

**Original Article**

# Construction of a human recombinant polyclonal Fab fragment antibody library using peripheral blood lymphocytes of snake bitten victims

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## ABSTRACT

Human snake bitten poisoning is a serious threat in many tropical and subtropical countries such as Iran. The best acceptable treatment of envenomated humans is antivenoms; however they have a series of economic and medical problems and need more improvements. In this study a combinatorial human immunoglobulin gene library against some of Iranian snakes venoms was constructed. Total RNA prepared from peripheral blood lymphocytes of two recovered snake victims. RT-PCR was used for cDNA synthesis and amplification of the heavy (Fd segment) and kappa light chains of IgG antibody. After digestion of the heavy chain with *SpeI* and *XhoI* and light chain with *XbaI* and *SacI* enzymes, inserted successively into the cloning vector pComb3x, and then recombinant vector transformed to TG1 bacteria to construct the Fab library. For determination insertion rate of Fab segment into cloning vector, plasmids of 12 clones of library were extracted and digested with *SfiI*. Length of amplified Fd and  $\kappa$  chains, were 650 - 750 bp. Primary library size was determined to contain  $4.9 \times 10^5$  members out of which half of them contained the same size of Fab fragment. This result is comparable to some researchers and shows that this method could be appropriate tool for the production of human polyclonal Fab fragment antibodies for management of poisonous snake bitten victims.

**Keywords:** Fab, Combinatorial library, snake, PComb3x, immunoglobulin

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## INTRODUCTION

Snake bitten is a serious threat for human being and the most dangerous snake envenomation occurs in many of subtropical and tropical countries such as Iran. Snakes are sparse in the world except New Zealand and Ireland. Many species of snakes are found in large parts of Iran. More than 21 species of

poisonous and semi poisonous snakes are in Iran and annually a great number of our people are damaged or die by the bite of venomous snakes (Amrollahi 2011). At the present, Razi Institute of Iran make antivenom against 6 poisonous snake venoms including, *Echis carinatus*, *Naja naja oxiana*, *Vipera lebetina*, *Vipera albicornuta*, *Agkistrodon halys* and *Pseudocerastes persicus*, which are used for management of

envenomated victims. The most acceptable systemically treatment of envenomated humans is antivenoms (Elda *et al* 2003). Generally the most of antivenoms are producing against crud venom in horses, in which there is a series of medical drawbacks such as life-threatening anaphylactic shock and serum sickness (Nicolas *et al* 2004). However, these products need more improvements. Current methods for producing of antibodies involve immunization of animals (such as horse, sheep, goat, rabbit) (Ibrahim *et al* 2003, Helen & Yvonne 1995), however, in recent years newer techniques such as phage display system as a major strategy for generation of antibodies have also been developed (André *et al* 2014). Phage display system, accompany antibody gene libraries, can prepare desired immunoglobulins. It was established by George P. Smith in 1985 and describes the presentation of exogenous peptides on the coat of phage particles (Carmela *et al* 2012). Display of peptides and proteins on filamentous phage display is an *in vitro* selection technique that enables polypeptides with desired properties to be extracted from a large collection of variants. A gene of interest is fused to that of a phage coat protein, resulting in phage particles that display the encoded protein and contain its gene, providing a direct link between the phenotype and genotype (Marjorie *et al* 2003). Antibody libraries can provide a rich source of antibody diversity and potentially providing hundreds of unique antibodies per target (Darren *et al* 2007). Indeed, it has been reported that recombinant neutralizing human antibodies to the snake venoms could be generated from human antibody phage display libraries. However it needs some hosts including *E. coli* for propagation of phages and expression of recombinant proteins. Today, antibody gene libraries in combination with phage display system are widely used to select host cells that express favorable immunoglobulin fragments (Achim & Ralf 2009). In this technique, the repertoire of V genes of one or more individuals is amplified with primers covering all or important parts of V gene

