

Targeting of cholera toxin A (*ctxA*) gene by zinc finger nuclease: pitfalls of using gene editing tools in prokaryotes

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Abstract

Background and purpose: The study was launched to use zinc finger nuclease (ZFN) technology to disrupt the cholera toxin gene (*ctxA*) for inhibiting CT toxin production in *Vibrio cholera* (*V. cholera*).

Experimental approach: An engineered ZFN was designed to target the catalytic site of the *ctxA* gene. The coding sequence of ZFN was cloned to pKD46, pTZ57R T/A vector, and *E2-crimson* plasmid and transformed to *Escherichia coli* (*E. coli*) Top10 and *V. cholera*. The efficiency of ZFN was evaluated by colony counting.

Findings/Results: No expression was observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blotting in transformed *E. coli*. The *ctxA* gene sequencing did not show any mutation. Polymerase chain reaction on pKD46-ZFN plasmid had negative results. Transformation of *E. coli* Top10 with T/A vectors containing whole ZFN sequence led to 7 colonies all of which contained bacteria with self-ligated vector. Transformation with left array ZFN led to 24 colonies of which 6 contained bacteria with self-ligated vector and 18 of them contained bacteria with vector/left array. Transformation of *V. cholera* with *E2-crimson* vectors containing whole ZFN did not produce any colonies. Transformation with left array vectors led to 17 colonies containing bacteria with vector/left array. Left array protein band was captured using western blot assay.

Conclusions and implications: ZFN might have off target on bacterial genome causing lethal double-strand DNA break due to lack of non-homologous end joining (NHEJ) mechanism. It is recommended to develop ZFNs against bacterial genes, engineered packaging host with NHEJ repair system is essential.

Keywords: *ctxA* Gene; Gene editing tools; *Vibrio cholerae*; Zinc finger nuclease.

INTRODUCTION

Cholera, caused by *Vibrio cholera* (*V. cholerae*) O1 and O139 strains, is one of the most prevalent infectious diseases in communities with poor water and sanitation infrastructure (1). The main implication of epidemic cholera is severe dehydration. Annually, 1.4 to 4.3 million individuals worldwide are affected, with an annual mortality of 28,000-143,000 (2). Cholera toxin (CT), an important pathogenesis factor for *V. cholerae* is produced through the integration of

CTX ϕ phage into its genome (3). In 1886, Koch proposed that the symptoms caused by *V. cholera* could be due to some poisons produced by this organism (4). In 1959, S. N. De showed that cell-free extracts from *V. cholerae* cultures induced fluid accumulation in rabbits when injected into ligated small intestinal loops. This test confirmed Koch's postulate (5).

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Later, evidences suggested the presence of a toxic protein product in *V. cholerae* cell-free supernatants (6). After the entrance of *V. cholerae* into small intestine, the ToxR regulon activates the expression of virulence gene through a regulatory cascade. CT and the toxin-co-regulated pilus (TCP) are the most important virulence genes stimulated by the ToxR regulon. CT enterotoxin is responsible for acute diarrhea and TCP is a type IV pilus essential for colonization (7,8). Disruption of the CTX ϕ phage could be an important target to eliminate toxigenesis of *V. cholera* and ultimately decrease pathogenesis of bacteria (9).

CT is an ADP-ribosylating toxin and belongs to the large family of A-B toxins that contains an AB₅ subunit structure. The B subunit (CTB) forms a pentamer that binds to the pentasaccharide part of GM1 gangliosides on the cell surface, and the A subunit (CTA) is cleaved by host proteases into A₂ subunit that attaches enzymatically to active A₁ subunit using a disulfide bond. A₁ subunit releases into cytosol and catalyzes the transfer of an ADP-ribosyl moiety to α subunit of Gs protein, that leads to adenylate cyclase stimulation and triggers cAMP-dependent intestinal fluid hypersecretion *via* ion channels and transporters (10,11).

