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λ-Red-Recombineering Live Attenuated *ΔipaD Shigella dysenteriae* from Iranian Isolates as A Candidate of Vaccine

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Abstract

Shigella species (spp.) are gram-negative bacteria that are responsible for shigellosis. Although it can be controlled via antibiotics, increasing number of antibiotic resistant isolates of *Shigella* have been reported. Therefore, other strategies such as production of specific vaccine against this organism could be a suitable therapeutic approach. Attenuated live vaccines produced by gene deletion are advised candidates of vaccine against *shigella*. The *ipaD* gene is responsible for *Shigella* invasion which act as a chaperone for some other invasion proteins such as IpaB and IpaC. We used λ-red recombination system to delete the *ipaD* gene in *shigella* that end to production of a live attenuated form of *Shigella dysenteriae*. This strain has a high prevalence in Iran that make it suitable target for vaccine production. Our results showed that live attenuated *ΔipaD* S*higella* was produced via λ-red recombination system. We suggest that application of *λ*-red recombination could be a simple and useful technique for production of live attenuated *ΔipaD Shigella* as a candidate of vaccine against shigellosis.

Keywords: *Shigella dysenteriae*; λ-red Recombination; *ipaD*; Attenuated live vaccine; Gene deletion

Introduction

Shigella species(spp.) are gram-negative, non-spore forming and entropathogenic bacteria [1]. *Shigella dysenteriae, flexneri, sonnei* and *boydii* are four species present in this genus. *Shigella spp.* are responsible for

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shigellosis [2], an acute endemic disease in tropical regions of developing countries, which causes difficulties requiring hospitalization, in adults and neonates. *Shigella spp* are attributed to 163 million diarrheal cases that end to one million deaths per year worldwide. Studies have shown that shigellosis incidence rate is lower in developed countries (1.5 million cases / year)[1] as compared with developing countries such as India [3, 4], south Africa [5] and Iran [6]. *Shigella* is an intestinal pathogen and its contribution to pathogenicity of low doses has been frequently reported [7, 8]. *Shigella* infection is transmitted by the fecal-oral route and via contaminated water or food [9]. The control and therapy of shigellosis is possible by antibiotics, although the high cost of antibiotics and increasing level of antibiotic resistance found in *Shigella* isolates [1]. High antibiotic resistancy of S*higella dysenteriae* and *Shigella sonnei* is well documented in *Iran* [10, 11].These groups of *Shigella* need to be controlled by different therapy methods, such as vaccination. It seems that vaccination against shigellosis is a suitable approach to solve this problem. Recent studies have suggested that attenuated live vaccines produced by gene deletion are advised [12].

After entrance, *Shigella* causes cellular invasion in colon and devastation of the colonic epithelium that leads to induction of acute inflammatory response [13]. Furthermore, in epithelial cells, the mentioned organism promotes its own entrance by eliciting a pinocytic episode [14, 15]. The process of *Shigella* pathogenicity is initiated by its contact with host cell [16, 17] followed by secretion of a variety of effector proteins which is led to dysentery [12, 15]. The mentioned effector proteins are encoded by a locus of genes (*ipa-mxi-spa*), homologous to genes in other pathogens [18]. The *ipa-mxi-spa* locus is in a 31 kb pathogenicity island on 186kb virulence plasmid. This locus consists of 20 genes, coding for a type III secretion system (TTSS). The TTSS forms a needle-like structure and contribute to secretion of invasion plasmid antigen (Ipas) proteins [19]. The Ipas are encoded by *ipa* operon [13] and contain five proteins, IpaA-D and -H. These proteins are vital for the bacterial entrance process into epithelial cells [20]. The Ipas translocate into the intracellular section of the intestinal cells through the TTSS. These proteins change cell cytoskeleton and signal transduction pathway in host cell [19, 21, 22]. In addition, there are other effector proteins like Ipas that modify host cell environment and cause intracellular endurance [23, 24]. Sansonetti and Egile [25] suggested that the secretion of IpaB, -C and –D proteins could be started by the bacterial contact with epithelial cell surface. As a result, they can form a complex that interacts with the target cell [26], and attach to the α5β1 integrin and CD44 on the cell surface [27, 28]. Subsequently different biomolecules such as pp60c-src [29, 30] and GTP-binding protein (Rho)-mediated are activated by the attachment of bacteria to the host cells [31, 32]. It has been shown that proliferation [33], colonization[34] and transmission [13, 35] of the bacteria are depended on these biomolecules [28, 36]. The *ipaD* gene encodes IpaD protein and play critical roles in *Shigella* invasion [37-39]. Moreover, not only IpaD acts as a chaperone for some other invasion proteins such as IpaB and -C proteins but also regulate secretion of them [40]. In addition, IpaD interacts with *TTSS* which is a key element for *Shigella* invasion, cell to cell migration and dysentery. In this report we have described the production of an attenuated live *ΔipaD Shigella dysenteriae* (1020) as a candidate vaccine.

