

Original Article

Production of a Human Recombinant Polyclonal Fab Antivenom against Iranian Viper *Echis carinatus*

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ABSTRACT

Venomous snakebite is a life-threatening injury in many tropical and subtropical areas including Iran. The gold standard treatment option for human envenomation is the use of antivenoms. Despite the unique effects of horse-derived antivenoms on the treatment of snakebite, they are not fully perfect and need improvements. In this study, human recombinant Fab fragment antivenom was produced in Rosetta-g bacterium using a gene library constructed in the previous study. The prepared Fab was purified in several steps, desalted, and lipopolysaccharide-depleted using ammonium sulfate solution and dialysis against phosphate buffer and Triton X-114 solution, respectively. Subsequently, the product was initially confirmed by the sodium dodecyl sulfate polyacrylamide gel electrophoresis and enzyme-linked immunosorbent assay (ELISA), respectively. Finally, the neutralization potency of the product was investigated in laboratory Syrian Mice. The obtained results showed corresponding reduced bands to Fab fragment with the molecular weight of about 28 kDa at a concentration of 3.1 mg/ml. There was a significant difference between the groups in terms of ELISA test ($P < 0.05$). The neutralization potency of the product against the venom of *Echis carinatus* (*E. carinatus*) was about 7 LD₅₀/ml (54.6 µg/ml) when tested on mice. Based on the results, the Fab fragment antivenom had the ability to neutralize the in vivo biological activity of the venom of Iranian *E. carinatus*. However, further studies are recommended to reach a suitable concentration of antivenom fragment.

Keywords: *Echis carinatus*, Fab fragment, Gene library, Antivenom, Polyclonal

Production d'un de Sérum Recombinant Humain de Fab Polyclonal contre *Echis carinatus* Iranien

Résumé: La morsure de serpent venimeux est une blessure mortelle dans de nombreuses régions tropicales et subtropicales, y compris Iran. L'etraitement de référence en cas d'envenimation humaine est l'utilisation d'antivenirs. Malgré l'efficacité uniques des sérums antivenimeux dérivés du cheval sur le traitement des morsures de serpents, ils présent néanmoins des limitations et doivent être améliorés. Dans cette étude, un

fragment de sérum recombinant humain de Fab Polyclonal a été produit dans la bactérie Rosetta-g en utilisant une bibliothèque de gènes construite dans l'étude précédente. Le Fab préparé a été purifié en plusieurs étapes, dessalé et appauvri en lipopolysaccharide en utilisant une solution de sulfate d'ammonium et une dialyse contre un tampon phosphate et une solution de Triton X-114, respectivement. Par la suite, le produit a été analysé par électrophorèse sur gel de polyacrylamide de dodécyl sulfate de sodium (SDS-PAGE) et par dosage immuno-enzymatique (ELISA), respectivement. Enfin, à capacité de neutralisation du produit a été étudiée chez des souris syriennes de laboratoire. Les résultats obtenus ont montré des bandes réduites correspondantes au fragment Fab avec un poids moléculaire d'environ 28 kDa à une concentration de 3,1 mg / ml. Il y avait une différence significative entre les groupes en termes de test ELISA ($p < 0,05$). La capacité de neutralisation de ce produit contre le venin *Echis carinatus* était d'environ 7 LD50 / ml (54,6 µg / ml) lorsqu'il a été testé sur des souris. Sur la base des résultats, l'antivenin du fragment Fab avait la capacité de neutraliser l'activité biologique in vivo du venin *E. carinatus* d'Iran. Cependant, des études complémentaires sont recommandées pour atteindre une concentration appropriée de fragment antivenin.

Mots-clés: *Echis carinatus*, Fragment Fab, Bibliothèque de Gènes, Antivenin, Polyclonal

INTRODUCTION

Globally, snakebite envenoming is a life-threatening disease with million cases each year. According to the latest available information, more than 10,000 snakebite cases occurred annually by various snake species in Iran (Motedayen, 2015). Although the mortality rate of snakebite in Iran is low compared to the number of the bites, the morbidity, necrotic wounds, severe pain, amputation, high costs, soft-tissue deformities, as well as physical and psychological complications are caused by the snake bite that cannot be compensated. Nowadays, therapeutic serums are usually produced in horses. The use of these antivenoms is the gold standard treatment option for the victims (Sapsutthipas et al., 2015). This therapeutic method rapidly reverses the symptoms of envenomation (Koh et al., 2006). Despite the high therapeutic value of this method, the use of conventional antivenoms may cause some unwanted side effects such as anaphylaxis or serum sickness. Many patients may be sensitive to horse blood proteins or already have anti-proteins antibodies (Ariaratnam et al., 1999). Serum sickness is a type III hypersensitivity reaction that results from the injection of a foreign protein in about two weeks after the exposure. The leading cause of this disease is the accumulation of immune complexes in certain tissues of the body (Bugli et al., 2008). Generated antivenoms may contain whole IgG, or F(ab')₂ and Fab

