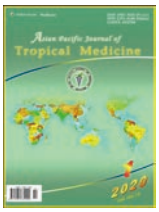

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Salivary gland antigens of laboratory-bred *Phlebotomus sergenti* and their immunogenicity in human volunteers in laboratory condition

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ABSTRACT

Objective: To investigate *Phlebotomus (P.) sergenti* Parrot, 1917 (Diptera: Psychodidae) salivary gland antigens and their immune response in human.

Methods: Human volunteers were exposed to sand flies' bites in the laboratory, and following each exposure the size of induration was recorded. The mean protein concentration of salivary gland lysate and specific anti-*P. sergenti* saliva IgG was measured. Sand fly salivary proteins were separated by SDS-PAGE and their immunoreactivity was examined by Western blotting assays.

Results: Individuals exposed to *P. sergenti* salivary gland lysate for 8 months showed both antibody and delayed type hypersensitivity responses, although exposure for one month did not provoke any immune responses. The trend of antibody fluctuated during the exposure time and dropped by the end of antigen loading. The mean protein content was (0.36±0.08) μg in each pair salivary glands. Salivary gland lysate showed 11 to 12 major protein bands and 3 to 6 of them were immunoreactive.

Conclusions: Our study showed that the salivary gland components of *P. sergenti* provoked both cellular and humoral immune responses in human. Furthermore, there are some immunogenic proteins in *P. sergenti* saliva which could be subjected for further investigation as vector-based vaccine candidate/s against anthroponotic cutaneous leishmaniasis.

KEYWORDS:

Phlebotomus sergenti; Antibody response; Delayed-type hypersensitivity; Human; Salivary gland antigen

1. Introduction

Leishmaniasis is a neglected protozoan parasitic disease, transmitted to human through sand flies bite, presents worldwide, affects 98 countries, and it is estimated 350 million people are at risk globally[1]. Cutaneous leishmaniasis due to *Leishmania tropica* Wright, 1903 (Kinetoplastida: Trypanosomatidae) is endemic in many parts of Middle East including Iran[2]. Recent advances in the field of vector-based vaccine introduce some new and promising approach to design new generation of vaccines against arthropod-borne disease including leishmaniasis[3]. Successful *Leishmania* infection is a result of long host-parasite-vector co-evolution, which

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is mostly linked to the ability of the parasite and vector to manipulate host immune response[4–5]. Interestingly, salivary active molecules facilitate establishment of *Leishmania* in the naive host[6]. In contrast, it was shown that pre-exposure to sand flies' saliva induces immune responses which reduce the risks of severe leishmaniasis in animal model[7,8]. Thus, sand fly salivary gland molecules might be considered as vector-based vaccine candidate. It is demonstrated that *Leishmania* major Yakimoff and Schokhor, 1914 (Kinetoplastida: Trypanosomatidae) co-injection with either *Lutzomyia (L.) longipalpis* Lutz & Neiva, 1912 (Diptera: Psychodidae) or *Phlebotomus (P.) papatasi* Scopoli, 1786 (Diptera: Psychodidae) salivary gland lysate caused larger lesions compared to the control group that received parasite alone[9,10]. Furthermore, several studies have shown that, different animals were repeatedly bitten by sand flies or exposed to salivary galnd lysate, induced different class of anti-saliva antibodies[11–14]. Antibody response against sand fly's salivary galnd lysate varies in genetically different individuals; also it might be affected by sand fly species. Sand flies population fluctuates during the active season in the endemic areas, so it affects antigens loading through biting, which results in antibody response fluctuation in the hosts[15]. In the case of anthroponotic cutaneous leishmaniasis, human known as the main reservoir host of the disease[16], thus studying the role of *P. sergenti*'s saliva in immunity and the trend of the disease can be a step toward to reach new vaccine candidate(s) against anthroponotic cutaneous leishmaniasis. To date, it is little known about *P. sergenti*'s saliva components and their antigenicity, especially in human. So that, current study aimed to study *P. sergenti*'s salivary gland profiles and their antigenicity in human.

