PRESENCE OF ANTIGENIC AND SPECIFIC HEAT SHOCK PROTEIN(S) OF *Shigella flexneri* AND *Shigella sonnei*

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by

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LIST OF ABBREVIATIONS

AP	Alkaline Phosphatase
ATCC	American Type Culture Collection
DNase	Deoxyribonuclease
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immunosorbent Assay
HSP	Heat Shock Protein
ICT	Immunochromatography
Ipa	Invasion Protein Antigens
kDa	Kilodalton
mA	Milliampere
MALDI-ToF	Matrix-assisted Laser Desorption/Ionization-Time of Flight
MW	Molecular Weight
OD	Optical Density
OMP	Outer Membrane Protein
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PPSP	Pusat Pengajian Sains Perubatan
RNase	Ribonuclease
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium-Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis
SRL	*Shigella* Resistance Locus
TEMED	N,N,N',N'-tetramethylethylenediamine
TSB	Tryptic Soy Broth
WHO	World Health Organization
°C	Degree Celcius
%	Percentage
±	Plus Minus
µg	Microgram
µl	Microliter
mg	Milligram
ml	Milliliter
g	Gram
h	Hour
V	Volt

KEHADIRAN PROTEIN KEJUTAN HABA YANG ANTIGENIK DAN SPESIFIK BAGI *Shigella flexneri* DAN *Shigella sonnei*

ABSTRAK

Shigellosis adalah penyakit berjangkit yang membimbangkan awam terutamanya di negara-negara yang sedang membangun dan ianya adalah disebabkan oleh *Shigella* spp.. Oleh kerana kebangkitan global spesies *Shigella* yang rintang terhadap antibiotik, pilihan agen antimikrob untuk rawatan shigellosis adalah sangat terhad. Pengenalpastian bakteria pada masa kini adalah dengan menggunakan kaedah pengkulturan konvensional dan ujian biokimia yang mengambil masa lebih kurang 2 hingga 7 hari. Justeru itu, terdapat keperluan untuk membangunkan ujian pantas yang boleh memberi keputusan yang tepat supaya rawatan segera dapat diberikan kepada pesakit shigellosis. Pembangunan ujian yang spesifik dan sensitif memerlukan penemuan penanda bio yang tidak bertindakbalas silang dengan bakteria lain dan spesifik hanya untuk *Shigella* spp. Protein kejutan haba (HSP) adalah protein yang diekspresi oleh bakteria semasa ia mengalami tekanan daripada persekitaran dan protein ini juga berpotensi untuk dijadikan sebagai penanda bio dalam bidang diagnostik. Oleh itu, tujuan kajian ini dilakukan adalah untuk mengenalpasti kehadiran HSP dan penanda bio daripada protein membran luar (OMP) terhadap *S. flexeri* dan *S. sonnei*.

Di dalam kajian ini, profil protein daripada strain ATCC dan strain klinikal *S. flexneri* dan *S. sonnei* didemonstrasi dengan menggunakan teknik SDS-PAGE. Profil OMP daripada sel *S. flexneri* dan *S. sonnei* yang diekspres pada suhu 37°C dibandingkan dengan profil yang diperolehi pada suhu 38.5°C dan 40°C (suhu pada pesakit yang mengalami demam semasa shigellosis) untuk menilai kesan suhu

terhadap ekspresi protein membran luar (OMP). Kajian ini menunjukkan bahawa tahap pengekspresian OMPs *S. flexneri* dan *S. sonnei* berubah dengan peningkatan suhu yang diuji.

Elektroforetogram protein tersebut seterusnya dianalisis dengan blot Western menggunakan serum pesakit yang dijangkiti oleh *S. sonnei* dan *S. flexneri* serta semua jangkitan lain yang berkaitan. Sera daripada jangkitan *S. flexneri* menunjukkan 11 jalur antigenik terhadap antibodi IgA dan IgG. Manakala, sera daripada jangkitan *S. sonnei* menunjukkan 14 dan 11 jalur antigenik terhadap antibodi IgA dan IgG masing-masing. Kesemua jalur antigenik diuji samada bertindak balas silang dengan serum daripada pesakit yang dijangkiti oleh *Salmonella* spp., enteropathogenic *Escherichia coli*, *Salmonella* Typhi, *Aeromonas hydrophila* dan *Campylobacter jejuni*. Tiga jalur protein (33.3, 43.8 and 100.3 kDa) daripada *S. sonnei* dan 2 jalur protein (25.6 and 63.2 kDa) daripada *S. flexneri* tidak menunjukkan tindak balas silang terhadap serum daripada jangkitan bakteria lain dan ini mencadangkan bahawa protein ini adalah spesifik terhadap *S. sonnei* dan *S. flexneri*.

Kehadiran protein kejutan haba yang menunjukkan kuantiti ekspresi yang tinggi telah dikenalpasti melalui kaedah MALDI-ToF-ToF. Protein kejutan haba bersaiz 18.4, 25.6 dan 57.0 kDa daripada *S. flexneri* dikenalpasti sebagai Dps, WrbA dan PepA melalui kaedah ini. Manakala, protein kejutan haba bersaiz 43.8 dan 100.3 kDa daripada *S. sonnei* telah dikenalpasti sebagai Pbp dan AceE. Peningkatan ekspresi protein-protein ini berkemungkinan bertindak sebagai mekanisma pertahanan oleh bakteria terhadap suhu tinggi dalam badan pesakit dan protein-protein ini berpotensi untuk digunakan sebagai penanda bio dalam pembangunan ujian diagnostik segera bagi shigellosis.

