



Identification of the immunogenic epitopes of the whole venom component of the *Hemiscorpius lepturus* scorpion using the phage display peptide library

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Abbreviations:

EDTA

Ethylenediaminetetraacetic acid

IgG

immunoglobulin G

Ph.D

phage display

ELISA

enzyme-linked immunosorbent assay

PEG

Polyethylene glycol

ABSTRACT

The venom of the *Hemiscorpius lepturus* scorpion contains mixtures of bioactive compounds that disturb biochemical and physiological functions of the victims. *Hemiscorpius lepturus* envenomation is recognized as a serious health concern in tropical regions. So far, there is no preventive procedure, and the main focus is on treatment of victims with an antiserum purified from hyper-immunized horses. Although antisera can neutralize the venom, they, in some cases, lead to anaphylactic shock and even death. Selection of peptides mimicking antigenic and immunogenic epitopes of toxins from random peptide libraries is a novel approach for the development of recombinant toxins and poly-epitopic vaccine. To achieve this aim, a phage display peptide library and three rounds of biopanning were performed on immobilized antibodies (IgGs) purified from the sera of hyper-immunized horses. The results show that the highest binding of the phage to immobilized horse antibodies occurred in the third round of biopanning. Over 125 individual clones carrying mimotopes of *Hemiscorpius lepturus* toxins were selected and subjected for sequencing. The sequencing results identified unique peptides mimicking the antigenic and immunogenic epitopes of *Hemiscorpius lepturus* toxins. The results of this study provide a basis for further studies and the development of a putative epitopic vaccine and a recombinant toxin.

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1. Introduction

The *Hemiscorpius lepturus* is a member of the *Hemiscorpiidae*

family and recognized as the Gadim scorpion in Khuzestan province (Dehghani and Arani, 2015). Its body colour is clear yellow, and it is found in Iran, Iraq, Pakistan, and Yemen (Kassiri et al., 2015). As many as 95% of mortality and morbidity relating to scorpion bites occur from the *Hemiscorpius lepturus* scorpion. The clinical symptoms in victims appear after 24 h–72 h of scorpion bite. Limiting lesions, such as mid pain and erythema, have been reported at the site of bite followed by systemic and neurologic symptoms such as

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dry mouth, fever, nausea, hyperthermia, infection, hypotension, tachycardia, muscle spasm, and seizure. The victims usually experience fatigue, dizziness, and vertigo. In fact, necrosis occurs in some cases. Depending on the circumstances and severity of scorpion bite, other symptoms are kidney failure and fatal haemoglobinuria, while death occurs in some victims (Pipelzadeh et al., 2015; Ghafourian et al., 2015). Currently, there is no preventive plan for scorpion bite and the main focus is on the treatment. Specific treatment includes injection of polyclonal F(ab')₂ fragment purified from the serum of hyper-immunized horse. Although antisera are able to neutralize the venom, there is a concern about their use in case of mild to severe scorpion bite. Sometimes the use of horse antiserum leads to anaphylactic shock and even death. Other limitations of antisera are as the following: low potency of serum, potential changes in serum to neutralize the toxin in different production batches, and lack of efficacy of the antiserum in case of delay in referring to health centres (Pipelzadeh et al., 2015). Although significant advances have been made in immunological studies of some toxins of scorpion venom, only a few research studies are available on subject. So, there is scant information on how to identify the whole venom component and develop vaccine candidates or novel therapies (Gazarian et al., 2005). Phage display is a new technique for displaying proteins on the surface of the bacteriophage. George Smith introduced this technique in the mid-1980s. Display of proteins on the phage surface is a reliable method to select rare genes encoding proteins with binding activity (Lee et al., 2007a, 2007b; O'Brien and Aitken, 2002; Barbas et al., 2001). A new approach to identify the components of the venom and the achievement of vaccine candidate is based on selection of mimotopes isolated from the random peptide phage display library. It is believed that peptide phage display libraries have great potential in the development of immunological studies of toxins and provide a convenient source for the vaccine candidate (Galfrè et al., 1995). Using a peptide library and the enrichment process, a population of phage mimicking the antigenic and immunogenic region of toxins is identified. In fact, with the phage-display technique, the important epitopes of antigens involved in antibody-antigen binding are recognized. According to a study, epitopes of toxins of NTX and Cn2 of *Centruroides* scorpions have been determined by using phage display peptide libraries and the enrichment process of antibodies purified from the immunized mice (Galfrè et al., 1995). In another study, dominant epitopes of Cn2 toxin were identified by the peptide phage display library (Gazarian et al., 2003). There is no complete information about the antigenic proteins, peptides, and raised antibodies to the venom of the *Hemiscorpius lepturus*. So, the main aim of the current study was to access the profile of peptides mimicking the antigenic component of *Hemiscorpius lepturus* venom. For that purpose, a horse was immunized with the crude venom of the *Hemiscorpius lepturus*. The peptide phage display library (Ph.D.C7C library) was enriched against purified IgGs (purified from horse sera) through three rounds of biopanning. The selected peptides subjected to Blastp in scorpion database and their corresponding proteins (phospholipase A2, phospholipase D, venom allergen, metalloproteinases, hyaluronidase, potassium channel toxins (KTx), venom protein, and calcin) were recorded. Our results, for the first time, revealed the venom component and epitopes of the *Hemiscorpius lepturus*.

2. Materials and methods

2.1. Materials

The serpentarium of the venom lab at Pasteur Institute of Iran, Karaj, Iran consists of two different rooms for keeping scorpions and snakes. The rooms contain ventilators and air conditioners to

keep temperatures between 23 and 25 °C. Scorpions are maintained in a room with several windows. Each type of scorpion is kept in individual polyethylene cages of 50 W × 75 L × 40 H cm. Each cage contains wet cotton (in plastic bowl) to provide water for scorpions. About 35–45 scorpions are kept in one cage, and feeding them and cleaning the cage is performed weekly. Venom extraction was performed every month according to the guideline set by the World Health Organization (WHO). All experiments were performed in accordance with the ethical committee of Pasteur Institute of Iran. Crude venom was extracted from the *Hemiscorpius lepturus* tail by electric shock, according to the method set by the WHO (World Health Organization W, 2010). Loop-forming heptapeptide phage display library was purchased from New England Biolab (Ph.D. C7C, NEB). Melon™ Gel IgG Purification Kit was purchased from Thermo Scientific. The whole venom component was identified by the RNA sequencing of the venom gland of the *Hemiscorpius lepturus* by using the Illumina next generation sequencing (unpublished data).

