EXPLORING THE IMMUNOMODULATION PROFILES OF THE THP-1 HUMAN MACROPHAGE-DERIVED CELL LINE MEDIATED BY *Shigella flexneri*

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by

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

˚C	Degree Celsius
Δ	Delta
µ	Micro
™	Trademark
®	Registered
×	Multiplication
× *g*	Relative centrifugal force
β	Beta
α	Alpha
γ	Gamma
±	Plus-minus
=	Equal to
<	Less than
amu	Atomic mass unit
CFU/mL	Colony forming unit per mililliter
µL	Microliter
µM	Micromolar
KDa	Kilo dalton
L	Liter
mL	Milliliter
mM	Milimolar
nm	Nanometer
rpm	Revolutions per minute

LIST OF ABBREVIATIONS

APCs	Antigen-presenting cells
BLASTp	Protein basic local alignment search tool
BSC	Biosafety cabinet
CD4+	Cluster of differentiation 4
CD8+	Cluster of differentiation 8
cDNA	Complementary deoxyribonucleic acid
CIP	Calf intestine phosphatase
CO_2	Carbon dioxide
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leukocyte antigen
IC_{50}	Half maximal inhibitory concentration
IgG	Immunoglobulin G
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-12	Interleukin-12
INF-γ	Gamma interferon
iNOS	Inducible nitric oxide synthase
Ipa	Invasion plasmid antigen
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
M cells	Microfold cells
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NaOH	Sodium hydroxide

NCBI	National Center for Biotechnology Information
NO	Nitric oxide
OMPs	Outer membrane proteins
ORF	Open reading frame
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PMA	Phorbol 12-myristate-13-acetate
PMN	Polymorphonuclear
PMSF	Phenylmethylsulfonyl fluoride
PRRs	Pattern-recognition receptors
RT-qPCR	Real time-quantitative polymerase chain reaction
RPMI	Roswell park memorial institute medium
SAPs	Surface-associated proteins
S. boydii	*Shigella boydii*
S. dysentriae	*Shigella dysentriae*
S. flexneri	*Shigella flexneri*
S. sonnei	*Shigella sonnei*
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH057	Virulent strain of *Shigella flexneri* 2a
SH062	Mild strain of *Shigella flexneri* 2a
SS	*Salmonella-Shigella*
TAE	Tris-acetate-EDTA
TFA	Trifluoroacetic acid
TNF-α	Tumor necrosis factor-alpha
THP-1	Human monocytic cell line derived from an acute monocytic leukemia patient
TLR	Toll-like receptors
V	Volts

LIST OF APPENDICES

PENEROKAAN PROFIL IMUNOMODULASI TITISAN SEL TERBITAN MAKROFAJ MANUSIA THP-1 YANG DIPERANTARAKAN OLEH *Shigella flexneri*

ABSTRAK

Shigellosis adalah punca utama cirit-birit yang teruk, terutamanya dalam kalangan kanak-kanak berumur kurang daripada lima tahun. Kejadian strain tahan pelbagai ubat spesies *Shigella* telah menyumbang kepada ketidakberkesanan rawatan sedia ada. Vaksinasi telah menjadi keperluan penting untuk mencegah shigellosis. Penciptaan vaksin berasaskan epitope telah mengarahkan pembangunan model reka bentuk vaksin baharu. Makrofaj ialah APC khusus yang memproses antigen diikuti dengan mempersembahkan antigen yang diproses kepada sel-T melalui molekul HLA. Profil imunomodulator makrofaj yang dijangkiti oleh strain klinikal *S. flexneri* 2a kekal tidak ditentukan. Makrofaj terbitan THP-1, sebagai model makrofaj manusia, telah dijangkiti secara bebas dengan strain ringan dan virulen *S. flexneri* 2a pada keadaan pertumbuhan standard. Tahap ekspresi gen mediator inflamasi dikenal pasti menggunakan qPCR, manakala pengeluaran NO diukur menggunakan kit ujian NO yang dikomersialkan. Regulasi tinggi TNFα, IL-1β, IL-6, IL-12, iNOS dan NO yang ketara menunjukkan tindak balas pro-radang terhadap jangkitan *S. flexneri* 2a. Ketegangan virulen juga menyebabkan peningkatan ketara dalam tahap ekspresi mRNA sitokin anti-radang. Ekspresi gen kelas II HLA juga disiasat untuk melanjutkan kajian imunopeptidomik dan mendapati pengawalseliaan ketara kelas II HLA oleh makrofaj yang dijangkiti *Shigella*. Strategi imunopeptidomik berasaskan LC-MS/MS digunakan untuk mengenal pasti peptida yang boleh digunakan sebagai calon berpotensi untuk pembangunan vaksin *Shigella* berasaskan epitope. Makrofaj terbitan

THP-1 telah dijangkiti secara individu dengan strain ringan dan virulen *S. flexneri* 2a. Kompleks kelas II HLA-peptida telah diimunopresipitasi dengan anti-HLA DR, DP, dan DQ dan dianalisis menggunakan LC-MS/MS. Penempatan jujukan telah diramalkan menggunakan tiga alat bioinformatik dan mendapati 14 peptida sebagai OMP. Selepas dianalisis menggunakan beberapa alat bioinformatik, serpihan daripada reseptor membran luar FepA dan reseptor yang bergantung kepada TonB diramalkan sebagai calon vaksin *Shigella* yang berpotensi. Rekombinan protein terpilih telah dibina untuk ujian imunogenisiti. Serum tikus yang dijangkiti *Shigella* diperoleh dengan mengimunkan tikus secara bebas dengan OMP dan SAP *S. flexneri*. Ujian imunogenisiti dilakukan dengan mengesan antibodi daripada serum tikus yang diimunisasi menggunakan protein rekombinan yang dibina oleh imunoblot dan ELISA. FepA dan TonB rekombinan adalah reaktif dengan serum tikus imunisasi OMP dan SAP. Kesimpulannya, FepA dan TonB rekombinan adalah calon yang sangat berpotensi untuk pembangunan vaksin *Shigella* berasaskan epitope, dan keberkesanannya boleh dinilai lebih lanjut dalam kajian imunisasi haiwan.