Protein-protein spesifik terhadap *S. flexneri* dan *S. sonnei* yang dikenalpasti boleh digunakan sebagai penanda bio untuk pembangunan ujian diagnostik yang membolehkan diagnosis yang tepat dan cepat bagi mengesan shigellosis untuk pengawalan wabak penyakit.

PRESENCE OF ANTIGENIC AND SPECIFIC HEAT SHOCK PROTEIN(S) OF *Shigella flexneri* AND *Shigella sonnei*

ABSTRACT

Shigellosis caused by *Shigella* spp. is a public health concern in developing countries. Due to global emergence of drug resistance to *Shigella* spp., the choice of antimicrobial agents to treat shigellosis is limited. Current identification of this pathogen is by conventional culture method and biochemical tests, which takes about 2 to 7 days to produce result. Hence, there is a need for a rapid and reliable test that would allow rapid management of shigellosis infections. Development of a specific and sensitive diagnostic test requires discovery of biomarker(s), which does not cross react with other bacteria and are specific only to *Shigella* spp .Heat shock proteins (HSP) are proteins expressed in bacteria during stress environment and these proteins have potential as biomarker in diagnostic field. Thus the aim of this study is to detect the presence of HSPs and biomarker(s) in the outer membrane proteins (OMPs) of *S. flexneri* and *S. sonnei*.

Protein profiles of OMPs from the ATCC strain and clinical isolate of *S. flexneri* and *S. sonnei* were demonstrated using the technique of Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE). The OMPs profiles of *S. flexneri* and *S. sonnei* expressed at 37°C was compared with the profiles of 38.5°C and 40°C (temperature in patients with fever during shigellosis) to assess the effect of temperature on the expression of the OMPs.

This study demonstrated that the expression level of OMPs of *S. flexneri* and *S. sonnei* varies with increasing temperatures.

The protein electrophoretograms were subjected to Western blot using serum from patients infected with *S. sonnei* and *S. flexneri* as well other related infections. Result of this study demonstrated 11 antigenic bands were detected when probed with sera from patients with *S. flexneri* infection against both anti-human IgA and IgG isotypes. A total of 14 and 11 antigenic bands were obtained against anti-human IgA and IgG respectively when probed with sera from *S. sonnei* infection. All the antigenic bands were checked for cross reactivity using sera from patients infected with *Salmonella* spp., Enteropathogenic *Escherichia coli*, *Salmonella* Typhi, *Aeromonas hydrophila* and *Campylobacter jejuni*. Three protein bands (33.3, 43.8 and 100.3 kDa) of the *S. sonnei* and 2 protein bands (25.6 and 63.2 kDa) of the *S. flexneri* did not cross reacted with sera from other infections, suggesting that these proteins are specific biomarker for *S. flexneri* and *S. sonnei.*

The identified HSPs showing prominent increased in expression were further identified by MALDI-ToF-ToF analysis. The HSPs of 18.4, 25.6 and 57.0 kDa in size from *S. flexneri* were identified as Dps, WrbA and PepA respectively. Whereas, the HSPs of 43.8 and 100.3 kDa in size from *S. sonnei* were identified as Pbp and AceE. The increased expression of these proteins probably is a mechanism of survival of the bacterium at higher temperatures in the host body and could be potential diagnostic biomarkers. Therefore these identified specific proteins for *S. flexneri* and *S. sonnei* can be used as biomarkers for development of diagnostic test which would allow rapid and accurate diagnosis of shigellosis to control the disease outbreak.

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CHAPTER ONE

INTRODUCTION

1.1 Introduction to *Shigella*

1.1.1 Historical perspectives and importance of *Shigella* discovery

Bacillary dysentery or also known as shigellosis is an acute infection caused by *Shigella* spp., a gram negative, non-motile bacillus. Shigellosis often characterized by abdominal cramps, fever and frequent mucoid bloody stools. Isolation of this bacterium from feces of individuals with acute dysentery was first described by Chantemese and Widel in 1888 (Lan and Reeves, 2002). Later, in 1898 a young Japanese microbiologist Kiyoshi Shiga had reported detailed description of the organism that caused the dysentery and named the organism as *Bacillus dysenteriae* (Yabuuchi, 2002). In late 1940, genus *Shigella* was classified into 4 species: *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei* and which are also designated as group A, B, C, and D respectively (Gangarosa *et al.*, 1970).

In the earlier classification, *Shigella* and *Escherichia coli* were classified in the same genus because of their genetic similarity. Several studies have reported that they share genetic homology greater than 90% (Boileau *et al.*, 1984; Faruque *et al.*, 2002). However, 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). *Shigella* strains were grouped in a different genus from *E. coli* because of their medical significance, human host interactions, pathogenicity, and physiology and serological characteristics (Escobar-Paramo *et al.*, 2003).

