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Full Length Research Paper

Construction and immunogenicity of a genetically mutant of a native *Shigella dysenteriae* type 1 isolated from an Iranian patient with diarrhea by recombineering

M. Heiat[1], M. Saadati[2], S. Mostafa Hosseini[3]*, T. Zahraei Salehi[4], B. Nayeri Fasaei[4], Y. Ahsani Arani[5], A. R. Salehi[5], M. Doroudian[5] and E. Hosseini[6]

[1]Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran.
[2]Department of Biology, Imam Hossein University, Tehran, Iran.
[3]Young Researchers Club, Science and Research Branch, Islamic Azad University, Tehran, Iran.
[4]Department of Microbiology, Faculty of Medical Sciences, University of Tehran, Iran.
[5]Young Researchers Club, Islamic Azad University, Tehran-Markaz, Iran.
[6]Young Researchers Club, Central Tehran Branch, Islamic Azad University, Tehran, Iran.

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Since protective immunity against shigellosis is serotype specific, it appears that construction of a live *Shigella dysenteriae* type 1 strain (attenuated by deletion of the *virG* gene as a native new vaccine candidate) is urgently needed. The *virG* gene from a native of *S. dysenteriae* type 1 was amplified and cloned into the pGEM-5Zf vector and sequenced. Alignment analysis indicated that the *virG* gene was 100% identical with other *shigella* serotypes. The polymerase chain reaction fragment carrying a chloramphenicol resistance cassette flanked by short (46 nt) regions homologous to the *virG* gene was electroporated into recipient *S. dysenteriae* strains expressing a highly proficient *γβexo* gene system. This cassette allowed successful disruption of one invasive plasmid locus. The results obtained showed that 46-nt homology between the PCR product and the *virG* gene is enough to promote homologous recombination via the λ Red system. The PCR and sequencing analysis demonstrated that 3220-nt of *virG* gene was successfully deleted. To intensify recombination efficiency, however, we used 232 ng of purified PCR-product. Upon Sereny test challenge with a native wild *S. dysenteriae* type 1, Δ*virG*-vaccinated animals were significantly protected against keratoconjunctivitis. The λ Red recombinase is a new tool, powerful, inexpensive, rapid and simple method for the construction of *in vivo* genetic engineering.

Key words: Iran*, Shigella dysenteriae* type 1, virG(icsA), λ red-recombineering*,* attenuation*.*

INTRODUCTION

Shigella dysenteriae type 1 belongs to Gram-negative bacterium, nonmotile, non-spore-forming, facultative intracellular anaerobes of the family Enterobacteriaceae. As few as 10 to 100 bacteria can cause disease in some adults that is characterized by diarrhea and discharge of bloody mucoid stools, accompanied by severe abdominal pain, nausea, vomiting and fever (Kotloff et al., 1999; Iwalokun et al., 2001; Morpeth and Thielman, 2006; Hien et al., 2007; Niyogi, 2007). *Shigella* was discovered by Japanese scientist Shiga gave a detailed description of bacillus dysentery as the causal organism for dysentery in 1898 (Ewing, 1949). *Shigella* species, none of H(flagellar) and K (capsular) antigens thereby, are classified serologically by their O antigen. The genus of *Shigella* species based on their O antigen is divided into four species: *Shigella*

*Corresponding author. E-mail: geneticman2005@gmail.com.

dysenteriae (subgroup A which is consisting of 13 serotypes), *Shigella flexneri* (subgroup B which is consisting of 14 serotypes), *Shigella boydii* (subgroup C which is consisting of 18 serotypes), and *Shigella sonnei* (subgroup D which has a single serotype) (Simmons and Romanowska, 1987; Cheah et al., 1991). Two species of the causative agent include *S. dysenteriae* type 1 and *S. flexneri* are responsible for acute diarrheal illness. *S. dysenteriae* type 1 is responsible for the predominant cause of epidemic dysentery. Among species of *Shigella*, *S. dysenteriae* type 1 secretes the shiga enterotoxin which contributes to a more severe clinical observation which is characterized by central nervous system abnormalities, seizures, hemolytic-uremic syndrome (HUS) and high case fatality rates accounts for prevalent and deadly epidemics in areas where sanitation of the personal and general hygiene are insufficient that allow contamination is propagated (Kotloff et al., 1999; Cherla et al., 2003).

Because Shigella strains are significant causes of diarrhea, especially in developing countries where they cause more than 150 million annual cases including 700,000 fatalities occurring annually, predominantly, about 70% of all patients are children of <5 years old (Kotloff et al., 1999; Chompook et al., 2005; Lee et al., 2005), human to human transmission of bacteria occurs through the fecal-oral route, but food-borne episodes have also been described. In the developing countries, *S. flexneri* is the prevalent species (60%), followed by *S. sonnei* (15%), and then *S. dysenteriae* (6%), while *S. dysenteriae* 1 is not as prevalent as the other species; it is more serious (Kotloff et al., 1999). *S. dysenteriae* is a major reason of shigellosis epidemics in areas of crowding such as refugee camps, while the other serotypes chiefly account for endemic outbreak. During the last decades, epidemic *S. dysenteriae* type 1 strains resistant to chloramphenicol, ampicillin, tetracycline, trimethoprim—sulfamethoxazole and nalidixic acid have been isolated with growing frequency in Asia and Africa (Mitra et al., 1990; Tuttle et al., 1995; Jahan and Hossain, 1997; Iwalokun et al., 2001; Bhattacharya et al., 2003; Ghosh et al., 2003; Taneja et al., 2005; Bercion et al., 2006). In a study was executed in Iran by Farshad et al. (2006) showed approximately 90.24% of the *Shigella* isolates were resistant to cotrimoxazole (Farshad et al., 2006).

