MUTATION ANALYSIS OF APYRASE GENE OF *Shigella flexneri* - TOWARDS THE DEVELOPMENT OF VACCINE STRAIN

by

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CERTIFICATE

This is to certify that the dissertation entitled

"Mutation analysis of *apyrase* gene of *Shigella flexneri* - towards the development of vaccine strain."

is the bona fide record of research work done by

Mr. Seah Chin Aun

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ABSTRACT

The discovery of the microbe *Shigella* was dated back about 100 years ago by a scientist named Kiyoshi Shiga. Virulent *Shigella* spp. are known to cause bacillary dysentery (shigellosis) in humans which is characterized by the passage of bloody stools. According to the WHO, an estimated of 1.1 million global population dies every year is due to the disease shigellosis. Despite much studies were carried out regarding this pathogenic bacteria, no vaccine is available for this infectious disease. Literatures have postulated that one of the virulence genes is the *apy* gene which encodes for apyrase. Therefore, in this study the gene of interest is the *apy* gene. We have successfully mutated the *apy* gene with a kanamycin gene cassette (*aphA*) and subcloned the construct into a conjugative suicide vector. The use of this antibiotic gene cassette is to facilitate the process of manipulating the genes as it serves as a selective marker. The creation of the construct (*apy::aphA*) is to facilitated the approach into the development of a potential vaccine strain of *Shigella;* as literatures have indicated that *apy* gene is one of the virulence genes. Furthermore, mutation of the virulence genes has been proven to be highly immunogenic and safe.

ABSTRAK

Microorganisma *Shigella* telah ditemui sejak lebih kurang 100 tahun yang lalu oleh seorang saintis bernama Kiyoshi Shiga. Bakteria *Shigella* telah dikenalpasti dimana ia mengakibatkan diarhea berdarah (bacillary dysentery). Penyakit yang dijangkiti oleh microorganisma ini dikenali sebagai "shigellosis". Menurut WHO (World Health Organization), lebih kurang 1.1 juta nyawa terkorban setiap tahun akibat penyakit tersebut disekitar dunia. Walaupun banyak penyelidikan mengenai bacteria patogenik ini, sehingga kini masih tiada vaksin untuk penyakit berjangkit ini. Ahli saintis telah mengenapasti yang salah satu gen virulen adalah gen *apy* yang mengekodkan enzim apyrase. Dengan demikian, tujuan projek ini adalah tertumpu kepada gen *apy* ini. Kami telah melakukan mutasi (insertional mutation) kepada gen *apy* ini dengan kaset gen kanamycin (*aphA*). Tujuan perlaksanaan mutasi dengan kaset tersebut adalah untuk menyenangkan proses pengenalpastian klon-klon yang mengalami mutasi daripada yang lain. Konstrak yang telah direkakan ini dapat menyumbang kepada penghasilan satu strain vaksin yang berpotensi untuk menyumbangkan imuniti terhadap bakteria *Shigella* ini. Tambahan pula, banyak penyelidikan telah menunjukkan kerberkesanan mutasi gen virulen dengan penghasilan vaksin yang imunogenik serta selamat untuk manusia.

1.0. INTRODUCTION

1.1. *Shigella* & Shigellosis

The microbe *Shigella* was discovered about 100 years ago by a scientist named Kiyoshi Shiga. *Shigella* is a small, gram-negative, encapsulated, non-motile, non-lactose fermenting bacterium that is bile salts resistant. In addition, unlike other bacterial pathogens *Shigella* is less susceptible to acid. *Shigella* is very infectious in which its infectious dose is 10-200 organisms by ingestion; partly this is due to the ability of the organism to survive the transit through the stomach (DuPont HL *et al.*, 1989). In addition, researchers have found that many strains of *Shigella* produce a toxin called "shiga toxin" which is very potent and destructive. Shiga toxin is very similar to the verotoxin of *E. coli* O157:H7. *Shigella flexneri* is a human intestinal pathogen that invades the epithelium of the colon; the invasion causes symptoms like fever which is similar to other invasive bacterial pathogens, enteric viruses, and cytotoxic organisms (DuPont HL, 1997). The disease cause by this virulent bacterium is called shigellosis, which is also known as classical bacillary dysentery.

Shigellosis is characterised by the frequent and painful passage of stools that consist largely of blood, mucus and pus, accompanied by fever and stomach cramps. At present, shigellosis can be treated with antibiotics; ampicillin, trimethoprim, or ciprofloxacin. At present, there is no vaccine available to prevent shigellosis. However, active research and studies into the development of *Shigella* vaccine are currently in the progress.

