# MOLECULAR CHARACTERIZATION AND PRODUCTION OF A SPECIFIC RECOMBINANT PROTEIN OF *Shigella flexneri*: TOWARDS DEVELOPMENT OF A RAPID IMMUNOCHROMATOGRAPHY DIAGNOSTIC TEST FOR DYSENTERY

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**By**

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## DEDICATIONS

**This thesis is dedicated to my husband, Lau Hut Yee for his encouragements and patience, and to my children Simran and Roshan who have been a great source of motivation and inspiration.**

**This thesis is also dedicated to all those who believe in the richness of learning.**

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| | |
|---|---|
| Amp | Ampicillin |
| AP | Alkaline Phosphatase |
| bp | Base pair |
| BSA | Bovine Serum Albumin |
| $CaCl_2$ | Calcium chloride |
| dATP | Deoxyadenosine triphosphate |
| DNA | Deoxyribonucleic acid |
| DNase | Deoxyribonuclease |
| dNTP | Deoxynucleotide triphosphates |
| DTT | Dithiothreitol |
| EDTA | Ethylene diamine tetra acetic acid |
| EIA | Enzyme Immunoassay |
| Ek | Enterokinase |
| ELISA | Enzyme Linked Immunosorbent Assay |
| FPLC | Fast Protein Liquid Chromatography |
| hCG | Human Chorionic Gonadotropin |
| ICT | Immunochromatography |
| IMAC | Immobilized metal affinity chromatography |
| IPTG | Isopropyl-beta-D-thiogalactopyranoside |
| kb | Kilobase |
| kDa | Kilodalton |
| LIC | Ligation Independent Cloning |
| mA | Milliampere |
| MW | Molecular Weight |
| Ni-NTA | Nickel-Nitrilotriacetic Acid |
| OD | Optical Density |
| OMP | Outer Membrane Protein |
| ompA | Outer membrane protein A |
| PCR | Polymerase Chain Reaction |
| *Pfu* DNA polymerase | *Pyrococcus furiousus* DNA polymerase |
| psi | Pounds per square inch |
| Rf | Relative mobility factor |
| SAP | Surface Associated Protein |
| SDS | Sodium Dodecyl Sulphate |
| SDS-PAGE | Sodium-Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis |
| TAE | Tris-Acetate EDTA |
| *Taq* DNA polymerase | *Thermus aquaticus* DNA polymerase |
| TEMED | N,N,N',N'-tetramethylethylenediamine |
| TSB | Tryptic Soy Broth |
| WHO | World Health Organization |
| λ | Wavelength |

**PENCIRIAN MOLEKULAR DAN PENGHASILAN PROTEIN REKOMBINAN YANG SPESIFIK UNTUK *Shigella flexneri*: KE ARAH PEMBANGUNAN UJIAN DIAGNOSTIK SEGERA BAGI PENYAKIT DISENTERI**

## ABSTRAK

Disenteri basilus atau juga dikenali sebagai shigelosis disebabkan terutamanya oleh bakteria *Shigella*. Shigelosis merupakan suatu penyakit yang serius di mana hampir 165 juta penduduk dijangkiti setiap tahun. Ia merupakan infeksi enterik invasif yang biasanya mempunyai gejala seperti demam, sakit abdomen dan diarea berdarah. *S. flexneri* merupakan spesies yang paling kerap dipencilkan dan biasanya menyebabkan shigelosis secara endemik di negara yang sedang membangun. Kaedah pengesanan rutin bergantung kepada teknik pengkulturan dan pengenalpastian *Shigella* spp. daripada tinja, adalah sukar dan memerlukan proses ujikaji yang cerewet, memakan masa 3 hingga 5 hari dan mempunyai sensitiviti yang rendah. Sehingga kini, tiada ujian diagnostik segera yang terdapat di pasaran bagi mengesan shigelosis. Oleh yang demikian, ujian diagnostik segera yang ringkas, sensitif dan spesifik perlu dibangunkan bagi mengesan jangkitan pada peringkat awal. Ujian diagnostik segera secara langsung akan membantu dari segi memberi pengendalian dan rawatan yang berkesan kepada pesakit serta dapat menyediakan strategi pencegahan awal perebakan penyakit yang sangat mudah ditularkan tersebut. Pembangunan ujian diagnostik segera berasaskan teknologi imunoasai generasi baru amat diperlukan supaya proses pengesanan dapat dilakukan di tepi katil pesakit dengan segera.

Kajian terdahulu telah mengenalpasti satu protein yang antigenik dan spesifik pada *Shigella* spp. berberat molekul 35 kDa dan berpotensi dalam pengesanan jangkitan *Shigella*. Sehubungan dengan itu, tujuan utama kajian ini adalah untuk membangunkan parameter asas yang penting bagi menghasilkan protein rekombinan

yang tulen dalam kuantiti yang tinggi ke arah pembangunan ujian imunokromatografi segera bagi pengesanan segera jangkitan *S. flexneri*.

Jujukan asid amino protein tersebut ditentukan dengan kaedah penjujukan asid amino. Seterusnya analisis homologi dilakukan terhadap pengkalan data jujukan lengkap genom *S. flexneri* untuk menentukan jujukan lengkap asid amino serta nukleotida gen yang mengkodkan protein 35 kDa tersebut. Hasil daripada analisis tersebut telah mengenalpasti gen yang mengkodkan protein ompA. Pengenalpastian protein tersebut seterusnya disahkan melalui teknik MALDI-ToF dan kehadiran peptida signal pada protein tersebut ditentukan. Plasmid rekombinan dihasilkan dengan menggunakan vektor pET 43.1 Ek/LIC untuk proses ekspresi protein rekombinan ompA di dalam *E. coli*. Klon rekombinan yang terhasil dipastikan mengandungi jujukan selitan yang betul dan proses ekspresi protein rekombinan dioptimakan bagi menghasilkan protein rekombinan yang antigenik dan spesifik dalam kuantiti yang tinggi. Kehadiran protein rekombinan ditentukan dengan menggunakan kaedah elektroforesis SDS-PAGE dan reaktiviti imun ditentukan dengan kaedah imunoasai blot Western.

Protein rekombinan yang mempunyai histidin ditulenkan dengan menggunakan teknologi IMAC. Proses penulenan protein dilakukan secara kecil pada awal ujikaji dan seterusnya ditingkatkan dengan menggunakan sistem FPLC. Kaedah penulenan dioptimakan terlebih dahulu dan didapati 150 mM imidazole adalah mencukupi untuk mengeluarkan protein daripada resin. Protein berkepekatan lebih daripada 4.0 mg/ml telah dihasilkan dengan menggunakan kaedah ini dan lebih kurang 88 mg protein dapat diperolehi daripada 3 L kultur bakteria. Kajian ini berjaya menghasilkan maklumat asas yang penting dan berguna untuk strategi menghasilkan protein rekombinan yang tulen.

Protein yang telah ditulenkan digunakan untuk membangunkan satu ujian imunokromatografi segera bagi mengesan antibodi IgA yang spesifik di dalam serum pesakit yang dijangkiti dengan *S. flexneri*. Ujian ini berprinsip bahawa antibodi IgA yang spesifik terhadap protein rekombinan yang telah disembur di atas membran akan dikesan dengan partikel yang dikonjugat dengan emas. Intergrasi antigen OMP daripada *S. flexneri* di dalam ujian tersebut didapati memainkan peranan sebagai antigen kontrol dalaman. Kajian pengoptimuman dilakukan terhadap kepekatan antigen yang digunakan supaya sensitiviti dan spesifisiti yang maksimum diperolehi. Kepekatan protein rekombinan 1 mg/ml dan antigen OMP 4 mg/ml adalah kepekatan yang optimum bagi ujian tersebut memandangkan ujian tersebut memberikan reaksi positif dengan serum *S. flexneri* dan reaksi negatif diperolehi dengan serum dari pesakit yang dijangkiti dengan *Salmonella typhi* dan serum daripada individu sihat. Kajian ini menunjukkan bahawa kuantiti dan posisi antigen yang disembur adalah kritikal untuk memperolehi keputusan ujian yang sebenar.