1.1.2 Global epidemiology of shigellosis

Shigellosis is a public health concern in developing countries and for travellers from industrialized nations (Vila *et al.*, 1994). The annual numbers of shigellosis episodes and deaths in Asia were estimated to be 91 million and 414,000, respectively, with *S. flexneri* and *S. sonnei* being the first and second most common serotypes (WHO, 2005). *Shigella* known as a disease of all age group but it is commonly seen in paediatric patients less than 5 years of age group (Arya *et al.*, 1977; Mutanda *et al.*, 1979; MoezArdalan *et al.*, 2003). Studies have reported that in developing countries, majority of *Shigella* infections are due to endemic shigellosis which causes approximately 10% of all diarrhoeal cases among children younger than five years old (Bennish and Wojtyniak, 1991).

In developing countries, the isolates of *S. flexneri*, *S. sonnei*, *S. boydii* and *S. dysenteriae* accounted for 60%, 15%, 6% and 6% respectively; and 16%, 77%, 2% and 1% respectively, in developed countries (Kotloff *et al.*, 1999). A study conducted in Singapore between 1986 and 1990 on total *Shigella* isolates had reported that *S. flexneri* was the most frequently isolated strains (60.3%) followed by *S. sonnei* (33.6%), *S. boydii* (3.2%) and *S. dysenteriae* (3%) (Lim and Tay, 1991).

Shigellosis outbreaks are mostly due to contaminated water, food, overcrowding communities, food handlers and flies (Kapperud *et al.*, 1995; Shears, 1996; Brooks *et al.*, 2003). A case control study conducted in rural Western Kenya to characterize the epidemiology of bloody diarrhea reported that 80% of bacterial pathogens isolated were due to *Shigella* spp., of which approximately 49% was caused by *S. flexneri* (Brooks *et al.*, 2003). Studies have also reported that, approximately 580,000

travellers from industrialized countries are infected annually by *Shigella* (Kotloff *et al.*, 1999).

In Malaysia, very few studies have been conducted to describe the prevalence of shigellosis. A retrospective study conducted on bacterial enteropathogens in childhood diarrhea in University Malaya Medical Centre (UMMC), Kuala Lumpur, from 1978 to 1997 showed that *Shigella* spp. was the third most common bacteria isolated from patient's stool samples (Lee and Puthucheary, 2002). *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* found to be the most frequently isolated species reported which constituted 74% of all *Shigella* isolates. A 9-year retrospective study conducted in Northeast Malaysia showed that the isolation rate was 9.99% of the total bacterial pathogens isolated from stool specimens, with *S. sonnei* and *S. flexneri* being the most common species isolated (Banga Singh *et al.*, 2011).

1.1.3 Classification

The genus *Shigella* is subdivided into four species based on the serological characteristics. Serotyping is based on the presence of O antigen component of lipopolysaccharides present on the outer membrane of the cell wall. *S. dysenteriae* consists of 13 serotypes. *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. *S. boydii* consist of 23 serotypes and is least frequently isolated species from cases of bacillary dysentery. *S. sonnei* consist of single serotype but may occur in two forms which is phase 1 and phase 2. (Rahaman *et al.*, 1975).

1.1.4 Bacteriology of *Shigella*

1.1.4.1 Physical characteristics

Shigellae are small, Gram-negative rods with a diameter of 0.3 to 1 µm and a length of 1 to 6 µm. They are non-motile, non-spore forming, noncapsulated and possess K and O antigens. The O antigen (somatic antigen) is useful in serological identification to classify the four species. The K antigen is the capsule antigen which occasionally interferes with agglutination by O antisera. Shiga toxin or verotoxin is an enterotoxin, which is produced only by *Shigella dysenteriae* type 1. It is a multi-subunit toxin 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 (Mata *et al.*, 1970).

1.1.4.2 Growth and culture characteristics

Shigella is a facultative anaerobe bacterium and is able to grow at temperatures ranging from 12^{o}C to 48^{o}C (optimum 37^{o}C), at a pH range of 5.0 to 7.0 (Tetteh and Beuchat, 2003; Zaika and Phillips, 2005). Generally, death of *Shigella* are enhanced with increased in temperature, NaCl concentrations and decreased in pH. The organism is acid resistant and can easily pass the gastric acid barrier. The common selective and 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). After overnight incubation, colonies on the MacConkey and DCA agar appears to be large, 2 to 3 mm in diameter, translucent and colourless (non-lactose fermenting) due to the absence of lactose fermentation. An exception is *S. sonnei* which is a late lactose

fermenter and forms pale pink colonies after 24 hours incubation. 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. On the HEK agar, the colonies appear to be blue greenish colour. *Shigella* does not produce hydrogen sulphide (H_2S) on the XLD, HEK and SS agar (Germani and Sansonetti, 2006).

1.1.4.3 Biochemical characteristic

Shigella does not ferment lactose but culture of *S. sonnei* ferments late lactose after 24 hours incubation. *Shigella* utilizes glucose and other carbohydrates, producing acid but not gas except for the *S. flexneri* serotype 6 and is oxidase negative. All *Shigella* species ferment mannitol except *S. dysenteriae.* Normally, indole is not produced and methyl red (MR) test is positive. On Triple Sugar Iron (TSI) agar, *Shigella* isolates are glucose positive (yellow butt), lactose negative (alkaline red slant) and do not produce H_2S gas. It is non-motile on Motility-Indole-Urea (MIU) agar. The suspected *Shigella* species will be further identified using serological test (Cheesbrough, 2006).

