Mutation of *hemA* gene of *Shigella flexneri*: Towards development of an oral live attenuated *Shigella* vaccine

By

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CHAPTERS

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approx. approximately

atm atmosphere

bp basepairs

kbp kilobasepairs

ABSTRAK

Shigella merupakan faktor yang terpenting yang menyebabkan penyakit *gastroenteritis* dan juga kematian sebanyak 3-5 juta kanak-kanak di bawah umur 5 tahun di negara-negara sedang membangun (Ashkenazi, 2004; Raqib *et al.*, 2006; Sur *et al.*, 2004). Walaupun rawatan antibiotik umumnya berkesan dalam merawat *shigellosis*, namun S*higella* kini dikatakan mempunyai rintangan terhadap antibiotik, walaupun terhadap antibiotik terkini (Ashkenazi *et al.*, 2003). Oleh yang demikian, Pertubuhan Kesihatan Sedunia (World Health organization, WHO) telah memberi keutamaan dalam pembangunan vaksin yang selamat dan berkesan terhadap *Shigella* (Kotloff *et al.*, 1999). Shigellosis yang mempunyai kesan global yang penting tidak dapat dikawal dengan berkesan berdasarkan langkah-langkah pencegahan and rawatan yang sedia ada. Salah satu cara untuk mencegah *shigellosis* adalah mengganggu laluan metabolik *Shigella. Shigella flexneri* mempunyai satu gen metabolik yang dinamakan *hemA.* Gen ini mengekodkan sintesis sejenis enzim yang disebut *glutamyl-tRNA reductase*. Enzim ini bertindak sebagai pemangkin dalam pembentukan glutamate-1-semialdehid daripada glutamyl-tRNA^{Glu} dan NADPH dalam sintesis porfirin. Oleh yang demikian, dalam kajian ini, *hemA* akan dimutasikan melalui teknik 'insertional mutation'. Kesan yang berkemungkinan besar dilihat dalam mutasi gen *hemA* ialah menghalang atau merencatkan pertumbuhan *Shigella flexneri* dalam keadaan *in vivo* secara aerobik. Hasil daripada mutasi gen ini akan mengaruh imuniti yang berkesan terhadap shigellosis. Dalam kajian ini, gen *hemA* berjaya dimutasikan melalui teknik 'insertional mutation' dengan menggunakan kaset gen kanamycin (kanamycin gene cassette) dan kemudiannya sub-klon ke dalam 'conjugative

suicide vector', pWM91. Penggunaan kaset gen antibiotik bertujuan untuk memudahkan proses pencarian gen *hemA* memandangkan ia berfungsi sebagai penanda yang 'selective'. Pembagunan *hemA::aphA* dengan ini akan membantu dalam pembangunan vaksin yang berpotensi terhadap *Shigella flexneri.*

ABSTRACT

Shigella is one of the most important causes of gastroenteritis and death of 3-5 millions of children under the age of 5 years in developing countries (Ashkenazi, 2004; Raqib *et al.*, 2006; Sur *et al.*, 2004). Even though antibiotics are generally effective against shigellosis, but *Shigella* are increasingly developing antibiotic resistance, even to the newest antibiotics (Ashkenazi *et al.*, 2003). Therefore the World Health Organization has given priority to the development of a safe and effective vaccine against *Shigella.* (Kotloff *et al.*, 1999) Shigellosis, which continues to have an important global impact, cannot be adequately controlled with the existing prevention and treatment measures. One of the ways of prevention is to interrupt the metabolic pathway of *Shigella. Shigella flexneri* poses a metabolic gene, *hemA* which encodes for glutamyl-tRNA reductase enzyme. This enzyme catalyzes the formation of glutamate-1-semialdehyde from glutamyl-tRNA^{Glu} and NADPH in prophyrin biosynthesis. Therefore, in this study *hemA* gene will be mutated by insertional mutation technique. The most likely effect of the *hemA* mutation is to block or retard the growth of *Shigella flexneri* in an aerobic *in vivo* environment. Such mutants will induce protective immunity against shigellosis. In the present study, *hemA* gene has been successfully mutated with a kanamycin gene cassette (*aphA*) and the construct was successfully subcloned into a conjugative suicide vector, pWM91. The use of the antibiotic gene cassette is to facilitate the process of manipulating the genes as it serves as a selective marker. The construction of *hemA::aphA* thus will facilitate the development of a potential vaccine strain of *Shigella flexneri.*

Chapter 1 Introduction

1.1 Importance of *Shigella*

Shigella are Gram-negative, non-spore forming, facultative anaerobic bacilli closely related biochemically and antigenically to *E. coli* (Alfredo, 2004). They cause a disease called dysentery (bacillary dysentery or shigellosis), an infection of the large bowel characterized by abdominal cramps, diarrhea and fever. Initially, the diarrhea may be copious and the liquid stools often contain blood and mucus. *Shigella* are pathogenic primarily due to their ability to invade intestinal epithelial cells. One of the virulence factor of *Shigella* is its smooth lipopolysaccharide cell wall antigen which is responsible for the invasive features. Another virulence factor is the shiga toxin which is both cytotoxic and neurotoxic. This shiga toxin causes watery diarrhea (Sur *et al.*, 2004). Various surveys carried out in treatment centers show that *Shigella* is associated with 5-15% of cases of diarrhea and 30-50% of cases of dysentery.