fragments (Thierry et al., 2004). The Fab and F(ab')₂ fragments are antibody structures that still bind to antigens with no Fc portion. Based on the classic method, the antibody is cleaved by Papain enzyme to give three 50 kDa fragments including two Fab and one Fc fragments. In contrast, F(ab')₂ fragment antibodies are prepared by pepsin digestion of whole IgG antibodies to remove the Fc region. The 110 kDa divalent F(ab')₂ fragments have two antigen-binding Fab portions linked together by disulfide bonds. Intact IgG has a prolonged plasma half-life with more allergic reactions in comparison to its fragment. Therefore, the use of intact IgG antivenom is limited. Fab fragment protein differs from F(ab')₂ due to greater velocity and diffuse volume. The use of F(ab')₂ antivenoms may be associated to the risk of initial unwanted reactions, probably due to the activation of the complement system through the presence of Fc residues or F(ab')₂ aggregates. The prevalence of these reactions is 0.8% and 10% in the use of Fab and F(ab')₂ antivenoms, respectively (Ariaratnam et al., 1999; Theakston et al., 2003). Whole immunoglobulin antivenoms are used in some countries like Costa Rica, while in Iran, polyvalent horse-derived F(ab')₂ fragment antivenoms are used (Morais and Massaldi, 2009). However, polyvalent Fab antivenom (CROFAB) is a type of antivenom with lower allergic reactions indicated for the management of minimal or moderate North American Crotalid envenomation

(http://accessemergency_medicine_mhmedical.com). The production of different antibodies using phage display gene libraries is a traditional approach that is applied in many fields of research for generating antibodies, and especially their components against the antigens (Wu et al., 2001; Peterson, 2005; Schofield et al., 2007; Hammers and Stanley, 2014; Motedayen, 2015; Rami et al. 2017; Zhu and Dimitrov, 2009; Shukra et al., 2014). Antibody phage display is the key method for the generation of immunoglobulin and selection. Diverse regions of the immunoglobulin genes can be engineered and generated using a gene library. The antibody genes can be conveniently shuttled into a variety of expression formats, which potentially provide hundreds of unique immunoglobulins per target (Schofield et al., 2007; Dantas-Barbosa et al., 2012; Roncolato et al., 2015). This technology allows the antibody genotype to come with its phenotype and lets the isolation of human antibody components with a wide variety and potential for application to all research, medical, and industrial areas (Carmen and Jermutus, 2002; Rahbarizadeh et al., 2003; Schofield et al., 2007; Pansri et al., 2009; Turunen et al., 2009; Zhu and Dimitrov, 2009; Motedayen, 2015). Phage display technique offers the generation of antivenoms without the need to the use of animals or antigens (Roncolato et al., 2015). In addition, this method is associated with some disadvantages. For instance, about 10% of the phages are produced in recombinant forms and the rest remain intact. The presence of two domains of light and heavy chains in the structure of antibodies makes it possible to combine distinct these chains when it comes out of the phage with poorer affinity. The aim of this study was to produce a recombinant polyclonal Fab fragment antivenom specific for Iranian *E. carinatus* using a gene library constructed in the previous research. This snake species was selected due to its wide distribution and is the most dangerous snake extended through Asia including in Iran (Kamyab et al., 2017).

MATERIAL AND METHODS

Fab library construction. Briefly, in the previous study, we prepared total RNA isolated from peripheral blood mononuclear cells of two recovered snake victims (Motedayen, 2015). Afterward, cDNA was synthesized using reverse transcription polymerase chain reaction technique. Then, the heavy (Fd segment) and kappa light chains of IgG with the following specific primers were amplified.

VH1a (Forward:5'-CAGGTGCAGCTCGAGCAGTCTGGG-3' and Reverse: CG1z 5'- GCATGTACTAGTTTTGTCACAAGATTTGGG-3');

VH3a (Forward:5'-GAGGTGCAGCTCGAGGAGTCTGG-3' and Reverse: CG1z 5'- GCATGTACTAGTTTTGTCACAAGATTTGGG-3');

VK1a (Forward:5'-GACATCGAGCTACCCAGTCTCA-3' and Reverse: CK1a, 5'- GCGCCGTCTAGAACTAACACTCTCCCCGTTGAAGCTCTTTGTGACGGCAAG-3');

VK3a, (Forward:5'-GAAATTGAGCTCACGCAGTCTCA-3' and Reverse: CK1a, 5'-GCGCCGTCTAGAACTAACACTCTCCCCTGTTGAAGCTCTTTGTGACGGGCAAG-3').

