

Allergenome profiling of *Vespa orientalis* venom by serum IgE in patients with anaphylactic reaction to this hornet sting[☆]

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ABSTRACT

Background: Hymenoptera stings are one of the most common causes of anaphylaxis. *Vespa orientalis* (red hornet) is a common and very aggressive hymenopteran endemic in central and southern areas of Iran. Allergy testing and venom immunotherapies are carried out with venom components which are expensive, have limited commercial availability, and often lack specificity. Although proteomic analysis of hymenopteran venom has been shown to be a powerful technique to identify allergens, data on the protein components of *V. orientalis* venom are lacking.

Aim: This study was designed to characterize the allergenome profile (proteome of allergenic proteins) of this local hornet venom.

Methods: Venom was extracted from *V. orientalis* worker venom sacs. The venom constituents were separated by two-dimensional gel electrophoresis (2DE). Protein components were blotted and probed with serum from 10 allergic patients by immunoblotting. Reactive spots were isolated and characterized by liquid chromatography with tandem mass spectrometry.

Results: A total of 195 protein spots were detected on the 2DE gels. Fifteen distinct venom proteins showed reactivity to IgE in patients' sera. Four major allergens in order of allergenicity in patients were identified as hyaluronidase, arginine kinase, phospholipase A1 (PLA1) and PLA1 magnifin.

Conclusions: Broadening our knowledge of *V. orientalis* venom constituents can contribute to improvements in diagnostic and immunotherapeutic techniques, both of which are dependent on the major allergens in venom extract. This information is also potentially helpful to develop medical uses of major allergens in this venom to improve the diagnostic specificity and the efficacy of immunotherapy.

1. Introduction

Allergic reactions caused by hymenopterans, an order comprising an estimated 120,000 species of insects, are of medical importance (Fitzgerald and Flood, 2006), particularly because Hymenoptera stings are one of the most common causes of anaphylaxis (Simons et al., 2015). Hymenopteran venoms are a complex mixture of enzymes, low-molecular-mass proteins, and amines; some of these components are recognized allergens, such as phospholipase A1 (PLA1), PLA2, hyaluronidase, antigen-5, and acid phosphatase (Elieh Ali Komi et al., 2017; Hoffman, 2006).

Hymenoptera stings mainly cause six classes of reactions in the host.

Local nonallergic reactions are characterized by skin signs such as pain, erythema, and swelling around the sting site (about ≤ 5 cm). Extensive local swelling expands beyond the sting site (>10 cm) and persists longer than 24 h with fever, malaise, headache, and chills. Generalized skin lesions that can appear include urticaria and angioedema. Systemic IgE-mediated anaphylactic reactions affect more than one organ system with generalized urticaria, wheezing, bronchospasm, hypotension, cardiovascular collapse, vomiting, diarrhea, and loss of consciousness. Rare systemic toxic reactions (non-IgE mediated) are caused by massive envenomation by the more aggressive species, and can lead to diarrhea, headache, hypotension, seizures, and multi-organ dysfunction, and may result in death. Lastly, unusual and infrequent reactions may appear but

[☆] This study was carried out in compliance with the ethical principles for medical research involving human subjects based on the Declaration of Helsinki and was approved by the Ethics Committee of the Shiraz University of Medical Sciences (approval code: IR.SUMS.REC.1399.1001).

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their underlying pathogenesis remains to be established. These reactions occur several hours after stinging, and can affect the eyes, myocardium, kidneys, and lungs (Elieh Ali Komi et al., 2017; Bilo et al., 2005; Ciszowski and Mietka-Ciszowska, 2007; Sookrung et al., 2014; Mingomataj et al., 2014).

The group of wasps commonly known as hornets are among the largest and most dangerous hymenopterans, and are widespread in different parts of the world. *Vespa orientalis* (red hornet, Oriental hornet) is widespread in southern and central Asia and also occurs in the Arabian Peninsula, and in northern and eastern Africa (Archer, 2006). This species is a common and very aggressive social wasp endemic in central and southern areas of Iran, where it causes hundreds of stinging accidents of medical importance every year. Red hornets are primary predators of honey bees, attacking bee colonies to obtain honey and proteins (Ebrahimi and Carpenter, 2012).