Although, outbreaks of shigellosis through antibiotic therapy is possible but the emergence of antibiotic resistant *Shigella* species and the high-cost of antibiotics, even to the newest antibiotics complicate treatment. Vaccination against shigellosis appears the most cost-effective approach in this particular situation. As well as, based on immunogenicity properties data, a native new vaccine for *S. dysenteriae* 1 is required to design and construct. All four serogroups including *Shigella flexneri*, *Shigella sonnei*, *Shigella boydii* and *Shigella dysenteriae* can cause dysentery, carrying a 220-kb plasmid which encodes the ‘invasive phenotype' of this species. The virulence plasmid of Shigella spp. encodes critical protein essential for epithelial cell invasion and intra and inter-cellular spreading shigella within host tissue. The VirG (IcsA) as one of the most important virulence protein that encoded on invasive plasmid have a key role in the intra- and intercellular spreading in the host's epithelial cells. This protein belongs to the autotransporter (AT) protein family (Pallen et al., 2003). The N-WASP (neural Wiskott-Aldrich syndrome) is one of the member of Cdc42-dependent mediators act as ligand for VirG\IcsA thereby recruits Arp2/3 complex for polymerized globular actin into F actin tail; also serves as link between nucleation de novo actin and signaling pathway that leads to achieve bacterial morphological changes and motility and finally, caused occurrence dissemination infectious in host (Rajakumar et al., 1997; Moss et al., 2000; Turner et al., 2001; Walker and Verma, 2002). The principal attenuating feature of WRSd1, as well as SC602 and WRSS1 is the loss of the VirG (IcsA) protein function preventing bacterial spread within hosts’ epithelial tissue. The application of live attenuated strains of *Shigella* as live oral vaccines has been demonstrated to induce protective efficacy and may be most effective against *Shigella* during infections (Jennison and Verma, 2004).

In healthy adult, volunteers WRSS1 or SC602 was effectively highly immunogenic at 10^4 CFU dose and safe, but more reactogenic at higher doses. In addition, the WRSS1 has completed Phase 1 trials in the US and in Israel and shown to be safe and highly immunogenic (Orr et al., 2005; Barnoy et al., 2011). Sansonetti and Arondel (1989) constructed a double mutat (Δ*virG*, Δ *iuc*) of *S. flexneri* serotype 5 (SC5700) and then evaluated in macaque monkeys. The SC5700 was shown to be protective and safe up to 70% after administering 3 doses in animal models were immunized intragastrically (Sansonetti and Arondel, 1989). Sadorge et al. (2008) evaluated a study of the SC599, a live attenuated *S. dysenteriae 1* strain with four deletion included ΔicsA, Δent, Δfep and ΔstxA. In Phase 1, clinical trial of immunogenicity and efficacy was shown maximum tolerated that was up to an oral dose of 10^8 CFU (Fontaine et al., 1990; Sadorge et al., 2008). The accessibility of a growing number of sequenced genomes has created the need for the improvement of efficient and effective methods that will allow us to generate knockout mutants through gene replacement by homologous recombination. Recently, a more efficient mutagenesis procedure that does not require cloning was developed and has been successfully utilized in various bacteria such as *Yersinia, Shigella, E. coli* and *Salmonella*. One of the most powerful approaches for developing a wide variety of DNA manipulations in *E. coli* has been termed "recombineering" (recombination mediated genetic engineering) (Derbise et al., 2003; Ranallo et al., 2006; Sawitzke et al., 2007).

The λ-Red recombineering system utilizes phage-encoded proteins, which those encoded by the *Red*

genes of lambda bacteriophage. These proteins including Gam (inhibiting the Exonuclease V activity of RecBCD of host cell), Bet (or *red*β which a ssDNA annealing protein), Exo (or *red*α which a 5'-3' dsDNA exonuclease) which are capable of promoting homologous recombination in short homologous regions between the electroporated linear DNA substrates and the target gene to be modied (Datsenko and Wanner, 2000). In this current research, the *S. dysenteriae* 1 as native vaccine candidate, a first-generation virG (*icsA*)-based vaccine with deletion in the virG gene was designed and has been compared to the well characterized and tested wild type strain. In addition, we report here, the application of the λ Red recombineering technique to inactivate *S. dysenteriae* 1 gene in which invasion plasmid single gene deletion were developed using a one-step PCR product containing 46-nt flanking sequences homologous to *virG* sequence. We further explored whether the ability of the live attenuated vaccine to protect immunized guinea pigs against infection with wild type.

MATERIALS AND METHODS

Bacterial strains and growth conditions, plasmids, media

S. dysenteriae serotype 1 strain isolated from patients with diarrhea from six hospitals in Tehran, it was used as a wild-type strain, its constructed mutant and the plasmids used in this study are listed in Table 1. This strain was characterized by biochemical tests and identified as type 1 serotype (MAST serotyping kit; MAST Group Ltd, Merseyside, UK). PCR amplifications of antibiotic cassette are performed by using expand high fidelity PCR system according to the manufacturer's instructions (Roche). Bacteria routinely were grown on LB media and Tryptic soy agar plates containing 0.05% Congo Red (w/v) as the standard rich broth. The antibiotic concentrations were 100 µg/ml ampicillin (Amp) and 12.5 µg/ml chloramphenicol (cm). The sulfur-oxidizing bacteria (SOB) medium without Mg2+ contained 1% tryptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl and 0.1% KCl. L-arabinose was used at concentrations of 50 µg/ml. Customized DNA primers designed by Gene Runner sofware, the nucleotide sequences of which are listed in Table 2 were purchased from CinnaClon. The DNA molecular weight was visually confirmed on agarose gel 1% and the DNA concentration was measured by spectrophotometer at 260 nm (Eppendorf).

Genomic DNA from *S. dysenteriae* type 1 was extracted using the genomic DNA purification kit (Qiagen, USA) and Accuprep plasmid midi purification kit (BioNEER Inc), respectively, as recommended by the manufactures.

