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Antigenic and enzymatic properties of sand fly saliva

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PhD Thesis

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Prague, 2008

I declare that Jitka Hostomská substantially contributed to writing of four manuscripts as well as to the experimental work in three projects presented in this thesis, specifically:

- Effect of hyaluronidase on *Leishmania* transmission
- Kinetics of canine antibody response to *Lutzomyia longipalpis* saliva
- Analysis of salivary transcripts and antigens of *P. arabicus*.

6th August 2008, Prague

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1 Introduction

1.1 Sand flies and leishmaniasis

Sand flies (Diptera: Phlebotominae) are tiny insects that can be found in tropical and subtropical regions. Female sand flies require bloodfeeding for the production of eggs. Two sand fly genera, *Lutzomyia* and *Phlebotomus*, transmit protozoan parasites of the genus *Leishmania* and are therefore of considerable importance for public health.

Sand flies may ingest *Leishmania* together with the bloodmeal when feeding on an infected mammal. In the lumen of sand fly gut, *Leishmania* amastigotes transform into procyclic flagellated promastigotes, multiply and differentiate in infective metacyclic forms. During next bloodfeeding, infective metacyclics can be regurgitated from the gut and *Leishmania* are thus transmitted to the mammalian host. Parasites deposited in host dermis are then taken up by phagocytic cells, macrophages being the target cells for intracellular replication. Once in the macrophage, parasites differentiate into amastigotes with retracted flagellum. Multiplying amastigotes emerge from ruptured parasitized cells and are ingested by other macrophages (reviewed by Molyneux & Killick-Kendrick, 1987; Sádlová, 1999; Kamhawi, 2006). Eventually, parasite multiplication in host tissue together with immunopathological processes causes leishmaniasis. The severity of clinical presentation in leishmaniases varies from mild cutaneous affection to visceral disease leading to death if untreated. Currently, leishmaniases are endemic in 88 countries, with estimated 12 million people infected and 2 million new cases considered to occur annually (www.who.int/leishmaniasis).

In my work, I was dealing mainly with two sand fly species, *Lutzomyia longipalpis* and *Phlebotomus arabicus*. *Lutzomyia longipalpis* is a species which shows by far the widest distribution of all New World sand flies, and is the only proven vector of visceral leishmaniasis in Latin America (Killick-Kendrick, 1999; Lainson & Rangel, 2005). *Phlebotomus arabicus* transmits cutaneous leishmaniasis caused by *L. tropica* (Jacobson et al., 2003) and is distributed in certain parts of the Middle East and Africa. In his revision of subgenus *Adlerius*, Artemiev (1980) lists only the type locality, Yemen; later specimens identified as *P. arabicus* were described from Saudi Arabia (Lewis & Büttiker, 1980), Egypt and Ethiopia (Lane, 1986), Jordan (Kamhawi et al., 1995), and Israel (Jacobson et al., 2003). In Ethiopia, sand flies designated by Ashford (1974) as *P. chinensis* conform with *P. arabicus*

description and recently *P. arabicus* females infected with uncharacterized *Leishmania* sp. were reported (Gebre-Michael et al., 2004).

1.2 Immunology of cutaneous leishmaniasis

In this chapter, the initial steps of immune response to *Leishmania* infection are briefly outlined. As noted earlier, infective *Leishmania* promastigotes are transmitted to the mammalian host during bloodfeeding. From the medical entomologist's point of view, the interplay between host immune system, transmitted pathogens and the insect vector-derived factors such as saliva is of special interest. It is obvious that the salivary components cannot remain active in the inoculation site indefinitely. Therefore, the following chapter is focused on the early stages of *Leishmania* infection whereas its later stages are somewhat neglected.