1.2. Epidemiology, pathogenesis and *Shigella* outbreak

Shigella is a highly contagious bacterium that causes bacillary dysentery. This genus consists of 4 species; S. *dysenteriae, S. flexneri, S. boydii, and S. sonnei.* All four species of *Shigella* cause disease in humans. The disease process involves invasion of colonic mucosal cells and induction of an intense inflammatory response, leading to the death of epithelial & immune cells and the formation of colonic mucosal ulcerations and abscesses. Shigellosis is an endemic throughout the world. It causes about 1.1 million deaths every year globally. To date, there are approximately 164.7 million cases world wide (WHO 2002). There may be many go unreported as only mild symptoms is involved while some have no symptoms at all. *S. sonnei* is predominantly isolated from patients of developed countries; *S. flexneri 2a* is predominantly found in developing countries; while *S. dysenteriae* is predominantly isolated in underdeveloped countries.

This contagious organism is spread by direct or indirect contact with infected individuals. Salads (potato, tuna, shrimp, macaroni, and chicken), raw vegetables, milk and dairy products, and poultry; contamination of these foods is usually through the fecal-oral route. Fecally contaminated water and unsanitary handling by food handlers are the most common causes of contamination. *Shigella* often causes outbreaks in daycare centers. Parents often acquire the illness from their children. Symptoms manifestation of *Shigella* include diarrhea which may be mild or severe, along with fever and nausea. The diarrhea may be watery or bloody. Vomiting and abdominal cramping may also occur. However, some infected people may not show any symptoms. *Shigella* infection is generally limited to the intestinal mucosa therefore bacteremia due to *Shigella* is extremely rare (Morduchowicz G *et al.*, 1987; Struelens MJ *et al.*, 1985). *Shigella* rarely occurs in animals; principally a disease of humans except other primates

such as monkeys and chimpanzees. The organism is frequently found in water polluted with human feces.

Although several Gram negative enteric pathogens are able to penetrate mammalian cells (including *Salmonella*, certain strains of *Escherichia coli*, and *Yersinia*), only *Shigella* and enteroinvasive *E. coli* escape from the phagocytic vacuole and utilize proteins of the host cell cytosol in the pathogenic process. Once in the intestinal tract of the human host, *Shigella flexneri* directs its own uptake into the colonic mucosa through membrane ruffling and macropinocytosis in a manner similar to *Salmonella* uptake (Adam T *et al.*, 1995; Clerc P *et al.*, 1987). After engulfment, the bacterium is surrounded by a membrane-bound vacuole within the host. However, the bacterium rapidly lyses the surrounding vacuole and is released into the cytosol, where it grows and divides (Sansonetti PJ *et al.*, 1986). Once the microbe has escaped from the vacuole, it quickly becomes coated with filamentous actin and ultimately forms an actin tail at one pole of the bacterium (Bernardini ML *et al.*, 1989; Ogawa H *et al.*, 1968). This actin polymerization propels the bacterium through the cytoplasm at speeds approximately 0.4 μm/sec (Zeile WL *et al.*, 1996). When the pathogen reaches the plasma membrane, it forms a long protrusion into the neighboring cell, which subsequently internalizes the microbe (Kadurugamuwa JL *et al.*, 1991). The bacterium again breaks out of the vacuole, thereby starting a new cycle of infection in a new host cell (Allaoui A *et al.*, 1992). This process allows *Shigella* to move from cell to cell without ever contacting the extracellular milieu.

Outbreaks of infection are common and usually occur in schools and institutions where people are in close contact. It has been suggested that in developing countries flies may spread the infection from person to person, as the disease is commonest at the time of year when the fly population is highest. Worldwide, there are about 165 million

cases of shigellosis a year and 1.1 million deaths, according to the World Health Organization. Outbreaks have been reported in men who have sex with men; under conditions of crowding; and where personal hygiene is poor, such as in jails, institutions for children, day-care centres and mental hospitals. Usually, more than one serotype is present in a community; occasionally mixed infections with other intestinal pathogens are seen (CDC, 2001). In recent years, multiple-antibiotic-resistant *Shigella* has surfaced in all areas of the world (CDC, 2001). The widespread use of antimicrobial drugs is often related in multiple drug resistant strains of bacteria.

According to the World Health Organization, 580,000 cases of shigellosis are reported among travellers from industrialized countries. A total of 69% of all episodes and 61% of all deaths attributable to shigellosis involve children less than 5 years of age. Since the late 1960s pandemic waves of *Shigella* dysentery have hit Central America, South and Southeast Asia and sub-Saharan Africa, often striking areas of political upheaval and natural disaster (WHO 2002).

