Original Article

Seasonal and Physiological Variations of *Phlebotomus papatasi* Salivary Gland Antigens in Central Iran

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Abstract

Background: Sand fly saliva helps parasite establishment and induce immune responses in vertebrate hosts. In the current study, we investigated the modulation of *Phlebotomus papatasi* salivary gland antigen expression by seasonal and biological factors.

Methods: Sand flies were grouped according to physiological stages such as unfed, fed, semi-gravid, gravid, parous, nulliparous, infected or non-infected with *Leishmania major* and based on the season in which they were collected. Salivary gland antigens (SGAs) were analyzed using SDS-PAGE and the antibody response against SGAs in *Rhombomys opimus* was determined by ELISA and Western blot.

Results: The highest protein content was found in the salivary glands of unfed sand flies. The saliva content was higher in parous compared to nulliparous, in summer compared to spring, and in *Leishmania*-infected compared to non-infected flies. The salivary gland lysate (SGL) electrophoretic pattern variations were observed among sand flies with various physiological stages particularly from 4–9 protein bands of 14–70 kDa. The SGL of unfed and gravid flies had extra protein bands compared to fed and semi-gravid sand flies. There was missing protein bands in SGL of parous compared to nulliparous; and in summer compared to spring collected flies. *Rhombomys opimus* serum reacted strongly with an antigenic band of around 28 kDa in the SGL of all sand fly groups.

Conclusion: Certain biological and environmental characteristics of wild populations of vector sand flies affect the protein content and antigenicity of saliva. This might have an important implication in the design of vector-based vaccines.

Keywords: Antibody response, *Phlebotomus papatasi*, *Rhombomys opimus*, Salivary gland antigens, Iran

Introduction

Zoonotic cutaneous leishmaniasis (ZCL) is a neglected tropical disease of public health importance in many rural areas of Iran (Yaghoobi-Ershadi et al. 1996, 2001, 2003, 2010). *Leishmania major* is the causative agent, *Phlebotomus papatasi* is the main

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vector and *Rhombomys opimus* (great gerbil) is the major reservoir host of the disease in Esfahan Province, which is a hyperendemic zone of ZCL in central Iran (Yaghoobi-Ershadi et al. 1995, Akhavan et al. 2010a, b, Yaghoobi-Ershadi 2012). The incidence rate of ZCL in Esfahan Province is reported around 2400 cases per year (communication from the Esfahan Center for Public Health) and is considered an underestimate of the actual incidence.

Saliva of phlebotomines consists of different molecules that are necessary for a sand fly to take successfully a blood meal (Ribeiro 1987). Additionally, previous exposure to sand fly saliva indirectly affects the establishment of *Leishmania* in vertebrate hosts (Oliveira et al. 2013). Mice previously exposed to saliva by injection or by uninfected sand fly bites showed both a humoral and a cellular immune response against salivary antigens that protected them against *L. major* infection (Belkaid et al. 1998, 2000, Kamhawi et al. 2000). Importantly, immunization of mice with defined molecules from saliva of vector species also conferred a strong protection against *L. major* infection (Valenzuela et al. 2001, Oliveira et al. 2008, Gomes et al. 2012). This suggests that sand fly salivary components may be considered as candidates for a cocktail vaccine against *Leishmania* infection. In the Esfahan hyperendemic focus of ZCL, the most abundant sand fly species is *P. papatasi* (Yaghoobi-Ershadi and Javadian 1997, 1999). Of relevance, antibodies against saliva of this vector species were demonstrated in the main animal reservoir of *L. major* in this area, *R. opimus* (Akhavan 2011).

Differences in the antigenic components of the salivary gland lysate (SGL) of various sand fly species, sex, and age have been reported (Volf et al. 2000). In the Esfahan hyperendemic focus, vertebrate hosts are bitten by *P. papatasi* with various physiological characteristics and under diverse environmental conditions. It is therefore important to address the effect, if any, of the variability of vector salivary gland components on *L. major* infections and the clinical outcome of the disease.

The aim of the current study was to determine the composition of salivary gland antigens (SGAs) of *P. papatasi* with respect to certain seasonal and biological factors in vector populations in the Esfahan hyperendemic focus, and to further characterize the *P. papatasi* SGAs reacting with *R. opimus* antibodies. The composition of the SGAs was studied with respect to physiological aspects of the collected sand flies comprising unfed, fed, semi-gravid, gravid, parous, nulliparous, infected or non-infected with *L. major*, and with respect to the season in which they were collected.

