



Biomarkers for exposure to sand flies bites as tools to aid control of leishmaniasis

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Intense research efforts so far have not been sufficient to reduce leishmaniasis burden worldwide. This disease is transmitted by bites of infected sand flies, which inject saliva in the host skin in an attempt to obtain a blood meal. Sand fly saliva has an array of proteins with diverse pharmacological properties that modulates the host homeostatic and immune responses. Some of these proteins are also immunogenic and can induce both cellular and humoral immune responses. Recently, the use of sand fly salivary proteins to estimate exposure to sand fly bites and consequently the risk of infection has emerged. Here, we review evidence that supports the use of the host immune responses against sand fly salivary proteins to estimate risk of infection. We also discuss how the use of recombinant salivary proteins can optimize serological surveys and provide guidance for the implementation of specific measures for disease control in endemic areas.

Keywords: leishmaniasis, sand fly, biomarkers, salivary proteins, vectors

Leishmaniasis is caused by infection with *Leishmania* parasites transmitted by bites of infected sand flies. The infection can result in a wide range of clinical manifestations varying from self-healing localized skin lesions to lethal visceral disease, and major determinants of the clinical outcome rely on the parasite strain and the host immune response (Murray et al., 2005). Despite ample basic and applied research, there is no effective vaccine to prevent leishmaniasis. As a consequence, the prophylactic strategies proposed by public health authorities are restricted mainly to vector control and consistent screening and elimination of potential reservoirs. In this scenario, understanding the nuances of the host–vector–parasite interactions becomes critical for the development of more reliable tools to adequately control leishmaniasis.

A critical event in *Leishmania* transmission is the sand fly bite. Female sand flies require hematophagy for nutrition, egg development, and survival. During blood feeding, sand fly saliva containing a number of pharmacologically active molecules with diverse effects on the host's hemostatic responses is delivered into the host skin (Andrade et al., 2005). There is strong evidence that components of the sand fly saliva play a major role driving both susceptibility to *Leishmania* infection and disease severity (Titus and Ribeiro, 1988; Belkaid et al., 1998; Morris et al., 2001; de Moura et al., 2007). Indeed, sand fly saliva can exacerbate lesions in experimental models of cutaneous leishmaniasis (CL) and this effect is considered, at least in part, a consequence of its immunomodulatory properties. Salivary proteins are also immunogenic and can elicit specific immune responses that can be detrimental for *Leishmania* establishment (Kamhawi, 2000; Valenzuela et al., 2001; Thiakaki et al., 2005; de Moura et al., 2007; Gomes et al., 2008; Oliveira et al., 2008; Collin et al., 2009; Rohousova et al., 2011; Tavares et al., 2011; Xu et al., 2011).

The idea of using antibodies against saliva from bloodsucking arthropods as markers of exposure has been proposed for different arthropod vectors. There is extensive work showing that humans and other vertebrates can develop antibodies against salivary components of different bloodsucking vectors like mosquitoes (Brummer-Korvenkontio et al., 1994; Palosuo et al., 1997; Remoue et al., 2006; Orlandi-Pradines et al., 2007; Andrade et al., 2009), ticks (Schwartz et al., 1990, 1991; Sanders et al., 1998), and triatomines (Volf et al., 1993; Nascimento et al., 2001; Schwarz et al., 2009, 2010, 2011). Specific antibodies and their dynamics in vertebrate hosts were described also against fleas and louse (Volf, 1991). As expected, exposure to sand fly bites or saliva also induces antibody production in humans and animal models (Barral et al., 2000; Volf and Rohousova, 2001; Gomes et al., 2002, 2007, 2008; Rohousova et al., 2005; Silva et al., 2005; Clements et al., 2010; Souza et al., 2010; Teixeira et al., 2010; Vlkova et al., 2011). In mice, the antibody isotype most induced by sand fly saliva is IgG1 (Silva et al., 2005) whereas IgG1, IgG2, IgG4, and IgE are more frequent in humans (Vinhas et al., 2007; Marzouki et al., 2011). Specific IgG1 and IgG2 isotypes are also highly induced in dogs bitten by sand flies (Hostomska et al., 2008; Vlkova et al., 2011). Thus, these antibody isotypes are obvious candidates for use as targets in serological surveys in endemic areas. Interestingly, two major patterns of clinical and serological responses to sand fly saliva are identified in human volunteers repeatedly exposed to *Lutzomyia longipalpis*, the vector of visceral leishmaniasis (VL) in South America (Vinhas et al., 2007). Individuals who develop delayed-type hypersensitivity (DTH)-like skin reactions after exposure usually display higher IgG/IgE ratios than those evolving mild erythematous reactions (Vinhas et al., 2007; **Figure 1**). These observations argue that the host response to sand