EXPLORING THE IMMUNOMODULATION PROFILES OF THE THP-1 HUMAN MACROPHAGE-DERIVED CELL LINE MEDIATED BY *Shigella flexneri*

ABSTRACT

Shigellosis is the primary cause of severe diarrhea, especially among children less than five years old. The occurrence of multi-drug resistant strains of *Shigella* species has contributed to the ineffectiveness of the existing treatment. Vaccination has become an essential requirement for preventing shigellosis. The invention of an epitope-based vaccine has directed the development of a new vaccine design model. Macrophages are specialized APCs that process antigens and then present the processed antigens to T-cells through HLA molecules. The immunomodulatory profiles of the macrophages infected by the clinical strains of *S. flexneri* 2a have remained undefined. THP-1-derived macrophages, as the model of human macrophages, were infected independently with mild and virulent strains of *S. flexneri* 2a at standard growth conditions. The gene expression level of inflammatory mediators was determined using qPCR, while NO production was measured using a commercial NO assay kit. The significant up-regulation of TNFα, IL-1β, IL-6, IL-12, iNOS, and NO indicates the pro-inflammatory reaction to *S. flexneri* 2a infection. The virulent strain also markedly increased the expression levels of the anti-inflammatory cytokine mRNAs. HLA class II gene expression was also investigated to extend the immunopeptidomics study and found the significant up-regulation of HLA class II by the *Shigella*-infected macrophages. LC-MS/MS-based immunopeptidomics strategy was employed to identify the peptides that can be used as potential candidates for the epitope-based *Shigella* vaccine development. THP-1-derived macrophages were

individually infected with the mild and virulent strains of *S. flexneri* 2a. The HLA class II-peptide complexes were immunoprecipitated with anti-HLA DR, DP, and DQ and analyzed using LC-MS/MS. The localization of the sequences was predicted using three bioinformatic tools and found 14 peptides as OMPs. After being analyzed using several bioinformatics tools, fragments from outer membrane receptor FepA and TonB-dependent receptors were predicted as the potential *Shigella* vaccine candidates. The recombinant of the selected proteins was constructed for immunogenicity assay. *Shigell*a-infected rat serum was obtained by independently immunizing rats with OMPs and SAPs of *S. flexneri*. The immunogenicity assay was performed by capturing the antibody from the serum of the immunized rat using the constructed recombinant proteins by immunoblot and ELISA. The recombinant FepA and TonB were reactive with the OMPs- and SAPs-immunized rat serum. In conclusion, recombinant FepA and TonB were highly potential candidates for epitope-based *Shigella* vaccine development, and the efficacy can be further evaluated in animal immunization studies.

CHAPTER 1

INTRODUCTION

This thesis provides six chapters and displays the second and third sub-chapter. The thesis starts with the introduction in Chapter 1, which outlines the overall thesis content and presents an overview of the knowledge and the area of the knowledge gap. The research questions and objectives of the study are clearly stated to ensure the direction of content in this thesis is focused on the area of interest.

1.1 Background

Diarrhea is a global human health issue. Although usually assumed as a less critical health problem, several studies focus on the significance of diarrhea as a continuing factor of mortality among children, adolescents, and adults in developing countries (Lamberti et al., 2012). While child mortality rates have been reduced in the previous few decades, diarrhea is still the second most common factor of death among children under five years old, which causes 1 in 11 death worldwide (Liu et al., 2015).

Shigella is a bacterial pathogen that contributes significantly to the global burden of diarrheal disease (Zaidi & Estrada-García, 2014). A study in seven developing countries in sub-Saharan Africa and South Asia identified *Shigella* as the primary cause of diarrheal disease among children under five years old (Kotloff et al., 2013). Another study conducted in eight countries in sub-Saharan Africa, South America, and South Asia found that *Shigella* was one of four pathogens related to the leading burden of diarrheal diseases during two years old (Platts-Mills et al., 2015). Nine-year research on diarrhea in the Northern region of Malaysia found the *Shigella* species is the third most common bacterial pathogen that can cause diarrhea among children in this region (BangaSingh et al., 2011).

Shigella is an etiological agent for shigellosis. It is a rod-shaped, Gram-negative bacteria and facultative enteric intracellular pathogen. Kiyoshi Shiga, a scientist from Japan, reported the first isolation of *Shigella* in 1897. Now, after more than 120 years of discovery, shigellosis is still a critical public health issue, particularly in unsafe water supplies and poor hygiene (Niyogi, 2005). Every year up to 165 million cases of shigellosis worldwide, of which around one million deaths are reported among children under five years old (Killackey et al., 2016). Besides affecting young children, *Shigella* also targets the elderly, immunocompromised, and malnutrition persons (Khan et al., 2013).

Shigellosis is an acute intestinal disease spread from person to person through the fecal-oral route by ingesting contaminated food or water. The ingestion of 10 to 100 bacilli is enough to cause severe shigellosis (DuPont et al., 1989). *Shigella* can survive in acidic conditions in the host stomach, partially related to the low infection dose of this bacteria (Gorden & Small, 1993b). *Shigella* can also induce the reduction of antibacterial peptides secreted continuously from the mucosal membrane of the gastrointestinal tract (Islam et al., 2001). The clinical manifestation of shigellosis includes vomiting, abdominal cramp, fever, and diarrhea with mucous and blood. The untreated shigellosis can precede more severe illness and long-term effects such as reactive arthritis and kidney failure (Burnett, 2017). Therefore, effective management and prevention are required to control shigellosis incidence long-term.

Prevention and management of shigellosis have currently become a significant public health challenge. Presently, the occurrence of the multi-drug resistance of *Shigella* makes the treatment of shigellosis increasingly difficult and challenging (Puzari et al., 2018). A new initiative to treat and prevent shigellosis is highly essential. Experiments proved that *Shigella* infection gives protective immunity. In the endemic

areas, shigellosis incidences are high among children under five years old and reduced after that age, indicating that immunity developed after repeated exposure to shigellosis during the first five years (Taylor et al., 1986). Compelling evidence shows the primary infection of *Shigella* presented 76% protective efficacy in response to the reinfection with the same serotype (Ferreccio et al., 1991). This finding indicates the *Shigella* vaccine could be a practical approach that significantly reduces the disease burden.

To date, several *Shigella* vaccines have been developed, including the killed bacteria, live-attenuated, and epitope-based vaccines. Unfortunately, no *Shigella* vaccine has so far been licensed (Zaidi & Estrada-García, 2014). The existing *Shigella* vaccines lack the right balance of robust immunogenicity and are not sufficiently attenuated (Ashkenazi & Cohen, 2013). The idea of forming a vaccine from selective few epitopes has emerged as a more logical approach since the conventional approaches need a longer time to be produced, and the selection of antigens is more or less random. Identifying immunogenic epitopes from *Shigella* is another choice for developing effective epitope-based *Shigella* vaccines.