Kajian penilaian awal di dalam makmal dilakukan dengan menggunakan 56 serum dari pesakit yang dijangkiti dengan *S. flexneri*, jangkitan berkaitan yang lain serta serum daripada individu sihat. Ujian ini menghasilkan keputusan positif apabila ditindakbalaskan dengan ke semua serum daripada pesakit yang dijangkiti dengan *S. flexneri* (n=6) dan memberi keputusan negatif kecuali satu serum daripada individu sihat, apabila ditindakbalaskan dengan serum daripada pesakit yang bukan dijangkiti dengan *S. flexneri* (n=50). Berdasarkan keputusan penilaian terhadap ujian yang dibangunkan, ujian tersebut adalah spesifik terhadap pengenalpastian jangkitan *S. flexneri* dengan 100% sensitiviti, 98% spesifisiti, 85.7% PPV dan 100% NPV. Sebagai kesimpulan, asai yang dibangunkan tersebut mempunyai potensi diagnostik bagi pengesanan jangkitan *S. flexneri*. Walau bagaimanapun, kajian penilaian lanjutan yang menggunakan serum yang lebih banyak dari negara yang endemik perlu dilakukan untuk menentukan potensi sebenar ujian tersebut.

**MOLECULAR CHARACTERIZATION AND PRODUCTION OF A SPECIFIC RECOMBINANT PROTEIN OF *Shigella flexneri*: TOWARDS DEVELOPMENT OF A RAPID IMMUNOCHROMATOGRAPHY DIAGNOSTIC TEST FOR DYSENTERY**

## ABSTRACT

Bacillary dysentery is caused mainly by infection with *Shigella* spp. which is also known as *s*higellosis. It remains a common and serious health problem throughout the world and has been estimated to infect about 165 million people worldwide annually. It is an acute invasive enteric infection often characterized by abdominal pain, fever and bloody diarrhea. *S. flexneri* is the most frequently isolated species causing endemic shigellosis in developing countries. The current method of diagnosis still depends on the traditional culture method, which is laborious, relatively insensitive and takes 3 to 5 days to produce result. To date, no rapid diagnostic methods are available commercially. Hence there is a need to develop a rapid, simple, sensitive and specific diagnostic test, which can diagnose *S. flexneri* at early stages of infection. A rapid and reliable diagnostic assay would significantly improve effective treatment, management and control of this highly infectious bacterium. There is a need for the development of the next generation immunoassay platform which provides a more rapid, sensitive and portable point-of-care assay.

In the effort to improve the diagnosis of shigellosis, a specific and antigenic protein of 35 kDa in size has been previously identified which has a diagnostic potential for detection of *Shigella* infections. The aim of this study was to develop important fundamental parameters to generate sufficient amounts of purified recombinant 35 kDa protein and to exploit its application in developing a rapid dipstick test, as an alternative diagnostic test for the detection of anti-*S. flexneri* IgA antibody.

The N-terminal amino acid sequences encoding the 35 kDa protein were determined by amino acid sequencing and subsequently homology search was performed against the *S. flexneri* genome sequence database to determine the complete amino acid and nucleic acid sequences. The identified ompA protein was validated by MALDI-ToF technique and presence of signal peptide was determined. A recombinant plasmid was constructed using pET 43.1 Ek/LIC vector for the expression of the recombinant ompA protein in *E. coli*. This recombinant clone was verified to contain the appropriate insert and important parameters were optimized to express the target protein in *E. coli* BL21 (DE3) to generate high concentrations of soluble, antigenic and specific recombinant protein. The target protein was verified by SDS-PAGE analysis and immunogenicity was determined by Western blot technique.

The recombinant protein was over-expressed and the histidine-tagged protein was purified using IMAC technology. Small-scale protein purification was performed initially using a batch column technique and consequently was up-scaled using FPLC system to generate high concentrations of purified protein in a large quantity. The purification parameters were optimized and the 150 mM of imidazole was sufficient to elute the target protein. Protein concentration above 4.0 mg/ml was generated and approximately 88 mg of total protein was eluted from 3 L of bacterial culture using the optimized procedure. This study provided important fundamental information useful for devising strategies to purify the soluble recombinant protein.

The purified recombinant protein was used to develop a rapid dipstick test for the detection of specific IgA antibody in sera from patients infected with *S. flexneri*. The test is based on the principle that the IgA antibodies against immobilized purified recombinant ompA antigens on a membrane are detected by a colloidal gold particle technique. Incorporation of OMP antigen of *S. flexneri* served as the internal control antigen in the dipstick test. Different concentrations of antigens were lined to obtain

maximum sensitivity and specificity. The recombinant protein concentration of 1 mg/ml and OMP concentration of 4 mg/ml was selected in this study since the test strip gave positive reactions when probed against the *S. flexneri* sera and produced negative reactions when probed against the non-*S. flexneri* sera. This study demonstrated that the positions and the amount of the antigens lined in the Test Line are critical in obtaining a true positive result.

A preliminary laboratory evaluation was carried out using 56 sera from patients with *S. flexneri* infections, other related infections and healthy individuals' sera. The test produced positive results in all patients infected with *S. flexneri* (n=6) and was found negative in all except one of the sera from non *S. flexneri* cases (n=50). This test was found to be specific in recognizing *S. flexneri* infections with 100% sensitivity, 98% specificity, 85.7% positive predictive value and 100% negative predictive value. The results of this assay showed promise with high reliability and accuracy and capable of discriminating non-*S. flexneri* cases in an endemic area. It includes benefits of user-friendly format that is rapid, simple, reliable, with built-in control and relatively inexpensive. In conclusion, the dipstick test has a diagnostic value and is a useful method when it is used together with the bacteriological gold standard culture methods in the diagnosis of this highly contagious disease. However, further studies with a larger set of patients from various endemic areas need to be investigated to evaluate the true performance of this dipstick test.

## LIST OF PUBLICATIONS

**NATIONAL DIAGNOSTIC CONFERENCE AND WORKSHOP, RENAISSANCE KOTA BHARU HOTEL, KELANTAN, 4th - 7th OCTOBER 2004.**

### Application of recombinant ompA protein in the development of a rapid immunodiagnosis of *Shigella flexneri* infection

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**Abstract**

*Shigella flexneri* is highly infectious agent that is transmitted by the faecal oral route. It is the most common etiological agent of bacillary dysentery contracted especially among children in developing countries. The current laboratory detection method is by culturing the patients' stool onto selective media. However, the major limitations of the culture method are low isolation rate, time-consuming and labour-intensive. The development of a rapid detection method for *Shigella* would greatly benefit the disease control and patient management. In an earlier study, ompA protein of *Shigella flexneri* exhibited high antigenicity and specificity. The purpose of this study was to produce recombinant ompA protein of *Shigella flexneri* and to use it to develop a rapid test to detect *Shigella flexneri* in patients' specimens. The specific protein was cloned and expressed to generate sufficient

amount of protein for the development of a specific test. PCR cloning was performed using pET Ek/LIC 43.1 vector and the specific recombinant protein was expressed in BL21 *E. coli* expression host. Protein expression profile was visualized via SDS-PAGE and antigenicity of the recombinant protein was determined using Western blot technique. The histidine-tagged protein was purified using the standard immobilized metal affinity chromatography technique (IMAC). The purified recombinant ompA protein was immobilized on a nitrocellulose membrane attached to a plastic film and cut into strips. The test strips were used to detect specific immunoglobulin A in the patients' sera by means of immunochromatography technique using gold conjugated anti-human IgA. Nucleotide sequence of the recombinant plasmid revealed an open reading frame encoding the appropriate ompA protein. Based on the SDS-PAGE analysis, the recombinant protein tagged with the NusA fusion protein had a molecular mass of about 100 kDa. The ompA protein was expressed abundantly in soluble form and was purified to near homogeneity. The protein was antigenic when probed with mouse polyvalent sera raised against the native ompA protein, as well as sera from patients infected with *S. flexneri.* Immunochromatography test was developed and optimized with sera from patients infected with *S. flexneri* as well as sera from other related infections. This preliminary study showed that the immunochromatography test developed is simple and rapid. This test can play a useful role as a complementary test in the diagnosis of *S. flexneri* infection. However, further optimization are required to improve its performance.