Genome editing technologies have become a promising and novel method to develop new therapeutic strategies to fight infectious and monogenic diseases (12-16). These technologies such as zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs), clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein 9 (Cas9) can target and modify the genome of an organism (17). ZFNs are the first class of these nucleases, which were discovered in 1996 and used for the first time in 2002 for genetic engineering of drosophila and mammalian cells (18,19). ZFNs are composed of two domains: a DNA binding site that is a tandem array of Cys₂-His₂ zinc-finger and usually contains 3-6 domains that each binds to 3-bp of DNA sequence (19-20) and a cleavage domain of bacterial restriction enzyme *FokI* which must dimerize in order to make double strand breaks (21) and subsequently stimulate DNA repair pathways

including homologous recombination or non-homologous end joining (NHEJ) (22). This research was conducted to investigate the ability of an engineered ZFN to create disruption in *ctxA* gene and consequently to inhibit CT toxin production in *V. cholerae*.

MATERIALS AND METHODS

Design of ZFN construct

The protein sequences coding for the left and right zinc finger protein arms were designed by zinc finger tools (<https://www.scripps.edu/barbas/zfdesign/zfdesignhome.php>). This ZFN targets the catalytic site of *ctxA* and its sequence was obtained from uniprot identifier.

The left and right ZFP arrays consist of three fingers that bind to two sites on *ctxA* gene. The designed protein sequences were then converted to nucleic acid sequence using the EMBOSS Backtranseq (http://www.ebi.ac.uk/Tools/st/emboss_backtranseq/). The heterodimeric variants of *FokI* were obtained from EENDB (<http://eendb.zfgenetics.org/>). The left and right ZFN arrays were linked to the *FokI* using linker peptide coding sequences. The order of gene construct is nucleic acid sequences of kanamycin resistance promoter-ATG-6xHIS tag-GLY SER GLY-ZF left array -FOK1 KKR-TGA-chloramphenicol resistance promoter - ATG-HA tag- GLY SER GLY- ZF right array - FOK1 ELD-TGA. GeneCust Company synthesized the pKD-ZFN sequence which was subcloned in pKD46 plasmid between *SacI* and *NcoI* restriction sites. It was transformed in chemically competent *Escherichia coli* (*E. coli*) Top10F' with CaCl₂ by heat shock protocol (23). The transformed bacteria were cultured on lysogeny broth (LB) agar medium containing 100 μ g/mL ampicillin. One colony was cultured overnight and the pKD-ZFN plasmid was extracted by SolGent plasmid extraction kit (SolGent, Korea).

Transformation

V. cholerae ATCC 16961 was purchased from Iranian Research Organization for Science and Technology. The bacteria were cultured at 37 °C in brain heart infusion (BHI) agar medium containing 1% NaCl and then transferred to LB broth for 24 h at 37 °C. The

competent *V. cholera* was prepared using CaCl₂ and transformed with pKD-ZFN through heat shock treatment, then spread on LB agar medium containing ampicillin (100 µg/mL) and incubated for 24 h at 37 °C.

Evaluation of ZFN expression using SDS-PAGE and western blot

To evaluate the ZFN expression, a single positive clone of *E. coli* Top10 containing pKD-ZFN, a clone of *E. coli* TOP10 containing pKD46 and *E. coli* Top10 without plasmid were inoculated in LB medium separately and were shaken overnight at 30°C and 200 rpm to achieve OD₆₀₀ of 0.6-0.8. Cell lysate preparation was performed using lysis buffer (containing 8 M urea, 0.01 M tris-base, 0.1 M NaH₂PO₄, pH:8, 0.1 g cell/0.4 mL buffer). In order to detect the left and right ZFN array expression, western blot was performed using anti-His antibody (Sigma-Aldrich, MI, USA) and anti-HA antibody (Cell Signaling Technology, USA), respectively (24).

