

Vaccination is another approach in controlling and managing spread of typhoid fever. Currently, there are three available vaccines that has been commercially used which are the live attenuated oral vaccine Ty21a, Vi capsular polysaccharide vaccine

(ViCPS) and Typhoid conjugate vaccine (TCV) (Morusupalli et al., 2020). (Coalition Against Typhoid, n.d.; WHO, 2003, 2018). The vaccines are given through oral or intramuscular injection, and they are recommended for travellers, food handlers, street vendors, immunocompromised individuals, and communities in typhoid endemic areas (Patel et al., 2021; Qadri et al., 2021; Shakya et al., 2021). In addition to these vaccine target groups, laboratory employees, individuals with household exposure to the disease and military personnel may benefit from typhoid immunisation (Milligan et al., 2018; The Australian Immunisation Handbook, 2021; WHO, 2018).

1.13 Problem statement

Human is the only host of typhoid fever. Therefore, infected individual can become carrier and continue spreading the disease. Until today typhoid fever still exists and the world is facing continuous challenge in controlling and prevention of this disease. Significant health burden has been documented in developing countries. However, alarming cases associated with travelers and domestic source of infection have been reported in industrial countries. Thus, typhoid fever is now recognized as a neglected re-emerging infectious disease in developing and industrial countries.

Clinical presentations of typhoid fever are unpredictable and resemble other infectious disease. Culture isolation is the gold standard for diagnosis of typhoid fever. However, culture isolation is laborious, time-consuming and lack of sensitivity. On the other hand, many diagnostic tests reported in literature are confined to detection of antibodies against limited antigens and hampered by lack of significant sensitivity and specificity. The alarming emergence of multi drug resistance strains, relapse, recurrence infections and lack of adequate diagnostic tools remains as the main hindrance for early life saving treatment.

1.14 Rationale for initiation of present study

Early conclusion of definitive diagnosis of typhoid fever is important for early life saving treatment. Currently available immunoassays for laboratory diagnosis are confounded by several limitations that hindered immediate treatment of typhoid fever. The diagnosis dilemma for typhoid fever is further complicated by the rise of iNTS cases, which has caused a global concern throughout the world. To date, scientists are engaged with the continuous struggle to develop an immunoassay for the rapid and definitive diagnosis of typhoid fever.

Therefore, this study aims to elucidate the role of whole cell protein, cell surface protein and whole cell-minus cell surface protein for the serodiagnosis of typhoid fever. For these reasons, the differentially extracted proteins derived from *S*.Typhi and *S*.spp were used as a cocktail of multi-antigens to develop an immunoassay for the detection of specific antibody isotypes comprising of IgM, IgG and IgA in serum from typhoid patient, healthy subjects, typhoid vaccinated subjects and subjects with other febrile diseases. The usefulness and the role of genus conserved and species-specific proteins against the antibody isotypes in the serodiagnosis of typhoid fever was determined by comparative analysis of dot EIA test results.

1.15 General Objectives

1. To perform differential extraction and characterization of whole cell proteins (WCP), cell surface proteins (CSP) and surface depleted whole cell protein (sdWCP) derived from *S*.Typhi and invasive non-typhoidal *Salmonella* species by SDS-PAGE.

2. To determine the synergistic effect of differentially extracted species and genus conserved antigens for the development of TYPHOIDYNE Microspot Multi-Antigen Arrays Enzyme Immunoassay.
3. To determine the antigenic profiles of the differentially extracted proteins by Western blot analysis based on molecular weight by SDS-PAGE.
4. To perform further immunological characterization of the differentially extracted protein by combined preparative liquid phase isoelectric focusing and SDS-PAGE.

The research methodology comprised of two parts of sequential strategies. Figure 1.4 describes the experimental procedures for the first part of the research undertaken to characterize the differentially extracted proteins derived from *S*.Typhi and *S*.spp and illustrates the usefulness of the differentially extracted proteins in the serodiagnosis of typhoid fever. The subsequent research strategy that focused on the application of preparative separation technique for purification, identification and immunological characterization of the differentially extracted proteins was illustrated in Figure 1.5.

