

MUTATION OF *APY* GENE OF *Shigella flexneri* 2A: TOWARDS THE DEVELOPMENT OF AN ORAL LIVE ATTENUATED *SHIGELLA* VACCINE

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INTRODUCTION

Shigella is a Gram negative bacterium which causes bacillary dysentery by invading colonic mucosa of humans. It is highly infectious as 10-100 microorganisms are sufficient to cause the disease. *Shigella* spp. is transmitted by faecal-oral route via ingestion of contaminated food and water (DuPont *et al.*, 1989). It is reported that *Shigella* species cause illness in over 150 million individuals and deaths in over 1 million individuals in the developing countries (Kotloff *et al.*, 1999). Choice of antimicrobial treatments are becoming limited due to increase reports on antibiotic resistance strains (Niyogi, 2005). Thus, there is an urgent need in developing a vaccine for *Shigella* as the disease burden is increasing. Several strategies have been used in attempts to develop a vaccine targeting shigellosis, but till to date there is no licensed vaccine available for *Shigella*. The pathogenesis of *Shigella* is contributed by the virulent genes located in the megaplasmid and chromosome of *Shigella* species (Jennison and Verma, 2004). The *apy* gene which is located in the megaplasmid of *Shigella* is reported to be involved in the killing of the host cells (Bhargava *et al.*, 1995). This gene encodes for apyrase enzyme which is reported to be a pathogenesis associated enzyme for *Shigella* species (Santapaola *et al.*, 2006). A few studies have been carried out on the structure function of apyrase enzyme and the involvement *apy* gene in *Shigella* pathogenesis, but no studies have been carried out on *apy* gene as a vaccine candidate for *Shigella*. Thus, the aim of this study is to develop an oral live attenuated *Shigella* vaccine by mutating the *apy* gene. A virulent *Shigella flexneri* strain was selected by Sereny test as a candidate for the *apy* gene mutation. The *apy* gene from the virulent strain was cloned into a PCR cloning vector and was mutated by inserting a kanamycin resistant gene cassette. Subsequently the mutant construct was subcloned into pWM91 suicidal vector and by homologous recombination process the wild *apy* gene was deleted from the *Shigella flexneri*. The

obtained mutant was further characterised for apyrase enzyme activity by colorimetric assay. The constructed mutant was then evaluated for its virulence by plaque assay and for protective efficacy using guinea pigs model.

METHODOLOGY

Determination of a virulent strain as candidate for vaccine construction work

This animal study was conducted under guidance from the Universiti Sains Malaysia Health Campus Animal Ethics Committee [ref: PPSG/07 (A)/044/ (2007) (26)]. Four *S. flexneri* strains (SH052, SH057, SH060, and SH062) were tested via inoculation into guinea pig eyes as follows. The bacterial cultures were grown overnight in LB broth and were brought to 10^8 CFU in normal saline (0.9%) by measuring optical density (OD) at 600 nm. Each eye of a Hartley guinea pigs ($n=3$)g was inoculated in the conjunctival sac with 10^8 CFU of one of the wild strains. Guinea pigs were examined daily for 5 days, and their inflammatory responses were graded according to Hartman and colleague (1991). Development of the disease was rated as follows: 0: no disease or mild; 1: mild conjunctivitis or late development and/or rapid clearing of symptoms; 2: keratoconjunctivitis without purulence; and 3: fully developed keratoconjunctivitis with purulence. The strain that showed the highest degree of conjunctivitis was used to construct the *apy* vaccine.

Construction of apy mutant

Wild apy gene was PCR amplified from *S. flexneri*, SH057, and it was cloned into pTZ57R PCR cloning vector. It was then mutated by inserting a kanamycin resistant gene cassette and subsequently transformed into pWM91 suicidal vector in BW20767 *E. coli pir* host. Conjugation was then carried out between BW20767 *E. coli pir* host and *S. flexneri*, SH057 to delete the wild *apy* gene.

Characterisation of apy mutant by colorimetric assay

Colorimetric assay was carried out by as described Sankaran *et al.* (2009) to determine the apyrase enzyme activity in the *apy* mutant.

To evaluate the virulence and protective efficacy of the constructed apy mutant

Plaque assay was carried out following methods by Oaks *et al.* (1985). The *apy* mutant with different concentrations (10^6 - 10^9) was infected into HeLa cell line and plaque formation was observed using an agarose layer containing antibiotic. Immunisation assay was carried out as described by Hartman *et al.* (1991). Ocular inoculation with vaccine strains, 3×10^8 to 4×10^8 CFU were carried out on days 0, 1, 14 and 15. Guinea pigs were challenged with virulent *S. flexneri*, SH057, 2 weeks after the last immunisation. One group of guinea pig was challenged without giving the booster dose on day 14 and 15. This was carried out to determine effect of booster dose that confers protection by the

vaccine candidate. Development of the disease was rated as follows: 0: no disease or mild; 1: mild conjunctivitis or late development and/or rapid clearing of symptoms; 2: keratoconjunctivitis without purulence; and 3: fully developed keratoconjunctivitis with purulence.

RESULTS

Determination of a virulent strain as candidate for vaccine construction work

Among the four *S. flexneri* strains tested (SH052, SH057, SH060, and SH062), SH057 gave the highest score with rating 3. Thus, this strain was chosen for this vaccine construction work.

Construction of apy mutant (SFUSM1)

The *apy* mutant was successfully constructed using a kanamycin resistant gene cassette. The mutation in *apy* gene was verified by PCR screening and DNA sequencing. By homologous recombination between BW20767 *E. coli pir* host with mutated *apy* gene and *S. flexneri*, SH057, the wild *apy* gene was deleted from the wild SH057.

Colorimetric assay

The apyrase enzyme activity was confirmed to be non-functional in the *apy* mutant, SFUSM1, but in the parental strain, SH057, the enzyme activity was shown to be functional.

Virulence of SFUSM1 on HeLa cells and protective efficacy of SFUSM1 in guinea pig

HeLa cells were inoculated with SFUSM1 (10^6 - 10^9 CFU). SFUSM1 formed smaller plaques compared to the wild *S. flexneri* strain, SH057. The smaller plaque with SFUSM1 indicates it has attenuation in virulence. For the safety studies of SFUSM1, guinea pigs inoculated with SFUSM1 showed no signs of irritation or disease throughout the experiment. Furthermore, when administered with a booster dose it elicited a good protection when challenged with wild *S. flexneri* SH057.

CONCLUSION

This mutation of the *apy* gene is a good approach towards the development of a potential live attenuated vaccine for shigellosis. Further studies need to be done to examine the serum antibody response of the vaccinated animals and to determine the protective efficacy of SFUSM1 against other *Shigella* species.

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