Materials and Methods

Shigella Strain

Wild-type *Shigella* strains were isolated from patients with dysentery from Mofid Hospital, Tehran, Iran. These strains were characterized by biochemical, monoclonal antibody and antibiotic resistancy tests.

Media and Reagents

Media used in this study were LB broth (Difco, USA), MacConkey Agar and TSB (MERCK, Germany). SOB and SOC media were prepared as described elsewhere [41]. *Taq* and *Pfu* polymerases (Fermentas, Germany) were used to generate *Cat* cassette which plays critical role in mutagenesis. Selection for presence of appropriate plasmids and desired markers were based on antibiotics (Sigma, Germany) at the following concentrations: ampicillin, 100μg/ml; chloramphenicol, 32μg/ml [42, 43].

Plasmids

The *E.coli* BW25113, BW25141 and DH5α strains were kindly provided by Dr. Naieri (Department of Veterinary medicine, University of Tehran) (Table 1). The *E.coli* BW25113 harbors plasmid pKD46 expressing λ-recombination proteins γ, β and exo under the control of the arabinose-inducible ParaB promoter (Fig. 1-a) (NATURE TECHNOLOGY CORPORATION, England). The BW25141 strain carries plasmid pKD3 and The DH5α strain harboring the pCP20 plasmid (E. coli Genetic Stock Center, Yale University, New Haven, Conn.).

Table 1. Bacterial strains and plasmids

Strains or plasmids	Related genotype or characteristics	Reference
Strains		
BW25113	*lacI^q rrnB3 ΔlacZ4787 hsdR514 DE(araBAD)567 DE(rhaBAD)568*	[45]
BW25141	*lacI^q rrnB3 ΔlacZ4787 ΔphoBR580 hsdR514 DE(araBAD)567 DE(rhaBAD)568 galU95 ΔendA9 uidA(DMluI)::pir(wt) recA1*	[45]
Specific DH5α	*amp^R , chl^R*,harboring pCP20 plasmid	This work
S.dysenteriae (1020)	*isolated from patient amp^S chl^S kan^R*	This work
SD146	*amp^R chl^S kan^R*	This work
SD1-46C+	*amp^S chl^R kan^R*	This work
SD1-D	*amp^s cm^s*	This work
Plasmids		
pKD46	*araBp-gam-bet-exo, bla(ApR), repA101(ts), oriR101*	[45]
pKD3	*FRT-cat-FRT, oriR6K$_\gamma$, bla(ApR), Amp^R, cat, rgnB(Ter), PS1, PS2*	[45]
pCP20	*FLP+, λ cI857+, λ p$_R$ flp, Amp^R, Cm^R*	[12]