After digestion of the heavy chains with *SpeI* and *XhoI* and light chains with *XbaI* and *SacI* enzymes, the chains inserted successively into the phagemid vector *pComb3X*, which contains an *f1* origin of replication from an *f1* phage. Afterward, the recombinant vector was transformed into TG1 bacterial cells to construct the primary Fab library.

Bacterial Stock Culture. In this study, 10 ml of 2xYT culture medium was poured into a sterile 50 ml Falcon tube. After adding 15 µl ampicillin (100 mg/ml) and 250 µl of sterile glucose solution with a final concentration of 1%, 65 µl of the bacterial stock containing the recombinant plasmid was added and incubated overnight at the temperature of 30 °C with shaking. Then, it was centrifuged at 4000 rpm (2600 xg) at 4 °C for 15 min. Sediment was dissolved in a new Falcon tube containing 10 ml of 2xYT fresh medium, 15 µl ampicillin (100 mg/ml) and 12.5 µl glucose solution (40% w/v) to achieve the final

concentration of 0.05% and incubated at 28 °C with shaking. The turbidity of the culture was measured at the wavelength of 600 nm. Upon reaching the optical density of 0.6, isopropyl β -D-1-thiogalactopyranoside (IPTG) (15 μ l of 1 M) was added to the culture, incubated at 27°C with shaking for 5 hours, and centrifuged (8200 xg, 4 °C, 15 min). The supernatant was transferred to a sterile 15 ml Falcon tube and stored in a refrigerator until use. The bacterial sediment was dissolved in 1.5 ml of phosphate buffered saline (PBS) and used for sonication.

Sonication of Rosetta-g bacteria sediment. Native lysis buffer containing 50 mM NaH_2PO_4 (pH=8.0), 300 mM NaCl, and 10 mM imidazole was added in 1:1 ratio to the bacterial suspension and sonicated with six 15 s bursts with 10 s cooling period between each burst (UP200H, Hielscher, Ultrasound Technology). The temperature of the bacterial suspension was controlled by surrounding the reaction Falcon with an ice bath. Meanwhile, a sample without IPTG with the same condition was sonicated as a negative control.

Protein determination. The amount of protein in the Rosetta-g cell culture medium was quantified by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) at the wavelength of 280 nm.

Enzyme-linked immunosorbent assay for expressed proteins. The wells of a microplate (Nunc MaxiSorp flat-bottom) were coated with 100 μ l (0.5 μ g/ μ l) of the venom of *E. carinatus* dissolved in sodium bicarbonate buffer (pH=9.6) and incubated overnight at 4°C. Meanwhile, in control wells (control 1, without antigen), 100 μ l of bicarbonate buffer was poured. The coating solution was removed and washed the plate twice by filling the wells with 300 μ l PBS. Then, 300 μ l/well fresh PBS with 5% non-fat milk (PBSM) was added to block the remaining protein-binding sites in the microplate, and ultimately it was incubated at 37 °C for 2 hours. The wells were discharged and washed twice with with PBST (PBS containing 0.05% of Tween 20). Thereafter, 50 μ l of the product was added to each well as primary antibody and incubated at 37

°C for 75 min. In addition, 50 μ l of PBS was poured into second control wells. The content of the wells was removed and washed six times with PBST. Thereafter, 50 μ l of newly developed Anti-His Tag HRP-conjugated antibody with the final concentration of 180 mU/ml was poured into all wells and incubated at 37 °C for 80 min. The content of the wells was removed and washed seven times (five times with PBST and twice with PBS), and then 50 μ l of tetramethylbenzidine (TMB) was poured into each well. The reaction was stopped by adding 25 μ L of 1M H_2SO_4 to each well. After 7 min, the absorbance values were read at 450 nm using an enzyme-linked immunosorbent assay (ELISA) reader.

