Synthetic peptides as a novel approach for detecting antibodies against sand fly saliva

Michal Sima1*, Blanka Ferencova1, Tapan Bhattacharyya2, Michael A. Miles2, Sergey V. Litvinov3, Asrat Hailu4, Gad Baneth5, Petr Volf1

1 Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic, 2 Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, United Kingdom, 3 Aptum Biologics Ltd, Southampton, United Kingdom, 4 Department of Microbiology, Immunology & Parasitology, Faculty of Medicine, Addis Ababa University, Addis Ababa, Ethiopia, 5 School of Veterinary Medicine, The Hebrew University of Jerusalem, Rehovot, Israel

* michal-sima@seznam.cz

Abstract

Background

Hosts repeatedly bitten by sand flies develop antibodies against sand fly saliva and screening of these immunoglobulins can be employed to estimate the risk of Leishmania transmission, to indicate the feeding preferences of sand flies, or to evaluate the effectiveness of vector control campaigns. Previously, antibodies to sand fly saliva were detected using whole salivary gland homogenate (SGH) or recombinant proteins, both of which also have their disadvantages. This is the first study on sand flies where short peptides designed based on salivary antigens were successfully utilized for antibody screening.

Methodology/Principal findings

Specific IgG was studied in hosts naturally exposed to Phlebotomus orientalis, the main vector of Leishmania donovani in East Africa. Four peptides were designed by the commercial program EpiQuest-B, based on the sequences of the two most promising salivary antigens, yellow-related protein and ParSP25-like protein. Short amino acid peptides were synthesised and modified for ELISA experiments. Specific anti-P. orientalis IgG was detected in sera of dogs, goats, and sheep from Ethiopia. The peptide OR24 P2 was shown to be suitable for antibody screening; it correlated positively with SGH and its specificity and sensitivity were comparable or even better than that of previously published recombinant proteins.

Conclusions/Significance

OR24 P2, the peptide based on salivary antigen of P. orientalis, was shown to be a valuable tool for antibody screening of domestic animals naturally exposed to P. orientalis. We suggest the application of this promising methodology using species-specific short peptides to other sand fly-host combinations.
Previously, two types of antigens were used for detection of antibodies to sand flies: 1) salivary gland homogenate (SGH), which requires maintaining a sand fly colony and laborious work to obtain a sufficient amount of antigen or 2) recombinant proteins with the need to use cell expression and a complicated purification procedure. In contrast, synthetic peptides have never been studied for sand flies despite it being easier to produce them in sufficient quantities and purity. In this study, we screened specific antibodies to sand flies in domestic animals using synthetic peptides based on the two most antigenic salivary proteins of *Phlebotomus orientalis*. This sand fly is the main East African vector of *Leishmania donovani*, causative agent of visceral leishmaniasis, and we detected specific anti-*P. orientalis* IgG in naturally exposed dogs, goats, and sheep from Ethiopia. We showed that, in dogs and goats, the peptide named OR24 P2 is more suitable for antibody detection then the recombinant proteins. Therefore, we recommend this peptide to replace SGH in larger epidemiological studies for evaluation of the effectiveness of vector control programmes or to estimate the risk of *Leishmania* transmission.

**Introduction**

The specific IgG antibody response against salivary proteins is induced in repeatedly exposed hosts after being bitten by the female sand fly (reviewed by Ribeiro and Francischetti [1] and Lestinova et al. [2]). In sand flies this antibody response is species-specific [3,4] and correlates with the biting intensity [5–8]. IgG values decrease after the hosts are protected against sand flies [9], therefore the detection of antibodies can be used for testing the efficacy of vector control campaigns [10,11].

Antibody detection with the whole salivary gland homogenate (SGH) as antigen is impractical in large epidemiological studies due to the possibility of crossreactivity with other insects [9], variability of saliva composition during sand fly aging [12,13], and the workload required to obtain sufficient quantity of the antigen. In the past decade, sand fly SGH was replaced by several antigenic recombinant proteins, expressed in bacterial or mammalian cells, and with various degrees of success (reviewed by Lestinova et al. [2]). In humans, successful detection of anti-sand fly IgG with recombinant proteins was described by Teixeira et al. [14] and by Souza et al. [15] for *Lutzomyia longipalpis* and by Marzouki et al. [16,17] and Mondragon-Shem et al. [18] for *Phlebotomus papatasi*. In domestic animals, using recombinant antigens, antibodies against sand fly saliva were detected in sera of dogs bitten by *L. longipalpis* or *P. perniciosus* [14,19,20] and in sera of dogs, sheep, and goats exposed to *P. orientalis* [21]. In wild animals these studies were performed with rabbits and hares bitten by *P. perniciosus* [22] and with foxes exposed to *L. longipalpis* [14].