2. Materials and methods

2.1. Sand fly maintaining and breeding

Phlebotomus sergenti was reared at Phlebotomine Insectary, School of Public Health, Tehran University of Medical Sciences, using the modified methods of Modi and Tesh and Killick-Kendrick and Killick-Kendrick[17,18]. The colony was maintained in the insectary at a mean temperature of 27 ℃-28 ℃, 80% relative humidity and 14:10 light and dark cycle (L:D) photoperiod. For larval feeding, a composted mixture of ground and dried rabbit feces and rabbit pellets was used, which was prepared through a specific procedure[19].

2.2. Volunteers and exposure

Ten human volunteers who had not lived in the endemic areas of *P. sergenti* and were willing to participate and signed an informed consent were recruited. The aims and procedures of the study were explained for each potential candidate. Individuals with specific conditions like anaemia, entomophobia, pregnant women, and individuals with positive antibody were excluded from the study.

To mimic monthly *Phlebotomus* activity in the field (from April to November), five volunteers (coded as A, P, G, E and F) were exposed to non-infected lab-bred *P. sergenti* bites once a week for eight months, and another five volunteers (coded as B, M, D, A and N were exposed to lab-bred non-infected *P. sergenti* four times during two weeks and with one month interval they were exposes four times during two weeks again, similar to the times of the peaks of vector density fluctuation in the field. Each individual was exposed to 20 to 50 female uninfected sand flies at each time. After each exposure at least three bite sites were traced by a permanent marker and the size of induration was measured using a digital Vernier calipers at 24 h, 48 h and 72 h post bite. The photographs were taken before and after exposure. To evaluate the anti-saliva IgG antibody, human sera were collected monthly up to a year after first exposure, and were kept at -20 ℃ until use.

2.3. Phlebotomus sergenti and their salivary gland lysates

Salivary glands of lab-bred female sand flies were dissected out by the means of fine forceps (# 5) and needles (# 28) into cold Phosphate Buffered Saline (PBS, pH7.4). Salivary glands were extracted from four generations (F3, F4, F5 and F18) and four to ten-days-old females. Groups of 20 dissected glands were transferred into 1.5 mL micro-tubes containing 20 μL PBS, and kept at -20 ℃ until use.

Just before use, glands were disrupted by three cycles of freeze/thaw in liquid nitrogen and boiling water bath, centrifuged at 18 000 × *g* for 10 min, and then the supernatants were used for the experiments.

2.4. Protein measurement

Protein concentration of the salivary galnd lysate was measured by Bicinchoninic acid assay (BCA) method using Pierce® BCA Protein assay kit (Thermo Fisher Scientific) according to the manufacture's instruction.

2.5. ELISA

Specific anti-*P. sergenti* saliva IgG was measured by indirect enzyme-linked immunosorbent assay (ELISA) technique. All the experiments were conducted in triplicate, and preliminarily to the main experiment, the assay was optimized by sera of experimentally bitten volunteers. In brief, wells of ELISA plate were coated with 50 μL of salivary galnd lysate (equivalent to about 0.25 gland per well) in carbonate-bicarbonate buffer (0.01 M, pH 9.6), and kept overnight at 4 ℃. The plates were then washed three times with PBS buffer including 0.05% Tween 20 (PBS-T), and blocked with 100 μL mixture of 5% BSA (Sigma-Aldrich) and 10% fetal bovine serum (FBS, Gibco) in PBS-T for two hours at 37 ℃. After another washing step, 50 μL of the volunteers' sera diluted 1:50 in 5% BSA/PBS-T were added to each well and incubated at 37 ℃ for 90 min. Following three times washing step, 50 μL of HRP-conjugated anti-

human IgG (Sigma-Aldrich), 1:16 000 dilution in PBS-T, was added to each well, and the plates were incubated at 37 ℃ for 90 min. This procedure was followed by another washing step, then 50 μL of substrate (3,3′,5,5′-Tetramethylbenzidine; TMB; Pishtazteb) was added, and the plates were incubated at room temperature for about 10 min. Finally, 25 μL of stopping solution (10% H2S04) was added to each well. The absorbance was measured using the BioTek ELISA reader (ELx808) at 450 nm wavelength. Sera from pre-exposed volunteers with positive antibody and 10-20 days old naïve infants (Prepared from the biobank of Children's Medical Center, TUMS) were used as positive and negative controls respectively. Two wells without first layer (coating), and two wells without second layer (human sera) were included in all ELISA tests. To determine the cut-off values, two standard deviations added to the mean optical densities of negative controls.

