DEVELOPMENT OF DNA-BASED DIAGNOSTIC TESTS FOR THE DETECTION OF *Shigella dysenteriae, Shigella flexneri* AND *Shigella sonnei*

By

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbols/Abbreviations	Definition
ג	Wavelength
%	Percentage
~	Approximately
°C	Degree celcius
µg	Micro gram
µl	Micro liter
A_{280}	Absorbance at 280 nm
BLAST	Basic Local Alignment Search Tool
bp	Base pair
cfu	Colony forming unit
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
EDTA	Ethylenediamine tetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme Linked Immunosorbent Assay
et al.	*Et alii*
GNB	Gram negative broth
H_2S	Hydrogen sulfide
HUSM	Hospital Universiti Sains Malaysia
IC	Internal Control
IMR	Institute of Medical Research
IpaB	Invasion plasmid antigens protein B
IpaC	Invasion plasmid antigens protein C
IpaD	Invasion plasmid antigens protein D
IpaH	Invasion plasmid antigens protein H
kb	Kilobase
kDa	Kilodalton
Kg	kilogram
L	Litre
LPS	Lipopolysaccharide
M	Molar
mg	Milligram
$MgCl_2$	Magnesium chloride
ml	Mililitre
mM	Milimolar
MW	Molecular Weight
NaCl	Natrium chloride
ng	Nano gram
NCBI	National Centre of Biotechnology Information
OD	Optical Density
OmpA	Outer membrane protein A
ORS	Oral Rehydration Salt
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction

pg	Pico gram
pmole	Pico mole
psi	Pounds per square inch
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolution per minute
SHET	*Shigella* Entero Toxin
spp.	Species
Ta ° C	Annealing temperature
TAE	Tris-Acetate EDTA
Taq	*Thermus aquaticus*
V	Volt
VP	Voges-Proskauer
WHO	World Health Organization

PEMBANGUNAN UJIAN-UJIAN DIAGNOSTIK BERDASARKAN DNA BAGI PENGESANAN *Shigella dysenteriae, Shigella flexneri* DAN *Shigella sonnei*

ABSTRAK

Shigelosis atau disenteri basilus disebabkan oleh *Shigella* spp. merupakan masalah kesihatan yang signifikan di seluruh dunia. Cara pengenalpastian dan pengesanan patogen tersebut daripada tinja yang diamalkan kini iaitu dengan kaedah pengkulturan adalah sukar, memerlukan proses ujikaji yang cerewet, mempunyai kadar sensitiviti yang rendah dan mengambil masa yang lama (2 hingga 5 hari) untuk mendapat keputusan. Dalam kajian ini, ujian tindakbalas rantai polimerase dengan reagen yang dihasilkan secara beku-kering atau stabil terma telah dicipta untuk pengesanan gen *ompA* pada *Shigella dysenteriae*, *Shigella flexneri* dan *Shigella sonnei*. Ujian-ujian yang dicipta mengandungi primer spesifik, reagen beku-kering tindakbalas rantai polimerase dengan kawalan dalaman (IC) dan pewarna muat turun jeli.

Optimasi parameter tindakbalas rantai polimerase dilakukan meliputi suhu pelekatan (Ta°C) dan kepekatan $MgCl_2$. Ujikaji kepekaan dan kespesifikan dijalankan dengan menggunakan kultur tulen *S. dysenteriae*, *S. flexneri*, *S. sonnei* dan bakteria enterik lain. Hasil ujikaji menunjukkan tiada tindakbalas dihasilkan daripada templat DNA bukan sasaran. Lima pg/µl DNA IC dimasukkan ke dalam ujian selepas ujikaji optimasi kepekatan templat DNA IC dilakukan. Amaun minima templat DNA genomik yang dapat dikesan oleh ujian-ujian yang dicipta adalah 0.39 ng/µl untuk *S.*

sonnei dan *S. flexneri* manakala 0.78 ng/µl untuk *S. dysenteriae*. Analisis kepekaan bilangan bakteria (cfu) pula mendapati kepekaan adalah 4.0 x 10^3 cfu setiap reaksi tindakbalas rantai polimerase untuk ujian *S. dysenteriae* manakala bagi *S. flexneri* dan *S. sonnei pula adalah* 4.0 x 10^4 cfu. Analisis kestabilan reagen stabil terma tindakbalas rantai polimerase yang dijalankan dengan menyimpan reagen berkenaan pada suhu bilik (25-26 °C) dan pada 4 °C mendapati reagen stabil disimpan sehingga 6 bulan. Ujikaji tempoh pengeraman optimum kultur tinja di dalam GNB yang dijalankan mencadangkan pengeraman perlu dilakukan selama 8 hingga 12 jam sebelum ujian tindakbalas rantai polimerase dibuat.

Kajian penilaian awal atau evaluasi awal di dalam makmal menggunakan tinja kanak-kanak yang dimasukkan kultur tulen *S. dysenteriae, S. flexneri, S. sonnei* dan bakteria enterik telah dilakukan (n=50). Berdasarkan keputusan ujikaji, didapati ujian yang dibangunkan untuk *S. dysenteriae* dan *S. sonnei* adalah 100% untuk spesifisiti dan sensitiviti, begitu juga dari segi NPV (Nilai Jangkaan Negatif) serta PPV (Nilai Jangkaan Positif) iaitu 100% . Manakala bagi asai yang dibangunkan untuk *S. flexneri* pula, sensitiviti adalah 100%, spesifisiti 92.8% dengan PPV 92.8% dan NPV 100% . Kajian ini telah berjaya menghasilkan ujian yang ringkas, pantas, sensitif, spesifik dan berkos rendah dengan kawalan dalaman bagi pengesanan *S. dysenteriae, S. flexneri,* dan *S. sonnei*. Namun begitu, kajian lanjutan menggunakan bilangan sampel tinja lebih banyak dan tinja orang dewasa perlu dilakukan untuk menilai potensi sebenar asai yang dicipta.