However, production of recombinant proteins requires cell expression and a complicated purification procedure. Therefore, we focused on linear B-cell epitopes (synthetic peptides, representing short amino acid sections of the antigenic proteins), which can be produced in large amounts with high purity. This approach was previously applied to mosquitoes as well as to tsetse flies. In *Anopheles gambiae* the peptide designed based on the salivary protein gSG6 was validated in many field studies [23–26] and promising results were also achieved with peptide based on the salivary protein of *Aedes aegypti* and human serum samples [27]. In tsetse flies, peptides originating from saliva of *Glossina palpalis gambiensis* and *G. morsitans* specifically bound anti-tsetse fly antibodies in human and cattle sera, respectively [28–30].

In this study we applied, for the first time, this novel approach to sand flies and used short peptides to detect specific IgG response in domestic animals (dogs, goats, and sheep) naturally exposed to *P. orientalis*. Our main aim was to compare the peptides with previously described
recombinant proteins [21] and to assess whether this methodology is also applicable to large scale surveillance.

Materials and methods

Ethical statement

BALB/c mice were maintained and handled in the animal facility of Charles University in accordance with institutional guidelines and the Czech legislation (Act No. 246/1992 coll. on Protection of Animals against Cruelty in present statutes at large), which complies with all relevant European Union and international guidelines for experimental animals. The experiments were approved by the Committee on the Ethics of Animal Experiments of the Charles University ( Permit Number: MSMT-10270/2015-6) and were performed under the Certificate of Competency (Registration Number: CZ 02457) in accordance with the Examination Order approved by Central Commission for Animal Welfare of the Czech Republic. Sera of domestic animals were collected within the study by Rohousova et al. [31]. Their collection was approved by the Ethiopian National Research Ethics Review Committee (NRERC) under approval no. 3.10/3398/04.

Host sera

Sera of domestic animals naturally exposed to *P. orientalis* in Ethiopia were obtained during the previous study by Rohousova et al. [31] and include 40 sheep, 94 goats, and 30 dogs. Sera from 10 sheep, 15 goats, and 10 dogs from non-exposed animals originating from the Czech Republic served as negative controls. More details of all the samples are provided by Rohousova et al. [31]. Twenty laboratory Balb/c mice were divided into four groups of five animals. Three groups were exposed at least ten-times to about 150 insectary-bred sand fly females (at two-week intervals) of either *P. orientalis*, *P. papatasi*, or *Sergentomyia schwetzi*; the fourth group was used as the non-exposed control.

Sand flies and salivary gland dissection

The *Phlebotomus orientalis* colony originating from Ethiopia (for more details see Seblova et al. [32]) was reared under standard conditions as described by Volf and Volfova [33]. Salivary glands were dissected from 4–6 day old female sand flies in 20mM Tris buffer with 150mM NaCl and stored at -20°C. Before use, salivary glands were disrupted by freeze-thawing three times in liquid nitrogen.

Peptide design and preparation

Peptides were designed from amino acid sequences based on the two most suitable recombinant proteins of *P. orientalis* (rPorSP24 and rPorSP65) as previously described [21]. Two peptides from each protein were selected in the software EpiQuest-B (Aptum Biologics Ltd., www.epiquest.co.uk). In EpiQuest-B, immunodominant parts of protein sequences were distinguished and their antigenicity indices were calculated based on three algorithms—the peptide immunogenicity, the probability of antibody-accessibility (exposure on the protein surface), and the uniqueness of protein sequence. The probability of peptide-antibody binding increases with the antigenicity index.

These four generated sequences (Table 1) were sent to a commercial laboratory (Genosphere Biotechnologies, France), where they were synthesised and conjugated with two molecules of polyethylene glycol, which acts as a spacer on ELISA plates and facilitates improved accessibility of antibodies. After the spacer, one molecule of biotin was added, which enabled
avidin-biotin peptide binding to ELISA plates coated with diluted avidin. Peptides were diluted in sterile PBS at a concentration 1 mg/ml and stored in -80°C.