Cloning, sequencing and design primer for recombineering

In order to perform gene knock out, two long PCR primers (66 nt) are required. Each primer has at its 5′end 45 nt matching the *S. dysenteriae* type 1 sequence homologues to *virG* gene, and at its 3′ end, a 20- sequence matching the right or left end of the antibiotic cassette (antibiotic cassette have the two priming sites P1 and P2 (Figure 1). Initially, we need to determine *virG* sequence of native *S. dysenteriae* type 1 strain that isolated from patient with acute diarrhea in Iran; thus, the *virG* gene sequence of *S. dysenteriae* type 1 (Accession No.CP000035) was retrieved from the Gen- Bank database and was amplified through the use of the following primers, $F_{up\text{-}virG}$ and $F_{down\text{-}virG}$. The PCR reaction was performed in 25 µl reaction volume containing 2.5 µl of 10X PCR buffer (CinnaGene, 1 ml), 0.75 µl of 50 mM $MgCl_2$ (CinnaGene, 500 µl), 0.5 µl of 10 mM deoxynucleoside triphosphates (CinnaGene, 100 µl), 1 µl of each of the upstream and downstream primers (10 pmol), 1.0 U of expand high fidelity polymerase (Roche, 2.5 u/µl) and 1 µl of DNA template (50 ng). The PCR program was carried out through 35 cycles in a thermocycler (TC 512 Techne) at 94°C for 1 min denaturation, 58°C for 1 min annealing and 72°C for 3.45 min extension. The DNA was denatured for 4 min at 94°C in the beginning and finally extended for 7 min at 72°C.The PCR products were analyzed in 1.5% agarose gel then stained with ethidium bromide (25 µg ml^{-1}) (CinnaClone) (Figure 2). The amplified PCR product was purified from the agarose gel using a Gel extraction kit (Fermentas) and the purified PCR product was ligated to commercial pGEM-5Zf (+) vector (Promega) at 16°C overnight. Ligated product was transformed into DH5α competent cells by heat shock treatment at 42°C for 90 s. The recombinant transformants were selected using ampicillin (100 lg ml^{-1}), X-gal (40 µg/ml) and 0.2 mM IPTG on LB agar plates.

The colonies were selected randomly and the plasmid was extracted from the transformed cells, and the virG insert was further confirmed by using $F_{up\text{-}M13}$ and $R_{down\text{-}M13}$ primers. The pGEMvirG were sequenced using ABI PRISMcc BigDyeTM terminator cycle sequencing kits. The samples were read using an ABI PRISMcc 3730XL automated sequencer (Macrogen Inc., Seoul, South Korea); sequencing was performed in triplicate. The similarity analysis of virG gene and protein sequence were carried out by using blast program at NCBI (http:// blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignment was performed with CLC sequence 6.2 viewer software.

Construction of a cat cassette and gene replacement protocol

The one-step PCR product was produced by using pKD3 as a template, which contains the 'cat gene' flanked by two FRT sites (Datsenko and Wanner, 2000). This cassette ware generated by using the following primers: $F_{up\text{-}vrgH1}$ and $R_{down\text{-}\ vrgH2}$. These primers contains 46-nucleotide (nt) homologous extensions of the virG to be inactivated. The PCR program was performed at 95°C for 3 min, followed by 35 cycles at 94°C for 30 s, 56°C for 35 s, 72°C for 1 min and final extension at 72°C for 10 min. For electroporation, the PCR products were purified using a high pure PCR product purification kit (Qiagen) and dissolved in 10 µl of 10% glycerol. An overnight culture of *S. dysenteriae* strain grown at 37°C with aeration was diluted with fresh LB broth 100 times and the cultivation continued up to 0.6 to 0.8 ($OD_{600\ nm}$). Cells from 8 ml were washed three times with a non-equal volume of 10% cold glycerol included 5, 2.5 and 1 ml respectively; then resuspended in 40 µl of 10% cold glycerol. Just before electroporation, 1 µl of pkD46 was added to the cell suspension. The procedure of plasmid electro-transformation was performed using the GenePulser and pulse controller ("BioRad", USA). The applied pulse parameters were: electric field strength of 2.0 kV, 25 mF and 200 Ω. After electroporation, immediately, 1 ml of LB medium was added to the cell suspension. Then the cells were cultivated under aeration at 30°C for 18 to 36 h and plated on LB-agar containing the ampicillin. All steps described earlier were exactly performed for control sample. A fresh colony of a *S. dysenteriae* strain carrying a temperature sensitive plasmid pKD46 which encodes the Bet, Exo and Gam proteins controlled by the araBAD promoter (Datsenko and Wanner, 2000) was inoculated in LB containing ampicillin and grown overnight at 30°C. The overnight culture was diluted in 25 ml LB containing ampicillin and L-arabinose (10 mM), and then grown for at least 3 h at 30°C with vigorous shaking. After incubation on ice for 20 min, the cells were collected by centrifugation at 4°C, washed three times with 25 ml

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Characteristics	Source or references
Strain		
S. dysenteriae type 1	A virulent wild-type from patients with diarrhea isolated 2008-2009 in Iran, Amps, Cms.	This study.
Δ*virG S. dysenteriae* type 1	ΔvirG::FRT::cat::FRT	This study
S. flexneri 2b	ATCC12022	Was a gift from Farshad et al. (2006).
E. coli DH5α	F–, φ80d, lacZΔM15, endA1, recA1, hsdR17(rK –mK –), supE44, thi-1, gyrA96, relA1, Δ(lacZYA-argF)U169	Laboratory strain.
Plasmids		
pGEM-5Zf(+)	Derived from the pGEM-3Zf(+) Vector and contains the origin of replication of the filamentous phage f1.	Promega.
pKD46	cZWJ16, hsdR514, ΔaraBADAH33,	Datsenko and Wanner (2000).
pKD3	FRT-cat-FRT, PS1, PS2, oriR6K, AmpR, CmR	Datsenko and Wanner (2000).
pCP20	FLP+, λ cI857+, λ p_R flp, Amp^R, Cm^R.	Cherepanov and Wackernagel (1995).

ice-cold 10% glycerol and finally resuspended in 200 µl ice-cold 10% glycerol.