Evaluation of mutation in *ctxA* gene

The ZFN transformed *V. cholera* was cultured in AKI medium. After 48 h, DNA was extracted and *ctxA* gene was amplified using forward primer: 5'ATGGTG-AAATTAATTTTTGATGTAC3' and reverse primer: 5'ATCACTTTCGTCTACTCCGTTA-C3'. Polymerase chain reaction (PCR) products were sequenced.

Evaluation of ZFN construct

The authenticity of gene construct was checked based on the presence of negative results in western blot. In order to evaluate ZFN construct, a single positive clone was inoculated in LB broth medium containing ampicillin, and plasmid was extracted. The extracted plasmid was amplified using following primers: PKD46 5'ATCACTTT-CGTCTACTCCGTTAC3' (forward) and PKD46 5'ATGGTGAAATTAATTTTTGAT-GTAC3' (reverse) and primers that amplified a segment of ampicillin resistant gene including Amp 5'CAGTGCTGCAATGATA-CCG3' (forward) and Amp 5'AATTAATA-GACTGG-ATGGAGGC3' (reverse).

Cloning of whole and left ZFN arrays

The nucleic acid sequences of whole ZFN construct (kanamycin resistance promoter-ATG-HIS tag - GLY SER GLY- ZF left array - FOK1 KKR-TGA - chloramphenicol resistance promoter - ATG-HA tag -GLY SER GLY- ZF right array - FOK1 ELD-TGA) were synthesized by GeneCust Company. It was received as a PCR product and was amplified by *Pfu* enzyme

The whole ZFN sequence (case group) was amplified by ZFN 5'ATCCATGGT-TTGTACATCAAAAATTAA3' (forward) and ZFN 5'TAGAGCTCTTTCCGGAA TCAG-AAG3' (reverse). The left array of ZFN (control group) was amplified by left ZFN 5'TTCCATGGGCAGACAGTTTTATTGTTT ATGATG3' (forward) and left ZFN 5'TAGAGCTCTTTCCGGAATCAGTAAG3' (reverse) primers from the main gene construct. PCR products were treated by *Taq* DNA polymerase to add an A nucleotide in 3' ends and cloned to T/A vector, then *E.coli* Top10 was transformed with it. In addition, PCR products were cloned to *E2-crimson* plasmid in *NcoI* and *SacI* sites and were used to transform *V. cholera*. Transformed bacteria were cultured on LB medium containing 100 µg/mL ampicillin and incubated 24 h at 37 °C and resulted colonies were counted.

Evaluation of left array ZFN expression using western blot

A single positive clone of *E. coli* Top10 containing left array of ZFN was inoculated in LB medium containing ampicillin (100 µg/mL) and was shaken overnight at 35 °C and 200 rpm to achieve OD₆₀₀ of 0.6-0.8. The cell lysate was prepared as mentioned above and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot was performed using anti-His antibody (Sigma-Aldrich, MI, USA).

RESULTS

Transformation

The transformation of pKD-ZFN into *E. coli* and *V. cholera* resulted several colonies.

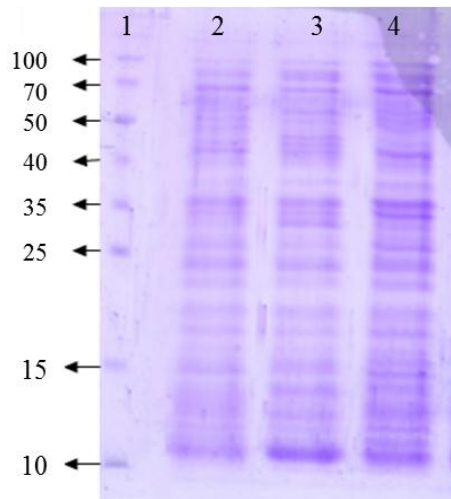


Fig. 1. SDS-PAGE on lysates of *Vibrio cholera* with and without pKD-ZFN. 1, Protein ladder of Thermo scientific lot No 00433557; 2, lysate of *Vibrio cholera* without plasmid; 3, lysate of *Vibrio cholera* contains pKD46; and 4, lysate of *Vibrio cholera* contains pKD-ZFN. There is not any change in protein bands. The expected band is about 32 and 64 kDa (dimer). SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ZFN, zinc finger nuclease.