1.3. Diagnosis of shigellosis

Diagnosis of a *Shigella* infection depends on a great deal microbiologic culture and sensitivity testing. Even though clinical presentation of the patient may evoke suspicion of shigellosis but the diagnosis solely relies on the isolation and identification of the bacterium from the feces or rectal swabs in the laboratory. Patients presenting with watery diarrhea and fever should be suspected of having shigellosis. Although blood is common in the stools of patients with amebiasis, it is usually dark brown rather than bright red, as in *Shigella* infections. Microscopic examination of stool smears from patients with amebiasis should reveal erythrophagocytic trophozoites in the absence of

PMN, whereas bacillary dysentery is characterized by sheets of PMN. Isolation of shigellae in the clinical laboratory typically involves an initial streaking for isolation on differential/selective media with aerobic incubation to inhibit the growth of the anaerobic normal flora. Commonly used primary isolation media include MacConkey, Hektoen Enteric Agar, and Salmonella-Shigella (SS) Agar. These media contain bile salts to inhibit the growth of other Gram-negative bacteria and pH indicators to differentiate lactose fermenters (Coliforms) from non-lactose fermenters such as shigellae. Following an overnight incubation, non-lactose fermenting colonies are streaked and stabbed into tubed slants of Triple Sugar Iron Agar. In this differential media, *Shigella* species produce an alkaline slant and an acid butt with no bubbles of gas in the agar. This reaction gives a presumptive identification, while slide agglutination tests with antisera for serogroup and serotype confirm the identification. This is usually accomplished using commercially available, absorbed rabbit antisera slide agglutination tests.

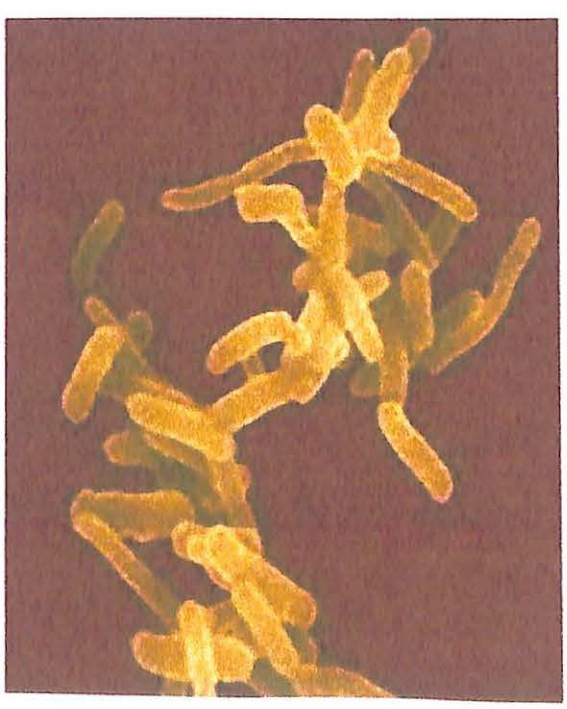

Figure 1.1.: Microscopic view of the infectious microbe Shigella flexneri.

1.4. Treatment

Regardless of the causative agents, treatment of diarrheal diseases is mainly to correct the abnormalities that result from isotonic dehydration, metabolic acidosis, and significant potassium loss though shigellosis seldom causes severe dehydration. Therefore the mainstay of treatment for shigellosis is the oral rehydration treatment developed by the World Health Organization which has been proven effective and safe in the treatment of acute diarrhea, provided that the patient is not vomiting or in shock from severe dehydration. With proper hydration, shigellosis is generally a self-limiting disease, and the decision to prescribe antibiotics is dependant on the severity of disease, the age of the patient, and the likelihood of further transmission of the infection. Some studies found that antimicrobial therapy is unnecessary unless to prevent further transmission. However, clinically it is found that effective antibiotic treatment reduces the average duration of illness from approximately one week to approximately 3 days and also reduces the period of *Shigella* excretion after symptoms subside. Absorbable drugs such as ampicillin (2 g/day for 5 days) are likely to be effective when the isolate is sensitive. Trimethoprim (8 mg/kg/day) and sulfamethoxazole (40 mg/kg/day) will eradicate sensitive organisms quickly from the intestine, but resistance to this agent is increasing. For the resistant strain, ciprofloxacin (1 g/day for 3 days) is effective against multiple drug resistant strains, but this antibiotic is not approved by the United States Food and Drug Administration (FDA) for use in children less than 17 years of age due to the risk of cartilage damage. Multiple drug resistant strain of *Shigella* is common in Manitoba, Canada; especially to cotrimoxazole, thus the antibiotic choice should be based on culture and sensitivity test results (CDC 2001).

1.5. Control measures & prevention

As for the control measures of the infected patients, education on the importance of proper hand wash and personal hygiene is essential in preventing further transmission. Infected patients should not provide food, child, and patient care until two consecutive stool cultures (collected 24 hours apart or 48 hours after antibiotic treatment has ended) are free of *Shigella* (CDC 2001). As a general prevention guideline for the public, proper personal hygiene education is very important in curbing fecal-oral-transmitted diseases. Other preventative measures are proper sanitary disposal of feces and toilet waste, drinking of safe and clean water supply, control of flies (may be the intermediate host), cleanliness in preparing and handling of food and milk, and breastfeeding for the infants as it provides good immunity to the newborns. Theoretically, this communicable disease can be curbed if proper preventative measures are undertaken. However, in recent years multiple drug resistant strains of such organisms have stirred the interest amongst scientists to a deeper approach into the development of effective vaccines and drugs.

