ORIGINAL ARTICLE

Cross-Reactivity and Neutralization Capacity of Polyspecific Antivenom Produced by Razi Institute against Three Species of *Buthidea* Family Scorpions

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Abstract

Background: Scorpion sting is a significant health problem in southern provinces of Iran. Many thousands of people are stung by different species of scorpions annually. More than 60 scorpion species have been reported to be present in Iran. However, Razi Institute manufactures polyvalent antivenom against only six species of scorpions, excluding *Buthacus macrocentrus*, *Apistobuthus susanae* and *Vachoniolus iranus*, wide spread scorpion species in the south region of Iran. Since the venom of these scorpion species is not included in the production of Razi polyvalent antivenom, the aim of this study was to check the neutralization capacity of our antivenom against these species.
Methods: In this research, after collecting scorpions (at night), milking by the electroshock method, the venom was lyophilized and the LD50 was determined. The fractions were then separated by gel chromatography and HPLC. Using SDS page electrophoresis, the fractional molecular weight was determined. At the end, the potency test of these scorpion venoms was carried out in the vicinity of the antiserum produced by Razi Institute on the animal. In the present work, we tried to investigate the cross reactivity of present antivenom against these excluded scorpion species.
Results: The antisera production of Razi Institute was able to neutralize the 33 LD50 of *Apistobuthus susanae* venom. This antivenom could neutralize *Buthacus macrocentrus* as well as *Vachoniolus iranus* scorpion venoms by 41 LD50 and 15 LD50, respectively.
Conclusion: Based on the results obtained in the present study that indicate the neutralization of the three species of scorpions' venom, not included in antivenom production, the present Razi polyvalent antivenom is able to be used in patients stung by these 3 species of scorpions.

Keywords: Antisera; Apistobuthus; Buthacus; Scorpion; Vachoniolus

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INTRODUCTION

Scorpion stings is a major public health problem in many tropical countries such as Africa, southern India and the Middle East (1, 2). Yearly thousands of people are stung by scorpions in Iranian provinces of Khuzestan, Hormozgan and Khorasan Razavi that induces morbidity and mortality (3, 4). Around 1,500 different species of scorpions have been identified so far; however, the largest family of scorpions in the world has been *Buthidea*, some of whom are medically important. The researchers have categorized Iranian scorpions into three families of *Buthidae*, *Scorpionidae* and *Hemscorpidae* (3). More than 90% of the species identified in Iran are from the *Buthidae* (3, 5, 6). Some members of the family have the deadly bite and some cause pain. Hence, *Odontobuthus, Hottentata, Compsobuthus, Apistobuthus, Androctonus, Mesobuthus, Orthochirus* and *Olivirus* are medically significant. Scorpionism has been reported in all provinces of Iran, especially in southern and southwestern regions. In Iran, anti-serum polyvalent that is obtained from the hyperimmune serum of the horse, cross-reaction among animal poisons, are used to make anti-serums. Thus, antisera has the ability to neutralize the venom of different species of scorpion based on the structural similarities of different species of venom (7). A report by Emerich et al. showed that there is a significant cross-reactivity between the polyclonal antibodies of the Tunisian scorpion and the Moroccan scorpion (8). Other scientists also studied the cross-reactivity between polyvalent antivenoms and homologues and heterologous venoms (8, 9, 10).

In Iran, polyvalent anti-serum is produced from the venom of six scorpion species. However, there are some scorpions present in Iran whose venoms are not used in immunization process. Hence, the purpose of this study was to perform

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cross-reactivity test for polyvalent antiserum against the venom of scorpions *Buthacus macrocentrus* (Figure 1), *Apistobuthus susanae* (Figure 2) and *Vachoniolus iranous*.

Figure 1. *Buthacus macrocentrus* scorpion.

Figure 2. *Apistobuthus susanae* scorpion.

METHODS

Preparation of scorpion venom

Apistobuthus susanae, Buthacus macrocentrus and *Vachoniolus iranus* scorpions were hunted at night with the help of the UV lamp. The scorpions were milked using the electroshock method, and the resulting freeze dried.

Preparation of venom

The venom dissolved in distilled water and centrifuged at 14,000 rpm for 15 minutes. The supernatant was collected and protein content was determined by the Bradford method (11).

