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Engineering of an Iranian *Bordetella pertussis* strain producing inactive pertussis toxin

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Abstract

Introduction. Differences between the genomic and virulence profile of *Bordetella pertussis* circulating strains and vaccine strains are considered as one of the important reasons for the resurgence of whooping cough (pertussis) in the world. Genetically inactivated *B. pertussis* is one of the new strategies to generate live-attenuated vaccines against whooping cough.

Aim. The aim of this study was to construct a *B. pertussis* strain based on a predominant profile of circulating Iranian isolates that produces inactivated pertussis toxin (PTX).

Methodology. The *B. pertussis* strain BPIP91 with predominant genomic and virulence pattern was selected from the biobank of the Pasteur Institute of Iran. A BPIP91 derivative with R9K and E129G alterations in the S1 subunit of PTX (S1mBPIP91) was constructed by the site-directed mutagenesis and homologous recombination. Genetic stability and antigen expression of S1mBPIP91 were tested by serially *in vitro* passages and immunoblot analyses, respectively. The reduction in toxicity of S1mBPIP91 was determined by Chinese hamster ovary (CHO) cell clustering.

Results. All constructs and S1mBPIP91 were confirmed via restriction enzyme analysis and DNA sequencing. The engineered mutations in S1mBPIP91 were stable after 20 serial *in vitro* passages. The production of virulence factors was also confirmed in S1mBPIP91. The CHO cell-clustering test demonstrated the reduction in PTX toxicity in S1mBPIP91.

Conclusion. A *B. pertussis* of the predominant genomic and virulence lineage in Iran was successfully engineered to produce inactive PTX. This attenuated strain will be useful to further studies to develop both whole cell and acellular pertussis vaccines.

INTRODUCTION

In spite of extensive vaccination programmes, pertussis (whooping cough) caused by *Bordetella pertussis* remains an endemic disease that results annually in 24·1 million pertussis cases and 160 700 deaths from pertussis in children younger than 5 years around the world [1]. In the 1950s, the use of whole-cell vaccines (wP) consisting of killed bacteria led to a significant reduction in pertussis incidence. However, its reactogenicity as evidenced by redness, pain and swelling at the injection site and joint or muscle pain resulted in decreasing vaccination coverage. In order to improve both safety and efficacy of pertussis vaccines, second-generation vaccines, such as acellular vaccines (aP) containing three–five antigens purified from *B. pertussis* have been developed in the early 1990s [2].

B. pertussis produces a range of toxins, including pertussis toxin (PTX), tracheal cytotoxin (TCT), adenylate cyclase toxin (ACT), heat-labile toxin and endotoxin or LPS. In addition, adhesions, including pertactin (PRN), filamentous haemaglotinin (FHA) and fimbriae (FIM), mediate ciliated epithelial cell attachment.

Most aP vaccines contain PTX, PRN, FHA and two fimbrial serotypes (FIM2 and FIM3), which have been exclusively used in many countries, mostly in Europe, the USA, Canada,

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Keywords: *Bordetella pertussis*; S1 subunit; homologous recombination; vaccine.

Abbreviations: aP, acellular vaccines; CHO, Chinese Hamster Ovary; Cm, Choloramphenicol; PTX, Pertussis Toxin; S1mBPIP91, S1 mutant BPIP91; wP, whole-cell vaccines.

Six supplementary figures are available with the online version of this article.

Australia and Japan [3]. However, neither wP nor aP vaccines prevented *B. pertussis* infection and transmission [4, 5], and pertussis has recently been on the rise in several countries. Several reasons may account for the pertussis recurrence, such as short duration of immunity, decreased vaccine efficacy and pathogen adaptation [6].

It was shown that alterations of genes encoding virulence factors are effective for *B. pertussis* to escape from aP vaccine-induced immunity. Genetic differences between circulating isolates and strains used for vaccine preparations have been documented in many countries [6–10]. There are different alleles of virulence factors caused by single-nucleotide polymorphisms among *B. pertussis* populations [11] that have occurred over the years since vaccination. Polymorphisms occurred in the PTX promoter region (*ptxP*) as well as in the PTX coding region, which may have changed B- and T-cell epitopes [7]. Moreover, proteomics studies of clinical and vaccine strains have illustrated the differences in general protein profiles between strains [12, 13].

Vaccine-induced immunity by current wP and aP is generated in response to antigens of *B. pertussis* strains that were isolated more than 50 years ago. Recent studies suggest that the use of modern strains for the composition of wP or aP vaccines can be an effective solution to decrease the incidence of pertussis and potentially eradicate *B. pertussis* [12, 14–16].

Beside improving the protective efficacy of current vaccines, many studies have proposed other types of pertussis vaccines, such as outer membrane vesicles [17, 18], novel vaccine formulations and adjutants [19, 20], novel vaccine antigens [21, 22] and live-attenuated nasal vaccines [23]. The live-attenuated vaccine BPZE1 has now successfully completed a first-in-human study [24]. BPZE1 was derived from the Tohama I strain, a well-known *B. pertussis* strain originally isolated in Japan in the 1950s and widely used for genetic studies and vaccine research. It was constructed by the genetically inactivating PTX, reducing TCT activity and removing dermonecrotic toxin (DNT) [23].

