TRANSCRIPTIONAL ANALYSIS AND IMMUNOLOGICAL CHARACTERIZATION OF IRON UPTAKE RECEPTORS OF *Shigella flexneri* 2a USM CLINICAL ISOLATES

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2022

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by

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Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science

June 2022

ACKNOWLEDGEMENT

First and foremost, Alhamdulillah. Special gratitude to Allah S.W.T for giving me full strength to be able to complete my master's degree within the years of study with several difficulties in completing all the research objectives. My sincere appreciation also to my supervisor, Dr. Leow Chiuan Yee who continuously guided and shared his wide knowledge throughout the study, all those efforts, constructive comments finally made me able to achieve the task completion. Also, to all my co-supervisors, Associate Prof. Dr. Kirnpal Kaur Banga Sigh, Dr. Khairul Mohd Fadzli Mustaffa, and Dr. Leow Chiuan Herng for assisting and motivating me until finished.

I also would like to express my gratitude to Amy Amilda Anthony, Ahmad Hafiz Murtadha, Nor Raihan Mohammad Shabani, Basyirah Binti Ghazali, Haslizai Binti Hassan, Badrul Syam Bin Mat Zainuddin, all lecturers, administrative and laboratories staff, and all post-graduate students of INFORMM who involved directly or indirectly in this research. Thank you for all the guidance, motivational support, and friendship made together with precious experience gained. Finally, my family has been my backbone and strongest motivation for continuous support especially both of my parents and my beloved wife, Che Rugayah Binti Che Awang, Ismail Bin Daud, and Nur Fairuz Binti Mohd Fauzey for their prayers, encouragement, and love. Alhamdulillah, I am deeply thankful to Allah S.W.T. for providing them in a good health.

This study has been supported by the Malaysian Ministry of Higher Education of the Higher Institutions Centre of Excellent Program under Grant (311/CIPPM/4401005) and Fundamental Research Grant Scheme (203/CIPPM/6171199).

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A	Absorbance
ABTS	2,2’-Azinobis [3-ethylbenzothamine-6-sulfomic acid] diammonium salt substrate
ANOVA	Analysis of variance
bp	Base pair
$CaCl_2$	Calcium chloride
CIP	Calf Intestinal
Ct	Cycle threshold
DEPC	Diethyl pyrocarbonate
ELISA	Enzyme-linked immunosorbent assay
g	Gram
HRP	horseradish peroxidase
IC_{50}	Inhibitory concentration
IgG	Immunoglobulin G
IPTG	Isopropyl-β-D-thiogalactopyranoside
IEDB	The Immune Epitope Database
LB	Luria-Bertani
mM	Millimolar
mg/mL	Milligram per milliliter
$MgCl_2$	Magnesium chloride
NaCl	Sodium Chloride
NCBI	National center for Biotechnology Information
Ni-NTA	Nickel-charged affinity resin
nm	Nanometers
OD	Optical density

OMP	Outer membrane protein
PCR	Polymerase chain reaction
PAGE	Polyacrylamide gel electrophoresis
rpm	Revolution per minute
SDS	Sodium dodecyl sulfate
V	Voltage
w/v	Weight by volume
x g	Relative centrifugal force (RCF)
%	Percentage
℃	Degree Celsius
µl	Microliter
µM	Micromolar

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TINDAK BALAS TRANSKRIPSI DAN PENCIRIAN IMUNOLOGI RESEPTOR ZAT BESI DARI ISOLAT KLINIKAL USM *Shigella flexneri* 2a

ABSTRAK

Shigellosis adalah penyakit yang membimbangkan kesihatan awam dengan kadar kematian dan morbiditi yang tinggi terutamanya di kalangan kanak-kanak di bawah umur kurang dari lima tahun. Tanpa penjagaan yang betul, penyakit ini boleh bertambah teruk seperti najis berair dan cirit-birit berdarah teruk. Peningkatan rintangan terhadap pelbagai ubat dalam kebanyakan bakteria gram-negatif termasuk spesies *Shigella* mengehadkan rawatan klinik yang sedia ada kerana kebanyakan antibiotik yang diberi kurang berkesan. Selain ketiadaan vaksin komersil atau yang diluluskan terhadap penyakit shigellosis dan laporan peralihan dominan dari *Shigella flexneri* kepada *Shigella sonnei*, pembuatan vaksin yang berkesan dan mampu untuk memberi perlindungan panjang amat diperlukan. Kebanyakan vaksin yang sedang dibuat dilaporkan memberi kesan sampingan seperti disentri dan keberkesanan perlindungan yang berbeza-beza di kalangan individu yang telah divaksin terutamanya dalam kalangan umur yang berbeza. Memandangkan perkara ini, pemahaman komprehensif tentang patogenesis di tahap molekul di kalangan individu yang telah dijangkiti adalah penting. Menariknya, molekul besi terkenal dengan peranan pentingnya dalam kelangsungan hidup bakteria dan penentu virulen dengan regulasi pelbagai reseptor protein pengankut besi semasa berada di dalam individual yang telah dijangkiti untuk memperoleh sumber besi yang mencukupi. Dalam kajian ini, Kedua-dua *S. flexneri* isolat klinikal dari Hospital USM (SH057 dan SH062) telah ditumbuhkan di dalam medium yang mempunyai 200 µM 2,2-bipyridyl. Ekspresi gen pengekodan reseptor pengambilan besi *S. flexneri* 2a, *fepA, fhuA, IutA, efeU,* dan *SitA*

diperhatikan dengan tindak balas rantai polimerase kuantitatif (qPCR). Semua reseptor pengambilan besi *S. flexneri* 2a selanjutnya dianalisis dengan menggunakan pendekatan bioinformatik untuk menyaring lebih lanjut calon vaksin yang berpotensi untuk digunakan terhadap penyakit Shigellosis. Reseptor pengambilan besi yang dipilih kemudian akan diteruskan dengan pembinaan protein rekombinan dan meramalkan antigenisiti protein rekombinan melalui dot-blot dan ELISA. Keputusan qPCR menunjukkan kadar ekspresi kebanyakan reseptor pengambilan besi termasuk *fepA, fhuA, IutA, efeU*, dan *sitA* bagi kedua-dua virulen (SH057) dan virulen ringan (SH062) *S. flexneri* 2a isolat klinikal adalah tinggi dalam keadaan kekurangan zat besi. Tambahan pula, analisis bioinformatik menunjukkan bahawa reseptor pengambilan besi FepA, FhuA, dan IutA mengandungi kawasan antigen yang menjanjikan untuk bertindak balas dengan sistem imun humoral serta selular. Di samping itu, analisis antigenik menunjukkan bahawa protein rekombinan yang sepadan dengan protein FepA, FhuA, dan IutA adalah antigenik terhadap antigen-antibodi kumpulan anti-*Shigella* dan serum manusia yang telah dijangkiti oleh *Shigella*. Penemuan ini boleh memberi kesan kepada pembuatan vaksin yang berkesan terhadap jangkitan *Shigella* dengan menggunakan reseptor pengambilan besi sebagai sebahagian daripada formulasi vaksin. Walau bagaimanapun, keberkesanan perlindungan dan keberkesanan protein ini terhadap shigellosis perlu dinilai terlebih dahulu dengan lebih lanjut melalui kajian in vivo.