2.6. SDS–PAGE

Sand fly salivary gland proteins were separated by SDS-PAGE on 10% gel using "Mini-PROTEAN® Tetra Cell" (Bio-Rad), under reducing conditions. Salivary galnd lysate proteins of ten salivary glands, which were extracted from *P. sergenti* females, loaded in one lane. The molecular weights of the proteins bands were estimated by loading a "PageRuler™" Prestained Protein Ladder (Fermentas). Finally, the protein bands on the gels were stained and visualized by silver following a special procedure of silver staining protocol.

2.7. Western blot

In order to investigate the immunogenicity of *P. sergenti* salivary galnd lysate antigens western blot analysis was used. After electrophoresis, separated proteins were electro-transferred onto a polyvinylidene difluoride (PVDF, Thermo Fisher Scientific) membrane using "Mini-PROTEAN® Tetra Cell" (Bio-Rad). After transferring, polyvinylidene difluoride membrane was temporally stained by "Ponceau S" (Abcam) and then the membrane was cut into strips. Following a washing step by PBS, the polyvinylidene difluoride membrane strips were blocked with 5% mixture of BSA and 10% FBS in PBS-T, and then were incubated overnight at 4 ℃. At the following, the strips were washed three times (15 min each time) with PBS-T buffer. ELISA positive sera were diluted 1:100 in PBS-T including 2.5% BSA, and then added individually to each strip. The strips were incubated with the serum samples at room temperature for 90 min with shaking. After several times washing in PBS-T, the strips were incubated at room temperature for 90 min with 1:64 000 (diluted in 0.05% PBS-T) of HRP-conjugated goat anti-human IgG. The procedure was followed by washing the strips in PBS-T for six times; each wash for 15 min. Finally the chromatic reaction was developed using Tetramethylbenzidine (Sigma-Aldrich), liquid substrate system for membranes. Sera from 10 to 20 days old infants were used as negative control.

2.8. Statistical analysis

The largest induration diameter was measured for delayed-type hypersensitivity response. Volunteers with at least one delayed-type hypersensitivity response was considered delayed-type hypersensitivity positive. Induration size of different time frame after exposure were compared by analysis of variance followed by Tukey's multiple comparison tests. Statistical analyzes were done using SPSS (version 20) and graphs were prepared by GraphPad Prism (version 5.0d).

2.9. Ethics approval and consent to participate

The consent forms were filled out by all volunteer participants and the experiments were approved by the Ethical Committee of Tehran University of Medical Sciences, Tehran, Iran (IR.TUMS.VCR.REC.1395.253).

3. Results

3.1. Evaluation of human IgG response

All five individuals (coded as A, P, G, E and F) exposed to *P. sergenti* bite for 32 weeks, showed positive reaction to *P. sergenti* salivary gland antigens. Although other five volunteers (coded as B, M, D, A and N) exposed to salivary galnd lysate of *P. sergenti* for eight times within four weeks with a month interval, unexpectedly did not showed any reaction.