DEVELOPMENT OF DNA-BASED DIAGNOSTIC TESTS FOR THE DETECTION OF *Shigella dysenteriae, Shigella flexneri* AND *Shigella sonnei*

ABSTRACT

Shigellosis or bacillary dysentery caused by *Shigella* spp. remains as significant health problem worldwide. Current detection and identification methods of the pathogens from stool specimens by culture method is relatively insensitive, generally time consuming (~ 2-5 days) and laborious. In this study, thermostabilised Polymerase Chain Reaction (PCR) assays were developed for the detection of *ompA* genes in *Shigella dysenteriae, Shigella flexneri* and *Shigella sonnei*. The tests contained specific primers for the detection of *S. dysenteriae, S. flexneri* and *S. sonnei* respectively, freezed-dried or thermostabilised PCR reagents with Internal Control (IC) and gel loading dye.

Optimization of PCR parameters including annealing temperature (Ta °C) and $MgCl_2$ concentrations was performed. In assessing specificity and sensitivity, the primers were challenged with known *S. dysenteriae, S. flexneri, S. sonnei* and other enteric pathogens. None of the DNA template from non-targeted organisms was amplified. Five pg/µl of IC was incorporated into the assays after optimization of IC DNA template concentration was done. Minimum concentration of genomic DNA that could be detected by the assays was 0.39 ng/µl for *S. sonnei* and and *S. flexneri* .As for the detection of *S. dysenteriae* was 0.78 ng/µl. At bacterial level, the sensitivity was found to be 4.0 $x10^3$ cfu/PCR reaction for the test developed for the detection of *S. dysenteriae* and 4.0 $x10^4$ cfu/PCR reaction for *S. flexneri* and *S. sonnei*.

Thermostabilised PCR mix periodic stability assessment for each test was done at ambient temperature (25-26°C) and 4°C. Results suggested that the reagent is stable until 6 months of storage at both temperatures. Optimization of stool culture in GNB (Gram Negative broth) was done in order to measure optimum incubation time prior to PCR assay. The optimum time was found to be 8 to 12 hours for all tests.

Preliminary evaluation of the developed tests was done using children seeded stool culture in GNB with *S. dysenteriae, S. flexneri, S. sonnei* and other enteric bacteria (n=50). The PCR results gave 100% for sensitivity and specificity, with 100% Positive Predictive Value (PPV) and Negative Predictive Value (NPV) for the test developed for the detection of *S. dysenteriae* and *S. sonnei*. As for the detection of *S. flexneri,* the sensitivity was found to be 100% and for specificity, 92.8%. Therefore, the PPV and NPV was 92.8% and 100% respectively. This study has developed simple, rapid, sensitive, specific and cost-effective tests with built-in control for the detection of *S. dysenteriae, S. flexneri, and S. sonnei*. However, further studies with a larger set of sample probably using adult stool need to be done to evaluate the true performance of the developed tests.

CHAPTER 1

INTRODUCTION

1.1 Introduction to *Shigella*

1.1.1 History and discovery of *Shigella*

Shigella is the primary causative agent of bacillary dysentery or shigellosis characterized by bloody stools containing mucus (Navia *et al.*, 2005). Isolation of this bacterium from faeces of individuals with acute dysentery was first described by Chantamesse and Widel in 1888 (Lan and Reeves, 2002). Later, Kiyoshi Shiga, a Japanese bacteriologist reported detailed description of the organism that caused the dysentery in 1898 (Hale, 1998; Niyogi, 2005). *Shigella dysenteriae* type 1, initially termed *Bacillus dysenteriae*, was the first *Shigella* spp. isolated by Shiga. Forty years after the discovery, three additional groups of related organisms were defined and taxonomically placed in the genus *Shigella.* They were named *Shigella flexneri, Shigella boydii* and *Shigella sonnei* in honor to the investigators Flexner, Boyd and Sonne. The 1950 Congress of the International Association of Microbiologists *Shigella* Commission adopted the generic name of *Shigella*, in honor of Shiga (Hale, 1998). Species subgroups A (*S. dysenteriae*), B (*S. flexneri*), C (*S. boydii*) and D (*S. sonnei*) were also designated by the commission. All of the *Shigella* species were found to be pathogens to human (Coimbra *et al.*, 2001).

In the earlier classification, *Escherichia coli* and *Shigella* were classified in the same genus because of their genetic similarity. *Escherichia coli* and *Shigella* spp. cannot be differentiated on the polynucleotide level in some reported DNA hybridization studies. They share greater than 90% homology by DNA-DNA reassociation analysis (Boileau *et al.*, 1984; Wang *et al.*, 1997; Faruque *et al.*, 2002; Lan *et al.* 2004).

However, recently comparative genomic studies between the two organisms revealed that 175 of the total 3235 open reading frames were exclusively for *S. flexneri* (Wei *et al.*, 2003; Lan *et al.*, 2004). *Shigella* spp. were allocated in a different genus from *E. coli* because of their medical significance, human host interactions, pathogenicity, physiology and serological characteristics (Escobar-Paramo *et al.*, 2003). *Shigella* spp. are non-motile, lactose negative. They ferment glucose and other carbohydrates without producing gas. These characteristics differentiate *Shigella* from typical *Escherichia coli* which are positive for those characteristics. The ability to cause acute bacillary dysentery to human by invading the colonic mucosa cells and produce tissue damage differentiated *Shigella* spp. from certain strains of *E. coli* (Brenner *et al.*, 1982).

1.1.2 Shigellosis epidemiology

Shigellosis has been reported to be a global human health problem. The annual global burden of shigellosis episodes throughout the world was estimated to be 164.7 million; 163.2 million cases (1.1 million deaths) were in developing countries and 1.5 million in industrialized countries (Kotloff *et al.*, 1999; Niyogi, 2005). The reported mortality rate was approximately 0.7%. In Asia, the burden was estimated from published studies initiated in Asia countries after the year 1990. The annual episodes and deaths were estimated to be 91 million and 414 000 respectively with *S. flexneri* as the commonest serotype followed by *S. sonnei* (Kotloff *et al.*, 1999; WHO, 2005). Majority of *Shigella* infections are due to endemic shigellosis especially in numerous developing countries. Studies carried out in developing countries had reported that *Shigella* is associated with 5% to 15% of the diarrheal cases and 30% to 50% of dysentery cases (Faruque *et al.*, 2002; Hiranrattana *et al.*, ;

2005). It is responsible for approximately 10% of all diarrheal episodes among children age below five years living in developing countries and up to 75% of diarrheal deaths (Kotloff *et al.*, 1999; Niyogi, 2005).