**ELISAs**

ELISA Clear Flat-Bottom Plates (3855: Thermo Fisher Scientific, USA) were coated with avidin (A9275: Sigma-Aldrich, UK) at a concentration 5 μg/well, diluted in 20mM carbonate-bicarbonate buffer (pH 9.5) and incubated overnight at 4˚C. Plates were washed three times with PBS-Tw (0.05% Tween 20), blocked with 6% blocking medium diluted in PBS (see S1 Table) for 2 hours at 37˚C and then washed twice. Peptides diluted in 2% blocking medium in PBS-Tw were added to the wells at a concentration 5 μg/well and the plates incubated for one hour at 37˚C. After washing three times, sera diluted in 2% blocking medium in PBS-Tw were incubated on the plates for one hour at 37˚C. Plates were washed five times and secondary antibodies diluted in PBS-Tw were added and incubated for one hour at 37˚C. Finally, plates were washed six times, the reaction developed with phosphate-citrate buffer (pH 5.5) in the dark for six minutes at room temperature and stopped with 10% sulfuric acid. The optical density was measured at 492 nm using the Infinite M200 microplate reader (Tecan, Switzerland). At each step, 100 μl of each solution per well was used and all serum samples were tested in duplicate.

When the salivary gland homogenate (SGH) was used as antigen, ELISA plates were coated with 0.2 gland/well [21]. The step with peptide incubation was replaced by incubation with 2% blocking medium in PBS-Tw and the rest of the protocol remained the same. Blocking media, sera and conjugate dilutions for individual host species are indicated in S1 Table.

**Statistical analysis**

The non-parametric Spearman test was used to assess correlations between total anti-SGH and anti-peptide IgG levels using GraphPad Prism version 6 (GraphPad Software, Inc., San Diego, CA, USA). For evaluation of the possible crossreactivity with other sand fly species the non-parametric Wilcoxon Rank-Sum test in GraphPad Prism version 6 was used. Statistical significance was considered when the p-value was < 0.05. Cut-off values were calculated from the mean optical density of control sera plus 3 standard deviations. The optical density values of anti-SGH antibodies were used as the gold standard to validate peptides in ELISA tests using positive (PPV) and negative predictive values (NPV), sensitivity, and specificity.

**Results**

**Peptide design**

For designing the peptide sequences, two of the most antigenic proteins previously tested in recombinant form were used: rPorSP24 (yellow-related protein) and rPorSP65 (ParSP25-like protein). The antigenicity was calculated for both protein sequences in EpiQuest-B and two

| Table 1. Peptide description. |
|-----------------------------|
| **Peptide** | **Peptide sequence** | **Original protein ACCN** |
| OR24 P1  | GVGQVEYKGDQKY PKGC | AGT96461 [34] |
| OR24 P2  | GDSRQICWNQRLPHGC | AGT96461 [34] |
| OR65 P1  | GTPSTANQYDHYNQIGC | AGT96466 [34] |
| OR65 P2  | DRGDHNTDHEEDYDSC | AGT96466 [34] |

Peptide names, sequences and accession numbers of the original proteins are indicated, with references.

https://doi.org/10.1371/journal.pntd.0007078.t001
peptides with the higher antigenicity indices (Fig 1) were chosen from each protein: OR24 P1, OR24 P2, OR65 P1 and OR65 P2.

Crossreactivity with sympatric sand fly species

First, the synthetic peptides were tested by ELISA for possible crossreactivity with antibodies against salivary antigens of sympatric sand fly species (*P. papatasi* and *Sergentomyia schwetzi*) using sera of experimentally bitten Balb/c mice. Five mice were exposed to single sand fly species–either *P. orientalis*, *P. papatasi*, or *S. schwetzi*, and five mice served as non-exposed controls. Significant differences in OD values were detected with sera of mice bitten by *P. orientalis* compared to all the other three groups, as shown in Fig 2. No differences were observed in non-exposed controls and mice exposed to *P. papatasi* or *S. schwetzi* (Fig 2).

ELISA experiments

The four aforementioned peptides were used as antigens in ELISA experiments to detect the specific anti-*P. orientalis* SGH antibodies from three animal species–dogs, goats, and sheep. Their antigenicities were compared with the whole SGH, and the statistical values calculated were cut-off, positivity, correlation coefficient, PPV, NPV, specificity, and sensitivity (Table 2). OR24 P1 showed the closest cut-off value to SGH with canine sera, as well as high correlation coefficient (> 0.75), PPV, specificity (> 0.65) and very high NPV and sensitivity (> 0.9).

With the goat sera, the correlation (0.6) was lower as were other statistical values (all between 0.6–0.7). With sheep sera, all statistical values were very high (> 0.9) except for the low sensitivity (0.25).