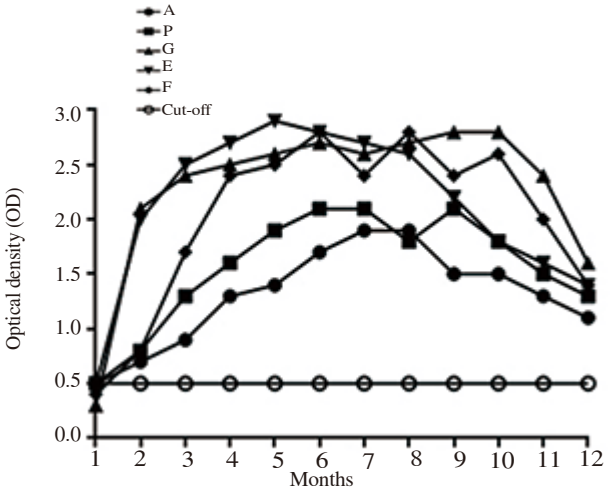

Figure 1. Fluctuation of IgG anti-*Phlebotomus sergenti* salivary gland antibodies in the volunteers (A, P, G, E and F) over 12 months post exposure. Cut-off values were determined by adding two standard deviations to the mean optical densities of negative controls.

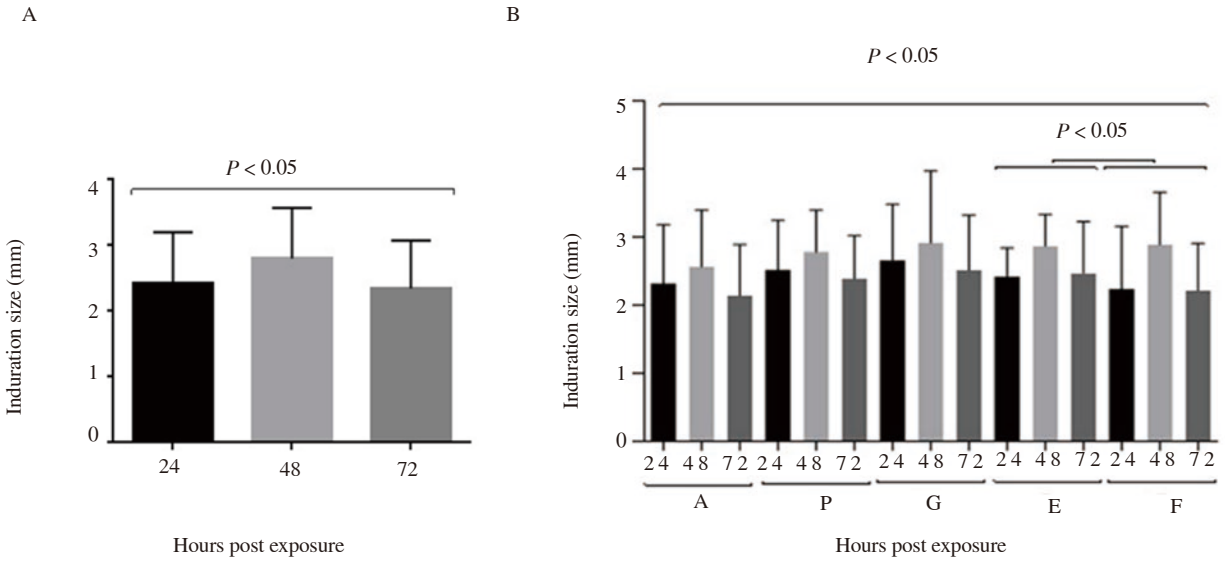

Figure 2. Delayed-type hypersensitivity response to uninfected sand fly bites in volunteers for eight months (32 times) in the laboratory condition. Following exposure to lab-bred sand flies, the diameter of the largest induration size at 24 h, 48 h and 72 h post bite were measured (±SD). (A) delayed-type hypersensitivity response indicated by the mean induration size at different time points. (B) delayed-type hypersensitivity response indicated by the mean induration size in each individual (A, P, G, E and F) at different time points.

Fluctuations of antibody responses in experimentally exposed individuals coded as A, P, G, E and F are shown in Figure 1. As the Figure shows, the trend of antibody from the first month of exposure to 4th or 5th month is increasing, afterward it showed some fluctuation from 6th to 8th month, following the ending of exposure (8 weeks), it showed a decreasing trend till 12th months which means that despite passing 4 months of the last exposure, all the participants remained IgG positive. But the optical density of all sera were all below the cut-off point before exposing to sand fly bite (Figure 1).