fly saliva may present some degree of divergence resulting from genetic variations that could influence susceptibility to *Leishmania* establishment. Furthermore, characterization of the humoral response against saliva can provide evidence regarding susceptibility to *Leishmania* infection in humans. In this case, characterizing the antibody profile of an exposed individual may be useful in predicting susceptibility to disease. Whether these immunological aspects are reproducible in individuals exposed to other species of sand flies, including those that transmit the cutaneous form of the disease is still unknown.

Several field studies in highly endemic areas for leishmaniasis indicate that natural exposure to non-infected sand fly bites can impact the epidemiology of this disease (Barral et al., 2000; Gomes et al., 2002; Rohousova et al., 2005; de Moura et al., 2007; Aquino et al., 2010; Marzouki et al., 2011; Vlkova et al., 2011). In these regions, residents are frequently bitten by both non-infected and infected sand flies. It is hypothesized that these frequently exposed individuals usually present mild and chronic disease manifestations while travelers from non-endemic areas, not previously exposed to sand flies or *Leishmania*, present a higher risk of developing severe clinical forms of leishmaniasis. The idea is that frequent exposure to sand fly bites leads to the production of neutralizing antibodies against salivary proteins and also to activation of cellular mechanisms that may have an adverse effect on *Leishmania* establishment. In this perspective, characterization of immune responses against sand fly saliva can help estimate both risk of infection and to some degree anti-parasite immunity. Although this hypothesis has been proven in animal models, additional large-scale clinical studies are necessary to validate it in humans. Individuals living in leishmaniasis endemic areas that are exposed to bites from uninfected sand flies over a long term still get infected and develop disease. Interestingly, protection against leishmaniasis in mice has been recently shown to be limited to short-term exposure to sand flies immediately before infection (Rhousova et al., 2011). This finding may explain the persistence of leishmaniasis in endemic areas, as individuals might experience periods without exposure to sand fly bites. Moreover, other factors besides exposure to sand fly bites might also be driving susceptibility to infection and disease severity in human leishmaniasis and the interaction between these determinants are still unknown. The characterization of the host's immune responses against sand fly saliva can lead to the identification of key salivary proteins with at least two remarkable potentials: (i) its use as surrogates for exposure to sand fly bites and the risk of infection and (ii) the identification of candidate vaccines against leishmaniasis. In fact, estimation of exposure to sand fly bites to infer risk of infection has emerged as an important tool to help drive strategic interventions to reduce disease transmission in endemic areas. Here, we review the current advances in the use of sand fly saliva as a helpful tool to estimate exposure to vector bites and risk for leishmaniasis. We also summarize the development of reliable tools to assess vector-exposure in endemic areas.