**1st NATIONAL COLLOQUIUM AND WORKSHOP IN PHARMACOGENETICS, KUBANG KERIAN, KELANTAN, 1st - 4th APRIL 2004.**

# Expression and purification of ompA protein of *Shigella flexneri* in *Escherichia coli*

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## Introduction

*Shigella flexneri* is the most common etiological agent of bacillary dysentery contracted especially among children in developing countries. The current laboratory detection method is by culturing the patients stool onto selective media. However, this pathogenic bacterium may be present in low numbers in stool and the culture method is time-consuming and labour-intensive.

## Objectives

Development of a specific diagnostic test requires the use of a specific antigen. In an earlier study, ompA protein of *Shigella flexneri* exhibited high antigenicity and specificity. The purpose of this study was to clone, express and purify the ompA protein of *Shigella flexneri*.

## Methods

PCR cloning was performed using pET Ek/LIC 43.1 vector and the specific recombinant protein was expressed in BL21 *E. coli* expression host induced with 1 mM IPTG. Protein

expression profile was visualized via SDS-PAGE and antigenicity of the recombinant protein was determined using Western blotting technique. The histidine-tagged protein was purified using the standard immobilized metal affinity chromatography technique (IMAC).

**Results**

Nucleotide sequence of the recombinant plasmid revealed an open reading frame encoding the appropriate ompA protein. Based on the SDS-PAGE analysis, the recombinant protein tagged with the NusA fusion protein had a molecular mass of about 100 kDa. The ompA protein was expressed abundantly in soluble form and was purified to near homogeneity using native conditions with 250 mM imidazole in the elution buffer. The protein was antigenic when probed with mouse polyvalent sera raised against the native ompA protein extracted from *Shigella flexneri*, as well as patients sera infected with *S. flexneri* when tested against IgA.

**Discussion and conclusion**

The production of large quantities of recombinant ompA protein using the expression and purification protocol described above would be useful for development of rapid detection test, crystallographic analysis, studies of pathogen-receptor interactions and potential for vaccine design.

**THIRD FEDERATION OF ASIA-PACIFIC MICROBIOLOGY SOCIETIES (FAPMS) CONFERENCE, KUALA LUMPUR, 15th - 18th OCTOBER 2003.**

# Characterization, cloning and expression of a specific membrane protein of *Shigella flexneri*

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## Abstract

Conventional laboratory diagnosis of shigellae (stool culture method) is relatively inefficient, time-consuming and laborious. Development of a specific diagnostic test requires the use of a specific antigen. In our earlier study, a specific and antigenic membrane protein of *Shigella flexneri* was determined. Purpose of this study was to characterize, clone and express the specific membrane protein. PCR cloning was performed using pET Ek/LIC 43.1 vector and the specific recombinant protein was expressed in BL21 *E. coli* expression host induced with 1 mM IPTG. Protein expression profile was visualized via SDS-PAGE and antigenicity of the recombinant protein was determined using Western blotting techniques. Based on the SDS-PAGE analysis, the protein of interest was expressed abundantly in soluble form, in which majority of the protein was in the supernatant of the sonicated lysed cells. The molecular size of the recombinant protein tagged with the NusA fusion protein was estimated at ~100 kDa. Nucleotide sequence of the recombinant plasmid revealed an open reading frame encoding the appropriate specific protein. Further analysis indicated that the protein

contained a 21 N-terminal amino acid residue predicted to be a signal peptide. The protein was antigenic when probed with mouse polyvalent sera raised against the native membrane protein extracted from *Shigella flexneri*, as well as patients infected with *S. flexneri* when tested against IgA.

**2nd INTERNATIONAL CONFERENCE ON BIOINFORMATICS (INCOB), PENANG, MALAYSIA, 7th - 10th SEPTEMBER 2003.**

# Application of bioinformatic tools for identification of gene encoding for a specific membrane protein (ompA) of *Shigella flexneri* and *Shigella sonnei*

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**Abstract**

*Shigella* is the most common etiological agent of bacillary dysentery contracted mainly among children in developing countries. Advances in bioinformatics tools are important for identification of partial or complete gene and protein sequence. In our previous study, we have identified a specific membrane protein against IgA in patients infected with *Shigella flexneri* and *Shigella sonnei*. Aim of this study is to determine the complete sequence of the gene encoding the specific protein for *Shigella flexneri* and *Shigella sonnei*. The pre-fractionation of membrane protein was performed by SDS-PAGE and the specific band was eluted via electro-elution. The purified protein from pre-fractionation was subjected to SDS-PAGE followed by electroblotting onto PVDF membrane for N-terminal amino acid sequencing. Homology search was performed for the amino acid sequence using BLAST at url: ncbi.nlm.nih.gov/BLAST and the complete gene sequence encoding the specific protein was identified as *ompA* gene of *Shigella flexneri*. To determine the gene encoding for the similar membrane protein in *Shigella sonnei*, PCR was performed with primers designed at 100 bases flanking of the gene with *pfu* polymerase and *Shigella sonnei*

genomic DNA as the PCR template. The purified PCR product was sequenced and the complete gene sequence was determined. We found that the gene sequence of *S. sonnei* is about 96% similar to *S. flexneri ompA* gene. Both gene sequences for *S. flexneri* and *S. sonnei* were submitted to GeneBank with accession numbers AY305875 and AY305874 respectively.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Introduction to *Shigella*

### 1.1.1 History and significance of *Shigella* discovery

*Shigella* is a Gram-negative bacterium that causes bacillary dysentery or also known as shigellosis. Shigellosis is an acute invasive enteric infection often characterized by abdominal pain, fever and bloody diarrhea (dysentery). Isolation of this bacterium from faeces of individuals with acute dysentery was first described by Chantemesse and Widel in 1888 (reviewed by Lan & Reeves, 2002). A detailed description of the organism that caused the dysentery was reported by Shiga in 1898 and named the organism as *Bacillus dysenteriae*. In late 1940, the genus *Shigella* was classified into four species; *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei* which were also known as *Shigella* subgroups A, B, C and D respectively (Ewing, 1986).

In the earlier classification, *Shigella* and *Escherichia coli* were classified in the same genus because of their genetic similarity. Recently, a comparative genomic study between the two organisms revealed that 175 of the total 3235 open reading frames were exclusive for *S. flexneri* (Wei *et al.*, 2003). However, *Shigella* strains were put in a different genus from *E. coli* because of their medical significance, human host interactions, pathogenicity, physiology (failure to ferment lactose or decarboxylate lysine) and serological characteristics (Escobar-Paramo *et al.*, 2003).

Shigellosis remains a common and serious health problem throughout the world (Echeverria *et al.*, 1992; Oyofo *et al.*, 2002; Kimura *et al.*, 2004). The global burden of *Shigella* infection was estimated by analyzing the number of cases worldwide

according to the world's population as categorized into developed and developing countries and into age groups (Kotloff *et al*., 1999). This study found that the annual number of *Shigella* episodes throughout the world was 164.7 million, of which 163.2 million occurred in developing countries, with 1.1 million deaths. Sixty-nine percent of the episodes and 61 percent of all shigellosis-related deaths involved children younger than 5 years of age. Shigellosis is endemic in many developing countries and occasionally occurs as an epidemic. *S. dysenteriae* serotype 1, also known as the Shiga bacillus has been recognized as the major cause of epidemic dysentery. *S. sonnei* and *S. boydii* usually causes relatively mild illness in which diarrhea may be watery or bloody. *S. flexneri* is the main cause of endemic shigellosis in developing countries (Faruque *et al.*, 2002; Lee & Puthucheary, 2003; Niyogi & Pazhani, 2003).

### 1.1.2 Epidemiology of shigellosis

Shigellosis is a global human health problem. It is the most important cause of bloody diarrhea worldwide, especially in developing countries with substandard hygiene and poor quality of water supplies (Stoll *et al.*, 1982; Bennish & Wojtyniak, 1991; Niyogi, 2005). Shigellosis is endemic in numerous developing countries (Echeverria *et al.*, 1991; Clemens *et al.*, 1999). Various studies carried out in developing countries showed that *Shigella* is associated with 5% to 15% of the diarrheal cases and 30% to 50% of dysentery cases (Echeverria *et al.*, 1991; Faruque *et al.*, 2002). Endemic *Shigella* is responsible for approximately 10% of all diarrheal episodes among children younger than five years living in developing countries (Bennish *et al.*, 1990; Kotloff *et al*., 1999).