PTX plays a key role in the pathogenesis of pertussis. It induces leucocytosis and lymphocytosis and activates innate and adaptive immune responses [25]. It is a member of the A-B family of toxins and contains five dissimilar subunits (S1–S5). These subunits are assembled into the holotoxin in the periplasm of *B. pertussis*. The S2–S5 subunits (B oligomer) attach the toxin to target-cell receptors and deliver the S1 subunit (A protomer) into the target cells. S1 expresses enzymatic ADP-ribosylation activity, which is responsible for the ADP-ribosylation of the α subunit of Gi proteins. Giα ADP-ribosylation by PTX interrupts interaction between the Gi protein and adenylate cyclase, resulting in increased cAMP levels in the target cells, which leads to a variety of biological effects [25–27].

Two key residues of the *S1* gene are Arg9 and Glu129 that are involved in substrate binding and catalysis, respectively. Substitution of Arg by Lys and Glu by Gly eliminates PTX enzymatic activity [23, 28–31]. These amino acid substitutions can be used to generate live-attenuated vaccines or to produce genetically inactivated PTX for wP or aP vaccines.

In Iran vaccination coverage with wP was high over the last 60 years, without any changes in vaccine strains, yet the pertussis incidence has been increasing and follows the global trend [32, 33]. Based on recent epidemiological studies on *B. pertussis* strains isolated from Iranian patients at the Pasteur Institute of Iran, approximately 50% of the strains have an identical genomic profile, as determined by PFGE, although they were isolated from different geographic regions, showing a wide spread of the same *B. pertussis* lineage in Iran. The predominant antigenic profile of the strains was *ptxP3*, *ptxA1*, *prn2*, *fim2-1, fim3-2*, which is completely different from the antigenic profile of the vaccine strains [33]. Therefore, one potential reason for the pertussis resurgence in Iran may be pathogen adaptation. Strikingly, this pattern is similar to the predominant pattern in Europe [33, 34].

The importance of new strategies to use circulating strains of *B. pertussis* in vaccine combination [12, 14–16] together with the successful attempt in developing live-attenuated *B. pertussis* vaccines [23, 30] convinced us to construct a *B. pertussis* strain producing inactive PTX, based on the predominant profile of circulating Iranian isolates that was significantly different from vaccine strains. We thus selected a dominant *B. pertussis* strain to genetically inactivate PTX by two critical amino acid substitutions in the S1 subunit. The *S1* gene was first substituted by the chloramphenicol-resistance gene (*cat*), then the mutated form of the *S1* gene was introduced back into its location replacing the *cat* gene by homologous recombination. The genetic stability, expression of virulence factors and function of PTX in recombinant strain were assessed.

METHODS

Bacterial strains and culture media

B. pertussis BPIP91 was selected from the biobank of the Pertussis Reference Laboratory, Pasteur Institute of Iran. Based on our previous study, among 100 clinical isolates of *B. pertussis* over the 7 years, BPIP91 is a representative of the predominant profile of circulating strains in Iran. The alleles of virulence factor genes include *ptxP3*, *ptxA1*, *prn2*, *fim2-1, fim3-2* [33]. Some features of BPIP91, such as growth rate and antibiotic susceptibility and infectivity in mice, including the LD_{50}, have been provided previously [35].

Regan Lowe agar (RG) and Bordet-Gengou agar (BG) (Difco) supplemented with 10 and 15% sheep blood, respectively, and 40 μg ml^{-1} cephalexin (Sigma-Aldrich) were used for the *B. pertussis* culture at 35 °C.

The *Escherichia coli* DH5α (prepared from Taragen Saz Vista Co, Pasteur Institute of Iran) and *E. coli* SM10 (kindly provided by Dr Locht, Institut Pasteur de Lille, France) were used as the cloning host and donor strain for conjugation, respectively. Luria–Bertani (LB) medium supplemented with

Table 1. Primers used in this study

Primer no.	Primer name	Restriction enzyme site	Primer sequence (5′–3′)	Reference
1	ptxAF	*Kpn*I/*Nde*I	TT**GGTACC**A**CATATG**CGTTGCACTCGGGC	This study
2	ptxAR	*Bam*HI	CC**GGATCC**TAGAACGAATACGCGATGCTTTC	This study
3	ptxF-R9K	–	CGCCACCGTATACAAGTATGACTCC	This study
4	ptxR-R9K	–	GGCGGGAGTCATACTTGTATACGG	This study
5	PTXF-E129G	–	CCACCTACCAGAGCGGGTATCTGGCACACCGG	[30]
6	PTXR-E129G	–	CCGGTGTGCCAGATACCCGCTCTGGTAGGTGG	[30]
7	DSF	*Bam*HI	TTA**GGATCC**ACCTGGCCCAGCCCCGC	This study
8	DSR	*Hin*dIII	ACT**AAGCTT**GGAACCAGGGCGGAAGATCGTCTC	This study
9	UPF	*Sac*I/*Nhe*I/*Cla*I	AC**GAGCTCGCTAGCATCGAT**GGTGCTGGTCACCAACC	This study
10	UPR	*Bmg*BI	AGC**CACGTC**AGCCAGCCTGTTCTTGCG	This study
11	CmF	*Bmg*BI	AGC**CACGTC**AACATAGTAAGCCAGTATAC	This study
12	CmR	*Bam*HI	TTA**GGATCC**CGTCAATTATTACCTCCAC	This study
13	5′UPF	–	GCAATGCGGCGCGGAC	This study
14	3′DSR	–	TCATAGAGACCGTATATGGACCAG	This study