1.2 Classification of *Shigella*

There are four different species of *Shigella*, divided on the basis of differences in the O antigen of their lipopolysaccharide and some biochemical reactions, such as indole production or mannitol fermentation. These different species of *Shigella* are named *S. dysenteriae* (13 serotypes), *S. flexneri* (15 serotypes), *S. boydii* (18 serotypes) and *S. sonnei*

(1 serotype). In general, *S. dysenteriae* accounts for lethal epidemics in developing countries, whereas *S. flexneri* and *S. sonnei* are responsible for endemic disease. The former being prevalent in the developing world whiles the latter being prevalent in developed countries. *S. boydii* accounts for most cases of infection in India and neighboring countries. Among these four species, the most extensively studied is *S. flexneri* (Alfredo, 2004). *S. flexneri* serotypes (serotype 2a is the most common) predominate as agents of endemic shigellosis.

1.3 Transmission of *Shigella*

Shigella are transmitted from an infected person to another who become infected. *Shigella* are present in the diarrheal stools of infected persons while they are sick for a week or two afterwards. Most of the infections occur as the result of the passing of bacterium from stools or soiled fingers of one person to the mouth of another person. This happens when basic hygiene and handwashing habits are inadequate. It is prone to occur among toddlers who are not fully toilet-trained. Family members and playmates of this children are at high risk of becoming infected.

Part of the reason for the efficiency of transmission is because a very small infectious dose (approximately 100 organisms) is sufficient to cause infection (Rahim *et al.*, 2007). As a result, spread can easily occur by the fecal-oral route and occurs in areas where hygiene is poor. Epidemics of *Shigella* may be food borne or waterborne. The infections can be acquired from eating food that has been contaminated by infected food handlers. Vegetables can be contaminated if they are harvested from a field with

contaminated sewage. *Shigella* infections can also be acquired by drinking or swimming in contaminated water. Water becomes contaminated when sewage runs into it, or even if someone with shigellosis swims or bathes or, much less, defecates, in it (Kenneth, 2005). *Shigella* can also be transmitted by flies. Flies can breed in infected feces and then contaminate the food.

1.4 Epidemiology of Shigellosis

Shigellosis is endemic in most developing countries and it is the most important cause of bloody diarrhea in worldwide. It is estimated to cause at least 80 million cases of bloody diarrhea and 700,000 deaths each year (WHO, 2005). 99% of infections caused by *Shigella* occur in developing countries, and the majority of cases (~70%), and of deaths (~60%), occur among children less than five years of age. In developing countries, the major burden of *Shigella* infection is among children of 1 to 4 years old. Probably less than 1% of cases are treated in hospital (WHO, 2005). *S. flexneri* serotypes were highly heterogeneous in their distribution from site to site, and even from year to year (Seidlein *et al.*, 2006). Worldwidely, approximately 165 million cases of shigellosis occur and 1,100,000 deaths are caused by the disease per year (Rahbar *et al.*, 2007). The median percentages of isolates of *S. flexneri*, *S. sonnei*, *S. boydii* and *S. dysenteriae* were 60%, 15%, 6%, and 6% (30% of *S. dysenteriae* cases were type 1) respectively in developing countries and 16%, 77%, 2%, and 1% in industrialized countries. In developing countries, the predominant serotype of *S. flexneri* is 2a, followed by 1b, 3a, 4a, and 6 (WHO, 1999). In industrialized countries, most isolates are *S. flexneri* 2a or other unspecified type 2 strains

(WHO, 1999). In Malaysia the prevalence of shigellosis has been increased from 8.5% in 1992 to 13% in 2003 (Lee *et al.*, 2003).