1.2 Clinical significance of shigellosis

1.2.1 Mode of transmission

The only natural hosts for *Shigella* infection is human and primates. The predominant mode of transmission is by fecal-oral route, in which *Shigella* is usually transmitted from person to person. The bacterium is highly infectious, since as few as 10 to 100 microorganisms are sufficient to cause disease (DuPont *et al.*, 1989) as they able to survive in acidic environment in the stomach (Small *et al.*, 1994). The

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 (Kothary and Babu, 2001).

1.2.2 Pathogenesis

The pathogenesis of shigellosis involves the invasion of the bacterium into the epithelial M cells of the colon. Upon contacting with the epithelium cell surface, *Shigella* releases Ipa (invasion proteins antigens) proteins through a specialized type III secretory apparatus (Sansonetti, 2001). The intrinsic invasive properties of the *Shigella* facilitate its invasion into the cell which damages the cell and cause inflammatory response (Zychlinsky and Sansonetti, 1997). Expression of various cells like M cells, epithelial cells, resident macrophages and polymorphonuclear leukocytes (PMNs) during *Shigella* invasion causes rupture, invasion and inflammatory destruction of the intestinal barrier (Sansonetti, 2001). The shigellosis pathogenic mechanism is complex which involves multiple genes during invasion and has been studied extensively by Sansonetti and colleagues (Goldberg and Sansonetti, 1993; Sansonetti *et al.*, 1996).

1.2.3 Clinical signs and symptoms

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 other invasive microorganisms such as *Campylobacter*, *Salmonella* and *Entamoeba histolytica*.

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 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). Therefore, clinical signs and symptoms are very important in helping to identify and treat patients with shigellosis.

1.2.4 Complications

Persistent life-threatening complications are often seen in malnourished infants and young children living in developing countries and also in elderly people who have weakened immune system (Bennish and Wojtyniak, 1991). Shigellosis usually resolves within five to seven days without sequelae (Steffen *et al.*, 1983). Complications include metabolic derangements, such as dehydration, hyponatraemia, hypoglycaemia, hypoproteinaemia and severe anorexia intestinal complications such as toxic megacolon, rectal prolapsed and intestinal perforation (Ashkenazi, 2004). HIV-infected and other immunocompromised patients have been reported as a risk factor associated with bacteraemia due to *Shigella*. Persistent diarrhea and

malnutrition are the most common chronic sequelae (Niyogi, 2005). Reactive inflammatory arthritis (Hannu *et al.*, 2005), conjunctivitis and urethritis known as Reiter's syndrome and anorexia has also been reported as complication due to shigellosis (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). Shigellosis has also been associated with acute appendicitis in children and endometriosis in females (Kodati *et al.*, 2008).

1.2.5 Antimicrobial treatment and prevention

World Health Organization has recommended that oral rehydration treatment is an effective and live-saving therapy for acute dehydrating watery diarrhea in the developing world. Treatment is critical in young children, the elderly or persons with chronic diseases, as shigellosis can be severe and lead to dehydration and other complication (Salam and Bennish, 1991). Antibiotic treatment also plays an important role in recovery from shigellosis. Appropriate antibiotic treatment, shorten the duration of excretion of *Shigella*, thus decrease the spreading of pathogens and prevent further complication. Multiple drug resistance plasmids are widely prevalent in *Shigella* spp. (Replogle *et al.*, 2000). The choice of antibiotic should be based on the sensitivity of the prevailing strain. The main antibiotics used for shigellosis are ampicillin, tetracycline, amoxicillin, nalidixic acid, cotrimoxazole, chloramphenicol and trimethoprim-sulfamethoxazole. Ciprofloxacin is recommended as the first line treatment and pivecillinam, ceftriaxone or azithromycin as the second line treatment for *Shigella* infection (Christopher *et al.*, 2010). However, treatment has become increasingly difficult due to emerging resistance to these commonly prescribed

antibiotics (Salam and Bennish, 1991; Replogle *et al.*, 2000; Ke *et al.*, 2011). Many studies have reported the emergence of multi-resistant strains to the commonly prescribed antibiotics occurring in the developing and developed countries (Replogle *et al.*, 2000; Taneja *et al.*, 2004; Pazhani *et al.*, 2008; Schultsz and Geerlings, 2012). The *Shigella* resistance locus (SRL), have been identified in *Shigella* strains which have been reported to mediate resistance to antibiotics (Turner *et al.*, 2001; Turner *et al.*, 2003). A study conducted by Lee & Puthucheary in Malaysia, reported that 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 and Puthucheary, 2002).

As the only significant source of *Shigella* infection is human and the mode of transmission is oral-faecal route, improving the personal and environmental sanitation are essential for disease prevention. The prevention measures include hand washing with soap, safe disposal of faeces, chlorination of water and protection of food from contamination. To date, there is no commercially vaccine available in the market to treat shigellosis (Das *et al.*, 2013).