F: forward primers. **R**: reverse primers. **UP**: upstream region of the *S1* gene. **DS**: downstream region of the *S1* gene. **Cm**: chloramphenicol. Bold letters are recognition sites of restriction enzymes.

kanamycin (50 µg ml^{-1}), ampicillin (50 µg ml^{-1}) or chloramphenicol (15 µg ml^{-1}) (Sigma-Aldrich) was used as selective media.

Plasmid and primers

Plasmids pUC18 (prepared from Taragen Saz Vista Co, Pasteur Institute of Iran) and pSS1129 (kindly provided by Dr Locht, Institut Pasteur de Lille, France) were used as cloning and allelic exchange vectors, respectively. Plasmid pSS1129, a suicide vector that cannot replicate in *B. pertussis* hosts, harbours ampicillin and gentamicin resistance genes [36]. Table 1 shows all primer sequences that were used. The PCR product purification kit (Roche, Germany) and Exprep Plasmid SV mini kit (GeneAll, Korea) were used for cloning purposes.

Construction of pU-*S1M*-D, pU-*cat*-D, pSS*S1M* and pSS*cat*

BPIP91 was grown on RG agar and after 72 h incubation at 35 °C the colonies were harvested and suspended in sterile PBS. After DNA extraction, primers ptxAF and ptxAR (Table 1) were used to amplify the *S1* gene from the chromosomal DNA of BPIP91 by PCR.

The PCR product was digested with *Kpn*I and *Bam*HI and inserted into pUC18 digested with the same enzymes. The mutated *S1* gene was generated by site-directed PCR mutagenesis as previously described with the following modification [30]. Primers three to six (Table 1) were used for replacement of AAG at position 127–129 and GGG at position 487–489 of the *S1* gene for R9K and E129G substitution, respectively.

Then, the upstream and downstream regions of the *S1* gene were separately amplified by PCR using the UPF/UPR and DSF/DSR primers (Fig. 1). The 1610-bp downstream fragment was digested with *Bam*HI and *Hin*dIII and inserted into pUC18 harbouring the S1 mutant (*S1M*). *Sac*I and *Bmg*BI were used to insert the 920 bp upstream fragment into this plasmid. The resulting plasmid pU-*S1M*-D (Fig. 1) was confirmed by PCR, restriction enzyme analysis and DNA sequencing (Macrogen Company, South Korea) (Figs S1 and S2, available in the online version of this article).

The *cat* gene and its promoter were amplified from pLysS (prepared from Taragen Saz Vista Co, Pasteur Institute of Iran) using primers CmF and CmR (Table 1). After digestion with *Bmg*BI and *Bam*HI and purification of the PCR product, the 1309 bp fragment was introduced into pU-*S1M*-D and substituted the *S1M* segment to produce pU-*cat*-D. In pU-*cat*-D, the *cat* gene was flanked by the upstream and downstream fragments of the *S1* gene (Fig. 1). Plasmid pU-*cat*-D was also confirmed by PCR (Fig. S1).

Next, pU-*cat*-D and pU-*S1*M-D were digested with *Cla*I (see UPF primer in Table 1) and *Hin*dIII and the resulting fragments were introduced into pSS1129 digested with the same enzymes to generate pSS*cat* and pSS*SIM* plasmids, respectively (Fig. S1). pSS*cat* and pSS*SIM* were transferred separately into the *E. coli* SM10.

Homologous recombination

Fresh colonies of *B. pertussis* BPIP91 (3 days' culture on RG agar) were suspended in PBS, adjusted to OD_{600}=1.5 and