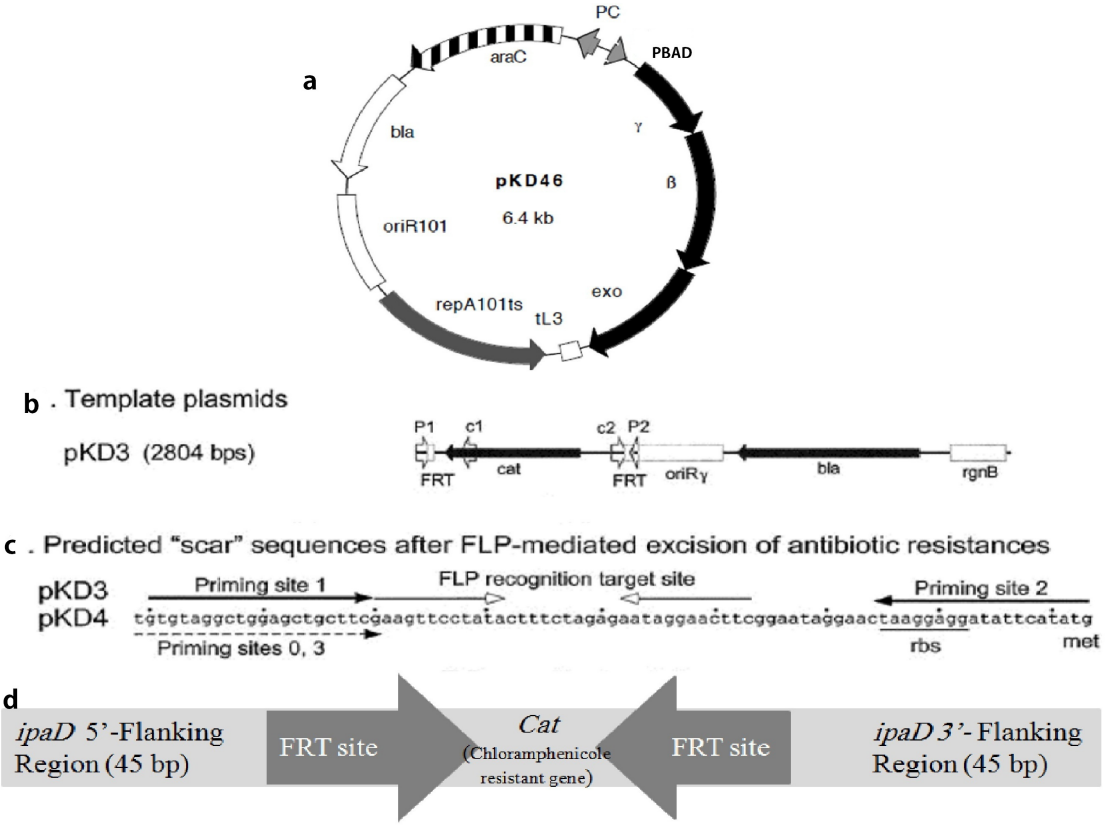

Figure 1. pKD46 plasmid scheme(a), pKD3 template plasmid (b), c-predicted scar sequences after FLP-mediated excision of antibiotic resistances (c), schematic view of *chloramphenicol* resistant construct , used in this study (d).

Table 2. Primers that used in this study

Primer name	Sequence*	Function
IpaDF	tcatgaattcagaacaacaaatcag	Used to confirm *ipaD* presence
IpaDR	tcttaagcttttaagtatatgaactaacg	
5FlD	tatggctatcggatattttgctttttaactcgctgtattctgttggtgtaggctggagctgcttcg	The template used in the PCR amplification was pKD3. The PCR product contained the cat cassette and the flanking regions of *ipa*
3FLD	ccacctcatcattcagcccaaacaataccaacggttcatcaaccgcatatgaatatcctccttagt	
AmpF	caacatttccgtgtcgccct	Amp recognition primers, used to confirm pKD46 presence
AmpR	tcccaacgatcaaggcgagt	
catF	agaaactgccggaaatcgtc	Used to confirm the *ipaD* deletion by sequencing
catR	ccagaccgttcagctggata	