1.3 Diagnosis of shigellosis

1.3.1 Clinical diagnosis

The diarrhoeal stage of the infection cannot be distinguished clinically from other bacterial, viral and protozoan infections (Youssef *et al.*, 2000). Therefore clinical diagnosis of shigellosis is not specific. The presence of fever suggests an invasive pathogen. Patients presenting with watery diarrhea and fever should be suspected of having shigellosis but the differential diagnosis should include infection caused by

enteroinvasive *E. coli* (EIEC), *Salmonella enteritidis, Campylobacter* species and *Entamoeba histolytica* (Goodman and Segreti, 1999; Sur *et al.*, 2004). The typical clinical feature of shigellosis is bloody and mucopurulent stool. Usually, fresh and bright red blood is present in the stools of patients infected with *Shigella* rather than dark brown blood which usually seen in amoebiasis (Niyogi, 2005).

1.3.2 Laboratory diagnosis

1.3.2.1 Conventional bacteriology techniques

1.3.2.1.1 Stool examination

Routine microbiological investigations in the laboratory diagnosis of shigellosis comprised of macroscopic and microscopic examinations of the suspected patients feces. Fecal specimens from patients with shigellosis will be watery, contains mucus and blood. The presence of red and white blood cells is an important characteristics in establishing the diagnosis of dysentery (Cheesbrough, 2006). Fresh and unstained suspensions of feces are 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 fecal secretion. A study has been reported that fecal blood or leukocytes are detectable in the stool in approximately 70% of shigellosis cases, whereas both fecal blood and leukocytes are detected in approximately 50% of cases (Echeverria *et al.*, 1992).

1.3.2.1.2 Stool culture

Stool culture remains the gold standard for establishing the definitive diagnosis of shigellosis. The laboratory diagnosis of *Shigella* infection is made by isolating the organism from stool specimen and serotyping the isolate. Pure isolates from culture

also required for antibiotic susceptibility testing. The successful isolation and identification of *Shigella* can be greatly enhanced when optimal laboratory media and techniques are employed. Fresh stool samples collected from patients before initiation of antibiotic therapy are preferred for microbiological tests as the chances of recovering the organisms are higher (Niyogi, 2005). The patients' specimens should be cultured within 2 to 4 hours after sample collection. The specimens that cannot be cultured within this time should be stored at 4°C in buffered glycerol saline or Cary-Blair medium for transportation and preservation because *Shigella* spp. is fastidious and survives poorly in stool samples with mixed flora at room temperature (Taylor and Schelhart, 1975).

1.3.2.1.2 (a) Inoculation on selective media

Isolation of *Shigella* in the clinical laboratory usually involves an initial streaking for isolation on differential or selective media with aerobic incubation to inhibit the growth of the anaerobic normal flora. The selective media are commonly used to isolate *Shigella* spp. as previously described. These selective media contain bile salts to inhibit the growth of other Gram-positive bacteria and pH indicators to differentiate lactose fermenters (coliforms) from non-lactose fermenters (*Shigella* spp.). Inoculation of stool specimens into liquid enrichment broth (Hajna Gram-negative broth or selenite F broth) may enhance the viability of the organisms before subculturing onto the selective media (Theron *et al.*, 2001). Selective media can be inoculated with a fecal swab, rectal swab or with a single drop of liquid fecal suspension and incubated at 35°C ± 2°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.

Identification of the suspected colonies can be achieved by performing biochemical and serological tests as described below.

1.3.2.1.2 (b) Biochemical screening tests

Biochemical tests are used for further identification of *Shigella* spp. 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, methyl red (MR), Voges-Proskauer (VP), nitrite reduction test, oxidase test medium, citrate agar and urea agar. *Shigella* spp. is usually MR positive and reduces nitrates to nitrites and negative for citrate, oxidase, urease, motility and VP test. *Shigella* produces 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 (Germani and Sansonetti, 2006).

1.3.2.1.2 (c) Serological identification

Serological testing is performed to confirm and classify the species of the *Shigella* isolates. Each species of *Shigella* contain a distinct type of O antigen. The identification of species is done by performing slide agglutination test using commercially available polyvalent O antigen grouping sera. In some cases, specific serotype identification is performed by testing with monovalent antisera for serotypes and sub-serotypes identification. For serotyping, agglutination test was carried out by emulsifying a colony from the surface of TSI or other non-selective agar on a drop of normal saline, on a clean glass slide. A drop of polyvalent or monovalent antisera is mixed with the bacterial suspension and the slide was tilted

back and forth for approximately 10 seconds. Clear agglutination indicates positive result while no agglutination indicates negative results. Cultures that react serologically and show strong suggestive results in the biochemical screening tests are reported as positive for *Shigella* (Cheesbrough, 2006).