2.2. Horse immunization by the crude venom of the *Hemiscorpius lepturus*

All experiments involving horses used for immunization and blood collection was approved by the ethical committee of animal care of Pasteur institute of Iran, Tehran, Iran (No; IR.PIL.REC.1394.42). A four-year-old male horse (Torkaman horse), weighing 300 kg, was subcutaneously injected at the neck with different concentrations of *Hemiscorpius lepturus* crude venom every two weeks for eight times. Concentrations of 100, 200, 400, and 500 µg were used for first to fourth injections, respectively. Subsequent injections were carried out with the concentration of 500 µg. Freund's complete adjuvant was used for the first injection and the rest were continued with Freund's incomplete adjuvant (ratio 1:1 of the crude venom volume). Horse immunization was monitored by ELISA on immobilized crude venom (10 µg/ml). Briefly, two weeks after the last injection, about 10 ml of horse blood sample was collected and serum isolated by centrifugation at 2000 rpm for 10 min. A 96-well (Nunc, Roskilde, Denmark), was coated overnight with 10 µg/ml of crude venom at 4 °C in sodium bicarbonate buffer (PH = 9.6). A well also was coated with sodium bicarbonate buffer at the same condition as negative control. Next day, the wells were blocked with skimmed milk 2% (in PBS) for 1 h at RT. Then, the wells were incubated with different dilutions of immunized horse serum and non-immunized horse serum (as control) (1/500 to 1/64000) for 1 h at RT. The wells were washed with PBST (0.05% (V/V) Tween 20 in PBS) for five times and incubated with rabbit anti-horse HRP conjugated antibody (1:2000 in PBS buffer) for 1 h at RT. Peroxides activity was detected by adding TMB (3,3',5,5'-tetramethylbenzidine). The absorbance of the wells was determined at 450 nm by the ELISA plate reader.

2.3. Biopanning of the phage display peptide library

To isolate peptides mimicking the immunogenic sites of *Hemiscorpius lepturus* toxins, biopanning of the phage display peptide library (Ph.D. C7C, NEB) was performed on immobilized horse IgGs. The library-cloning vector was M13KE, which was derived from the common cloning vector M13mp19. Immunized and non-immunized horse IgGs was purified from sera sample by the Melon™ Gel IgG Purification Kit (Thermo Scientific) in accordance with the manufacturer's instructions. A polystyrene 96-well was coated overnight at 4 °C with purified IgGs (immunized horse) in the sodium bicarbonate buffer. A well was also coated at the same condition with IgGs isolated from non-immunized horse as negative control. The wells were blocked with the blocking buffer (0.1 M

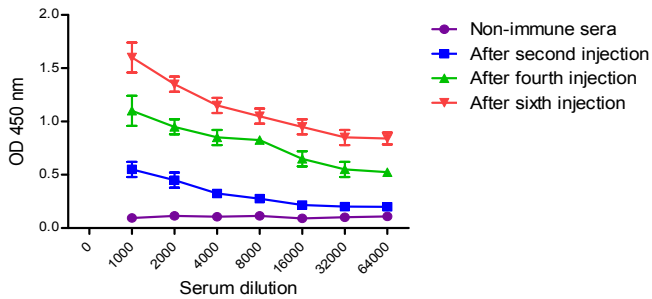


Fig. 1. Horse immunization response. Before each injection sera purified and monitored by ELISA. As can be seen, antibody is raised against the crude venom of the *Hemiscorpius lepturus* after the second injection. The graph indicates triplicate assay $\pm$ SD.

NaHCO₃ (pH 8.6), 5 mg/ml BSA) for 1 h at RT. Then, 10^{12} CFU (Colony Forming Unit) of peptide library was added to the well coated with the IgG of the non-immunized sample (to remove false positive phage) and incubated for 1 h at 37 °C. After 1 h, unbounded phage (in the supernatant) was collected and added to the wells coated with the IgG of the immunized sample and incubated for 1 h at 37 °C. The wells were washed three times by PBST to remove unbounded phage. The bounded phage to immobilized IgGs (output phage) was eluted by incubation of the wells with 100 μ l of 100 mM triethylamine (TEA, pH 10.0) for 10 min. Neutralization of the wells was performed by adding 100 μ l of 1 M Tris-HCL (pH 8.0). A total of 200 μ l of phage were collected in a vial, of which 10 μ l had been used for titration of output phage through the infection of *E. Coli* ER2738 (F proA + B + lacI q Δ (lacZ)M15 zcf::Tn10(Tet R)/fhuA2 glnV Δ (lac-proAB) thi-1 Δ (hsdS-mcrB)) and plating on the LB/IPTG/Xgal medium containing tetracycline, 180 μ l used for re-amplification of phage, and the rest 10 μ l stored at –80 freezers as backup. For re-amplification of output phage, 180 μ l of eluted phage was infected with 20 ml of *E. Coli* ER2738 at the early log phase, OD₆₀₀ ~ 0.05 and incubated with vigorous shaking (250 rpm) for 4.5 h at 37 °C. Bacterial cells were pelleted by centrifugation at 5,000 g for 15 min. Phage particles in the supernatant were precipitated overnight by 20% PEG/2.5 M NaCl at 4 °C. These particles were collected by centrifugation at 14,000 rpm for 2 min, and re-suspended in PBS and used as input phage for the next round of the biopanning process. The biopanning was carried out for three consecutive rounds.

2.4. Polyclonal phage ELISA

Polyclonal phage ELISA was performed on achieved phage from different rounds of biopanning to monitor the progress of biopanning. A 96-well plate was coated overnight at 4 °C with 10 μ g/ml of IgG samples purified from the immune sera in the sodium bicarbonate buffer (positive well). The control well was also coated with IgGs of non-immune sera and incubated at the same condition of the positive well. After blocking with skimmed milk 2% for 1 h at RT, the wells were incubated with 10^{12} CFU of phage of different rounds of biopanning for 1 h at 37 °C. Then, the wells were washed

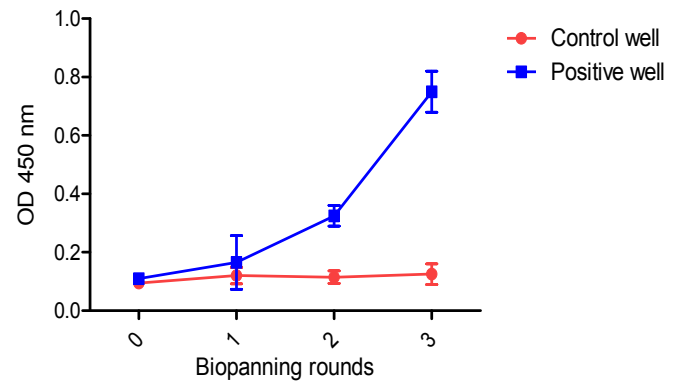


Fig. 2. Polyclonal phage ELISA results. Phage eluted from each rounds of biopanning were monitored by phage ELISA. Positive well: well containing IgGs isolated from the sera of the immunized horse. Control well: well containing IgGs isolated from the sera of the non-immune horse. The error bar represents the triplicate of each assays $\pm$ SD.

three times with PBST and incubated with anti M13-HRP antibody (Roche) (1:2000 in PBS) for 1 h at 37 °C. The wells, subsequently, were washed three times with PBST and incubated with TMB for 10 min at RT. The reaction was stopped by adding 2N H₂SO₄ to the wells. The absorbance of each well was measured at 450 nM.