1.2.1 Experimental models of murine cutaneous leishmaniasis

Immune response to cutaneous *Leishmania spp.* has been studied in great detail in mice. Traditionally, experimental leishmaniasis is induced by subcutaneous inoculation of high doses of promastigotes (10^5-10^7) in murine footpad. Not only were studies employing this model very useful in determining the mechanisms of protective immunity to *Leishmania,* but they brought some very interesting results for basic immunological research as well. For example, the model of murine experimental *Leishmania major* infection has frequently been used for functional analyses of Th1 / Th2 responses (Scott et al., 1988). However, in nature, the mammalian host is infected intradermally by far lower number of promastigotes, supposedly 10-1000 (Warburg & Schlein, 1986; Rogers et al., 2004). The only study attempting to quantify *L. major* transmitted by a single sand fly bite in murine skin reported a wide range (10-100 000) in the dose of transmitted parasites, the majority of the flies transmitting <600 parasites (Kimblin et al., 2008). Moreover, apart from the parasites, the natural inoculum contains also sand fly saliva and parasite-derived promastigote secretory gel (PSG), both of which have been shown to promote *Leishmania* infectivity (Titus & Ribeiro, 1988; Rogers et al., 2004). Taken together, the route of infection, infection dose and inoculum composition are different in experimental and natural leishmaniasis. Thus, recent studies focusing on cutaneous leishmaniasis immunology often use low dose inocula ($\leq 10^3$) injected intradermally to mimic natural infection (e.g., Belkaid et al., 2000a; Courret et al., 2003; Lang et al., 2003; Baldwin et al., 2004; Woelbing et al., 2006). Comparison of these different models and analysis of disease outcome in immunodeficient mice reveals that the immune

mechanisms responsible for control of *L. major* infection are the same in the conventional model as well as in the low-dose intradermal infection model. These include interleukin (IL)-12-driven, CD40/CD40L-dependent $CD4^+$ T cell activation, IFN-γ production and nitric oxide (NO)-mediated killing of intracellular parasites (Belkaid et al., 2000a). IFN-γ is needed for proper activation of macrophages and induction of nitric oxide synthase. Its source in *Leishmania*-infected tissue can either be Th1 cells or NK cells. IL-12 produced by activated macrophages or dendritic cells (DC) is a crucial factor for both determining Th1 differentiation of activated $CD4^+$ T cells and the induction of IFN-γ release by NK cells (Scharton-Kersten & Scott, 1995). When interpreting the results from studies employing low-dose intradermal infections, we should, however, be aware that even in these studies the infectious inoculum rarely contains sand fly saliva or PSG. In addition, extrapolation of knowledge based on experimental mouse models to dogs or humans is limited.

1.2.2 Anti-*Leishmania* immune response

After intradermal infection of mice with low dose inoculum, anti-*Leishmania* response ensues, which can be separated into several distinct phases. The following description of these events is mostly based on experiments in genetically resistant strains of mice, e.g. C57BL/6 or C3H. It is beleived that in these strains the course of infection resembles human infections more than in susceptible ones. In humans, similar to resistant murine strains, *L. major* infection most often results in a self-healing lesion, which resolves within months post infection (Griffiths, 1987).

In the first, „silent“ phase of *Leishmania* infection which takes place after parasite entry into host skin, infective promastigotes are phagocytosed by resident macrophages. They persist and replicate inside macrophage phagolysosomes. Multiple mechanisms of resistance to this hostile environment and active impairment of antimicrobial capacity have evolved in *Leishmania* (reviewed by Sacks & Sher, 2002; Gazzinelli & Denkers, 2006). Two different ways of *Leishmania* infection of macrophages were described. Firstly, *Leishmania* promote their own phagocytosis by exploiting complement receptor CR3 on macrophages (Mosser & Edelson, 1985). Generally, CR3 ligation results in downregulation of IL-12 production (Marth & Kelsall, 1997), as accordingly does *L. major* promastigote binding to macrophages (Carrera et al., 1996; Belkaid et al., 1998a). In effect, infection of macrophages with *Leishmania* via CR3 does not lead to cellular activation. Secondly, *Leishmania* promastigotes can quietly arrive at macrophage phagosome by infecting neutrophils first and so avoiding direct contact with macrophage surface receptors (van Zandbergen et al., 2004). Neutrophils serve as

transient host cells for *Leishmania* promastigotes, producing monocyte-attracting chemotactic factors such as macrophage inflammatory protein 1-β (MIP1-β/CCL4) upon infection and undergoing apoptosis while still supporting *Leishmania* survival. Macrophages were shown to take up parasitized apoptotic neutrophils and allow *Leishmania* multiplication (van Zandbergen et al., 2004).