1.3.2.2 Other diagnostic techniques

1.3.2.2.1 DNA based technique

A variety of new molecular technologies are available for rapid diagnosis of shigellosis. The conventional methods for determining the presence of certain species are time-consuming and labor-intensive. Therefore, the application of molecular methods such as PCR may compensate the limitation encountered in conventional method for the early diagnosis of shigellosis. Most researchers started to utilize PCR for their studies in detection and identification of pathogenic microorganism, evaluation of emerging novel infections and surveillance, early detection of biological agents and antimicrobial resistance profiling (Lukinmaa *et al.*, 2004). Studies have reported that PCR technique is more sensitive and specific than conventional culture technique and has the potential to be employed in routine diagnosis of dysentery in clinical centres as well as in epidemiological studies (Dutta *et al.*, 2001; Aranda *et al.*, 2004; Vu *et al.*, 2004; Ojha *et al.*, 2013). Having highlighted advantages and positive aspects in the molecular technique, there are also several drawbacks that are encountered during PCR assay. The PCR assay requires specialized instrument, trained personnel and relatively expensive reagents. Thus, the application of this technology is currently restricted to research laboratories and larger clinical laboratories. Besides that, this method also requires careful selection of clinical specimen and specimen processing to extract the genetic material. There

are also many studies reported the protocol for selective enrichments of the target organism need to be carried out in clinical samples containing low number of organisms (Feder *et al.*, 2001; Palladino *et al.*, 2003; Sails *et al.*, 2003).

1.3.2.2.2 Protein based technique

The antibody-based assays such as latex agglutination, immunodiffusion and enzyme immunoassays (EIA) format are the rapid methods being used for bacterial identification in most laboratories. The EIA is the most common assay used for testing large number of samples and is suitable for automation and quantification. There are many different EIA methods have been developed for diagnosis of shigellosis. A study in Kuwait, has developed a monoclonal antibody-based ELISA using a 43 kDa invasion plasmid-coded protein antigen (IpaC) to identify EIEC and *Shigella* strains in fecal samples from children (Pal *et al.*, 1997). ELISA techniques have been widely utilized for the detection of serum and urine antibodies of various immunoglobulin classes developed against *Shigella* serogroup specific LPS (Cohen *et al.*, 1991; Cohen *et al.*, 1996). A rapid latex agglutination procedure, also known as Wellcolex Colour *Shigella* tests, have been developed for grouping *Shigella* using an antibody attached to multicoloured latex particle (Bouvet and Jeanjean, 1992). Another simple test that has been utilized for detection is dot-EIA, in which specific antigens or antibodies are immobilized on a nitrocellulose membrane and the reaction is observed by enzyme activity. Previous study has demonstrated an antigenic membrane protein of 35 kDa in size, which is specific to IgA in sera of patients infected with *Shigella* spp. (Banga Singh, 2001). A simple dot-EIA was developed using this protein and diagnostic result of this test could be obtained within 3 hours to diagnose shigellosis.

1.4 The importance of heat shock proteins (HSPs)

Heat shock protein (HSP) or also known as stress protein belong to a family of essential protein synthesized by a variety of microorganisms including Gram negative and Gram positive bacteria, yeast, plant, human cells and even archaebacteria (Zhang *et al.*, 1998; Kanagasabai *et al.*, 2011). HSP has been reported to be the most highly conserved molecules in living organisms (van Eden, 2012). The heat shock response was first discovered in 1962 by Ferrucino Ritossa and his co-workers. They demonstrated an unusual profile of gene expression in the polytene chromosomes of salivary glands induced in *Drosophila melanogaster* larva (Ritossa, 1962). The first product of the gene was identified as a heat shock protein (Tissieres *et al.*, 1974).

HSPs constitute of a large family of proteins that are often classified based on their homology, related function and molecular weight (Chitradevi *et al.*, 2013). HSPs that have been classified based on their molecular weight are hsp10, hsp40, hsp60, hsp70, hsp90 and hsp100. HSPs are expressed constitutively and their synthesis is up-regulated following exposure to various forms of stress. The stress situation includes environmental (exposure to heat, heavy metal or UV radiation), pathological (infection or fever, malignancies, inflammation or autoimmunity) or physiological stress (growth factor deprivation, cell differentiation, hormonal stimulation or tissue development). HSPs synthesis varies with temperatures but it increases rapidly after sudden increase in temperature or in response to other environmental stresses to protect the cells from damage (Santoro, 2000; Kiriyama *et al.*, 2001).

HSP function as a molecular chaperon to prevent the proteins aggregation in folding and unfolding of protein (Kilic and Mandal, 2012). Many molecular chaperones described so far are members of hsp60 and hsp70 families. Hsp70 proteins in the cytosol are associated with newly formed polypeptides and are directly involved in protein transport between different intracellular compartments that lie across membranes (Frydman *et al.*, 1994; Hartl, 1996). In *E .coli*, the hsp70 is a homolog to Dnak which stabilizes the newly synthesized proteins and promotes the assembly of proteins into multimeric complexes as well during their disassembly process (Hartl, 1996). Besides the role as a molecular chaperon, hsp70 and hsp90 proteins were also reported as immunogens to elicit innate and adaptive immune responses (Basu and Srivastava, 2000; Srivastava and Amato, 2001).