1.5 Pathogenesis of Shigellosis

The intestinal manifestations of *Shigella* infection range from uncomplicated watery diarrhea to dysentery with frequent passage of small-volume stools containing gross blood and mucus. Dysentery is also accompanied by abdominal cramps and rectal tenesmus. Shigellosis usually includes constitutional symptoms such as fever and headache (Katz *et al.*, 2004). It causes disease by invading the epithelium of the colon, resulting in an intense acute inflammatory response (Nhieu *et al.*, 1999). The prominent pathogenic feature of *Shigella* is its ability to invade a variety of host cells, including macrophages, dendritic cells and epithelial cells, which leads to a severe inflammatory response in intestinal tissue. When *Shigella* reaches the colon and rectum, they translocate through the epithelial barrier via M cells that overlie the solitary lymphoid nodules (Wassef *et al.*, 1989; Sansonetti *et al.*, 1996). Once they reach the sites underlying M cells, *Shigella* encounter resident macrophages, and the phagocytosed bacteria escape from the phagosome into the cytoplasm of macrophages. *Shigella* multiplies in the cytoplasm and induces cell death through activation of caspase-1. The activation of caspase-1 is mediated by *IpaB* protein. This in turn leads to the maturation and release of IL-1ß (Weinrauch *et al.*, 1999; Navarre *et al.*, 2000). However, recent work has shown that macrophages undergo pro-inflammatory necrotic cell death and that the necrosis and caspase-1 activation are due to components common to Gram-negative bacteria (Suzuki *et al.*, 2005). Another study has reported that *Ipaf*, a member of the pathogen-associated molecular pattern-sensing Nod family proteins,

has a role in caspase-1 activation in *Shigella*-infected macrophages (Mariathasan *et al.*, 2005). *Shigella* released from killed macrophages enters the surrounding enterocytes through their basolateral surface by inducing membrane ruffling and macropinocytosis (Nhieu *et al.*, 2000). The invasion eventually results in extensive inflammation and tissue destruction of the villous epithelium (Perdomo *et al.*, 1994).

1.6 Treatment and prevention

1.6.1 Treatment

Shigellosis can usually be treated with antibiotics. The antibiotics commonly used for treatment are trimethoprim/sulfamethoxazole and ciprofloxacin (Joyann, 2007). Trimethoprim/sulfamethoxazole inhibits the bacterial growth by inhibiting synthesis of dihydrofolic acid. Another antibiotic recommended for shigellosis is ciprofloxacin. Ciprofloxacin is a fluoroquinolone that inhibits the bacterial DNA synthesis and consequently, the growth of the bacteria (Joyann, 2007). Ciprofloxacin is not recommended for use of individual younger than 18 years of age except in exceptional circumstances. The oral route is preferred except for seriously ill patients. ampicillin (but not amoxicillin) is still the drug of choice if the isolate is susceptible to this drug (Jaya, 2006). Amoxicillin is less effective than ampicillin because of its rapid absorption in the gastrointestinal tract. If ampicillin and trimethoprim/sulfamethoxazole resistant strain is isolated or if susceptibility is unknown, parenteral ceftriaxone sodium, a fluoroquinolone (eg, ciprofloxacin, ofloxacin), or azithromycin dihydrate will be the drugs of choice (Jaya, 2006).

Appropriate treatment kills the *Shigella* bacteria that might be present in the patient's stools and shortens the illness. Unfortunately, some *Shigella* bacteria have become resistant to antibiotics and using antibiotics to treat shigellosis can actually make the germs more resistant in the future. Persons with mild infections will usually recover quickly without antibiotic treatment. Therefore, when many persons in a community are affected by shigellosis, antibiotics are sometimes used selectively to treat only the more severe cases.

1.6.2 Prevention

Until now, there is no WHO-recommended vaccine that is effective for preventing infection by *Shigella*. Several candidate vaccines, mostly against *S. flexneri,* are currently under development (WHO, 2005). However, the spread of *Shigella* from an infected person to other person can be stopped by frequent and careful handwashing with soap. Frequent and careful handwashing is important among all age groups. Supervised handwashing of all children should be practiced in day care centers and at homes with children especially those who are not completely toilet-trained (including children in diapers). When possible, young children with a *Shigella* infection who are still in diapers should not be in contact with uninfected children. Preparation of food for others by people who have shigellosis should be avoided until they have been shown to no longer be carrying the *Shigella* bacterium. If a child in diapers has shigellosis, everyone who changes the child's diapers should be sure that the diapers are disposed of properly in a closed-lid garbage can, and should wash his or her hands carefully with soap and warm water immediately after changing the diapers. After use, the diaper changing area should be wiped down with a disinfectant such as household bleach, Lysol® or bactericidal wipes. Shigellosis can also be prevented by basic

food safety precautions and treatment of regular drinking water. Safe water supplies and adequate sanitation combined with improved hygiene are likely to reduce the shigellosis burden in the future.