Materials and Methods

Study area

This investigation was undertaken during 2012–2013 in the three villages of Parvaneh-Aliabadchi, Habibabad and Abbasabad, Esfahan Province, central Iran (Fig. 1). The villages of Habibabad and Parvaneh-Aliabadchi are located 25–40 km north of the city of Esfahan (32°39′ 35" N, 51°40′ 17"E) at an altitude of around 1550 m and Abbasabad is located 5 Km from Badroud city (33° 42′ N, 52° 2′ E) at an altitude of 1056 m, in the foothills of Karkas Mountains. The biotope of the selected areas is desert with hot summers and cold winters. In 2013, the maximum and minimum monthly relative humidity in Esfahan city was 81 % and 9.1 % in November and July, respectively. The minimum monthly temperature was -5.3 °C in December and the maximum was 39.8 °C in July. In Badrood district, the minimum and maximum monthly temperatures were -2.9 °C in December and 43 °C in July, respectively. The maximum relative humidity was 62 % in December and the minimum was 20 % in

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February. Total annual rainfall was 84.6 mm in Esfahan city and 77.5 mm in Badrood district (Esfahan Metrological Organization).

Sand fly collection and rearing

Phlebotomines were aspirated from resting places throughout the selected study villages during the active season of sand flies in 2012–2013. Sand flies were identified according to morphological characters using a valid systematic key (Seyedi-Rashti and Nadim 1992). Females of *P. papatasi* were separated from other species for inclusion in the study and categorized into ten groups according to certain seasonal and biological factors: accessory glands status, parous and nulliparous, unfed, fed, semi-gravid and gravid. Two groups of sand flies were collected throughout spring and summer and analyzed according to their *L. major*-infection status. Salivary gland antigens of parous versus nulliparous, *L. major*-infected versus non-infected, spring versus summer collection and unfed versus fed, semi-gravid and gravid were compared.

Sand fly colonies were reared at the Department of Medical Entomology and Vector Control, Tehran University of Medical Sciences and the Esfahan Center for Public Health according to the methods of Modi and Tesh (1983) and Killick-Kendrick and Killick-Kendrick (1991) with modifications. *Phlebotomus papatasi* colonies were reared on a 14:10 LD photoperiod, at 26–28 ºC and around 80 % relative humidity. Adult sand flies were fed on 20 % sucrose and females were blood fed on a white small BALB/c anesthetized with Ketamine hydrochloride (60 mg/kg) and Xylazine (5 mg/kg).

Preparation of salivary gland lysates of *Phlebotomus papatasi*

Sand fly salivary glands were dissected in cold phosphate buffered saline (PBS), pH 7.2, and stored in fresh PBS at -20 ºC until used. Salivary glands were disrupted by three cycles of freeze/thaw in liquid nitrogen and boiling water just before use. The SGL was centrifuged at 18000 g for 10 min, and the supernatants were used for experiments (Volf and Rohousova 2001, Rohousova et al. 2005, Akhavan 2011). The concentration of SGAs was determined using the BCA protein assay kit according to the manufacturer's instructions (Pierce Biotechnology, Rockford, USA). Standards were prepared from bovine serum albumin (BSA) in sodium azide saline.

Animal sera

Great gerbils were collected from Badrood rural district using Sherman live traps. Collections were carried out during the active sand fly season when the gerbils are supposedly repeatedly bitten by sand flies. Animals were anesthetized with Ketamine hydrochloride (60 mg/kg) and Xylazine (5 mg/kg) and the isolated sera were kept at -20 ºC until use (Akhavan 2011).

HRP- conjugated anti-*Rhombomys opimus* antibody production

Rhombomys opimus antibodies were purified from animal sera by HiTrap Protein G chromatography. The antibodies were then injected intramuscularly in the hind legs of rabbits and the induction of anti-*R. opimus* antibodies was checked using ELISA. Anti-*R. opimus* antibodies were purified from rabbit sera and conjugated to horseradish peroxidase (HRP) then the titer of HRP-conjugated anti-*R. opimus* antibodies was determined by ELISA (Akhavan et al. 2011).