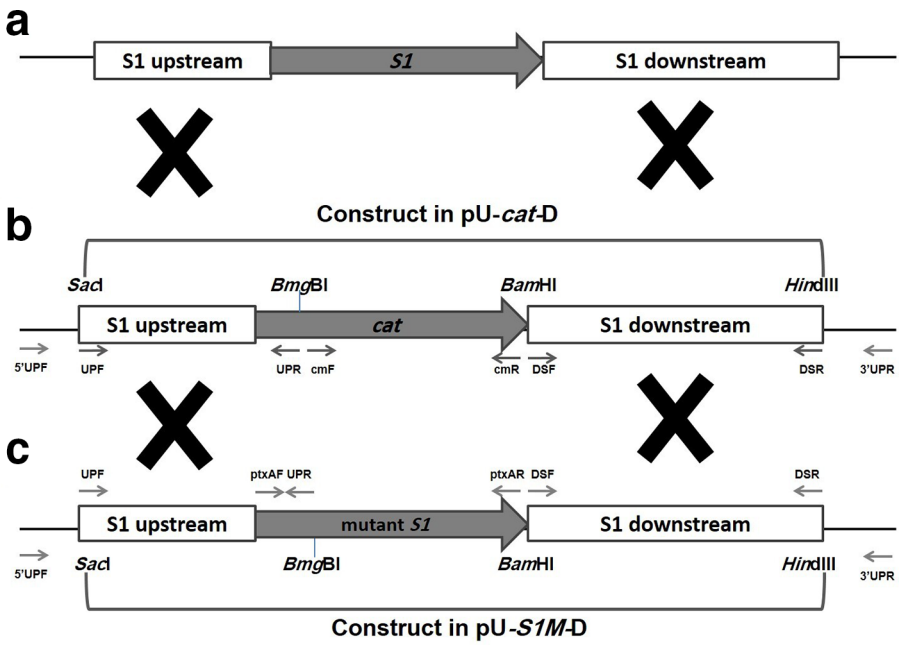

Fig. 1. Schematic representation of the constructs in this study. Primers are shown by the arrows and the crosses show the homologous recombination sites. *S1* upstream and downstream fragments were amplified and placed 5′ and 3′ of the *S1* mutant (R9K and E129G) allele to create pU-*S1M*-D. The chloramphenicol-resistance gene (*cat*) was amplified and inserted into the vector to replace the *S1* gene, generating pU-*cat*-D. Homologous recombination between the *B. pertussis* BPIP91 chromosome (a) and pU-*cat*-D (b) generated Cm^RBPIP91. Homologous recombination between the Cm^RBPIP91 chromosome (b) and pU-*S1M*-D (c) generated the *S1* mutant strain S1mBPIP91.

mixed with *E. coli* SM10 harbouring pSS*cat* (overnight culture on LB broth containing kanamycin, gentamicin and chloramphenicol) with the same OD_{600} at a ratio 1:3. The bacterial mix was incubated for 5 h on BG agar plates without antibiotics containing 10 mM $MgCl_2$. The mix was then swabbed and spread onto RG agar containing 5 µg ml^{-1} chloramphenicol and 40 µg ml^{-1} cephalexin to select against the donor strain. Chloramphenicol-resistant *B. pertussis* colonies were then cultured on the RG agar containing 5 µg ml^{-1} chloramphenicol for the second event of homologous recombination. After that, the gentamicin-susceptible (Gen^S) colonies were selected by replica plating. The Cm^R and Gen^S colony was named Cm^RBPIP91.

To ensure integration of the *cat* gene in the correct position of the BPIP9 genome and the replacement of the *S1* gene, PCR was performed using primers 5′UPF and 3′UPR, which specifically bind respectively to the upstream and downstream flanking regions of the insert (Table 1 and Fig. 1). The PCR products obtained with 5′UPF, 3′UPR and the primers of the *cat* gene (CmF and CmR) showed the correct location of the *cat* gene in the chromosome (Fig. S3).

Generation of S1 mutant *B. pertussis* S1mBPIP91

In order to insert the mutant *S1* gene into the BPIP91 chromosome, the donor strain *E. coli* SM10 harbouring pSS*SIM* and recipient strain Cm^RBPIP91 were mixed on BG agar plates without antibiotics containing 10 mM $MgCl_2$. The mix was then swabbed and spread onto RG agar containing 40 µg ml^{-1} cephalexin to prevent growth of the donor strain. As described above, the colonies were screened by replica plating to obtain Cm^S and Gen^S S1mBPIP91. The gene substitution in the correct location on the chromosome of S1mBPIP91 was verified by PCR using primers 5′UPF and 3′UPR and the primers of the *S1* gene (ptxAF and ptxAR) (Fig. S3). A PCR product of the *S1* gene was sequenced for final confirmation of the two mutations leading to the R9K and E129G substitutions.

Genetic stability tests

B. pertussis S1mBPIP91 was serially passaged 20 times on RG agar and grown at each passage for 72 h [37]. The genomic DNA of S1mBPIP91 at passages 10 and 20 was extracted and used as a template. PCR was performed using ptxAF and ptxAR (Table 1) and the amplicons were sequenced (Macrogen Company, South Korea).

The entire genomic profile of S1mBPIP91 in comparison with its parent strain (BPIP91) was examined by PFGE as previously described [33]. The genomic DNA of S1mBPIP91 and BPIP91 was digested with *Xba*I and PFGE was performed with a CHEF DRIII device (Bio-Rad, USA) with initial switch time of 5 s and final switch time of 45 s and the run time of 21 h.