2.5. Plaque amplification and phage DNA isolation for sequencing

After confirmation of progress of biopanning by polyclonal phage ELISA, 125 individual blue colonies (which carries the lacZ α gene) were randomly selected from the output phage of Round 3 of biopanning. Each clone was separately infected with *E. Coli* ER2738, which was at early -log phase, OD₆₀₀ ~ 0.05 and incubated for 5 h at 37 °C with vigorous shaking. The bacterial pellet was collected with centrifugation at 14,000 rpm for 30 s. The phage particle in the supernatant was precipitated by using 20% PEG/2.5 M NaCl at 4 °C. The phage was pelleted by centrifugation at 14,000 rpm for 10 min. The phage pellet was then re-suspended in 100 μ l of the iodide buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 4 M sodium iodide (NaI)), after which 250 μ l of ethanol was added and incubated for 10 min at RT. The single-stranded phage DNA was precipitated by centrifuging at 14,000 rpm for 10 min at 4 °C. The phage DNA pellet was washed with 500 μ l of 70% ethanol and pellet dried under vacuum. Finally, the phage DNA was suspended in 30 μ l of the TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) and sequenced with 96 gIII sequencing primer 5'- HO CCC TCA TAG TTA GCG TAA CG –3'. The obtained nucleotide sequences were translated into amino acid sequences and subjected to Blastp (<http://blast.ncbi.nlm.nih.gov/Blast>) in a non-redundant protein database to achieve peptide-mimicking epitopes of scorpion.

Table 2

The main antigens identified in *Hemiscorpius lepturus* venom involved in stimulation of immune responses.

	Antigen name	Peptide frequency
1	Phospholipase D	26/100
2	Phospholipase A2	21/100
3	Venom allergen	18/100
4	Metalloproteases	16/100
5	Hyaluronidase	7/100
6	Venom protein	4/100
7	Potassium channel toxin (KTx)	3/100
8	Calcin	5/100

Table 1

Phage titration results. Titration of output and input phage was performed to monitor the progress of biopanning.

Biopanning rounds	Input phage (pfu)	Output phage (pfu)
1	10^{12}	3×10^4
2	10^{12}	5×10^5
3	10^{12}	4×10^7

3. Results

3.1. Horse immunization results

Immunization of horse against *Hemiscorpius lepturus* venom was confirmed by ELISA. Before each injection blood sample was collected, sera were isolated, diluted, and monitored for raised immune response to *Hemiscorpius lepturus* crude venom. The immunization results showed raised antibody response to *Hemiscorpius lepturus* venom. As shown in Fig. 1, an increased antibody response was observed after the second injection of *Hemiscorpius lepturus* venom and immune response remained at a high level in

diluted sera. The results suggested success in the immunization process.

3.2. Biopanning and phage ELISA results

Three rounds of biopanning were performed on immobilized isolated horse IgGs to enrich peptide display phage library towards horse IgGs and isolated phage particles with high affinity and specificity to target. The output phage increased after each panning round, which indicated progress in the panning procedure. The enrichment of the phage library was estimated by comparing output phage in positive (well-coated by immunized horse IgGs)

PLD id in <i>Hemiscorpius lepturus</i> transcriptome	Aligned PLDs	Identified Peptide	Peptide frequency
HLPLD2	MRIGSYDNNKKRRYDRTIKTVALIAIVSTV--VAEDNLD--NDPLLRPIWNIGHMANSKY		
HLPLD4	-----MVNSLP		
HLPLD1	MA-HCYYNKRGKCNRMKTVALVVLITV--MVEESRGDSQEDKKRPIWNIGHMVNAVK		
HLPLD2	E-----ITKFLLELGANGVEAD-----ISFNDGKALWTYH---GRPCDCG----	1- VFDDNGR	1/100
HLPLD4	Q-----IDFPLNLGANALEAD-----FAFDDGKAYKWTYH---GYPCDCF----	2- RENDNGS	1/100
HLPLD1	Q-----IEEFLDLGANALEAD-----VTFDDNGNPKWTYH---GTPCDF----	3- WYTCNGN	1/100
HLPLD2	RNCTMRENVDDY-LDYVGD-----RPDSSSFLS-----HFTLIILDMKISHL		
HLPLD4	RSCKRYERVDDY-LRYVRE-----RGSPKFRS-----DFVLLQIDLKISGL	4- HTYDTT	2/100
HLPLD1	RDCLRWEYVDEY-LKRIRE-----PGSSKFRK-----GFILLMLDLKISKL	5- FDTDEA	2/00
HLPLD2	SNVAFNSAGKD-----IAEKIVRHLWNG		
HLPLD4	SKEAKHNAGVD-----VAVKLIRHLWSG		
HLPLD1	SDNAKSKAGKE-----IADMI IKRLWSG	6- TDKSKAGFE	3/100
HLPLD2	EGE-----MSQVWILFSIPSTEASEFVVGKKEI-----		
HLPLD4	KDP-----KSQLWILLSFPYTTDIDFVEGFLPTL-----		
HLPLD1	SGE-----KAQLYIVLSFPYVNDIEFVRAFRERV-----		
HLPLD2	-----TKLGYGKYYHDKIGWDIWDYFYVRETYKRLNVSDNVWLSGNTNCLY-----	7- KGLH	2/100
HLPLD4	-----RANGYHN-MQSRIGWDISGKGLIDKKTYQRLGISNSVWQGDGITNCLP-----	8- GLKH	2/100
HLPLD1	-----KSKGFASEAEKRIGWDISGKGLGKRDAYQKLGITDNVWQSDGITNCLT-----		
HLPLD2	---RFH--RSLSRAGKAV---KKRDSGESKWKF--AKKVYVWTIDEBELLGNAISLKVDG	9- KSDAEWF	3/100
HLPLD4	---RSI--KRLV--DAI---YRRD-FDAWEF--LKKVYYWTLDKSSMRQALRVGVDA		
HLPLD1	---RSH--DRLA--EAV---CKRD-SDKEWPS--LKKVYYWTVDKQSSMKEALKVGVGD		
HLPLD2	I-----VTR-----FTLHG--LLT	10- VTWDF	2/100
HLPLD4	I-----TTH-----FDFVS--VLA	11- VSHDF	1/100
HLPLD1	M-----TTL-----EDLVA--VL-	12- WVGLDF	2/100
HLPLD2	TTEFAYLGRLANRLDDPKK-----WKSPYE-----	13- TDRWQKT	2/100
HLPLD4	SDEFSKSHRLATIRDNFK-----QQPQRYLSQYTANVDY--VFYECNPEQ	14- ASWQKT	2/100
HLPLD1	-NEFSGTHRLANINDSPK-----PRPKSNC-----		

Fig. 3. Multiple alignment of identified Sphingomyelin phosphodiesterase D (newly named as phospholipase D) in *Hemiscorpius lepturus* transcriptome. Epitopes mimicking antigenic site highlighted with different colours. The sequences of PLDs released to the gene bank of NCBI with the following accessions: HLPLD1 (KX932445), HLPLD2 (KX932446), and HLPLD4 (KX932448). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