TRANSCRIPTIONAL ANALYSIS AND IMMUNOLOGICAL CHARACTERIZATION OF IRON UPTAKE RECEPTORS OF *Shigella flexneri* 2a USM CLINICAL ISOLATES

ABSTRACT

Shigellosis is a public health concern disease with high mortality and morbidity rate, especially among children under the age of five. Without proper care, the disease worsens from watery stool to acute bloody diarrheal. The rise of multi-drug resistance strain in most gram-negative bacteria including *Shigella* species limits the current clinical treatment as most of the antibiotic administrated is less effective. Besides the unavailability of commercial or approved vaccines against shigellosis disease and the report of the predominant shift from *Shigella flexneri* to *Shigella sonnei*, the development of an effective and long-protection vaccine is urgently needed. Most current developed vaccines are reported to cause side effects such as dysentery and the protection efficacy varies among vaccinated individuals at different groups of ages. In view of this, a comprehensive understanding of molecular pathogenesis among infected individuals is of the utmost importance. Interestingly, iron molecules have been known to play a critical role in bacterial survival and virulent determinant by upregulation of multiple iron uptake receptor proteins within the infected individuals to acquire enough iron sources. In this study, both of *S. flexneri* 2a Hospital USM clinical isolates (SH057 and SH062) were grown under the presence of 200 µM of 2,2-bipyridyl. The expression of gene encoding iron uptake receptors of *S. flexneri* 2a, *fepA, fhuA, IutA, efeU*, and *SitA* were observed by quantitative polymerase chain reaction (qPCR). All iron uptake receptors of *S. flexneri* 2a were further analyzed by using the bioinformatic approach to further screening the potential target vaccine

candidates against shigellosis disease. The selected iron uptake receptors then proceeded for construction of recombinant protein and predict the antigenicity of the recombinant protein corresponding to iron uptake receptors using dot-blot and ELISA approach. The qPCR result showed the expression rate of majority iron uptake receptors including *fepA*, *fhuA*, *IutA*, *efeU*, and *sitA* of both virulent (SH057) and mild virulent (SH062) of *S. flexneri* 2a clinical isolates were high under iron-depleted condition. Furthermore, the bioinformatics analysis showed that iron uptake receptors of FepA, FhuA, and IutA contain promising antigenic regions to elicit both humoral as well as cell-mediated immune responses. In addition, antigenicity analysis demonstrated that the recombinant proteins corresponding to FepA, FhuA, and IutA protein were antigenic against anti-*Shigella* group antigen-antibody and *Shigella*-infected human serum. The findings could impact the development of an effective vaccine against *Shigella* infection by the utilization of iron uptake receptors as part of vaccine formulation. However, the protective efficacy and the effectiveness of these proteins against shigellosis needs to be further evaluated through in vivo study.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Diarrheal is one of the major causes of illness and mortality in children under the age of five and persons over the age of 70 (Troeger et al., 2018, Bernadeta Dadonaite, 2018). In 2019, approximately 370 000 children globally were reported death as a result of diarrheal disease (WHO, n.d.). The burden of this disease is affected by low- and middle-income countries. In Malaysia, acute diarrheal is the second leading cause of death in children with at least 13 million episodes occurring annually (Abdul Rahim et al., 2020). Diarrheal disease is defined as a clinical presentation of frequent loose stool within 24 hours while acute or chronic diarrheal episodes up to several weeks (Talley et al., 1991). Besides, the acute diarrheal cases also were presented by bloody diarrheal, known as dysentery. The cause of this clinical presentation is varied, including either being infected with bacteria, viral, or parasitic organisms that spread through feces-contaminated food or water source.

Shigellosis is one of the leading causes of severe diarrheal disease worldwide and is among the deadliest diarrheal case reported among children under the age of five (Kotloff et al., 2018). The disease remains endemic around the world, including Malaysia, where the prevalence of shigellosis has been reported to occur throughout the year, disregarding the seasonal patterns compared to other Asian regions (Singh et al., 2011). Shigellosis requires an exceptionally low dose (10-100 microorganisms) to initiate an infection. The ability to survive under gastric acid harsh environment makes this pathogen a great challenge to treat. Furthermore, this pathogen is intracellular and was classified as a highly contagious pathogen as it can be transmitted from person to person (Schnupf et al., 2020). So far, there is no approved or commercial vaccine

specifically targeting the *Shigella* serotype, and the uncontrolled use of antimicrobial agents had led to the rise of multiple antibiotic-resistant strains, hindering the appropriate selection of current antibiotics (Papić et al., 2021, de Alwis et al., 2021). This poses a threat to the public due to the non-effective of existing antibiotics to treat this infectious disease (Puzari et al., 2018, Leow et al., 2020a). A dominant shift pattern from *S. flexneri* to *S. sonnei* also has been reported in the developing countries, possibly due to this strain exhibiting an exceptional ability to acquire antimicrobial resistance genes from commensal and pathogenic bacteria (Thompson et al., 2015). Therefore, the development of a long-term effective vaccine is critically needed that can elicit both humoral and cell-mediated immune responses.

1.2 Rationale of study

Multiple approaches in finding effective vaccines to resolve the infection either through the utilization of whole attenuated, killed bacteria, or bacterial-based components that take part in bacterial virulence. Among the challenges encountered for vaccine development against shigellosis includes the vaccine candidate is either not sufficiently attenuated or inconsistent in terms of protection and efficacy among vaccinated individuals, especially different groups of age (Pasetti et al., 2020, Ashkenazi and Cohen, 2013). Thus, a comprehensive understanding of bacterial pathogenesis and bacterial-host interaction is urgently required for the discovery of novel potential antigens for the improvement of vaccine formulation. Given this, the iron uptake system possessed by *Shigella* is known to be critically important in bacterial pathogenesis (Wei and Murphy, 2016). Iron uptake receptors have widely been reported to be up-regulated under the tight control of the iron level in the infected host. Without these transporters, bacteria are not able to acquire iron molecules and

survive within the infected host. These proteins are known to be exposed on the outer membrane layer of the bacteria cell, exposing a direct contact with the host's immune system for recognition and triggering an immune response (Larrie-Bagha et al., 2013). Therefore, targeting the iron-inducible outer membrane proteins (IROMPs) that can trigger the production of antibodies to block the uptake of an iron molecule by the bacteria, directly contributes to the prevention strategy by the immunized individuals. Furthermore, outer membrane proteins are highly antigenic and they are shown to be capable of triggering an immunological response in the animal model through a dynamic interface during host-pathogen interaction (Mani et al., 2016). Given the importance of iron uptake receptors for bacteria iron regulation and homeostasis, the present study was aimed to (1) investigate the molecular role of iron uptake receptors in *Shigella flexneri* 2a and (2) to determine the antigenicity of iron uptake receptors in *Shigella flexneri* 2a, as part of the efforts in developing vaccine formulation against *Shigella* species.