Growth curve

To compare the growth rates of S1mBPIP91 and BPIP91 strains, both strains were cultured in modified SS (Stainer Schoulte) media [38]. Then, 20 h after the primary incubation, bacteria were inoculated into fresh SS media at OD_{600nm}=0.05 under shaking (150 r.p.m.) at 35 °C. The OD_{600} of the samples was recorded to draw the growth curves.

Western blotting

The expression of the most important virulence factors of S1mBPIP91 in comparison with BPIP91 was analysed by Western blotting as previously described [39]. Cell lysates of both strains were subjected to SDS-PAGE using a 12 % polyacrylamide gel and then electro-transferred to a polyvinylidene difluoride (PVDF) membrane (Figs S5 and S6). PRN, FHA and PTX monoclonal antibodies (NIBSC no. 97/558, 97/564 and 97/572, respectively) were used as primary antibodies at 1:1000 dilution in PBS. The 1:500 dilution of a rabbit anti-sheep horseradish peroxidase-conjugated (HRP) antibody (PADZA Company, Iran) was used as a secondary antibody. Finally, membranes were developed with Metal Enhanced DAB Substrate (Thermo Fisher Scientific, Waltham, MA, USA).

Chinese hamster ovary (CHO) cell-clustering test

The lack of toxicity of genetically inactivated PTX of S1mBPIP91 was checked as previously reported [40]. Briefly, 200 µl of CHO cell (CCL61; Cell Bank of Pasteur Institute of Iran) suspension at a density of 3×10^4 cells ml^{-1} in Ham's. F12 medium (Nano Zist Arrayeh Company, Iran) was seeded in 96-well cell culture plates. After 24 h of incubation at 37 °C under 5% CO_2, 50 µl of the supernatant of BPIP91 or S1mBPIP91 suspension in PBS after inactivation at 56 °C was added to the cells. As a positive control, 25 µl of DTP vaccine (Serum Institute of India, PVT. LTD) containing *B. pertussis* ≥8 IU ml^{-1} was added to the cells in tenfold serially dilutions. The clustering of the cells was recorded after fixing with 15% formaldehyde for 30 min and staining with 5% Giemsa for 1 h.

RESULTS

Plasmid construction

By inserting the *S1* gene into pUC18, followed by site-directed mutagenesis, two amino acid substitutions (R9K and E129G) were successfully done in the S1 subunit of PTX (Fig. S2). The S1 flanking regions were inserted into pUC18 by separately inserting the upstream (862 bp) and downstream (1615 bp) regions, yielding pU-*S1M*-D (Fig. 1). pU-*cat*-D was created by inserting the *cat* gene and its promoter (1309 bp) into pU-*S1M*-D via replacement of *S1M* with the *cat* gene Figs 1 and S1). The two inserts in pU-*S1M*-D and pU-*cat*-D were then successfully transferred to the allelic exchange vector pSS1129 to generate pSS*S1M* and pSS*cat*, respectively.

All these constructs were verified by restriction enzyme analysis to confirm the correct size of fragments (Fig. S1).

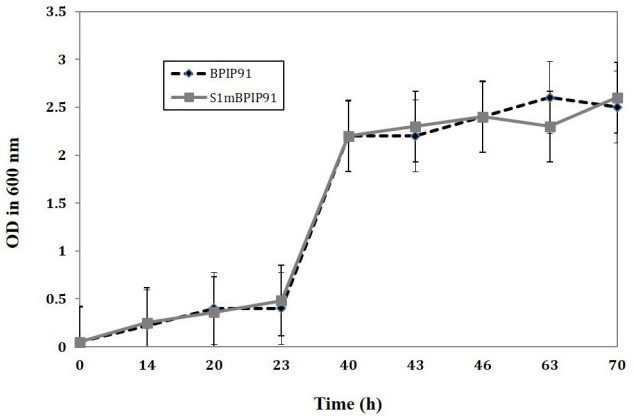

Fig. 2. Growth curves of S1mBPIP91 and BPIP91. Inoculations were done in triplicates and the OD was recorded at 600 nm.

Construction of S1mBPIP91

First Cm^RBPIP91, containing the *cat* gene instead of the *S1* gene (Cm^R and Gen^S phenotype) was constructed by homologous recombination between *E. coli* SM10 and *B. pertussis* and characterized by PCR and phenotype (Fig. S3). The PCR showed that the *cat* gene and its promoter were successfully inserted at the S1 location on the chromosome of BPIP91. The *cat* gene of Cm^RBPIP91 was substituted by the mutated *S1* gene using pSS*SIM*. The resulting strain S1mBPIP91 was found to be sensitive to chloramphenicol and gentamycin. The genetic confirmation and phenotype of S1mBPIP91 are shown in Fig. S3. The comparison of the growth rates of S1mBPIP91 and BPIP91 showed that the mutation in the *S1* gene has not affected the growth rate (Fig. 2).

DNA sequencing of the PCR product of the *S1* gene confirmed the two desired mutations in S1mBPIP91 (Fig. S2).