Numerous studies reported that the potential of HSP as a therapeutic agents and their role in host defence mechanisms are being exploited in the protective vaccine development especially in cancer and infectious disease. Most studies reported that hsp60 is a potent vaccine candidate against many infectious diseases (Gomez *et al.*, 1995; Noll and AutenriethIb, 1996; Lee *et al.*, 2006; Carrillo *et al.*, 2008). The immunization of mice with recombinant hsp60 from *Histoplasma capsulatum* induced protection against pulmonary histoplasmosis (Gomez *et al.*, 1995). Another study proved that the vaccination of mice with recombinant hsp60 protected the animal against subsequent infection and development of gastroduodenal disease (Ferrero *et al.*, 1995). Several other scientists reported the protective efficacy of HSPs against various microorganisms such as *Porphyromonas gingivalis* (Lee *et al*., 2006*)*, *Piscirikettsia salmonis* (Wilhelm *et al*., 2005), *Yersinia enterocolitica* (Noll and AutenriethIb, 1996), *Paracoccidioides brasiliensis* (de Bastos Ascenço Soares *et*

al., 2008), *Leishmania infantum* (Carrillo *et al.*, 2008) *Mycobacterium tuberculosis* (Lowrie *et al.*, 1997), *Candida albicans* (Matthews and Burnie, 1992) and *Brugia malayi* (Dakshinamoorthy *et al*., 2012).

Recently, a study conducted in India to investigate the cross protective efficacy of recombinant hsp60 of *Salmonella* Typhi against various bacterial pathogens such as *S. dysenteriae* type 1, *S. flexneri*, *S. boydii*, enteropathogenic *E. coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Chitradevi *et al.*, 2013). This study reported that, the vaccination of mice with recombinant hsp60 of *Salmonella* Typhi not only conferred protection against *Salmonella* infections but also elicited cross-protection against all the tested pathogens. Furthermore, the findings concluded that the recombinant hsp60 has the potential to be developed as a single novel vaccine candidate against multiple pathogens.

1.5 Rationale of study

Shigellosis is endemic throughout the world and it is a major health concern in most impoverished areas, particularly in paediatric population (Phalipon *et al.*, 2008). Major obstacles in controlling shigellosis include the ease with which *Shigella* spreads from person to person due to its low infectious dose and its rapidity in emergence of antimicrobial resistance strains (Replogle *et al.*, 2000). The clinical management of the disease has become difficult due to rapid escalation in antibiotic resistance of *Shigella* spp. even to the newest antibiotics (de Paula *et al.*, 2010). As developing antimicrobial resistance to multiple antibiotics causes serious therapeutic problems, it is crucial to develop a rapid method to identify the bacteria in order to provide appropriate treatment and control the spreading of the disease. The early conclusion of definitive diagnosis of shigellosis is crucial to ensure proper clinical treatment and management of the infected patient. This would avoid the unnecessary prescription of antibiotics and thus would limit the emergence of antibiotic resistance.

The current culture method remains as a gold standard to diagnose shigellosis from stool samples. Among the drawbacks of this method are time consuming, labour intensive, competition in isolation from other commensal organisms and inappropriate sample collection. Furthermore, samples are collected after the antibiotic therapy, the growth of the pathogen may be impaired as a result of sublethal injuries by the antibiotics (Sethabutr *et al.*, 2000). As a result, shigellosis remains undiagnosed in a significant number of patients. To overcome these problems, an alternative diagnostic method for the early identification of this pathogen is needed to control the disease outbreak.

The outer membrane of Gram negative bacteria plays many roles in cellular function in addition to the classical role of transporting ions through the porin proteins (Jyothisri *et al.*, 1999; Silhavy *et al.*, 2010; Galdiero *et al.*, 2012). Protective immune response stimulated by OMPs has been documented for several Gram negative organisms (Isibasi *et al.*, 1988; Achouak *et al.*, 2001; Galdiero *et al.*, 2003). Bacteria exist widely in the diverse environment. During the course of infection, growth of bacteria is influenced by various environmental factors including pH, osmotic strength, oxygen, iron availability, and temperature, which may change dramatically (Guiney, 1997). Thus, the bacteria must adapt to and survive in the new environment. Temperature has been described as one of the main factor which regulated the expression of microbial protein (Mekalanos, 1992). Studies have been reported that certain proteins were overexpressed in response to high temperature such as HSPs and serve as defence mechanisms against various environmental stresses (Ellis, 1996; Stewart and Young, 2004). Research has also shown that the HSPs are usually associated to the virulence of the pathogens (Lathigra *et al.*, 1991; Gophna and Ron, 2003).

A previous study has reported that *Shigella* spp. is only capable in invading the epithelial cell of human at elevated temperature but not at 37°C (Hale, 1991). As such, the HSPs that expressed during higher temperatures may play a role in the bacteria to regulate the expression of virulence related proteins. Moreover, the immunogenic properties of HSPs have been widely reported in infectious diseases. Various studies have proven the capability of HSPs as a protective vaccine candidate against bacterial infection (Wilhelm *et al.*, 2005; Lee *et al.*, 2006). In an immunoproteomic study conducted in China, the authors discovered the presence of

an immunoreactive hsp70 in *S. flexneri* 2a (Ying *et al.*, 2005). This hsp70 from *S. flexneri* was shown to have potential in providing protection against shigellosis. To date, there is no commercially vaccine available to prevent shigellosis. Thus the identification of antigenic HSPs can be potential candidate for vaccine and diagnostics development.