1.7 Antibiotic resistance of *Shigella spp*

All *Shigella* species have shown increasing resistance to multiple antimicrobial drugs formerly useful for treatment (Health and Science Bulletin, 2004). The predominant *Shigella* strains isolated in Karaj, Iran was *S. flexneri* and the least common antimicrobial resistance belonged to Nalidixic acid (Zali *et al*). In Kashan, Iran the most sensitive antibacterial agent for *Shigella* was ciprofloxacin (Afzali *et al.*, 2001). A *Shigella* outbreak investigation in India (Siliguri, Diamond Harbour, Kolkata, and Aizwal) and Bangladesh has shown high level of antibiotic resistancy even to norfloxacin, ciprofloxacin and ofloxacin (Sarkar *et al.*, 2003; Sur *et al.*, 2003; Datta *et al.*, 2003; Bhattacharya *et al.*, 2003). Only ceftrioxone and azithromycin are now clinically effective (Khan *et al.*, 1997; Niyogi *et al.*, 2001) for the treatment of multi drug resistant shigellosis. The proportion of resistant *S. flexneri* also increased. For instance, resistance to nalidixic acid increased from 4% in 1986 to 66% in 2003. Nevertheless *S. flexneri* strains have remained susceptible to mecillinam and ciprofloxacin (Health and Science Bulletin, 2004).

1.8 Candidate Vaccine for *Shigella spp*

The advent of genetic engineering technology has provided opportunities in *Shigella* vaccine development. In recent years, a number of attenuated *Shigella* strains have

been constructed and evaluated as candidates for use in vaccines (Levine at al., 1997; Coster *et al.*, 1999; Jennison *et al.*, 2004). Live attenuated vaccine strains of the *S. flexneri* and *S. dysenteriae* have also been developed (Seidlein *et al.*, 2006). The primary role of a *Shigella* vaccine is to protect against clinical disease. An additional benefit would be to interfere with infection and colonization of *Shigella.* The most important *Shigella* strains to be targeted for vaccine development are *S. flexneri* 2a, *S. dysenteriae 1 and S. sonnei.* However, the possible emergence of new serotypes has been emphasized. Mutations in chromosome or virulence plasmid have been introduced to generate noninvasive live vaccine strains, but a high bacterial dose and long period of vaccination are needed to achieve protective immunity (Jennison *et al.*, 2004).

1.8.1 Live attenuated vaccine

Two prototype attenuated vaccine strains of *S. flexneri 2a* and *S. dysenteriae 1* have been developed at the Pasteur Institute, Paris. These two vaccines are live attenuated *S. flexneri* 2a strain (SC602) and an *S. dysenteriae* type 1 strain (SC599) which carry the mutations in their *icsA*, *iuc*, *int* and *toxA* genes (Girarda *et al.*, 2006).

SC602, a candidate vaccine strain with a deletion of the *iscA* (or *virG*) plasmid virulence gene (Coster *et al.*, 1999) has been tested in adult volunteers in the USA and in adults and children in Bangladesh. It has been demonstrated to be safe, highly immunogenic in stimulating secretory IgA (sIgA) antibodies and protective in the guinea-pig challenge model (Sereny test). Phase 1 trials of *S. flexneri* 2a SC602, has been carried out. SC602 was the first example of a live oral *Shigella* vaccine tested in human trials

where deletion of a specific virulence gene that is critical for pathogenesis has been used as the major attenuating feature. *Shigella* with this attenuating mutation is fully invasive for tissue culture monolayers but is unable to spread to contiguous host cells (Bernardini *et al.*, 1989). SC602 has proceeded to phase 2 trials in humans (Coster *et al.*, 1999). *S. flexneri icsA* mutant causes only asymptomatic nodular abscesses after intragastric challenge of rhesus monkeys (Sansonetti *et al.*, 1989). Such mutants also fail to elicit keratoconjunctivitis in the guinea pig Sereny test (Katz *et al.*, 2004).

S. dysenteriae type 1 vaccine strain SC599 was constructed at the Pasteur Institute in Paris (France) by creating deletions in several genes including *icsA, ent, fep* and *stxAnegative: HgR.* A Phase I clinical trial of a single escalating dose for safety and immunogenicity of vaccine strain SC599 was performed in 28 healthy adult volunteers at St. George's Vaccine Institute in London (United Kingdom). The vaccine was well tolerated, with no significant side effects reported. In general, SC599 was highly attenuated and was well tolerated at doses up to 10^8 CFU (WHO Weekly Epidemiological Report, 2006).

The future development of SC602 and SC599 was presented. A Phase IIa study is planned for SC599 to compare the immunogenicity of 2 doses (10^5 and 10^7 CFU) versus a placebo in healthy volunteers. An additional assessment of the clinical tolerance and evaluation of duration of shedding will be performed. Future studies of SC602 may include phase I and dose-ranging clinical trials in adults, and then in adolescents in endemic countries to determine the safety and reactogenicty of the higher doses. A multivalent vaccine trial has been planned to occur as early as 2009 (WHO Weekly Epidemiological Report, 2006).

Live attenuated *Shigella* vaccine candidates which targeting *S. flexneri* types 2a, 3a and 6, *S. sonnei*, and *S. dysenteriae* type 1, were also in development at Center of Vaccine Development. A series of strains were made auxotrophic for aromatic amino acids synthesis *(amA)* and guanine synthesis *(guaBA)* with progressive deletions of virulence genes *virG*, *set* (SHET1) and *sen* (SHET2) (CVD1203, CVD 1204) were engineered, culminating in strain CVD1208S, which has entered Phase I clinical trials (Kotloff *et al.*, 2007).