Electroporation was carried out with a bio-rad gene pulser and pulse controller (Bio-Rad) using cuvettes (0.1-cm gap) filled with 50 µl electrocompetent cells and 10 µg PCR product and the following settings: 1.8 kV, 25 mF and 200 Ω. The electroporated cells were added to 1 ml LB broth incubated for at least 3.5 h at 37°C with vigorous shaking and then spread onto LB agar containing Cm at 37°C. The resulting Cm-resistant transformants were checked for loss of pKD46. We verified it by their inability to grow on ampicillin -containing medium. This mentioned process is briefly described in Figure 1.

Elimination antibiotic resistance cassette and sequencing analysis

The Cm cassette was removed using a temperature-sensitive helper plasmid, pCP20 which expressed the FLP recombinase (Cherepanov and Wackernagel, 1995). Strains containing a FRT flanked cat insertion were transformed with pCP20, recovered and plated on LB agar containing amplitude at 24°C. Transformants were streaked onto LB agar containing amplitude at 28°C, re-streaked non-selectively onto LB agar at 42°C and then tested for loss of an antibiotic resistance cassette. The majority lost the FRT-flanked cat gene and pCP20 simultaneously. To verify for gene disruption on the invasive plasmid of *S. dysenteriae*; PCR analyses were performed by using external primers ($F_{up\text{-}E1}$/$R_{down\text{-}E2}$). These external primers were designed in 300 bp flanks of virG gene in intragenic regions. A freshly isolated colony was suspended in a PCR working solution. The PCR program was as follows: 95°C for 5 min, followed by 30 cycles at 94°C for 30 s, 56°C for 35 s and 72°C for 1 min. The expected sizes of the PCR products are shown in Figure 3. Finally, to check for chloramphenicol cassette deletion, 300-nt regions upstream and downstream of the mutant were sequenced.

In order to calculate the recombination efficiency, we used a form of single experiment which has any verified positive recombinants (Table 3).

Animal immunization

The native attenuated *Shigella* live strain was used to immunize guinea pigs individually. In a first experiment, overnight 'tryptic soy agar plates' containing 0.05% Congo Red (w/v) cultures were brought to the appropriated concentration of bacteria in phosphate buffered saline (PBS) by optical density at 600 nm. Hartley guinea pigs were inoculated in the conjunctival sac with 10^8 CFU of a native wild-type strain for the protection studies. Guinea pigs were checked out daily for 5 days and their inflammatory responses were arranged. The scoring and individual analyzing of guinea pigs daily were performed and the results was blinded as to which of them had received and what each eye had been inoculated. When eyes were inoculated for immunization efficacy studies of vaccine candidate, immunization was executed twice at 2-weeks intermissions. 4 weeks after the last immunization,

Table 2. Primers used in this study.

Primer name	Sequence (5′to 3′ direction)
Fup-virG	ATCTTTTCAGGGGTTTATCAACC
Rdown-virG	TGGGTCCCAGAGAAATGCA
Fup-M13	GTAAAACGACGGCCAGT
Rdown-M13	CAGGAAACAGCTATGAC
Fup-vrgH1	ATGAATCAAATTCACAAATTTTTTTGTAATATGACCCAATGTTCAGTGTAGGCTGGAGCT GCTTCG
Rdown- vrgH2	ATCAGAAGGTATATTTCACACCCAAAATACCTTGGGTGTCTCTGTCATATGAATATCCTC CTTAGT
Fup- E1	ACTGAAAAGTTGCGGTCTGA
Rdown- E2	GCATTAGTTTCTGCAATACC

guinea pig eyes were challenged with a native wild-type strain and scored for progression of disease and protection. Development of disease was rated as follows: 0, no disease or mild irritation; 1, mild conjunctivitis or late development and/or rapid clearing of symptoms; 2, keratoconjunctivitis without pus; 3, fully developed keratoconjunctivitis with pus. Percent protection is defined as follows: full, percent of eyes rated 0; partial, percent of eyes rated 1; combined, amount of complete and partial (Noriega et al., 1996).

RESULTS

PCR to determine extent of virG sequence

The PCR primers used in this study amplified a 3309 bp fragment of virG gene in *S. dysenteriae* 1 strain native of Iran. The other bacterial species included *S. typhimurium* were showed as negative strain by PCR (Figure 2). The PCR product was cloned into pGEM-5Zf vector using $F_{up\text{-}virG}$ and $F_{down\text{-}virG}$ into DH5α as host cells. After ampicillin and white colonies selection, positive colonies were screened for the insert using the same primers and PCR conditions. Positive colonies showed about 3339 bp amplicon. Restriction enzyme analysis of purified plasmid showed the expected bands. Comparison of the virG gene from a native strain with previous virG sequences (CP000035) from other serotypes to the database demonstrated that *S. dysenteriae* type 1 native of Iran was 100% identical to other previously published gene and protein sequences of VirG from *shigella* serogroup.

virG disruption on the *S. dysenteriae* type 1 by recombineering and mapping virG deletion

The gene knockdown was made by electroporating a PCR product that contains a cat cassette flanked by 46 bp homology to the virG gene into *S. dysenteriae* type 1 expressing the λ Red system. The presence of Bet, Exo and Gam promotes the virG gene deletion of the invasive plasmid via homologous recombination between the target region and the flanking sequences of the PCR product. In order to utilize this system in shigella, we first introduced the λ Red operon into the native *shigella*. Then, $F_{up\text{-}\ vrgH1}$ and $R_{down\text{-}\ vrgH2}$ was designed in order to amplify the 46-nt upstream and downstream respectively, short homologous extensions of virG gene [(H1: homology arm upstream and H2: homology arm downstream (Figure 1)] while each primer contains at its 3' end contained a 21 bp-region homologous to the priming sites [(P1: priming site 1 and P2: priming site 2, Figure 1)] of pKD3 as template. The 1.1 kb PCR fragment was electroporated into *shigella* containing pKD46 previously, induced by L- arabinose for the expression of the λ Red operon. Aliquots of 10 µg of electroporated DNA allowed the selection of 12.5 Cm resistant recombinants. The ΔvirG::cat mutant as revealed by their ability to grow on chloramphenicol medium. The correct integration of the chloramphenicol cassette was then confirmed by PCR in five independent colonies of *Shigella*.