Gel electrophoresis of the venoms

The Polyacrylamide gel electrophoresis (SDS-PAGE) (Bio Rad-Mini Protean tetra System) was performed by LaemmLi method to get the protein pattern of the scorpion venom. The concentration of the gel separator was 15%, the density of the gel condensing 4%, and the molecular marker 6.5 to 200 kDa. Staining of protein bands was done by Coomassie Brilliant Blue. The molecular weight of the proteins was calculated using the LaemmLi method (12).

HPLC pattern of venom

High performance liquid chromatography (HPLC) pattern of venom Analytical RP-HPLC (Amersham Biosciences) was carried out using a C18 column with a flow rate of 0.5 ml/min, using a 60 min linear gradient from 0 to 100% of solvent B (CH3CN containing 0.1% TFA) in solvent A (H2O containing 0.1% TFA). Elution was monitored by absorption at 280 nm (13).

Venoms lethal toxicity (LD_{50})

The ethical code of this study is RVSRI. REC. 9800004.

The venoms lethal toxicity was assayed by using intravenous injections of each venom into mice. Venoms were subjected to 4-5 serial dilutions by factor of 1.25 using sodium chloride solution. Then, an aliquot of 0.5 ml of each dilution was intravenously injected into 4 mice. Dilutions range was covering the entire mortality range (from 0%-100%). Deaths up to 24h after injections were considered as toxicity. The LD50 was calculated using the Reed–Muench method (14). To calculate the LD50 the following formula was used.

$\log_{10}$ 50% end point dilution = $- (x_0 - d/2 + d \sum r_i/n_i)$

x_0 = $\log_{10}$ of the reciprocal of the highest dilution (lowest concentration) at which all animals are positive;

d = $\log_{10}$ of the dilution factor;

n_i = number of animals used in each individual dilution (after discounting accidental deaths);

r_i = number of positive animals (out of n_i).

Summation is started at dilution x_0.

Antivenom neutralization Potency

Potency tests is an *in vivo* test to evaluate the potential of a antivenom for neutralizing a venom. In this study, the neutralization capacity of polyvalent antivenom was assayed against the venom of scorpions *Apistobuthus susanae*, *Buthacus macrocentrus,* and *Vachoniolus iranus* in mice weighting 18-20 grams. Briefly, the mixtures of venoms and polyvalent antivenom were incubated at 37 ºC for 60 minutes, centrifuged and the supernatants injected intravenously into groups of 3 mice of uniform weight, age, sex and strain. The overnight mortality (24 hours) was determined. The mixture that contains the highest concentration of venom and does not kill the mice represents the neutralizing power of the antivenom (or its titer) against that venom (15).

Statistical analysis

The neutralization potency of polyvalent antivenom against each scorpion venom was repeated 3 times. Data obtained were analyzed with statistical software SPSS version 16.0. using one-way ANOVA. The results expressed as means±SD and $P > 0.05$ was considered as significant (10).

RESULTS

Venoms lethal toxicity

When LD_{50} of venoms was carried out on mice, wide differences were found in values. The LD_{50} for the venom of *Apistobuthus susanae* scorpion was found to be 14.8±1.28 µg/mice and the LD_{50} for the venom of *Buthacus macrocentrus*

scorpion was 12.2±1.8 µg/mice. Interestingly, when LD_{50} was calculated for the venom of *Vachoniolus iranus* scorpion it was more than 3 times greater (44.2±3.6 µg/mice) than that of the other two scorpion species.

Electrophoretic Profile

The marker used to identify each band of electrophoretic pattern contained molecules ranging from 6.5 to 200 kDa. As Figure 3 shows, the molecular range of proteins in crude venom is from less than 6KD to about 200KD, with a large amount to peptides with molecular weight of less than 14 KD.