An *S. sonnei* vaccine candidate (WRSS1) has also been developed with a single deletion of the *VirG* gene and tested in a Phase I study conducted at Center of Vaccine Development. This vaccine was found to be markedly immunogenic and elicited a significant immune response in the volunteers (Kotloff *et al.*, 2002), generating strong serum anti-LPS and anti-LPS IgA antibody-secreting cell responses (James P Nataro, 2004). These dose selection trials of a second *icsA* (*virG*) candidate (*S. sonnei* WRSS1) have yielded promising safety and immunogenicity results in phase 1 trials (Kotloff *et al.*, 2002).

A recent clinical trial indicated that the *set* and *sen* enterotoxin genes contribute to the symptoms of fever and diarrhea observed with live *Shigella* vaccine strains. Based on these findings, a *S. flexneri* 2a vaccine candidate, WRSf2G11, with deletions in the *virG* (*icsA*), *set* and *sen* genes has been constructed using the lambda *red* recombinase system. The immunogenicity and protective efficacy of WRSf2G11 compares favorably with SC602 following either intranasal (IN) or ocular (OC) immunization of guinea pigs. The data collected has indicated that second generation *virG*-based *Shigella* vaccine strains

which lack enterotoxin genes, such as WRSf2G11, is expected to show lower levels of reactogenicity without hampering the strong immune responses achieved with previous live vaccines (Ryan *et al.*, 2007)

Another candidate vaccine for *Shigella* has also been developed based on the auxotrophic mutant of *S. flexneri* Y strain. The *S. flexneri* Y strain was mutated by Lindberg and colleagues at the *aroA* locus (James P. Nataro, 2004). The resultant vaccine candidate, SFL124, was generally well-tolerated and immunogenic in volunteers. This vaccine elicited *Shigella*-specific, anti-LPS responses in serum, stool and antibody-secreting cells in peripheral blood (Li *et al.*, 1992). It has been proved to be safe and immunogenic (Li *et al.*, 1993). Another *S. flexneri* Y strain vaccine with *aroD* mutation has also been constructed (James P. Nataro, 2004). This vaccine strain was named SFL1070. It produced adverse reactions in volunteers in a dose-dependent fashion (Karnell *et al.*, 1995) eventhough it was at least 100 000-fold attenuated compared with its wild-type parent strain 2457T (Karnell *et al.*, 1995).

1.8.2 Subunit vaccines

1.8.2.1 Conjugate vaccines

Detoxified O-specific polysaccharide protein conjugate vaccines have been developed as *S. flexneri* 2a-rEPA and *S. sonnei*-rEPA. These vaccines have been tested in clinical trials In Israel and found to be safe and highly immunogenic after a single dose in young adults (Passwell *et al.*, 2001). Antibody levels persisted at high levels up to 2 years

and at strong (40–50%) levels up to 4 and 5 years post vaccination. Serum IgG levels were higher following vaccination than following natural infection. LPS-specific IgA ASC responses were elicited at high levels. The *S. sonnei* conjugate demonstrated a good level of protective efficacy (WHO Weekly epidemiological record, 2006).

1.8.2.2 Proteosome vaccines

The term "proteosome" refers to purified preparations of meningococcal outer membrane proteins (OMPs) that form multimolecular vesicular structures with antigens noncovalently complexed to them, generally (but not exclusively) via hydrophobic interactions (Lowell *et al.*,1997). It is used as mucosal vaccine to induce systemic and mucosal immune response. Proteosome-based LPS vaccines for *Shigella* have been tolerated well by several animal species and have shown protective activity in the Séreny test and in a murine lethal pneumonia model when delivered via mucosal routes (Orr *et al.*, 1993; Mallett *et al.*, 1995; Lowell *et al.*, 1997). After nasal or oral immunization in mice or guinea pigs with *S. flexneri* or *S. sonnei* proteosomes, high levels of antibodies against LPS has been induced in intestinal and lung secretions as well as in serum. Monkey studies showed that intratracheal administration was more efficient than nasal spray, and nasal spray is more efficient than oral delivery. Good manufacturing practice (GMP) production of *S. sonnei* and *S. flexneri* vaccines has yielded candidate vaccines for human clinical trials. The initial phase I trial of the proteosome *S. sonnei* vaccine, a dose-escalating study, has shown a dose-dependent immune response after two intranasal spray administrations, whereas oral immunization gave only minimal antibody-secreting cell responses. A phase I

trial of *S. flexneri* 2a proteosome vaccine has been planned, as well as expanded nasal or nasal-oral combination prime and boost studies.