ASSOCIATION BETWEEN EXPOSURE TO SAND FLY SALIVA AND VISCERAL LEISHMANIASIS

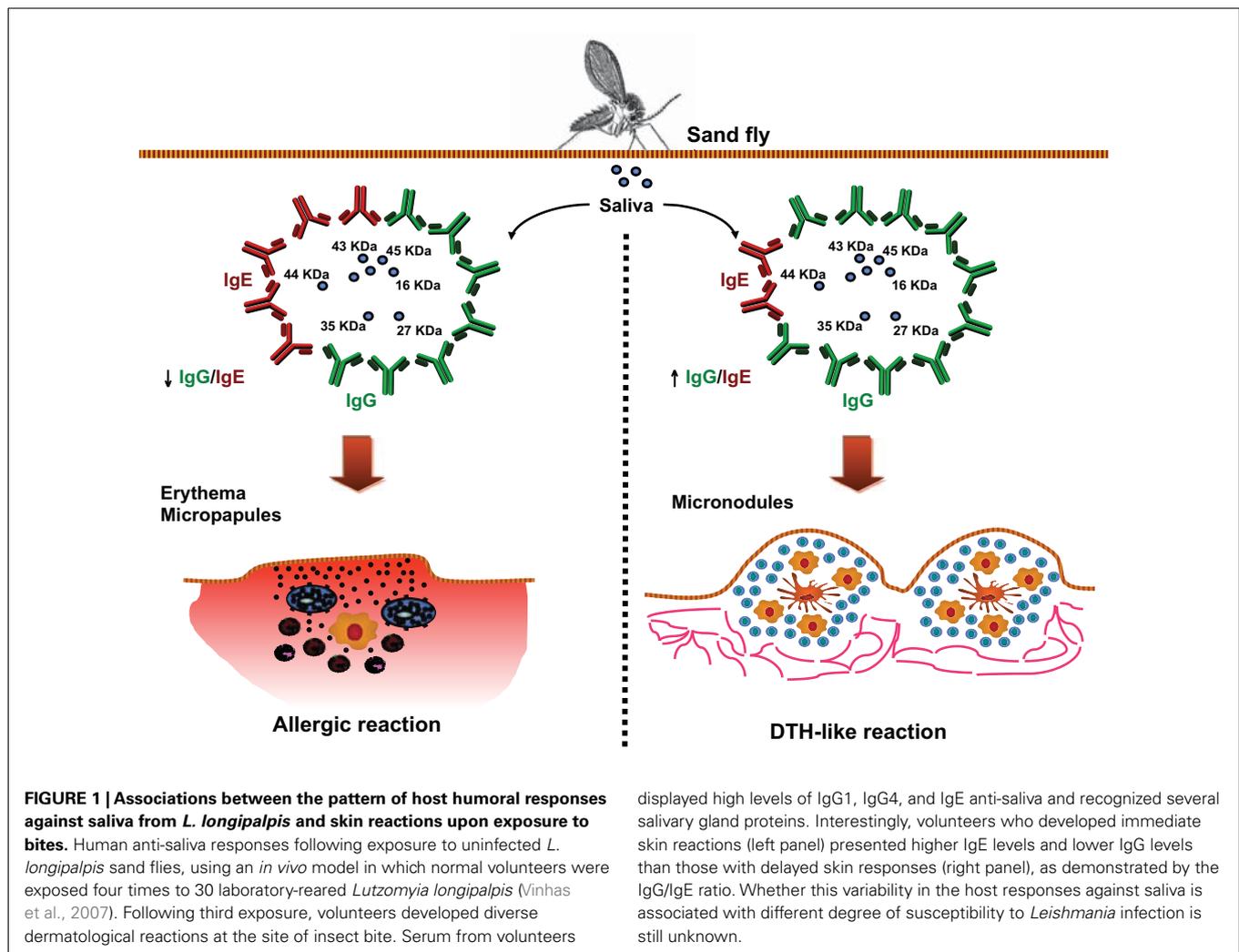
Visceral leishmaniasis in Latin America is transmitted by the bite of *L. longipalpis* sand flies infected with *Leishmania infantum chagasi*.

One of the first reports showing the immunogenicity of sand fly saliva in exposed individuals was carried out in a highly endemic VL area in Brazil, where inhabitants differentially recognize salivary gland proteins (Barral et al., 2000). Interestingly, a positive correlation was observed between recognition of salivary proteins and anti-*Leishmania* DTH reaction to parasite antigens and protection, but not to anti-*Leishmania* serologic status. Furthermore, children who experienced anti-*Leishmania* DTH conversion had an increase in anti-*L. longipalpis* saliva antibodies, suggesting that development of anti-parasite DTH coincides and is directly related to the development of anti-saliva antibodies (Gomes et al., 2002). More recently, in the same endemic area, the rate of a positive anti-*Leishmania* DTH was higher in individuals that had anti-*L. longipalpis* antibodies at the baseline of a 2-year follow-up study (Aquino et al., 2010). On the other hand, these results could simply suggest that DTH is also dependent on parasite challenges which was not specifically measured in either studies. Other factors that might contribute to the susceptibility to infection in endemic areas such as vector density and reservoir infection rates still need to be addressed.