The global burden was determined from various locations estimated that shigellosis accounts for 164.7 million cases, of which 99% (163.2 million cases) occurred in developing countries (Kotloff *et al*., 1999). The burden of shigellosis in Asia was estimated from published studies that were initiated in Asian countries. The annual

number of *Shigella* episodes and deaths in Asia was estimated to be 91 million and 414 000 respectively. *S. flexneri* is the most common serotype, followed by *S. sonnei* (WHO, Weekly Epidemiological Report, 2005). In Bangladesh, dysentery due to *Shigella* causes 75 000 deaths among children annually during peak epidemic years and estimated 35 000 deaths occur during non-epidemic years (Bennish & Wojtyniak, 1991). In general, both the incidence and the fatality rates are highest among very young children and the elderly. The highest incidence of shigellosis is in young children, usually those ages less than 5 years (Taylor *et. al.*, 1986; Bennish *et al.*, 1990; Minh *et al.*, 1998; Kotloff *et al*., 1999; Vargas *et al.*, 2004).

*S. flexneri* has been the most frequently isolated species in developing countries (Taylor *et. al.*, 1991; Faruque *et al.*, 2002; Niyogi & Pazhani, 2003). *S. flexneri* serotype 2a was found to be the major endemic serotype in developing countries (Kotloff *et al*., 1999). The median percentages of isolates of *S. flexneri*, *S. sonnei*, *S. boydii* and *S. dysenteriae* were 60%, 15%, 6%, and 6% respectively, in developing countries; and 16%, 77%, 2%, and 1% respectively, in developed countries (Kotloff *et al*., 1999). A case-control study to characterize the epidemiology of bloody diarrhea in rural Western Kenya reported that 80% of the bacterial pathogens isolated were due to *Shigella* spp., of which approximately 49% was caused by *S. flexneri* (Brooks *et al.*, 2003). Another study in Pakistan reported that *Shigella* spp. were a more commonly occurring enteric pathogen in children having diarrhea and showed that *S. flexneri* was the most frequently identified species compared with *S. dysenteriae, S. boydii* and *S. sonnei.* (Khalil *et al.*, 1998). A study in Bangladesh reported that the fatality rate for 970 inpatients with shigellosis was 11%, with most deaths occurred among malnourished children infected with *S. flexneri* (Bennish & Wojtyniak, 1991). Studies have also reported that *Shigella* is one of the most important pathogen causing traveller's diarrhea (Jiang *et al.*, 2002; Ruiz *et al.*, 2002). It has been reported that approximately 580 000 travellers from industrialized countries are infected annually (Kotloff *et al*.,

1999). In the United States and Europe, children in day-care centres, migrant workers, travellers to developing countries are frequently infected (Niyogi, 2005). *Shigella* spp. continue to be responsible for morbidity and mortality in high-risk populations such as children under five years of age, senior citizens, toddlers in day-care centres and people with a weakened immune system. Rapid spread occurring in closed environments, such as day care centres have been reported (Weissman *et al.*, 1975; Chiou *et al.*, 2001). Outbreaks are more associated with contaminated water, food, overcrowding communities, food handlers and flies (Kapperud *et al.*, 1995; Shears, 1996).

A study by Lee & Puthucheary (2002) on bacterial enteropathogens in childhood diarrhea in a Malaysian urban hospital showed that *Shigella* spp. was the third most common bacteria isolated from stool samples. This retrospective study conducted among children admitted with diarrhea in University of Malaya Medical Centre (UMMC), Kuala Lumpur, from 1978 to 1997, reported that *Shigella* spp. was isolated from 1.4% (386 isolates) of total stool specimens, corresponding to 13% of the 2986 isolates positive for bacterial pathogens. *S. flexneri* was found to be the most frequently isolated species reported which constituted 74% of all *Shigella* isolates (Lee & Puthucheary, 2003). Another retrospective analysis conducted among children admitted to the Hospital USM, Kelantan, Malaysia, showed that the isolation rate of *S. flexneri* was 2.28% out of the 1097 stool samples studied (Ludin *et al.*, 2003). Not much information was available for the prevalence of shigellosis in other parts of Malaysia

### 1.1.3 Classification

The genus *Shigella* belongs to the tribe Escherichia in the family Enterobacteriaceae. The four species of *Shigella* are subdivided into serotypes based on the O antigen component of lipopolysaccharide present on the outer membrane of the cell wall: *S.*

*dysenteriae* (consisting of 13 serotypes), *S. flexneri* (consisting of 8 serotypes), *S. boydii* (consisting of 18 serotypes) and *S. sonnei* (single serotype). *S. flexneri* is subdivided into eight serotypes that are 1, 2, 3, 4, 5, 6, X and Y, with eleven sub-serotypes that are 1a, 1b, 1c, 2a, 2b, 3a, 3b, 4a, 4b, 5a and 5b.

### 1.1.4 Properties of *Shigella*

#### 1.1.4.1 Physical characteristics

It is a small, non-spore forming rod with a diameter of 0.3 to 1 µm and a length of 1 to 6 µm. It is Gram-negative, non-motile (no H antigens), non-capsulated and possess the K and O antigens. O antigen (somatic antigen) is useful in serological identification to classify the four species. K antigen is the capsule antigen which occasionally interferes with O antigen determination. Shiga toxin, also called as verotoxin, is produced by *Shigella dysenteriae* type 1. The toxin has a molecular weight of 68 kDa. It is a multi-subunit protein made up of an A subunit (32 kDa), responsible for toxic action of the protein and five molecules of the B subunit (7.7 kDa), responsible for binding to a specific cell receptor.

#### 1.1.4.2 Growth and culture characteristics

*Shigella* is a facultative anaerobe bacterium and is able to grow at temperatures ranging from 12°C to 48°C (optimum 37°C), at a pH range of 5.0 to 7.3 (Tetteh & Beuchat, 2003; Zaika & Phillips, 2005). Generally, deaths of *Shigella* are enhanced with increased in temperature, decreased in pH and increased in NaCl concentrations. The organism is acid resistant and can easily pass the gastric acid barrier.

The common selective/differential agar media used for the recovery of *Shigella* are MacConkey (MAC), Xylose Lysine Deoxycholate (XLD), Hektoen (HEK) and *Salmonella-Shigella* (SS) and Deoxycholate Citrate Agar (DCA). It has typical non-lactose fermenting characteristic colonies on lactose enriched media such as on MAC, DCA and SS agar. *Shigella* is resistant to bile salts and this characteristic is usually

useful in the selective media. Colonies on the MacConkey and DCA agar appears to be large, 2 to 3 mm in diameter, translucent and colourless (non-lactose fermenting). Whereas, on the XLD agar, colonies appear to be much smaller (1 to 2 mm diameter) and red in colour as lysine is decarboxylated producing alkaline end products which raises the pH and cause the agar to turn into deep red colour. *Shigella* does not produce hydrogen sulphide ($H_2S$) on the XLD, HEK and SS agar.

#### 1.1.4.3 Biochemical characteristics

*Shigella* is biochemically much less reactive than their close relatives, members of the genus *Escherichia* (Ewing, 1986). *Shigella* does not ferment lactose but cultures of *S. sonnei* utilize this substrate after 24 hours incubation. *Shigella* utilizes glucose and other carbohydrates, producing acid but not gas (with the exception of *S. flexneri* 6) and is oxidase negative. Normally, indole is not produced and methyl red (MR) test is positive. Lysine is not decarboxylated and ornithine is decarboxylated only by *S. sonnei* and *S. boydii*. Serogroups A, B and C are very similar biochemically while *S. sonnei* can be differentiated from the other serogroups by positive beta-D-galactosidase and ornithine decarboxylase biochemical reactions. Neither citrate nor malonate is used as the sole carbon source for growth, and the organisms are inhibited by potassium cyanide.