3.2. Delayed-type hypersensitivity

In all individuals (coded as B, M, D, A and N) were exposed eight times, no delayed-type hypersensitivity response was developed. However, all the volunteers coded as A, P, G, E and F which exposed 32 times, developed a delayed-type hypersensitivity positive response. The statistical analysis showed a significant difference (P<0.05) between three time frames. The induration size increased after 24 h and reached to its peak in 48 h and subsided after 72 h post exposure. The induration size showed a significant difference between different times (Figure 2A). On the other hand, comparing mean induration size in each individual was significant in two volunteers (Figure 2B).

3.3. Protein assay

The average protein amount calculated to be (0.36±0.08) μg (mean ± SD) in each pair of salivary glands.

3.4. SDS-PAGE

In 10% poly acrylamide gel 11 to 12 major protein bands with molecular masses of 10 kDa - 70 kDa were observed. In Figure 3, *P. sergenti*'s salivary galnd lysate electrophoretic pattern of lab-bred generations of 3th and 18th are shown. As Figure 3 shows, there was a slight difference between electrophoretic pattern of 3th and 18th generations in protein bands of 55 kDa. In addition, in electrophoretic pattern of 3th generation, about eight major and four faint protein bands of 35 kDa to 38 kDa were detectable. On contrary, electrophoretic pattern of 18th lab-bred generations showed ten major protein bands of 10 kDa to 70 kDa and three faint proteins of 35 kDa to 38 kDa.

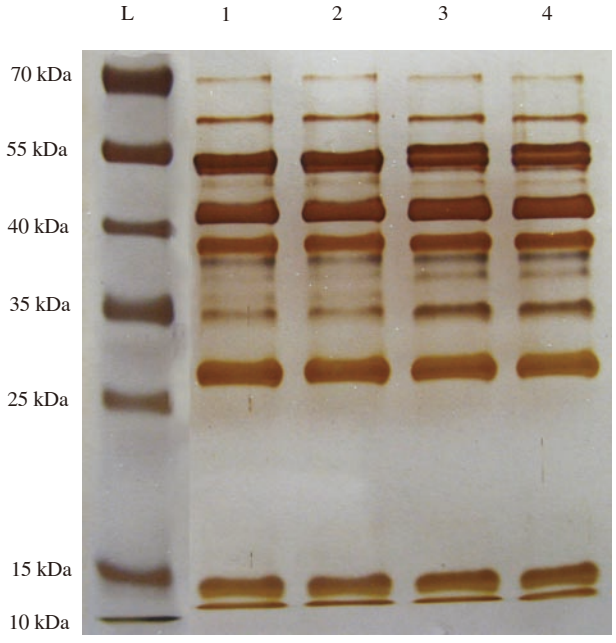

Figure 3. SDS-PAGE analysis of salivary glands lysates of *Phlebotomus sergenti*, L: PageRuler™ Prestained Protein Ladder (Fermentas), lane 1 and 2: lab bred (F3), lane 3 and 4: lab bred (F18) *Phlebotomus sergenti* originated from Dehbakri village, Bam County, Kerman, Iran

3.5. Western Blotting

Individuals three (stripe 3) and four (stripe 4) had the highest and lowest reaction against salivary galnd lysate proteins which produced max and min of eight and three antigenic bands, respectively. Strips of 6, 7 and 8 have shown immunoreactivity of sera from 10 to 20 days years old infants against salivary galnd lysate of *P. sergenti* as negative controls (Figure 4).

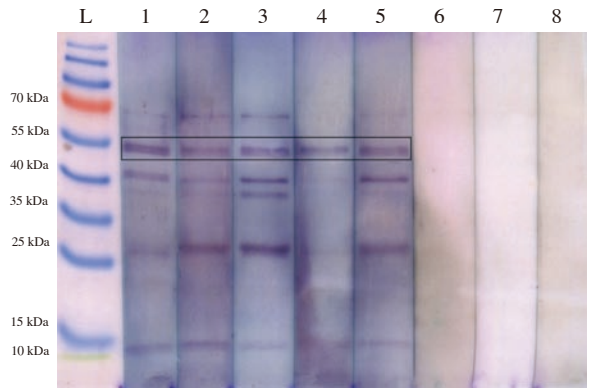

Figure 4. Immunoblots of *Phlebotomus sergenti* salivary galnd lysate incubated with serum of human volunteers, exposed to *Phlebotomus sergenti* bite experimentally. L: PageRuler™ Prestained Protein Ladder (Fermentas), lane1-5: sera of human volunteers, exposed to *Phlebotomus sergenti* bite, lane 6-8: sera of 10-20 day old naïve infants. Marked immunoblots inside rectangle show the same immunoreactivity toward different previously exposed human sera.