1.6. Vaccine candidates under development

In contrast to other more established diseases like cholera and typhoid fever, there are no licensed vaccines currently available for shigellosis. Until recently, scientists have carried out vaccine development in search of *Shigella* vaccines. Candidate vaccine strains have been developed in many research laboratory facilities. However, as of present none has been approved by any federal agency.

In search of the shigellosis vaccines, two approaches have been undertaken for the development: 1) live attenuated bacteria with genetically engineered mutations in a

virulence gene (*icsA*) and/or a synthetic pathway gene (*iuc, ent, aroA, aroD, guaBA*); and 2) subunit vaccines that elicit antibody responses against the O-specific polysaccharide component of the LPS. Scientists at the Institut Pasteur have developed live-oral vaccine candidates against *Shigella flexneri* 2a (SC602) and against *Shigella dysenteriae* type 1 (SC599) by creating precise mutations in the *icsA* (or *virG*) gene, which is a virulence plasmid gene encoding a 120 mDa outer membrane protein; the *iuc* and *ent* genes, which are related to a chromosomal locus encoding aerobactin and enterocholin; and *stxA* gene, which encodes the A subunit of Shiga toxin. The advancement in molecular biology has enabled extensive development of the live attenuated oral vaccine candidate. The SC602 vaccine candidate has been tested in Phase 1 and 2 studies in North American adult volunteers in which they are given a single dose of 10^4 cfu. The findings are positive; that is not only does the vaccine candidate proved high immunogenic but also safe for oral dose.

Another *Shigella* vaccine candidate in the development by Kotloff *et al.*, 1999; known as CVD1207, is a live attenuated *Shigella flexneri* 2a vaccine candidate with specific deletion mutations in *virG, sen, set,* and *guaBA*. The vaccine candidate CVD 1207 strain still has the ability to invade epithelial cells but cannot effectively spread intercellularly after invasion (Δ*virG*), does not produce enterotoxin (Δ*sen* and Δ*set*), and has limited proliferation in vivo (Δ*guaBA*). In this research work, Kotloff *et al.* have successfully produced the CVD1207 strain which is highly attenuated in humans. Other than the CVD1207 and SC602 strains, another vaccine candidate strain that is currently under developmental progress is the SFL 124 (Serotype Y). It is said to be a promising live oral vaccine candidate, which has been shown to be safe and immunogenic in human volunteers. To change the serotype of this vaccine strain, Guan and Verma, 1998 inserted a serotype conversion gene cluster into the chromosome of SFL124 by using a

bacteriophage-based site-specific integration system. By cloning an integrase gene (*int*), an attachment site (*attP*) and a glucosyl transfer gene cluster from bacteriophage SfX into a suicide vector, and subsequently introducing this construct into *S. flexneri* SFL124, Guan and Verma, 1998 obtained a *S. flexneri* strain (designated SFL1213) expressing the serotype X somatic antigen specificity.

1.7. The importance of *Apyrase* gene

Many studies & research have shown that one of the major virulence factors is the *apyrase* gene (Bhargava *et al.*, 1995, Babu *et al.*, 2002, Santapaola *et al.*, 2002). The presence of a nucleoside triphosphate hydrolyzing activity of *S. flexneri* is catalyzed by the enzyme apyrase (ATP diphosphohydrolase). The enzyme apyrase has the ability to sequentially hydrolyze nucleoside triphosphates to corresponding diphosphates and then monophosphates. Babu *et al.* reported that *Shigella* apyrase acts on both organic and inorganic pyrophosphates and does not require metal ions. Having such characteristics, Babu *et al.* suggested that apyrase is a novel cytotoxin that indiscriminately hydrolyzes cellular NTPs and helps *Shigella* take control of the host cells. Furthermore, its activity is also seen in actin polymerization (Babu *et al.*, 2002). This has suggested that perhaps the apyrase has a role in the rate of actin polymerization which is essential for the intracellular spread of the bacterium. From the above citations of many researchers, apyrase encoded by the *apy* gene is indeed a virulence associated gene found in the megaplasmid as well as in the genome of the *Shigella flexneri*. To date, no studies have been done in the mutation of the *apy* gene. Therefore, whether or not a *apy*-mutated *S. flexneri* strain will still elicits similar pathogenicity as the wild type still remains debatable.

2.0. LITERATURE REVIEW

Bacillary dysentery in humans is often caused by the virulent *Shigella* spp. or the enteroinvasive *Escherichia coli* (EIEC). Since the advancements of molecular biology, extensive studies of the virulence genes associated with the pathogenesis of *Shigella* spp. have been carried out by scientists through out the world. While all these studies have led to a better understanding of the overall pathogenesis by the bacteria, little is known regarding the biochemical basis of the cytotoxicity by *Shigella* (Bhargava *et al.*, 1995).