Anti-*Phlebotomus papatasi* saliva antibodies assessed by ELISA

Anti-saliva antibodies were measured by ELISA. SGL was prepared from 2–6 day old sand flies. ELISA wells were coated with 50 µl SGL (equal to 0.5 gland per well) in carbonate-bicarbonate buffer (0.01 M, pH 9.6) overnight at 4 ºC. Wells were washed three

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times with PBS-Tween 1X buffer. Each well was treated with 50 µl *R. opimus* serum and incubated for 1 hour at 37 ºC. After 3 washes, 50 µl of HRP-conjugated anti-gerbil antibodies (1: 1000 in PBS-Tween) was added to each well, and incubated for 1 hour at 37 ºC. The wells were washed and 50 µl of substrate (3, 3', 5, 5'-Tetramethylbenzidine, TMB) added to each well and incubated for 15 minutes at room temperature. The stopping solution (20% H2So4) was added and the optical density measured by an ELISA reader at 450 nm. Negative sera were obtained from lab-bred *R. opimus* not bitten by any sand fly. The cut-off value was calculated by adding two standard deviations to the mean optical densities of negative controls.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Salivary glands were removed from collected sand flies. A SGL prepared from 10–14 pooled glands was loaded into each lane to separate and visualize proteins or glycoproteins. SGL from 8 salivary glands were loaded into each well for western blots. Electrophoresis was carried out at room temperature under reduced conditions on 13 % Tris-Glycine gels of 1mm thickness and a 100V constant voltage, using Mini-Protean III (Biorad, Munich, Germany). Prestained Protein Ladders (PageRuler, Fermentas) were used and the gels were silver stained according to the methodology of Heukeshoven and Dernick (1985).

Western Blot analysis

After SDS-PAGE, one part of the gel was silver-stained and the second part was electro- transferred to PVDF (polyvinylidene difluoride) membrane (Roche, pore size 0.45 µm) using a Mini Protean Tetra Cell (Biorad) under constant voltage 100V for 75 min. The PVDF membrane was blocked using 5 % skim milk in PBS-Tween and incubated overnight at 4 ºC. The PVDF membrane was treated with *R. opimus* positive serum, which was detected by ELISA. The second part of PVDF membrane was treated with naïve *R. opimus* negative serum as a control.

HRP-conjugated anti-*R. opimus* antibodies were added and the membrane was incubated for 1 hour at room temperature. Positive bands were visualized using Luminata Forte (Millipore, Billerica, MA) immunochemical staining (Akhavan 2011).

Detection and identification of *Leishmania* species in *Phlebotomus papatasi*

DNA was extracted from individual sand flies using Exgene^{TM} Tissue SV (plus) kit (GeneAll Biotechnology, Korea). Nested PCRs were done on individual sand flies using specific primers: outer forward primer (5′-AAA CTC CTC TCT GGT GCT TGC-3′), outer reverse primer (5′-AAA CAA AGG TTG TCG GGG G-3′), inner forward primer (5′- AAT TCA ACT TCG CGT TGG CC-3′), inner reverse primer (5′-CCT CTC TTT TTT CTC TGT GC-3′) (Akhavan et al. 2010a). PCR-RFLP was carried out to confirm the identity of the *Leishmania* species in positive samples (Akhavan et al. 2010b). Additionally, PCR products of a limited number of specimens were sequenced for species identification. The composition and protein concentration of SGAs were compared between infected and non-infected sand flies regardless of the physiologic state of the sand flies.

Ethical consideration

The protocol was approved by the Ethics Committee of Tehran University of Medical Sciences (No. 18511/16.5.2012).

Results

Protein concentration of sand fly salivary gland proteins

The average protein content per pair of glands was 0.2, 0.1, 0.1 and 0.1µg for unfed, fed, semi-gravid and gravid sand flies, re-

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spectively. The protein content per pair of glands for sand flies collected in the spring and summer was <0.1 and 0.2 µg, respectively. In SGL of parous and nulliparous sand flies, the protein content was 0.1 and <0.1 µg per pair glands, respectively. The protein content in *L. major*- infected and in non-infected sand flies was 0.1 and <0.1 µg per pair of glands, respectively.

Leishmania infection in collected *Phlebotomus papatasi* sand flies

Nested PCR and RFLP analyses were used to detect and identify *Leishmania* infection in *P. papatasi* sand flies.

Overall, 49 (32.2%) out of 152 *P. papatasi* females were infected with *L. major*. Of those, 44 (28.9%) were infected with *L. major* alone, 1 (0.7%) sand fly was infected with *L. major* and *L. turanica* and 4 (2.6%) were infected with *L. major*, *L. turanica* and *L. gerbili*. Three (2%) sand flies were infected with *L. turanica* and *L. gerbili*.

Determination of the presence of anti-*Phlebotomus papatasi* saliva antibody in *Rhombomys opimus* serum

Serum samples were obtained from 13 *R. opimus*, collected during the active sand fly season when the gerbils are supposedly repeatedly bitten by sand flies and tested for antibodies against saliva of *P. papatasi* by ELISA. All of the 13 gerbil sera examined were positive.