1.3 Research hypothesis

Iron uptake receptors expressed by the *S. flexneri* 2a clinical isolates may be upregulated under iron starvation conditions to acquire enough iron sources. Besides, iron uptake receptors of *S. flexneri* 2a also may be comprised on antigenic epitope throughout the protein sequence capable of inducing both B- and T-cells immune response during bacterial pathogenesis.

1.4 Research objectives

1.4.1 General objective

The main objective of this study is to evaluate the transcription response of iron uptake receptors of *S. flexneri* under iron-depleted conditions and assess their antigenicity using *Shigella*-infected sera.

1.5 Specific objectives

The sub-objective of this study is divided into three sections:

1. To compare the transcription profile of iron uptake receptors under normal and iron-depleted growth conditions of both virulent and mild virulent clinical isolates strain.
2. To identify the antigenic regions within iron uptake receptors that are capable of inducing B- and T-cell as potential vaccine construct against shigellosis through bioinformatic analysis.
3. To evaluate the antigenicity of recombinant proteins, correspond to iron uptake receptors of *S. flexneri* using Shigella-infected sera.

1.6 Flowchart of experimental design of this study

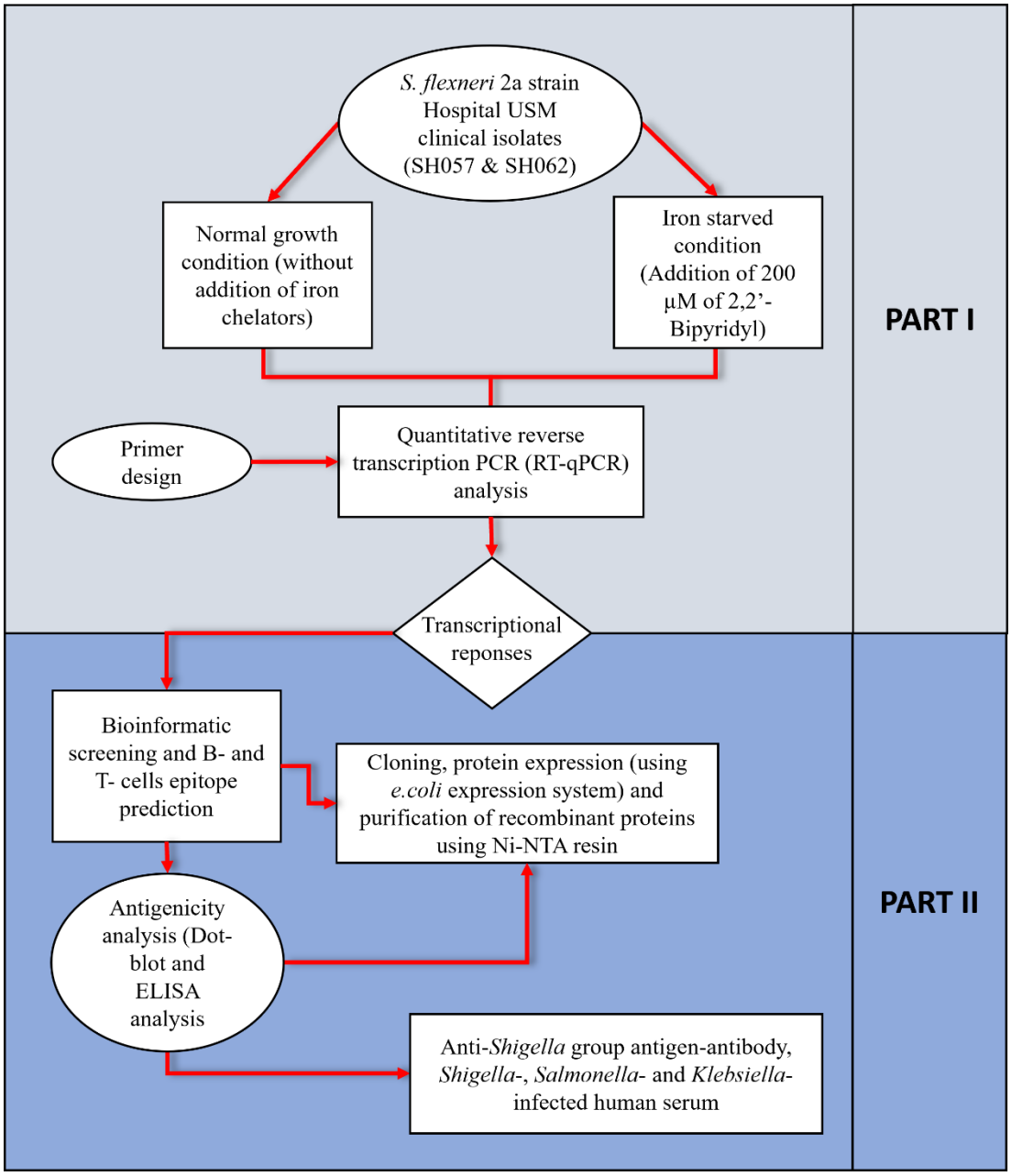

Figure 1.1 The experimental design used to accomplish the research objectives in the current study. This study comprises of two parts: Part 1 comprise of transcription analysis of iron-uptake receptors of *S. flexneri* 2a clinical isolate under the presence of iron chelators. Part II comprises bioinformatic analysis, construction of recombinant protein corresponding to iron-binding proteins as well as antigenicity analysis of iron-uptake receptors of *S. flexneri* 2a as potential vaccine candidates.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to shigellosis

Shigellosis is an intestinal infection caused by *Shigella* species ranging from mild to acute infection. Without proper treatment, the infected person can lead to death. This endemic disease remains a major health problem and becomes a burden to low- and middle-income countries, including Asia, with an estimation of 80 – 165 million cases with 600 000 death occurring annually worldwide (Bowen, 2017, Edward et al., 2020). More than 1 million deaths have been reported among children under the age of 5 (Bowen, 2017, Vinh et al., 2011). Nevertheless, the elderly and immunocompromised individuals are also prone to *Shigella* infection. Although the diarrheal incident reveals to decline by 4% per year, the morbidity and mortality rates are still relatively high. Bacillary dysentery occurs mainly due to poor water quality, sanitation, and lack of food safety. Thus, low-income countries with improper hygiene settings and inadequate water supply expose a higher risk of infection. Shigellosis usually is characterized by several clinical presentations such as mild watery diarrhea, bloody mucoid diarrhea, together with abdominal cramps and fever (Schnupf et al., 2020).