The diagnosis of and immunotherapy for hymenopteran venom anaphylaxis is complicated in tropical countries due to the lack of standardized commercial allergen extracts. In Iran, Hymenoptera venom allergy is diagnosed mainly on the basis of clinical history and skin testing (considered the gold standard) performed with either skin prick tests or the intradermal injection of allergen extracts. Venom extracts from three species (honey bee, yellow jacket and wasp) are available in Iran thanks to importation from abroad. However, extracts for diagnosing wasp venom allergies are heterologous to causative wasp species that are local to the Middle East. The main disadvantages of these diagnostic reagents are the high cost and limited supply of these extracts, and misidentification of the venom components that cause allergy. Moreover, designing efficient immunotherapy approaches for allergic patients is complicated because of the nonspecific nature of these venom preparations. Another obstacle to prescribing venom immunotherapy (VIT) is that approximately half of the patients allergic to Hymenoptera venom show positive reactions to multiple venom extracts. In these situations, VIT is often carried out with multiple allergens, leading to an increased risk of adverse effects and sensitization to new antigens. In contrast, VIT with shared allergens will be adequate in some patients, given that positive skin test results may be due to cross-reactive venom allergens (Golden, 2005; Caruso et al., 2007). One way to circumvent the problem of allergen cross-reactions is to produce recombinant Hymenoptera allergens (Jakob et al., 2017; Frick et al., 2015).

Because allergic responses such as anaphylaxis after a *V. orientalis* sting can be life threatening, and given the importance of accurate diagnosis and effective treatment for allergic patients, this study was designed to identify the allergenome profile (proteome of allergenic proteins) of *V. orientalis* venom.

2. Materials and methods

2.1. Patients and controls

This study was approved by the Ethics Committee of the Shiraz University of Medical Sciences (approval code: IR.SUMS.REC.1399.1001) and carried out in compliance with the ethical principles of the Declaration of Helsinki for medical research involving human subjects. The study sample was selected from patients with a history of anaphylactic reactions to *V. orientalis* sting who were referred to the Asthma and Allergy Clinic of Ghotb Al-Din Hospital in Shiraz. After patients provided written informed consent, the skin prick test was done with commercial wasp (*Polistes* spp) venom extract (Jubilant HollisterStier LLC, Spokane, WA, USA), and then 10 patients with positive skin reactions were included in the study. Ten nonallergic individuals with no previous history of reaction to Hymenoptera stings and negative skin tests were included as a negative control group. Background information was recorded, i.e. clinical symptoms after the stinging event according to the classes of reactions to hymenopteran stings in the host as described above. Patients and controls were then examined for other

hymenopterans venom allergies by skin prick tests with commercial honey bee (*Apis mellifera*) and yellow jacket (*Vespula* spp) venom extracts (both from Jubilant HollisterStier).

Blood samples were collected from patients and negative control participants, and serum samples were isolated and stored at -20°C for further analysis.

2.2. Venom preparation

About 500 *V. orientalis* workers were captured with traps when they approached to attack beehives in Dariun (located 35 km east of Shiraz), placed in plastic containers, and transported to the immunology laboratory. Female hornets were verified as *V. orientalis* by an experienced entomologist.

Venom was isolated with a method described previously elsewhere (Sookrung et al., 2014; dos Santos et al., 2010). Briefly, to obtain venom sacs, the hornets were anesthetized by freezing for 3 min at -20°C . Forceps were used to remove the sting apparatus, and the venom sac was detached with small scissors. Venom sacs were transferred to microtubes (10 sacs per tube) on ice, washed with PBS, and suspended in a solution containing PBS and protease inhibitor cocktail. The venom reservoirs were punctured with micropipette tips and centrifuged at 10,000 *g* for 10 min at 4°C . The supernatants (crude venom extract) were collected and kept at -80°C until use. The protein concentration of venom extract was determined with the Bradford assay using bovine serum albumin (BSA) as the standard (Bradford, 1976).