Worldwide, the four species of *Shigella*; *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei* have been reported to be responsible for mortality and morbidity for bloody diarrhea especially in developing countries with substandard hygiene and poor quality of water supplies (CDC, 2005; Niyogi, 2005). In the United States and Europe, *Shigella* spp. continue to be responsible for morbidity and mortality in high risk population in association with over crowding and poor personal hygiene such as senior citizens, people in prisons and crowded refugee camps, patients in mental hospitals, toddlers in day-care centres and among homosexual men (Niyogi, 2005).

The geographical distribution of the four serogroups of *Shigella* is varying, as is their pathogenicity (Opintan and Mercy, 2007). The predominant serogroups of *Shigella* circulating in a community appear to be related to the level of socioeconomic development. Epidemic and endemic disease being caused by *S. dysenteriae* type 1 occur worldwide. In most developed countries or industrialized nations, infections by *Shigella* are usually due to *Shigella sonnei* .(WHO, 2009). In United States, most of shigellosis outbreaks were due to *S. sonnei*. Meanwhile, *S. flexneri* remains the leading species that cause shigellosis in most of developing countries and resource-poor countries. In Singapore, a study conducted by Lim and Tay (1991) had reported that *S. flexneri* was the most frequent isolated strains (60.3%) followed by *S. sonnei* (33.6%), *S. boydii* (3.2%) and *S. dysenteriae* (3%) among total isolated *Shigella* (506) in four years, from 1986 to 1990. Studies done in five resource poor countries;

Bangladesh, China, Pakistan, Indonesia and Vietnam found that *S. flexneri* was the most frequently isolated species whereas in Thailand, which is rapidly becoming industrialized *S. sonnei* was found to be the most common species (Lorenz Von S. *et al.*, 2006). The fourth species, *S. boydii* was first found in India and uncommonly encountered except in the Indian subcontinent. The median percentage of isolates of *S. flexneri, S. sonnei, S. boydii* and *S. dysenteriae* in developing countries were 60%, 15%, 6% and 6% respectively while in developed countries were 16%, 77%, 2% and 1% respectively (Kotloff *et al.,* 1999). In Iran, a study conducted involving Iranian children less than 5 years old with acute diarrhea showed that out of 555 faecal samples, *Shigella* spp. (26.7%) was the most prevalent bacterial pathogen isolated (Jafari *et al.*, 2009).

In Indonesia, *Shigella* spp. was reported to be the most frequently isolated pathogen associated with diarrhea; led by *S. flexneri*, followed by *S sonnei* and *S. dysenteriae (*Buhari *et al., 2002).* A case-control study to characterize the epidemiology of bloody diarrhea in rural Western Kenya had reported that 80% of the bacterial pathogens isolated were due to *Shigella* spp and 49% was caused by *S. flexneri* (Brooks et al., 2003). Generally, the incidence and fatality rates due to shigellosis are highest among very young children and the elderly. The highest incidence of shigellosis is in young children especially under 5 years of age (Kotloff *et al.*, 1999; Lorenz von S. *et al.*, 2006). A study conducted in Pakistan had reported that *Shigella* spp. were commonly isolated among children having diarrhea. *S. flexneri* was identified as the most frequently isolated species compared to *S. dysenteriae, S. boydii* and *S. sonnei* (Kausar et al., 1998).

In Malaysia, there are lack of reports on the prevalence of shigellosis. In a study done by Lee and Puthucheary (2002) on bacterial Enteropathogens in childhood diarrhea in urban hospitals, *Shigella* spp. was the third most common bacteria isolated from stool samples. This retrospective study was conducted among children admitted due to diarrhea at the University of Malaya Medical Centre (UMMC), Kuala Lumpur, from year 1978 to 1997. *Shigella* spp. was isolated from 1.4% (386 isolates) of the total stool specimens, which corresponded to 13% of the 2986 isolates found positive for bacterial pathogens. *S. flexneri* was found to be the most frequently isolated species reported of all *Shigella* isolates from 1970s to early 1990s, followed by *S. sonnei* (Lee and Puthucheary, 2002). However, from 1993 to 1997, the number of *S. sonnei* strains isolated surpassed *S. flexneri*. A year after that, another retrospective analysis was conducted among children admitted with diarrhea but this time to Hospital Universiti Sains Malaysia (HUSM), Kelantan, Malaysia. The study showed that the isolation rate of *S. flexneri* was 2.28% out of 1097 stool specimens studied (Ludin *et al.*, 2003).

1.1.3 *Shigella* classification

Shigella belongs to the tribe Escherichia in the family of Enterobacteriaceae. The genus of *Shigella* was classified into four species based on biochemical basis and serological differences; *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei*. *S. dysenteriae* consist of 13 serotypes, *S. flexneri* consist of 8 serotypes, *S. boydii* consist of 18 serotypes and *S. sonnei* consist of 1 serotype. Serotyping was based on the presence of O antigen component of lipopolysaccharide on the outer membrane of the cell wall.

1.1.4 *Shigella* properties

1.1.4.1 Physical characteristics

Shigella is a gram negative small rod bacteria which is non-capsulated with a size 0.3 to 1 µm in diameter with length 1 to 6 µm. It has no flagella and non-motile (no H antigens). This bacteria posses K and O antigens. The O antigen is useful in serological identification to classify the four species. The K antigen is the capsule antigen which occasionally inteferes with the determination of O antigen. *Shigella* strains produce 3 distinct enterotoxins. *SHET 1* a chromosome encoded *Shigella* enterotoxin 1 present in all *S. flexneri 2a* but rarely found in other *Shigella* serotypes. *SHET2*, *Shigella* enterotoxin 2 located on a large plasmid associated with virulence of *Shigella.* The enterotoxin produced by *S. dysenteriae* only is called Shiga toxin or verotoxin. The toxin is neurotoxic, cytotoxic and enterotoxic encoded by chromosomal genes with 2 domains, 1-A (32 kDa) and 5-B (7.7 kDa).