In this step, the PCR reaction was performed by $F_{up\text{-}\ E1}$ and $R_{down\text{-}\ E2}$; these two primers amplified a 1600 and 3308 bp fragments from ΔvirG::FRT::cat::FRT and wild type strain, respectively Figure 3.

Removal of the cat resistance cassette flanked by FRT sites from invasion plasmid of *S. dysenteriae* type 1 mediated Flp protein

Finally, the cat gene was eliminated using a pCP20 which contains λ_{pR} promoter under the control of the thermal induction of λcI857 repressor. PCR analysis with sequences flanking the disrupted virG was used to ensure that the flanking sequences remained unchanged after the recombination events. To verify for cassette

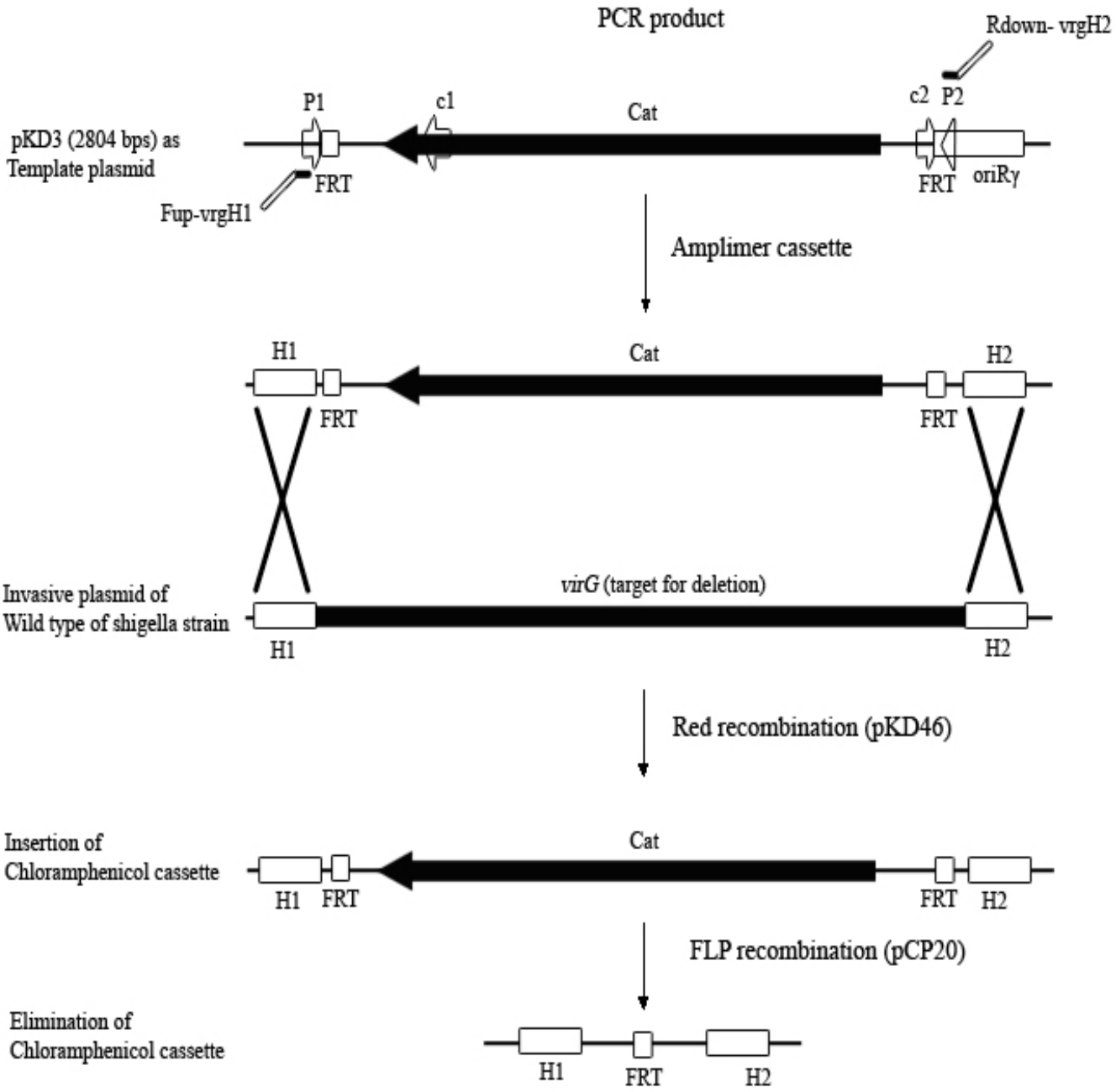

Figure 1. Scheme of a construction of single gene disruption using the λ Red recombineering system. The protocol was used to construct the amplimers containing short homologous extension to the virG gene while it was replaced by the antibiotic resistance gene. The figure shows the protocol used for the cat gene.

elimination on an invasive plasmid of a native mutant strain, sequencing were performed using external primers ($F_{up-\ E1}$/$R_{down-\ E2}$) which anneal to specific locations on invasion plasmid depending on the location of the virG gene. The result of PCR reaction with external primers confirmed the loss virG sequence (Figure 3, lanes 1 and 2). The data of sequencing confirmed 3220 bp of wild virG gene was removed. Furthermore, the FLP-mediated excision of the FRT-flanked antibiotic resistance gene was shown to leave behind an ~ 84 bp 'scar' sequence in-frame with the virG gene. This residual fragment (H1 + P1 + FRT + H2 + P2) includes a single FRT site.

Transformations with pKD46 containing *Shigella* strain typically generated 0 to 150 antibiotic resistant colonies. PCR examination to recognize correct recombination events were anywhere from 0 to 100% positive for gene disruption. The following results are mentioned in Table 3.