Studies have found that virulent *Shigella* codes for the soluble apyrase. The apyrase is found to be expressed in all the four species of *Shigella* and EIEC. Experiments and studies done by Bhargava *et al.* (1995) have pointed out that invasion caused by virulent *S. flexneri* were indicative of a disruption of cellular respiration and fermentation. These findings were carried out an invasion assay using J774 macrophages as the host. A rapid decrease in the intracellular concentration of adenosine triphosphate (ATP), an increase in pyruvate and a decrease in lactate concentration were observed in J774 macrophage cells invaded by virulent *S. flexneri.* In the study, Bhargava *et al.* have identified the protein responsible for the ATPase activity of *Shigella*; ATP diphosphohydrolase or apyrase. Apyrase is a 25kDa enzyme that catalysed the hydrolysis of both ATP and ADP to AMP through sequential release of inorganic phosphate (Pi). The enzyme was found to be active in the presence of high concentration of EDTA and this indicated that it did not require divalent metal ions for its activity. On the other hand, the activity is inhibited by various divalent cations. Bhargava *et al.* also reported that the apyrase enzyme has the highest activity between pH 7.0 and 7.5. However, at temperature 30°C or at 42°C the activity is greatly reduced.

This is the temperature at which *Shigella* phenotypically behave as non-virulent bacteria.

Another study by Babu *et al.* (2002) revealed that the virulence-associated ATP diphosphohydrolase (apyrase) activity of *Shigella* was found to be similar to bacterial non-specific acid phosphatases in primary structure up to as high as 73% similarity. In this study, Babu and colleagues obtained the 3D structure of *Shigella* apyrase by threading using Swiss-Model with EBPase as PDB template. However, bacterial phosphatases such as acid phosphatase of *Escherichia blattae* are predominantly a phosphatase whilst *Shigella* apyrase showed negligible phosphatase activity. According to Babu *et al.*, given the account that *Shigella* apyrase has no significant phosphatase, haloperoxidase, peroxidase, or catalase activities, it should be classified as a new variant in the bacterial acid phosphatase family; exo-pyrophosphatase. Distinct from other known bacterial pyrophosphates, Babu *et al.* reported that *Shigella* apyrase acts on both organic and inorganic pyrophosphates and does not require metal ions. Having such characteristics, Babu *et al.* suggested that apyrase is a novel cytotoxin that indiscriminately hydrolyzes cellular NTPs and helps *Shigella* take control of the host cells. Similar to other bacterial acid phosphatase, *Shigella* apyrase has a poly-proline sequence. The actual significance of the poly-proline region is not well understood in *Shigella*. However, previous study by Niebuhr *et al.* (1997) has indicated that the poly-proline region may have interactions with actin. Coincidently, very intense actin polymerization activities are central to *Shigella*'s pathogenesis. Actin polymerization, also known as treadmilling; is essential for the organisms in intracellular spread. Therefore, *Shigella* apyrase mght be involved in modulating actin polymerization rates (Babu *et al.*, 2002).

In a study on the expression of the virulence plasmid carried genes, Berlutti *et al.* (1998) found that the expression of the *apy* gene was temperature regulated which is similar to other virulence genes. To determine whether the expression of apyrase was controlled by temperature at the transcriptional level, Berlutti *et al.* performed a northern hybridization and dot blot analysis of total RNAs from strains grown at 30 and 37°C with a 627bp PCR product of internal fragment (coding region) of the *apy* gene as a probe. The RNA blot results demonstrated that the transcription of *apy* was strictly regulated by temperature. They found that it was 12-fold higher at 37°C than at 30°C. This has led them to further study the possibility of the *apy* gene expression is under the regulation of H-NS, and VirF & VirB regulatory cascade. In this experiment, Berlutti *et al.* assayed sonic extracts and performed RNA blot analysis of the Δ*hns* deletion derivative strains; HN680 & HN280/32 and a pINV-intergrated derivative of wild-type EIEC. They then found that the *apy* gene expression is negatively regulated by H-NS, and VirF & VirB regulatory cascade at the transcriptional level. The findings on the regulatory mechanisms of the *apy* gene by Berlutti *et al.* have strongly suggested that the expression of apyrase may be important, especially when bacteria are inside susceptible host cells, probably during the phase of intracellular multiplication.

Previous studies have suggested and indicated that the expression of the virulence-plasmid (pINV)-carried potential pathogenesis associated *apy* gene is regulated by the same regulators that govern the expression of virulence genes. These findings have led a group of scientists (Santapaola *et al.*, 2002) into sequencing an 8032bp *Pst*I fragment of the pINV of EIEC strain HN280, in which it encompasses the *apy* gene as well as the adjacent genes. In this study, the methods used involved Northern hybridization, RT-PCR, sequence analyses, and primer extension experiment. Data & results obtained from these experiments have indicated that *ospB* and *apy* genes

are co-transcribed as a 2kb bicistronic, temperature regulated mRNA from an upstream promoter that precedes *ospB*. The *ospB* gene encodes a secreted protein of unknown activity and is located immediately upstream of the *apy* gene. In their study, they have also found that sequences resembling promoter elements were not found in the DNA region upstream of the 5' end of *apy*. Furthermore, data findings were consistent with their hypothesis of an *ospB-apy* bicistronic transcript starting from a promoter located upstream of *ospB*. These have led to their postulation that the 1kb transcript encoding *apy* alone must have result from post-transcriptional processing of the 2kb transcript at the identified 5' end, within the 328bp *ospB-apy* intercistronic region. In addition, the *ospB* and *apy* genes are expressed from a single transcript and located in a highly conserved DNA region in both *Shigella* and EIEC have indicated that such a gene organization perhaps provide certain advantages to the organisms over the others.