## 1.2 Clinical manifestations, pathogenesis, treatment and prevention of shigellosis

### 1.2.1 Reservoirs and mode of transmission

Humans and primates are the only natural hosts for *Shigella*. The predominant mode of transmission is by faecal-oral route, in which *Shigella* is usually transmitted from person to person and occurs in areas where personal hygiene is poor (Smith, 1987; Shears, 1996; Chiou *et al.*, 2001; Chen *et al.*, 2003). Part of the reason for the efficiency of transmission is because *Shigella* is highly infectious, as 10 to 200 organisms are sufficient to cause infection (DuPont *et al.*, 1989). This infective dose of

*Shigella* is lower than that reported for most other enteric pathogens such as for *Vibrio* spp. and *Salmonella* spp. which require at least $10^4$ to $10^5$ organisms to cause infection. The low infectious dose suggests that *Shigella* are capable of surviving in highly acidic environments such as in gastric secretions (Gorden & Small, 1993; Lin *et al.*, 1995; Waterman & Small, 1998).

Shigellosis is usually acquired from consumption of contaminated food and water (Islam *et al.*, 1993; Villalobo & Torres, 1998). It is usually acquired by eating food that has become contaminated by infected food handlers (Rosalie *et al.*, 1999), consumption of raw vegetables harvested in fields where sewage was used as fertilizer (Sur *et al.*, 2004) or associated with eating oysters (Terajima *et al.*, 2004). Transmission also occurs through contaminated fomites or by accidentally drinking contaminated water in the swimming pools (Shears, 1996; Fleming *et al.*, 2000). In certain settings where disposal of human faeces is inappropriate, flies, particularly *Musca domestica*, may serve as vectors for the transmission of shigellosis (Alan & Olsen, 1998; Todar, 2005). During dysentery, *Shigella* are excreted in large numbers in the stool, up to $10^6$ to $10^{10}$ bacteria per gram of stool (Rowe, 1990). The organism can be found in the faeces for weeks after symptoms have ceased (Sur *et al.*, 2004). *Shigella* have been documented to survive in soiled linen for up to seven weeks, in fresh water for 5 to 11 days, in salt water for 12 to 30 hours, in dust at room temperature for 6 weeks, in sour milk for 4 weeks and in kitchen refuse for 1 to 4 days (reviewed by Todar, 2005).

### 1.2.2 Incubation period

The incubation period is 1 to 4 days, which is usually followed by the sudden onset of acute symptoms (Hale & Formal, 1987). In mild cases, the disease may be self limiting but severe cases require appropriate medication. Shigellosis usually resolves within 5 to 7 days (Hale *et al.*, 1991).

### 1.2.3 Pathogenesis and immune responses

*Shigella* infection is generally limited to the intestinal mucosa. Pathogenesis involves the penetration of the bacterium into the epithelial M cells of the colon (LaBrec *et al.*, 1964; Mantis *et al.*, 1996). *Shigella* uses the type III secretion system to invade the epithelium cells and involves approximately 20 proteins which are encoded by the 210 kb virulent plasmid (Maurelli & Sansonetti, 1988; Suzuki & Sasakawa, 2001; Mavris *et al.*, 2002a; Mavris *et al.*, 2002b). Among the proteins secreted are VirA, OspB to OspG and invasion plasmid antigens (Ipa) proteins such as IpaB, IpaC, IpaD and IpaH.

The ability to cross the epithelial lining, induce apoptosis of phagocytic cells, escape into the cytoplasm of epithelial cells and followed by cell-to-cell spread is the key determinant of the disease that elicited acute inflammatory reaction (Anand *et al.*, 1986; Sansonetti *et al.*, 1995; Islam *et al.*, 1997). The pathogenic mechanism of shigellosis is complex and has been studied extensively by Sansonetti and colleagues (Goldberg & Sansonetti, 1993; Sansonetti *et al.*, 1996).

Besides the development of acute inflammation, the adaptive immune response is elicited to provide protection against the infection (reviewed by Phalipon & Sansonetti, 2003). *Shigella* is not an obligatory intracellular pathogen, thus the humoral immunity response is largely due to its extracellular activity. IgA is secreted by IgA secreting plasma cells in the lamina propria of submucosal layer. It is then endocytosed through the polymeric immunoglobulin receptor (pIgR), which is then transported through the mucosal epithelial cells into the mucosal secretions as secretory IgA (sIgA) [Lamm, 1998]. In serum, IgA exist predominantly in monomer form, whereas in mucosal secretions it is mostly in dimers form (two monomeric IgA units linked by a J chain), mainly in the form of sIgA (present with a secretory component).

Studies have reported that the circulating IgA antibody secreting-cells (ASC) were significantly higher in the *S. flexneri* infected patients 5 to 7 days after the onset of the disease (Minh *et al.*, 1996; Rasolofo-Razanamparany *et al.*, 2001). Although the mechanism of immunity against shigellosis remains unclear, it has been reported that natural and experimental infections with *Shigella* elicited specific systemic and mucosal immune responses (Cohen *et al.*, 1989, Cohen *et al.*, 1991, Robin *et al.*, 1997). The highest IgA and IgG titres were seen 1 week and 2 weeks after the onset of diarrhea, respectively (Van De Verg *et al.*, 1996). Several studies have suggested that serum IgA reactivity reflected that of intestinal IgA since these antibodies were derived from the same source (Oberhelman *et al.*, 1991; Van De Verg *et al.*, 1996). A study conducted by Minh and colleagues (1996) showed that patients infected with *S. flexneri* had significantly higher serum IgA and IgG titres against LPS and Ipa proteins compared to the healthy controls, at day 4 after the onset of diarrhea and prolonged for 2 weeks. The highest serum IgA and IgG titres were seen on day 7 and day 14 after the onset of diarrhea, respectively. This study also found that *S. flexneri* infected patients developed intestinal sIgA responses against these antigens, which significantly increased 4 days after the onset of diarrhea and reached its peak in 2 weeks. The IgA antibody was reported to be most frequently detectable between 10 and 20 days after the onset of infection (Oberhelman *et al.*, 1991).

### 1.2.4 Clinical signs and symptoms

Clinically, the disease begins within 24 to 48 hours of ingestion of the organism (Hale *et al.*, 1991). Shigellosis is characterized by frequent small volume passage of stools that consist largely of blood, mucus and pus, accompanied by fever and stomach cramps. Blood, mucus and pus cells in the stools are the signs of colorectal inflammation (Li, 2000). These symptoms are also common in infections caused by *Campylobacter, Salmonella* and *Entamoeba histolytica*, but usually generate self-limited illness that is rarely as serious as shigellosis. Apart from bloody stools, patients

with dysentery often have rectal pain, fatigue, malaise and anorexia. However, in some cases, *Shigella* causes acute non-bloody diarrhea that cannot be distinguished clinically from diarrhea caused by other enteric pathogens. Shigellosis can be serious in some cases, especially involving young children and the elderly, which require hospitalization of the patients. Severe cases may become life threatening and contribute to substantial mortality. Severe infections with high fever have been associated with seizures among children (Khan *et al.*, 1999). Patients with severe infection may pass more than 20 dysenteric stools in one day. Dysentery is also characterized by the daily loss of 200-300 ml of serum protein into the faeces, which exacerbates malnutrition and growth stunting in young children living in developing countries (Alam *et al.*, 2000). Depletion of immune system also increases the risk of shigellosis and contributes to substantial mortality (Simor *et al.*, 1989; Gassama *et al.*, 2001). Clinical symptoms usually persist for 10 to 14 days or longer (Li, 2000). The use of clinical signs and symptoms is therefore very important in helping to identify patients with shigellosis.