Phlebotomus papatasi salivary gland protein profile

On polyacrylamide gels, the number of visualized protein bands was different between sand fly groups varying in the status of their accessory glands and physiological stages as well as by their collection seasons and presence or absence of *Leishmania* infection. Overall, 4–9 protein bands were observed with molecular weights ranging from 14 to 70 kDa. The electrophoretic pattern of SGL of all ten groups of *P. papatasi* is shown in Figure 2.

The SGL of parous and nulliparous separated into 5 and 6 major protein bands with molecular masses of 14 to 70 kDa, respectively, and one faint band of about 30 kDa in both parous and nulliparous flies. The difference was a missing protein band from SGL of parous flies with molecular weight of around 42 kDa (Fig. 2a).

In the SGL electrophoretic profile of unfed, fed, semi-gravid and gravid groups of sand flies, 3 major protein bands with molecular weights of 14–17 kDa were observed. The groups of unfed and gravid flies differed from fed and semi-gravid flies in showing the extra protein bands in their electrophoretic profiles. In SGL of gravid sand flies 2 faint bands with molecular weights of around 28 and 42 kDa and in unfed sand flies one faint band with around 28 kDa were also visible. The SGL of unfed and gravid flies differed also in the intensity of bands of 14–17 kDa. These protein bands in unfed and gravid groups of sand flies were stronger than fed and semi-gravid groups (Fig. 2b).

The SGL of sand flies collected throughout spring separated into 6 strong proteins bands with molecular weights of 14–42 kDa and one weak band of around 36 kDa. Sand flies collected throughout summer had a SGL pattern of 5 protein bands with molecular weights ranging from 14 to 30 kDa. The SGL of both spring and summer groups of sand flies, showed 5 protein bands of around 14–30 kDa. The difference was in the presence of 2 protein bands of around 36 and 42 kDa in SGL of spring flies which were missing from SGL of flies collecting in summer (Fig. 2c).

The SGL profiles of *L. major*-infected and non-infected sand flies were similar with 5 proteins bands ranging from 14–30 kDa. Just a little difference was seen in the intensity of mentioned protein bands, which were a bit stronger in non-infected sand flies (Fig. 2d).

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Rhombomys opimus antibody response to *Phlebotomus papatasi* salivary proteins

The antibody response of *R. opimus* against SGAs of the ten different sand fly groups was determined by Western blot analysis (Fig. 3). Negative serum did not react with any of the SGAs in the ten sand fly groups. The positive serum reacted variably to the SGAs of the different groups. Western Blot analyses revealed 6–9 antigenic bands with molecular masses of 14–72 kDa.

The immunoreactions were stronger with SGAs around 17, 22, 24 and 28 kDa in the SGL of parous flies and with SGAs around 14, 22, 24 and 28 kDa in the SGL of nulliparous flies, respectively (Fig. 3a).

The serum reacted with 9 SGAs of unfed, fed, semi-gravid and gravid sand flies. The strongest reactions were observed against antigens of gravid sand flies. A strong reaction was observed against a 28-kDa antigen in unfed, fed, semi-gravid and gravid sand flies (Fig. 3b).

The *R. opimus* serum recognized 6 antigenic bands, five common antigens were between 14–28 kDa in SGL of both spring and summer collected sand flies. One antigenic band of 44 and one band of 69 kDa was specifically recognized in spring and summer collected sand flies, respectively (Fig. 3c).

Anti *P. papatasi* serum from *R. opimus* reacted similarly with SGL of *Leishmania* infected and non-infected sand flies. Six antigenic bands from 14 to 44 kDa and one faint band of 68 kD were recognized. The serum reactions differed only in intensity with non-infected flies recognizing a 22 kDa and a 24 kDa antigen more strongly than infected flies (Fig. 3d).

Fig.1. Geographical location of Abbasabad, Habibabad and Parvaneh-Aliabadchi in Esfahan Province, central Iran