1.8.2.3 *Shigella* nucleoprotein (ribosomal) vaccine

Shigella ribosome-based vaccines (SRV), considered among the potent vaccine candidates, are composed of O-antigen and ribosome isolated from *S. flexneri* 2a (Shim *et al.*, 2007). This approach has been studied in the United States. Parenteral vaccination with the non-covalent complexes of O-polysaccharide and ribosomal particles from *Shigella* induces an intense systemic O-antibody response in experimental animals. It also elicited a significant response of the secretory immune system with IgA antibodies appeared in tears, milk (guinea pigs), bile (rats) and saliva (monkeys). One subcutaneous injection of the vaccine protects 70% to 90% of animals from the challenge with homologous *Shigella* in the guinea-pig model. Lyophilized *Shigella* ribosomal vaccine has been demonstrated to be very stable and can be produced at low cost. A GMP protocol for vaccine production on a large scale is currently under development.

1.9 Disease burden and need of a vaccine

The development and availability of *Shigella* vaccines should be a major public-health priority. Industrialized and developing countries have different dominant serotypes. Therefore different vaccines (or components) may need to be developed to cover different strains in different geographic areas.

1.12 Importance of *hemA* gene

HemA is the gene which encodes for glutamyl-tRNA reductase. This enzyme catalyzes the formation of glutamate-1-semialdehyde from glutamyl-$tRNA^{Glu}$ and NADPH in prophyrin biosynthesis. The enzyme glutamyl-tRNA reductase (*HemA*) catalyzes the first committed step of the heme biosynthetic pathway, the reduction of charged glutamyl-$tRNA^{Glu}$ to form glutamate-1-semialdehyde, an unstable intermediate which is then converted to 5-aminolevulinic acid (ALA) by the product of the *hemL* gene (Jahn D. *et al.*, 1992). Therefore this gene is expected to be a target of regulation. Mutation of the *hemA* gene encoding glutamyl-tRNA reductase resulted in the absence of detectable amounts of heme.

1.13 Justification of study

Existing antimicrobial treatments are becoming increasingly compromised because of the growing occurrence of antibiotic resistance among *Shigella* spp. Study has shown that ampicillin and cotrimoxazole no longer have a place in the treatment of shigellosis. Nalidixic acid was recommended by the WHO as the first-line treatment against shigellosis until year 2004, when it was replaced by ciprofloxacin (WHO, 2005). Resistance to nalidixic acid is now common in South Asia, and frequent in Eastern and Southern Africa. In addition, strains of *Shigella* resistant to nalidixic acid show some degree of cross-resistance to ciprofloxaci (WHO, 2005). Complete resistance of *Shigella spp* to nalidixic acid in China and high levels of resistancy in Bangladesh have clearly reduced the usefulness of this drug, at least in these two countries (Lorenz *et al.*, 2006). In addition, the

cost of treating shigellosis with antibiotics, particularly in the developing world, is impractical and stresses the need for an efficient vaccine against this disease. Currently, however, there is no vaccine available that can provide adequate protection against the many different serotypes of *Shigella*. Therefore, both the development of new treatments and the design of innovative vaccines for the prevention of shigellosis rely on an improved understanding of the pathogenesis of the disease (Dana *et al.*, 2000).

In addition, microbiological surveys in areas where diarrheal disease is endemic implicate *Shigella* species as etiologic agents in at least 20% of diarrheal cases. *Shigella flexneri* 2a is usually the most prevalent species and serotype (Rosemans, 1988; Ferreccio *et al.*, 1991; Henry, 1991). Therefore by doing this study of *hemA* mutation, perhaps it might facilitate the development of potential oral, live attenuated *S.flexneri* 2a vaccine strain.

1.14 Objectives

1. To clone *hemA* gene of *Shigella flexneri* into a cloning vector, pTZ57R.
2. To mutate the *hemA* gene by insertional mutation using kanamycin gene cassette.
3. To subclone the mutated *hemA* gene into a conjugative suicidal vector, pWM91.