2.3. Two-dimensional gel electrophoresis

To separate the protein components of the venom extract, two-dimensional gel electrophoresis (2DE) was done. For the first dimension, a mixture of 320 μg venom extract and rehydration buffer [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.5% (v/v) IPG buffer, 0.002% (w/v) bromophenol blue, 1% (w/v) DTT] was transferred to a channel of an isoelectric focusing (IEF) tray. An 18-cm IPG strip (pH 3–10 nonlinear) (GE Healthcare, Chicago, IL, USA) was placed gel side down in the channel and actively rehydrated for 16 h at 20°C . IEF was conducted at 60,000 Vh and 20°C with an end voltage of 10,000 V. Prior to running the second dimension, the focused strip was first equilibrated in the reduction buffer [6 M urea, 75 mM Tris-HCl pH 8.8, 29.3% (v/v) glycerol, 2% (w/v) SDS, 2% (w/v) DTT] for 15 min at room temperature, and then alkylation buffer [6 M urea, 75 mM Tris-HCl pH 8.8, 29.3% (v/v) glycerol, 2% (w/v) SDS, 2.5% (w/v) iodoacetamide] for a further 15 min at room temperature. The second dimension was generated with 12.5% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) at 15 mA for 10 min, followed by 25 mA for 6 h at 25°C . Coomassie Brilliant Blue (CBB) G-250 (Merck KGaA, Darmstadt, Germany) was used for total protein staining, and the 2DE gel was then scanned with a densitometer.

2.4. Immunoblotting

To transfer the protein spots to a PVDF membrane (Amersham, London, UK), blotting was done for 1 h at a constant voltage of 22 V in a semidry transfer cell. Then immunodetection was started: the membrane was blocked with 5% skim milk in PBS-T (phosphate buffered saline with 0.05% Tween-20) at pH 7.4 overnight at 4°C . Then the membrane was incubated with the serum sample at a 1:10 dilution in blocking buffer for 2 h at room temperature. After washing with PBS-T, the membrane was incubated with HRP-conjugated goat anti-human IgE (Invitrogen, Carlsbad, CA, USA) at a 1:2000 dilution in blocking buffer for 1 h at room temperature. After washing, the membrane was developed with ECL solution (Bio-Rad Laboratories Inc., Hercules, CA, USA) for 5 min, and reactive protein spots were visualized with a chemiluminescence detection system.

2.5. Spot selection

Protein components were blotted onto a PVDF membrane and probed with serum from 10 allergic as well as 10 nonallergic individuals by immunoblotting. The PVDF membranes representative of reactive proteins were analyzed with Image Lab software (version 6.0.1, Bio-Rad). To identify the same reactive spots on PVDF membranes and match them to protein spots in the 2DE gel, we used Progenesis SameSpots software (version 1.0, Nonlinear Dynamics, UK).

To identify the proteins, corresponding reactive spots were excised from the 2DE gel and transferred to separate microtubes carefully to avoid keratin contamination. For protein identification, the gel pieces were sent to the Proteomics Core Facility of Ottawa Hospital Research Institute, Canada.

2.6. Sample preparation

Excised pieces were dehydrated in 50% acetonitrile, rehydrated in 50 mM Tris pH 8.0, and reduced with 10 mM DTT for 45 min at 65°C. The pieces were then alkylated with 15 mM iodoacetamide and incubated for 30 min at room temperature with protection from light. The remaining iodoacetamide was quenched by the addition of 10 mM DTT. The pieces were dehydrated again with 50% acetonitrile and rehydrated in Trypsin/Lys-C mix solution. Digestion was carried out overnight at 37°C; then the peptides were extracted by dehydration with 5% formic acid in 50% acetonitrile for 30 min. The extracted peptides were purified by reversed-phase chromatography and prepared for peptide analysis.

2.7. Mass spectrometric analysis

Protein were separated and identified by LC-MS/MS (liquid chromatography with tandem mass spectrometry) with a microliquid chromatography system (Eksigent ekspert microLC, Eksigent Technologies, Redwood City, CA, USA) coupled to TripleTOF 6600 mass spectrometer (AB Sciex, Foster City, CA, USA) equipped with an electrospray ionization interface with a 25 µm iD capillary. Analyst TF (version 1.8, AB Sciex) was used for data processing and acquisition. Identifications were done in information dependent acquisition (IDA) mode. The peptides were separated on a reversed-phase Kinetex XB-C18 column with an internal diameter of 0.3 mm, particle size of 2.6 µm and a length of 150 mm (Phenomenex, Torrance, CA, USA) which was maintained at 60°C. Five microliters of sample was injected into the column by loop overfilling; the micropump generated a flow rate of 3 µL/min. Peptides were separated in a 15 min LC gradient with the mobile phase consisting of solvent A (0.2% formic acid and 3% DMSO in water) and solvent B (0.2% formic acid and 3% DMSO in Ethanol). Lastly, peptides in the elute were analyzed by MS at the following parameters: source voltage 5.5 kV maintained at 325°C, curtain gas 45 psi, ion source gas one 25 psi, ion source gas two 25 psi.