The interaction between antigen-presenting cells (APCs) and T-cells is considered a critical collaboration between the innate and adaptive arms of the immunity. Both immune responses are necessary to defend the host from the *Shigella* infection (Toapanta et al., 2018). Macrophages are the APCs mainly known for triggering host defense by engulfing foreign particles, degrading the particles, and presenting small parts of the foreign peptide on major histocompatibility complex (MHC) molecules to T-cell receptors to activate adaptive immune response (Neefjes et al., 2011). The activation of the cell-mediated immunity primarily against any

foreign or non-self antigens relies on identifying epitopes or peptides presented on the HLA of the APCs (Mellman & Steinman, 2001).

Knowledge about *Shigella*-derived molecules or epitopes that induce T-cell-specific responses is critical to understand the protection against shigellosis and developing further effective vaccines. Outer membrane proteins (OMPs) of *Shigella* are appropriate immunogenic proteins and effectively induce the immune response of the host (Pore et al., 2011). Identifying specific and antigenic OMPs in *Shigella* is essential to determine the suitable candidate for the *Shigella* vaccine development. The mass spectrometry-based immunopeptidomics approach offers a method to identify peptide sequences derived from the OMPs of *Shigella* presented by HLA class II of *Shigella*-infected macrophages. The identified immunopeptidomes are a highly versatile group of peptides presented on the surface of the macrophages to T-cell receptors, which after their recognition, activate T-cells.

Understanding the immunomodulatory mechanism of macrophages mediated by *Shigella* is crucial for disease prevention and therapeutic intervention. The present study investigates the immunomodulation roles of activated macrophages mediated by *Shigella*. The study will also explore the processed peptides presented by *Shigella*-infected macrophages. The peptides signify the epitopes that are naturally processed and present in a form identifiable by the immune system of the host.

1.2 Problem statement

Shigella is among the most common pathogens accountable for the burden of diarrheal illness and death among children under five years old. Antibiotic treatment is recommended to reduce disease severity. However, due to the risk of antibiotic resistance, shigellosis cannot be continuously treated by mass drug administration. The

prevention of shigellosis would primarily depend on developing a vaccine to strengthen the immune system and fight this microbial pathogen. Before developing the vaccine, a comprehensive understanding of the shigellosis-associated immune response is critically essential. Developing an effective vaccine that induces T-cell-specific responses may provide long-lasting memory and reliable immunity against shigellosis. In the present study, acquiring valuable knowledge about immunomodulatory roles of the human macrophages and peptides presentation by activated macrophages-mediated *Shigella* is crucial for future immunotherapy development. The peptides are potentially the candidate for developing the epitope-based *Shigella* vaccine.

In the present study, *S. flexneri* 2a mild (SH062) and virulent (SH057) strains isolated from Malaysian patients are the strain of choice instead of the commercial reference bacteria. The use of clinical strains gives a similar expression as the real infection and the proteins presented by the HLA molecules of macrophages derived from the isolated strains. These strains were previously determined for the protection efficacy and the OMPs expression conditions (BangaSingh et al., 2016; Harikrishnan et al., 2013). The differences between both clinical strains of *S. flexneri* 2a were previously investigated by BangaSingh et al. (2016). The virulent strain is the wild type of *S. flexneri* 2a and showed resistance to tetracycline (BangaSingh et al., 2016). Sereny test was performed to study the ability of both clinical strains to invade and multiply within the corneal epithelium of guinea pig, as the experiment closely mimics the invasion process in the intestinal epithelium (Powell et al., 1991). The virulent strain showed more virulence in all the guinea pig eyes tested than the mild strain in this test.

The immunomodulation profiles of the human macrophages mediated by the clinically isolated mild and virulence strains of *S. flexneri* 2a need to be identified. Data from this study can be used to study the heterologous protection of these strains against other virulent *Shigella* species.

1.3 Research questions

i. What are the gene expression profiles of inflammatory mediators secreted by macrophages in response to *Shigella* infection?

ii. What peptides are bound to HLA class II of macrophages upon *Shigella* infection?

iii. What are the immunoreactivity profiles of the rats immunized with OMPs and SAPs of *S. flexneri*?

iv. What is the immunogenicity status of the selected immunopeptidomes from the *Shigella*-infected macrophages?

1.4 Objectives

1.4.1 General objective

To investigate the immunomodulatory profile of macrophages in response to *Shigella* infection, which leads to the identification of immunogenic proteins presented by the HLA class II of macrophages.

1.4.2 Specific objectives

i. To identify the gene expression profiles of inflammatory mediators secreted by *Shigella*-infected macrophages.

ii. To investigate the peptides bound to HLA class II of macrophages upon *Shigella* infection.

iii. To determine the immunoreactivity profiles of rats immunized with OMPs and SAPs of *Shigella*.

iv. To identify the immunogenicity status of selected immunopeptidomes from *Shigella*-infected macrophages.

1.5 Significance of the study

Shigella infection exemplifies an exciting model for the immune response and inflammation imbalance. These bacteria also developed several strategies to escape from killing by the immune cells of the host. The antimicrobial peptides expressed in phagocytic cells and at epithelial surfaces are the effector molecules of innate immunity. However, it has been reported that *Shigella* can turn off the expression of antimicrobial peptides, subsequently resulting in severe shigellosis. Like many other tropical diseases, treatment with an antibiotic is considered to manage shigellosis.

Nevertheless, the existence of multi-drug resistance of *Shigella* strains complicates the treatment, causing the prevention of infection is essential. Although the vaccine is often known to offer excellent protection in the host, there is still a lack of appropriate vaccines that can be used to prevent shigellosis. Therefore, in acquiring valuable knowledge for developing the epitope-based *Shigella* vaccine, the discovery of peptide presentation by activated macrophages mediated *Shigella* is increasingly required to reduce the worldwide impact of shigellosis.

1.6 The layout of the thesis

The following chapters describe the details of this study. In chapter 2, the gap in the knowledge discussed in Chapter 1 is explicitly explained through a literature review. The content connects the global incidences of shigellosis, which leads to the need to develop a *Shigella* vaccine. The theoretical background about shigellosis is briefly outlined, and the past work relevant to the host immune response against *Shigella* infection was briefly explained in this chapter. A clear hypothesis about the accountability of macrophages in engulfing pathogen and presenting the peptide to the T-cells strengthens the need to use these cells to identify the peptides as candidates for the development of epitope-based *Shigella* vaccine.