1.1.4.2 Growth and culture characteristics

Shigella is a facultative anaerobe. This bacterium is able to grow at temperatures ranging from 12°C to 48°C with an optimum growth temperature at 37°C and at a pH range of 5.0 to 7.3. *Shigella* spp. are acid resistant and can pass the gastric acid barrier but are sensitive to the increment of temperature, decrement of pH and increment of NaCl concentrations that will lead to their deaths. A number of commercially prepared plating media are appropriate for culturing *Shigella*. MacConkey (MAC), Xylose Lysine Desoxycholate (XLD), Hektoen (HEK), *Salmonella-Shigella* (SS) and Desoxycholate Citrate Agar (DCA) are among the common selective or differential agar media used for the recovery of *Shigella* (Clemens *et al.,* 1999). However, *S. dysenteriae type 1* and *S. sonnei* do not grow

well on *Salmonella-Shigella agar. Shigella* spp. has typical non-lactose fermenting characteristic colonies on lactose enriched media such as on MAC, DCA and SS agar. Colonies on MAC and DCA agar appears to be 2 to 3 mm in diameter, translucent and colorless or non-lactose fermenting. On XLD agar, the colonies appear to be much smaller; 1 to 2 mm in diameter and red in colour as lysine is decarboxylated to produce alkaline end products which raised the pH and change the agar into deep red colour. On the HEK agar, the colonies appear to be blue-greenish in colour.

1.1.4.3 Biochemical characteristics

All *Shigella* spp. do not produce hydrogen sulphide (H_2S) on the XLD, HEK and SS agar. All serotypes do not ferment lactose but cultures of *S. sonnei* utilize this substrate after prolonged incubation (more than 24 hours incubation). *Shigella* utilizes glucose and other carbohydrates, producing acid with no gas except for certain biotypes of *S. flexneri* and is oxidase negative. Indole is not produced and methyl red (MR) test is positive. *Shigella* is negative for lysine decarboxylase. Most isolates of *S. sonnei* are able to produce ornithine decarboxylase. Rare isolates of *S. boydii* are ornithine positive. Other serotypes are not able to produce ornithine decarboxylase. Serogroups A, B and C are similar biochemically except for *S. sonnei*. Positive beta-D-galactosidase and ornithine decarboxylase biochemical reactions differentiated *S. sonnei* from other serotypes (Cheesbrough, 1984; Clemens *et al.*, 1999).

1.2 Shigellosis by *Shigella* spp.

1.2.1 Reservoirs and modes of transmission

Natural hosts for *Shigella* are humans and primates. The predominant mode of transmission is by faecal-oral route where personal hygiene is poor. The infective dose is very low, varying from 10 to 10^3 organisms renders shigellosis a highly contagious disease (Du Pont *et al.*, 1989; Villabo and Torres, 1998; Navia *et al.*, 2005). The infective dose is lower than other enteric pathogens such as *Vibrio* spp., *Salmonella* spp. and *Campylobacter* spp. which require at least 10^4 to 10^5 organisms to cause infection. Persons symptomatic with diarrhea were reported to be primarily responsible for the transmission (García-Fulgueiras *et al.*, 2001). The disease is usually acquired by consumption of contaminated water and food but sometimes through contact with contaminated inanimate object and sexual contact; mainly among men who have sex with men (Lampel *et al.*, 1990; Villabo and Torres, 1998; Lindqvist, 1999; Hamamoto *et al.*, 2000; Niyogi, 2005). Consumption of raw or uncooked vegetables where sewage was used as fertilizer (Sur *et al.*, 2004) and eating of cheese and oysters that has been contaminated by infected food handlers has been reported to be the cause of shigellosis (García-Fulgueiras *et al.*, 2001; Jun *et al.*, 2004).

Transmission may also occur through contaminated fomites. In areas where disposal of human faeces is inappropriate, flies, specifically *Musca domestica*, may serve as vectors for the transmission of shigellosis (Sur *et al.*, 2004; Niyogi, 2005). Infected people may excrete *Shigella* in large numbers in the stools, up to 10^5-10^9 bacteria per gram stool (Kausar *et al.*, 1998; Ahmed *et al.*, 2003). The organism can be found in the faeces for weeks after symptoms have ceased. It has been reported that this

organism is able to survive in soiled linen for up to seven weeks, in salt water for 12 to 30 hours, in dust for 6 weeks at room temperature, in refrigerator for at least 1 week , in fresh water for 5 to 11 days, in sour milk for 4 weeks and in kitchen refuse for 1 to 4 days (Lampel *et al.*, 1990; Sur *et al.*, 2004).

1.2.2 Incubation period

Shigellosis typically evolves through several phases. Manifestations of *Shigella* infection vary with the infecting species, the presence of risk factors, the age of the host and the specific immune status of the host. The incubation period is typically 1 to 4 days and sometimes may be as long as 8 days with *S. dysenteriae* infection. Severe cases require immediate appropriate medication. In mild cases, the disease sometimes may be self limiting which will resolve within 5 to 7 days without sequelae (Hale, 1991; Hale, 1998).

1.2.3 Pathogenesis

The fundamental event in the pathogenesis of *Shigella* is the ability to invade and colonize the human intestinal epithelium. The disease is characterized by the destruction of the colonic epithelium provoked by the inflammatory response that is induced upon invasion of the mucosa by *Shigella* (Parsot, 2005). Infection is generally localized or limited to the intestinal mucosa areas. After oral inoculation, *Shigella* passes the terminal ileum and colon where it invades and proliferates within epithelial cells and spread cell to cell. Those abilities are the key determinants of the disease that elucidated acute inflammatory reaction (Islam *et al.*, 1997; Alfredo, 2004).

The organism can enter both erythrocytes and M cells, which are special epithelial cells overlying mucosal lymphoid follicles. The infection process involves multiple steps including macropinocytosis, escape into the cytosol followed by multiplication and passage to the adjacent cells. *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. Among the proteins secreted are *VirA*, *OpsB* to *OpsG* and invasion plasmid antigens (Ipa) proteins such as *IpaB, IpaC, IpaD* and *IpaH*. Most of the virulence determinants responsible for invasion of epithelial cells are encoded by a 213 kilobase (kb) plasmid that is unique to virulent *Shigella* and enteroinvasive *E. coli* (EIEC) strains. Pathogenic mechanism of shigellosis has been extensively studied and published (Hale, 1991; Tran Van Nhieu and Sansonetti, 1999; Sansonetti, 2001; Fernandez and Sansonetti, 2003).