The genetic stability of S1mBPIP91 and expression of virulence factors

Sequencing of PCR amplicon of the *S1* gene confirmed the presence of the mutations causing the R9K and E129G substitutions in the S1 subunit after 10 and 20 *in vitro* passages of S1mBPIP91. No other nucleotide changes were observed compared to BPIP91. The PFGE analysis showed that the *Xba*I digested DNA patterns of the entire genome of S1mBPIP91 and BPIP91 were identical (Fig. S4). This pattern is the dominant genomic pattern in Iran [33].

PRN, FHA and PTX production by S1mBPIP91 was determined by Western blot analysis. The 220, 69 and 26 KD protein bands related respectively to FHA, PRN and the S1 subunit of PTX were readily distinguishable on the PVDF membrane (Figs 3 and S6). Thus, the expression of these antigens was demonstrated in BPIP91 and its S1 mutated variant S1mBPIP91.

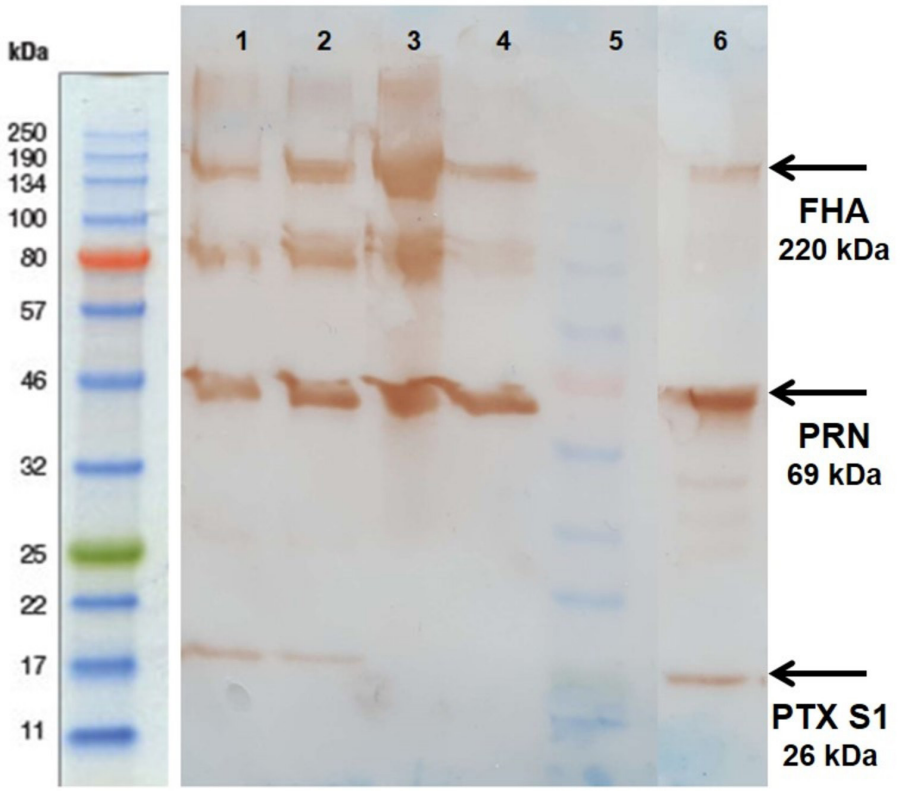

Fig. 3. Western blot analysis of pertussis antigen (FHA, PRN and PTX) production in S1mBPIP91 and BPIP91. Lanes 1 and 2: cell lysates of BPIP91 and S1mBPIP91, respectively. Lanes 3 and 4: cell lysates of the chloramphenicol-resistant strain lacking the *S1* gene (Cm^RBPIP91). Lane 5: protein ladder (ab116028). Lane 6: cell lysate of Tohama I.

Assessment of PTX inactivation

The effect of the R9K and E129G substitutions of the S1 subunit on the reduction of toxicity by S1mBPIP91 was revealed by the CHO cell-clustering assay (Fig. 4). As evidenced by the absence of CHO cell clustering in the presence of S1mBPIP91 supernatants, we demonstrate that the toxicity of S1mBPIP91 has been reduced compared to that of its original strain BPIP91. As the clustering of CHO cells as a result of the ADP-ribosylation activity of PTX did not occur with S1mBPIP91, we conclude that the two amino acid replacements in the *S1* subunit have successfully inactivated PTX in S1mBPIP91. DTP vaccine, as a positive control induced clustering of the CHO cells at a concentration of 0.08 IU ml^{-1}.

DISCUSSION

This study was designed to generate the S1 mutant *B. pertussis* strain named S1mBPIP91, which was successfully mutated so that two important residues of S1 subunits that are both critical for its ADP-ribosyl transferase activity Arginine 9 and Glutamate 129 were replaced by Lysine and Glycine, respectively (Fig. S2). These mutations resulted in PTX toxicity reduction without any change in the genomic pattern, growth rate and PTX expression (Figs 2, 3 and S4).