Purification of the product. Ammonium sulfate (14% w/v) was added to 95 ml of the product and stirred at 54 °C for one hour and then centrifuged (6000 xg, 4 °C, 7 min). The pH of the supernatant was adjusted to 7.2, and then ammonium sulfate (18% w/v) was added and rotated on the stirrer for 35 min and centrifuged (10700 xg, 25 °C, 7 min). The precipitate was poured into a dialysis bag (10 kDa), which was placed in a 1 L glass beaker containing 800 ml deionized water, incubated at 4°C overnight, and stirred for 24 hours to remove the ammonium salt. The water of glass beaker was changed twice during 48 hours, and the content of the dialysis bag was placed in a refrigerator after pH adjusting (pH=6.8). Afterward, Triton X-114 was added to 15 ml of the product with the final concentration of 1% and incubated at 4 °C for 30 min with stirring and then incubated (43 °C for 2 hours) and centrifuged (10000 xg, 15 min) to remove lipopolysaccharide. The supernatant was transferred to a refrigerator (Zhang et al., 2013). To determine the correspondence to Fab fragment antibody bond in the reduced form (25 kDa) of the product, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 12% w/v separating and 5% w/v stacking gels. Electrical current was set as 40 mA, the voltage was self-adjusted, and the time was 2 hours.

Potency assay of the product. To assess the antivenom potency, venom stock solution at doses of 4, 6 and 7 LD₅₀ was poured in three tubes for three groups of 3 NMRI mice with the mean weight of 18±2 g. Additionally, each tube received 1 ml of the product, and they were filled up to 2 ml with physiological serum and incubated at 37 °C for 30 min for the antigen-antibody reaction. The route of injection was intravenous (0.5 ml/mouse) and after injection, all the mice were kept for 24 hours in the experimental animal house according to the modified method of Theakston and Reid (Theakston and Reid, 1983).

Statistical analysis. Results of the expressed proteins and ELISA were analyzed using t-test. In all the measurements, P-value less than 0.05 was considered statistically significant.

RESULTS

Expression of Fab fragment antibody in Rosetta-g bacterium. The amount of protein in the Rosetta-g medium was measured by NanoDrop spectrophotometer at the wavelength of 280 nm. As shown in Table 1, the content of protein was found in sample 1 to be 0.53 mg/ml that increased by more than 4.5 folds when the concentration of IPTG was increased by two-fold. The rise in protein content was found to be more than 7 folds when the concentration of IPTG increased to 2 mM. After the sonication of bacterial sediments, the amount of protein in the supernatant was raised to 5.6 mg/ml, which had a significant difference with the control sample (2.43 mg/ml) with IPTG concentration of zero (Table 1). Moreover, after the purification of the product, the concentration of the protein was 3.1 mg/ml.

Table 1. Amounts of crude protein in Rosetta-g medium

Sample	Concentration of IPTG* (mM)	Mean protein concentration (mg/ml)	P-value
1	0.25	0.53±0.33 ^a	-
2	0.5	2.55±1.56 ^b	>0.01
3	1	2.74±1.14 ^c	>0.04
4	1.5	3.53±0.217 ^d	>0.002
5	2	3.69±0.281 ^e	>0.001
6	1	5.61±7.79 (sonicated)	>0.01
7	0	2.43±1.38	>0.01

* Isopropyl β-D-1-thiogalactopyranoside

Enzyme-linked immunosorbent assay of the expressed protein. The supernatant of Rosetta-g medium containing bacterial expressed proteins was used in ELISA. The wells of ELISA plate were coated with venom antigens. The results of the experiment were read at the wavelength of 450 nm (Table 2, Figure 1).

Table 2. Enzyme-linked immunosorbent assay results of the protein expressed in Rosetta-g medium

Samples	Optical density (Mean±SD)	P-value
Test sample	1.114±0.51	-
Sample without antigen (control 1)	0.6±0.37	>0.05
Sample without primary antibody (control 2)	0.67±0.40	>0.04

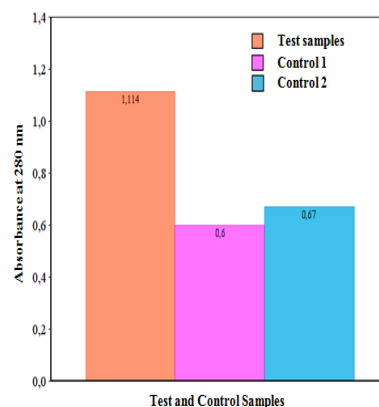


Figure 1. Comparison of Enzyme-linked immunosorbent assay results of the product

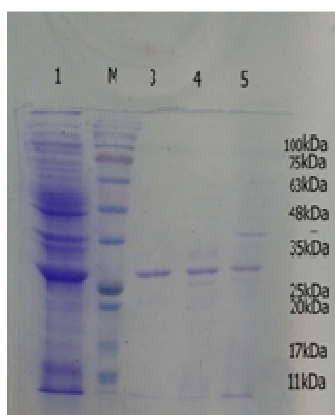


Figure 2. Performing reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis to verify the presence of the Fab like fragment antibody expressed in the Rosetta-g medium. The unpurified product (lane 1) and purified with ammonium sulfate are seen in the lanes 3, 4, and 5. Lane 2 is a ladder.