Peptide OR24 P2 had a high correlation coefficient with the SGH for dog and goat sera. It reached comparable PPV, NPV, specificity, and sensitivity as OR24 P1 for dogs and the highest
Correlation and PPV (<0.45) were low for sheep sera. OR65 P1 showed similar statistical values as the previous two peptides for dogs but the specificity was slightly lower (0.5). With goat sera, there was a very high cut-off value and a low correlation coefficient (0.55). The second highest correlation (0.7) was achieved with this peptide and with sheep sera but the PPV and sensitivity (<0.35) were low.

Despite high statistical values for OR65 P2 and canine sera (0.8), high cut-off and the lowest correlation coefficient (0.65) were observed with this host species. In contrast, OR65 P2 was the second best antigen for goats, with the lowest cut-off value, high correlation, PPV, NPV, specificity, and sensitivity (all above 0.65). Although high PPV, NPV, and specificity (>0.9) were detected with sheep sera, this peptide achieved low correlation and NPV (<0.6).

The correlation analysis for the most promising peptide (OR24 P2) with dogs and goats is shown in Fig 3.

**Discussion**

Previously, anti-sand fly IgG was detected by using SGH or recombinant proteins prepared based on the most antigenic proteins (reviewed by Lestinova et al. [2]). In this study, we focused on the novel approach of detecting IgG with short amino acid chain synthetic peptides. These peptides can be synthesised in large amounts with very high purity and without the need for cell expression of recombinants. We designed four peptides, two from each of the most promising *P. orientalis* salivary proteins: yellow-related protein (PorMSP24, ACCN: AGT96461) and ParSP25-like protein (PorMSP65, ACCN: AGT96466), and tested them with sera of domestic animals naturally exposed to *Phlebotomus orientalis*.
| HOST         | SGH      | OR24 P1 | OR24 P2 | OR65 P1 | OR65 P2 |
|--------------|----------|---------|---------|---------|---------|
| Dogs (n = 30 + 10) |          |         |         |         |         |
| Cut-off      | 0.417    | 0.481   | 0.587   | 0.607   | 0.883   |
| Positivity (%)| 50.0     | 46.7    | 46.7    | 46.7    | 40.0    |
| Correlation  | N.A.     | 0.764 ***| 0.770 ***| 0.774 ***| 0.661 ***|
| PPV          | 0.737    | 0.737   | 0.667   | 0.889   | 0.800   |
| NPV          | 0.909    | 0.909   | 0.533   | 0.800   |         |
| Specificity  | 0.667    | 0.667   | 0.933   | 0.800   |         |
| Sensitivity  | 0.933    | 0.933   | 0.933   | 0.800   |         |
| Goats (n = 94 + 15) |        |         |         |         |         |
| Cut-off      | 0.499    | 0.546   | 0.656   | 1.111   | 0.532   |
| Positivity (%)| 45.7     | 29.8    | 36.2    | 24.5    | 34.0    |
| Correlation  | N.A.     | 0.631 ***| 0.784 ***| 0.550 ***| 0.662 ***|
| PPV          | 0.636    | 0.773   | 0.697   | 0.681   |         |
| NPV          | 0.700    | 0.820   | 0.672   | 0.766   |         |
| Specificity  | 0.686    | 0.804   | 0.804   | 0.706   |         |
| Sensitivity  | 0.651    | 0.791   | 0.535   | 0.744   |         |
| Sheep (n = 40 + 10) |        |         |         |         |         |
| Cut-off      | 0.208    | 0.354   | 0.300   | 0.312   | 0.318   |
| Positivity (%)| 10.0     | 2.5     | 7.5     | 2.5     | 5.0     |
| Correlation  | N.A.     | 0.793 ***| 0.426 ***| 0.742 ***| 0.571 ***|
| PPV          | 1.000    | 0.333   | 0.333   | 1.000   |         |
| NPV          | 0.923    | 0.968   | 0.919   | 0.947   |         |
| Specificity  | 1.000    | 0.833   | 0.944   | 1.000   |         |
| Sensitivity  | 0.250    | 0.750   | 0.250   | 0.500   |         |

Spearman-Rank Correlation Matrix test for optical densities between sera tested against *P. orientalis* SGH and against each peptide, where the SGH was used as a golden standard. This table provides host species (with numbers of Ethiopian animals + non-exposed controls), cut-off values, positivity—the percentage of samples with OD values above cut-off, correlation coefficients, PPV, NPV, specificity, and sensitivity for four peptides and *P. orientalis* SGH. Asterisks (*** *) indicate significant correlation *p* < 0.001 and N.A. means not applicable.

https://doi.org/10.1371/journal.pntd.0007078.t002

![Fig 3. Correlation analyses between anti-*P. orientalis* IgG detected by SGH and by peptide OR24 P2 in ELISAs.](https://doi.org/10.1371/journal.pntd.0007078.g003)