PLA2 id in <i>Hemiscorpius lepturus</i> transcriptome	Aligned PLA2s	Identified Peptides	Peptide frequency
Hemilipin	----MTF-----LILTILATV--TPSLYSHVVQREL---RVNFEPLAGQDSWPVAR	1- RQQRNGW	1/100
HLlipin-1	----MEF-----VFLIVFATV--ISTSYTHVVRREL---YLSFEPVPGQDSWPAWR	2- GQRYEPL	1/100
HLlipin-2	----MPF-----LIFFLFAGM--AALCYSRFPRTTEL---RVHFEPLPNQKNSWPLAR		
HLlipin-3	----MTA-----WALLVFALL--KYVAAELNPDNEL---YVSFESLPGQRGSWPLAR		
HLlipin-4	----MAV-----RTLLALALL--KYIAAELNTDNEL---YVNFEPLPGQRDQWPVAR		
Hemilipin	AAMVTFDARSEKAR---EFSECRMINSMHLSRELMDSPHEVTVKFASKKEEMDDLVRQCSG		
HLlipin-1	AAVVSNDARSKRGK---TFSDCRMLNSMEDIARETNELTGITTKRISKQMDVLQTRCSG	3- PKRISKR	2/100
HLlipin-2	VALVNYDAS-----RDSLECRMLSSIEDISRQGIHLPATRIEALIQEMDALEKRCSS	4- IFISKRI	2/100
HLlipin-3	AVRMQFTRRSEEGREYRSFEGCQLLESDDIAREATRPQQAICRISKKEEMQLFEGRCQR	5- SHDSKLW	3/100
HLlipin-4	AVRMQFTRRSEEGREYRSFEGCQLLESDDIAREATRPQQAICRISKKEEMQLFEGRCQR		
Hemilipin	SA-----E-GRSWFIWEDKFCGPGTDAKNESDLGPL-EADK		
HLlipin-1	LD-----E-GR-----WFGFKCGPGNSAKNYSDLGTLVEADV	6- ITHMRV	2/100
HLlipin-2	FA-----EAGRQTITYGKFCGDSNAANYSDLGPL-EADK	7- DCGATDG	2/100
HLlipin-3	MG-----DA---ERTVWGKFCGAGSAENEGDLGYFSNLDI	8- CGVSDTE	1/100
HLlipin-4	MG-----DA---ERTVWGKFCGAGSAENEGDLGYFSNLDI	9- CGANQHEY	1/100
Hemilipin	CCRTHDHCYDI-----GA-GETKYGLTNKS-----FFTKLNCKCEAAF		
HLlipin-1	CCRDHDHCDNI-----AS-GETKYGLKNKR-----PFTILNCVCDEAF	10-KDET KYC	3/100
HLlipin-2	CCRDHDHCDGI-----LS-GETKYNLTNEG-----MFTLLNCECEAL		
HLlipin-3	CCRDHDHCDNI-----GA-GETKYNLTNTG-----TFTMMNCDEVAF		
HLlipin-4	CCRDHDHCDNI-----GA-GETKYNLTNTG-----TFTMMNCDEVAF		
Hemilipin	DQCLKESIDRA-EGSASMSMEGLHSFYFNTYSPECYEVKCSRKR---DAECTNGIAWK	11-SAKSTYP	1/100
HLlipin-1	DKCLTDAYEKV---KKKGIQDIKEFYFDYKPKCYVLTCDKRSEKKLECNPIALKE	12-CPAINFT	1/100
HLlipin-2	DNCFKETARNETDAFHKLTVVLRLLIYFELYSPKCYVLTCDNSTEPNC-----VGWRD	13-IVSWRD	1/100
HLlipin-3	KNCLDSIPG---FWTKKAIAAVKMVYFNLYGNGCYLNKCSGGRSENF-----RIGGCAS		
HLlipin-4	KNCLDSIPG---FWTKKAIAAVKMVYFNLYGNGCYLNKCSGGRSENF-----RIGGCAS		
Hemilipin	SYKS-----		
HLlipin-1	SYKS-----		
HLlipin-2	GYEDA-----		
HLlipin-3	GVAEYTGEGT-P----FA-KIVNIG-----		
HLlipin-4	GVAEYTGEGT-P----FA-KIVNIG-----		

Fig. 4. Multiple alignment of identified phospholipase A2 in *Hemiscorpius lepturus* transcriptome was performed by CLUSTALW 2 program. Epitopes mimicking the antigenic site of PLA2s were highlighted with different colours. The selected peptides from the phage display peptide library (Ph.D.C7C) that mimicked the antigenic epitopes of phospholipase A2 of *Hemiscorpius lepturus* venom. They are: Hemilipin (KX924472), HLlipin-1 (KX924473), HLlipin-2 (KX924474), HLlipin-3 (KX924475), and HLlipin-4 (KX924476). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and negative wells (well-coated by non-immune horse IgGs) (Table 1). Polyclonal phage ELISA was used for quality assessment of biopanning, with results showing increases in the signal intensity

after each round of the biopanning procedure (Fig. 2). The highest signal value was observed in the third round of biopanning. Therefore, 125 individual clones containing phage carrying