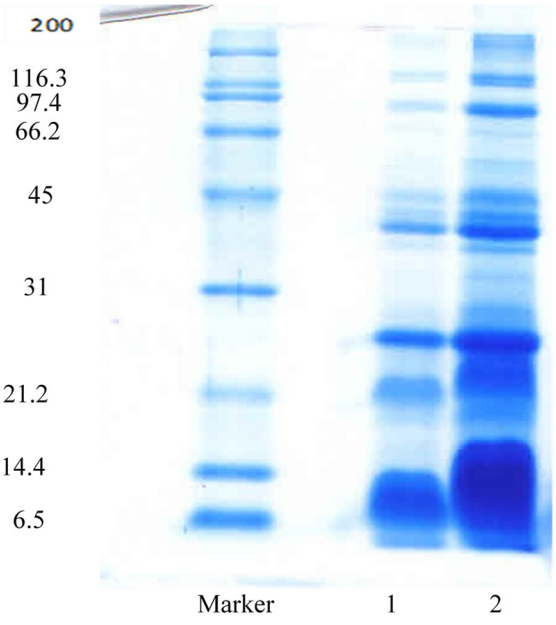

Figure 3. Comparison of scorpion venoms batches by 15% SDS-PAGE (conditions: 20 µg of protein/well, Coomassie stain). Arrows indicate visual differences between protein bands of three species of scorpion venoms. 1: *Apistobuthus susanae,* 2: *Buthacus macrocentrus.*

There exists a similarity between the venom of these species. However, some of mark differences also can be seen in a few bands. It seems the electrophoretic pattern of *Buthacus macrocentrus* is much more different from that of the other two scorpions.

RP-HPLC pattern

Figure 4 shows the HPLC pattern of the *Apistobuthus susanae* venom. The separation of peptide peaks based on polarity of peptide groups. As shown in the figure, the total protein fractions (area/total area) eluted from the column within 10 to 20 min of retention time was found to be 12.76. However, when the same zone determined in the venom of *Buthacus macrocentrus* and *Vachoniolus iranus* were found to be 5.66 and 11.01, respectively (Figure 5 and 6).

The total venom fractions (area/total area) eluted by RP-HPLC column at retention time within 25 to 40 min in scorpion (*Apistobuthus susanae*) venom was calculated and it was found to be 12.49. The same zone of fractions was determined in venom of *Buthacus macrocentrus and Vachoniolus iranus (*Figure 5 and 6). As shown in the figure, the sum of the fractions was 14.26 and 36.12, respectively.

Potency test

The potency test of polyvalent scorpion antivenom was determined in mice with average weight of 18-20 grams. Based on the mice mortality rate obtained within 24 hours of incubated venom and antivenom, the neutralizing potency of the antivenom in case of scorpion *Apistobuthus susanae* venom was found to be 488 µg (33 LD_{50}). The neutralizing capacity of polyvalent antivenom was found to be 500 µg (41 LD_{50}) for scorpion *Buthacus macrocentrus* venom as well. However, the neutralization of the same polyvalent antivenom for scorpion *Vachoniolus iranus* venom was found to be 660 µg (15 LD_{50}).

DISCUSSION

Polyvalent scorpion antivenom is produced against a few species but according to cross-reaction ability to neutralize multiple scorpion venoms. Our results show that antisera made by Razi Institute have the ability to neutralize the range of scorpion species found in Iran, as one of the countries with

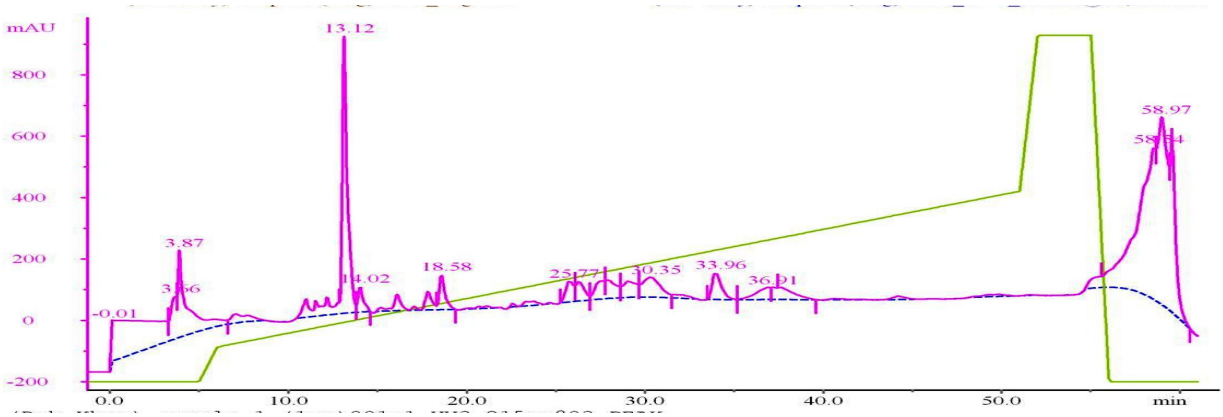

Figure 4. Revered-phase HPLC of the crude venom in *Apistobuthus susanae* scorpion was carried out using a C18 column with a flow rate of 0.5 ml/min. The crude venom was equilibrated with solvent A (H2O, 0.1% TFA), and eluted with a concentration gradient of solvent B (acetonitrile, 0.1% TFA) from 0 to 100%.