* Underlined Nucleotides are homologous to *ipaD* adjacent regions

PCR Amplification and Construction of Cat Cassette

Genomic DNA was extracted using BioNEER Genomic DNA extraction kit (BioNEER, South Korea) according to manufacturer's instruction. In order to verify the presence of *ipaD* gene, PCR amplification of *ipaD* gene was conducted using IpaDF & IpaDR primers (Table 2). Also, PCR amplification, using 5FLD and 3FLD primers (Table 2), was performed to generate the *Cat* cassette containing 3' and 5' flanking regions of *ipaD* gene. 5FLD and 3FLD primers consist of two features, FRT sites and 45bp homologous flanking regions. FRT sites are substrates for *FLP recombinase* and act both as primers, and homologous regions are main players in recombination (Table 2 and Fig.1- d). The pKD3 plasmid was used as a DNA template for the mentioned PCR reactions (Fig. 1-b, c).

Preparation of Electrocompetent Cells and Generation of Recombinants

S. dysenteriae cells were cultured at 37°C with shaking (220rpm) in SOB medium until late log phase (O_{D600} = 0.6). The cells were centrifuged at 5500g for 5min at 4°C [44], washed three times using ice-cold 10% glycerol. Then, cells were resuspended in 10% glycerol [45]. Electrocompetent cells were transformed by 300ng of pKD46 plasmid using eppendorf gene pulser (eppendorff, Germany) (field strength 1.8 kV, 25µF, 4ms) as described previously [43-46]. The transformed cells were incubated at 30°C for four hours in TSB. Subsequently, cultured cells were screened on MacConkey Agar plates containing appropriate concentration of ampicillin. Selected colonies (Amp^+ colonies) were characterized by PCR using AmpR and AmpF primers (Table 2). The resultant cell carrying plasmid pKD46 named *SD146*, were cultured at 30°C shakingly (220 rpm) in SOB medium containing 100 µg/ml ampicillin and L-arabinose (0.2%) until late log phase (O_{D600} = 0.6) [41]. 55µl of *SD146* competent cells were transformed by 300ng of purified *Cat* cassette PCR products via eppendorf gene pulser (eppendorf,). Afterward, the cells were plated on MacConkey Agar supplied with an appropriate concentration of chloramphenicol. The recombinant bacterial cells were examined using MacConkey Agar containing chloramphenicol and PCR amplification by 5FLD and 3FLD primers. Deletion of *ipaD* gene in recombinant cells was confirmed by PCR using IpaDF and IpaDR primers and PCR-product sequencing by catF primer. The sequencing results were searched using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). The verified recombinant cells which are knock out for *ipaD* gene called *SD1-46C+*.

Deletion of Cat Gene from SD1-46C+ Strain

The *SD1-46C+* cells were cultured at 37°C with vigorous shaking. Electrocompetent cells were prepared from *SD1-46C+* cells and electrotransformed by pCP20 plasmid. The cells were cultured in TSB media at 30°C for four hours in shaker incubator, then *Shigella* cells were selected using LB agar containing 100µg/ml ampicillin and followed by incubation at 30°C. Finally, to eliminate the pCP20 plasmid the cells were cultured on MacConkey Agar plates at 37°C and the genetic engineered cells were characterized by PCR using IpaD and FLD primer sets (Table 1).

Results

In this study, *Shigella spp.* strains fidelity were

confirmed by biochemical and antibody tests. The *Shigella dysenteriae* (1020) serotype1, sensitive to ampicillin and chloramphenicol antibiotics was chosen as a target strain for manipulation. Firstly, the presence of *ipaD* gene in the *Shigella dysenteriae* (1020*)* serotype1 was demonstrated through PCR strategy (data not shown). Electrocompetent cells were transformed by purified pKD46 as described in materials and methods section. The transformed *Shigella dysenteriae (1020)* cells harboring pKD46 plasmid were able to grow on ampicillin supplemented media. We verified this result using PCR amplification (Fig. 2).