The methods employed to respond to the research questions and to test the hypothesis of the study was elaborated in Chapter 3. This chapter mentions every step involved in conducting this research. The chapter presented a figure illustrating the summary of the overall procedure to explore the research questions. The preparations of reagents, buffers, and media are included in this chapter. A detailed explanation of the research methodology and the data analysis techniques are outlined. The collected data are displayed in chapter 4. The raw data are not presented in this chapter, but the way to collect, process, and analyze the data are briefly described. The data are presented in the bar chart, pie chart, graph, and table. The results are subsequently appraised, and explanations are suggested for particular outcomes in the data.

Chapter 5 discusses the central results and the potential implications of the study. The results are interpreted, and the theoretical and clinically relevant implications of the findings are also considered. In this chapter, the choice of methods is also discussed, in which the possible influence of methodological biases and errors

on data validity. The result discussions are also connected to the theory outlined in the literature review forming the basis of the conclusions.

Chapter 6, the final chapter, summarizes the answer to the research questions, and conclusions are drawn. A strong statement is made in this chapter based on the finding. The content addresses the strengths and general limitations of the study. The proposed areas for future research are also recommended in this chapter.

CHAPTER 2

LITERATURE REVIEW

2.1 A general overview of *Shigella* and shigellosis

In developing countries, infectious diarrhea is the second most common cause of death among children under five years old (Walker et al., 2012; Liu et al., 2015). Diarrhea is generally understood to describe the passage of usually watery or loose feces at least three times per day (Hellard et al., 2003). Every year, nearly 1.7 billion cases of childhood diarrhea are reported by the World Health Organization globally, with an estimated 525,000 deaths yearly. These deaths occur primarily in South Asia and Africa ("WHO. Factsheet on Diarrhoeal disease," 2017).

Numerous studies aimed to determine the species of pathogens in diarrheal stools. With *Salmonella* species and *Entamoeba histolytica*, *Shigella* species are among the primary pathogens of diarrhea isolated from patients in many countries (Walker, Sack, and Black, 2010). A research finding by Zaidi and Estrada-García (2014) also points to *Shigella* as one of the most common causes of diarrheal illness among children, significantly contributing to the burden worldwide.

2.1.1 *Shigella*

Shigella bacteria are Gram-negative bacillus, non-motile, non-spore-forming, non-lactose fermenter, and facultative anaerobes that belong to the Enterobacteriaceae family. The morphology of *Shigella* on the *Salmonella-Shigella* (SS) agar plate and under the light microscope is showed in Figure 2.1. This bacterium shares many common properties with *Escherichia coli* (*E. coli*) strains, and their genetic similarity strongly suggests that they represent a subtype of *E. coli* (Mattock & Blocker, 2017).

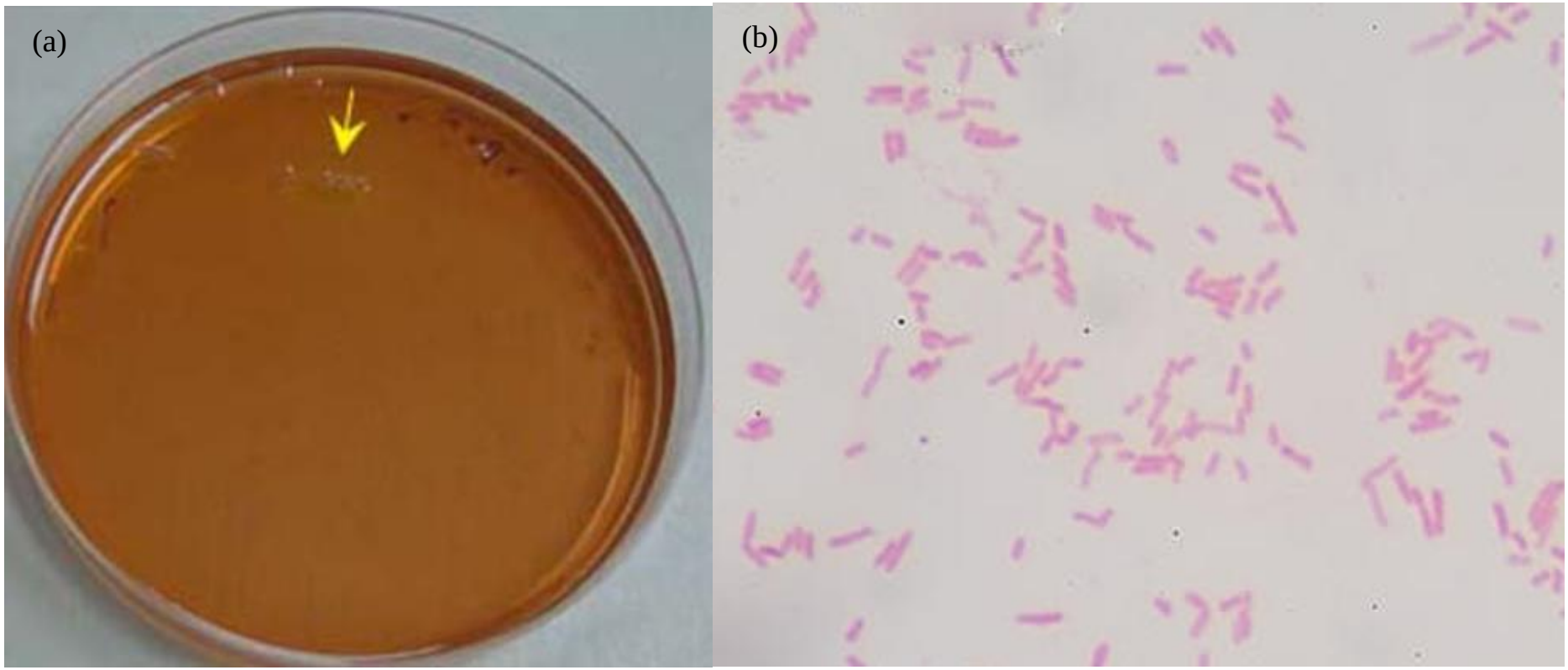

Figure 2.1: (a) The morphology of *Shigella* colonies on SS agar plate. The colonies showed smooth, colorless, without black-centered. (b) *Shigella* was stained with Gram's stain and observed under the light microscope with a 40× objective lens. The shape of the bacteria is rod-shaped and stained with pink color, indicating the Gram-negative bacteria.