2.2 The etiological agent responsible for shigellosis

Kiyoshi Shiga firstly identified *Shigella* species in 1898 during the high mortality rate of dysentery in Japan (Trofa et al., 1999). The bacteria were known as Shiga bacillus in the first place, later were taxonomically classified as *Shigella* species. This bacterium is closely related to *Escherichia coli*, and it is a facultative anaerobic bacterium that belongs to the Enterobacteriaceae family. The non-motile, gram-negative bacteria differentiated into several serotypes based on O-antigen structure

repeats (O-Ag), which are *S. dysenteriae, S. flexneri, S. boydii*, and *S. sonnei* (Figure 2.1) (Trofa et al., 1999, Schnupf et al., 2020, Kotloff et al., 2018). *Shigella* species is a highly contagious bacterial agent as it only requires a minimum mass of 10 to 100 microorganisms to establish the infection and colonize the target host. The capabilities of this bacterium to survive in an acidic environment make it difficult to control. Besides, his disease is capable of being transmitted from person to person, and it is an intracellular pathogen.

2.3 Epidemiology of shigellosis

Shigellosis is endemic in temperate and tropical climates region. Based on previous cases, the spread of *Shigella* species is influenced by geographical location and economic development in a particular region (Anderson et al., 2016). A distribution pattern study shows that *Shigella* species are predominant in the Asian region and are the leading cause of childhood diarrhea among children less than five years old besides *Campylobacter, Escherichia coli*, and *Salmonella* spp. (Hien et al., 2008, Lee et al., 2017). In China, bacillary dysentery demonstrates the seasonal pattern where the prevalence peak is reported during October (Zhao et al., 2021), while in Bangladesh, the seasonal peak is in May (Ekdahl and Andersson, 2005, Chao et al., 2019).

In Malaysia, *Shigella* species has been known as the third most common isolated strain among patients infected with diarrheal and the prevalence study of this disease in the Northeast region shows the incidence of shigellosis occurs throughout the year from 2001 to 2009, especially from May to August, followed by the rainy season. The most isolated *Shigella* species among diarrheal patients is highest in June, followed by November as the second peak (Singh et al., 2011). However, the latest prevalent data obtained from Hospital HUSM, the *Shigella* species isolated was reported less than 10

cases yearly from 2017 to 2020 (unpublish data from Department of Medical Microbiology and Parasitology, School of Medical Science, Universiti Sains Malaysia). The reported cases were shown to be decreasing as previously reported by Singh and colleagues. The seasonal pattern is not observed in Malaysia due to consistent environmental conditions with exceptional monsoon season during November and December as compared to other seasonal countries (Singh et al., 2011, Toro et al., 2015).

In the aspect of serotypes, *S. flexneri* is the established serotypes as a common etiological agent responsible for diarrhea in low-, middle-income, and developing countries for over the past 30 years, accounting for 60% of endemic diseases among infants and children (Hosangadi et al., 2019, Connor et al., 2015). On the other hand, *S. sonnei* is the second most common *Shigella* species isolated in low- and middle-income countries accounting for 25%. However, this strain is most prevalent, and the leading species is isolated in high-income regions. Besides, *S. sonnei* has been reported to associate with diarrheal disease among travelers (Moreno-Mingorance et al., 2021, Shad and Shad, 2021). The remaining strains are least common globally, but *S. boydii* and *S. dysenteriae* remain endemic in South Asia and Sub-Saharan Africa. *S. dysenteriae* type 1 is known as the most virulent strain as it has caused the population to experience turmoil outbreaks with cases of death among all ages (Levine et al., 2007). This bacterium can generate a severe and prolonged illness due to the association of potent Shiga cytotoxin with the development of hemolytic-uremic syndrome, HUS (Williams and Berkley, 2018, Taylor, 2008).

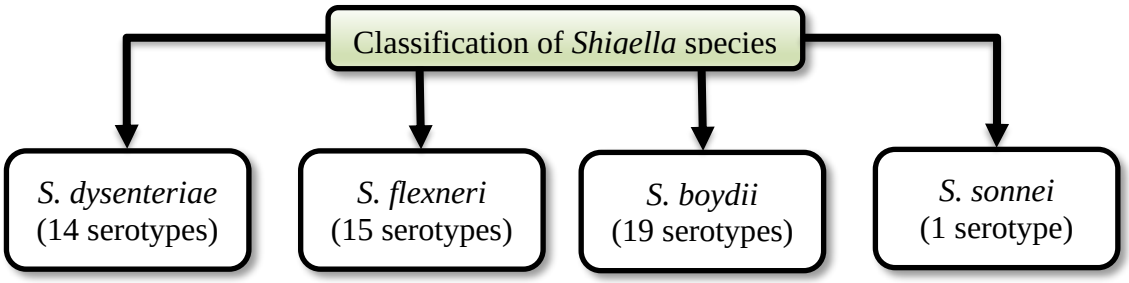

Figure 2.1 The classification of *Shigella* species and the total number of its sub-serotypes (Kotloff et al., 2018). In general, *Shigella* species were classified based on components of the lipopolysaccharide O antigen repeats expressed on the outer membrane layer. *Shigella dysenteriae* has 14 serotypes, *Shigella flexneri* has 15, *Shigella boydii* has 19, while *Shigella sonnei* has just one serotype.

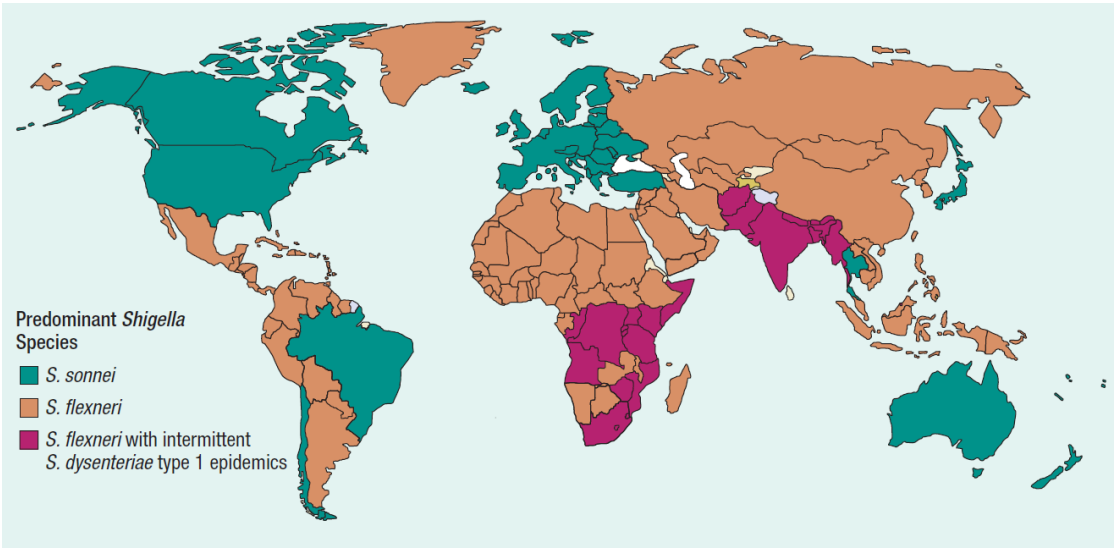

Figure 2.2 The global distribution of *Shigella* species is labeled by their serotype (Bennish and Ahmed, 2020). *S. sonnei* is predominant in industrialized countries while *S. flexneri* is predominant in relatively poor countries. The shift of predominant from *S. flexneri* to *S. sonnei* infection may be a marker of the country's social and economic development. for the case of *S. dysentariae*, the type 1 serotype occurred in South Asia and central and east Africa in the last decade of the 20th century. however, this serotype is rarely found worldwide. For *S. boydii*, it has been reported that this serotype was found to commonly occur in South Asia, accounting for minority infection even in an endemic area.