Venom allergens id in <i>Hemiscorpius lepturus</i> transcriptome	Aligned venom allergens	Identified Peptides	Peptide frequency
HLAllergen-3	-----MRRG-----RLATMML		
HLAllergen-6	MDLFGTISCL---LLVFAPYISGVILWQIPLDNTRGD---STANVV---S-----		
HLAllergen-2	MNVLL-----GILYLLASLFPFTLCEGQRCNSTEYGNVDRASHRKYTRDEKNFP		
HLAllergen-1	-----MDFVLLS-----		
HLAllergen-7	MKLLY-----TTSWYLLPMVLPFLRLSDS---TTRFGTYDTISRE-----ANLD		
HLAllergen-3	RLLFIAHFLITVS---SKGFDKTAPYI-IRPDFNKDGCKIQEADCSK---HLDLQQH	1- PTISEKN	1/100
HLAllergen-6	----RDGATFVAEEK-RNLETKDCPEIKYKRYSTRHIFCLEPR--CQTEQKFTLTSLKDN	2- REFYISD	1/100
HLAllergen-2	N-SWVKTVRKYNDEET-WISEKDECPSEYLYRSADPTYCKTST--CDIISBGVNEEDKKL	3- SGHP EYL	1/100
HLAllergen-1	----CLVLI---PC-RSVLGQSCP IYLRFSKDHTYCRHSD--CNVKSQGVTESDKEV	4- GAD E EKL	1/100
HLAllergen-7	D-AWHASRSD---LE-NSRNSDSCP ELYQRFSKEHTYCRKST--CNVLDKGVSKKEKEI		
HLAllergen-3	IINLLNAQRYSISSGLG-EFKKVSFAINMLYLEGDP E L I N K A Q T F V E T C F Q Q P - T L Q G C V	5- P E E F I G	1/100
HLAllergen-6	IVRMHNELRSSLARGATTQFSLFPRAANMMELEW E E L A R L A E A E A K L C P E S L D E K P K A S	6- P E D D P G L	1/100
HLAllergen-2	ILEIHNNFRSKLASGNESRYREL P A A D M M E L E W E E L A K I A Q A H A S L C V F K H D T - G Q R T	7- H H T P A A N	1/100
HLAllergen-1	ILKMHNEFRNKVALGRETTVPVQ P A A N M M Q M E W E E L A R V A Q A H A N L C K F E H D T G P Q R Q	8- H H T P A A N	1/100
HLAllergen-7	ILGIHNNFRSKLATGRETTCHQLPS A A N M M E M E W D E L A R I A Q A H A N L C V F N H D E S N D R A		
HLAllergen-3	APGETEQDGLVIYEQIYPIDTLFFAE-----RIEALMKSDISKF--P----	9- I N E N D R M	1/100
HLAllergen-6	-----IAIGNFSSIGQILFN-----ERWMNDLMQRIEKEA-SSFY	10- D T S D E N R	1/100
HLAllergen-2	-----VDEFQ I G N L I L T M - Y S P -----K D W N - I L E M L Y K E E V - C F Y L		
HLAllergen-1	-----VENFS I G N L F I S M M T K T -----I D W R - K T A M W Y T S E V - K D F Y		
HLAllergen-7	-----VENF I G N M V Y T T - S S K -----K N W R - A I E S W Y K E E V - C F Y T		
HLAllergen-3	SRFVESYDPT-LTDDKIQNFAKIAHARVWV G G G F A D F I I A D F V D S K A R A R F K E V V V C F F	11- H T E V G C N	1/100
HLAllergen-6	PGYINQFHL---K-DDYENFAQVAWAETWK M C S R A D V V D G -----E N I K R S Y F I C N Y	12- R T R F K C G	2/100
HLAllergen-2	PENNEPFLP---S-KDYGHFTQMTWAKTWK V G C G C T L F R R Y -----G E - - Q H A L Y T C N Y		
HLAllergen-1	PEYREPFVF-----GTYGHFSQVIWAETWK V G C G V A W Y Y D D -----R E R M D K I L Y T C N Y		
HLAllergen-7	PDIVPSFRS-----GEYNHFSQVTWANTWK V G C G Y A E Y N D G -----G K - - K K G L Y T C N Y		
HLAllergen-3	GPPKLRKGEKLYMTGSC E A D C P R G T S C L T E G K F S G G L C R R Q A -----	13- M T C P P W F	1/100
HLAllergen-6	GPKGNIEGKVKPG-K E C S Q C P K N T E C S K D Y ---PGLCKSKTSDGPQRER-PPVEDFLL	14- Y E C S G P E	1/100
		15- H L E S T V G	1/100

Fig. 5. Multiple alignments of identified venom allergens in *Hemiscorpius lepturus* transcriptome. The antigenic epitopes of venom allergens that stimulated the immune response of horse were determined by the phage display peptide library. The identified peptides and their corresponding epitopes were highlighted with different colours. They are: HLAllergen-1 (KX924489), HLAllergen-2 (KX924490), HLAllergen-3 (KX924491), HLAllergen-6 (KX924494), and HLAllergen-7 (KX924495). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Hemiscorpius lepturus-mimicking peptides were randomly selected from the output phage of the third round of biopanning, plaque amplified and phage DNA isolated for sequencing.

3.3. Sequencing results

Sequenced peptides were subjected to the Blastp (protein-protein BLAST) of non-redundant protein sequences (nr) database of NCBI for the scorpion (taxid: 6855). Then, the corresponding proteins that showed hit with the peptides were manually searched in *Hemiscorpius lepturus* transcriptome. The proteins matched with at

least two different selected peptides. Twenty-five among 125 peptides did not show any significant hit in the scorpion database. Epitope-mimicking peptides and the antigenic profile of the *Hemiscorpius lepturus* venom component was investigated and reported in Table 2. The main antigens involved in the stimulation of horse immune response were as follows: phospholipase D (Fig. 3), phospholipase A2 (Fig. 4), venom allergen (Fig. 5), metalloproteinases (Fig. 6), hyaluronidase (Fig. 7), potassium channel toxins (KTxs) (Fig. 8), venom protein (Fig. 9), and calcium channel inhibitor (calcin) (Fig. 10). Among 100 peptides, 21 were related to PLA2, 26 peptides were for PLD, 18 peptides for venom allergen, 16

HLAllergen-2	GPSGNEWYGKFTYEG-E PCSR CPPTKCSLEY---PGLCKSQTEDGPKLKR-PPSDDFVF		
HLAllergen-1	GPAGNMQGGTLYIKG-S PCSR CPKNTQCSNEY---EGLCKSMTDPGPQQDLSISSDDFLL		
HLAllergen-7	GPEGNFRFRPHYVAG-E PCSR CPKNTCESTSY---AGLCKSKTEDGPMQNR-PSSNDYIL		
HLAllergen-3	-----R---K---EH		
HLAllergen-6	YCDFS-----EDDPVACRTLKITGDRNFTTKHIYSGNYKSVVLR---E		
HLAllergen-2	FCDFS-----EQDPERCNEVEVTGSRNFSTKRIYIGDYKTVVQLQ---A		
HLAllergen-1	YCDFS-----ANEQSECKNVEITGSKPFETRKIYSGEYKTVVLH---G		
HLAllergen-7	YCDFS-----DDDPSECSSVGKSRNFSTRHIYGGNYKTVVLN---G		
HLAllergen-3	EQTIWKCDLKRYSDGCSVERKCAHHTVNKTGGFKYVVVS SS CVTVSLFIKAIHVEQPSC	16- SS ATCM	1/100
HLAllergen-6	GEKVG---IDLI-PVDYEGGYCPFLYIRAGSNNAAD SAGP -----	17- LEFT SAG	1/100
HLAllergen-2	GESII---IDFG-KVYNSEGFPCPFVYTRFGSNRAND SAGS -----		
HLAllergen-1	GESIT---IKLG-KAQDNRGICPFIYGSFGPNRDGAQAS-----		
HLAllergen-7	GESVE---IDVG-WAQDKKGICPFLYLRFPGPNNAKDTKSS-----		
HLAllergen-3	FFFRYMKSGDLLLQ-TKVIAFVSVDVSHHGHSTDVGKRTDASEWTDVTLDISPFG---N		
HLAllergen-6	-----MLKI---YSI---DETEMA-----ER-LTGGGV		
HLAllergen-2	-----VVEF---NIG---R-----YRIPYRP---PRE		
HLAllergen-1	-----AVSI---GFS---A-PGIF-----FGDPVKI-QHSS		
HLAllergen-7	-----SLTL---AFY---TSPGAP---V-----LTTALNVPEMSK		
HLAllergen-3	GK-----A-----IQGVVVRSYPTSDGKQLIKVRDPEVVAGRCLQQPA-----		
HLAllergen-6	EWFTFRGVDEWKRKTRQVRIQLWVDN-GYSPQYCDIKAWGIRKGRCT-----DF		
HLAllergen-2	PVFTFMAAHMIANLELQCKVILRVDE-DAAPQYFDIKAWGISKGDCI-----NA		
HLAllergen-1	SDFWPIGIHQFDQEMKSTLKLEAYP-GADPQYFNIAFGIGKGCPC-----KF		
HLAllergen-7	NSFTIRGMHMKFGGKLKSVFTYKANE-GAAPQYIDIKAFGITRGPCQ-----TS		

Fig. 5. (continued).

peptides for metalloprotease, seven peptides for hyaluronidase, five peptides for calcin, four peptides for venom protein, and three peptides for KTx (Table 2).