With the introduction of enteroinvasive *E. coli,* the distinction between *Shigella* and *E. coli* has become even more difficult. Enteroinvasive *E. coli* are strains that exhibit some of the biochemical properties of *E. coli* and can produce dysentery utilizing the same invasion mechanism as *Shigella*. Enteroinvasive *E. coli* is more closely linked to *Shigella* than non-invasive *E. coli*, according to the sequencing of numerous housekeeping genes (Ragupathi et al., 2018). Within *E. coli*, *Shigella* and enteroinvasive *E. coli* originated from the same origin and formed a single pathovar. A polymerase chain reaction (PCR) targeting the ipaH-gene, a multicopy gene present solely in *Shigella* and enteroinvasive *E. coli*, could be used to distinguish *Shigella* and enteroinvasive *E. coli* from other *E. coli* (Van Den Beld & Reubsaet, 2012).

Shigella is classified into four species from more than forty *Shigella* serotypes. The four *Shigella* species are *Shigella flexneri* (*S. flexneri*; 19 serotypes and two variances, serogroup B), *Shigella sonnei* (*S. sonnei*; one serotype, serogroup D), *Shigella dysenteriae* (*S. dysenteriae*; 15 serotypes, serogroup A), and *Shigella boydii* (*S. boydii*; 20 serotypes,s serogroup C) (Asbury et al., 2018). *Shigella* are divided into different serotypes based on their O antigen molecule of lipopolysaccharide (LPS) found in the outer membrane (Mattock & Blocker, 2017). LPS is one of the major components of the outer membrane of Gram-negative bacteria, a complex molecule consisting of a lipid A anchor, a polysaccharide core, and chains of carbohydrates. Sugars in the polysaccharide chains confer serologic specificity. The *S. flexneri*, *S. dysenteriae,* and *S. boydii* are physiologically similar, while *S. sonnei* differs from the other species based on biochemical reactions. The endemic disease is mainly caused by *S. flexneri* and *S. sonnei*, while the epidemic disease is commonly due to *S. dysentriae* (Niyogi, 2005).

2.1.2 Shigellosis incidences worldwide

Children under five years old are vulnerable to *Shigella* infection, and this age group is the top proportion of shigellosis and death incidence (Asbury et al., 2018). However, during the first six months of life, shigellosis is rare due to the relatively low direct interaction with the environment and the presence of maternal immunity. After the six months, the incidence increases, peaking at 12 to 23 months and decreasing moderately afterward (Kotloff et al., 2013).

The PCR data show a higher *Shigella* disease burden than the conventionally practiced estimate (Livio *et al.*, 2014). A study that isolates *Shigella* from feces and food samples by conventional culture methods and PCR proves the utilization of the PCR method enhanced the identification rate of *Shigella* in food samples from 2.1% to 8.6% and feces samples from 6.7% to 14% (Mokhtari et al., 2012). The other study that diagnoses shigellosis using the quantitative polymerase chain reaction (qPCR) found that the rate of diarrhea cases related to *Shigella* almost doubled, from 9.6% by conventional culture methods to 17.6% by qPCR (Lindsay et al., 2013). This finding confirms that conventional culture methods may underrate the global burden of shigellosis.

Shigella strains have similar pathogenic properties, unique epidemiological characteristics, and varied dissemination arrangements in different geographical regions (Puzari et al., 2018). Shigellosis incidences are mainly in Africa, Asia, and Latin America, where limited access to clean water and poor hygiene promotes the transmission of *Shigella* (Bennish & Ahmed, 2020). Particular *Shigella* species are more dominant than the others in a particular region of the world, as shown in Figure 2.2.

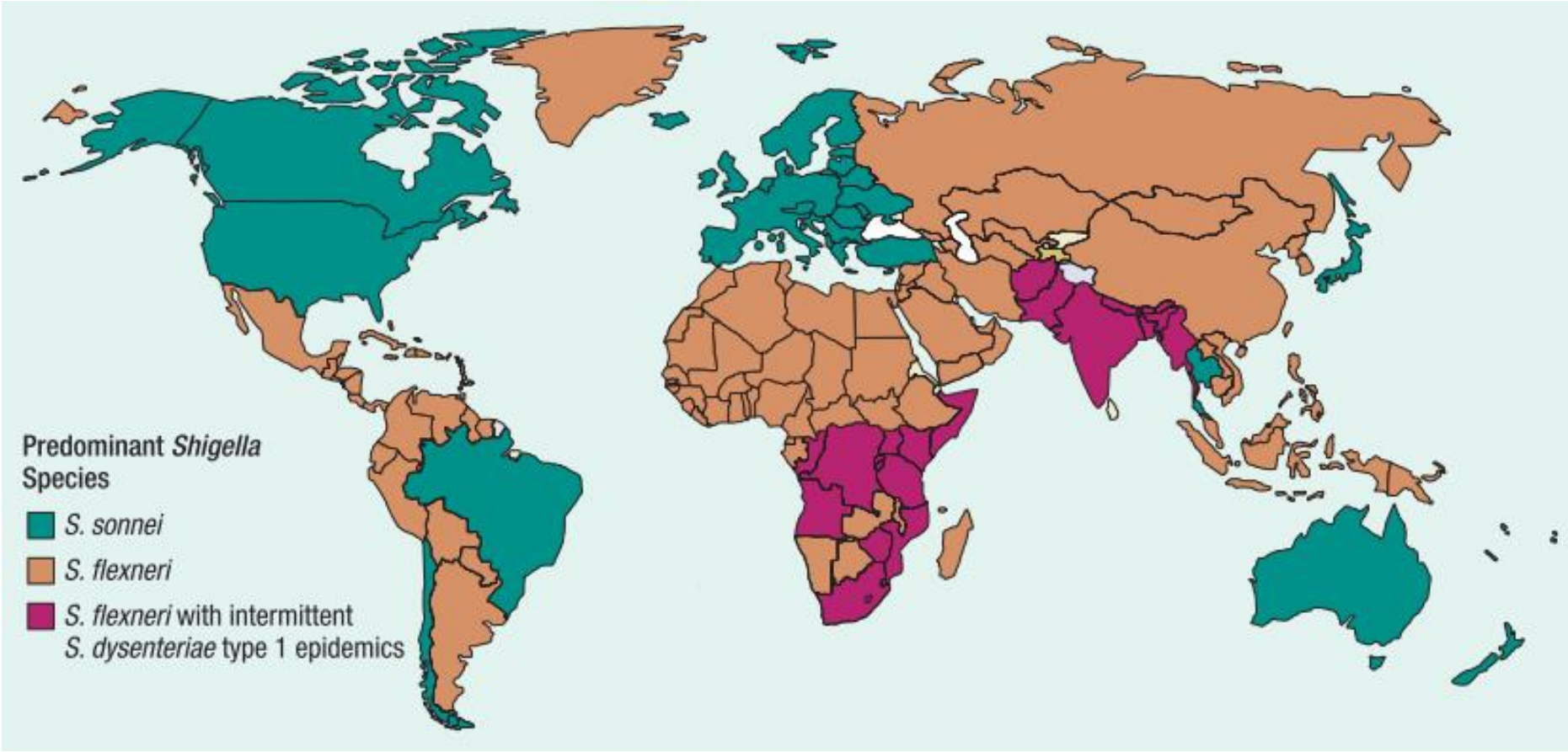

Figure 2.2: The global distribution of *Shigella* species. Shigellosis caused by *S. boydii* is not displayed because it is uncommon. Data source: (Bennish & Ahmed, 2020).