Parasite phagocytosis may also be indirectly supported by factors chemotactic for neutrophils and/or macrophages, thus making more host cells available for *Leishmania* infection at primary site. Neutrophil recruitment might be enhanced by a chemotactic *Leishmania* factor, as seen in *in vitro* migration assays (van Zandbergen et al., 2002) or by skin-resident mast cell-derived tumour necrosis factor-α (TNF-α) (Von Stebut et al., 2003). Resident mast cell activation was observed within hours post *L. major* inoculation (Maurer et al., 2006). Preformed mast cell-derived TNF-α was responsible for recruiting neutrophils and macrophages to inflamed skin site (Von Stebut et al., 2003) and the authors speculate that mast cell degranulation after *Leishmania* infection has similar effects. Silently, without lesion development and inflammatory reaction, the parasites multiply in the inoculation site. Parasite load in inoculation site increases gradually over several weeks and peaks at the end of the first phase of infection (Belkaid et al., 2000a).

The second phase of *L. major* infection is marked by the simultaneous occurence of lesion and the onset of parasite clearance. It was suggested that after a threshold level of released *Leishmania* antigen or infected macrophages is reached, skin cells are activated to produce IL-12 (Belkaid et al., 2000a). The supposed source of IL-12 are epidermal Langerhans cells, a distinct subset of dendritic cells (Belkaid et al., 2000a) or immature Gr-1$^+$ myeloid cells recruited from the bloodstream (dos Santos et al., 2008). Accumulation of neutrophils, macrophages and eosinophils was observed in the dermal site coincident with the occurence of lesion. Dendritic cells as well as CD4$^+$ and CD8$^+$ cells also appear in lesional tissue (Belkaid et al., 2000a). Unlike macrophages, DC only take up amastigote stages of *L. major* and do so via Fcγ receptors. This route of infection leads to cellular activation, upregulation of major histocompatibility complex (MHC) and costimulatory molecule expression, IL-12 production and presentation of *Leishmania* antigen in the context of both MHC I and MHC II molecules (Von Stebut et al., 1998; Belkaid et al., 2002b; Woelbing et al., 2006). CD4+ and CD8+ T-cell priming by antigen-presenting cells leads to IFN-γ production by T cells, as confirmed by analysis of cytokine production in cultured cells from draining lymph nodes of infected animals (Belkaid et al., 2000a; Belkaid et al., 2002b).

Dendritic cells are thus crucial players in the initiation of anti-*Leishmania* protective response; it remains to be elucidated which DC subset is responsible for this phenomenon. While Langerhans cells were long believed to be central in the control of *L. major* infection (Moll et al., 1993), current hot candidates include lymph node-resident DC (Iezzi et al., 2006) and monocyte-derived inflammatory DC (Leon et al., 2007).

Eventually, parasite killing by NO from IFN-γ -activated macrophages follows (Green et al., 1990; Liew et al., 1990), marking the third, resolving phase of infection (Belkaid et al., 2000a). Key molecular targets of nitric oxide in *Leishmania* include enzymes such as glyceraledhyde-3-phosphate dehydrogenase and aconitase which are involved in glycolysis and citric acid cycle, respectively. Damage to the active sites of the enzymes results in lethal metabolic inhibition of the parasite and cell death with morphological features of apoptosis (Lemesre et al., 1997; Holzmuller et al., 2006).