The *Cat cassette* transformed *SD146* cells were analyzed by two strategies including culturing the cells on MacConkey Agar plates containing an appropriate concentration of chloramphenicol (Fig. 3) as well as PCR amplification of *ipaD* (344bp) and *Cat cassette* (1103bp) (Fig. 3) using specific primers (Table 2). In addition, we employed direct PCR product sequencing to confirm the results were obtained from above strategies. Sequencing data revealed 50% homology with *Shigella dysenteriae* SD197 invasion plasmid sequence (in neighboring regions of *ipaD* gene) and 50% homology with the pKD3 plasmid chloramphenicol resistance gene.

To eliminate the *Cat cassette* gene from the SD1-46+ strain, the pCP20 plasmid was used as a helper. Culturing the transformed cells, under specific conditions (as described in materials and methods section), indicated that pCP20 plasmid is present in transformed cells. It had FLP recombinase and ampicillin resistance activities (Fig. 4) that end to absence of *Cat cassette* in the genetic engineered cells. The elimination of the pCP20 plasmid was performed by culturing the cells at 37°C instead of 30°C.

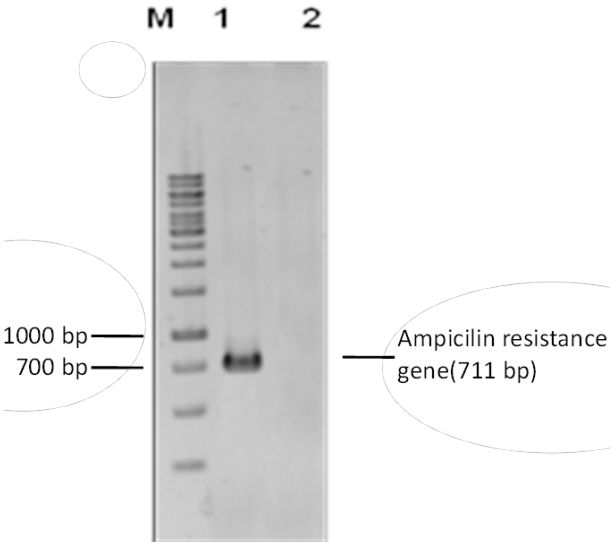

Figure 2. Lane M -size marker Lane 1- ampicillin PCR product using AmpF and AmpR as primers and SD146 cells as a source for DNA template Lane 2- ampicillin PCR product using AmpF and AmpR as primers and wild type *Shigella dysenteriae* as a source for DNA template.

Discussion

The considerable incidence of shigellosis as an acute endemic disease causes major public health problems in

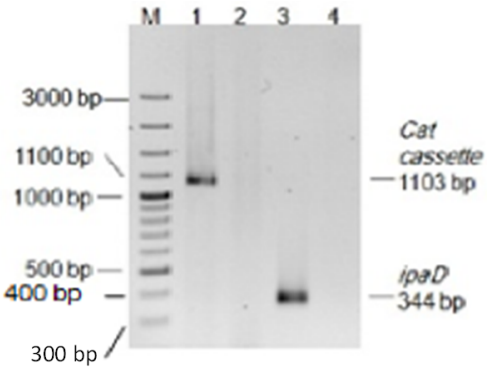

Figure 3. Lane M-DNA size marker Lane 1- PCR amplification using FLD primer set and SD1-46C+ cells as a DNA template Lane 2- PCR amplification using FLD primer set and SD146 cells as a DNA template Lane 3- PCR amplification using IpaDF and IpaDR primers and SD146 cells as a DNA template Lane 4- PCR amplification using IpaDF and IpaDR primers and SD1-46C+ cells as a DNA template.