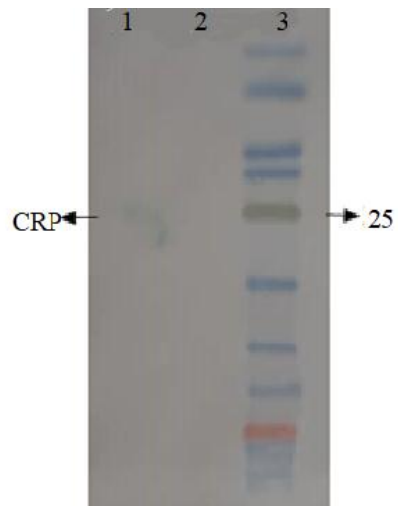


Fig. 2. Western blot for ZFN protein on *Vibria cholera* lysate that transformed with pKD-ZFN. 1, CRP protein as a positive control; 2, lysate of *Vibria cholera* that transformed with pKD-ZFN, there was no band (expected bands were 32 and 64 kDa); 3, pre-stained protein ladder of fermentas. ZFN, zinc finger nuclease; CRP, C-reactive protein.

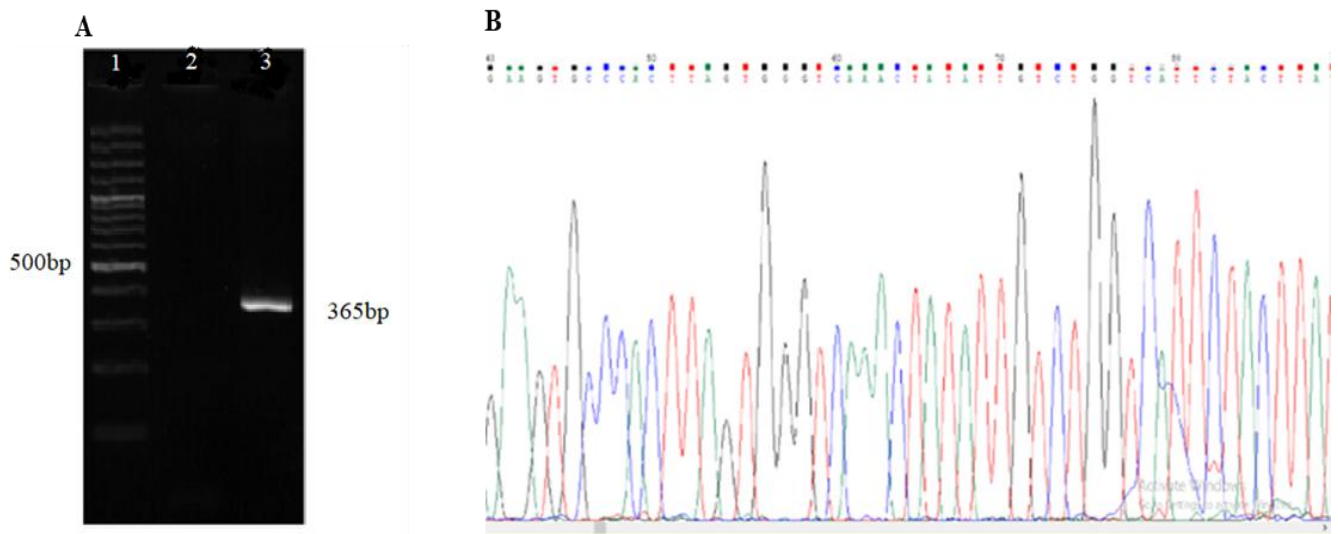


Fig. 3. (A) PCR of *ctxA* gene on *Vibrio cholera* genomic DNA. 1, 100 bp DNA ladder of fermentas; 2, negative control (distilled water); 3, PCR of *ctxA* (365 bp), and (B) sequencing result of *ctxA* gene. PCR, Polymerase chain reaction; *ctxA*, cholera toxin A.