### 1.2.5 Complications

Most episodes of shigellosis in healthy individuals are self-limited and resolve without sequelae. Life-threatening complications are most often seen in malnourished infants and young children living in developing countries, the elderly and people who have a weakened immune system (Bennish & Wojtyniak, 1991; Baer *et al.*, 1999; Alam *et al.*, 2000; Gassama *et al.*, 2001). Complications include metabolic derangements, such as dehydration, hyponatraemia, hypoglycaemia, hypoproteinaemia and severe anorexia intestinal complications such as toxic megacolon, rectal prolapsed and intestinal perforation (Bennish & Wojtyniak, 1991; Ashkenazi, 2004). Bacteraemia due to *Shigella* is relatively rare but does occur (Strulens *et al.*, 1985; Heyking & McInintosh 1997). Bacteraemia due to *Shigella* have been reported to be common among HIV-infected and other immunocompromised patients (Batchelor *et al.*, 1996). Persistent diarrhea and malnutrition are the most common chronic sequelae (Niyogi, 2005). Other

complications seen primarily in adults following infection with *S. flexneri* are reactive inflammatory arthritis (Gaston, 2005; Hannu *et al.*, 2005), conjunctivitis and urethritis which is known as Reiter's syndrome which occurs most commonly in adults with HLA-B27 histocompatibility antigen (Finch *et al.*, 1986). A variety of unusual extraintestinal manifestations may occur. Some children under 2 years can have neurological symptoms, including febrile seizures which usually occur in the presence of fever (Ashkenazi, 2004). Microangiopathic haemolytic anaemia can complicate infection with Shiga toxin-producing organisms, manifesting as the haemolytic uraemic syndrome (HUS) in children and as thrombotic thrombocytopenic purpura in adults (Koster *et al.*, 1978). *Shigella* has also been associated with chronic vulvovaginitis in children (Namdari *et al.*, 2002).

### 1.2.6 Treatment and prevention

Though fluid and electrolyte replacement by either oral hydration or intravenous fluid therapy is the treatment of choice for acute diarrhea, antibacterial agents are indicated for treatment of suspected shigellosis. Antibiotic treatment for *Shigella* infection is recommended to decrease the duration of illness and person-to-person spread. Treatment in malnourished children especially in the developing countries is likely to reduce the risk of worsening malnutrition morbidity following shigellosis. Treatment is critical in the young children, the elderly or persons with chronic diseases, as shigellosis can be severe and lead to dehydration and other complications.

The main antibiotics used are ampicillin, tetracycline, amoxicillin, cotrimoxazole, chloramphenicol and trimethoprim-sulfamethoxazole. However, treatment has become increasingly difficult due to emerging resistance to these commonly prescribed antibiotics (Martinez-Salazar, 1986; Salam & Bennish, 1991; Replogle *et al.*, 2000). A study using 369 isolates showed that 59% were resistant to trimethoprim-sulfamethoxazole, 63% were resistant to ampicillin, 85% were resistant to tetracycline, 1% was resistant to cefixime, and 0.3% was resistant to nalidixic acid and none of the

isolates were resistant to ciprofloxacin (Replogle *et al.*, 2000). Ciprofloxacin and nalidixic acid are now recommended as the first line treatment for *Shigella* infection. Many recent studies have reported multi-resistant strains to the commonly prescribed antibiotics occurring in the developing and developed countries (Replogle *et al.*, 2000; Taneja *et al.*, 2004). The *Shigella* resistance locus (SRL), have been identified in *Shigella* strains which mediates resistance to antibiotics (Turner *et al.*, 2001; Turner *et al.*, 2003). In Malaysia, out of 241 isolates tested for antibiotic sensitivity, 58% were resistant to at least one antibiotic, and 42% were multi-resistant to three or more antibiotics (Lee & Puthucheary, 2003).

The spread of *Shigella* from an infected person to other persons can be stopped by increasing the personal hygiene. Hand washing with soap, safe disposal of human waste as well as safe food handling and processing are the basis of personal hygiene that will minimize the transmission of *Shigella*.

## 1.3 Diagnosis of shigellosis

### 1.3.1 Clinical diagnosis

Clinical diagnosis of shigellosis is not specific. The diarrhoeal stage of the infection cannot be distinguished clinically from other bacterial, viral and protozoan infections (Youssef *et al.*, 2000). The presence of fever suggests an invasive pathogen. Fever is not usually associated with organisms that act solely by means of enterotoxin production, so this finding is helpful for narrowing down the number of organisms under consideration. Patients presenting with watery diarrhoea and fever should be suspected of having shigellosis (Echeverria *et al.*, 1991; Goodman & Segreti, 1999). Studies have reported that the height of the fever correlated with the severity of the illness (Mackowiak *et al.*, 1992). The typical clinical feature of shigellosis is bloody and mucoid stool, but the differential diagnosis should include infection caused by enteroinvasive *Escherichia coli* (EIEC), *Salmonella enteritidis*, *Campylobacter* species

and *Entamoeba histolytica* (Goodman & Segreti, 1999; Sur *et al.*, 2004). Usually, fresh and bright red blood is present in the stools of patients infected with *Shigella* rather than dark brown blood in *Entamoeba histolytica* infections (Niyogi, 2005).

### 1.3.2 Laboratory diagnosis

#### 1.3.2.1 Haematology

The total white blood cell count is often within reference range. However, in some cases, leucopenia, anaemia and thrombocytopenia may occur.

#### 1.3.2.2 Bacteriology

##### 1.3.2.2.1 Stool examination

Macroscopic examination of stool characteristics such as for consistency (liquid, semisolid or formed stool) and atypical components (mucus, blood, non-bloody stools) are the key features in determining the potential pathogens causing the diarrhea. Moreover, routine microscopic examinations for the presence of red and white blood cells are important characteristics in establishing the diagnosis of dysentery. Fresh and unstained suspension of faeces is examined directly under the microscope. Invasion of the intestinal wall and the consequent inflammatory reaction resulted in the infiltration of the polymorphonuclear leukocyte and red blood cells in the faecal secretion. Studies have demonstrated that faecal blood or leukocytes are detectable in the stool in approximately 70% of shigellosis cases, whereas both faecal blood and leukocytes are detected in approximately 50% of cases (Echeverria *et al.*, 1991). However, confirmation of laboratory diagnosis is achieved by stool culture.

##### 1.3.2.2.2 Stool culture

Stool culture is the method of choice. A definite diagnosis of *Shigella* infection is made by isolating the organism from stool specimens and serotyping the isolate. Culture is also required for antibiotic susceptibility testing. Fresh stool samples collected from patients before initiation of antibiotic therapy are preferred for microbiological tests

because the chances of recovering the organisms are higher. *Shigella* is usually isolated from faecal specimens by conventional culture methods followed by identification by biochemical tests and serological agglutination assays (WHO, 1987). Ideally, patients' specimens should be analyzed within 2 to 4 hours after sample collection. Specimens that cannot be processed within this time should be stored at 4°C in transport media (buffered glycerol saline or Cary-Blair medium). Isolation and identification of *Shigella* can be greatly enhanced when optimal laboratory media and techniques are employed. Since *Shigella* is a very fastidious organism, appropriate collection, rapid transport to the laboratory and rapid plating of the sample are important criteria for successful isolation (Shears, 1996). Such conditions are often difficult to attain, especially in developing countries. Therefore, the recovery rate of *Shigella* is usually low. As a result, shigellosis remains undiagnosed in a significant number of patients (Echeverria *et al.*, 1992).

#### 1.3.2.2.2 (a) Inoculation of selective media

Isolation of *Shigella* usually involves an initial streaking for isolation on differential or selective media with aerobic incubation to inhibit the growth of the anaerobic normal flora. Faeces specimens or rectal swabs are inoculated onto primary isolation media such as MAC, DCA, XLD, HEK or SS agar. However, *S. dysenteriae* type 1 and *S. sonnei* do not grow well on SS agar. These selective media contain bile salts to inhibit the growth of other Gram-negative bacteria and pH indicators to differentiate lactose fermenters (Coliforms) from non-lactose fermenters (*Shigella* spp.). Liquid enrichment broth (Hajna Gram-negative broth or selenite F broth) may also be inoculated with the stool specimen and subcultured onto the selective agar after a short growth period.

For optimal isolation of *Shigella*, two different selective media should be used. This normally include a low selective general purpose plating medium such as MAC agar, and a more selective plating medium, such as DCA, XLD or HEK agar. Selective media

may be inoculated with a faecal swab, rectal swab or with a single drop of liquid faecal suspension and incubated at 37°C for 18 to 24 hours. Characteristics of the colonies appearance on the selective media are as described in the section 1.1.4.2. Suspected colonies were then identified using biochemical and serological tests.

#### 1.3.2.2.2 (b) Biochemical screening tests

Suspected well-isolated colonies from the selective media are inoculated into appropriate screening media such as Kligler iron agar (KIA) or triple sugar iron agar (TSI), motility medium, MR test medium, citrate agar and urea agar (WHO, 1987). *Shigella* produces an alkaline slant and an acid butt due to the inability to ferment lactose aerobically in the slope and the anaerobic fermentation of glucose in the butt, and fail to produce $H_2S$ gas. However, a few strains of *S. flexneri* serotype 6 and very rare strains of *S. boydii* produce gas in KIA or TSI. *Shigella* is negative for motility, citrate and urea test, and positive reaction is observed in the MR test.