In endemic areas, the incidence of shigellosis peaks during the first 5 years of life and declines thereafter, suggesting that immunity develops after repeated exposures during childhood (Taylor *et al.* 1986; Ferrerico *et al.*, 1991). Antibody responses to the somatic antigens of *Shigella* develop early in infection and follow the typical course for anti-LPS antibodies, which is IgM response that peaks within weeks and wanes after 1-2 years (Sur *et al*., 2004). Serotype specific natural immunity was reported by a longitudinal study of a cohort of Chilean children in whom primary *Shigella* infection conferred 76% protective efficacy against reinfection with the same serotype (Cohen *et al.*, 1992). Adult volunteers who were experimentally infected with either *S. sonnei* or *S. flexneri* were found to be significantly protected against shigellosis following rechallenge with the homologous strains (Herrington *et al.*, 1990; Kotloff *et al.*, 1995).

1.2.4 Clinical manifestation

There are several phases in shigellosis. The incubation period is 1 to 4 days after oral inoculation but in certain cases may be as long as 8 days with *S. dysenteriae* (Sur *et al.*, 2004). Clinical disease typically begins within 24-48 hours of ingestion of a few hundred to a few thousand organisms (Hale, 1998). Among the first symptoms to occur are fever with other constitutional symptoms such as malaise, fatigue, stomach cramps, headache, anorexia and occasional vomiting. These will be associated with frequent passage of small stool volume that consist of blood, mucus and pus. Patients with severe infection may pass more than 20 dysenteric stools in a day. Blood, mucus and pus cells found in the stools suggest colorectal inflammation due to the infection (Li, 2000). Infections by *Entamoeba histolytica*, *Campylobacter* spp. and *Salmonella* spp. also manifest similar symptoms but usually generate self-limited illness that is rarely as serious as shigellosis. There were some cases that shigellosis cannot be distinguished clinically from diarrhea caused by other enteric pathogens. This is due to acute non-bloody diarrhea that may be caused by certain strains of *Shigella*. Clinical symptoms usually persist for 10 to 14 days and sometimes longer (Alfredo, 2004).

In some severe shigellosis cases, the infection may become life threatening and contribute to substantial mortality especially among young children. A study conducted by Khan and colleagues had reported that severe infections of shigellosis may cause high grade fever associated with seizures among children 5 years of age or less (Khan *et al.*, 1999). Studies on the effects of diarrhea on the growth of children living in developing countries had suggested that dysentery caused by *Shigella* spp. is associated with significant growth retardation (Alam *et al*., 2000).

Daily loss of 200-300 ml of serum protein into the faeces during the infection exacerbates malnutrition and growth stunting. The loss of serum proteins results in depletion of nitrogen stores (Alam *et al.*, 2000). Decreament of immune system also increases the risk of shigellosis and contributes to substantial mortality.

1.2.5 Complications

Complications due to shigellosis are rare but may happen in severe cases. Shigellosis may be associated with a large number of mild to severe life-threatening complications, particularly due to *S. dysenteriae* type 1. Most episodes of shigellosis in healthy individuals are self-limited and resolve within 5 to 7 days without sequlae. Persistent diarrhea and malnutrition are the most common chronic sequlae (Niyogi, 2005).

Life threatening complications are most often seen in malnourished infants and young children living in developing countries. These include metabolic derangements; dehydration, hyponatraemia and hypoglycaemia, intestinal complications; toxic megacolon, rectal prolapse, intestinal perforation and rarely sepsis. HIV-associated immunodeficiency and immunocompromised patients have been reported to have severe shigellosis, including persistent or recurrent intestinal disease and bacteremia (Kristjansson *et al.*, 1994; Angulo and Swerdlow, 1995). Reactive inflammatory arthritis (Hannu *et al.*, 2005), conjunctivitis, urethritis known as Reiter's syndrome and anorexia also has been reported as complications due to severe shigellosis. Microangiopathic haemolytic anaemia can complicate infection with Shiga toxin-producing organisms, manifesting as the haemolytic uraemic syndrome (HUS) in children and as thrombocytopenic purpura in adults (Niyogi,

2005). Shigellosis was also reported to cause acute appendicitis in children (Tayman, 2008) and associated to chronic vulvovaginitis in children (Namdari *et al.*, 2002).

1.2.6 Management (treatment and prevention)

In order to treat shigellosis patients, rehydration treatment developed by the World Health Organization (WHO) has proven to be effective and is the treatment of choice for acute diarrhea. In acute dehydration, oral rehydration salt (ORS) is recommended while in severe dehydration, intravenous fluids usually Ringer's lactate solution is recommended (Sur *et al.*, 2004; Niyogi, 2005).

In the management of shigellosis, antibiotics treatment also plays an important role. Use of appropriate antibiotic hastens recovery from shigellosis, shorten the duration of excretion of *Shigella,* decrease spreading of the pathogen and possibly prevent complications. Treatment in malnourished children especially in developing countries will reduce the risk of worsening malnutrition morbidity due to shigellosis (Alam *et al.*, 2000; Ashraful *et al.*, 2005). A variety of antibacterial agents are effective for treatment of shigellosis. Commonly prescribed antibiotics in treating shigellosis are ampicillin, tetracycline, amoxicillin, cotrimoxazole, chloramphenicol and trimethoprim-sulfamethoxazole. Nevertheless, options are becoming limited due to globally emerging drug resistance (Vrints et al., 2009). *Shigella* resistance to sulfonamides, tetracyclines, ampicillin and trimethoprim-sulfamethoxazole has been reported worldwide (Lima *et al.,* 1995; Replogle *et al.*, 2000; Pickering, 2004; Temu *et al.*, 2007; Sire *et al.*, 2008). Studies in Asian regions such as Thailand, Vietnam (Isenbarger *et al.*, 2002), Singapore (Lim and Tay, 1991), Indonesia (Tjaniadi *et al.*, 2003) and Malaysia (Jegathesan, 1984; Thong *et al.*, 2002; Lee and Puthucheary,

2003) also have reported the emergence of multi-resistant strains of *Shigella*. The *Shigella* resistance locus (SRL), have been identified in *Shigella* strains which mediates resistance to antibiotics (Turner *et al.*, 2003). In Malaysia, out of 100 clinical strains of *Shigella* tested, 73% were resistant to at least one antibiotic (Thong *et al.*, 2002). Another study done had reported that out of 241 isolates of *Shigella* tested, 58% were resistant to at least one antibiotic and the rest were multi-resistant to three or more antibiotics (Lee and Puthucheary, 2003). Antimicrobial susceptibility and pulsed-field gel electrophoresis study done by Thong and colleagues on 62 *Shigella sonnei* strains isolated in Malaysia from year 1997 to 2000 revealed that multi-drug-resistant strains were circulated in different parts of Malaysia.