1.15 Experiment Overview

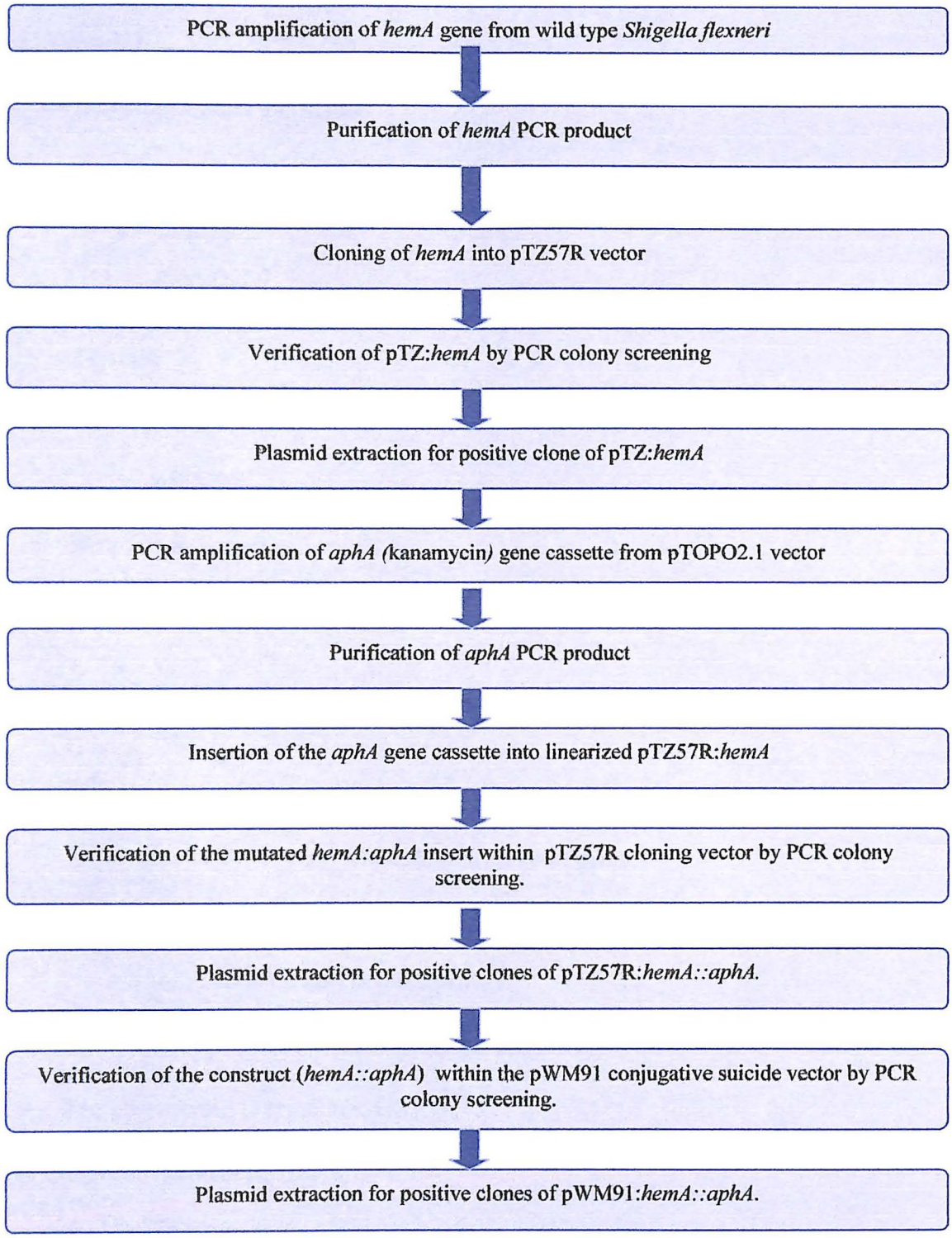

Figure 1.1 Experiment overview

Chapter 2 Material and Methods

2.1 Material:

- *Shigella flexneri* 2a, SH060
- *E.coli* host
  - TOP10
  - BW20767
- Plasmid
  - pTZ57R
  - pWM91
- Restriction Enzymes
  - *NdeI*
  - *SacI*
  - *SmaI*
- Primers
  - *HemA* F_1,forward and *hemA* R_1, reverse primer
  - kanamycin forward, KanFse2F and kanamycin reverse,KanFseR primer
  - *Sac B* forward and reverse primer
- PCR Thermal Cycle, PT200 (MJ Research, USA)
- Taq polymerase (Fermentas, USA)
- dNTPs (Fermentas, USA)
- Agarose Gel Electrophoresis
- Plasmid Extraction Kit (Fermentas, USA)

- InsTAclone™ PCR Cloning Kit (Fermentas, USA)
- UV Transilluminator, Chemilmager™ 5500 (Alpha Innotech)
- LB
    - Agar
    - Broth
- Antibiotic
    - ampicillin
    - kanamycin

2.2 Method

2.2.1 Media Preparation

2.2.1.1 LB agar

Content	Amount
Peptone	7.5 g
Yeast extract	2.5 g
Sodium Chloride	5.0 g
Deionized Distilled Water	500 ml
Agar powder	7.5 g

Table 2.1 Materials for LB Agar

To prepare 500 ml of LB agar, 7.5 g of tryptone (Merck), 2.5 g of yeast extract (Oxoid), 5.0 g of sodium chloride (Amresco), and 7.5 g of agar (Amresco) were first dissolved in 500 ml of deionized distilled water and stirred well in a beaker until it is completely dissolved. After that, the pH was adjusted to 7.2 by either adding drops of 1M sodium hydroxide or 1M hydrochloric acid. The agar powder was then poured into a bottle. The mixture with pH 7.2 was then poured into the bottle containing agar powder. The mixture was mixed well until the agar powder was completely dissolved. It was then sent for autoclaving (121°C, 15 minutes, 1 atm). After autoclaving, the media agar was poured into clean, sterile Petri plates with approximately 25 ml in volume each. The plates were left in room temperature for solidifying and can be kept in cold room for future use.