Apart from IFN-γ activation, the activity of inducible nitric oxide synthase (iNOS) in macrophages can also be triggered by toll-like receptor ligands together with other Th1 cytokines such as TNF-α, or Th1-favoured chemokines, including MIP-1α/CCL3, macrophage chemoattractant protein-1 (MCP-1/CCL2), and gamma interferon-inducible protein-10 (IP-10/CXCL10) (reviewed by Wanasen & Soong, 2008). *Leishmania major*-induced inhibition of IP-10/CXCL-10 production by macrophages thus can be interpreted as an additional strategy for the parasite to escape immune defense of the host (van Zandbergen et al., 2002). This is supported by the finding that IP-10/CXCL10 response to *L. major* can be elicited in DC from *L. major*-resistant, but not susceptible mice (Steigerwald & Moll, 2005). Similarly, correlation of MCP-1/CCL2 expression with parasite elimination was documented by preferential expression of MCP-1/CCL2 and IP-10/CXCL10 in draining lymph nodes of *L. major*-resistant mice (Zaph & Scott, 2003) or MCP-1/CCL2-mediated triggering of leishmanicidal activity in human monocytes (Ritter & Moll, 2000). It should be noted, however, that macrophages can also kill *Leishmania* by nitric oxide-independent mechanisms such as reactive oxygen species production which can be triggered by above-mentioned chemokines. (Ritter & Moll, 2000).

Control of the clinical infection, be it self healing or chemotherapy, does not result in sterile cure. On the contrary, the parasites persist even after lesion involution. It was shown that IL-10 promotes parasite persistence, which is vital for the maintenance of memory T cell population (Belkaid et al., 2001). Several distinct cellular populations were reported to produce IL-10 at later stages of *Leishmania* infection. These include macrophages stimulated

with IgG-opsonized amastigotes (Kane & Mosser, 2001; Miles et al., 2005), $CD4^+CD25^+FoxP3^+$ natural regulatory T cells (Belkaid et al., 2002a), and even $CD4^+CD25^-CD103^-Foxp3^-$ T cells (Anderson et al., 2007). Persistence of parasites generally leads to life-long immunity, on the other hand, it is also the cause of reactivation of disease as seen e.g. in visceral leishmaniasis reactivation in HIV patients (Alvar et al., 1997) or in immunocompromised patients with cured *L. major* infections (Vardy et al., 1999). Concerning the epidemiology of leishmaniases, asymptomatic infected humans or animals are important for the perpetuation of the disease. This was documented by transmission experiments with reservoir hosts showing no signs of disease and appropriate sand fly vector species, e.g. *L. tropica*-infected rats and *P. sergenti* sand flies (Svobodova et al., 2003) or *L. infantum*-infected dogs and *P. perniciosus* or *L. longipalpis* sand flies (Molina et al., 1994; Michalsky et al., 2007). In all cases, transmission of *Leishmania* to the vector was accomplished.

1.3 Saliva of bloodsucking insects

Salivary glands of nematoceran Diptera are typically sac-like structures consisting of one or more lobes, deposited in the thorax. They are composed of a single cell layer and surrounded by a basal membrane. Salivary ducts bring saliva to the feeding site with the help of salivary pump, a structure located at the base of the mouthparts. Regulation of salivary gland secretion is hormonal and/or neuronal (Stark & James, 1996). While in ticks it is possible to stimulate salivation by topical application of substances such as pilocarpine, in most other insects this is not feasible. Salivary material can be obtained from biting midges by letting the insects feed through plastic film, or from kissing bugs by mechanically forcing the bug to salivate into a capillary. Therefore, salivary gland homogenates are frequently employed in studies that actually aim to analyse the properties of insect saliva. Another technical limitation might be the small size of some insect species which does not allow the dissection of salivary glands. In these cases, thoracic or even body homogenates are used (e.g., Chen et al., 2005). It should be noted that the composition of saliva may differ with sex, age, and physiological status of the insect (Volf et al., 2000; Prates et al., 2008) and therefore only saliva or salivary glands from insects that are ready for bloodfeeding should be used in experiments.

In non-parasitic arthropods, saliva serves as a medium for dissolving and solubilizing food and provides enzymes that initiate digestion (Stark & James, 1996). This is obviously the case for bloodsucking Diptera as well; some species feed also on sugar, and accordingly, amylases and glucosidases were detected in mosquito and sand fly salivary glands (e.g., James & Rossignol, 1991; Jacobson & Schlein, 2001). In addition, saliva of bloodsucking insects contains a host of pharmacologically active molecules which enable the insect to accomplish the bloodfeeding (reviewed by Andrade et al., 2005).