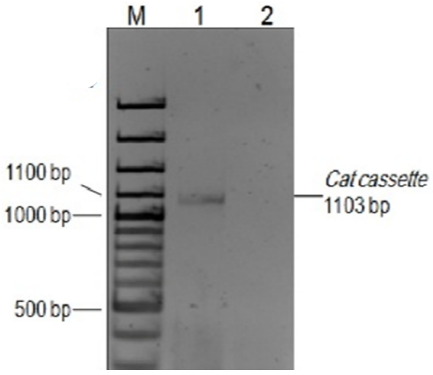

Figure 4. a-SD1-D cells on an ampicillin riched plate b-SD1-46C+ cells on another ampicillin riched plate c-Lane M- DNA size marker Lane 1- PCR amplification using FLD primer set and SD1-46C+ cells as a DNA template Lane 2- PCR amplification using FLD primer set and SD1-D cells as a DNA template.

tropical regions such as Iran. Due to considerable prevalence of *S.sonnei* (54%) and *S.dysenteriae* (24%)[6] and their high antibiotic resistance rates in Iran [47], research centers have been encouraged to develop and generate new strategies for prevention of shigellosis. Therefore, production of an effective vaccine against shigellosis seems to be a valuable approach. Although various vaccines against shigellosis are available, most of them are not efficient. Among these vaccines, live attenuated strains are advised vaccines and they enable to promote some degrees of protective immunity [48]. Some primary approaches such as serial passage and chemical mutagenesis had been used for production attenuated *Shigella* strains. Using these strategies, *S.flexneri* 2a Istrati T32 that is the most successful of these types of vaccines, was produced [49]. Characterization of *S.flexneri* 2a Istrati T32 indicated that at least three critical virulence loci (*ipaA-D*, *invA* and *virG*) of invasion plasmid were deleted [18]. In spite of the fact that the *S.flexneri* 2a Istrati T32 had 100% safety and 85% protection for human beings, this vaccine was too expensive to be cost effective for developing countries [48]. According to literature some disadvantages including reversion to wild type and unspecified deletions for production of this type of vaccine reported.

To date, various advanced techniques such as suicide vectors system, gene goreging and recombineering are currently used for gene deletion [50, 51].

Live attenuated *S.flexneri, S.dysenteriae* and *S.sonnei* strains have been generated through suicide vectors and phage based systems [52-54]. Despite the ability of the suicide vectors system in generation of live attenuated vaccine, there are some disadvantages such as time consuming. Furthermore, the construction of recombinant plasmids by this technique is prone to error. So, to address these problems new emerging methods which are promoting allelic exchange via short homologous recombination has been introduced [50, 51]. Short homologous recombination can be performed using Exo, Beta and Gam, lambda (λ) bacteriophage proteins. In the present study we produced a live attenuated Shigella strain using λ-red recombination system to delete the ipaD gene in shigella. Several gene deletions using λ-red recombineering technique revealed a high capability of this method for production of live attenuated cells.

In a study the λ-red recombineering technique was conducted to eliminate five O157-specific islands (O-islands) of EHEC [55]. In addition, Ranallo and coworkers deleted *virG* λ-red recombineering technique and produced different mutant stains such as WRSf2 (*S. flexneri*) [56], WRSd1 (*S. dysenteriae*)[57] and WRSs (*S. sonnei*) [56]. Here we have described the details of production of a live attenuated *Shigella* stain by deletion of the *ipaD* using λ-red recombineering technique. The *ipaD* is a self chaperonin protein and it is present at the tip of a needle like structure of *Shigella spp*. Also, it is a part of TTSS, a secretory protein, and plays an important role in IpaB and IpaC secretion. The IpaA-D proteins functions are involved in *Shigella* penetration into epithelial cells and cell-cell migration. We suggest that elimination of *ipaD* might inhibit the secretion of IpaD, IpaB and IpaC proteins subsequently resulted in suppression of *Shigella* invasion. To evaluate *ΔipaD Shigella* as candidate vaccine properties in terms of its safety, protection and efficiency in vivo studies are required to be carried out.

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