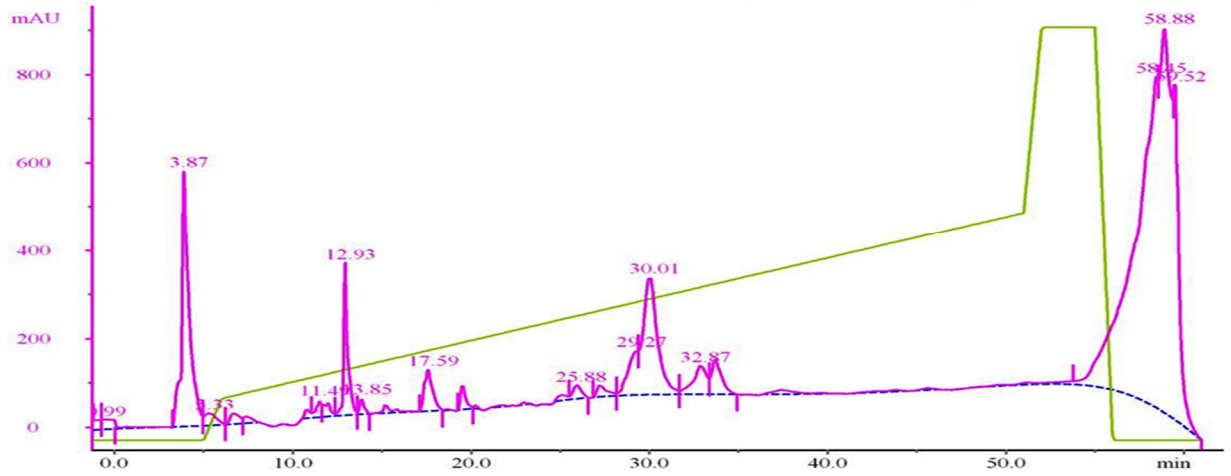

Figure 5. R P-HPLC of the crude venom in *Buthacus macrocentrus* scorpion was carried out using a C18 column with a flow rate of 0.5 ml/min. The crude venom was equilibrated with solvent A (H2O, 0.1% TFA), and eluted with a concentration gradient of solvent B (acetonitrile, 0.1% TFA) from 0 to 100%.