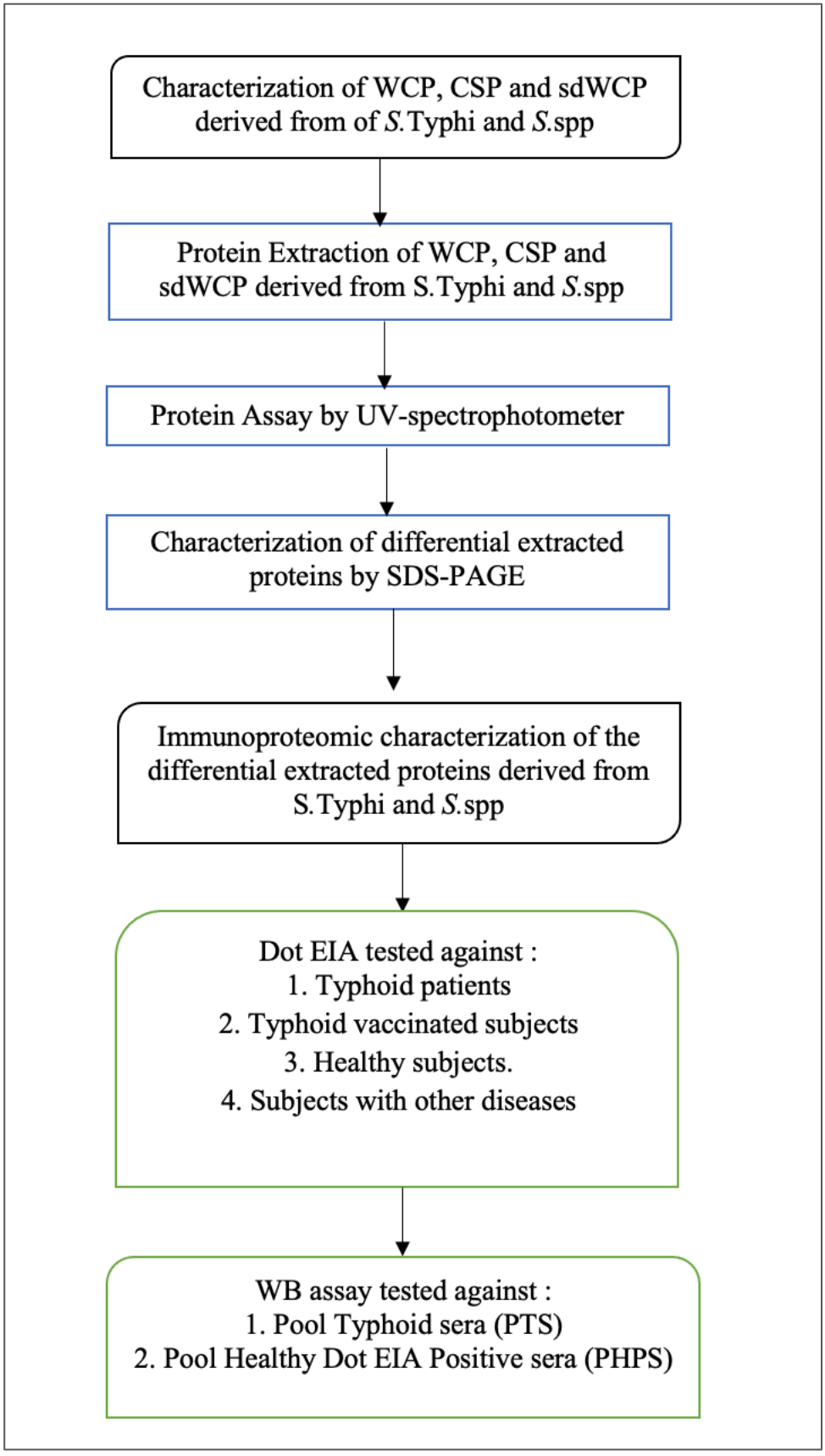

Figure 1.4 Flowchart illustrating the proteomic and immunological characterization of differentially extracted proteins derived from *S.*Typhi and *S.*spp