1.3.1 Fighting host haemostasis

Parasitic insects utilize two different strategies for finding blood: solenophagy (or vessel feeding) and telmophagy (or pool feeding). In vessel feeders, the feeding fascicle cannulates a blood vessel, while in pool feeders the mouth part stylets slash through the skin, and the insect sips blood that oozes out from the haemorrhage. It is conceivable that different feeding strategy might be reflected by differences in saliva composition (Ribeiro et al., 1998).

The most prominent pharmacological features of bloodfeeding insect saliva are shared regardless of the feeding strategy. Most importantly, saliva of all bloodsucking insects impairs host haemostasis (reviewed by Champagne, 2005). In case of injury, mammalian hosts avoid blood loss by vasoconstriction, platelet aggregation, and blood coagulation. Haematophagous insects have devised multiple ways of subversion of all three basic haemostasis mechanisms. Examples of salivary vasodilators include maxadilan from sand flies of the genus *Lutzomyia* (Ribeiro et al., 1989), AMP and adenosine in *Phlebotomus papatasi* (Ribeiro et al., 1999), or sialokinins in *Aedes aegypti* mosquitoes (Champagne & Ribeiro, 1994). Platelet aggregation is avoided by bloodfeeding insects especially by antagonising the effects of ADP on platelets. Salivary apyrases cleaving ADP are widespread in bloodfeeding insects, although two different and unrelated groups of enzymes were detected. Thus, apyrases from mosquitoes and kissing bugs are not homologous to those from sand flies and bed bugs (Champagne, 2005). Finally, although tissue injury inflicted by insect mouthparts is rather small and platelet aggregation would seem to be sufficient to fight possible blood loss, salivary inhibitors of coagulation cascade were detected, e.g. in mosquitoes (Valenzuela et al., 1999), biting midges (de Leon et al., 1998), black flies (Abebe et al., 1994), horse flies (Kazimirova et al., 2001) or body lice (Mumcuoglu et al., 1996).

Other activities detected in saliva of bloodfeeding insects are not directly related to inhibition of haemostasis. As mentioned earlier, enzymes cleaving carbohydrates are present in saliva. Lysosyme and defensin activities found in mosquito salivary glands are supposed to

protect the anterior portion of the mosquito digestive tract against bacterial infection (Moreira-Ferro et al., 1998; Calvo et al., 2006; Dixit et al., 2008). Another notable example is hyaluronidase activity, which was reported in salivary gland extracts of black flies (Ribeiro et al., 2000), sand flies (Ribeiro et al., 2000; Cerna et al., 2002) and horse flies (Xu et al., 2008). Hyaluronidase activity of sand fly saliva has been studied in our laboratory and is one of the topics covered by this thesis. Therefore, general properties of hyaluronidases and their possible function in bloodfeeding insect saliva are discussed in more detail in the following section (1.3.2). Another separate section (1.3.3) is dealing with immunomodulatory properties of saliva of haematophagous insects, focusing mainly on of sand fly saliva.

1.3.2 Hyaluronidases and hyaluronan

Hyaluronidases are a family of enzymes that degrade hyaluronan (HA) and, at a slower rate, chondroitin and chondroitin sulfate. Three groups of hyaluronidases were described with respect to their mechanism of action. Insect hyaluronidases are related to vertebrate enzymes and belong to the same class (EC 3.2.1.35). Hyaluronan is a non-sulfated unbranched glycosaminoglycan consisting of repeating disaccharide units of [-D-glucuronic acid-β1,3-N-acetyl-D-glucosamine-β1,4-]$_n$ that can reach 10^7 in size. The β1,4 glycosidic bond is cleaved by vertebrate type of hyaluronidases. Hyaluronan is a prevalent component of extracellular and pericellular matrix in vertebrates and is ubiquitously expressed by various cell types in most animal tissues. Soft connective tissues are especially abundant in hyaluronan, e.g. in rats, skin contains about one half of total body hyaluronan (reviewed by Laurent & Fraser, 1992; Stern & Jedrzejas, 2006).