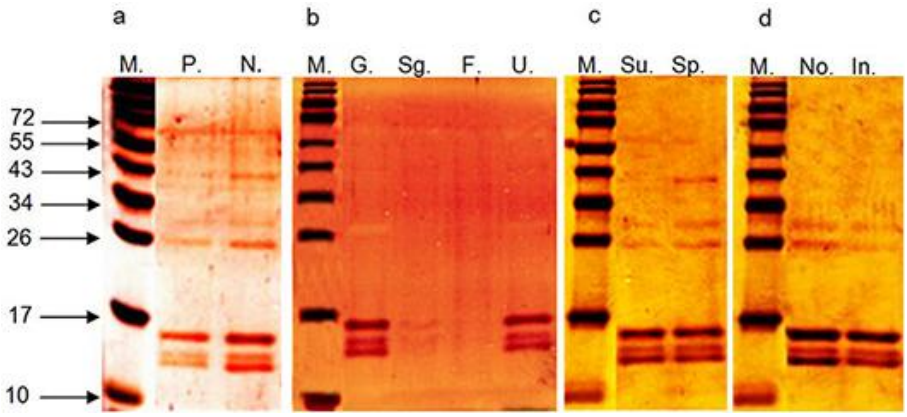

Fig. 2. SDS-PAGE analyses of salivary gland antigens of sand flies collected from Esfahan Province, Central Iran. a: parous(P) and nulliparous(N) groups, b: unfed(U), fed(F), semi-gravid(Sg) and gravid(G) groups, c: spring(Sp) and summer(Su) collections, d: infected(In) and non-infected(No), M: Prestained protein marker

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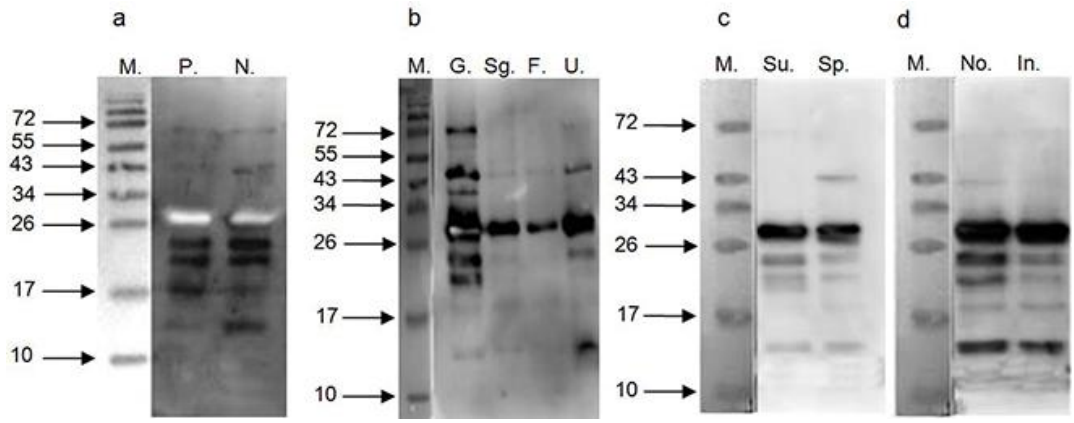

Fig. 3. Western blot analyses of salivary gland antigens (SGAs) collected from Esfahan Province, central Iran. a: SGAs of parous(P) and nulliparous(N) groups, b: SGAs of unfed(U), fed(F), semi-gravid(Sg) and gravid(G) groups, c: SGAs of spring(Sp) and summer(Su) collected sand flies, d: SGAs of infected(In) and non-infected(No) sand flies, M: Prestained protein marker

Discussion

Salivary glands composition depends on various characteristics of sand flies such as sex, age, generation and geographical location (Volf et al. 2000, Ben Hadj Ahmed et al. 2010, Akhavan 2011).

Mice pre-immunized with salivary gland extract (SGE) of F 29 laboratory-bred female *P. papatasi* were protected against *L. major* co-inoculated with the same type of SGE while the mice pre-immunized with SGE of wild-caught or F1 sand flies were not protected (Ben Hadj Ahmed et al. 2010). However, in field conditions, humans naturally exposed to *P. duboscqi* sand fly bites reacted with a Th1-like response to the bites of colonized sand flies, suggesting that humans react in the same manner to colonized and field-collected sand fly salivary proteins (Oliveira et al. 2013).

The composition of sand fly salivary glands is therefore an important element of *Leishmania* infectivity, where the amount of SGAs may affect their antigenicity and the outcome of infection with *L. major*. In this study, the salivary protein content differed in the various physiological stages of sand flies. The highest content of SGL protein was seen in unfed sand flies. Our results also showed that the number and the intensity of protein bands in the SGL of unfed sand flies was more than of fed and semi-gravid sand flies. This might be due to the deposition of saliva as an obligatory part of the sand fly blood feeding strategy, reducing the amount of saliva in blood fed flies. Compatible with this, the reactivity of antigens was stronger in the SGL of unfed and gravid sand flies than in semi-gravid and fed sand flies.