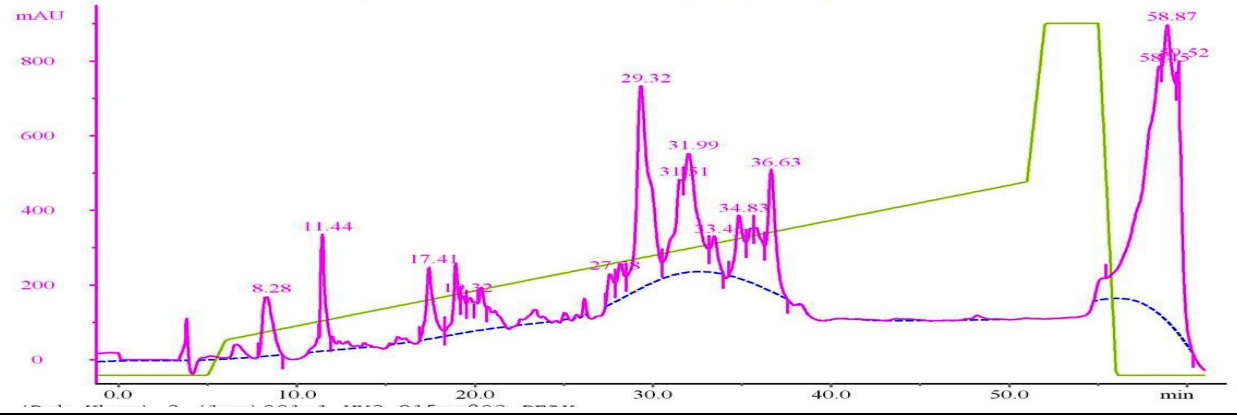

Figure 6. R P-HPLC of the crude venom in *Vachoniolus iranus* scorpion was carried out using a C18 column with a flow rate of 0.5 ml/min. The crude venom was equilibrated with solvent A (H2O, 0.1% TFA), and eluted with a concentration gradient of solvent B (acetonitrile, 0.1% TFA) from 0 to 100%.

high rates of scorpion stings. In southern regions of Iran, about 50,000 cases are referred to health centers annually (5).

The *Buthidae* family of scorpions has one of the most medically important species present in Iran (3). In this country, Razi Vaccine and Serum Research Institute, based in Hesarak, Karaj, is responsible for producing polyvalent antivenoms which are able to neutralize venoms of six species of scorpion, including *A. crassicauda, M. eupeus, O. doriae, H. saulcyi, H. schach and H. lepturus*. The treatment for a scorpion patient generally involves the use of antiserum, as well as medical care and clinical treatments (3). Serotherapy is a well-tested pharmaceutical method safely used in patients around the world (6, 15, 16).

In the present study, three scorpion venoms were analyzed by HPLC and electrophoresis methods to find the similarities and differences in protein composition of each venom (13). Due to limitation of venom, we were not able to study the electrophoretic pattern of the *Vachinous iranous* scorpion venom. A report on the protein profile of the Egyptian scorpions, by electrophoretic studies of crude venom showed variation similar to what was observed in our study (17). The difference in composition of venom could be due to the geographical diversity of the animal's sex and genetic characteristic of each species. The study of venom protein compounds is a complementary tool for identifying different species. To study the similarities and differences between the venoms of these three species the RP-HPLC technique was also used. The use of HPLC in the purification and venom study is a useful and practical method. The advantage of this method is using very low amounts for isolation, and determination of biological properties of venom (18). It is also a rapid and sensitive method. Considering the RP HPLC pattern of venom in three species of scorpion, it is possible to study the similarities and differences between them so that the peaks obtained in all three species can be plotted in three periods of time from 10 to 20 minutes, 25 to 30 minutes and after 30 minutes. However, in any of these periods there is one different species of scorpion. The cross-reactivity of

polyvalent antibody produced by Razi Vaccine and Serum Research Institute was tested against these three scorpions which are not included in antivenom production. Antivenom cross-reactivity can be assessed using several methods, all providing different levels of throughput. Traditionally, the *in vitro* methods used for determination of cross-reactivity have involved immunoblotting, immunodiffusion, and ELISA. However, the cross-neutralization test by *in vivo* method is the most reliable method and it is recognized as the golden standard for determining venom neutralization (10). Results of the present study reveal that this polyvalent antivenom is capable of neutralizing all three scorpion species *Apistobuthus susanae* (33 LD_{50}), *Buthacus macrocentrus* (41LD_{50}), and *Vachoniolus iranus* (15 LD_{50}). The cross-reactivity of antivenom and venoms are reported in several published papers (9, 11). Devaux et al. (9) reported producing antibodies against a synthetic polypeptide representing a conserved region in a set of several scorpion toxin sequences. These antibodies have been proved to cross-react with many species of scorpion toxins in different serotypes and to neutralize their pharmacological effects and biological activities. Hence, in many cases, it is quite necessary to develop species-specific scorpion antivenoms (2). The scorpion venom toxins are composed of peptides of low molecular weight (3 to 10 kDa). The genes encoding these toxins often carry post translation changes. In addition, there are many non-toxic toxins in the venom, which have many structural similarities to toxins. However, they are immunogenic, and the antibodies made against these toxins have the ability to neutralize even a few LD50s (10). Crossover reactions based on building similarities were even in the two different animal venoms, so that the scorpion venom has a cross-reaction with imported fire ant venom (19).

CONCLUSION

Based on the results obtained in the present study that indicate the neutralization of the three species of scorpions' venom, not included in antivenom production, the present Razi polyvalent antivenom is able to be used in patients stung by these 3 species of scorpion.

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