In insects, hyaluronidases are well-known from venoms of Hymenoptera and represent clinically important allergens of honey-bees, wasps and hornets (King et al., 1996; Markovic-Housley et al., 2000; Bilo et al., 2005). In Diptera, salivary hyaluronidases were detected in several bloodsucking species as noted in chapter 1.3.1. In addition, sequences homologous to hyaluronidase were found in cDNA libraries of salivary glands of various bloodsucking insects (Charlab et al., 1999; Ribeiro et al., 2004; Campbell et al., 2005; Xu et al., 2008).

The effects of salivary hyaluronidases on the host may be diverse. Hyaluronidases are frequently called “spreading factors“ (Kreil, 1995). In the case of bloodsucking insects, hyaluronidases increase tissue permeability for other salivary components by degrading HA abundant in host skin. These molecules serve as antihaemostatic, vasodilatory or anti-inflammatory agents (see sections 1.3.1 and 1.3.3). Thereby hyaluronidases play an important

role in blood meal acquisition. Their activity facilitates the enlargement of the feeding lesion and the insect acquires the bloodmeal more rapidly.

In addition, HA fragments were shown to have immunomodulatory properties (reviewed by Mummert, 2005). As bloodsucking insects represent the most important vectors of infectious diseases, it is tempting to speculate that local immunomodulation of the vertebrate host may enhance the infection establishment. For example, low molecular weight HA fragments affect dendritic cell maturation as documented by upregulated expression of TNF-α, costimulatory molecules and lymphoid homing receptor CCR7 (Tesar et al., 2006). TLR-4 signalling is at least partly responsible for phenotypic and functional DC maturation in response to HA fragments (Termeer et al., 2002; Tesar et al., 2006). TLR-4 engagement was also reported in HA fragment-induced IL-8 production by human endothelial cells and the production of the related cytokines MIP-2 and KC *in vivo* in mice (Taylor et al., 2004). Interaction of low molecular weight HA with macrophages induced upregulation of CD36 (Tabata et al., 2007), a thrombospondin receptor implicated in clearance of apoptotic cells by macrophages (Savill, 1997). Induction of iNOS and chemokine secretion was reported in macrophages stimulated by HA fragments, although it should be noted that the size of the fragments used by the authors ($2x10^5$ Da) was rather large (McKee et al., 1996; McKee et al., 1997; Horton et al., 1998). Similarly, iNOS induction in macrophages treated with "low molecular weight HA" in murine air-pouch inflammation model was actually due to HA fragments the size of $1\text{-}5x10^5$ Da (Cabrera et al., 2004). Yang et al. infected macrophages with lesion amastigotes of *L. amazonensis* and showed the requirement for simultaneous stimulation with HA ($2x10^5$ Da) to induce IL-10 production in macrophages (Yang et al., 2007). To sum up, HA fragments can be generated in the skin of a vertebrate host upon feeding of certain bloodsucking insect species. These fragments may then act on diverse targets, resulting in modulation of local immune and inflammatory milieu.

1.3.3 Immunomodulatory properties of saliva, with emphasis on sand fly saliva

Host haemostasis and inflammatory mechanisms are closely related. Therefore, it is conceivable that insect salivary factors subverting vasoconstriction, platelet activation and aggregation, and blood coagulation affect immune functions as well. The importance of immune modulation by insect saliva is underlined by the fact that many bloodfeeding arthropods represent important vectors of infectious diseases affecting humans. These include arboviral fevers such as yellow fever, Dengue, West Nile or Chikungunya, bacterial infections like Lyme disease, plague, and rickettsioses, protozoan diseases like malaria, sleeping

sickness, and leishmaniasis, and even filariases, heminth infections. Livestock farming is threatened by diseases like bluetongue disease, vesicular stomatitis, cowdriosis, babesiosis, anaplasmosis, or nagana. A role for saliva in transmission of some of these diseases was implied and, in several cases, confirmed (Titus & Ribeiro, 1988; Osorio et al., 1996; Edwards et al., 1998; Vaughan et al., 1999; Caljon et al., 2006b). Due to the importance of saliva-caused immunomodulation in disease transmission, immunomodulatory properties of bloodfeeding insect saliva have been the subject of intensive research, results of which are summed up in several recent reviews (Gillespie et al., 2000; Kamhawi, 2000; Andrade et al., 2005; Rohousova & Volf, 2006; Titus et al., 2006).