The protein content of saliva is not constant and varies with age, reaching the full electrophoretic pattern in 3–5 days flies (Volf et al. 2000), but in the sand flies collected from the field, age is not defined, very young sand flies as well as very old ones have very low amounts of SGA proteins. This may account for the overall five to ten times lower salivary gland protein content observed in this study compared to the 1ug reported for 4-day-old colonized *P. papatasi* (Volf et al. 2000, Valenzuela et al. 2001). The protein content of sand fly saliva also varies by geographical location and in different species (Volf and Rohousova 2001). The salivary protein content in *P. papatasi* from

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a Cyprus colony was 0.51 µg per gland, for *P. papatasi* from a Turkish colony was 0.33 µg/gland, for *P. duboscqi* was 0.78 µg/gland, for *P. halepensis* was 0.41 µg/gland, and for *P. sergenti* from a Turkish colony was 0.23 µg/gland and for *Lutzomyia longipalpis* was 0.18 µg/gland (Cerana et al. 2002).

In our study, the salivary protein content was higher in parous compared to nulliparous sand flies, in summer compared to spring collected sand flies, and in *Leishmania*-infected compared to non-infected sand flies. Nulliparous sand flies collected in this study might also be too young with their glands containing an insufficient amount of saliva. Additionally, the protein content of sand fly salivary glands collected in the summer was higher than in spring-collected sand flies, which seems to be due to environmental conditions. In the study area, the climate conditions change between seasons, in summer, the relative humidity is lower and the relative temperature is higher compared to spring, and there is almost no rainfall during summer. In a previous study, the effect of ecological characteristics on salivary gland gene expression was determined; expression levels of five salivary gland genes of *P. papatasi* were up regulated in September due to a water deficiency, which resulted in a reduction of sugar sources for sand flies (Coutinho-Abreu et al. 2011).

In the current study, the climate variations supposedly change the type and the amount of vegetation in the study area which effect on the phytophaghy of sand flies. In SGL of sand flies collected through spring there were 2 more protein bands of around 36 and 42 kDa compared to those collected in the summer, showing the influence of ecological factors.

The higher amount of saliva in infected sand flies in the current study might be explained by the fact that infected sand flies probe longer to obtain a blood meal and need to inject more saliva during blood meal to benefit from the biological and immunomodulatory effects of saliva (Kamhawi 2006).

In the current study, the SGL of the different groups of *P. papatasi* showed 4–9 protein bands with molecular weights ranging from 14 to 70 kDa. In a study performed by Volf and Rohousova (2001) from 5 to 8 prominent protein bands with molecular weights from 28 to 50 kDa were recognized. In another study SDS-PAGE analysis showed 14 major protein bands with 12–70 kDa molecular weights (Rohousova et al. 2005). In a previous study in our lab, 7 major protein bands of *P. papatasi* salivary gland lysate with molecular masses of around 12-40 kDa and 3 faint bands of 20, 55 and 65 kDa were observed (Akhavan 2011). This difference might reflect geographical distance of *P. papatasi* subpopulations (Hamarsheh et al. 2009) or be due to the generation of sand fly (Ben Hadj Ahmed et al. 2010).

In our study Western Blot analysis of *R. opimus* serum revealed 6–9 antigenic bands with molecular masses of 14–72 kDa compared to 8 antigenic bands from *P. papatasi* collected from Borkhar and Sejzi rural district in Iran (Akhavan 2011), 4–9 major antigenic bands for colonized *P. papatasi* Scopoli against BALB/c mice (Volf and Rohousova 2001) and 4–6 major antigenic bands for colonized *P. papatasi* from Turkey against BALB/c mice (Rohousova et al. 2005). These differences may be because the reservoir species was not the same and highlight the specificity of the immunogenicity of SGAs in various hosts.

The antigenic profile of spring and summer collections was different by one band only which seems to be due to environmental factors. The serum reactions with SGL of *Leishmania* infected and non-infected sand flies were similar and the difference was only in the strength of the reaction which may indicate that *Leishmania* infection do not affect saliva components. Importantly, the *R. opimus* serum reacted strongly with an

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antigenic band of around 28 kDa in the SGL of all sand fly groups collected in this study. Further studies are needed to confirm the immunogenicity of this protein in a larger sample of *R. opimus* to assess its potential as a marker of exposure to *P. papatasi*. This protein may be PPSP32, a protein that is highly recognized by humans bitten by *P. papatasi* (Marzouki et al. 2012).

Conclusion

In our study, some faint protein bands in the SGL profile reacted strongly with the serum of *R. opimus*. Conversely, some major protein bands in the SGL profile reacted weakly in the Western blot analysis. This should be considered in saliva-based vaccine development.