2.4 The shift pattern of dominant *Shigella* serotypes

A shift pattern of dominance from *S. flexneri* to *S. sonnei* is seen in developing countries. The dominance of *S. sonnei* is likely associated with the growth of the economic development of the country. However, the shift from *S. flexneri*, possibly due to driven by the multidrug-resistance lineage. In China, a clinical surveillance study shows that there is an increase of *S. sonnei* isolates from 17.4% in 2003 to 58.2% in 2004 due to rapid industrialization (Mao et al., 2013, Qiu et al., 2015). Bangladesh also shows the same increasing pattern from 7% in 2001 to 2.5% in 2011, even the sanitation and water quality has been improved (Das et al., 2013, Hulland et al., 2013). The same shift pattern also occurs in Malaysia, where the isolation of *S. sonnei* out-numbers the domination of *S. flexneri* (Singh et al., 2011, Mao et al., 2013, Qiu et al., 2015, Das et al., 2013, Hulland et al., 2013). A gradual decrease trend of *S. flexneri* was reported in 2008, while *S. sonnei* remain a dominant species in 2009 (Singh et al., 2011).

2.5 Mode of transmission

Unlike other endemic pathogens, human is the only reservoir for *Shigella* species. Transmission of shigellosis can occur in various ways, either via direct or indirect, including fecal-oral route, person-to-person contact, through household flies, contaminated food or water supply, and via inanimate object (Christopher et al., 2010, Black et al., 1989, Kirk et al., 2015, George et al., 2015). Fecal-oral route and person-to-person transmissions are usually associated with inadequate sanitation and hygiene setting. Contaminated water and food sources accelerate the disease spread faster within the community (Bardsley et al., 2020, Jaffee et al., 2018). On the other hand, houseflies are classified as mechanical vectors in disease transmission in poor hygiene and sanitation regions(Farag et al., 2013). The dissemination of this disease also occurs

among travelers who are returning from developing countries where the shigellosis is endemic. Besides the transmission through men-to-men sexual activity, the transmission also could occur either among infected persons or asymptomatic carriers. Poor hygiene day-care centers for children less than five years old also can be a high-risk factor for the dissemination of this pathogenic bacteria (Toro et al., 2015).

2.6 Bacterial pathogenesis

The fecal-oral route is a common way of disease transmission due to the consumption of contaminated food and water sources. The *Shigella* species can survive in an extremely acidic environment in the host's stomach due to the presence of effective acid resistance systems. During pathogenesis, *Shigella* species utilizes a type III secretion system (T3SS) that serves as a needle-like molecular syringe, anchored in the bacterial cell wall. The T3SS is usually activated through the contact of the needle tip with the host's plasma membrane leading to the formation of a direct channel on the host's cytoplasm. This allows the *Shigella* species to inject bacterial effectors to facilitate bacterial infection and dissemination (Mattock et al., 2017).

The infective stage of *Shigella* species is initiated through a multistep process where it begins through the uptake by microfold cells (M cells), crossed the intestinal epithelium as an entry port. The *Shigella* spp. are transcytosed and delivered to the underlying mucosal lymphoid tissues (Man et al., 2004, Zychlinsky et al., 1996, Wassef et al., 1989). This is where the invading *Shigella* species encounter the resident antigen-presenting cells, including macrophages. Unfortunately, *Shigella* species can survive after being phagocytosed by the macrophage and induce rapid apoptosis to allow their survival (Zychlinsky et al., 1996, Islam et al., 1997, Zychlinsky et al., 1992). Before that, *Shigella* species rapidly lyse the phagosomal compartment of macrophages

through the T3SS system (Schnupf et al., 2020). The lytic cell death of infected macrophages leads to the release of pro-inflammatory cytokines IL-1β and IL-18, which subsequently cause the acute and massive inflammatory response, respectively. The excretion of the IL-1β signal triggers intense intestinal inflammation. At the same time, the release of IL-18 induces a cascade immune reaction through the activation of natural killer (NK) cells and promotes the release of gamma interferon (IFN-γ) to amplify the innate immune response (Schnupf et al., 2020, Way et al., 1998). After the infected macrophages undergo apoptosis, the *Shigella* species invade epithelial cells from basolateral sides and replicate within the cytoplasm.

In the cytoplasmic layer, *Shigella* species move by directed polymerization of actin, allowing them to infect adjacent epithelial cells without being exposed to extracellular components of the host immune defense (Sansonetti et al., 1986, Bernardini et al., 1989, Monack and Theriot, 2001, Stevens et al., 2006). However, the release of the Nod1-mediated intracellular surveillance system activates the nuclear factor κB (NF-κB) when they encounter bacterial peptidoglycan fragments from the *Shigella* species. This triggers the release of chemokines IL-8 to mediate massive recruitment of polymorphonuclear neutrophil leukocytes (PMN) to the side of infection (Pédron et al., 2003, Girardin et al., 2003, Philpott et al., 2000, Sansonetti et al., 1999, Singer and Sansonetti, 2004). Unfortunately, the infiltration of PMN leads to the destruction of the epithelial lining, directly allowing the luminal *Shigella* species to reach the submucosa without the need for M cells as an entry port. The severe damage of epithelial lining causes the tissue lesion, and impaired absorption of water, nutrients as well as solutes leading to watery diarrheal, bloody or mucus in stools (Schroeder and Hilbi, 2008, Perdomo et al., 1994).

2.7 Clinical manifestation and treatment

The diarrheal infection caused by *Shigella* species occurs mostly among malnourished infants or children, the elderly, and HIV-infected individuals (Tickell et al., 2017). Shigellosis is a self-limiting illness, typically resolved within 4-7 days without any treatment. However, in some cases, severe or fatal cases happen due to the avoidance of proper treatment. Upon infection, among the symptoms observed from an infected individual can be fever, headache, malaise, anorexia, and vomiting, followed by watery diarrhea. Diarrheal cases are correlated with grossly bloody stools, more often to have rectal prolapse or abdominal tenderness. It also can lead to severe dehydration, but this case rarely occurs among children (Kotloff et al., 2018, Khan et al., 2013). The asymptomatic infection possibly occurs among previously infected individuals.