Evaluation of ZFN expression using SDS-PAGE and western blot

SDS-PAGE protein patterns of *V. cholera* containing pKD-ZFN, *V. cholera* containing pKD46, and *V. cholera* without plasmid did not show detectable changes (Fig. 1) and specific bands (64 kDa) related to left and right ZFN

arrays were not captured on western blot (Fig. 2).

Evaluation of mutation in *ctxA* gene

The PCR product for *ctxA* gene was sequenced and aligned with *ctxA* gene of a normal bacteria and no mutation was observed (Fig. 3).

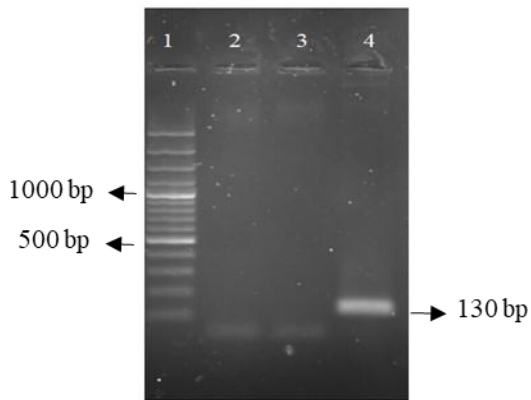


Fig. 4. Electrophoresis of PCR products on plasmid which extracted from *Escherichia coli* containing pKD-ZFN. 1, 100 bp DNA ladder of fermentas; 2, negative control; 3, PCR with forward and reverse primer of PKD46 and PKD46; and 4, PCR on ampicillin resistant gene. PCR, Polymerase chain reaction; ZFN, zinc finger nuclease.

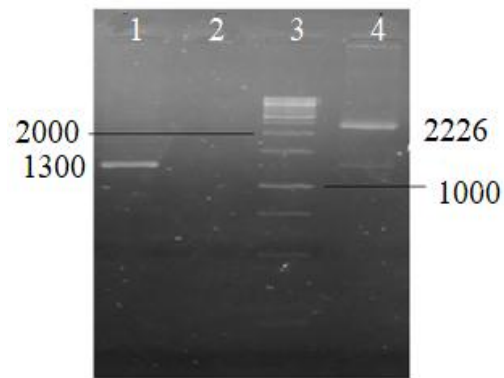


Fig. 5. PCR products of whole ZFN construct and left ZFN array. 1, PCR of left ZFN (1300 bp); 2, negative control; 3, 1kb DNA ladder of fermentas; and 4, PCR of whole ZFN construct (2226 bp). PCR, Polymerase chain reaction; ZFN, zinc finger nuclease.

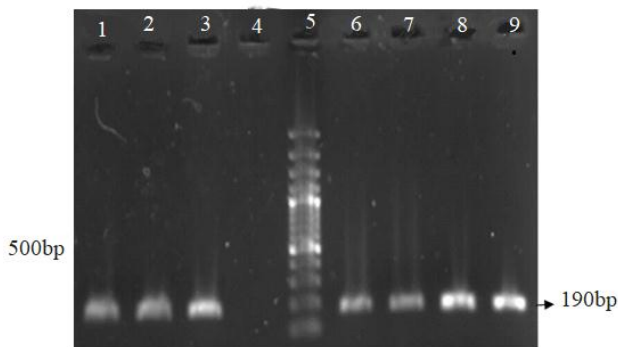


Fig. 6. Colony PCR on *Escherichia coli* transformed by pKD-ZFN (whole construct). Lines 1-3, and 6-9 are self-ligate because PCR bands are about 190 bp; line 4, negative control and line 5, is 100 bp DNA ladder of fermentas. PCR, Polymerase chain reaction; ZFN, zinc finger nuclease.