Since it is important to illustrate that the genetic manipulations of S1mBPIP91 are stable and reversion of these mutations does not occur, genetic stability has been evaluated upon continuous serial *in vitro* passages. We found that the mutations leading to the R9K and E129G substitutions in S1mBPIP91 are stable and do not reverse, even after 20 *in vitro* passages. In fact, it has been demonstrated by *in vivo* and *in vitro* experiments that reversion of both mutations to active residues is virtually impossible [37, 41]. Genomic profiling obtained by PFGE is a gold standard method to determine genetic similarities between the bacterial isolates. Our PFGE analysis validated that the *Xba*I-digested genomic pattern of S1mBPIP91 is exactly the same as that of its original strain. This pattern is the predominant pattern of strains circulating in Iran [33]. The R9K and E129G substitutions in S1 has also not affected the growth rate of S1mBPIP (Fig. 2).

The clustering effect of PTX on CHO cells is a sensitive and specific test for rapid diagnosis of pertussis [40]. In this study, S1mBPIP91 and its original strain were examined to determine the inactivation of PTX in S1mBPIP91 by morphological changes of CHO cells. We could determine that the S1mBPIP91 strain did not induce morphological changes of CHO cells compared to BPIP91 and DTP vaccine as a positive

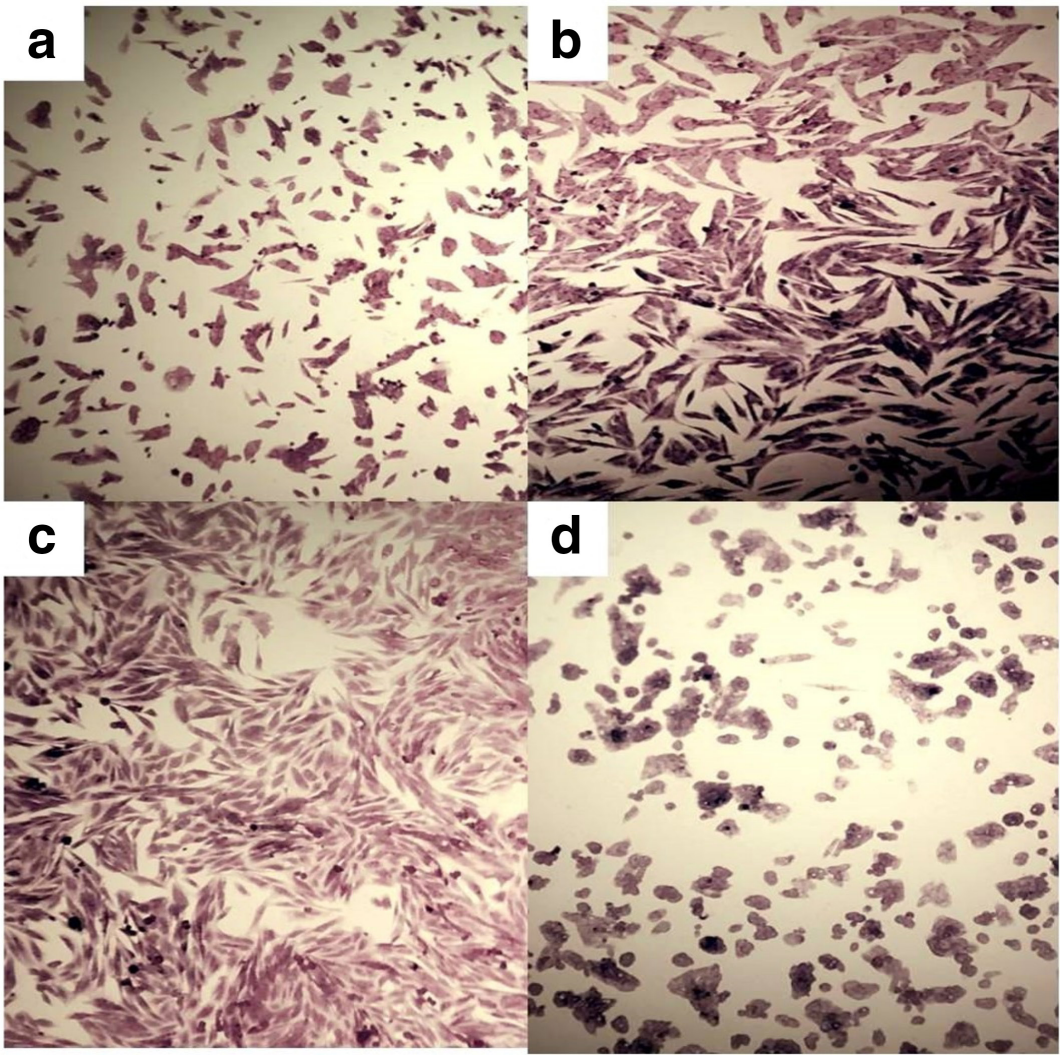

Fig. 4. Cytotoxic effect of *B. pertussis* on CHO cells. Clustering of the cells by PBS suspension of BPIP91 (a) or S1mBPIP91 (b). Monolayer of non-treated CHO cells (c) and clustering of the cells after treatment with DTP vaccine at 0.08 IU ml^{-1} (d).

control (Fig. 4). These findings confirm PTX inactivation in S1mBPIP91.