2.8. Data analysis

All mass spectra were processed and analyzed with ProteinPilot (version 5.0.2, AB Sciex). The acquired data was searched against the Uniprot/Swissprot protein database (accessed May 2021) for protein identification. To accurately assess the reliability of the results, global protein false discovery rate (FDR) was set to 1%. Only peptides identified with above 95% confidence were reported as detected. The candidate proteins were ranked with Pro Group Algorithm by calculating the Unused ProtScore.

3. Results

Background information on patients with anaphylactic reactions to *V. orientalis* venom and nonallergic individuals is summarized in Table 1.

After the protein components of *V. orientalis* venom were separated

Table 1

Background information of patients with anaphylactic reactions to *Vespa orientalis* venom and nonallergic individuals.

Subject ^a	Sex	Age (years)	Clinical symptoms ^b	Allergy to other hymenopterans ^c
P1	M	31	S, CV, RS	YJ
P2	M	14	S, RS	HB, YJ
P3	M	36	S, CV, RS, N	YJ
P4	M	55	S, CV, RS	–
P5	F	39	S, CV, RS, N	YJ
P6	M	17	S, CV, RS, GI	YJ
P7	M	23	S, CV, RS	HB, YJ
P8	F	26	S, CV, RS, N	–
P9	M	58	S, CV, RS, N	YJ
P10	M	13	S, CV, RS, N, UR	YJ
C1	M	25	–	–
C2	M	26	–	–
C3	M	59	–	–
C4	M	15	–	–
C5	M	36	–	–
C6	F	25	–	–
C7	F	37	–	–
C8	F	35	–	–
C9	F	60	–	–
C10	F	14	–	–

^a P: Patient, C: Negative control.

^b S: Skin (urticaria, angioedema), CVS: Cardiovascular (hypotension, cardiovascular collapse), RS: Respiratory (wheeze, bronchospasm), N: Nervous (loss of consciousness), GI: Gastrointestinal (nausea and vomiting, diarrhea) UR: Unusual Reactions (eye involvement).

^c HB: Honeybee, YJ: Yellow Jacket.

by 2DE and stained with CBB dye, a total of 195 spots were detected visually with a pI ranging from 3 to 10 and molecular mass between 10 and 130 kDa. After the protein spots were blotted onto the PVDF membrane and probed with allergic patients' sera, 15 distinct reactive spots were seen, demonstrating the high allergenic potential of this venom (Figure S1A–J). To test the allergenic capacity of these spots, blotted membranes were also probed with sera from one nonallergic individual separately and two groups of pooled serum samples, one containing serum from four and another from five nonallergic participants. Probing with normal samples detected no reactive spots on the corresponding membranes (Figure S1K–M). Reactive spots that recurred most frequently among different patients were chosen as major allergens for further characterization (Fig. 1). An example of the 2DE-IgE immunoblotted membrane with a serum sample from patient no. 10 in comparison to a negative control is shown in Fig. 2.

According to the LC-MS/MS results, the allergen profile obtained from the crude venom of *V. orientalis* separated by 2DE comprised four main proteins with sequence coverage between 42.8% and 94.6%. These major reactive proteins were identified as hyaluronidase (one spot with 80% allergenicity), arginine kinase (three spots with 70% allergenicity), PLA1 (one spot with 70% allergenicity), and PLA1 magnifin (one spot with 50% allergenicity), in order of the patients' sensitization frequencies. Detailed findings for these spots are summarized in Table 2.

4. Discussion

Insects of the order Hymenoptera cause thousands of stinging events leading to anaphylaxis worldwide. During the last one hundred years, different approaches have been tried to diagnose and treat venom allergies, especially those with systemic effects. The main focus of research in the diagnosis and treatment of venom allergies has changed over the years from whole venom to venom-specific or cross-reactive components.