Among the *Shigella* species, *S. flexneri* is the most predominant species in the endemic and developing countries (Lin, 2002; Lee and Puthucheary, 2003; Singh *et al.*, 2011). *S. flexneri* 2a and *S. dysenteriae* 1 is the most commonly isolated species of *Shigella* in Asia and Africa, whereas *S. sonnei* is the most commonly isolated species in developed countries, including Europe and the United States (Salam & Bennish, 1991). *S. dysenteriae* is accountable for epidemic dysentery in the developing world. The fourth species, *S. boydii,* accounts for 6% or less of *Shigell*a infections (Kotloff et al., 1999).

In developing countries, the distribution percentage of *S. flexneri, S. sonnei*, *S. boydii,* and *S. dysenteriae* is 60%, 15%, 6%, and 6%, respectively (Kotloff et al., 1999). The most notable *Shigella* species for developing vaccines are *S. flexneri* 2a, *S. dysenteriae* 1, and *S. sonnei* (Sansonetti, 2008). Bennish and Wojtyniak (1991) explain that *S. flexneri* 2a causes more mortality rate than the other species of *Shigella* in the developing world. Kotloff et al. (1999) found a similar finding in the study that reported *S. flexneri* 2a is the predominant serotype of *S. flexneri*. This serotype is the most prevalent species that causes bacillary dysentery or shigellosis.

2.1.3 Shigellosis incidences in Malaysia

In Malaysia, floods are regular natural disasters. It takes place during the monsoon season almost every year. Figure 2.3 shows the Northeast monsoon from October to March and the Southwest monsoon from May to September yearly (Diya et al., 2014). The East Coast of the Peninsular Malaysia and parts of Sabah and Sarawak are the most affected places during the monsoon season (Bamaiyi et al., 2016). Besides causing life loss and property damage, floodwater is biologically responsible for water contamination (Khan et al., 2014).

Figure 2.3: The map shows the monsoons that hit Malaysia every year. Southwest monsoon (⋯→) arises from May to September, and Northeast monsoon (⟶) occurs from October to March. The figure was created with BioRender.com.

The floodwater is a rich source of various microorganism species with different degrees of antibiotic resistance (D/iya et al., 2014). *Shigella* is a type of bacteria transported in the floodwater from one place to another during the flood (Bamaiyi et al., 2016). Floodwater sampling from 15th December 2014 until 3rd January 2015 showed the presence of *S. flexneri* in the flood areas along the East Coast of Peninsular Malaysia (Bamaiyi et al., 2016; Basri et al., 2015). The microbiological evaluation of floodwater in this region is consistent with the finding in Thailand, the north of Peninsular Malaysia. During the flood in Thailand in 2011, the presence of *Shigella* species in floodwater and tap water was reported (Chaturongkasumrit et al., 2013).

Nine-year research on shigellosis in the Northern region of Malaysia found that the *Shigella* species is the third leading bacterial agent that can cause diarrhea in children (BangaSingh et al., 2011). Isolates of *Shigella* species in Malaysia show resistance to multiple types of drugs. Seventy-three percent out of a hundred samples of *Shigella* isolated in Malaysia showed resistance to at least one antibiotic (Lin et al., 2002). Most *Shigella* species were resistant to kanamycin, trimethoprim-sulphamethoxazole, trimethoprim, chloramphenicol, ampicillin, tetracycline, and streptomycin (BangaSingh et al., 2011; Lin et al., 2002). The high prevalence of multi-drug-resistant *Shigella* and the high incidence of shigellosis during the flood event in Malaysia indicate the need for strategic control against the infection of this disease. Besides improving public hygiene and avoiding the excessive use of current antimicrobial agents, developing a vaccine is an excellent alternative to prevent shigellosis.

2.1.4 Transmission of *Shigella* and clinical manifestation of shigellosis

Shigellosis is associated with malnutrition and poor hygienic standards. This disease is a highly infectious enteric bacterial disease. An infected person who

experiences diarrhea is primarily responsible for transmitting shigellosis (Levine and Levine, 1991). There is no recognized reservoir for *Shigella*. The infection is spread from person to person, most commonly through fecal-oral contamination (Carayol and Nhieu, 2013). The ingestion of contaminated water and food is the common cause of *Shigella* infection (Asbury et al., 2018). A study by Levine and Levine (1991) reported a relationship between the transmission of *Shigella* infection to humans through fly vectors. The flies land on human excrement, which contains bacterium, followed by delivering the disease to fomites such as a dish or doorknob that, in turn, leads to social inoculation.

The transmission control of *Shigella* is difficult as the ingestion of as few as 10 to 100 bacilli is sufficient to cause dehydration caused by severe diarrhea (Schmid-Hempel & Frank, 2007). This low infectious dose affects the disease to spread quickly (Sethuvel et al., 2017). The incubation period depends on the infecting strains, ranging from 6 to 96 hours. No group of individuals is immune to the *Shigella* infection, but a specific person is at higher risk. In a healthy person, the infection is self-limiting, but in malnourished children or immunocompromised individuals, the illness stays for several days to weeks or even months (Kotloff et al., 2013).

The invasion of *Shigella* to the human intestine affects a spectrum of clinical presentations and begins with symptoms such as malaise, fatigue, fever, and short-lasting watery diarrhea. Shigellosis can precede illness from mild to severe diarrhea with the recurrent passage of mucus and blood, abdominal cramping and pain, nausea, high fever, chills, vomiting, and in a few cases, bacteremia (Replogle et al., 2000). The range of clinical symptoms of shigellosis among the elderly above 65 is as severe as in children (Casburn-Jones & Farthing, 2004). Diarrhea can cause the body to dehydrate and lack the salts necessary for survival (Carayol & Nhieu, 2013). The die

incidence of diarrhea has resulted chiefly from severe dehydration and fluid loss (WHO, 2013).