In this study we first obtained by homologous recombination the S1-deficient strain Cm^{R}BPIP91 that contains the *cat* gene instead of the *S1* gene in its chromosome (Fig. 1 and S1). In recent years, PTX-deficient strains were found among circulating strains of some countries [11, 42, 43]. Cm^{R}BPIP91 may potentially enable us in the future to study immune responses against *B. pertussis* in the absence of PTX in mouse models.

Most importantly, S1mBPIP91 is derived from a clinical strain of *B. pertussis*, BPIP91, with the predominant alleles of virulence factors (*ptxP3*, *ptxA1*, *prn2*, *fim2-1*, *fim3-2*) allowing it to spread in Iran despite high vaccination coverage. Epidemiological data on *B. pertussis* circulation and new awareness about whooping cough showed that vaccination strategies may need to be modified [44]. It is important to notice that both current wP and aP vaccines are derived from *B. pertussis* strains with alleles of virulence genes that are not dominant in most communities [33, 45, 46]. Therefore, the use of recently circulating strains of *B. pertussis* showing the predominant circulating virulence profile would be preferable for the development of new vaccine strategies against *B. pertussis* infections.

In spite of the several attempts to solve the pertussis problem, including booster vaccinations, cocoon vaccination and maternal vaccination, developing new generation vaccines against *B. pertussis* is necessary. One of these efforts is the development of a live-attenuated vaccine [3], such as BPZE1, as a nasal single-dose attenuated vaccine [23]. The advantages of such vaccines make them acceptable for future planning of vaccination. As *B. pertussis* is a strictly mucosal pathogen, nasally applied live-attenuated *B. pertussis* can help to induce both mucosal and systemic immune responses [41]. This kind of vaccine has low production cost, which makes it accessible

to all countries, even resource-limited countries, which cannot prepare or buy aP.

We decided here to select the predominant strain in Iran to construct a S1 mutant strain (S1mBPIP91) with the following objectives. First, the genetically inactivated *B. pertussis* clinical strain expressing predominant alleles of virulence factors can be very valuable to develop wP vaccines in new combination strategies. The use of predominantly circulating strains in wP vaccine production may potentially decrease pertussis incidences in the vaccinated populations.

Second, S1mBPIP91 may also be used in aP vaccine preparations, without the need of any chemically inactivation of PTX. In most current aP vaccines, PTX needs to be chemically inactivated, which is not a simple approach and also causes PTX denaturation [47].

While other double alterations of the S1 subunit of PTX, such as 13L/129G and 26I/129G, have been described, 9K/129G are the best well characterized and suggested as a candidate for pertussis vaccines [28, 29]. Purified genetically inactivated PTX was previously proposed as a vaccine candidate with good safety and efficacy results [29]. In 2012, researchers in Thailand successfully constructed a strain derived from Tohama I, which expressed increased amounts of PTX and PRN and could be useful for the manufacturing of affordable aP vaccines. That strain has two copies of the mutated *S1* gene and of the gene encoding PRN [30]. PTX is encoded by genes of the *ptx-ptl* operon and mature toxin cannot be assembled and secreted in recombinant *E. coli* [48]. Thus, S1mBPIP91 with enzymatically inactivated S1 may be a good candidate for future aP vaccine production [49]. On the other hand, this strain contains an inactivated form of the *ptxA1* allele, which is the dominant type in the world.

The third and main aim of this research was to obtain a S1 mutant strain for the construction of a live-attenuated pertussis vaccine [23]. A live-attenuated pertussis vaccine derived from Tohama I with three genetic changes in *B. pertussis* has been constructed previously by Locht and co-workers [3, 23, 24]. It has successfully completed the first phase of clinical development in human volunteers, which showed its safety and immunogenicity, and a phase II for preliminary efficacy is under way [50].

S1mBPIP91 was constructed as a first step in the development of a live-attenuated vaccine based on the circulating strains in Iran. However, the limitations of this study were that this research only included 100 Iranian isolates over the 7 years to choose a predominantly circulating strain based on PFGE results and allele typing of only five main virulence factors of *B. pertussis* (*ptxP, ptxA, prn, fim2* and *fim3*). It would be interesting to characterize and compare some of the other predominant strains resulted from continued monitoring of *B. pertussis* circulating strains.

It is obvious that in order to use new strains in vaccine-production strategies, the selected strains should be well characterized and have a fully documented history. So it is necessary to conduct further studies such as the genetic characterization by whole-genome sequencing that analyse the entire genomic DNA sequence of the strains, and also determination of safety and immunogenicity of the strains, including colonization and antibody production in animal models and investigation of stability of the selected strains to appreciate the full potential of the chosen strains as a vaccine candidate.

We hope that the results of this study based on the use of a predominantly circulating *B. pertussis* strain, which was significantly different from vaccine strains, will be a small step forward in obtaining future tools for pertussis prevention.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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