Studies of Hymenoptera venom components have used a variety of methods, including different types of chromatography, histamine release assay, Edman degradation, genome sequencing, electrophoresis, and most recently, 2DE-based proteomics (Hoffman, 2006; Vincent

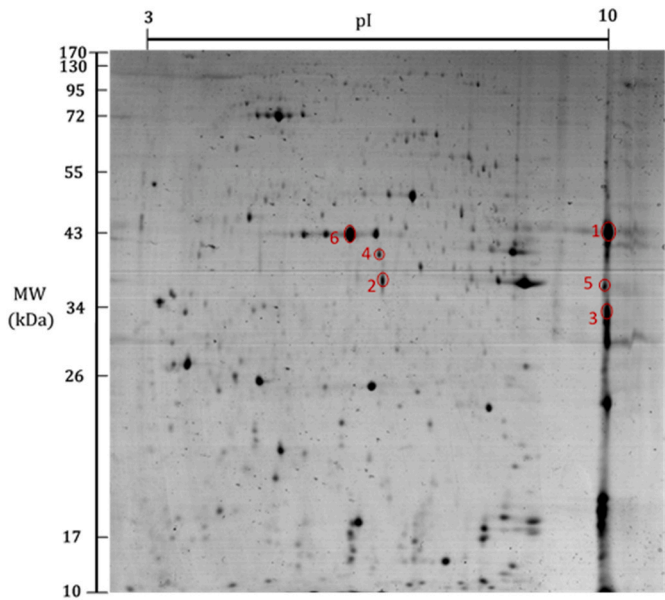


Fig. 1. Representative 2DE gel of *Vespa orientalis* venom stained with Coomassie Brilliant Blue G-250. Reactive spots are ordered according to their frequencies in patients as determined in immunoblotted membranes shown in Figure S1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

et al., 2010; Peiren et al., 2005, 2006; Sobotka et al., 1976; Shipolini et al., 1971). Modern proteomics methods have led to the characterization of a considerable number of major venom allergens from honey bees and some types of vespids. To improve allergy diagnosis and component-based immunotherapy, some of these allergens are being produced with recombinant biotechnological techniques (M ü ller,

2003; Perez-Riverol et al., 2015).

Studies of differential diagnostic methods and VIT have focused primarily on honey bees, yellow jackets and some vespid species, mainly those that are local to the western hemisphere and Europe. Diagnosis and immunotherapy for patients with venom allergies in the Asian and African continents, as well as in Iran, are carried out with neither local nor specific venom components, on the assumption that these components may be cross-reactive with nonlocal ones. This problem is especially evident in patients with allergy to *V. orientalis* venom, the most common local hymenopteran causing anaphylaxis, because of the limited geographical distribution of this species.

In the present study we provide initial insights into the allergenome of *V. orientalis* with data from 2DE immunoblotting. The proteins identified in the red hornet allergenome are major allergens as documented by the frequency of specific IgE reactions in the patients we studied. Our results show that hyaluronidase, arginine kinase, PLA1, and PLA1 magnifin are the main allergens in this species, and are in line with information from earlier studies that reported PLA1 and hyaluronidase as common allergens in hymenopterans (Bilo et al., 2005; M ü ller, 2010). Our data share some similarities with the major allergens described by dos Santos et al. in their proteomic analysis of *Polybia paulista* venom: these authors reported a group of six proteins, including PLA1, hyaluronidases and arginine kinase, as the main allergens (dos Santos et al., 2010). The information we report here on the immunoproteome profile of *V. orientalis* venom bears some resemblance to that provided by Sookrung et al. for *Vespa affinis* venom. Venoms from these two hornets show similarities not only in their 2DE patterns but also in their major allergens. The *V. affinis* venom allergenome consists of a group of nine allergens with allergenicity greater than 50%: PLA1 with 100% allergenicity (four protein spots reacted with all patients' sera), arginine kinase with 73.3% allergenicity (eight protein spots reacted with 11 out of 15 patients' sera), PLA1 magnifin with 60% allergenicity (two protein spots reacted with nine serum samples), and hyaluronidase with 53.3% allergenicity (two spots reacted with eight serum samples). However,