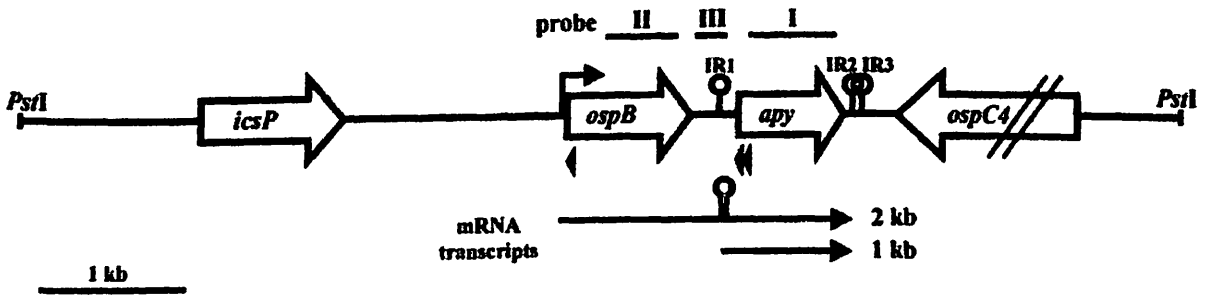

Figure 1.2.: Genetic and transcriptional organization of the ospB-apy operon.

The study of the *apy* gene found in all the literatures have postulated that *apy* is a potential pathogenesis-associated gene, located on the pINVs of virulent *Shigella* spp. and related EIEC strains (Berlutti *et al.*, 1998; Bhargava *et al.*, 1995; Buchrieser *et al.*, 2000; Venkatasen *et al.*, 2001). It is also strongly suggested by Berlutti *et al.* (1998) that the expression of apyrase may be essential especially when the bacteria are within the susceptible host cells during multiplication. The *apy* gene has been strongly indicated as

one of the virulence genes. However, questions like whether the periplasmic apyrase acts as a generalized cytotoxin, is involved in the utilization of exogenous nucleotides which mush be dephosphorylated to nucleosides to cross the impermeable cytoplasmic membrane, or does it play a role in some unknown bacterial metabolic functions that are not directly involved in the pathogenesis is still a mystery.

2.1. Lacunae in the literature

The *apy* gene was discovered recently as one of the major virulence genes that is present within the genome and the megaplasmid of the virulent *Shigella* (Bhargava *et al.*, 1995). Many studies have also cited the pathogenicity role of the apyrase encoded by the *apy* gene. According to Bhargava *et al.* (1995), the apyrase of *Shigella* may be a general cytotoxin involved directly or indirectly in the actual killing of the host cells. Therefore, the mutation of this *apy* gene seems to be a promising method of producing a highly immunogenic yet safe vaccine candidate. Furthermore, the *apy* gene is expressed in all the four species of *Shigella.* This has postulated the possibility of producing a vaccine that produces high immunogenicity against all the four species.

Although the roles of the pathogenicity of the *apy* gene are established, up to date there is no published work on the mutation of this *apy* gene. Researchers currently working on producing the potential vaccine strains for *Shigella* are targeting at other virulence genes, such as the *icsA* gene.

3.0. OBJECTIVES

- To clone the *apyrase* gene of *S. flexneri* into pTZ57R plasmid.
- To clone the Kanamycin gene cassette (*aphA*) from pTOPO 2.1
- To mutate the *apyrase* gene by insertional mutation with Kanamycin gene cassette (*aphA*).
- To subclone the mutated Apy::aphA into a conjugative suicide vector, pWM91.
- To screen the successful construct by PCR method and DNA sequencing.

4.0. MATERIALS AND METHODS

4.1. Materials

- *Shigella flexneri* SH043 strain
- *E. coli* host
  - TOP10 (*F'merA Δ(mrv hsdRMS mcrBC)ϕ80lacZΔM15 Δlac X74 deOR recA1araD139 Δ(ara-leu)7697 galU galKrpsL(str^R) end*A1 *nupG*)
  - DH5α λ pir (F-ϕ80d *lacZ*Δ m15Δ(*lac*ZYA-*arg*F) U169 deOR *rec*A1 *end*A1 *hsd*R17 (r_k^-, m_k^+)*pho*A suppE44 *thi*-1 *gyr*A96 *rel*A1)
- Plasmid
  - pCRTOPO2.1
  - pTZ57R
  - pWM91
- Resrtiction Enzymes
  - *Ksp*A1 (*Hpa*I)
  - *Sac*I
  - *Sal*I
  - *Sma*I
- *apy* Forward & Reverse primers
- Kanamycin (*aphA*) Forward & Reverse primers
- Plasmid extraction kit
- PCR thermal cycler
- Agarose gel electrophoresis
- Luria Bertani
  - Agar
  - Broth
- Antibiotic
  - Ampicillin
  - Kanamycin

Table 4.1. List of materials used.