The presence of antibodies to sand fly salivary proteins was also demonstrated in canids (dogs and foxes), important reservoirs that serve as a source of parasites to sand flies in endemic regions. In dogs experimentally exposed to *L. longipalpis*, the induced anti-saliva antibodies can recognize up to six different sand fly salivary proteins (Hostomska et al., 2008). Comparable results were also found in naturally exposed animals captured in a highly endemic area of VL in Brazil. Anti-*L. longipalpis* saliva antibodies were detected in naturally infected dogs and foxes, suggesting that these animals were in close contact with the vector (Bahia et al., 2007; Gomes et al., 2007). In a follow-up study of dogs experimentally exposed to *Phlebotomus perniciosus*, the main vector of canine VL in Europe, a positive correlation between the production of specific antibodies against vector saliva and the number of blood fed sand flies was shown. Notably, dogs naturally exposed to bites during the sand fly season that tested positive for *Leishmania* had decreased systemic levels of anti-*P. perniciosus* saliva IgG2 antibodies compared to healthy dogs (Vlkova et al., 2011). Such studies suggest that the detection of anti-saliva antibodies can be valuable to evaluate vector control programs. Indeed, in a recent study in VL areas in India and Nepal, the efficacy of insecticide treated nets (ITN) was evaluated in terms of vector-exposure by measuring anti-*P. argentipes* saliva antibody concentrations in the inhabitants before and after the intervention. This approach showed that exposure to *P. argentipes* was reduced by up to 12% in people living in villages where nets were used compared to controls (Clements et al., 2010; Gidwani et al., 2011). In summary, both observational and interventional field studies in VL areas strongly suggest that the production of antibodies to specific sand fly salivary proteins could be directly associated to correlates of protection.

ASSOCIATION BETWEEN EXPOSURE TO SAND FLY SALIVA AND CUTANEOUS LEISHMANIASIS

Cutaneous leishmaniasis is transmitted by the bite of a number of sand flies species with diverse geographic distributions. There is also evidence suggesting that individuals that live in endemic areas



for CL also develop specific antibodies to saliva of sand fly species that transmit the disease.

In an endemic area for *Leishmania tropica* in Turkey, the antibody response against saliva of *P. sergenti* (the vector of *L. tropica*) and *P. papatasi* (the vector of *L. major* elsewhere) was compared between healthy individuals and patients with active CL lesions (Rohousova et al., 2005). It was observed that approximately 40% of the study population carried IgG antibodies to the saliva of both sand fly species. Interestingly, individuals with active lesions had higher anti-*P. sergenti* IgG levels compared to healthy individuals, strongly suggesting that production of anti-saliva antibodies could be used as a marker for risk of developing CL (Rohousova et al., 2005). In a recent study performed in Tunisia, sera from children living in a CL endemic area of *L. major* showed a consistent production of anti-*P. papatasi* saliva antibodies after two transmission seasons. Anti-saliva IgG antibodies were significantly higher and correlated with lesion development, arguing that anti-saliva antibodies were strongly associated with the disease development (Marzouki et al., 2011). A similar finding was described for *Lutzomyia intermedia*, an important vector of CL in South America (de Moura et al., 2007). Residents from an

endemic area in Brazil presenting active CL displayed higher titers of antibodies against *L. intermedia* saliva compared to healthy individuals from the same area, indicating that in CL a humoral immune response to *L. intermedia* saliva is a marker of risk of disease (de Moura et al., 2007). Similarly to the studies performed in VL areas, these conclusions need to take into account other important determinants such as percentage of infected vectors, the biting rate and the presence of potential reservoirs in the area that could also influence the outcome of the host's immune response.

These observations indicate that the detection of specific humoral immune response against salivary proteins from different species of sand flies should be carefully evaluated. Altogether, these findings indicate that exposure to saliva of distinct species of sand flies can be correlated with two different outcomes: susceptibility to disease or protection. In VL, the findings were observed in cross-sectional and longitudinal prospective studies showing that the development of both types of immunity occurs simultaneously (Barral et al., 2000; Gomes et al., 2002; Aquino et al., 2010). In CL, however, the development of anti-saliva antibodies and susceptibility to disease are closely related and recent work