For dogs (n = 40) and goats (n = 109) the peptide with the highest statistical values is displayed. Correlation coefficients and *p*-values from Spearman-Rank analysis are indicated.
Phlebotomus orientalis is the main East African vector of Leishmania donovani—the causative agent of visceral leishmaniasis [35]. Previous studies revealed that salivary antigens of P. orientalis belong to several protein families, specifically yellow-related proteins, odorant-binding proteins, apyrases, antigen 5-related proteins and ParSP25-like proteins [34]. Recombinant yellow-related proteins and ParSP25-like proteins were used to replace P. orientalis SGH for antibody screening of domestic animals in Ethiopia [21].

Application of recombinant yellow-related proteins of Lutzomyia longipalpis was also described for canine, fox, and human sera [14,15], and of P. perniciosus for hare, rabbit, and canine sera [19,20,22]. However, so far, the recombinant ParSP25-like protein has not been used for other sand fly species except P. orientalis [21].

Peptides based on salivary antigens have not previously been used for studies on sand flies but they have been applied to detection of specific antibodies in hosts bitten by mosquitoes or tsetse flies. For mosquitoes, the peptides were first used for Anopheles gambiae, to study specific IgG responses among humans living in different foci of Plasmodium falciparum transmission [23,25,36], to correlate IgG levels with the risk of malaria transmission [26], and to monitor the effect of vector control campaigns [24]. Ndille et al. [27] used salivary peptide of Aedes aegypti to describe a positive correlation between specific IgG responses in humans with rainfall and mosquito seasonality. In tsetse flies, differences in anti-salivary peptide IgG titers were observed between two human populations with diverse abundance of Glossina palpalis gambiensis [28], and before and after vector control [29]. Soma et al. [30] suggested that the peptide based on salivary protein of G. morsitans was not suitable for IgG screening of domestic animals in areas with high tsetse fly abundance, because it was only recognized by sera of cattle with low exposure. Our study is the first, for sand flies, in which antigenicity and IgG detection are compared for peptides, whole SGH and recombinant proteins.

The peptides designed for P. orientalis are species-specific; no crossreactivity was observed with sera of mice exposed to the sympatric sand fly species P. papatasi and Sergentomyia schwetzi or with sera of non-exposed controls; there was similar high species specificity for the SGH and recombinant proteins of P. orientalis [21].

Previous work on dogs with recombinant proteins showed that the best protein-SGH performing recombinant (ParSP25-like protein) had very low specificity. This implied high probability of false positivity among non-exposed animals. Higher specificity was achieved with recombinant yellow-related protein [21]. Comparable correlation with recombinant yellow-related protein was detected with three peptides—OR24 P1, OR24 P2, and OR65 P1. The first two of these peptides also showed much higher specificity (0.7) than both of the recombinants.

With goats, low correlation was observed with both recombinant proteins [21]. In contrast, peptide OR24 P2 based on the sequence of yellow-related protein reached high correlation with SGH (0.8) as well as values > 0.75 for PPV, NPV, specificity, and sensitivity.

Results with sheep sera were difficult to interpret due to the very low positivity with SGH (10%). Similar positivity was found with all four peptides: even a relatively small change in the number of false positives or false negatives would significantly change calculation of PPV, NPV, specificity, or sensitivity. Although the correlation with two peptides (OR24 P1 and OR65 P1) was above 0.7, we therefore do not recommend these peptides for screening sheep sera. However, promising results for sheep sera have previously been achieved with both recombinant proteins [21].

In summary, we tested four short amino acid sequence peptides, designed based on two most antigenic P. orientalis salivary proteins, for detection of antibodies to sand fly saliva, in three species of domestic animals from Ethiopia. One of the peptides, OR24 P2, showed promising results with sera of dogs and goats. We therefore suggest that this peptide may replace SGH or recombinant proteins in surveillance for anti-P. orientalis IgG. As it was shown,
synthetic peptides might work only for some host species. For future detection of human anti-
odies to sand fly saliva, we recommend comparison of the efficacy of recombinant proteins
and synthetic peptides.

Supporting information

S1 Table. Dilutions in ELISA experiments. Host species, used blocking media and conju-
gates, sera and conjugate dilutions are indicated in this table.