2.2.1.2 LB ampicillin and LB ampicillin/kanamycin agar

Preparation of LB ampicillin and LB ampicillin/kanamycin agar plate utilizes the same protocol as in the preparation of LB agar plate. The only difference is that after autoclaving, the media agar was let to cool down to approximately 40°C (before solidifying) before the addition of any antibiotic to prevent denaturation of the antibiotic. For 500 ml of agar, 500 µl of ampicillin and kanamycin is required. ampicillin and kanamycin were added into LB agar as a selective marker for screening of positive clones carried by pTZ57R and pWM91 vector. These vectors carry ampicillin resistant gene.

Antibiotics	Final concentration
ampicillin	100 μg / ml
kanamycin	50 μg / ml

Table 2.2 Concentration of antibiotics used in LB agar and LB broth

2.2.2 Polymerase Chain Reaction (PCR)

2.2.2.1 Preparation of bacterial lysate for PCR

Isolated single bacterial colony was picked up from LB agar plate using wire loop and the colony was diluted with 20 μl of deionized distilled water in a microcentrifuge tube. The tube was then put into boiling water for 5 minutes. After 5 minutes, the microcentrifuge tube was centrifuged at 12,000 rpm for 45 second. The clear supernatant was collected and used as DNA template.

2.2.2.2 Principle of PCR

In the PCR procedure trace amounts of DNA can be quickly and repeatedly copied to produce a quantity sufficient to investigate using conventional laboratory methods. The polymerase chain reaction serves to copy DNA. It uses repeated cycles, each of which consists of three steps: first, the reaction solution containing DNA molecules, polymerases, primers and nucleotides was heated to 95°C. This causes the two complementary strands to

separate. This process is known as denaturation. The second step is the annealing step. The lowering of the temperature to 56°C causes the primers to bind to the DNA template. The resulting bonds are stable only if the primer and DNA segment are complementary, i.e. if the base pairs of the primer and DNA segment match. The polymerases then begin to attach additional complementary nucleotides at these sites, thus strengthening the bonding between the primers and the DNA. The third step is the extension step. In this step the temperature is again increased, this time to 72°C. This is the ideal working temperature for the polymerases used. Further nucleotides will be added to the developing DNA strand. Each time these three steps are repeated the number of copied DNA molecules will be doubled. After 20 cycles about a million molecules are cloned from a single segment of double stranded DNA.

2.2.2.3 Sequence of primers used in this study

- ***hemA* forward primer, *hemA* F_1:**

5' AGA ATC TAA CGG CTT TCG GC 3'

- ***hemA* reverse primer, *hemA* F_2:**

5' TCT TCA TGG CGT TCA TGC AG 3'

- **kanamycin (*aphA*) forward primer, KanFse2F:**

5' AGC GGC CGG CCG CTT ACA TGG CGA TAG CTA G 3'

- **kanamycin (*aphA*) reverse primer, KanFseR:**

5'ATA GGC CGG CCT CAG AAG AAC TCG TCA AGA A 3'

- ***Sac B* gene forward primer:**

5' ATG AAC ATC AAA AAG TTT GCA AAA 3'

- ***Sac B* gene reverse primer:**

5' TTA TTT GTT AAC TGT TAA TTG TCC 3'

2.2.2.4 Composition of PCR reaction:

Content	Amount (for 1 reaction) (µl)
dH_2O	11.53
10x Taq buffer	2.0
$MgCl_2$ (25 mM)	2.0
dNTPs (10 mM)	0.32
Forward Primer (20 pmol/µl)	1.0
Reverse Primer (20 pmol/µl)	1.0
Taq polymerase (1 unit/ µl)	0.15
DNA template	2.0
Total	**20.0**

Table 2.3 Reagents for PCR mixture

2.2.2.5 PCR amplification

	Temperature	Duration
Initial Denaturation	95°C	3 minutes
Denaturation	95°C	1 minute
Annealing	56 ~ 64°C (depending on Ta of the primers)	30 seconds
Extension	72°C	40 seconds
Loop 29 cycles (Total = 30 cycles)		
Final extension	72°C	5 minutes

Table 2.4 PCR program used in PCR

2.2.3 Agarose gel electrophoresis

2.2.3.1 Principle

Gels allow separation and identification of nucleic acids based on charge migration. Migration of nucleic acid molecules in an electric field is determined by the size and conformation, allowing nucleic acids of different sizes to be separated. However, the relationship between the fragment size and rate of migration is non-linear, since larger fragments have greater frictional drag and are less efficient at migrating through the