families, giving rise to human antibodies. The library is generated by a random combination of variable light (VL) and variable heavy (VH) chain genes produced as antigen binding (Fab) or single chain variable (scFv) antibody fragments (Carmela *et al* 2005). Afterward, recombinant M13 phages contained Fab antibody fragments are generated in *E. coli* bacteria. Many eukaryotic secretory proteins such as antibodies, requires oxidative environment for promote disulfide bonds formation and their stability. Such environment prepared in periplasmic of this bacterium, where the immunoglobulin can assemble together forming the mature antibody. In the present study, we developed a combinatorial Fab phage display library from a randomly combined kappa (VL+CL) and Fd (VH+CH1) chains of antibody genes of two snake bitten victims. So we could generate a polyclonal recombinant human antibody Fab fragment to the snake venom toxins from this library. For screening of the library, favorable antibodies as Fab fragments attached to minor coat protein III of filamentous phages were chosen.

## MATERIALS AND METHODS

In order to construct the natural immune Fab antibody phage display library, a total of 80 ml of peripheral blood from two victims who recovered from snake bitten were collected in 4ml hematological tube contained K2 EDTA 7.2 mg. Mononuclear cells were isolate from the whole peripheral blood by ficoll gradient centrifugation method and RPMI 1640 medium. Total RNA purified from lymphocytes using Total RNA Extraction Kit (PP-210S) and cDNA synthesis performed by using AccuPower RocketScript RT Premix, 0.2 ml tubes (K-2101) kit and Random hexamer oligonucleotide primer (dN6) was subjected to PCR reactions. Genes coding the light (K1 and K3 segments) chain and Fd region of the heavy (H1 and H3 segments) chain were amplified using AccuPower PCR Premix, 0.2 ml tubes (K-2012). The premix was then subjected to 35 rounds of amplification at 95 °C for 3 min, 94 °C for 1 min, 61.5 °C for 1.5 min and 72

°C for 2 min followed by a 72 °C for 10 min as final extension for all of four pairs of primers (table 1). Five microliter samples of PCR products were run on 1% agarose gel for verifying of desired bands. For amplification of light and Fd chains, we used two sets of three primers including VH1a and VH3a as 5' primers for amplification of IgG cDNA heavy chain (Fd), with *XhoI* site and CG1z (table 1) as the 3' primer for the Fd and corresponds to part of the hinge region with *SpeI* site for cloning into the pComb3x vector (Figure 1). And also VK1a and VK3a (table 1) as 5' primers for amplification of the kappa light chain with the *SacI* site and CK1a primer as a 3' primer corresponding to the 3' end of the kappa light chain, with the *XbaI* site for cloning into the same vector. The antibody library was created by sequentially cloning a repertoire of heavy Fd fragment genes into the pComb3x vector via the *SpeI* and *XhoI* and followed by cloning of kappa light chain genes into the same recombinant cloning vector via the *XbaI* and *SacI* restriction sites (Darren et al 2007).

**Table1.** Oligonucleotide primers used for construction of human Fab library

#### Heavy chain (Fd segment)

Forward primers

VH1a 5'-CAGGTGCAGCTCGAGCAGTCTGGG-3'

VH3a 5'-GAGGTGCAGCTCGAGGAGTCTGGG-3'

Back primer

CG1z 5'-GCATGTACTAGTTTTGTACAAGATTTGGG-3'

#### Light chain (Kappa)

Forward primers

VK1a 5'-GACATCGAGCTCACCCAGTCTCCA-3'

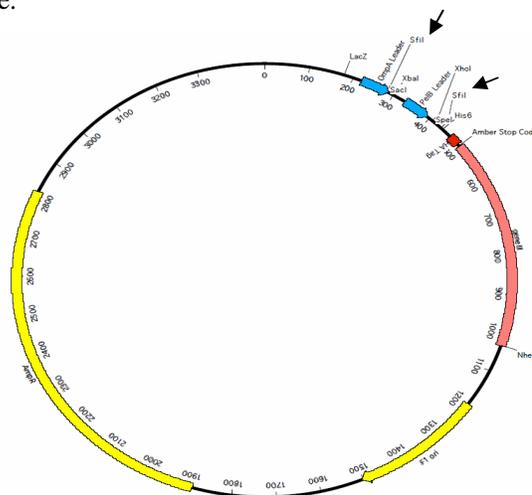
VK3a, 5'-GAAATTGAGCTCACGCAGTCTCCA-3'