(DOCX)

Acknowledgments

We are grateful to Aysheshm Kassahun, Carla Maia, and Jan Votypka for participating in field
sample collection. We also especially thank Professor Alon Warburg for his support. We
would like to thank Andrew Falconar for valuable advices on peptide sequences. We appreciate
the excellent technical and administrative support from Helena Kulikova and Jana Glogarova.
Sera of control domestic animals (non-exposed to sand flies) were generously provided by
Prof. David Modry (Faculty of Veterinary Medicine, University of Veterinary and Pharmaceut-
tical Sciences Brno, Czech Republic) and Dr. Kamil Sedlak (State Veterinary Institute Prague,
Czech Republic).

Author Contributions

Conceptualization: Michal Sima, Petr Volf.
Data curation: Michal Sima.
Formal analysis: Michal Sima.
Funding acquisition: Petr Volf.
Investigation: Michal Sima, Blanka Ferencova.
Methodology: Michal Sima, Blanka Ferencova, Tapan Bhattacharyya.
Resources: Asrat Hailu, Gad Baneth, Petr Volf.
Software: Michal Sima, Sergey V. Litvinov.
Supervision: Petr Volf.
Visualization: Michal Sima.
Writing – original draft: Michal Sima.
Writing – review & editing: Michal Sima, Michael A. Miles, Asrat Hailu, Gad Baneth, Petr
Volf.

References

1. Ribeiro JMC, Francischetti IMB. Role of arthropod saliva in blood feeding: sialome and post-sialo-
me perspectives. Annu Rev Entomol. 2003; 48: 73–88. https://doi.org/10.1146/annurev.en-
to.48.060402.102812 PMID: 12194906
2. Lestinova T, Rohousova I, Sima M, de Oliveira CI, Volf P. Insights into the sand fly saliva: Blood-feeding
and immune interactions between sand flies, hosts, and Leishmania. PLoS Negl Trop Dis. 2017; 11:
e0005600. https://doi.org/10.1371/journal.pntd.0005600 PMID: 28704370
3. Volf P, Rohousova I. Species-specific antigens in salivary glands of phlebotomine sandflies. Parasitol-
ey. 2001; 122: 37–41. PMID: 11197762
4. Rohousova I, Ozensoy S, Ozbel Y, Volf P. Detection of species-specific antibody response of humans and mice bitten by sand flies. Parasitology. 2005; 130: 493–499. PMID: 15991492

5. Hostomska J, Rohousova I, Volfova V, Stanneck D, Mencke N, Volf P. Kinetics of canine antibody response to saliva of the sand fly *Lutzomyia longipalpis*. Vector Borne Zoonotic Dis. 2008; 8: 443–450. https://doi.org/10.1089/vbz.2007.0214 PMID: 18260789

6. Vikova M, Rohousova I, Hostomska J, Pohankova L, Zidkova L, Drahota J, et al. Kinetics of antibody response in BALB/c and C57BL/6 mice bitten by *Phlebotomus papatasi*. PLoS Negl Trop Dis. 2012; 6: e1719. https://doi.org/10.1371/journal.pntd.0001719 PMID: 22802977

7. Drahota J, Martín-Martín I, Sumova P, Rohousova I, Jimenez M, Molina R, et al. Recombinant antigens from *Phlebotomus perniciosus* saliva as markers of canine exposure to visceral leishmaniasis vector. PLoS Negl Trop Dis. 2014; 8: e2597. https://doi.org/10.1371/journal.pntd.0002597 PMID: 24392167

8. Martín-Martín I, Molina R, Jimenez M. Kinetics of anti-*Phlebotomus perniciosus* saliva antibodies in experimentally bitten mice and rabbits. PLoS One. 2015; 10: e0140722. https://doi.org/10.1371/journal.pone.0140722 PMID: 26569103

9. Clements MF, Gidwani K, Kumar R, Hostomska J, Dinesh DS, Kumar V, et al. Measurement of recent exposure to *Phlebotomus argentipes*, the vector of Indian visceral leishmaniasis, by using human antibody responses to sand fly saliva. Am J Trop Med Hyg. 2010; 82: 801–807. https://doi.org/10.4269/ajtmh.2010.09-0336 PMID: 20439958

10. Dinesh DS, Das P, Picado A, Davies C, Speybroeck N, Ostyn B, et al. Long-lasting insecticidal nets fail at household level to reduce abundance of sandfly vector *Phlebotomus argentipes* in treated houses in Bihar (India). Trop Med Int Health. 2008; 13: 953–958. https://doi.org/10.1111/j.1365-3156.2008.02096.x PMID: 18482197