The maintenance of hydration and electrolyte balance is the cornerstone of controlling *Shigella* infection treatment through oral rehydration and intravenous fluids (Kotloff et al., 2018). Also, the administration of antibiotic therapy can reduce the severity of dysentery ranging from mild to severe infection. The administration of fluoroquinolones and azithromycin are the drugs of choice to treat shigellosis (Bennish and Ahmed, 2020). A combination of antibiotic treatment and oral dehydration leads to a rapid resolution among infected patients in reducing the prologue clinical presentation. In addition, the supplementation of zinc, Vitamin A, and magnesium sulphate has been reported to assist in reducing the severity of diarrheal disease (Bennish and Ahmed, 2020).

2.8 The rise of antibiotic resistance strain among *Shigella* species

The increase in consumption of antibiotic therapy either among individual patient-level or community leads to the emergence of antibiotic-resistant lineage among *Shigella* species, including ampicillin, tetracycline, and chloramphenicol (Cui et al., 2015, Khaghani et al., 2014, Ahmad, 2019). This situation not only restricts the available treatment but also exposes a higher risk among infected individuals. Admission of oral cephalosporins, ampicillin, trimethoprim-sulfamethoxazole, nalidixic acid, and tetracycline has been reported no longer useful against *Shigella* species due to the acquisition of multiple-antibiotic resistant genotypes such as R-plasmid (Bennish and Ahmed, 2020). A study conducted in Northern Ethiopia shows the isolated *Shigella* strain is resistant to ampicillin, amoxicillin, and cotrimoxazole with a percentage of 100, 86.7, and 66.7 % (Gebrekidan et al., 2015). In Malaysia, Koh and colleagues report that *Shigella* strain isolated from stool patients in the Malaysian region is resistant against streptomycin, tetracycline, and trimethoprim-sulfamethoxazole with a resistance rate of 67.5%, 40%, and 37.5% (Koh et al., 2012). On the other hand, a study conducted by Singh and colleagues reports that clinical isolates of *Shigella* are resistant against tetracycline and trimethoprim-sulfamethoxazole at a rate of 58.4% and 53.8% respectively (Singh et al., 2011). This explains the urgent need to develop an effective vaccine against the rise of multi-drug resistant strains as bacteria will always find a way to block the action of antimicrobial agents, rendering them to be non-effective.

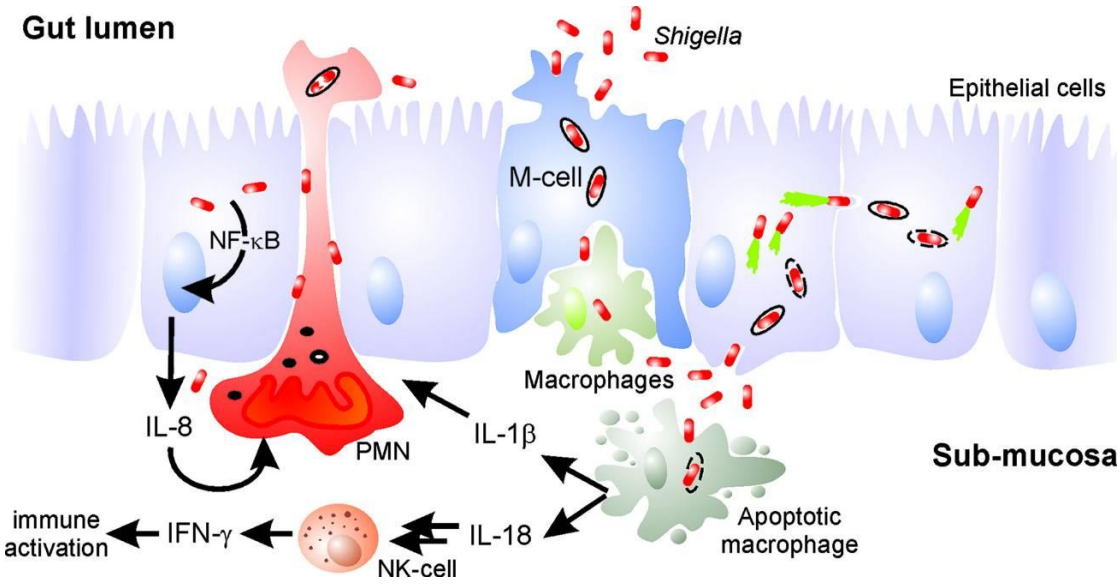

Figure 2.3 The cellular pathogenesis of *S. flexneri* during in vivo infection by utilizing the microfold cells (M cells) as an entry port. Later, the invading *Shigella* triggers the cascading immune response to assist their invasion by triggering the infiltration of PMN cells destroying the integrity of epithelial lining (Schroeder and Hilbi, 2008). The destruction of epithelial lining causes a clinical presentation such as watery or bloody diarrheal.

2.9 The status of the *Shigella* vaccine and its challenges

The advanced approach in developing promising vaccine candidates to resolve the issues of shigellosis has been performed through several ways including oral administration of inactivated or live attenuated *Shigella* strain, O-specific polysaccharide-based protein conjugates, a subcellular complex containing lipopolysaccharides (LPS) protein, invasion plasmid antigens (Ipa) and outer-membrane based protein (Pasetti et al., 2020, Luo et al., 2016, McKenzie et al., 2006, Kotloff et al., 2002, Kotloff et al., 2004, Rahman et al., 2011, Cohen et al., 1996, Passwell et al., 2010, Riddle et al., 2016, Riddle et al., 2011, Obiero et al., 2017). Immunization of whole-cell vaccine is capable of triggering both B- and T-cells response as the antibody and B-cell targeting specifically O-antigen or Ipas are raised in orally immunized individuals. While in the case of T-cells response, it is reported that T-cell is associated with the production of cytokines, IFNγ by antigen-stimulated peripheral blood mononuclear cells and $CD4^{\pm}$, and $\alpha4\beta7^{\pm}$ $CD8^{+}$ T effector memory cells (Pasetti et al., 2020). Most of the immunogenicity data from historical and recent under-developing vaccines pertain to humoral immunity response by the whole-cell vaccine, IgA and IgG are expected to be a significant contribution to confers protection by interfering with the invasion of bacteria within the intestine and facilitating complement-mediated killing and phagocytic killing. In contrast to the O-polysaccharide conjugate vaccine, the efficacy of this vaccine depends mainly on the high production of LPS-specific serum IgG, which resembles the bactericidal activity (Pasetti et al., 2020, Riddle et al., 2016).