The main aim of this study is to identify the presence of HSPs in the outer membrane protein of *S. flexneri* and *S. sonnei* as biomarker(s). These proteins can be used to develop rapid and reliable protein based diagnostic tests for the identification of *Shigella* spp. which would significantly improve effective management of the disease especially among young children.

1.6 Objectives of the study

1. To study the outer membrane proteins (OMPs) profiles of *S. flexneri* and *S. sonnei* expressed at 37°C, 38.5°C and 40°C by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

2. To study the antigenic profiles of the OMPs expressed at 37°C by Western blot analysis using patient's sera positive for *S. flexneri* and *S. sonnei* toward the identification of potential biomarker(s) for serodiagnosis of shigellosis.

3. To identify the OMPs of *S. flexneri* and *S. sonnei* that showed prominent increase in expression at 40°C by mass spectrometry method.

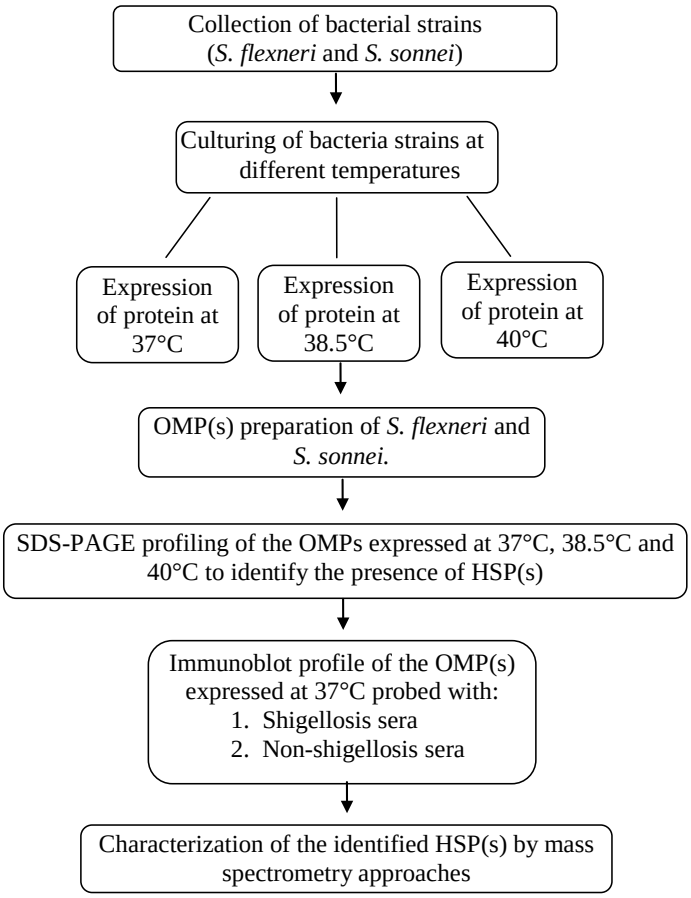

Figure 1.1: Flow chart of this study

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacteria

Clinical strains, *S. flexneri* SH057 and *S. sonnei* SH080 strains used in this study were obtained from the Department of Medical Microbiology and Parasitology, School of Medical Sciences, Health Campus, USM. *S. flexneri* ATCC 12022 and *S. sonnei* ATCC 25931 were used as the reference strain in this study.

2.1.2 Growth and maintenance of bacterial strains

S. flexneri ATCC 12022, *S. sonnei* ATCC 25931 and the clinical isolates were maintained in TSB-glycerol (10%) and kept at -20°C. For experimental purposes, the organism was grown in nutrient broth at 37°C for 18 hours and was subcultured on blood agar to check for its purity. *Shigella* spp. was confirmed by biochemical tests such as TSI (Triple sugar iron agar), SIM (sulfide-indole motility medium), urease, and methyl red (MR) and citrate utilization test. For serological testing, antisera *Shigella flexneri* 2a and *Shigella sonnei* (Denka Seiken, Japan) were used. In order to perform biochemical test, a single translucent colony was picked up from MacConkey agar. For serotyping purpose, colonies were picked from TSI agar and it was resuspended in a drop of normal saline on a glass slide. A drop of antisera was mixed with the bacterial suspension and the slide was tilted back and forth for 10 seconds. Clear agglutination shows positive results while no agglutination indicates negative results.

2.1.3 Sera samples

Prior to the collection of samples, permission was obtained from patients using consent letter as shown in the (Appendix A). Samples were collected from consented patients admitted in the ward at Hospital Universiti Sains Malaysia, Kelantan, immediately after the laboratory culture results were confirmed. The collected sera samples were stored in aliquots at -20°C until needed. Sera samples used in this study are as listed in Table 2.1. In the Western blot analysis, panel of sera were obtained from patients infected with *S. flexneri*, *S. sonnei* and other infections (*Salmonella* Typhi, *Salmonella* spp, enteropathogenic *Escherichia coli* (EPEC), *Aeromonas hydrophila* and *Campylobacter jejuni.*

2.1.4 Chemicals and media

All the chemicals, reagents and media used in this study as listed in the Table 2.2 were electrophoresis, molecular or analytical grade.

2.1.4.1 Media

The appropriate media were autoclaved at 121°C, 15 psi for 15 minutes and tested for sterility by incubating at 37°C overnight to check for contamination. The media were stored at 4°C prior to use.