4.2. Experiment overview

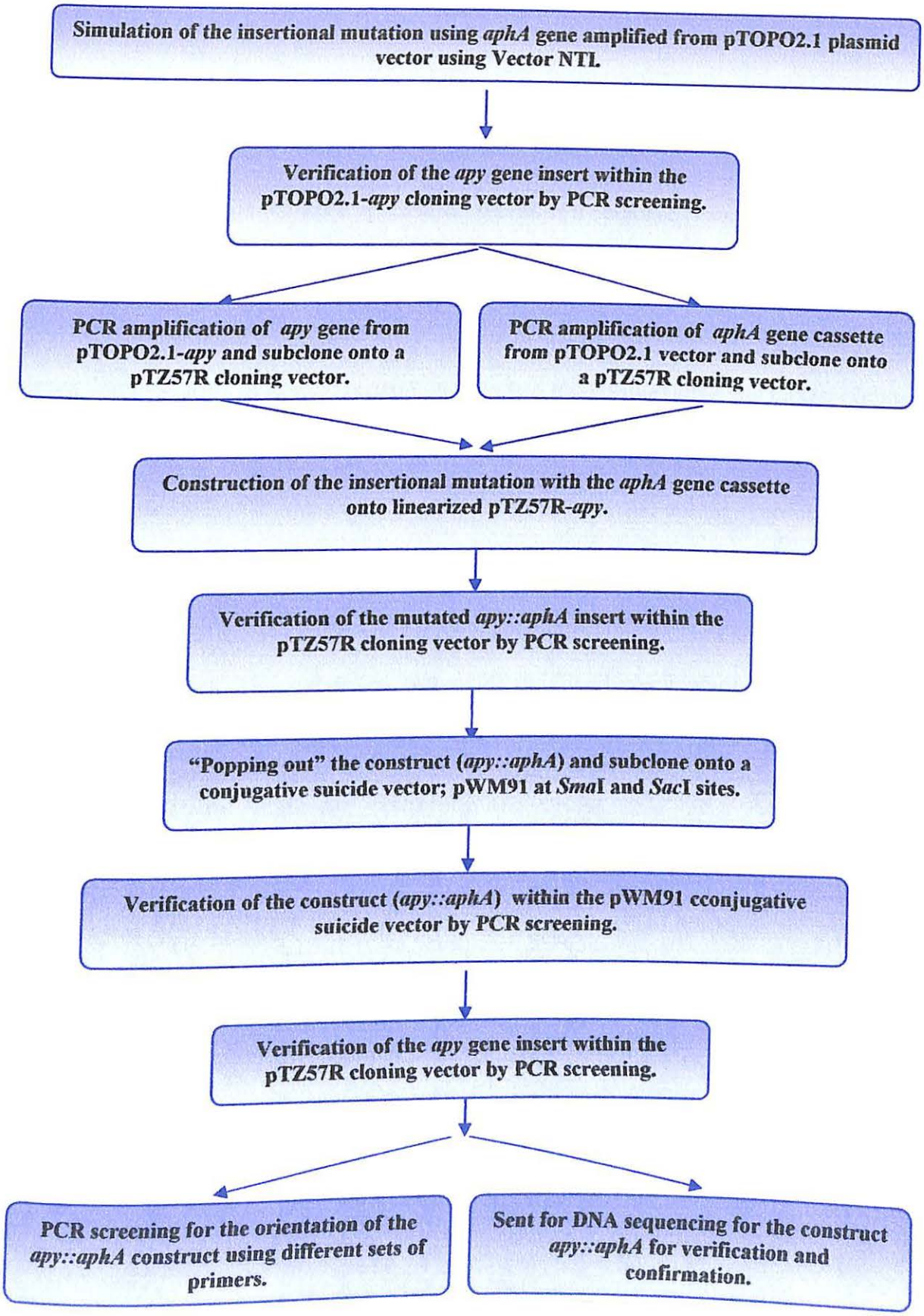

Figure 4.1.: Experiment overview

4.3. Methods

4.3.1. Virtual cloning using Vector NTI

By using the famous bio-informatic application such as the Vector NTI ver. 9.0, I was able to manipulate the sequence profile obtained very efficiently and conveniently on the computer. This versatile application serves as a database manager for a large data or collection of protein and nucleic acid sequence profiles. Many functions can be simulated using this software, for example oligonucleotide designing, locating of certain genes, identification of unique restriction enzymes recognition sites, gel electrophoresis and many more.