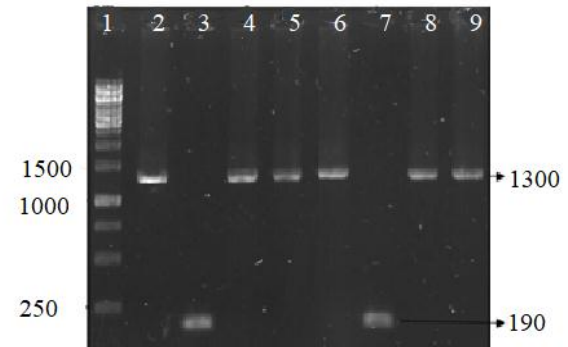


Fig. 7. Agarose gel electrophoresis of PCR on colonies resulted from transformation of *Escherichia coli* with T/A cloning of left arm of ZFN. Line 1, 1 kb DNA ladder of fermentas; lines 2-6, 8, and 9, colonies with insert left arm of ZFN, and lines 3 and 7, self-ligated colonies. PCR, Polymerase chain reaction; ZFN, zinc finger nuclease.

Evaluation of ZFN construct

The extracted plasmid was checked by PCR that amplified ZFN and ampicillin resistant sequence (Fig. 4). The PCR of ZFN using PKD46 primer did not result in amplification product, but ampicillin resistant gene showed a 130 bp band as expected (Fig. 4).

Electrophoresis of PCR product of the whole ZFN and left array showed 2226 and 1300 bp bands, respectively (Fig. 5).

The results of cloning

The number of *E. coli* Top10 colonies containing whole ZFN in the case group (seven self-ligated colonies) was significantly lower compared to the *E. coli* Top10 colonies (24 colonies of which 6 were self-ligated and 18

contained left array of the ZFN) in the control group in LB medium containing ampicillin (100 µg/mL). This was also repeated on LB medium without antibiotic selection which showed a considerable decrease in the number of colonies in case group.

The colony count of experiment on *V. cholera* that was transformed with *E2-crimson* plasmid containing ZFN was zero and 17 colonies contained the left array of ZFN.

Colony PCR on *E. coli* Top10 colonies in the case and control group showed that all colonies in the case group contained self-ligated plasmid and had no ZFN (Fig. 6), whereas many colonies in the control group had plasmid containing left array (Fig. 7). For *V. cholera*, all 17 colonies contained the plasmid with insert.

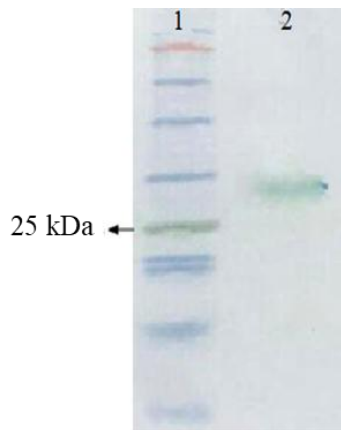


Fig. 8. Western blot of left arm of ZFN. 1, Prestained protein ladder; 2, left arm of ZFN (about 32 kDa).

Evaluation of left array ZFN expression using western blot

A detectable band was seen in western blotting of left array of ZFN in *E. coli* Top10 at about 32 kDa (Fig. 8).

DISCUSSION

Cholera, a severe and potentially life-threatening diarrheal disease with high morbidity and mortality, is a major public health concern (25,26) and its prevention and control has always been a challenge. CT, the most important virulence factor of *V. cholera* (27), induces the secretion of water and salts, main components of stool, from the epithelial cells. Traditional strategies for the prevention and treatment of infectious diseases are vaccination and antibiotic therapy. ZFN, TALEN, and CRISPR are gene editing tools (GETs) that have been widely used to target pathogenic island, toxin, and antibiotic resistant genes (28-32). There are several reports about GET application against eukaryotic organism from yeast to human (33,34).