A combination of both whole-cell vaccine and recombinant subunit vaccine can potentially provide a formidable precedent for an effective vaccine to prevent enteric

diarrheal diseases. This method has been proven successfully against cholera infection where the vaccine is constituted of inactivated whole cells of *Vibrio cholerae* with recombinant cholera toxin B subunit and live-attenuated CVD 103-HgR (Vaxchora) (Pasetti et al., 2020). However, in the case of *Shigella*, each of the under-developing vaccine candidates possess both advantages and disadvantages. The whole bacterium is found to be able to confer protection with high efficiency, but it causes side effects given some of the vaccines carry a heat-stable ST toxoid, and the protection is inconsistent among different individuals as the higher dosage is required for the conferral of protective efficacy. Hence, it also leads to diarrheal, emesis, and other mild constitutional symptoms (McKenzie et al., 2008, Tennant et al., 2016, Pitisuttithum et al., 2016, Venkatesan and Ranallo, 2006). Despite, O-polysaccharides, LPS- or membrane antigen-based vaccine, the efficacy of the vaccine is varying among different ages of immunized individuals with a range of 28% – 70% which contrast to preclinical test as the administration of the vaccine in challenged mice were reported to confer protection (Riddle et al., 2016, Camacho et al., 2013, Pore and Chakrabarti, 2013). Among the vaccine candidates, the subunit conjugated vaccine targeting *Shigella* species developed by John Robbin and colleagues is reported to be the most promising candidate and it is currently in phase III in the clinical trial. This vaccine is an O-specific polysaccharide covalently attached to succinylated mutant Pseudomonas aeruginosa exotoxinA (rEPAsucc) or succinylated Corynebacterium diphtheria toxin mutant (CRM9 or CRM9succ) (Passwell et al., 2001, Passwell et al., 2010, Pasetti et al., 2020). Upon immunization, this vaccine confers 70% protection to administrated adults and children of 3-4 years old. However, the administration of this vaccine towards children younger than three years old shows a drop of efficacy to 28%. In the case of bioconjugate *S. flexneri* 2a vaccine candidates Flexyn2a also shows 40% efficacy when

immunized in adult human volunteers. Thus, it can be concluded that a deep understanding of what constitutes a protective immunity within the infected host is crucial to construct an efficient, safe, as well as long-lasting protection.

2.10 Iron as an essential growth factor and virulent determinant

Iron is the fourth most abundant metal in the earth's crust, and it is an important transition metal in most living organisms for survival including bacteria. Under physiological conditions, iron exists in two oxidative states, the oxidized ferric (Fe^{3+}) and reduced ferrous (Fe^{2+}), making it suitable for serving as a co-factor by forming a part of the prosthetic group of proteins for a variety of biological processes including redox reaction, substrate binding, and activation, regulation of gene expression, central metabolism, respiration, and DNA repair mechanism (Frawley and Fang, 2014, Johnson et al., 2005, Crespo-Rivas et al., 2019). Ferrous iron was the dominant species within the anaerobic and at low physiological pH environment, where the iron was very soluble for direct utilization by the microorganism for iron source. Unfortunately, these free iron molecules were bound to the host's iron uptake receptors such as heme, transferrin, and lactoferrin (Andrews et al., 2003, Finkelstein et al., 1983, Cornelissen and Sparling, 1994). On the other hand, ferric iron was reported to be dominant in the oxygenated environment but poses low solubility to be utilized by microorganisms with an aerobic lifestyle (Andrews et al., 2013). This is unfavorable for enteropathogenic bacteria as iron is maintained at very low concentrations during pathogen invasion by the action of the host's iron uptake receptor protein, which further limits the chance of enteropathogenic survival in the host (Nairz et al., 2018). Besides, according to Fenton chemistry, the presence of free iron causes the production of highly reactive hydroxyl radicals which directly leads to DNA and protein damage (Imlay, 2003). This is one of

the reasons for the tight regulation of iron molecules within the host system. Enteropathogenic bacteria escape the unfavored condition by expressing a high-affinity iron uptake system to scavenge iron for survival followed by the initiation of its pathogenicity (Clarke et al., 2001, Andrews et al., 2003). Iron deprivation during colonization could impair the function of iron-requiring proteins such as eukaryotic ribonucleotide reductase, an enzyme responsible for deoxyribonucleotides (dNTPs) synthesis. dNTPs are a precursor required during DNA replication and repair. Lacking dNTPs will lead to an increase in the risk of DNA mutation and cell death (Zhang, 2014). Conceptually, the iron uptake receptors are assumed to be over-expressed during the bacterial colonization process to invade the infected host.

Bacterial pathogenesis is heavily relying on the ability to scavenge iron present in the host's environment to grow and initiate bacterial infection. It is considered a vital process as iron is one of the essential nutrients due to the critical function of iron itself (Ratledge and Dover, 2000, Harvie and Ellar, 2005, Luck et al., 2001). The oxidation state of iron depends on the presence of oxygen molecules as the ferric form is the most abundant in an oxygenated environment compared to ferrous iron, which is dominant under an anaerobic and non-physiological pH environment. The challenge begins among pathogenic bacteria when the fact that iron is readily oxidized to form ferric iron. This is because ferric iron (Fe^{3+}) is extremely insoluble in its oxidized form with approximately 10^{-18} M at pH 7, far below the minimum requirement of iron for bacterial survival, and it is inaccessible to be utilized readily by bacteria (Pi et al., 2012, Bullen et al., 1978). Most of the environmental irons are present in their insoluble ferric state under neutral pH. During bacterial infection, host-binding protein is being released, such as transferrin, ferritin, and lactoferrin, to control the iron concentration at an optimal level because excess iron molecules cause a detrimental effect on the host's cell.

The tight regulation of iron homeostasis in the host leads to low bioavailability of iron in the mammalian's body fluid or tissue with approximately 10^{-24} M (Andrews et al., 2013). The sequestration process in regulating the excess free iron in the body was termed nutritional immunity, which directly limits the growth of any invading pathogens through iron starvation. Unfortunately, bacteria found a way to tackle the iron-depleted issues to survive by developing various multiple iron uptake systems.

2.11 The expression of multiple iron acquisition systems by pathogenic bacteria to fulfill the iron requirement

Most invading pathogens encounter a variety of environmental conditions during transmission and natural infection. Thus, the ability of bacteria to scavenge iron is one of the critical virulent traits to encounter iron-restrict conditions (Wei and Murphy, 2016, Andrews et al., 2013). Without iron molecules, the iron-dependent pathogen is not able to survive and achieve its critical mass to cause bacterial infection in vivo. The presence of iron in the environment serves as a signal to trigger the regulation of the virulence factors, including genes responsible for the expression of the iron acquisition system by the ferric uptake regulator (*Fur*) gene (Litwin and Calderwood, 1993). Most Gram-negative bacteria including *Shigella* species evolve several systems for the acquisition of various iron sources within the hostile environment.