2.1.5 The emergence of multi-drug resistant *Shigella*

Healthy individuals with mild *Shigella* infection usually recover with oral rehydration and home remedies. Dehydration due to diarrhea of any etiology could be corrected by rehydration therapy, and this treatment has dramatically decreased the number of death caused by diarrhea (Niyogi, 2005). However, *Shigella* that invades the intestinal epithelium, which responds to moderate or severe shigellosis, needs special treatment to cure. Antimicrobial therapy is an effective treatment to recover shigellosis by decreasing the shedding of organisms, reducing the severity, and preventing the possibility of lethal complications (Sur et al., 2004). Without antimicrobial treatment or an ineffective antimicrobial is given, an episode of shigellosis lasts from two to 10 days or longer. The risk of serious complications or death increases significantly (Niyogi, 2005).

However, antimicrobial agents become less effective since most pathogens attempt to obviate antibiotics which cause harm and death to the microorganisms (Dhama et al., 2015). This condition results in the increasing failure rates of treatment for bacterial infectious disease as the antibacterial agent is no longer effective against the bacteria (Puzari et al., 2018). The primary mechanism of drug resistance bacteria might be drug modification, alteration of a metabolic pathway, alteration of the target site increasing the active drug efflux across the cell surface, or decreasing drug permeability resulting in reduced drug accumulation (Blair et al., 2015).

Shigella species are included in multi-drug-resistant bacteria in developing countries (Pazhani et al., 2008). *Shigella* showed resistance to primarily used antimicrobial agents for a previous couple of decades. The drug choices become

limited because of the global occurrence of multi-drug-resistant of *Shigella*, and the treatment of shigellosis becomes complicated (DeRoeck et al., 2005). Sulphonamide was the first to treat shigellosis, followed by chloramphenicol and tetracycline. When *Shigella* established resistance against all those antimicrobial agents, the treatment was moved to co-trimoxazole and ampicillin. *Shigella* developed resistance again to the previous drug; thus, treatment turned to nalidixic acid. The following resistance capacity to nalidixic acid introduced the next antimicrobial agent to *Shigella*, fluoroquinolones. However, isolation from various sources currently shows the resistant strains of *Shigella* to fluoroquinolones (Puzari et al., 2018). Ceftriaxone, pivmecillinam, and azithromycin were the antimicrobial agents used to replace fluoroquinolones to treat the infection (Taneja & Mewara, 2016). Unfortunately, isolates are resistant to ceftriaxone and azithromycin (Rahman et al., 2007).

This multi-drug-resistant *Shigella* has become a therapeutic challenge and a considerable concern worldwide. Therefore, innovative strategies are needed to overcome the global impact of shigellosis as the existing prevention and treatment measures are inadequate to control the incidence. Several approaches to prevent this infectious disease are generalizable, such as improving water and cleanliness and basic hygiene practices (Asbury et al., 2018). Other than that, there could be a generous benefit to developing a vaccine versus the most prevalent serotypes of *Shigella* (Niyogi, 2005). Before the vaccine development, a comprehensive understanding related to the disease-causing mechanisms of *Shigella* is essential to assist in this vaccine development (Jennison & Verma, 2004).

2.1.6 Pathogenesis of shigellosis

All serotypes of *Shigella* follow a similar pathogenesis pathway. *Shigella* have neither flagella nor adherence factor. The primary basis of the pathogenesis of

shigellosis is the ability of the bacteria to penetrate and reproduce in the intestinal epithelium, resulting in epithelial inflammation and destruction (Niyogi, 2005). *Shigella* has developed several strategies to escape from being destroyed by the immune cells and invading the neighboring cells (Ismail et al., 2002). The epithelial cells of the intestine act as a barrier to prevent the pathogen in the lumen of the gut from entering the epithelial layers. Unfortunately, *Shigella* can penetrate the colonic epithelial layers and access the lamina propia (Jennison & Verma, 2004). They can invade the intestinal epithelium by secreting virulent factors into the host cell through a type III secretion system, resulting in the down-regulation of the expression of active components, cathelicidin, and beta-defensin hence regulating innate signaling (Ashida et al., 2015). Besides, it has been reported that *Shigella* can turn off the endogenous antimicrobial proteins expression, the essential effector molecules of innate immunity expressed in phagocytes and at epithelial surfaces, resulting in severe shigellosis infection (Gudmundsson et al., 2010).

The *Shigella* infection involves multiple steps that start with the translocation through a particular epithelial cell called microfold cells (M cells). As showed in Figure 2.4, the M cells can carry the whole body of *Shigella* from the intestinal lumen to the underlying mucosal layer through transcytosis. After the transcytosis, the resident macrophages engulf *Shigella* by phagocytosis (Sansonetti and Phalipon, 1999). However, instead of killing the bacteria by phagosome, the *Shigella* can escape the phagosome through invasion plasmid antigens (Ipa) B-mediated phagocytic vacuole lysis (High et al., 1992). This IpaB attaches and stimulates pro-apoptotic cysteine proteases, causing the macrophages to undergo apoptosis (Chen et al., 1996).