This study shows that certain biological and environmental characteristics of wild populations of vector sand flies affect the protein content and antigenicity of saliva. This might have an important implication in the design of vector-based vaccines.

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References

Akhavan AA, Mirhendi H, Khamesipour A, Alimohammadian MH, Rassi Y, Bates P, Kamhawi S, Valenzuela JG, Arandian MH, Abdoli H, Jalali-zand N, Jafari R, Shareghi N, Ghanei M, Yaghoobi-Ershadi MR (2010a) *Leishmania* species: detection and identification by nested PCR assay from skin samples of rodent reservoirs. Exp Parasitol. 126: 552–556.

Akhavan AA, Yaghoobi-Ershadi MR, Khamesipour A, Mirhendi H, Alimohammadian MH, Rassi Y, Arandian MH, Jafari R, Abdoli H, Shareghi N, Ghanei M, Jalali-zand N (2010b) Dynamics of *Leishmania* infection rates in *Rhombomys opimus* (Rodentia: Gerbillinae) population of an endemic focus of zoonotic cutaneous leishmaniasis in Iran. Bull Soc Pathol Exot. 103: 84–89.

Akhavan AA (2011) Immune response of great gerbil against *Phlebotomus papatasi* saliva: Immune response of *Rhombomys opimus* against *Phlebotomus papatasi* saliva and its role in *Leishmania major* infection. Lambert Academic Publishing, Germany.

Akhavan AA, Ghods R, Jeddi-Tehrani M, Yaghoobi-Ershadi MR, Khamesipour A, Mahmoudi AR (2011) Production and purification of anti-*Rhombomys opimus* immunoglobulins. Iran J Arthropod Borne Dis. 5: 69–76.

Belkaid Y, Kamhawi S, Modi G, Valenzuela JG, Noben-Trauth N, Rowton E, Ribeiro JM, Sacks DL (1998) Development of a natural model of cutaneous leishmaniasis: powerful effects of vector saliva and saliva pre-exposure on the long-term outcome of *Leishmania major* infection in the mouse ear dermis. J Exp Med. 188: 1941–1953.

http://jad.tums.ac.ir
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Belkaid Y, Valenzuela JG, Kamhawi S, Rowton E, Sacks DL, Ribeiro JM (2000) Delayed-type hypersensitivity to *Phlebotomus papatasi* sand fly bite: an adaptive response induced by the fly? Proc Natl Acad Sci USA. 97: 6704–6709.

Ben Hadj Ahmed S, Chelbi I, Kaabi B, Cherni S, Derbali M, Zhioua E (2010) Differences in the salivary effects of wild-caught versus colonized *Phlebotomus papatasi* (Diptera: Psychodidae) on the development of zoonotic cutaneous leishmaniasis in BALB/c mice. J Med Entomol. 47: 74–79.

Cerna P, Mikes L, Volf P (2002) Salivary gland hyaluronidase in various species of phlebotomine sand flies (Diptera: psychodidae). Insect Biochem Mol Biol. 32: 1691–1697.

Coutinho-Abreu IV, Mukbel R, Hanafi HA, Fawaz EY, El-Hossary SS, Wadsworth M, Stayback G, Pitts DA, Abo- Shehada M, Hoel DF, Kamhawi S, Ramalho-Ortigao M, McDowell MA (2011) Expression plasticity of *Phlebotomus papatasi* salivary gland genes in distinct ecotopes through the sand fly season. BMC Ecol. 11: 24.

Gomes R, Oliveira F, Teixeira C, Meneses C, Gilmore DC, Elnaiem DE, Kamhawi S, Valenzuela JG (2012) Immunity to sand fly salivary protein LJM11 modulates host response to vector-transmitted *leishmania* conferring ulcer-free protection. J Invest Dermatol. 132: 2735–2743.

Hamarsheh O, Presber W, Yaghoobi-Ershadi MR, Amro A, Al-Jawabreh A, Sawalha S (2009) Population structure and geographical subdivision of the *Leishmania major* vector *Phlebotomus papatasi* as revealed by microsatellite variation. Med Vet Entomol. 23: 69–77.

Heukeshoven J, Dernick R (1985) Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. Electrophoresis. 6: 103–112.

Kamhawi S, Belkaid Y, Modi G, Rowton E, Sacks DL (2000) Protection against cutaneous leishmaniasis resulting from bites of uninfected sand flies. Science. 290: 1351–1354.

Kamhawi S (2006) Phlebotomine sand flies and *Leishmania* parasites: friends or foes? Trends Parasitol. 22: 439–445.