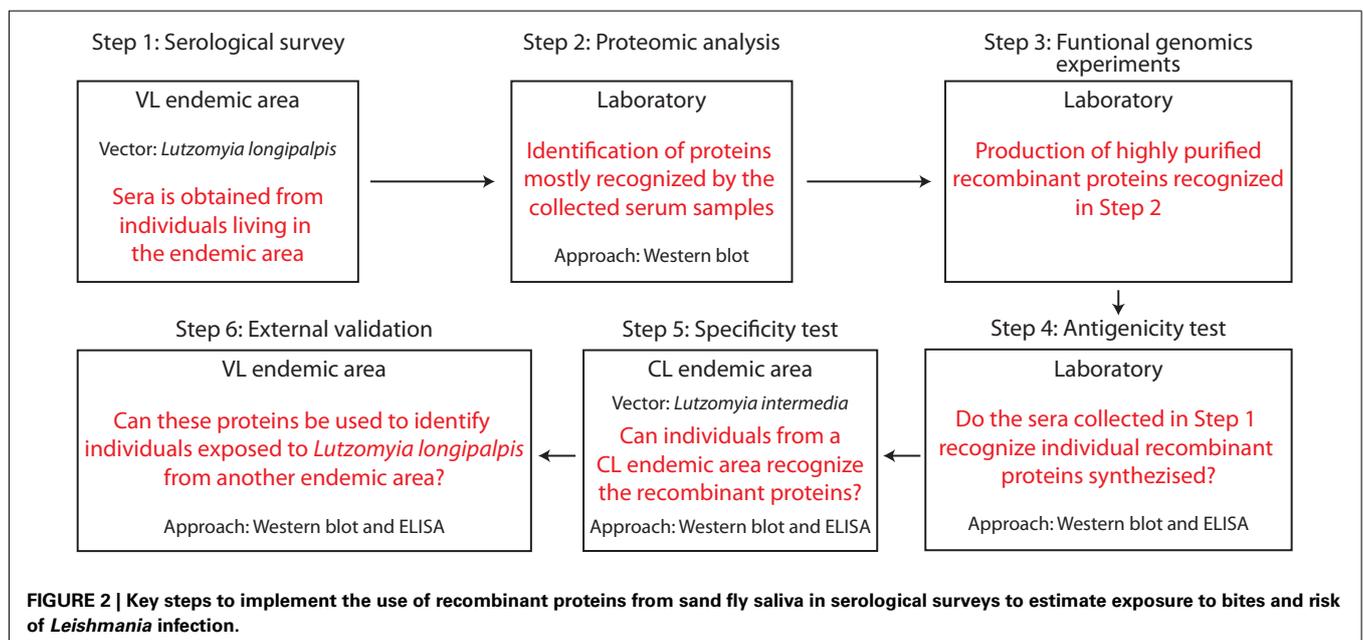
shows an association with a Th2 phenotype (Rohousova et al., 2005; de Moura et al., 2007; Marzouki et al., 2011). Although production of antibodies against vector saliva suggests an increased chance of encountering an infected bite and developing disease, constant exposure to non-infected bites in endemic areas could possibly modulate the host's immune response to affect a successful settlement of the parasite. Whereas production of antibodies against sand fly saliva can be used as biomarker of disease susceptibility more studies are required to identify salivary proteins that can affect the development of anti-*Leishmania* immunity.

TOOLS TO ESTIMATE EXPOSURE TO SAND FLY BITES

The work done so far with detection of anti-sand fly saliva antibodies have shown enough evidence to prove the relevance of using salivary proteins as tools to address the level of exposure to vector bites bringing a new perspective to the field. Although the use of antibody response to sand fly saliva is promising, an approach in which a large number of samples from different vertebrate hosts could be quickly and specifically tested is highly desired. The majority of work evaluating saliva of sand flies as potential biomarkers of vector-exposure used whole salivary extracts. While whole salivary extract has advantages as it represents the complete repertoire of crude secreted salivary proteins, it also has limitations that could restrict their application. Technical limitations to the use of large quantities of whole salivary extracts in population surveys include restrictions on the number of sand flies available for salivary gland dissection, homogeneity of the salivary content, and conservation of the proteins without degradation. These limitations impact the size and extent of field studies. Additionally, the use of total saliva reduces the specificity of detection due to a higher likelihood of cross-reactivity with saliva from other sympatric non-vector sand fly species. For instance, salivary antigens do not cross-react between *L. longipalpis* and *Phlebotomus* species

but some cross-reactions were demonstrated between diverse *Phlebotomus* species in both mice (Volf and Rohousova, 2001) and humans (Rohousova et al., 2005). Hence, the identification of specific and highly immunogenic salivary proteins that can also be produced in large quantities as quality-controlled recombinant proteins is of utmost importance for larger epidemiological studies.