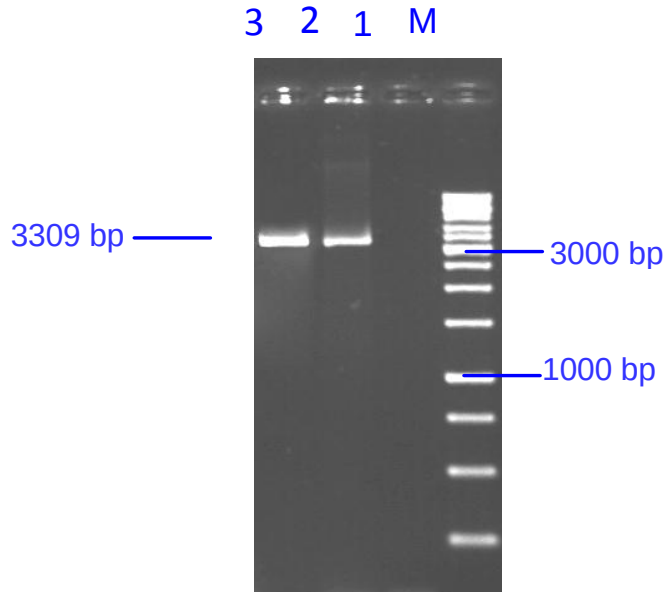

Figure 2. PCR amplification of virG gene. Marker 1 Kb (lanes M), *S. typhimurium* as negative control (lanes 1), *S. flexneri* 2b ATCC12022 as positive control (lanes 2) and *S. dysenteriae* type 1 (Lanes 3).

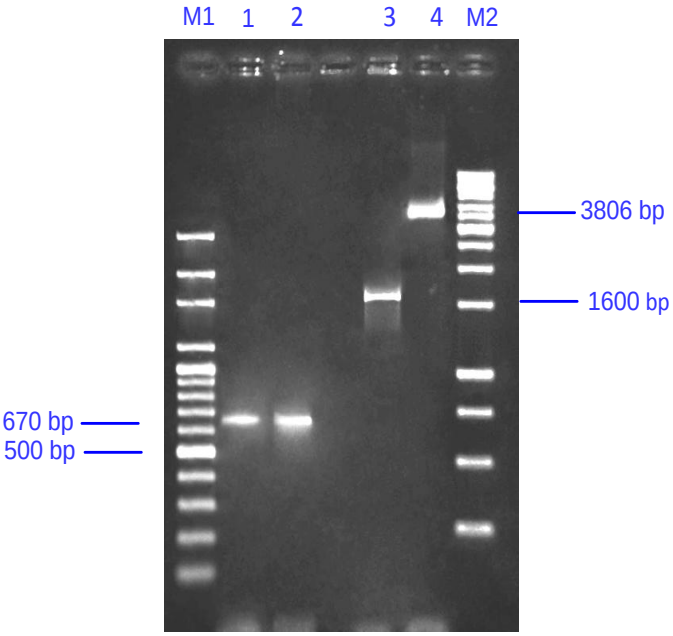

Figure 3. Three different sizes of PCR product with the same external primers. Marker 500 bp and 1 Kb (lanes M1 and M2, respectively), the mutant strain after cat cassette deletion (lanes 1, 2), the mutant strain before cat cassette deletion (lanes 3) and virG gene in the wild type strain (lanes 4).

Safety and immunization test of a native vaccine candidate in guinea pig eyes

In order to compare the effectiveness of the ΔvirG deletion mutation with that of the wild type strain and to assess the safety and immunogenicity of a native *S. dysenteriae* type 1 (ΔvirG) as native vaccine, a randomized and keratoconjunctivitis safety test (Sereny test) was performed comparatively with guinea pigs and a blinded observer. 24 h after a high-dose (> 10^9 CFU per eye) inoculation, the incidence and severity of the initial inflammatory response were significantly different between the ΔvirG strain and the native wild type strain, although this mild inflammation resolved faster in the animals inoculated with ΔvirG strain (Table 4). At 48 h, 3 of 8 animals inoculated with ΔvirG strain but all of the animals inoculated with a native wild type strain had residual signs of inflammation (Table 4). At 72 h postinoculation, the (blinded) observer grading the inflammatory response in the guinea pigs could distinguish 2 of 8 animals that received the attenuated mutant (ΔvirG strain) from the animals that challenged by wild-type strain which had full-blown purulent keratoconjunctivitis (Table 4).

DISCUSSION

Previous studies have demonstrated that the host's immune response and protection against shigellosis is serotype-specific. In the last decades, a number of shigella vaccine candidates have been constructed and evaluated in human adults which in the majority of them have focused on characterization and immunization with protein's bacterial encoded by virulence genes located on invasive plasmid that have key roles in pathogeneticity of shigella strains (Fries et al., 2001; Jennison and Verma, 2004). *Shigella* is highly infectious for its ability to cause clinical disease in adult volunteers after ingestion of as few as 10 organisms. One approach to immunization against shigellosis has been develop to constructing the live attenuated vaccine, generally based on the elimination of the virulence genes such as entrotoxins, virulence genes and introduction of mutations in metabolic genes is lead to limitation in bacterial growth. An example of a very highly attenuated strain, CVD 1207, an enterotoxin-deleted *S. flexneri* 2a candidate vaccine also attenuated for intercellular spreading by virG (an alternative designation for icsA) deletion when the wild-type of *S. flexneri* 2a strain (2457T) is administered to volunteers as little as 10^3 CFU consistently causes diarrhea in 80 to 90% of the volunteers (DuPont et al., 1972). The unique inclination of *S. dysenteriae* type 1 to cause epidemics of severe shigellosis with high attack rates makes a convincingness case for the development of a monovalent live attenuated vaccine that could be administered to refugees and aid workers in high-risk and poor hygiene situations (Kotloff et al., 1995). Initially, the aim of this research was to identify, clone and sequence the virG gene and then constructing a native *S. dysenteriae* type 1 (ΔvirG) and finally to assess the safety, immunogenicity and protective efficacy in guinea

Table 3. Overview of mutant in an Iranian native *S. dysenteriae* type 1 (ΔvirG) by use of recombineering.

Strain	Serotype	Function	Efficiency (%)*
A native *S. dysenteriae* (ΔvirG)	Type 1	Cell- to- cell spread	~ 76

*Efficiency calculated by the following relation: $effeciency = \frac{\text{positive Colonies}}{\text{total tested}} \times 100$.