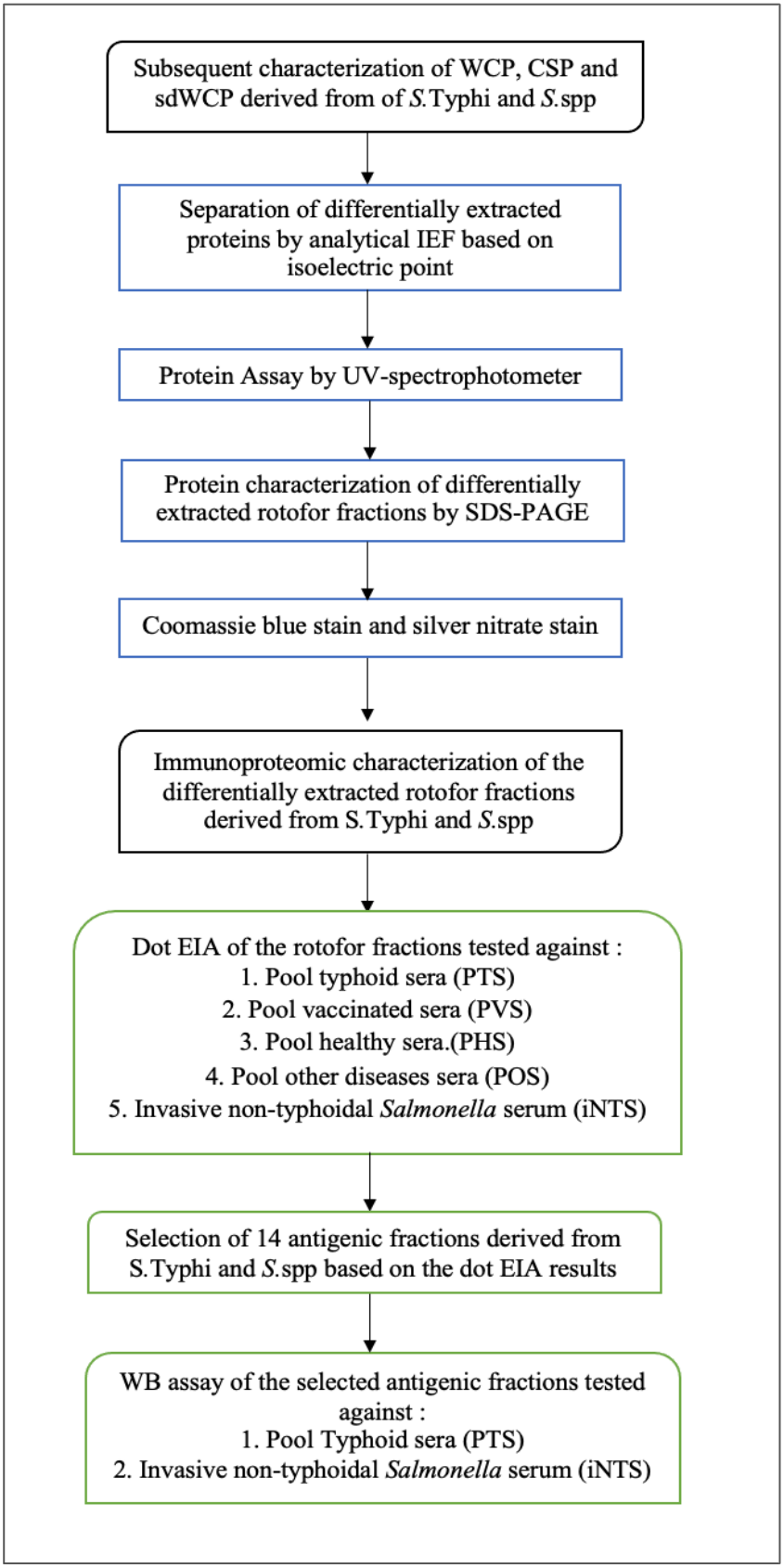

Figure 1.5 Flowchart illustrating the application of preparative analytical separation technique for purification, identification and characterization of antigenic rotofor fractions derived from *S.*Typhi and *S.*spp

CHAPTER 2

CHARACTERIZATION AND COMPARATIVE ANALYSIS OF DIFFERENTIALLY EXTRACTED WHOLE CELL PROTEIN DERIVED FROM *S.*Typhi AND *S.*spp BY SODIUM DODECYL SULPHATE–POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

2.1 Introduction

Typhoid fever is a potentially fatal re-emerging global infectious disease. The disease is acquired through faecal oral route by consumption of contaminated food and water (Marineli et al., 2013). The clinical presentation of typhoid fever is unpredictable and mimics many other acute undifferentiated febrile illnesses (AUFI). Therefore, clinical based diagnosis can be challenging and complicated in typhoid cases. Typhoid fever is not an uncommon infection in immunocompetent individuals. However, communities with poor sanitation, children, and immunocompromised individuals due to primary and secondary immunodeficiency or malnutrition are more susceptible to typhoid fever with severe outcome (VanMeter and Hubert, 2016; World Health Organization (WHO), 2018). Therefore, timely treatment is essential to prevent complications and fatal outcomes. If untreated the disease can progress to severe complications involving multi organ systems and lead to death within one month of infection. The survivor may be left with long term neurological comorbidities (B. Lee and Rose, 2018; Parry, 2004). The standard antimicrobial drug of choice for treatment of typhoid fever are fluoroquinolones, azithromycin and cephalosporin depending on the severity of disease (John L. Brusch and Michael Stuart Bronze, 2022; Parry, 2004). However, treatment for typhoid fever remain challenging due to the global widespread of the extensively drug resistance stains (XDR). The global spread of XDR strains limiting the choice of antibiotic treatment and may lead to scenario of untreatable typhoid fever (Tanmoy et al., 2018; Wolman-Tardy, 2018).