4. Discussion

Hemiscorpius lepturus venom causes inflammation and skin necrosis with ulcers resistant to therapy. Haemolytic anaemia, haemoglobinuria, and renal failure are common complications of *Hemiscorpius lepturus* envenomation (Valavi and Ansari, 2008; Ghafourian et al., 2016). *Hemiscorpius lepturus* venom is lethal for humans; it consists of various toxic peptides and proteins. Until now, less than 2–3 per cent of the venom component of the *Hemiscorpius lepturus* has been identified. Thus, our information on the whole range of toxins in *Hemiscorpius lepturus* venom is not complete. The phage display peptide library (Ph.D-C7C, NEB) was used to access the immunogenic profile of *Hemiscorpius lepturus* venom. The length of epitopes that could be recognized by antibodies is between 4 and 6 amino acids. Therefore, heptapeptide random libraries are a suitable choice for displaying all linear

epitopes of target proteins (Bastas et al., 2008). The enrichment of the Ph.D-C7C library towards *Hemiscorpius lepturus* venom was performed on immobilized IgGs (purified from immune and non-immune horse sera) by using biopanning. To select phage with high affinity to immobilized IgGs, biopanning was performed through three consecutive rounds. The enrichment of the library was monitored by polyclonal phage ELISA on eluted phage from different rounds of biopanning. The maximal enrichment occurred in the third round of biopanning. Therefore, 125 single colonies from third-round phage of biopanning were randomly selected and subjected to sequencing. The sequencing results identified unique peptides mimicking various epitopes of *Hemiscorpius lepturus* toxins. To the best of our knowledge, this is the first report on analysing the immunogenic profile of *Hemiscorpius lepturus* venom by using the peptide displayed phage library. All components of *Hemiscorpius lepturus* venom, identified by the peptide displayed phage library, are described as follows: All amino acid sequences of proteins and peptides showing here were achieved by a transcriptome analysis of the venom gland of the *Hemiscorpius lepturus* by using Illumina high seq 2000 Next Generation Sequencing

Metalloproteinase id in <i>Hemiscorpius lepturus</i> transcriptome	Aligned Metalloproteinase	Identified Peptides	Peptide frequency
HLMP1			
HLMP3			
HLMP2	MLVLVALLLVGVVAVEPVYLGDLPMENKGLFEGDII LRPRPRYVARSGAPYLHLRWPYGR -----MQME-----GGT : ** *		
HLMP1			
HLMP3			
HLMP2	IPYVIDRRLWTKQKAIIGAMNEYHKTCIRFIPRTTERDYIRIYKGKGCSSGVGKWVQGO IPYVIDRRLWTKQKAIIGAMNEYHKTCIRFIPRTTERDYIRIYKGKGCSSGVGKWVQGO I-----GICYVGGICDY-----WYKV- * .:*.:* * :		
HLMP1		1- IDGPPPN	1/100
HLMP3		2- LEVRPPP	1/100
HLMP2	LLSLGEGC--YSRGIILHELGHAI GFYHEHKRSDRDKYLRVHWGNIRKGFEEEFIM--- LLSLGEGC--YSRGIILHELGHAI GFYHEHKRSDRDKYLRVHWGNIRKGFEEEFIM--- GISMDDGSRFDGCSTVAHELCHNCGCPHDGD P -- P SYIGGSPGAESCP N LYGYMMSYIE *:.:*. . . : *** * * * *: . . *: * .: : : *	3- V WD P MYT	2/100
HLMP1		4- YLVNGDE	2/100
HLMP3		5- REFNGVL	2/100
HLMP2	KAPRQNIL-----YTAFDYQSIM----LYGETA K DGRSKTL-TAKNGAR-LLPV KAPRQNIL-----YTAFDYQSIM----LYGETA K DGRSKTL-TAKNGAR-LLPV RDTQRQYRFSECSKQCVHEMFTIASCМКVRN V NGAIDL S R D P G EFVSDQLGV K EDAPY : ** : * * * : * :*: . : : : *: *	6- GN K GD 7- Q PGFRCY 8- L DEAKTH	1/100
HLMP1		9- T CR R HGP	2/100
HLMP3		10- LQM Y YIR	2/100
HLMP2	YKKTGLSK-----SDAEDVNRMRYCRRNARE----- YKKTGLSK-----SDAEDVNRMRYCRRNARE----- YNQ R C ERREDLLLYKVPGETCSYCCREPTNKEGWFY Y YIMKCLDGEPCASGTKVCENG I *:.: * ** . : :		
	----- ----- CKDRVK		

Fig. 6. Multiple alignments of the identified metalloproteinase in *Hemiscorpius lepturus* transcriptome. Peptides mimicking *Hemiscorpius lepturus* metalloproteinase epitopes were obtained by the phage display peptide library and were highlighted. They are: HLMP1 (KX924496), HLMP2 (KX924497), and HLMP3 (KX924498).

(unpublished data).

Heminecrosis was the first hemolytic toxin isolated from *Hemiscorpius lepturus* venom (Borchani et al., 2011a). The most pathological effect of *Hemiscorpius lepturus* venom is related to Heminecrosis activity. In fact, Heminecrosis is the major protein in *Hemiscorpius lepturus* venom and mediates its function through phospholipase D activity (Borchani et al., 2013). It has been reported that Heminecrosis solely is able to stimulate immune response to *Hemiscorpius lepturus* venom (Borchani et al., 2011b). However, produced antibodies against Heminecrosis only neutralize the dermonecrotic effect of *Hemiscorpius lepturus* venom. Inflammatory effects are neutralized by antibody against the whole range of venom of the *Hemiscorpius lepturus* (Borchani et al., 2011b). In agreement with the study of Borchani et al. (2011b), we identified 26 peptides that mimicked epitopes of PLDS (KX932445, KX932446 and KX932448) (Fig. 2). In parallel to the study at our laboratory, the whole component of *Hemiscorpius lepturus* venom was identified by Illumina Next Generation Sequencing (NGS). On the other hand, a bioinformatics analysis of identified peptides from the peptide phage display library (Ph.D -C7C) revealed 26 peptides corresponding to PLD in scorpion databases. These peptides were manually searched in *Hemiscorpius lepturus* transcriptome to find their corresponding PLD. However, multiple PLD alignments were performed to identify the involvement of epitopes in serum antibody recognition. In their study, Batista Oliveira et al. showed that two peptides FDDNANPEY-TYHGIP and DKVGHDFSGNDDISDVGK were good candidate for stimulation of protective antibody response to sphingomyelinase D from *Loxosceles intermedia* spider venom (Oliveira et al., 2016). One of the identified peptides in the current study, VFDDNGR, mimicked similar amino acids on PLDs (underlined amino acids). Hemilipin is another protein isolated from *Hemiscorpius lepturus* venom. Hemilipin is a heterodimer-secreted phospholipase A2 (sPLA2) that hydrolyses phosphatidylcholine. Hemilipin inhibited all crucial steps of angiogenesis both *in vitro* and *in vivo*. Inflammatory and apoptosis effects of scorpion venom is due to the function of phospholipase A2 (Iridi et al., 2015). For example, Phaiodactylipin is