Back primer

CK1a, 5'-GCGCCGTCTAGAACTAACACTCTCCCCTGTT  
GAAGCTCTTTGTGACGGGCAAG-3'

**Construction of pFd recombinant vector.** For pFd (pComb3x contains Fd chain) construction, the amplified and mixed Fd (VH+CH1) chains of two victims and pComb3x vector, after agarose gel purification were separately and simultaneously digested with restriction enzymes of *XhoI* and *SpeI* and then were ligated together by T4 DNA ligase (Fermentas) according to the manufacturer's protocol.

For propagation purposes, the pFd construct was transformed into TG1 calcium chloride competent bacteria cells on LB/2xYT ampicillin medium agar and incubated at 37 °C overnight. For control, an aliquot of same competent TG1 cells without transforming with pComb3x vector was plated on a LB-ampicillin agar plate.



**Figure 1.** pComb3x vector. Spaces allocated for cloning of light chain between the *XbaI* and *SacI* and also Fd chain between *SpeI* and *XhoI* restriction enzymes sites. Incision sites of *SfiI* restriction enzyme for cloning/discharge Fab segment are demonstrated (arrows).

**Construction of pFdK recombinant vector.** The amplified and mixed  $\kappa$  (VL+CL) light chains of two victims and pFd, after gel purification, same as Fd chains, were digested with *XbaI* and *SacI* restriction enzymes and then ligated together by T4 DNA ligase according to protocol of its manufacturer's for construction of pFdK (pComb3x contains Fd and kappa chains). The product was then transformed into competent TG1 cells for propagation same as described above. Library size was detected to be  $4.9 \times 10^5$  which was determined by summing the number of transformed colonies on 2xYT agar/ampicillin plate. Insertion rate of Fab fragment into pComb3x vector were detected by digestion of pFdKs extracted plasmids from 12 library members with *SfiI* restriction enzyme according to its recommended protocol.

## RESULTS

**Fd and  $\kappa$  chain genes amplification.** The total RNAs of the PBMCs of two recovered victims were used for RT-PCR. Fd and  $\kappa$  immunoglobulin chains amplified separately, using four pairs of specific primers which contained one site for the restriction enzymes corresponding to the vector pComb3x. The PCR products of Fd and  $\kappa$  chains were about 650-750 bp (Figure 2) (Bao *et al* 2001, Carmela *et al* 2005, Hogrefe & Shopes 1994).

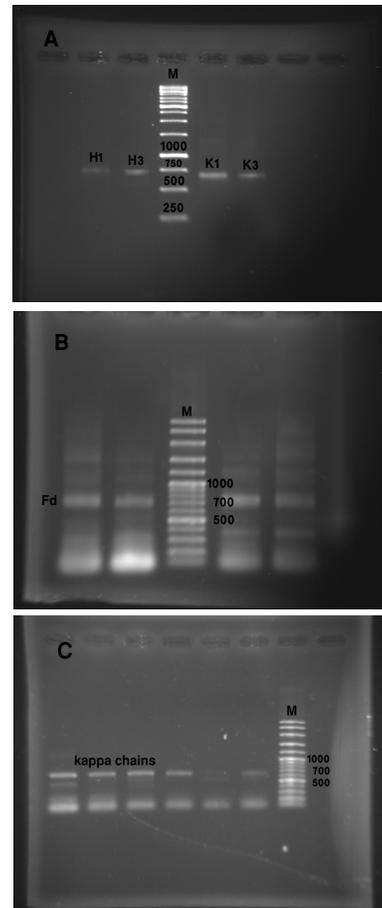
**Recombinant pFd Construction.** The recombinants of pFd were constructed by cloning mixed Fd fragments of two victims into prepared pComb3x vector. After transforming the competent TG1 cells, the Fd gene library was formed and the size of the transformed bacteria reached  $1.2 \times 10^5$ . Two of five colonies showed the fragments of 650 -750 bp. So recombination frequency was 40%, hence the practical quantity of Fd library was calculated to be  $4.8 \times 10^4$ . All the primary products of PCR amplification of the Fd and light chains (Figure 2) ranged from 650 to 750 bp in size (Carmela *et al* 2005).