Electrophoresis of the expressed protein. The electrophoresis of produced protein-like the molecular weight of Fab fragments is demonstrated in Figure 2. The molecular weight of the expressed protein in

reduced form was 28 kDa, corresponding to reduced Fab fragment antibody on electrophoresis. Furthermore, it indicated that the purity of the sample highly increased by using ammonium sulfate for purification.

Potency assay of the product. The results obtained in the present study clearly showed that the produced Fab was able to neutralize the *E. carinatus* venom. Further, 1 ml of the purified product was able to neutralize about 54.6 μg (7 LD50) of the venom.

DISCUSSION

Snake envenomation is a serious life-threatening injury. The global estimate of envenomation is about 2.5 million people every year, more than 100,000 of whom die (Koh et al., 2006). There are different species of venomous and semi-venomous snakes in Iran. According to the results of a study conducted by Dehghani et al. in 2014, in a 10-year report, there were more than 53,000 registered snakebite victims in medical centers of Iran. The annual incidence of snake bites in 100,000 of population varied from 4.5 to 9.1 people during this decade. In the present study, the previous constructed Fab fragment gene library was used for the expression of Fab segment in Rosetta-g bacteria (Motedayen, 2015). Rosetta-gTM host strains are BL21 derivatives, which are designed to enhance the expression of eukaryotic proteins containing codons rarely used in *E. coli*. Our results showed the expression of the protein with molecular weight of 28 kDa, in reduced form, which recognizes venom antigens in ELISA assay with a significant difference with the control groups. As demonstrated in Table 1, the amount of the protein in the test groups, simultaneously with the addition of IPTG concentration in culture medium from 0.25 mM to 2 mM leading to increasing the amount of protein production from 0.53 to 3.69 mg/ml, however, this difference was significant only between groups of 1, 1.5 and 2mM with 0.25 mM. It is well known that increasing IPTG concentration in the culture medium can raise foreign protein production, generally insoluble, in the host bacteria.

However, the production of essential bacterial proteins decreases, which in turn reduces the proliferation of the bacterium and enhances the cost of the experiment (Malakar and Venkatesh, 2012). In agreement with other studies, in this study, IPTG (1 mM) was used, which was more suitable for protein production (de Haard et al., 1999; Wu et al., 2001; Aubrey et al., 2004; Kwong and Rader, 2009; Fu et al., 2011). Moreover, when the bacterial sediment was sonicated, the amount of protein increased further up to 5.6 mg/ml and 3.1 mg/ml (purified form) due to the release of bacterial proteins to the medium (Table 1). Regarding the evidence, the production of Fab can fluctuate from 0.1 to 10 $\mu\text{g}/\text{ml}$ mainly based on its amino-acid residues in cultivation flask (Griffiths et al., 1994; Kwong and Rader, 2009; Restaino et al., 2013), 100 to 500 $\mu\text{g}/\text{L}$ (Humphreys et al., 2002; Zhu and Dimitrov, 2009), 580mg/L, (Itoh et al., 2001) up to 1000mg/L in fermenter cultivation (Kwong and Rader, 2009). In this study, the mean protein concentration in our samples was higher to some extent than the other studies. It may be because of the difference in purification methods and using the NanoDrop A280 measurement for the estimation of protein concentration in this study. A280 measurement is a fast, cheap, and primary method. Nevertheless, this method is highly dependent on the percentage of aromatic amino acids in the sample. Therefore, it may absorb everything in the sample and finally lead to raising the results. The potency determination of snake antivenoms is usually performed in mice (Nalbantsoy et al., 2012; Yap et al., 2015). Furthermore, *Echis carinatus* is wide spread and the most dangerous snake in Asia including Iran (Al-Maliki et al., 2015). Accordingly, this snake is used for the present study. In this study, 1 ml of Fab can be used to neutralize 54.6 μg of the venom. Although this is an initial result, it is important and with more purification and concentration, its potency will increase.

Based on the results obtained in this study, it can be concluded that the Fab fragment of antivenom can be expressed in *E. coli* (Rosetta-g) effectively. Furthermore, it could neutralize to some extent venom of *E.*

carinatus viper snake. However, further studies are recommended to increase the potency of produced Fab by Rosseta-g bacteria to be commercially suitable for specific antivenom production.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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