#### 1.3.2.2.2 (c) Serological identification

Serological testing is performed for confirmation and species classification of the *Shigella* isolates (Ewing & Lindberg, 1984). It is achieved by slide agglutination test using commercially available polyvalent O antigen grouping sera. However, in some cases, specific serotype identification is performed by testing with monovalent antisera for serotypes and sub-serotypes identification. Each species of *Shigella* contain a distinctive type of O antigen. Agglutination tests is carried out using a clean glass slide by emulsifying a portion of the growth from the surface of KIA, TSI, or other non-selective agar media in a drop of physiological saline. Colonies from selective media such as MAC or DCA are not advised to be used for this purpose because it may produce false-negative results. A small drop of polyvalent or monovalent antiserum is mixed with the suspension to observe for the agglutination reaction. *Shigella* polyvalent antiserum will agglutinate strains of the same serogroup and monovalent antiserum will

agglutinate the specific serotype or sub-serotype. Cultures that react serologically and show no conflicting results in the biochemical screening tests are reported as positive for *Shigella*.

However, in more than 50% of the patients, the exact pathogen is not identified by stool cultures. Recent advances in diagnostic technology make detection faster, more convenient, more sensitive and specific than the conventional culture method.

#### 1.3.2.3 Other diagnostic techniques

##### 1.3.2.3.1 DNA-based method

Recent advances in molecular biology have introduced new approaches for the rapid and sensitive diagnosis of bacterial infections by detecting the presence of pathogen-specific DNA sequences in clinical specimens without the need for culturing the bacteria. Polymerase chain reaction (PCR) represents a powerful tool in studying bacterial infections that can amplify the target DNA by a million fold in less than 2 hours. Moreover PCR technology provides information on the current infection status and is independent of the host's immune competency.

In the earlier studies, application of DNA technology to diagnosis *Shigella* spp. is based on identification of DNA segments of a 120 to 140 MDa virulent plasmid that is necessary for attachment and invasion of epithelial cells (Sansonetti *et al.*, 1982; Sansonetti, 1991). Several sensitive and rapid PCR techniques directed towards the virulent genes for the detection of invasive *Shigella* and EIEC have been reported (Frankel *et al.*, 1989; Frankel *et al.*, 1990). These techniques utilize gene probes or PCR primers directed towards the specific DNA fragments of the invasion plasmid locus (*ial*) or the genes encoding for the *ipaH*, and were detected with an alkaline phosphatase-labelled oligonucleotide probe. A few studies have utilized the immunomagnetic separation techniques to specifically isolate *Shigella* from faeces and

subsequently identified by PCR (Islam & Lindberg, 1992; Achi-Berglund & Lindberg, 1996; Peng *et al.*, 2002). Application of ELISA method to detect PCR products of *ipaH* gene sequences from *Shigella* and EIEC in diarrheal stool samples have been exploited (Sethabutr *et al.*, 2000). The PCR-ELISA technique allowed screening of larger number of specimens and avoided the use of mutagenic reagents. Gaudio and colleagues (1997) used the same *ipaH* PCR system in an epidemiology study of shigellosis among dysentery patients and family contacts in Thailand.

Various PCR protocols for the detection of *Shigella* and EIEC in faeces (Frankel *et al*. 1990; Sethabutr *et al*. 1993; Thiem *et al*., 2004), food (Lampel *et al*., 1990; Lampel *et al*., 2000; Li *et al*., 2005) and environmental samples (Theron *et al*., 2001) have been published. In addition, a PCR assay based on the *rfc* gene capable of distinguishing the various *Shigella* serotypes and EIEC in faecal samples had been described (Houng *et al*., 1997). A multiplex PCR assay was developed by Aranda and colleagues (2004) to classify the diarrheagenic *E. coli* organisms and *Shigella* spp. based on the *ipaH* gene sequences.

However, limiting issues that prevent DNA technology from general use in the diagnostic laboratory is that it is expensive to perform and requires sophisticated equipment, which may not be available in the developing countries. This is of particular concern, as most of the *Shigella* infections are endemic in these parts of the world. This technology is both technically demanding and labour-intensive, thus is poorly suited for routine practice. Moreover, the presence of PCR inhibitors in the complex samples, such as faeces, food and in culture media can inhibit amplification of the target genes, thus limiting the usefulness of the PCR technique in diagnosis. Moreover, in most cases, cultural enrichment is required prior to analysis. Although PCR products visualized with the agarose electrophoresis technique is simple, rapid and more sensitive than the conventional diagnostic method, the result is subjective, usually

involving the use of a mutagenic ethidium bromide reagent to illuminate the bands, requiring special equipment and a limited number of specimens can be processed at any one time.

Various formats of DNA and oligonucleotide microarray techniques have been developed and applied to screen multiple microbial organisms in diagnostic assays (Wu *et al.*, 2001; Cho & Tiedje, 2002). However, more meticulous and systematic assessment and development are needed to understand the full potential of microarrays for diagnostic studies. Moreover, such technology requires sophisticated instruments to prepare and the result can only be read by laser scanner, thus limiting its broad application in common laboratories (reviewed by Zhou, 2003).

#### 1.3.2.3.2 Immunological assay

Immunological assays have been the most rapid methods for specific identification of bacterial infections. These tests employ antibody or antigen recognizing only one or a group of bacteria. Recent advances in immunoassay technology make detection faster, more convenient, more sensitive and specific than the conventional culture method. The highly specific binding of antibody to antigen, plus the simplicity and versatility of this reaction, has facilitated the design of a variety of antibody assays. The antibody-based assays comprise the largest group of rapid methods being used in bacterial identification. To date, no reliable rapid method is commercially available for shigellosis identification and none are in use routinely in the laboratory.

There are several basic formats of antibody-based assays such as latex agglutination, immunodiffusion and enzyme immunoassays (EIA) format. Of the wide range of immunoassays available, EIA is the most common format used for testing large numbers of samples and is suitable for automation and quantification. There are many different EIA methods that have been developed for diagnosis of shigellosis. For example, a monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA)

has been developed using a 43 kDa invasion plasmid-coded protein antigen (IpaC) to identify EIEC and *Shigella* strains in faecal samples from children in Kuwait (Pal *et al.*, 1997). ELISA techniques have been employed for the detection of serum and urine antibodies of various immunoglobulin classes developed against *Shigella* serogroup specific LPS (Cohen *et al.*, 1989; Cohen *et al.*, 1996; Shamlal *et al.*, 1997). It has been reported that the increase in the level of serum IgA to *Shigella* LPS indicates a recent infection, in which IgA levels were highest 2 weeks after infection and declined to initial levels within 2.5 months (Cohen *et al.*, 1989). Another ELISA method was developed using purified recombinant IpaD protein for identification of *Shigella* spp. (Oaks *et al.*, 1996). Besides that, simple and inexpensive ELISA methods have been developed, which provides an alternative to the conventional virulent tests such as keratoconjunctivitis or *in vitro* penetration of the cultured mammalian cells (Pal *et al.*, 1983; Pal *et al.*, 1985). A rapid latex agglutination procedure, the Wellcolex Colour *Shigella* tests, have been developed for grouping *Shigella* using an antibody attached to multicoloured latex particles (Bouvet & Jeanjean, 1992). To indicate the related *Shigella* species, blue or red colour agglutination is observed when the corresponding antibodies coated to the latex particles recognize the specific antigen. Besides that, colony blot immunoassays have been developed to detect EIEC and *Shigella* in stool and water samples (Szakal *et al.*, 2001; Szakal *et al.*, 2003).

Another simple format is the dot-EIA test, in which specific antigens or antibodies are immobilized on a membrane (usually nitrocellulose) and the reaction is observed by enzyme activity. In a previous study, an antigenic membrane protein of 35 kDa molecular weight, which is specific to IgA in sera of patients infected with *Shigella* spp. had been demonstrated (Kirnpal-Kaur, 2001). A dot-EIA was developed using this protein and laboratory diagnostic result of this test could be obtained in 3 hours to diagnose *Shigella* infection.