4. Discussion

Here we showed naïve individuals experimentally exposed to salivary galnd lysate of *P. sergenti* provoked both humoral and cellular immunity. Average protein concentration of *P. sergenti*'s saliva per gland was reported as 0.18 μg. It was shown that the protein concentration and composition of saliva were affected by the sex and physiological status of the sand flies[20]. Previously, the amount of protein concentration in lab-reared *P. sergenti* reported much more than the current study, as a study reported 0.2 μg[21], and also another reported 0.23 μg[22] per gland. Proteins of sand flies' salivary galnd lysate differ both in concentration or composition even between colonies of the same species from different countries[23]. In a previous study the max protein concentration of sand flies' salivary galnd lysate reported for *P. duboscqi* (0.78 μg), *P. papatasi* (0.51 μg) from Cyprus colony, *P. halepensis* Theodor, 1958 (Diptera: Psychodidae) (0.41 μg) and *P. papatasi* (0.33 μg), from Turkish colony respectively[22]. Recently, Akhavan *et al*. reported 0.3 μg per gland protein concentration for wild-caught of *P. papatasi* in Iran[15]. Saliva protein profile varies based upon the physiological state of adults, sex, age, generation, species and also geographical distribution of the sand fly[24]. In the current study, electrophoretic pattern of SDS-PAGE showed 10 to 12 major protein bands with molecular mass of 10 to 70 kDa for *P. sergenti* salivary galnd lysate. Although, a slight difference was seen between salivary galnd lysate protein profile of 3th and 18th generation in molecular mass of 55 kDa. It is hypothesized, these differences is the result of population bottle neck which cause genetic diversity in lab-bred colonies. Previous study reported more than 12 protein bands with molecular mass of 20-60 kDa and same to our results, two protein bands with molecular mass of 14 kDa were reported as well[23]. Furthermore, in a recent study electrophoretic patterns of lab-reared *P. sergenti* salivary galnd lysate proteins from distinct geographical origins were compared, and results revealed different levels of variation. Although, same to the results of the current study, these variations were reported even in a colony with the same origin[23].

In the individual exposing part of the study we have faced some limitations to deal with. As many people had phobia of biting by flies, and also many voluntary individuals excluded from the outset because they came from endemic area of *P. sergenti*. This study revealed that short exposure to salivary galnd lysate of *P. sergenti* do not induce immune response in its host. At the beginning of *P. sergenti* bite exposure, IgG level had increasing trend, since then maintained almost in a same level, and by the end of antigen loading it was followed by a decreasing trend (seroconversion). The point is that, despite passing four month of the last exposure, all the participants remained IgG positive. A study conducted in Turkey showed, high percent of inhabitant living in endemic area of anthroponotic cutaneous leishmaniasis, had antibody against sand flies' salivary galnd lysate[24]. A recent study completed in Iran showed, the desert gerbil [*Rhombomys opimus* Lichtenstein, 1823 (Rodentia: Cricetidae)], the reservoir host of zoonotic cutaneous leishmaniasis, had the highest antibody titer in the fall and the lowest antibody titer in the winter against salivary galnd lysate of *P. papatasi*[15].

In another study, the antibody responses (IgG) of dogs against salivary galnd lysate of *L. longipalpis* maintained up to six months after the last exposure[25], conferring our results considering longtime maintenance of antibody against *P. sergenti* bite. Also it was shown, five to six weeks after the last exposure to sand flies bites the IgG and IgG2 reached to the peak[25].