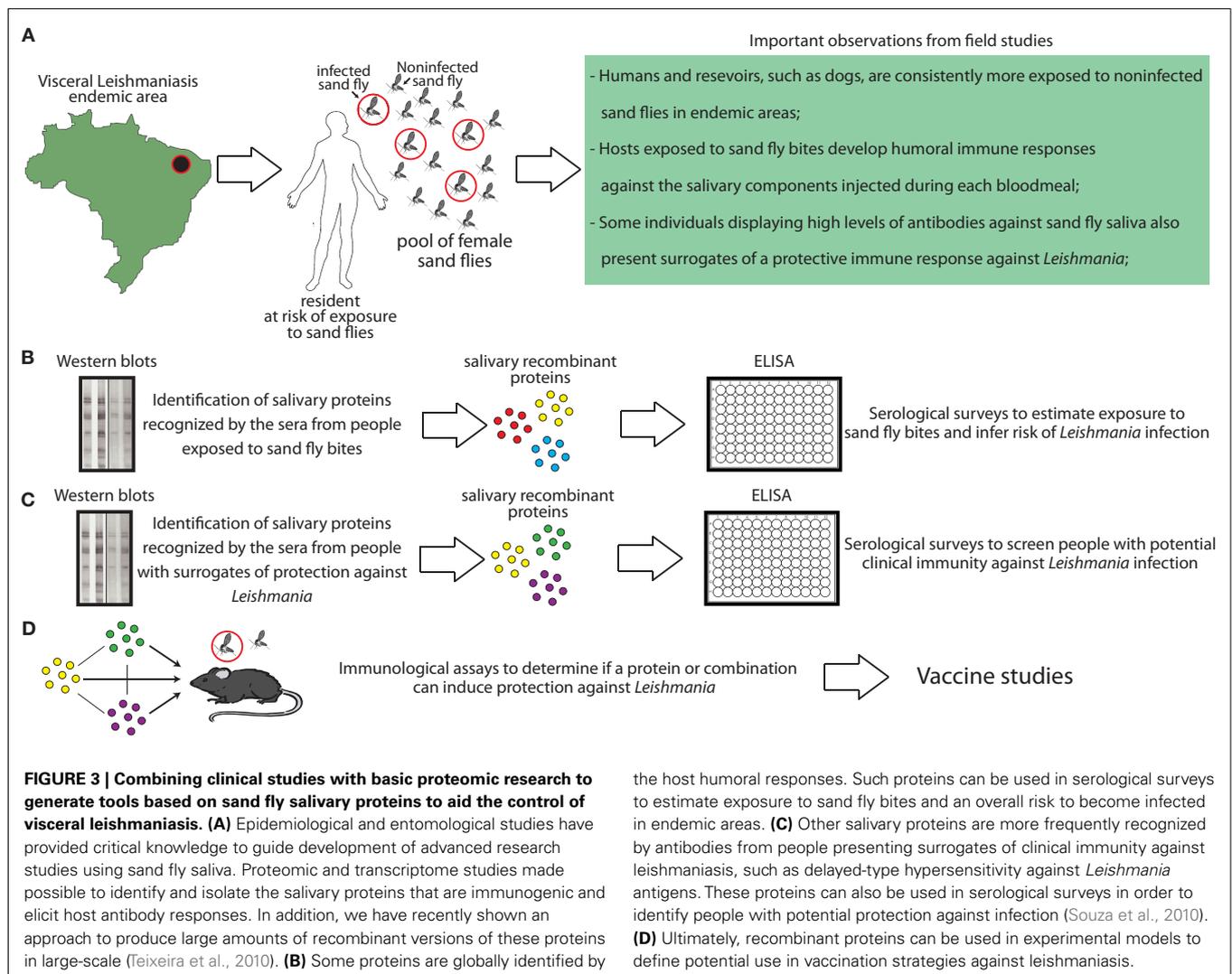
The first attempt to identify, produce, and test salivary recombinant proteins for serological surveys was recently demonstrated for *L. longipalpis* sand flies (Souza et al., 2010; Teixeira et al., 2010). These work describe a practical and functional approach for identification of salivary proteins most recognized by sera from humans and canids residing in endemic areas for VL in Brazil and, more importantly, how to obtain highly pure and immunogenic recombinant proteins. Initially, sera from humans, dogs, and foxes from VL endemic areas were screened against the whole salivary extract from *L. longipalpis*. The most recognized proteins were in the range of 15–65 kDa. Based on a *L. longipalpis* salivary transcriptome, nine transcripts that corresponded to proteins within the predicted molecular weight in the desired range were selected as potential candidates. To characterize and confirm the antigenicity of the candidates, each recombinant salivary protein was produced in a mammalian expression system, purified, and tested individually for their recognition by sera from individuals residing in a VL endemic area. Two of the nine salivary recombinant proteins, LJM11 and LJM17, were highly recognized by humans exposed to *L. longipalpis*, demonstrating their potential as markers for epidemiological studies. To verify their specificity, the two recombinant proteins were further tested against sera from individuals exposed to *L. intermedia*. Both recombinant proteins were only recognized by sera from individuals exposed to *L. longipalpis* but not to *L. intermedia* bites, confirming their potential as specific markers for *L. longipalpis* exposure. Finally, to validate LJM11 and LJM17 as