11. Gidwani K, Picado A, Rijal S, Singh SP, Roy L, Volfova V, et al. Serological markers of sand fly exposure to evaluate insecticidal nets against visceral leishmaniasis in India and Nepal: A cluster-randomized trial. PLoS Negl Trop Dis. 2011; 5: e1296. https://doi.org/10.1371/journal.pntd.0001296

12. Volf P, Tesarova P, Nohynkova E. Salivary proteins and glycoproteins in phlebotomine sandflies of various species, sex and age. Med Vet Entomol. 2000; 14: 251–256. PMID: 11016431

13. Prates ADB, Santos LD, Miranda JC, Souza APA, Barral A. Changes in amounts of total salivary gland proteins of *Lutzomyia longipalpis* (Diptera: Psychodidae) according to age and diet. J Med Entomol. 2008; 45: 409–413. PMID: 18533433

14. Teixeira C, Gomes R, Collin N, Reynoso D, Jochem R, Oliveira F, et al. Discovery of markers of exposure specific to bites of *Lutzomyia longipalpis*, the vector of *Leishmania infantum* chagasi in Latin America. PLoS Negl Trop Dis. 2010; 4: e638. https://doi.org/10.1371/journal.pntd.0000638 PMID: 20351786

15. Souza AP, Andrade BB, Aquino D, Entringer P, Miranda JC, Alcântara R, et al. Using recombinant proteins from *Lutzomyia longipalpis* saliva to estimate human vector exposure in visceral leishmaniasis endemic areas. PLoS Negl Trop Dis. 2010; 4: e649. https://doi.org/10.1371/journal.pntd.0000649 PMID: 20351785

16. Marzouki S, Abdeladhim M, Abdessalem CB, Oliveira F, Ferjani B, Gilmore D, et al. Salivary antigen SP32 is the immunodominant target of the antibody response to *Phlebotomus papatasi* bites in humans. PLoS Negl Trop Dis. 2012; 6: e1911. https://doi.org/10.1371/journal.pntd.0001911 PMID: 23209854

17. Marzouki S, Kammoun-Rebai W, Bettaieb J, Abdeladhim M, Hadj Kacem S, Abdelkader R, et al. Validation of recombinant salivary protein PsPSP32 as a suitable marker of human exposure to *Phlebotomus papatasi*, the vector of *Leishmania major* in Tunisia. PLoS Negl Trop Dis. 2015; 9: e0003991. https://doi.org/10.1371/journal.pntd.0003991 PMID: 26368935

18. Mondragon-Shem K, Al-Salem WS, Kelly-Hope L, Abdeladhim M, Al-Zahrani M, Valenzuela JG, et al. Severity of Old World cutaneous leishmaniasis is influenced by previous exposure to sandfly bites in Saudi Arabia. PLoS Negl Trop Dis. 2015; 9: e3448.

19. Kostalova T, Lestinova T, Sumova P, Vikova M, Rohousova I, Berriatua E, et al. Canine antibodies against salivary recombinant proteins of *Phlebotomus perniciosus*: A longitudinal study in an endemic focus of canine leishmaniasis. PLoS Negl Trop Dis. 2015; 9: e0003855. https://doi.org/10.1371/journal.pntd.0003855 PMID: 26111018

20. Kostalova T, Lestinova T, Maia C, Sumova P, Vikova M, Willen L, et al. The recombinant protein rSP03B is a valid antigen for screening dog exposure to *Phlebotomus perniciosus* across foci of canine leishmaniasis. Med Vet Entomol. 2017; 31: 88–93. https://doi.org/10.1111/mve.12192 PMID: 27718267

21. Sima M, Ferencova B, Warburg A, Rohousova I, Volf P. Recombinant salivary proteins of *Phlebotomus orientalis* are suitable antigens to measure exposure of domestic animals to sand fly bites. PLoS Negl Trop Dis. 2016; 10: e0004553. https://doi.org/10.1371/journal.pntd.0004553 PMID: 26986566

22. Martín-Martín I, Molina R, Rohousova I, Drahota J, Volf P, Jiménez M. High levels of anti-*Phlebotomus perniciosus* saliva antibodies in different vertebrate hosts from the re-emerging leishmanioses focus in

PLOS Neglected Tropical Diseases | https://doi.org/10.1371/journal.pntd.0007078 January 24, 2019 10 / 11
23. Poinsignon A, Cornelie S, Ba F, Boulanger D, Sow C, Rossignol M, et al. Human IgG response to a salivary peptide, gSG6-P1, as a new immuno-epidemiological tool for evaluating low-level exposure to Anopheles bites. Malar J. 2009; 8: 198. https://doi.org/10.1186/1475-2875-8-198 PMID: 19674487