The individuals with short time exposure (eight times in four weeks) did not elicit any delayed-type hypersensitivity response. Although, the individuals with long time (32 times during eight month) exposure elicited a strong delayed-type hypersensitivity response. Furthermore, delayed-type hypersensitivity response gradually appeared at 24 h, reached to its peak at 48 h, and subsidized following 72 h post exposure. A study accomplished in Mali, showed 64% of individual living in endemic area of cutaneous leishmaniasis had delayed-type hypersensitivity response to *P. duboscqi* bite, and same to our study, the delayed-type hypersensitivity response reached to its peak at 48 h, and subsidized after 96 h post exposure[26]. In another study delayed-type hypersensitivity response against the salivary gland components

of *L. longipalpis* in dogs reached to its highest size at 48 h post sand fly bite exposure in naïve animals[27]. Furthermore, delayed-type hypersensitivity response to *P. papatasi* exposure in human and mice have been well demonstrated[28].

Human immune response encountering various antigens is not always uniform due to some reason such as genetic resource of the host, immunity background *etc.*, so that responses might be different against same and even against different epitopes of the same antigen. Therefore as the current study confirmed, individual diversity in the level of antibody and immunologic reactions led to individual specific antigenic electrophoretic patterns. As mentioned previously sand fly saliva enhance *Leishmania* multiplication which led to establishment of *Leishmania* infection in the naïve host[6,25]. On the one hand, pre-exposing to some molecule of sand flies saliva conferred a strong protection against *L. major* in the corresponding host. In the current study, western blotting revealed variable pattern of antigenic bands, though there were two (about 55 kDa) proteins which strongly reacted to all the sera. It seems they could be candidates to further vector-based vaccine investigation. The approach of vector-based vaccine has been already proven in laboratory animals for single proteins like SP15 from *P. papatasi*[30] and or LJM19 from *L. longipalpis*[31,32]. Therefore looking for new protein/s from other vectors such as *P. sergenti* with strong immunoreactivity is a fundamental approach to reach a vector-based vaccine for controlling leishmaniasis.

In a previous study immunogenicity of sera from indigenous people in endemic foci of anthroponotic cutaneous leishmaniasis against *P. papatasi* and *P. sergenti*, same to the current study, showed one to six antigenic bands in molecular mass of 20 kDa to 70 kDa. In the case of *P. papatasi*, there was a 16 kDa protein which strongly reacted to all the sera. On the other hand, reactions to the salivary galnd lysate proteins of *P. sergenti*, similar to our study, were individual specific[33]. In another study, immunoreactivity of indigenous people from a visceral leishmaniasis region against *L. longipalpis* revealed six antigenic bands; three showed stronger antigenicity[34]. A recent study in Iran showed, sera of *R. opimus* strongly reacted to *P. papatasi* salivary galnd lysate and western blotting detected eight antigenic bands[15]. In another recent study accomplished in Iran showed antigenicity of *P. papatasi* salivary galnd lysate against *Rhombomys opimus* in different seasons and physiological status were variable[35].

In agreement with the results of previous studies, our results showed that the salivary gland components of *P. sergenti* provoked both cellular and humoral immune responses in human, although short exposure do not induce both immune responses. Furthermore it was shown that the humoral immune response was transient to salivary galnd lysate of *P. sergenti* and the level of antibody was dropped by the end of antigen loading. As mentioned above the immunogenicity of salivary galnd lysate components not only varied person to person but also that was individual specific. However, there were some proteins which reacted strongly to all individual sera. Therefore these proteins can be candidates for further studies on vector-based vaccine against anthroponotic cutaneous leishmaniasis.

Conflict of interest statement

The authors claim they have no conflict of interest.

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Authors' contributions

AV, ARM, AK and AAA designed the study and experiments. AV, AK and AAA wrote the manuscript, with input from MRY, MJ, YR, AZ and NH. AV and DR undertook the statistical analysis. AV and ARM undertook all laboratory experiments. AV and MF reared the sand flies and dissected the salivary glands. All authors read and approved the final manuscript.

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