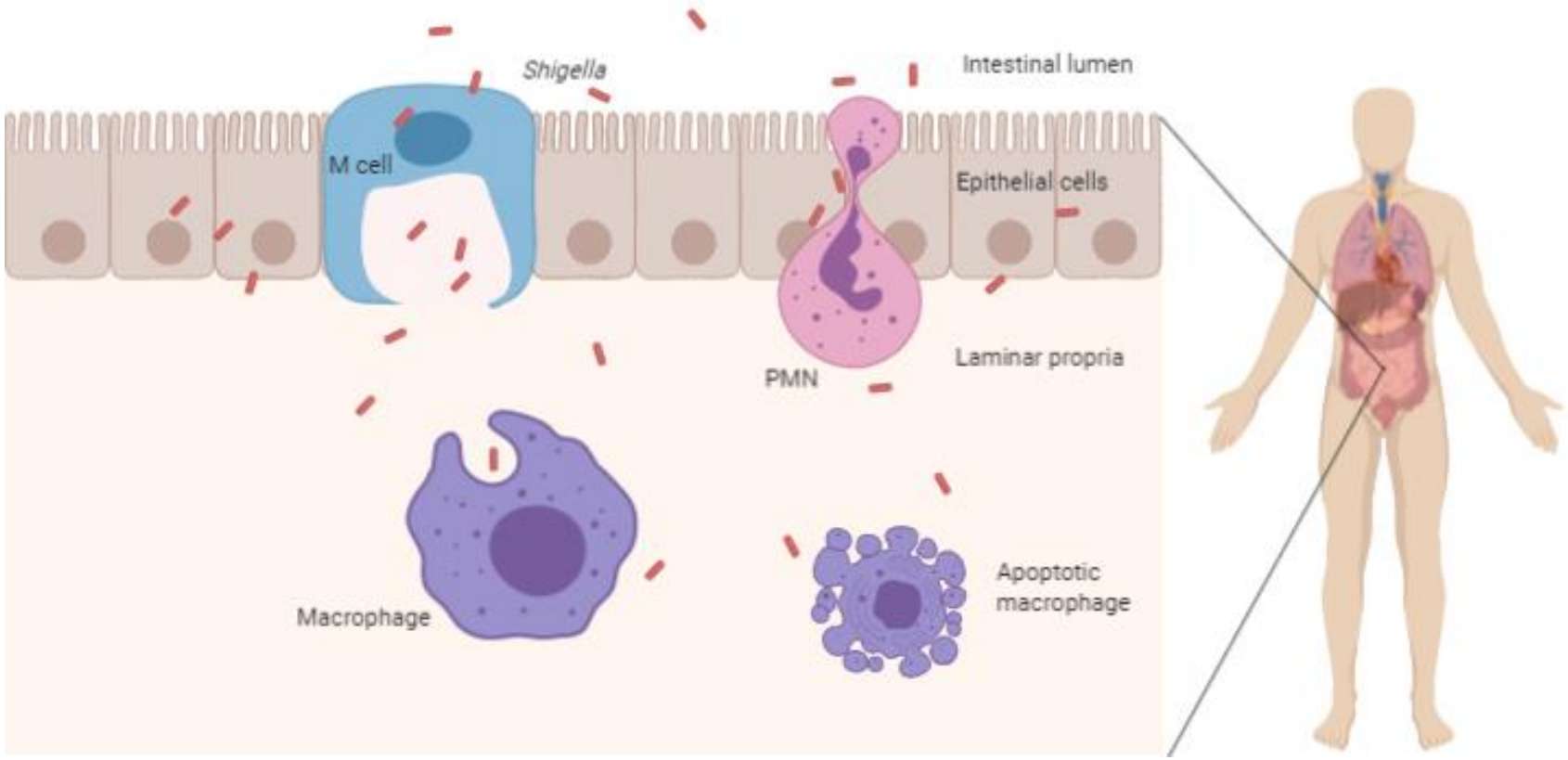

Figure 2.4: *Shigella* invasion during shigellosis. The process begins when *Shigella* invades the epithelial barrier through M cells, followed by the uptake by the resident macrophages. However, *Shigella* produce a variety of virulent factors to escape the phagosome and induce macrophage apoptosis. The apoptotic macrophages produce cytokines, which leads to the recruitment of PMN cells. This causes the destabilization of the epithelial layer, subsequently allowing the invasion of more *Shigella* through a basolateral route and dissemination within the mucosa. The figure was created with BioRender.com.

During apoptosis, the macrophage cell death is attended with the secretion of a large number of cytokines, including interleukin (IL)-1β and IL-8, which is mainly to recruit polymorphonuclear (PMN) cells to the infection site (Beatty & Sansonetti, 1997). PMN recruitment compromises the integrity of the epithelial layer, which triggers the destabilization of the epithelial layer, subsequently permitting the invasion of *Shigella* to intestinal epithelial cells through a basolateral route (Carneiro et al., 2008). In this early stage of infection, the tissue alteration facilitates impaired adsorption of water, nutrients, and solutes that subsequently causes watery diarrhea (Niyogi, 2005).

Once internalized, *Shigella* begins to rapidly reproduce while engaging the host actin to drive the *Shigella* within the intracellular host environment. This allows efficient intracellular movement and accelerates cell dissemination (Schroeder & Hilbi, 2008). The characteristic of *Shigella* pathogenesis that makes it particularly detrimental to infect an individual is the rapid spreading of the bacteria via the epithelial layer without the requirement to re-enter the basolateral pocket to infect the neighboring cells (Schroeder & Hilbi, 2008). Consequently, *Shigella* can spread and replicate within the epithelial layer of the intestine while escaping exposure to the immune cells and extracellular environment (Jennison & Verma, 2004).

2.2 Immune responses against shigellosis

The hallmark of shigellosis is stimulating the host immune response to combat the *Shigella.* The immune system of a host is divided into innate and adaptive immunity. The interaction of bacteria with the mucosal epithelial cells induces multiple processes that start with innate immune recognition of the microbial pathogen. Briefly, the innate immune response indirectly alerts the immune cells of

the host to the infection. It ultimately triggers the adaptive immune response, mediated by T-cells and B-cells. This specific immunity is responsible for clearing the infection and developing memory components of the disease (Janeway & Medzhitov, 2002).

2.2.1 Innate immunity

The first line of defense against *Shigella* infection is the mucosal intestinal epithelial layer of the host (McGhee & Fujihashi, 2012). This epithelial barrier protects the host from the invasion and systemic spread of commensal and pathogenic microorganisms (Phalipon & Sansonetti, 2007). The viscosity, thickness, and high acidity of this mucosa protect the host by preventing microbial adherence and interpenetration (Silva et al., 2013). However, one study proved that *Shigella* could survive in the stomach with high acidity better than other enteric pathogens. The *Shigella* isolates could survive at pH 2.5 for more than two hours, while not a single *Salmonella* isolate can survive (Gorden & Small, 1993).

Antimicrobial proteins in the intestinal environment are essential in the bacterial host defense (Hancock & Scott, 2000). Antimicrobial proteins are lytic enzymes that disrupt microbial membrane structure and function (Ganz, 1999). The primary classes of antimicrobial proteins in humans are cathelicidins and defensins. A report about the expression of human intestinal defensin in transgenic mice proved the importance of this defensin as an intestinally-secreted antibiotic peptide to attack the infection from *Salmonella typhimurium* (Salzman et al., 2003). However, S*higella* develop a strategy to inhibit the generation of these antimicrobials (Phalipon & Sansonetti, 2007).

Once *Shigella* have invaded the epithelial barrier of the host, body defense depends initially on innate or nonspecific mechanisms (Nauciel, 1999). In the early defense, the innate immune response involves immune cells against foreign