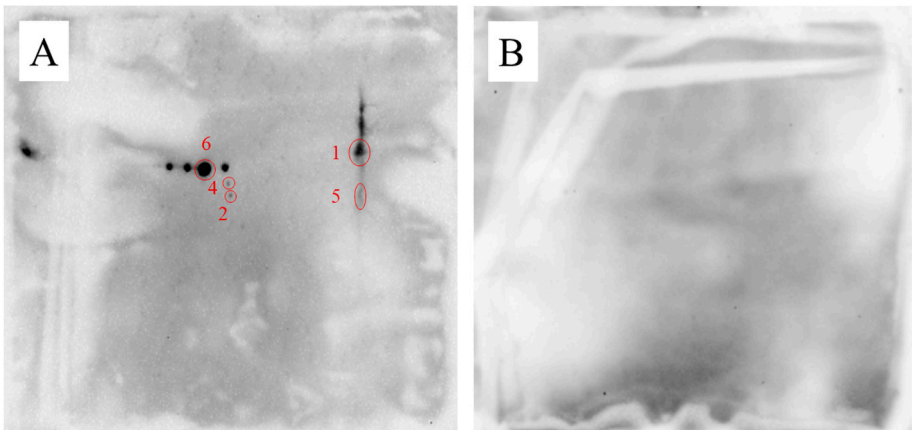


Fig. 2. Representative results of 2DE-IgE immunoblotting. (A) Reactive protein spots from *Vespa orientalis* venom revealed by probing with sera from allergic patient no. 10. This patient's serum-specific IgE reacted to major spots no. 1, 2, 4, 5, and 6. (B) A nonallergic individual serum served as a negative control.

Table 2
Components of *Vespa orientalis* major allergens detected in this study.

Spot no.	Protein	Accession no.	Coverage (%)	Peptides ^a (95%)	Patient no (s).	Allergenicity ^b (%)
1	Hyaluronidase	COHLL4	85.6	74	1,2,3,5,6,7,9,10	80
2	Arginine kinase	A0A2Z4LYY3	42.8	12	1,4,6,7,8,9,10	70
3	Phospholipase A1	P0DMB4	65.3	64	1,2,3,5,6,7,9	70
4	Arginine kinase	A0A2Z4LYY3	94.6	60	1,4,6,7,8,9,10	70
5	Phospholipase A1 magnifin	P0CH47	74.2	68	5,6,7,9,10	50
6	Arginine kinase	A0A2Z4LYY3	94.6	69	7,10	20

^a Identified peptides with at least 95% confidence.
^b The potential to induce allergen-specific IgE in patients studied here.

unlike their finding that PLA1 was the protein with the highest allergenicity in their patients, we found hyaluronidase to be the main allergen (Sookrung et al., 2014). In addition, an analysis of *Apoica pallens* (American paper wasp) venom is consistent with our results in that hyaluronidase was found to be the main allergen in this vespid venom (Mendonça et al., 2019). To compare different hymenopteran venom major allergens, we provide information about major allergens reported previously in well-studied species in Table S1.

Hyaluronidase is an enzyme belonging to the glycosyl hydrolase family, which acts on hyaluronic acid. This acid is a major constituent of the extracellular matrix, which holds cells together with its adhesive properties; degradation of this protein by hyaluronidase results in increased tissue permeability and facilitates the rapid diffusion of other venom components in the host tissue. Because of this activity, hyaluronidase is referred to as a “spreading factor” (Hoffman, 2006; Dotimas and Hider, 1987). This enzyme is a known allergen in ant (*Solenopsis invicta*) (Baer et al., 1979; Hoffman et al., 1988), honey bee, and vespid venoms, and exhibits some degree of cross-reactivity to IgE in patients allergic to different Hymenoptera species (Lu et al., 1995; Gmachl and Kreil, 1993; Arbesman et al., 1976). In addition to its ability to facilitate diffusion, this hydrolase can also act as an inflammatory mediator at the stinging site (dos Santos et al., 2010). Our findings with 2DE-IgE immunoblotting showed that *V. orientalis* venom hyaluronidase reacted to serum IgE in 80% of our allergic patients.

Arginine kinase, a member of the phosphotransferases, catalyzes the reversible transfer of phosphate from ATP to arginine residues (Müller, 2010). This enzyme is found in invertebrates and acts as a buffering system for energy maintenance (Wang et al., 2009; Laino et al., 2017). However, it is not known whether its substrates are self-venom components, host proteins, or both (dos Santos et al., 2010). Arginine kinase is known as a pan-allergen, and its allergenic effect has been documented in hymenopterans such as, *Polybia paulista* (dos Santos et al., 2010), *V. affinis* (Sookrung et al., 2014), and the endoparasitoid wasp *Pteromalus puparum* (Zhu et al., 2010). This allergen is also found in truncated form in the venom of the solitary spider wasp *Cyphononyx dorsalis*, and exerts paralytic activity in sting victims (Yamamoto et al., 2007). In the present study we identified three spots with arginine kinase characteristics (Fig. 1, spots no. 2, 4 and 6) with molecular mass ranging from 34 to 43 kDa as major allergens in sensitized patients. Regarding spot no. 6, although it reacted with only two patients' serum, these two were among seven patients allergic to arginine kinase as shown by spots no. 2 and no. 4. It appears that these three spots have the same protein core but different sizes and pIs possibly because of post-translational modification, or they may be three different isoforms (Rogowska-Wrzesinska et al., 2013). Thus, we included spot no. 6 as one of the main allergens in *V. orientalis* venom as a complement to the other two spots identified as arginine kinase. Overall, arginine kinase reacted with seven patients' serum, which makes it the second most frequent allergen in *V.orientalis* venom.