In this study, the Vector NTI was used to identify the unique restriction enzyme site in the *Shigella flexneri apy* gene. Using the Vector NTI, we simulated the analysis of restriction enzyme sites of the *apy* gene by using some of the more commonly used restriction enzymes. The restriction pattern of *apy* gene was examined to identify the unique restriction enzyme which restricts the *apy* gene somewhere in the middle of its open reading frame while not restricting the vector (pTZ57R). After which, an insertional mutation simulation was done using the software. The *aphA* gene sequence was obtained from pTOPO2.1 vector, in which it's then used to introduce the insertional mutation into the *apy* gene. The *aphA* gene which confers kanamycin resistant serves as a selective marker to facilitate the selection of successful clones.

4.3.2. Media preparation

4.3.2.1 SOB medium

Contents	Amount / volume
Tryptone	20g
Yeast extract	5g
Sodium chloride	0.5g
Potassium chloride (250mM)	10ml
Deionized distilled water	750ml

Table 4.2.Ingredients for preparation of SOB medium.

The dry ingredients were added into 750 ml of deionized distilled water and the pH was adjusted to 7.0 using 1N HCl/ 1M NaOH. The volume was made up to 990 ml with deionized distilled water. The medium was sterilized by autoclaving at 121°C for 15 minute and stored at room temperature (21-23°C). Just before use, 10 ml of sterile 1M magnesium chloride solutions was added to 990 ml of the medium.

4.3.2.2. SOC Medium

Contents	Amount / volume
SOB medium	1L
D-Glucose (1M)	20ml

Table 4.3. Ingredients for preparation of SOC medium.

Sterile solution of glucose was added to SOB medium and the medium was used immediately.

4.3.2.3. Luria Bertani (LB) agar

To prepare a 500 mL volume of Luria bertani (LB) agar, 7.5g of tryptone (Merck), 2.5g of yeast extract (Oxoid), 5.0g of sodium chloride (Amresco), and 7.5g of agar (Amresco) are the ingredients required. The first 3 ingredients are first dissolved in

about 300 mL of deionized distilled water and stirred well. The agar is poured into a separate bottle without adding water. As the 3 ingredients are dissolved top up the rest of the deionized distilled water to reach the final volume of 500 mL and stir until all the ingredients are completely dissolved. After that, the pH was adjusted to 7.0 by either adding drops of 1M sodium hydroxide or 1M hydrochloric acid to reach the neutral pH. Then, the mixture was poured into the bottle containing the agar powder and sent for autoclaving (121°C, 15 minutes, 1 atm). After the autoclaving process, the media agar was let cool down to approximately 40°C (before solidifying) before the addition of any antibiotic to prevent denaturation of the antibiotic. Then the media agar was poured into clean, sterile Petri plates with approximately 25 mL in volume each. The plates are left in room temperature for solidifying and can be kept in the cold room for about a month.

4.3.3. Concentration of antibiotics

In this research study, only 2 antibiotics were used; ampicillin and kanamycin. These antibiotics were used for the isolation and presumptive identification of successful clones depending on the antibiotic resistant genes carried on the plasmid backbone or the insert. The addition of the antibiotics in the media agar has to be within the concentration required to provide effective isolation of sensitive and resistant strain.

Antibiotics	**Final concentration**
Ampicillin	100 µg / ml
Kanamycin	50 µg / ml

Table 4.4.: Concentration of antibiotics used in LB agar and LB broth

4.3.4. Restriction enzyme digestion

4.3.4.1. Restriction endonucleases

Restriction endonucleases (REases), are very important tools in studies of many fields of molecular genetics and molecular biology. This is due to their high specificity and ease of use in molecular work. These restriction enzymes are sometimes known as the molecular scissors. Without these restriction enzymes, the spectacular advances in molecular genetics over the past two decades could not have taken place (Sharma *et al.*, 2003). Among the different classes of restriction enzymes (types I, II, III, and IV), Type II have been found to have extensive applications in molecular biology (Sharma *et al.*, 2003). To date, more than 3500 type II REases, exhibiting 241 different specificities have been isolated. These enzymes are isolated from bacteria in which they serve as a defense mechanism against viral infection. As the particular sequence occurs along the chromosome, the enzyme cleaves the sugar phosphate backbones of DNA and produces fragments. The restriction enzymes used in this study were obtained from Fermentas, USA as shown in Table 2 below:

Restriction enzymes	**Cleavage site**	**Optimal buffer**	**Optimal temperature**
Hpa I (*Ksp* A1)	5'...GTT|AAC...3' 3'...CAA▼TTG...5'	Buffer B	37°C
Sac I	5'...GAGCT|C...3' 3'...C▼TCGAG...5'	Buffer *Sac*I	37°C
Sal I	5'...G|TCGA C...3' 3'...C AGCT▼G...5'	Buffer O	37°C
Sma I	5'...CCC|GGG...3' 3'...GGG▼CCC...5'	Buffer Tango	30°C

Table 4.5.: Restriction enzymes used in this study