As in the case of other enteric infections, the spread of *Shigella* from an infected person to other persons can be stopped by increasing personal hygiene. Appropriate hand washing with soap, safe disposal of human waste as well as safe food processing and handling are the basis of personal hygiene that will contribute to minimize the transmission of *Shigella*. A study carried out in Bangladesh had reported that good personal hygiene practice greatly reduced the incidence of shigellosis (Khan, 1982).

1.3 Shigellosis diagnosis

1.3.1 Clinical diagnosis

The diarrhoeal stage of the infection cannot be distinguished clinically from the other bacterial, protozoan and viral infections. Therefore clinical diagnosis of shigellosis is not specific. Bloody, mucoid stools are highly indicative of shigellosis (WHO, 2009). The presence of fever suggests an infection by invasive pathogen. Patients presenting

with watery diarrhoea and fever should be suspected of having shigellosis, but the differential diagnosis should include infection caused by enteroinvasive *Escherichia coli* (EIEC), *Salmonella enteritidis*, *Yersinia enterolitica*, *Campylobacter* spp. and *Entamoeba hystilotica* (Ashraful *et al.*, 2005; Niyogi, 2005). Usually, fresh and bright red blood is present in the patients with shigellosis rather than dark brown in stools of patients with amoebiasis (Niyogi, 2005).

1.3.2 Laboratory diagnosis

1.3.2.1 Bacteriology

1.3.2.1.1 Stool examination

In order to diagnose the dysentery, macroscopic and microscopic examinations have to be done to the faeces of the suspected patients. Faecal specimens from patients with shigellosis may be watery, contain blood and mucus in the early stage of infection. In the later stages of infection, pus and blood mixed with mucus can be seen in almost entirely of specimens. In macroscopic examination, characteristics of faeces are examined which include consistency (formed stool, liquid stool or semisolid) and presence of atypical components (such as mucus and blood). As for microscopic examination, presence of hemoglobin and leukocytes are examined from fresh and unstained suspension of faeces (Cheesbrough, 1984). Invasion of the intestinal wall and the consequent inflammatory reaction resulted in the infiltration of the polymorphonuclear leukocyte and hemoglobin in the faecal secretion. It has been reported that faecal blood or leukocytes are detectable in the faeces in approximately 70% of shigellosis cases, whereas both faecal blood and leukocytes are detected in approximately 50% of the cases (Echeverria *et al.*, 1991).

1.3.2.1.2 Stool culture

Although clinical signs may evoke the suspicion of shigellosis, diagnosis is dependent upon the isolation and identification of *Shigella* from the stool specimen. The isolation usually done by conventional culture methods followed by identification by biochemical tests and serological agglutination assays (WHO, 1987). Culture is required for antibiotic susceptibility testing. Specimens are collected in the early stage of the disease, preferably before antibiotic treatment is begun. During that stage, the pathogens usually present in high number in the stool therefore chances of recovering the organisms are higher (Niyogi, 2005). *Shigella* spp. is fastidious and survive poorly in stool samples with mixed flora at room temperature (Taylor and Schelhart, 1975). Ideally, stool samples should be processed immediately, within 2 to 4 hours after collection. Specimens that cannot be processed within the optimum time should be stored at 4°C in appropriate transport media such as buffered glycerol saline or Cary-Blair medium. Appropriate specimens collection, rapid transport to the laboratory and rapid processing of the sample with ideal media used for isolation are important criteria for successful recovery of *Shigella* (Clemens *et al.*, 1999; Sur *et al.*, 2004). Those criteria are usually difficult to be attained resulting shigellosis undiagnosed (Dutta *et al.*, 2001).

1.3.2.1.2 (a) Inoculation on selective differential media

Isolation of *Shigella* typically involves an initial streaking on differential or selective media with aerobic incubation to inhibit the growth of the anaerobic normal flora. Primary isolation media that commonly being used are MacConkey (MAC), Xylose Lysine Desoxycholate (XLD), Desoxycholate Citrate (DCA), Hektoen Enteric (HEK) and Salmonlla-Shigella (SS) agar as mentioned in section 1.1.4.2. These

selective media contain bile salts that will inhibit the growth of other Gram-negative bacteria and have pH indicators to differentiate lactose fermenters organisms (coliforms) from non-lactose fermenters (*Shigella*). Inoculation of stool specimens into liquid enrichment broth such as Hajna Gram-negative broth or Selenite F broth may enhance the viability of the organisms before subculturing onto the selective media (Clemens *et al.*, 1999; Theron *et al.*, 2001). Inoculation on selective agar media is done by dropping a single drop of liquid faecal suspension or by streaking directly using rectal swab or faeces swab. The inoculated agar media should be incubated at 37°C for 18 to 24 hours. Characteristics of *Shigella* colonies on those selective agar media are as mentioned in section 1.1.4.2. Suspected colonies were chosen and further identified using biochemical and serological tests.

1.3.2.1.2 (b) Biochemical screening tests

Biochemical tests are used for further identification of *Shigella* spp. These tests are performed by inoculating the suspected colonies into different biochemical screening media. The media used are Kigler iron agar (KIA) or triple sugar iron agar (TSI), motility medium, MR (Methyl Red) -VP (Voges-Proskauer) test medium, oxidase test medium, citrate agar and urea agar (WHO, 1987). The *Shigella* spp. is usually positive for MR test and negative for citrate, oxidase, urease, motility and VP test. Growth of *Shigella* on TSI agar produces alkaline slant and acid butt due to ability to ferment lactose aerobically in the slope and the anaerobic fermentation of glucose in butt, with no production of gas and H_2S. However, a few strains of *S. flexneri* serotype 6 and very rare strains of *S. boydii* produce gas in KIA or TSI (Clemens *et al.*, 1999).