markers for exposure, they were tested in a large cohort with over a thousand people from a VL area (Souza et al., 2010). Both recombinant proteins (LJM11 and LJM17) were assessed individually and in combination and were able to detect anti-saliva seroconversion but the use of a combination of these two proteins showed a more powerful discrimination equal to the seroconversion observed against whole saliva extract. A summary of the approach used in these two studies is illustrated in **Figure 2**. Notably, this method used for identification of salivary proteins applied in ELISA assays could also be assessed to identify potential markers from the saliva of other arthropod vectors. The use of ELISA has shown to be a practical tool in different studies where whole vector's saliva was used. In several epidemiological studies, this approach has been used to determine the levels of specific anti-vector saliva antibodies including *Ixodes pacificus*, *P. argenteipes*, *Aedes caspius*, and *Aedes albopictus* (Lane et al., 1999; Clements et al., 2010; Fontaine et al., 2011; Gidwani et al., 2011; Vlkova et al., 2011; Doucoure et al., 2012), clearly demonstrating its potential to support control strategies programs for vector-borne diseases.

CONCLUDING REMARKS

Research focused in the interplay between vector saliva and host immune responses has been very successful in the identification of molecules with diverse biological and pharmacological activities and has contributed impressively to the understanding of the immunopathogenesis of an array of vector-borne diseases. Regarding leishmaniasis, different groups have shown that sand fly saliva plays a critical role in the establishment of *Leishmania* infection and have also described host immune responses against salivary proteins in mice, dogs, and humans. Interest in the field is empowered by recent observations arguing that it is possible to track human exposure to vectors using salivary recombinant proteins. The use of recombinant proteins in serological surveys performed in leishmaniasis endemic areas is critical to standardize the quality and reproducibility of results from different studies and also to optimize procedures (**Figure 3**). Unfortunately, although extensive data regarding the quantification of host antibody responses against sand fly saliva has been published, few studies provide validation of their results using large cohorts or multi-center approaches. Moreover, multi-center approaches



are also critical to identify salivary proteins that have conserved expression in different endemic areas, as variation in salivary content among the same species has been described in different geographical regions (Lanzaro et al., 1999). An expanded effort for studying salivary content of species from different parts of the world will certainly increase the chances to find common molecules that could function as markers or as candidates for a wide-ranging vaccine. This approach is critical as specificity to saliva-mediated protection against *Leishmania* infection was demonstrated in the mouse model arguing that immunization with salivary components from one species of sand fly can be ineffective to induce protection against other species (Thiakaki et al., 2005). This finding also suggests that immunity against *Leishmania*

is elicited by unique salivary antigens and not conserved molecules between various species of sand flies. Definitely, coordinated initiatives are necessary to design robust validation studies toward generating standardized guidelines. Ultimately, the use of sand fly salivary proteins in epidemiological studies can provide a fundamental tool to help public health authorities in planning key strategies to reduce the burden of leishmaniasis.

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