Table 4. Sereny test in guinea pigs of a native vaccine candidate strain.

Total of animals with degree of inflammation* at:															Sum of no. animals	Genotype†	Strain
72 h					48 h					24 h							
4	3	2	1	0	4	3	2	1	0	4	3	2	1	0			
4	0	0	0	0	4	0	0	0	0	4	0	0	0	0	4	Wild $^{CR+}$	A native of *S. dysenteriae* type 1
0	0	1	1	6	0	0	2	1	5	0	1	2	1	4	8	Wild $^{CR-}$	A native of *S. dysenteriae* type 1 (ΔvirG) as vaccine candidate.

†Genotype: Congo red positive, CR+; Congo red negative, CR-. *Inflammation degrees: 0, normal; 1, palpebral edema; 2, palpebral edema with conjunctival hyperemia only; 3, conjunctival hyperemia plus slight exudate; 4, full-blown purulent keratoconjunctivitis.

pigs of ΔvirG strain as vaccine candidate. It is clear that the attenuation in the ΔvirG strain may be due to both reduction in invasion and a severe limitation in its intracellular spreading. The vaccine candidates, which is constructed by 'aro' or 'gua' auxotrophic mutants through introducing disruption in these genes, demonstrated that these strains have exhibited some residual reactogenicity when administered in high doses to volunteers (Noriega et al., 1996). In the past, there are many methods that were used for attenuating virulent bacteria and viral pathogens included serial passage and chemical mutagenesis through nonpermissive hosts (Ranallo et al., 2006; Durnev, 2008; Chalker and Davis, 2010).

Overall two strategies are engaged: at first the disruption of identified virulence factors such as invasions and toxins, but as this may inadequately attenuate the bacteria, the possible secondary introduction of mutations that limit bacterial growth and replication in host such as metabolic pathway (Kotloff et al., 1995). We believe that the ΔvirG mutations in a native S*. dysenteriae* will bring us close to a suitable balance between immunogenicity and minimal reactogenicity. One strategy for developing a native vaccine candidate is to generate a strain in which the fundamental mutation is a deletion in plasmid-located virG (also known as icsA). The immune response of host to infection by shigellosis is serotype specific and protective against reinfection by the same serotype, thus development of effective novel vaccination strategies is urgently required. The ΔvirG mutant that was constructed in this study can be used in development of a native attenuated *Shigella* vaccine in future. Noriega and colleagues surveyed the attenuating effects of chromosomal disruption affecting enzymes employed in the distal purine biosynthesis pathway. Initially, they generated CVD 1204 by homologous recombination. When directly compared with a ΔaroA mutant strain, CVD 1204 was significantly less invasive in HeLa cells and caused remarkably less conjunctival inflammation in the guinea pig keratoconjunctivitis test (Noriega et al., 1996). The CVD 1205 as live attenuated candidate vaccine obtained fewer inflamed eyes than did CVD 1204, although this difference was not statistically significant. In a next effort, in order to minimize diarrheal reactogenicity, CVD 1207 was more attenuated compared with previous constructs such as (ΔaroA ΔvirG) CDV1203 or (ΔguaB-A ΔvirG) CVD105, which harbors deletion mutations in the *S. flexneri* 2a (Noriega et al., 1996; Kotloff et al., 2000). Our findings revealed that our constructed vaccine is a notably immunogenic vaccine candidate. Although the detailed immune responses that correspond with protection against shigellosis are not known, there are observations that type-specific immunity is of critical importance.

The probability of person-to-person transmission of a native vaccine candidate should be precisely evaluated in future vaccine trials such as detection presence of shigellae in feces through molecular procedures. Also, our data confirm the results of previous studies with ΔvirG *S. flexneri* 2a strain SC602 that recommended that small doses of a strain ($\leq 10^5$ CFU) with a single mutation in virulence gene (virG) may be enough to attenuate *S. flexneri* 2a for humans. The balance of immunogenicity in contrast to attenuation remains a challenge for generation of live attenuated vaccine candidate (Jennison and Verma, 2004). WRSd1 vaccine strain that was derived from clinical isolate 1617 represented live, oral *S. dysenteriae* type 1 vaccine candidates primarily attenuated by the loss of the virG (icsA) gene. This candidate vaccine has been manufactured at the Walter Reed Army Institute of Research, Maryland. Based on

the finding of gene disruption, it appears that WRSd1 will be protected at doses that demonstrated clearly reactogenic for WRSS1 and SC602 (Coster et al., 1999; Teska et al., 1999; Rahman et al., 2011). The approach we have adopted is to construct a live attenuated *S. dysenteriae* type 1 and vaccine carrying disruption in virG as important and major gene in pathogeneticity of *S. dysenteriae*. At first, we should be aware of the nucleotide sequence of virG in order to utilize the recombineering method. The current research reveals the similarity between the predicted virG gene from *S. dysenteriae* type 1 and those from different *shigella* serotypes.

Application of original techniques including chemical mutagenesis and serial passage for construction attenuating virulent bacteria are nonspecific and random method. Gene replacement in bacteria can be achieved by a variety of techniques. Approaches are based on homologous recombination and genetic engineering has been replaced with early techniques. In *shigella* Spp, the method traditionally used to inactivate invasive plasmids and chromosomal genes requires several DNA cloning steps into a suicide vector in the bacteria. The considerable approaches have been exploited during recent years; they expanded our knowledge about *Shigella* pathogenesis and allowed to design live attenuated *Shigella* strains which induce protective systematic and mucosal immune responses in human clinical trials after oral delivery without reactogenicity. The targeted disruption of virulence genes has been utilized to construct strains with deletions in both metabolic and virulence-associated genes such as *S. flexneri* (Fontaine et al., 1990; Kotloff et al., 1995; Jennison and Verma, 2004; Katz et al., 2004), *S. dysenteriae* (Venkatesan et al., 2002) and *S. sonnei* (Hartman and Venkatesan, 1998). Generally, different methods were developed in attenuation of vaccine strains, dosing schedules, requirement of growth and animal model may explain the differences in efficacy (Jennison and Verma, 2004). A native vaccine which can provide broad protection against bacillary dysentery occurred by *shigella* requires the construction of live invasive vaccine which can be able to generate long lasting protection in comparison with the other vaccines such as killed vaccines. One of the important methods to construct mutation in bacteria is λ red recombineering technique.