a PLA2 isolated from *Anuroctonus phaeodactylus* venom that showed inflammatory and anticoagulant activity (Valdez-Cruz et al., 2004). Here 21 peptides corresponded to the Hemilipin and PLA2s of *Hemiscorpius lepturus* venom. As mentioned above, the full amino acid sequence of hemilipin and other PLA2(s) was identified in our previous study (unpublished data). Metalloproteases are other classes of enzymes found in *Hemiscorpius lepturus* venom (Seyedian et al., 2010). Metalloproteinases are the leading cause of haemorrhage and fibrinolysis of the victims (Xia et al., 2013). The Existence of metalloproteases has been proven in various studies (Ortiz et al., 2014; Rendón-Anaya et al., 2012; Alvarenga et al., 2012). It has been proposed that metalloproteinases could be as potential targets for the development of a novel anti-venom therapy (Ortiz et al., 2014). In agreement with the literature, 16 peptides identified here corresponded to the metalloproteinase in *Hemiscorpius lepturus* venom. These 16 peptides mimicked eight different regions of metalloproteinase in *Hemiscorpius lepturus* venom. Venom allergens have been identified in the venom of scorpions and other venomous animals (Bordon et al., 2015; Van Vaerenbergh et al., 2015; Zobel-Thropp et al., 2013; Bouzid et al., 2013; Rodríguez-Pérez et al., 2014; González-Morales et al., 2014). Allergens are responsible for allergic reaction to envenomed victims. In the current study, 18 peptides were related to epitopes of venom allergens. However, the presence of venom allergens in the venom of *Hemiscorpius lepturus* has not been evaluated until now. The existence of venom allergens in the venom of *Hemiscorpius lepturus* suggests that some portions of allergic reactions of *Hemiscorpius lepturus* venom may contribute to the venom allergens. The hyaluronidase activity of *Hemiscorpius lepturus* venom was confirmed in the study of Seyedian et al. (2010). In fact, hyaluronidases are a class of enzymes that hydrolyse hyaluronic acid to increase the distribution of the toxic components of *Hemiscorpius lepturus* venom (Díaz-García et al., 2015). The presence of hyaluronidases was confirmed in our study and seven peptides were identified that matched the hyaluronidases of *Hemiscorpius lepturus* venom. Venom proteins are other proteins found in the venom of various scorpions (Luna-Ramírez et al., 2015). In our

Hyaluronidases id in <i>Hemiscorpius lepturus</i> transcriptome	Aligned Hyaluronidases	Identified Peptides	Peptides Frequency
HLHyaluronidase-3	MATAALLSLIITVAL-DITEANFDIIWNVPTFMCSSGSLNLTkdLLKYNILVNNKESFV		
HLHyaluronidase-2	MNWNLSLGLVLFNGASTIVGTDfKIYWNVPSSNCSErFGINVTETLVNNGVLVNTDEKFE		
HLHyaluronidase-1	MNWRTLIGIVLNFgVNIvAGTNfKIYWNVPSSLCsVKFGINVTETLISNGVLVNSKEEFL		
HLHyaluronidase-3	GDKIAVIYDYKMGTYPKIDSKDgDINGGIPRLDRLEKHLKIAKMNISQLISNPdFSGLGV	1- MTdING	2/100
HLHyaluronidase-2	GDKIVLFYQTKFGKYPYIDNEGHdINGGLPELGNLKKHLEVAQDIKKVIPNSrFNGLAV		
HLHyaluronidase-1	GDKIVIFYQNKGKYPYIDG-GNdINGGLPQLGNLTeHLRVSEDDINQIILNSSFdGLGI		
HLHyaluronidase-3	IDWEAWRPSWDYLWGPLKIYQNRtIKLIEKdNPSSDRDIESAAKDLWEETAKQWMLRTL		
HLHyaluronidase-2	IDWEKWRPTWLFNWGMQIYRNrTLELMTKKHPAWSRKRLTASIRWEEtARKWMQKTI		
HLHyaluronidase-1	IDWERWRPTWRfNWANMHVYKARTLALMRQKHPDWSWEQVvNASIDWWEETtKQWMLETL		
HLHyaluronidase-3	QLAKRLRPKGRWCYyHfPDCYNYgYGRHESQYFCSDrTSSINDRLSWMWDESTALCPSIY	2- YGKDNF	2/100
HLHyaluronidase-2	KLGGKMRPKGRWCYyTFPDCYNYgYGRSFESQYFCNSrTVKHENDRLFWLWDSATALCPSIY	3- RgKDQYC	1/100
HLHyaluronidase-1	ELAKKMRHEGKWCYyHfPDCYNYAgKNEEDQFHCSSIVKdNENDRLFWLWNSTtALCPSIY		
HLHyaluronidase-3	LHEEQFEYNNKSQQVWYTFGRLAeAVRVSrPQTkiYPYMNyLVHKSrVfVSKVSFRIRLAL	4- PGYNKTE	2/100
HLHyaluronidase-2	DGPKQEKYNNETQEVWYLYGRLEAKRVSRPEMPVYAYINyRMLDRLHDVPKKHfWLMMSY		
HLHyaluronidase-1	YDPRQGYNNETQKVWYLYGRLEATRVsQPKTPIYPYINyRMRNGLNDVPKDHfPLMSY		
HLHyaluronidase-3	VASMGldGLVIWGSsYFPdKNACEDLKTYVnKVIGPSAVtISNNVNRCsKEICNGRGKC		
HLHyaluronidase-2	AASLGldGVVIWGSSEfVETKEDCEDLETYVKVIAPAArKISTTVEWCGKKfCDNNGLC		
HLHyaluronidase-1	VASLGldGVVIWGSSEYVKSsKNCELETYVKEVIAPAArTNITSTASWCGETfCfNNNGLC		
HLHyaluronidase-3	TWPSEPYTSWMyFIDEK-QNFDSRNIVCKCHIGKGRYCQRES-----		
HLHyaluronidase-2	TWPQqPFTSWKCLiYPASCTfSAKDITCrcQIHEGRYCNITK-----		
HLHyaluronidase-1	TWPQqPYTSWKCLIDPTACsfNPEDIACrcQVHEGRYCNITDSYSTDL		

Fig. 7. Multiple alignments of the identified hyaluronidases in *Hemiscorpius lepturus* transcriptome. Peptides mimicking *Hemiscorpius lepturus* hyaluronidases epitopes are shown as highlighted. It, in this case, is: HLHyaluronidase-1 (KX932442).

study, four peptides partially matched the venom proteins of *Hemiscorpius lepturus* venom. Hemitoxin is a potassium channel toxin (KTx) isolated from *Hemiscorpius lepturus* venom (Srairi-Abid et al., 2008). Hemitoxin injected through the ICV route results in neurologic symptoms in mice (Srairi-Abid et al., 2008). Three peptides from the library mimicked an epitope of hemitoxin and other KTxs. Hemicalcin is a calcium channel blocker (ryanodine-sensitive channels) purified from *Hemiscorpius lepturus* venom (Shahbazzadeh et al., 2007). Administration of hemicalcin via the ICV route causes neurologic symptoms in mice (Shahbazzadeh et al., 2007). Here, five peptides were identified that corresponded to two epitopes of hemicalcin.