In general, the iron-acquisition system of *Shigella* species was categorized into three groups: (i) the acquisition of ferric iron, (ii) heme-bound iron, and (iii) ferrous iron uptake. The combination of iron uptake systems is varied among *Shigella* serotypes and is not necessarily for *Shigella* species to express all iron-uptake systems due to shifting mutation. During the stage of infection, an iron chelator known as siderophores

is being synthesized and secreted into the surrounding by bacteria to capture ferric iron either free- or bound to host-binding proteins. Since siderophores have a potent affinity towards insoluble ferric iron compared to the host's binding proteins thus allow it to seize iron from the host's binding proteins (Golonka et al., 2019). The ferric-siderophore complex was then transported across outer membrane proteins transporter into periplasm with the aid of the *TonB-ExbB-ExbD* complex for energy supply. The subsequent uptake from periplasm across cytoplasm is mediated by ATP-binding cassette (ABC) transport system (Postle and Larsen, 2007, Krewulak and Vogel, 2008, Krewulak et al., 2011). In the cytoplasm, the ferric-siderophores complex bind to periplasmic binding proteins to initiate the transportation across the cytoplasmic membrane layer with aid of energy from cellular ATP. Once the iron-complex enters the bacterial cells, iron is released by either reduction from ferric iron to ferrous iron molecules through the action of ferric iron reductase, or intracellular proteolysis of siderophore's peptide backbone to release iron into cells (Miethke, 2013, Miethke and Marahiel, 2007, Schröder et al., 2003). The iron molecule is ready to be used for different biological processes.

Relatively, the infected host found a way to prevent the uptake of ferric iron by inducing the secretion of siderocalin from leukocytes, epithelial cells, and macrophages. The synthesis of siderocalin is stimulated by Toll-like receptors on the immune cell to prevent the utilization of iron-bound siderophores by specifically binding to it, such as enterobactin siderophores. To escape the action of the host's immune system upon bacterial siderophores, pathogenic bacteria secrete a stealth siderophore with a different chemical structure, such as salmochelin and aerobactin (Wei and Murphy, 2016, Fischbach et al., 2006). On the other hand, when anaerobic and low pH conditions were introduced, insoluble ferric iron can switch into a more soluble ferrous form. In this

case, a different ferrous iron transport system is needed. Initially, the ferrous molecule was said to diffuse freely through non-specific outer membrane porin into periplasm followed by subsequent transportation into the cytoplasm through different periplasmic binding proteins, either *Feo*, *Sit*, or *Efe* system (Lau et al., 2016).

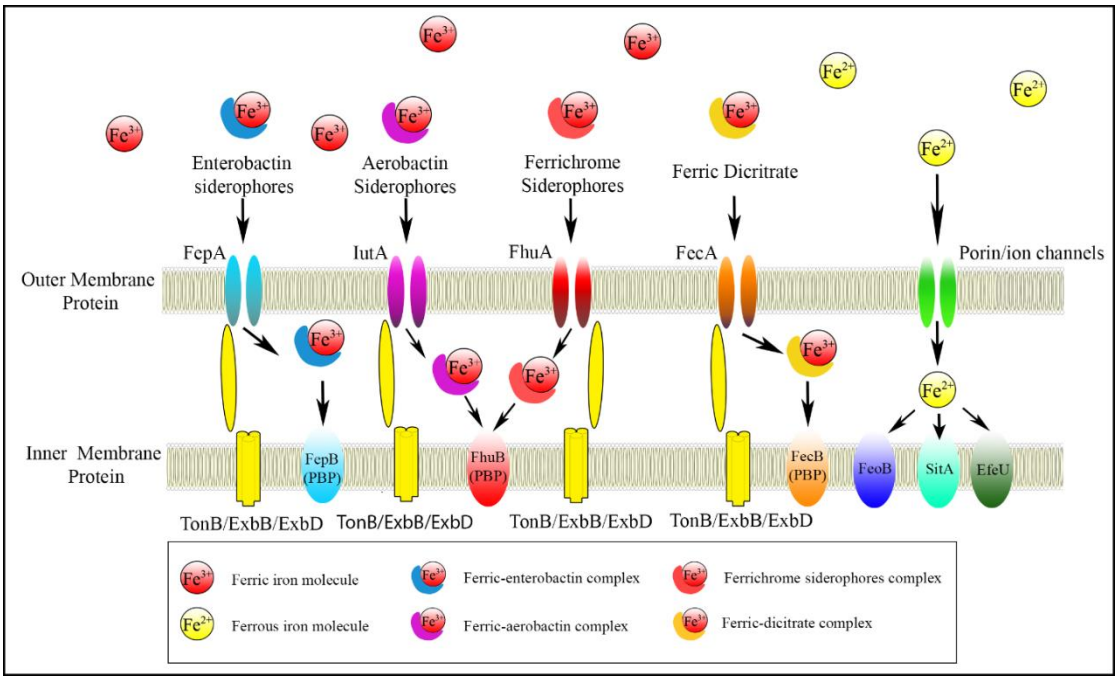

Figure 2.4 *Shigella* poses multiple iron uptake systems to adapt to different hostile environments during pathogenesis. The figure above illustrates the ferrous and ferric uptake mechanisms by secretion of siderophores to capture iron molecules and later were transported across the outer membrane proteins. For ferrous iron uptake, the ferrous molecule was transported across the non-specific porin, followed by the uptake through periplasmic binding proteins.

2.12 Iron-regulated outer-membrane proteins (IROMPs) as potential vaccine construct

Outer-membrane protein-based vaccine approach has been widely applied due to their critical participating roles in vivo, besides confers protection to the bacteria under stress environment. It is also well known for its immunogenicity, which is capable of invoking the host's immune response, and the location of these proteins that were exposed on the outer surface allows for direct recognition by the host (Luo et al., 2016, Ningqiu et al., 2008, Wang et al., 2012). Most of the important etiological agents is relying on iron-transport surface receptors that specifically bind iron molecules to support their growth and colonization in vivo. The invading pathogen responds to an iron-starved condition in the host through the expression of various iron uptake systems to suites different environmental conditions during invasive infection (Chan et al., 2018, Hayes et al., 2013). Due to the critical roles of iron-regulated outer membrane proteins (IROMPs) in bacterial pathogenesis and physiological function, this protein can be a promising vaccine candidate as this protein is a surface-exposed, antigenic, and capable of inducing an immunological response within the host (Todhunter et al., 1991, Larrie-Bagha et al., 2013). Besides, the iron uptake system also was recognized as one of the virulence factors that abundantly present in the genome of all Shigella strains (Wang et al., 2019). A raised antibody specifically targets IROMPs because of cascade immunological reaction may confer protection against the invading bacteria by the action of the host's antibody that blocks on that receptor. Interestingly, all the siderophores-mediated iron uptake receptors were reported to conserve in all *Shigella* serotypes, which can potentially resolve the serotype-specific immunological response within the host (Wei and Murphy, 2016, Williams and Berkley, 2018).