However, there are many limiting factors that prevent the immunoassay tests for routine laboratory diagnostic application. Two major factors that determine the efficacy of immunoassay is the efficiency of antigen-antibody complex formation and the ability to detect these complexes. Regardless of the format, the specificity of an immunoassay is dependent on the reaction between antibody and antigen. The limitations of EIA involve the specificity and affinity of the antibodies used, cross-reactivity, the specific activity of the enzyme and substrate employed, and efficiency of the signal detection. Some of the assays require growth in an enrichment medium before analysis. Besides that, the disadvantages of EIA are that interpretation of the test results requires experienced staff, and is time consuming, therefore fewer tests can be run per day. The major disadvantages of ELISA are that it is relatively time-consuming, labour-intensive and to some extent is temperature-dependent. The sensitivity and specificity of the assays depend mainly on the type of antigens used. Assays that employ recombinant protein or synthetic peptide antigens tend to be more specific than using whole or disrupted particles.

Thus, the need for a more rapid, sensitive and portable assay remains as urgent as ever, providing the impetus for the development of next generation technologies. Recently new rapid, membrane-based antibody assay, which is based on the technology developed for home pregnancy tests have been devised for diagnosis of infectious agents. It is known as the immunochromatography test (ICT), lateral flow assay or dipstick test. ICT have been available on the commercial market for more than 20 years, which was first developed to detect abused drugs and for pregnancy testing. It is simple to use and require minimal training, thus resulting in saving labour, time and materials. The detecting antibody is usually coupled to coloured latex beads or to colloidal gold instead of enzyme conjugates. In this test, reactants move by capillary action along a narrow rectangular membrane strip. The sample is applied at one end and traverses the strip, coming into contact first with detecting antibodies (conjugated

to gold particles, normally 10 to 60 nm diameter) and subsequently with capture antigens or antibodies that have been dried onto the membranes. If the sample contains the corresponding antibodies or antigens, it will form a visible line as it accumulates at the position of the captured lines. The test results can be read by the appearance of the bands directly with eye, which ensures the convenience of the assay on-site. Studies have reported that immunochromatography test is extremely simple, require no washing or manipulation, reliable and rapid that the test can be completed within 5 to 15 minutes (Paek *et al.*, 2000; Shyu *et al.*, 2002; Chiao *et al.*, 2004). A number of the immunochromatography tests have been developed for the detection of bacterial or parasitic infections such as *TYPHIRAPID*™ for diagnosis of typhoid, *BrugiaRapid*™ for diagnosis of filariasis (Rahmah *et al.*, 2001; Lammie *et al.*, 2004), brucellosis (Smits *et al.*, 1999b; Smits *et al.*, 2003) and malaria (reviewed by Moody, 2002).

### 1.4 Rationale of the study

*Shigella* spp. are one of the most important etiological agents of dysentery for people who are living in developing countries and travellers from industrialized countries. *S. flexneri* has been the most frequently isolated species especially in the developing countries (Taylor *et. al.*, 1991; Faruque *et al.*, 2002; Lee & Puthucheary, 2003; Ludin *et al.*, 2003; Niyogi & Pazhani, 2003). Major obstacles in controlling this pathogen are the high contagiousness of the disease and the emergence of multi-resistance characteristic to the commonly prescribed antibiotics. As shigellosis is highly contagious, it is crucial to develop a rapid method for identifying the bacteria in order to limit and control outbreaks. Immediate identification of the pathogen in clinical samples is critical to ensure proper clinical treatment, management of the patients and for epidemiological investigations.

Current laboratory diagnostic method used to diagnose *Shigella* infection relied on the time-consuming growth in culture media, followed by isolation, biochemical and serological identification. Nevertheless, the selection of the pathogenic bacterial colonies on selective media is time consuming and could be missed if their numbers are low in the sample particularly due to the delay in sample transportation. The sensitivity of this culture method is relatively low, thus increasing the need for alternative diagnostic methods. Various studies have reported that the sensitivity of the culture method is 54% (Dutta *et al.*, 2001) and 74% (Islam *et al.*, 1998), when compared to other diagnostic methods. As mentioned earlier, there are a number of DNA-based tests and immunoassay tests that have been developed for the diagnosis of shigellosis. Detection methods based on nucleic acid, such as the PCR have shown tremendous potential and have been increasingly exploited. However, among the limitations of DNA-based tests and immunoassays are relatively time-consuming, labour-intensive and require growth in enrichment medium before analysis.

The relatively low sensitivity and the difficulty in performing the current diagnostic procedure for shigellosis have called for an alternative diagnostic method for this disease. A rapid, simple and reliable diagnostic test is highly desired. One such method involves detection of specific antibodies in clinical specimens. There is a need for the development of next generation immunoassay technologies which provide a more rapid, sensitive and portable assays. The ICT technology which is based on the membrane-based antibody assays has been shown to be a potential tool for the diagnosis of pathogens (Rahmah *et al.*, 2001; Smits *et al.*, 2003; Lammie *et al.*, 2004). The advantages of the immunochromatography test over culture method are rapidity, simplicity, do not require expensive equipment, do not require cold chain for transportation, enhanced sensitivity and specificity for early diagnosis and the test can be performed at point of care. However, there is no report on diagnostic applications

employing the immunochromatography concept that have been developed for diagnosis of shigellosis.

A rapid and reliable diagnostic assay would significantly improve effective management of the disease especially among young children and the elderly. Although oral rehydration therapy has a role to play in the treatment of dysentery, it is often necessary to treat patients with specific antibiotics at the early stage to control outbreaks of this contagious disease and prevent further complications. This information is important in reducing the morbidity and mortality and in the selection of appropriate antibiotics in the management of extraintestinal complications.

In a previous study, an antigenic membrane protein that is specific to IgA in the sera of patients infected with *Shigella* had been demonstrated. A dot-EIA was developed using this specific protein and laboratory diagnostic result can be attained in 3 hours. In view of the fact that the current global diagnostic trend is moving towards rapid immunochromatography platform, there is a need to strategically convert this test to achieve a more rapid laboratory diagnosis. This study will be focused on the *S. flexneri* since it is the most common species isolated from patients in endemic area.

### 1.5 Objectives of the study

The ability to produce indigenous diagnostic tests for shigellosis that are novel, specific yet cost-effective would now provide a huge impact on public health management not only in Malaysia but also for all developing countries affected. Thus the main aim of this study is to optimize the conditions needed to provide sufficient purified recombinant 35 kDa protein and to exploit its application in developing a rapid immunochromatography test, as an alternative diagnostic test for the detection of anti-*S. flexneri* IgA antibody.

To achieve these goals, specific objectives of the study include:

1. To determine the complete amino acid and nucleic acid sequences encoding for the 35 kDa protein. Amino terminal (N-terminal) amino acid sequence will be determined and homology search will be performed against the *S. flexneri* whole genome sequence database. The identified amino acid sequence will be further validated by MALDI-ToF technique. The amino acid sequence of the protein will be further characterized to determine the presence of signal peptide.

2. To construct and evaluate a recombinant plasmid vector for the expression of the recombinant 35 kDa protein of *S. flexneri* in *E. coli*. This study will describe the cloning process of the gene encoding the 35 kDa protein of *S. flexneri* using the pET 43.1 Ek/LIC vector. This recombinant clone will be verified by restriction enzyme digestion and DNA sequencing technique. Optimization studies of the protein expression in *E. coli* BL21 (DE3) will be performed to generate high concentrations of recombinant protein. The target protein will be verified by SDS-PAGE analysis and its immunogenicity will be determined by Western blot technique.

3. To purify the recombinant 35 kDa protein of *S. flexneri*. In this study, immobilized metal affinity chromatography (IMAC) technology will be used to purify the histidine-tagged recombinant protein. Initially, small-scale protein purification will be performed using a batch column technique. The purification conditions will be optimized in advance and consequently the protein purification protocol will be up-scaled using FPLC system to generate high concentrations of purified protein in a large quantity.
4. To exploit the usage of the purified recombinant protein in the development of immunochromatography test for the detection of anti-*S. flexneri* IgA antibody in