Phospholipases are lipolytic enzymes that cleave ester bonds within phospholipids. PLA1 is widely distributed among hymenopterans and is well known for its allergenic activity (King et al., 1978; Pantera et al., 2003; Perez-Riverol et al., 2019). This protein from different vespid venoms is reported to have sequence similarities that indicate evolutionary conservation (Baumann et al., 2018), and was shown to be the major cause of sensitization in comparison with other hymenopterans (Perez-Riverol et al., 2019). In addition to its causal role in allergy induction, PLA1 also causes direct toxic effects in the host by disrupting phospholipid packing in biological membranes, which results in pore formation, and eventually leads to cell lysis and tissue damage (Dotimas and Hider, 1987; Aoki et al., 2007). The action of phospholipases can also lead to the generation of free polyunsaturated fatty acids, which are precursors of compounds involved in inflammation (Balboa and Bal-sinde, 2021).

Another enzyme with PLA1 activity was discovered in 2008 and named PLA1 magnifin. This protein, purified from *Vespa magnifica*

venom, induced in vitro platelet aggregation in a dose-dependent manner (Yang et al., 2008). Rare but life-threatening effects of wasp venom such as aortic thrombosis, heart failure, and contraction of the trachea are thought to result from the PLA1 activity of magnifin (Yang et al., 2008; Chen et al., 2004).

In the present study, two protein spots (nos. 3 and 5) with a molecular mass between 30 and 40 kDa were identified as phospholipase. Eight patients were found to be sensitive to either PLA1 or PLA1 magnifin, a finding that illustrates the high percentage (80%) of allergenicity exerted by these enzymes. Along with hyaluronidase, PLA1 enzymes were found to be the major cause of allergic reactions in *V. orientalis* venom.

Patients no. 4 and 8 in the present study were allergic only to arginine kinase (Table 2). Intriguingly, these patients were only allergic to *V. orientalis* venom and their skin tests for yellow jacket and honey bee venom were negative (Table 1). It can be hypothesized that the arginine kinase isoforms of red hornet venom share fewer similarities with other species in which this enzyme acts as an allergen. More specific studies designed to simultaneously compare hornet, yellow jacket and honey bee venom components and determine their uniqueness or similarity are needed to further clarify the properties of these proteins.

The present study provides insights into the immunological aspects of allergy to red hornet venom. Together with allergic effects, the toxic effects of hymenopteran venom components are also of medical importance particularly in persons who experience a massive number of stings or massive envenomation as described earlier. The characterization of toxic components can facilitate the development of specific therapies such as treatment with antivenom. In 2016, a phase I/II clinical trial was started to treat victims of massive Africanized honey bee stings with apilic antivenom. The main allergens and toxic proteins were identified and phospholipase A2 and melittin were found to be the main toxic components with little or no allergic effect (Barbosa et al., 2017). This trial ended in 2018 and showed promising safety and efficacy results (Barbosa et al., 2021). For its part, venom whole proteome research holds potential to yield insights regarding all components whether toxic or allergic. Therefore, future studies should be designed to characterize additional protein spots from *V. orientalis* venom in order to elucidate the nature of these constituents along with their biological roles. Whole proteome research would also make it possible to characterize species-specific and cross-reactive proteins with distinct functions.

5. Conclusion

The present study used conventional 2DE immunoblotting to obtain the first dataset on the allergenome of Middle Eastern red hornet venom. The proteome pattern of this venom, major allergens, and their corresponding allergenicity were determined. These findings can contribute to improvements in diagnostics and venom-specific immunotherapy in the near future based on the use of these allergens in either native or recombinant form.

Author statement

Shirin Farjadian: Conceptualization, Funding acquisition, Writing – review & editing. **Sajjad Nejabat, Mohammad Reza Haghsheenas and Shirin Farjadian:** Investigation, Methodology, Formal analysis and Writing – original draft.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.toxicon.2022.05.039>.

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