24. Drame P M, Poinsignon A, Besnard P, Cornelie S, Le Mire J, Simondon F, et al. Human antibody responses to the Anopheles salivary gSG6-P1 peptide: A novel tool for evaluating the efficacy of ITNs in malaria vector control. PLoS One. 2010; 5: e15596. https://doi.org/10.1371/journal.pone.0015596 PMID: 21197476

25. Sagna AB, Sarr JB, Gaayeb L, Drame PM, Ndiath MO, Senghor S, et al. gSG6-P1 salivary biomarker discriminates micro-geographical heterogeneity of human exposure to Anopheles bites in low and seasonal malaria areas. Parasit Vectors. 2013; 6: 68. https://doi.org/10.1186/1756-3305-6-68 PMID: 23497646

26. Londono-Renteria B, Drame PM, Weitzel T, Rosas R, Gripping C, Cardenas JC, et al. An. gambiae gSG6-P1 evaluation as a proxy for human-vector contact in the Americas: a pilot study. Parasit Vectors. 2015; 8: 533. https://doi.org/10.1186/s13071-015-1160-3 PMID: 24086785

27. Ndille EE, Doucouré S, Damien G, Drame M, Cornelie S, Noukpo H, et al. First attempt to validate human IgG antibody response to Nterm-34kDa salivary peptide as biomarker for evaluating exposure to Aedes aegypti bites. PLoS Negl Trop Dis. 2012; 6: e1905. https://doi.org/10.1371/journal.pntd.0001905 PMID: 23166852

28. Dama E, Cornelie S, Camara M, Somda MB, Poinsignon A, Ilboudo H, et al. In silico identification of a candidate synthetic peptide (Tsgf1_18-43) to monitor human exposure to tsetse flies in West Africa. PLoS Negl Trop Dis. 2013; 7: e2455. https://doi.org/10.1371/journal.pntd.0002455 PMID: 24086785

29. Courtin F, Camara M, Rayaisb JB, Kabagoudou M, Dama E, Camara O, et al. Reducing human-tsetse contact significantly enhances the efficacy of sleeping sickness active screening campaigns: A promising result in the context of elimination. PLoS Negl Trop Dis. 2015; 9: e0003727. https://doi.org/10.1371/journal.pntd.0003727 PMID: 26267667

30. Somda MB, Cornelie S, Bengaly Z, Mathieu-Daudé F, Poinsignon A, Dama E, et al. Identification of a Tafl1_52-75 salivary synthetic peptide to monitor cattle exposure to tsetse flies. Parasit Vectors. 2016; 9: 149. https://doi.org/10.1186/s13071-016-1414-8 PMID: 26979518

31. Rohousova I, Talmi-Frank D, Kostalova T, Polanska N, Lestinova T, Kassahun A, et al. Exposure to Leishmania spp. and sand flies in domestic animals in northwestern Ethiopia. Parasit Vectors. 2015; 8: 380. https://doi.org/10.1186/s13071-015-0975-1 PMID: 26152578

32. Seblova V, Volkova V, Dvorak V, Pruzinova K, Votycka J, Kassahun A, et al. Phlebotomus orientalis sand flies from two geographically distant Ethiopian localities: biology, genetic analyses and susceptibility to Leishmania donovani. PLoS Negl Trop Dis. 2013; 7: e2187. https://doi.org/10.1371/journal.pntd.0002187 PMID: 23638207

33. Volf P, Volkova V. Establishment and maintenance of sand fly colonies. J Vector Ecol. 2011; 36: S1–S9. https://doi.org/10.1111/j.1948-7134.2011.00106.x PMID: 21366760

34. Vikova M, Sima M, Rohousova I, Kostalova T, Sumova P, Volkova, et al. Comparative analysis of salivary gland transcriptomes of Phlebotomus orientalis sand flies from endemic and non-endemic foci of visceral leishmaniasis. PLoS Negl Trop Dis. 2014; 8: e2709. https://doi.org/10.1371/journal.pntd.0002709 PMID: 24587463

35. Elnaiem DE. Ecology and control of the sand fly vectors of Leishmania donovani in East Africa, with special emphasis on Phlebotomus orientalis. J Vector Ecol. 2011; 36: S23–S31. https://doi.org/10.1111/j.1948-7134.2011.00109.x PMID: 21366778

36. Poinsignon A, Cornelie S, Lanfrancotti A. Novel peptide marker corresponding to salivary protein gSG6 potentially identifies exposure to Anopheles bites. PLoS One. 2008; 3: e2472. https://doi.org/10.1371/journal.pone.0002472 PMID: 18575604