All GETs have off target and this can cause side effects in targeted cells (34). Usually the engineered *E. coli* like Top10 and DH5 α are used to amplify GET constructs. Eukaryotic expression cassettes of ZFN, TALEN, and CRISPR/Cas9 are under control of eukaryotic promoter and not expressed in prokaryotic host, although prokaryotic ones can be expressed in *E. coli* and cause double-strand DNA break (DSB) in host genome. This is lethal for *E. coli* because it does not have NHEJ repair (35). For

this reason, two arms of ZFN, TALEN or Cas9, and sgRNA cassette should be prepared and amplified in separate clones before transformation in targeted host. Co-transformation of bacteria with two arms of ZFN, TALEN or Cas9, and sgRNA is lethal for bacteria and if the aim is homologous recombination, donor DNA should be transformed with them (36). Other studies have tried to disrupt the ampicillin resistant gene in *E. coli* using ZFN (32). In these investigations, after transformation of bacteria, ZFN proteins were not detected by western blot assay (31,32). The target gene was episomal and the probability of off target presence was ignored in the bacterial genome. In our recent study a ZFN construct was designed against a pseudomonas gene that does not have homolog in *E. coli*. The ZFN construct could not be synthesized in *E. coli* after several attempts. It may be due to off target presence in *E. coli* that ZFN created DSB in *E. coli* genome and led to bacterial cell death. In this study the ZFN construct was designed against CTX toxin and ordered to the Gene Cust Company for synthesis. This gene does not exist in *E. coli* genome. ZFN array band was not captured in western blot and ZFN expression was not confirmed, a correct construct was not achieved, and the final construct had some deletions.

It was postulated that ZFN has off target on the genome of the bacterium and killed it and the colonies containing mutated plasmid grew, therefore ZFN could not be expressed. To confirm the result, both ZFN arrays (whole ZFN) and left array were amplified using PCR, separately and cloned in the vector. After transformation of the vector containing left array in *E. coli* and *V. cholera*, some colonies grew, but the bacteria that were transformed with vector containing both ZFN arrays did not grow and all of the resulted colonies in case group were self-ligation and did not have insert. It seems that ZFN has off target on bacterial genome that causes DSB and is lethal for them due to lack of NHEJ mechanism.

Inducing DSB in prokaryotes to target pathologic and antibiotic resistant micro-organism is an attractive field in GETs technology, but there are some pitfalls (29). In

the past decade, there was no report about NHEJ in prokaryotes, although, recently NHEJ repair is known in some bacteria (37). Next generation sequencing showed two proteins that run NHEJ in prokaryotes, KU and ligase D, are not present in *E. coli* K12 and *V. cholera* (38). This subject confirms and explains the result of ongoing study.

CONCLUSION

In conclusion, targeting pathologic islands, antibiotic resistance, and toxin genes through engineered armed phage is ideal at least for accessible infections like skin and mucosal infections. Engineered armed phages with GETs could target special pathogenic bacteria and MDR strains (39). Dispersing some special armed phage in environment especially in hospital and urban waste water system could regress population of antibiotic resistant gene, multidrug-resistant, and extensively drug-resistant strain. Packaging of these phages needs some special packaging host that is protected against them. Engineered packaging host with NHEJ repair system is essential and it would be implemented by insertion of KU and ligase D in the genome of packaging host.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest for this study.

AUTHORS' CONTRIBUTION

N. Hosseini contributed in experimental work, interpretation of data, and drafting the paper, H. Khanahmad, B. Kazemi, M. Bandeh

Pour, and B. Nasr Esfahani contributed in study design and managing the project, L. Shariati contributed in ZFN design, and N. Zahedi contributed in experimental work. All authors read and approved the final manuscript

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