1.3.2.1.2 (c) Serological identification

Serological identification is performed to confirm the isolation and further species classification of the *Shigella* isolates (WHO, 1987). Each species of *Shigella* owns a distinctive type of O antigen. The identification is done by performing slide agglutination test using commercially available polyvalent O antigen grouping sera. In some cases, specific serotype identification can be done by using monovalent antisera for serotypes and sub-types identification. The test is carried out by emulsifying the colonies from the surface of KIA or TSI or other non selective agar media in a drop of physiological saline on a clean glass slide. Colonies from selective agar media such as MAC are not advisable because it may produce false-negative results. A small drop of polyvalent or monovalent antisera is mixed with the colonies suspension to observe for agglutination reaction. *Shigella* polyvalent antisera will agglutinate strains of the same serogroups and monovalent antisera will agglutinate the specific serotype or subtype. Cultures that react serologically (agglutinates) and show strong suggestive results in the biochemical screening tests are reported as positive for *Shigella* (Cheesbrough, 1984).

1.3.3 Other Diagnostic techniques

1.3.3.1 Immunological assay

Immunological assays have been used for the detection of specific identification of bacterial infections in most laboratories. The antibody based assays comprise the largest group of rapid methods being used in bacterial identification. Several basic formats of antibody-based assays are latex agglutination, immunodiffusion and enzyme immunoassays (EIA). EIA is the most common format since it can be used for testing large numbers samples and it is suitable for automation and quantification.

There are many different EIA methods developed for the diagnosis of shigellosis. A study done in Kuwait has developed a monoclonal antibody-based enzyme-linked Immunosorbent assay (ELISA) using a 43 kDa invasion plasmid-coded protein antigen (*IpaC*) to identify EIEC and *Shigella* strains in faecal samples from children (Pal *et al.*, 1997). Another ELISA method was developed using purified recombinant *IpaD* (Oaks *et al.*, 1996). ELISA technique has also been used for the detection of serum and urine antibodies of various immunoglobulin against *Shigella* serogroups specific LPS (Cohen *et al.*, 1991; Cohen *et al.*, 1996). A rapid latex agglutination procedure, the Wellcolex Colour *Shigella* tests, had been developed for *Shigella* grouping (Bouvet and Jeanjean, 1992). Colony blot immunoassays were also developed by researchers in detection of *Shigella* and EIEC from stool and water samples using *IpaC* specific monoclonal antibody (Szakal *et al.*, 2001; Szakal *et al.*, 2003). Another EIA format is dot-EIA test. In this format, specific antigens or antibodies are immobilized on a membrane usually nitrocellulose and the reaction is observed by enzyme activities. 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 test was developed using this protein.

There are many factors that prevent the usage of immunoassay tests in routine laboratory diagnostic application. Two major factors that determine the efficacy of immunoassay tests is the efficiency of formation of the antigen-antibody complex and the ability to detect these complexes. The specificity of an immunoassay test is dependent on the reaction between antibody and antigen. The sensitivity and specificity of the assay depend mainly on the type of antigens used. 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 detection of signal. Besides, the test needs well trained and experienced staff for interpretation of results. The test is relatively time consuming, labour-intensive and is temperature dependent. As for colony blot immunoassay, the sensitivity of this diagnostic approach is limited by the number of colonies chosen from individual specimens for the assay. Furthermore, mutations resulting in the loss of the virulence protein (such as *IpaC* and *IpaD*) expression could lead to a false negative immune assays (Szakal *et al.*, 2003).

1.3.3.2 DNA-based method

Technology advances in molecular biology has been introduced that will allow the genetic detection and identification of some microbes. Unlike traditional culture techniques, these methods are based on the detection of the genetic material of the targeted bacteria. In earlier studies, the DNA-based methods developed for the diagnosis of *Shigella* spp. were more on DNA-hybridization techniques (Venkatesan *et al.*, 1989; Echeverria *et al.*, 1991; Sansonetti, 1991).

Amplification technology brings new possibilities for rapid detection of specific pathogens in a sample, including viruses, slowly growing bacteria, fastidious or uncultivable bacteria, fungi and protozoa (Vaneechoutte and Van, 1997). Most researchers started to utilize PCR (Polymerase Chain Reaction), the most promising DNA-based technique for their studies in detection and identification of pathogenic microorganisms (Lukinmaa, 2004), evaluation of emerging novel infections, surveillance, early detection of bio-threat agents and profiling of antimicrobial resistance (Yang and Rothman, 2004). Important advantages of PCR are speed, a

good detection limit, selectivity, specificity, sensitivity and great potential for automation. It relies on the *in vitro* amplification of a targeted DNA fragment by a million fold in less than 2 hours. Moreover, the technology provides information on the current infection status and is dependent of the host's immune competency.

Several PCR protocols for detection of pathogenic microorganisms have been published (Bej *et al.*, 1990; Lampel *et al.*, 1990; Josephson *et al.*, 1993; Monteiro *et al.*, 2001). Numerous studies reported that PCR technique is relatively more sensitive and specific than the conventional culture technique and has the potential to be employed in routine diagnosis of dysentery in clinical centers as well as in epidemiologic studies (Islam *et al.*, 1998; Dutta *et al.*, 2001; Luo *et al.*, 2002; Aranda *et al.*, 2004; Thiem *et al.*, 2004). Several PCR protocols for the detection of *Shigella* spp. in faeces (Frankel *et al.*, 1989; Sethabutr *et al.*, 1993; Houng *et al.*, 1997), environmental waters samples (Theron *et al.*, 2001; M. Du Preez *et al.*, 2003) and food (Lampel *et al.*, 1990; Villabo and Torres, 1998) have been published. The application of DNA technology to diagnose *Shigella* spp. is based on the identification of DNA segments of a 120 to 140 MDa virulent plasmid that is necessary for attachment and invasion of epithelial cells. Most of the protocols developed were directed towards the specific DNA fragments of the invasion locus (*ial*) or the genes encoding for the *ipaH*. The invasion-associated plasmid antigen, *ipaH*, has been described as the genes that are found in multiple copies on both the invasion plasmid and on the chromosome of both *Shigella* spp. and EIEC (Hartman *et al.*, 1990). These protocols showed PCR cross reactions with (EIEC) since the primers used were designed to amplify the gene that shared by EIEC and *Shigella* spp. Differentiation cannot be done between *Shigella* spp.