In conclusion, the immunogenic profile of *Hemiscorpius lepturus* venom was determined with the phage display peptide library and three rounds of biopanning. The biopanning on purified antibodies from immune-sera is an effective way to study the epitopes involved in antibody-antigen binding. Identification of peptides that correspond to identical amino acids of related proteins suggests raising of cross-react antibodies to these proteins. Most peptides recognized by horse antibodies mimicked conformational epitopes on the venom of the *Hemiscorpius lepturus*. Taken together, the results suggested that a pool of epitopes requires the covering diversity of *Hemiscorpius lepturus* venom and a single epitope cannot neutralize the whole venom. This study provides the basis

Alpha-KTx id in <i>Hemiscorpius lepturus</i> transcriptome	Aligned alpha-KTxs	Identified Peptides	Peptide frequency
HLKTx4	MNK-----ACAVAVS-----VASTGGCS-----DSCKGYCKKNKYNGKCDGTNCKCSG---	1- AKCRNTC	3/100
Hemitoxin	MNT-----KVVTTTMDT-----TAVAKCT-----SKDCYSCKKT-GCRAKC-NRNCICYGCSG		
HLKTx2	MNS-----KAATVMMAND-ARVNSACV-----TSDCYKCGKAYNCRAKC-MNRNCYANC-		
HLKTx3	MNS-----KAATVMMANV-AA-HTKCR-----YSNCHDCRKT--GSSKC-MNGCTCYR---		

Fig. 8. Multiple alignments of the identified alpha-KTx in *Hemiscorpius lepturus* transcriptome. Peptides mimicking *Hemiscorpius lepturus* alpha-KTx are shown as highlighted. Earlier, hemitoxin was identified in *Hemiscorpius lepturus* venom. These are: Hemitoxin (KX874530), HLKTx2 (KX874532), HLKTx3 (KX874533), and HLKTx4 (KX874534).

Venom protein id in <i>Hemiscorpius lepturus</i> transcriptome	Aligned venom proteins	Identified Peptides	Peptide frequency
HLVPr10	-MVKVVLVTLVVLAVSLRATIGLRCEGTYECPNCPAGTVKDVNCNCIVCAKGLN		
HLVPr7	MASKLCLNIVLFAIIS-TMALSCRFCDSSQCFPTNCPVGTVRDVCGCCLVCAKLN	1- FCPTEID	1/100
HLVPr3	MPSRFCLITLLFGIFLR-TMSLKCRVCGTYDCKTPENCVPVGIKDVNCNCDVCGKNLN	2- EGHFLTW	3/100
HLVPr6	MVAKFFLITLIMLGVTLS-TTALSCRRCGTYECPPLPADCPWGTVKDPCFCCLVCAKGLN		
HLVPr1	MELRLFA----LAVLIASCHCWPRKH-CTEPCGPFPPNDCEGVTKDYDGCAICAKGSG		
HLVPr9	MWLR LAVAYLLIESIHSLSCPCWKDHS----ICKAPTECELGLTKDACGCCDVCFKISG		
HLVPr8	MWLR LALVLTIVSIHSLSCPCWRDRS----ICRAPTECKLGLTKDACGCCDICFKISG		
HLVPr4	MWLR LALICLLGSCVYSLSCPCWRDRENLDKYCGTEPEECALGQTFDACGCCVCFKISG		
HLVPr2	-MLRLVLLSTLVVSIYSLSCPCWRERDK-TKFCGTEPCPLGTTHDACGCCTVCFKISG		
HLVPr5	-MFRLVLLSSLAVTIYSLSCPCWEQEDK-TDYCGTEPCPLGTTSDVCGCCPVYKVLG		
HLVPr10	EECGPWL LAGKCG-NGLTCVKRTTS-----PNARGCRRN-----		
HLVPr7	PCGGFP GIGKCG-KGLKCVYEPNV-----IVFHGICKKA-----		
HLVPr3	VCGGPWNIIGKCG-EGLKCV EEDH-----FESKGICQKE-----		
HLVPr6	ETCGGVWNISGKCG-EGLKCV KDGGSDIR-----SFIEAGRCQKI-----		
HLVPr1	ECGGLWNDYGVCGV-DLVC-----ETNGEASSEYDLPRGVCVPARRFSTRNIVKMLR		
HLVPr9	WSMGHQRKMWRKFEVRKLEGGCNRPCCKTRGRL-----		
HLVPr8	KCGGPWGTSGRCG-EGLECVAPKPEKAEDVPQHIAHQEGVCKPK-----		
HLVPr4	DCGGPWKVYKCG-EGLTCAKPDHPE----KLIRDQLTGVCVKKQQ-----		
HLVPr2	VCGGPWNVRGVCG-EGLYCKEVVVE--EG--FDQYDGVCEPKE-----		
HLVPr5	SCGGMWGTMGQCG-PGLFCKRDVESS--ENEYTYENS DGVCIAQDKKTSRS-----		

Fig. 9. Multiple alignments of the identified venom proteins in *Hemiscorpius lepturus* transcriptome. Peptides mimicking *Hemiscorpius lepturus* venom proteins are shown as highlighted. These are: HLVPr1 (KX924479), HLVPr2 (KX924480), HLVPr3 (KX924481), HLVPr4 (KX924482), HLVPr5 (KX924483), HLVPr6 (KX924484), HLVPr7 (KX924485), HLVPr8 (KX924486), HLVPr9 (KX924487), and HLVPr10 (KX924488).

Calcine id in <i>Hemiscorpius lepturus</i> transcriptome	Aligned calcines	Identified Peptides	Peptide frequency
Hemicalcin	-----MRASLFIVIFVVSFITISCLSTD-----DEEARWIEKRG---	1- EYETEG	1/100
Hemicalcin-2	MPPHQVPASIGEMNNATKAS-FCLLMIALIVTAAVVEADSSFEQYEGEGARTLPLSGDYE	2- DYERLFM	2/100
Hemicalcin	-----DCLPHLKLCKADKDCSKKCRREKRCR	3- SCVSTNP	2/100
Hemicalcin-2	CVRPRCKPHL-VCNSQQICVDPKKGK-----		

Fig. 10. Multiple alignments of identified calcines in *Hemiscorpius lepturus* transcriptome. Peptides mimicking *Hemiscorpius lepturus* calcines are shown as highlighted. Hemicalcin was previously identified in *Hemiscorpius lepturus* venom. These are: Hemicalcin (KX874539) and Hemicalcin-2 (KX874540).

for further research on the development of recombinant toxins as well as epitopic vaccine candidates.

Ethical statement

All experiments involving horse used for immunization and blood collection was approved by ethical committee of animal care of Pasteur institute of Iran, Tehran, Iran (No; IR.PII.REC.1394.42).

Conflict of interest

There is no conflict of interest to disclose.

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