Killick-Kendrick M, Killick-Kendrick R (1991) The initial establishment of sand fly colonies. Parasitologia. 33: 315–320.

Marzouki S, Abdeladhim M, Ben Abdessalem C, Oliveira F, Ferjani B, Gilmore D, Louzir H, Valenzuela JG, Ben Ahmed M (2012) Salivary antigen SP32 is the immunodominant target of the antibody response to *Phlebotomus papatasi* bites in humans. PLoS Negl Trop Dis. 6(11): e1911.

Modi GB, Tesh RB (1983) A simple technique for mass rearing *Lutzomyia longipalpis* and *Phlebotomus papatasi* (Diptera, Psychodidae) in the laboratory. J Med Entomol. 20: 568–569.

Oliveira F, Lawyer PG, Kamhawi S, Valenzuela JG (2008) Immunity to distinct sand fly salivary proteins primes the anti-*Leishmania* immune response towards protection or exacerbation of disease. PLoS Negl Trop Dis. 2(4): e226.

Oliveira F, de Carvalho AM, de Oliveira CI (2013) Sand fly saliva-*Leishmania*-man: the trigger trio. Front Immunol. 4: 375.

Ribeiro JM (1987) Role of saliva in blood-feeding by arthropods. Annu Rev Entomol. 32: 463–478.

Rohousova I, Ozensoy S, Ozbel Y, Volf P (2005) Detection of species-specific antibody response of human and mice bitten by sand flies. Parasitol. 130: 493–499.

Seyedi-Rashti MA, Nadim A (1992) The genus *Phlebotomus* (Diptera: Psychodidae: Phlebotominae) of the countries of the

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Eastern Mediterranean Region. Iran J Public Health. 21: 11–50.

Valenzuela JG, Belkaid Y, Garfiela MK, Mendez S, Kamhawi S, Rowton E, Sacks DL, Ribeiro JM (2001) Toward a defined anti-*Leishmania* vaccine targeting vector antigens: characterization of a protective salivary protein. J Exp Med. 194: 331–342.

Volf P, Tesarova P, Nohynkova E (2000) Salivary proteins and glycoproteins in sand flies of various species, sex, and age. Med Vet Entomol. 14: 251–256.

Volf P, Rohousova I (2001) Species-specific antigens in salivary glands of phlebotomine sand flies. Parasitol. 1222: 37–41.

Yaghoobi-Ershadi MR, Javadian E, Tahvildare-Bidruni GH (1995) *Leishmania major* MON-26 isolated from naturally infected *Phlebotomus papatasi* (Diptera: Psychodidae) in Isfahan Province, Iran. Acta Trop. 59: 279–282.

Yaghoobi-Ershadi MR, Javadian E (1996) Epidemiological study of reservoir hosts in an endemic area of zoonotic cutaneous leishmaniasis in Iran. Bull World Health Organ. 74: 587–590.

Yaghoobi-Ershadi MR, Javadian E (1997) Studies on sandflies in a hyperendemic area of zoonotic cutaneous leishmaniasis in Iran. Indian J Med Res. 105: 61–66.

Yaghoobi-Ershadi MR, Akhavan AA (1999) Entomological survey of sandflies (Diptera: Psychodidae) in a new focus of zoonotic cutaneous leishmaniasis in Iran. Acta Trop. 73: 321–326.

Yaghoobi-Ershadi MR, Hanafi-Bojd AA, Akhavan AA, Zahrai-Ramazani AR, Mohebali M (2001) Epidemiological study in a new focus of cutaneous leishmaniasis due to *Leishmania major* in Ardestan town, central Iran. Acta Trop. 79: 115–121.

Yaghoobi-Ershadi MR, Akhavan AA, Zahraei-Ramazani AR, Abai MR, Ebrahimi B, Vafaei-Nezhad R, Hanafi-Bojd AA, Jafari R (2003) Epidemiological study in a new focus of cutaneous leishmaniasis in the Islamic Republic of Iran. East Mediterr Health J. 9: 816–826.

Yaghoobi-Ershadi MR, Hakimiparizi M, Zahraei-Ramazani AR, Abdoli H, Akhavan AA, Aghasi M, Arandian MH, Ranjbar AA (2010) Sand fly surveillance within an emerging epidemic focus of cutaneous leishmaniasis in southeastern Iran. Iran J Arthropod Borne Dis. 4: 17–23.

Yaghoobi-Ershadi MR (2012) Phlebotomine sand flies (Diptera: Psychodidae) in Iran and their Role on *Leishmania* transmission. J Arthropod Borne Dis. 6: 1–17.

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