**Fab (pFdK) gene recombinant Construction.** The recombinant pFdk were constructed by cloning, mixed and digested  $\kappa$  light chains of two victims, into purified and digested pFd extracted vectors. After transforming competent TG1 cells with pFdk vectors, the Fab gene library was formed and the quantity of the transformed TG1 cells reached  $4.9 \times 10^5$ . Following extracting the plasmids from twelve random TG1 colonies and digestion with *Sfi*I restriction enzyme, half of the plasmids discharged the fragments about 1.4kb (Figure 3). The recombination frequency of fragments Fd together with  $\kappa$  chain insertion were 50%, hence the primary quantity of Fab library was calculated as  $2.45 \times 10^5$ .

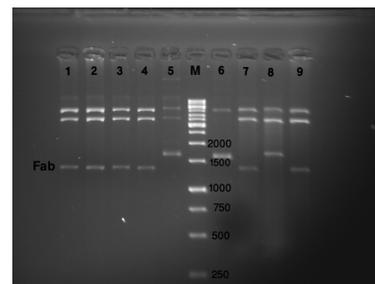
## DISCUSSION

Human envenomation with poisonous snake venoms is a serious threat. Several poisonous and semipoisonous snake species are found in Iran and annually a great number of our people are damaged and

in severe envenomation may result in death by bite of venomous snakes (Amrollahi 2011).



**Figure 2.** Agarose gel electrophoresis of PCR products. A. The purified bands are about 700 bp in the left and right of the ladder represented the Fd (H1/H3) segment of heavy chain and kappa light chain (K1/K3) respectively. B and C represented unpurified PCR products of Fd chain (B) and kappa light chain (C).



**Figure 3.** Digestion of 9 random TG1/pFdK colons with *Sfi*I restriction enzyme. As can be seen in figure, colons 1-4, 7 and 9 discharged a 1400 bp segment which represented a Fab fragment.

The use of horse origin antivenin is the treatment of choice for snake bitten victims and results in rapid amelioration of symptoms in victims who well tolerate the medication. However using traditional antivenoms have some drawbacks such as early anaphylactic and late serum sickness which may occurs in the patients who treated with these antivenoms. Serum sickness, a type III hypersensitivity reaction, results from the injection of foreign proteins and develops within 2 weeks after serum injection. It is mainly caused by the deposition of immune complexes at certain tissue sites. The syndrome usually lasts only a few days but its severity depends on both the amount of serum injected and the previous immune state of the patient (Francesca *et al* 2008). Phage display technique exhibiting antibody libraries is another way for production of immunoglobulins. In phage display methodology, genotype of antibodies linked to its phenotype (Sara & Lutz 2002) and allows the selection of human antibody fragments with potential use in clinical applications. Phage display antibody technology can provide a rich source of antibody diversity and become increasingly popular for creating binding sites for use in all areas of research, as well as in medical and industrial applications (Potjamas *et al* 2009, Darren *et al* 2007). During phage display, only the Fd chain is fused to the phage PIII coat protein, whereas the kappa light chain gets attached to Fd segment in the *E. coli* periplasm, where the immunoglobulin chains can assemble together and forming the mature antibody. In addition to existence of an oxidizing environment, the periplasm of *E. coli* also contains enzymes that promote protein folding and formation of disulfide bonds between heavy and light chains (Ka & Christoph 2009, Robert *et al* 2003). Antibody libraries may be non-immunized or immunized. An antibody repertoire from immunization is generally restricted to generating antibodies against the antigen of the original immunogenic response (Sara & Lutz 2002). However non-immunized library may create more antibodies against favorable antigens but affinity of these antibodies for their antigens, is generally less than the latter. In this study a