One of the highly attenuated strain which deleted an enterotoxin gene of *S. flexneri* 2a candidate vaccine (CVD1207) also attenuated for intercellular spreading by virG deletion, furthermore attenuated for *in vivo* proliferation by deletions of guaA and guaB was able to colonize healthy US recipients, and was tolerated at doses up to 10^{10} CFU, but an isogenic derivative (designated CVD 1204) with a single deletion in the guaBA operon of 2457T generates a strain that is notably attenuated compared to its wild type parent (*S. flexneri* 2a strain 2457T) but one that continues to cause shigellosis in 35% of recipients who ingest 10^7 10^8or 10^9 CFU with buffer (Kotloff et al., 2000). The *S. flexneri* Δ*icsA* and Δ*iuc* vaccine SC602 was immunogenic in US volunteers at an oral dose of 10^3 CFU, fully protective against shigellosis after a challenge with an oral dose of 10^4 CFU as the majority of volunteers developed the illness with an oral dose of 10^8 CFU (Rahman et al., 2011). One of the first generation of live attenuated vaccines that were produced against shigellosis were T32- ISTRATI and *S. flexneri* streptomycin dependant strains (SmD) which is generated by serial passage as original technique. Venkatesan et al. (1991) characterised T32-ISTRATI that was derived from *S. flexneri* serotype 2a as parent strain and they found at least three critical virulence loci of invasive plasmid included ΔipaBCDA, ΔinvA and ΔvirG have been deleted (DuPont et al., 1972; Venkatesan et al., 1991). The early approaches have several disadvantages such as nonspecificity and came back to wild type possibility. These original methods have been replaced by improved techniques, which are based on homologous recombination including suicide system, gene goreging (Herring et al., 2003) and recombineering (Court et al., 2002). In post genomics era, targeted deletion of virulence-associated genes has been expedite by more complete genome sequencing laborious, increase understanding our knowledge about detailed mechanism of pathogeneticity of bacteria and emerging new techniques that are based on homologous recombination event. Numerous suicide vectors and phages based systems are available in order to manipulating bacterial genomes through combination.

The main advantage of suicide systems in comparison to serial passage, it provides independent estimation frequency at which disruption of the target gene should occur and therefore, allow us to one explain events that have occurred in bacteria. The suicide plasmids pIB279 and pKTN701 was manipulated by Noriega et al. (1996) to insert aroA and virG deletion mutations in *S. flexneri* 2a strain. Construction of ΔvirG2 has a 212-bp deletion in the virG has been engineered by several steps into pCVD442 as suicide vector and was used to construct virG deletion in WRSS1 (*S. sonnei*) and WRSd1 (Hartman and Venkatesan, 1998; Venkatesan et al., 2002). Despite all advantages of suicide system in modifying bacterial genome, but an extensive *in vitro* engineering for constructing recombinant plasmids, more time consuming, low frequency of recombination efficiency and longer regions of homology, using this system was complicated. Thus, this approach has been replaced with new emerging methods which promoting allelic exchange through homologous recombination using Exo, Bet and Gam from lambda bacteriophage (Court et al., 2002). Since the recombineering system represented one of the best characterized recombination systems which can be used to create mutation, therefore we chose this technique. Ranallo et al. (2006) have

previously generated that the WRSf2 (*S. flexneri 2a*), WRSd1 (*S. dysenteriae* type 1) and WRSs (*S. sonnei*) which based on virG mutant by using the different expression vector systems (pKM208) and they were able to successfully construct gene deletions in virulence genes of *shigella* Species (Ranallo et al., 2006). But in this research, we used another expression system which the λ red (gam, bet and exo) genes from lambda phage under the control of the AraC repressor which is induced by L-arabinoes. Here, we report gene inactivation frequencies produced by different treatments of plasmid to construct precise genetically defined deletions of *shigella virG.* Our results indicated that 46-nt homology between the PCR product and the target gene is sufficient to occur correctly recombination. Explanations of the undesirable recombination events are difficult because it might have several reasons.

The presence of regions with similarity to the other location of *shigella* genome caused the integration of antibiotic resistance cassette into unexpected sites. Sequencing of several *shigella* genomes shows the wide-spread occurrence of insertion sequences (IS elements) (Hamilton et al., 1989; Link et al., 1997) is very important to reduce efficiency of correctly recombination. In addition, one of the difficulties we have encountered included the screening hundreds of colonies observed after elimination antibiotic cassette in order to find a strain with the true recombination occurrence. This differentiating immunogenicity among different live attenuated oral Shigella vaccine candidates aforementioned could be attributed to background immunity against the vaccine strains in the participating population and to different population characteristics. However, this mutant can be suitable for constructing a multivalent *Shigella* vaccine. In conclusion, recombineering (recombination-mediated genetic engineering) is a new tool, powerful, inexpensive, rapid, simple method for the construction of *in vivo* genetic engineering that allows creation of DNA modification to be made efficiently and easily. Production of vaccine strains including multiple gene disruption can be obtained by using this technique. Safety and immunogenicity of a native genetically mutant was needed for testing in animal model.

During recent years, as more bacterial genome have been published, the study of *shigella* genomics displayed there are many of the gene having uncharacterised or unknown role, this method can be used to understand role them. Furthermore, systematically disrupt of virulence associated genes of *shigella* was reached quickly by recombineering. The A native *S. dysenteriae* type 1 is a relatively weak vaccine candidate that went through one deletion. Shigella vaccine has been followed for decades with proportionately less favorable outcome. Another consideration for future trials is to construct engineered *S. dysenteriae* type 1 strain which is more attenuated and then to assess their immunogenicities and reactogenicities in relation to the current native vaccine.

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