A few studies reported the utilization of the immunomagnetic separation techniques to specifically isolate *Shigella* from faeces and subsequently identified by PCR (Islam and Lindberg, 1992; Luo *et al.*, 2002; Peng *et al.*, 2002). The PCR protocols targeted shiga toxin genes and conserved regions of the 16S rRNA in *Shigella* spp. Applications of ELISA method to detect PCR products from amplification of *ipaH* gene sequences in EIEC and *Shigella* in diarrhoeal stool samples also have been exploited. The technique developed allowed screening of larger number of specimens and avoided the use of mutagenic reagents in preparing agarose gel for electrophoresis (Sethabutr *et al.*, 2000). In Thailand, Gaudio and colleagues (1997) also utilized the ELISA-PCR for their study of shigellosis among dysentery patients and family contacts.

Another PCR technique with incorporation of digoxigenin-11-dUTP was also reported to detect Shiga toxin and shiga-like toxin type I (Sht/SLT-I)-producing *S. dysenteriae* and *E. coli* (Jackson, 1991). Labeled PCR products were hybridized to specific gene sequences which immobilized on a nitrocellulose membrane and detected by a few enzymes and substrates. Real-time PCR is a significant advancement in the era of PCR technology. It has the potential to provide a faster and more sensitive method for detection of microorganisms. In another study, real-time-fluorescence-based PCR assays was reported able to detect shiga toxin producer genes from *S. dysenteriae* and *Escherichia coli* (Belanger *et al.*, 2002). For the detection of *Shigella* spp. in environmental water, seminested PCR was developed which also target the *ipaH* gene shared *by Shigella* spp. and EIEC. The studies suggested enrichment step prior to PCR assay to minimize PCR inhibitors (Theron *et al.*, 2001; M. Du Preez *et al.*, 2003).

A multiplex PCR assay was then reported for the detection of *Shigella* spp. and diarrheagenic *E. coli* using pure cultures isolated from stool specimens (Aranda *et al.*, 2004). Later, a multiplex PCR assay was designed for simultaneous detection of chromosomal and plasmid-encoded virulence genes (*set1A, set1B* and *ipaH*) in *Shigella* spp. (Thong *et al.*, 2005). In an earlier study, a multiplex PCR assay based on the *rfc* and virulent plasmid genes capable of distinguishing the various *Shigella* serotypes and EIEC had been described. The *rfc* gene is the gene that encodes for O-antigen polymerase that polymerizes the O-antigen subunit into lipopolysaccharide (LPS) chains (Houng *et al.*, 1997).

Molecular study for the detection, identification and differentiation between enteroinvasive *Escherichia coli* and *Shigella* spp. was done in United States (US) utilizing PCR-Restriction Fragment Length Polymorphism (RFLP) method (Kingombe *et al.*, 2005). The detection, identification, and differentiation of *Shigella* spp. and EIEC were achieved by analyses of the PCR patterns and RFLP types. It combined a multiplex PCR for the amplification of two virulence genes, *iuc* (222 bp) and *ipaH* (629 bp), amplification of the *ial* gene (a 1,038-bp amplicon) located within a large plasmid and RFLP of the *ial* gene amplicons for the two organisms.

Recently, a few studies had reported the development of various formats of DNA and oligonucleotides microarray techniques. It is applied to screen multiple microbial organisms in diagnostic assays (Wu *et al.*, 2001; Cho and Tiedje, 2002). In 2006, a DNA microarray assay based on the target genes *wzx*, *wzy* and *wfa U* (genes encoding glycosyltransferase) of *Shigella* spp. and pathogenic *E. coli* serotypes was developed in China. The limitation of the test is its inability to distinguish *S. sonnei* and *S. flexneri* 2a from other strains that share the same O antigen structure and corresponding O antigen gene cluster (Li *et al.*, 2006). Later, the same format of

diagnostic test was developed targeting O serotype-specific genes in all 34 distinct O antigen forms of *Shigella*, including *Shigella boydii* types 1–18, *Shigella dysenteriae* types 1–13, *Shigella flexneri* types 1–5 and 6 and *Shigella sonnei*. The microarray developed is the first reported microarray assay for serotyping all O antigen forms of *Shigella* (Li *et al*., 2009). More systematic and meticulous assessment and development is needed in understanding the full potential of microarrays for diagnostic studies. Furthermore, such technology requires sophisticated instruments in preparing and producing results for example array plate can only be read by laser scanner. In addition, it needs well trained personnel in handling the equipment with special setup, thus limiting its broad application in common laboratories (Zhou, 2003).

1.4 Rationale of the study

Current laboratory diagnostic methods used in detection and identification of *Shigella* spp. relied on the relatively time consuming growth in culture media, followed by isolation, biochemical screening and serological identification. It takes approximately 2 to 5 days to produce results. Immediate identification of the pathogen in specimens is very critical to ensure proper clinical treatment, management of the patients, controlling the spreading of the disease and for epidemiological studies. Nevertheless, the sensitivity is relatively low because the suspected bacterial colonies might be missed during the isolation step due to the delay in sample transportation prior processing. Furthermore, this conventional method really needs labor intensive culture technique for isolation of *Shigella* spp.

As mentioned earlier, there are number of DNA-based tests and immunoassay tests that have been developed for the diagnosis of shigellosis. The immunoassay methods developed needs well trained and experienced staff for interpretation of results, relatively time consuming, labour-intensive, temperature dependent and solely depends on the reaction between antibody and antigen used. Meanwhile, the DNA microarrays were time consuming in several post-PCR steps for detection of the organisms such as the hybridization of the PCR products to the DNA arrays which requires ~ 16 hours and needs special laboratory setup with expensive equipments.

Detection methods based on nucleic acid such as PCR have shown tremendous potential and have been increasingly exploited. However, limiting issues in using PCR assays in diagnostic laboratory is that it is technically demanding; would need skilled personnel, requires many pipetting steps to add PCR reagents therefore prone to give erroneous results and need cold-chain for transportation and storage. Moreover, the presence of PCR inhibitors in the complex samples such as faeces, food and culture media may inhibit the amplification of the target genes thus limiting the usefulness of the PCR technique in diagnosis.

Previously reported PCR protocols targeted virulence plasmid and toxin genes in *Shigella* spp. Since mutations, instability and loss of the genes may occur; it is seemly not reliable to use the virulence or toxin genes for the detection of *Shigella* spp. especially from environmental samples. The plasmid is known to be unstable even after long storage in culture collections and can be easily lost after overnight culture under nonselective or competitive condition. Strains may lose plasmids that harbour the target genes therefore give false negative results (Lampel *et al.*, 1990). In