combinatorial immunized human immunoglobulin gene library against Iranian poisonous snake venoms was constructed. For this, we cloned genes coding for the human antibody light and Fd gene repertoires of snake bitted victims into a pComb3x vector for the isolation of immunoglobulins against snake venom antigens by phage display technique in the next steps. Libraries may have some similarities and differences in the points of size, form of inserting light and heavy chains in cloning vector and also their immunity. Our library size ( $4.9 \times 10^5$  members) is bigger than some libraries reported, however it is smaller than the other reported libraries for human with more than  $10^6$  members (Carmela *et al* 2005). For example, Jinye Liu *et al* (Jinye *et al* 2006) constructed an immune library with  $4.8 \times 10^4$  members for Fab segment antibody production with consequently cloning of light and heavy chains in the vector, Bao-Ping Wu *et al* (Bao *et al* 2001) constructed an immune library with  $1.48 \times 10^6$  members for isolation of Fab fragment antibody against colorectal cancer cells where heavy and light chains was consequently inserted into pComb3 cloning vector, Carmela Dantas Barbosa (Carmela *et al* 2012) constructed an immune Fab library with  $1.45 \times 10^8$  members in which heavy and light chains were ligated together and then inserted to the cloning vector. Rahbarizadeh *et al.* (Rahbarizadeh 2003) constructed two immune libraries with more than  $10^4$  members for isolation of  $V_{HH}$  segment antibodies against MUC1 antigens. Hans J. de Haard (Hans *et al* 1999) generated a Fab non-immune library with  $3.7 \times 10^{10}$  members by using two separated cloning vectors for increasing of its diversity. One important reason for lower size of our library as compared with some other libraries can be the use of heat shock in our study, because the efficacy of DNA transformation into bacteria has always been the limiting step for constructing large libraries. While in other studies, electroporation has been showed to have a high efficacy, hence has been frequently used to make large bacterial libraries (Zhongyu & Dimiter 2009). One of the important factors in library construction is diversity. We attempted to increase the

diversity by adoption of four items :i) using PBMCs of two snake bitten victims for total RNAs isolation and two different cDNAs synthesis; ii) use of random hexamer primers for first strand cDNA synthesizing. It was previously reported that the diversity of the library could be maximized by using random hexamers for cDNA synthesis so that all five antibody classes could potentially be represented (Potjamas *et al* 2009). iii) use of four of 5' primers based on the leader exons and FR1 sequences and also two 3'primers within the constant regions during amplifying the V genes of Fd and kappa light chains; and iv) separately amplifying of common segments of VH (VH1, VH3) and VK genes (VK1, VK3) of different families with the introduction of cloning sites at their extremities in each of related victims and pooled the same purified PCR product in the cloning steps. The later item is similar to Hans et al and Bao-Ping Wu et al which in order to obtain larger size diversity Fab library, used a two-step cloning procedure; heavy and light chain variable genes were first separately cloned as digested PCR products (Hans *et al* 1999, Bao *et al* 2001). Ligation efficiency plays an important role in creation a suitable size library. Observed ligation efficiency of our study was 50%, which means that this is compatible with the other researcher found the ligation efficiency 30-50 percent (Hogrefe & Shopes1994).

Our library is the first report of a polyclonal Fab antibody library against snake venom antigens which was constructed by PBMCs of two envenomated victims, and according to the size, diversity items and origin, it seems to be suitable for isolating Fabs which capable of neutralizing of Iranian poisonous snake venom antigens.

### 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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