Leishmaniases represent the most extensively studied group of diseases with respect to the effect of vector saliva on pathogen transmission and infection establishment. In addition, the interactions between *Leishmania*, sand flies and the vertebrate hosts are studied in our laboratory. Therefore, this section focuses mainly on immunomodulatory properties of sand fly saliva, although several other insect species will be mentioned as well.

Multiple immune mechanisms, including both innate and adaptive immunity, were shown to be influenced by saliva of bloodsucking insects, or rather salivary gland extracts. In all species studied, inhibition of inflammation and modulation of cytokine production was observed. As inflammation is a common feature of immune response and cytokines are key mediators of this response, salivary components might prevent the sensitization of vertebrate host to insect feeding. In line with this hypothesis is the fact that in several insect species, as well as in ticks, bias in host immune response toward Th2 was observed (e.g., Mbow et al., 1998; Caljon et al., 2006a; Skallova et al., 2008) athough this response was not necessarily highly polarized (Kovar et al., 2002; Wasserman et al., 2004). Basophil and eosinophil blood counts were elevated in guinea pigs exposed to feeding of kissing bug *Triatoma protracta* or sand fly *Lutzomyia longipalpis*; these cellular types are usually related to Th2-like response (Brown & Rosalsky, 1984). On the other hand, it was shown that sand fly feeding induces a strong delayed-type hypersensitivity (DTH) associated with increased blood flow in mice and humans, enabling the flies to feed more rapidly. Judging by the analysis of cellular infiltrate mobilized in the skin, the response was of a relatively mixed Th1/Th2 type. It was suggested that only a small proportion of salivary molecules is responsible for the DTH effect (Belkaid et al., 2000b). This opinion was later supported by studies of Oliveira et al. (2006; 2008), who immunized mice with individual plasmids encoding salivary proteins of *P. ariasi* or *P. papatasi*. Only 3 out of 24 tested *P. ariasi* plasmids and 3 out of 10 tested *P. papatasi* plasmids induced DTH in mice after subsequent intradermal challenge with SGE of the given

sand fly species. Moreover, it remains to be investigated whether hosts in the field develop DTH even after prolonged, even life-long exposure to sand flies.

Salivary immunomodulators act either on immunocompetent cells or other components of immune system, such as complement cascade or the kinin system. Anti-complement activity was detected in *Lutzomyia longipalpis* sand flies, interfering with both classical and alternative pathways of complement activation (Cavalcante et al., 2003). The authors suggest that sand fly anti-complement activity may aid the inherent anti-complement activity of *Leishmania* (Mosser & Brittingham, 1997) and thus promote infection establishment. The classical pathway could also be inhibited by salivary gland material of several triatomine bug species (Cavalcante et al., 2003). Interference with kinin system resulting in inhibition of bradykinin release can occur due to lipocalins of triatomine bugs (Isawa et al., 2007) or salivary D7 proteins of *Anopheles* mosquitoes (Isawa et al., 2002). Bradykinin is a primary mediator of acute inflammatory responses, causing vasodilation, microvascular permeability increase, and nociception enhancement. Along with an unrelated anti-kinin protein identified in *Haemaphysalis* ticks (Kato et al., 2005), these salivary factors developed independently as a result of convergent evolution of salivary proteins in bloodfeeding arthropods.

Of interest is the effect of insect saliva on host macrophages. In the case of *Leishmania* infection, these are one of the earliest cells to encounter the parasites and at the same time their nursing cells. Later in the infection, activated macrophages fight the infection mostly by producing reactive nitrogen intermediates (Green et al., 1990; Liew et al., 1990). Indeed, *in vitro, P. papatasi* saliva downregulates expression of the iNOS gene and reduces NO production in murine macrophages (Hall & Titus, 1995; Waitumbi & Warburg, 1998; Katz et al., 2000). Similar results were reported with *Lutzomyia longipalpis* SGE in *Leishmania amazonensis*-infected macrophages (Norsworthy et al., 2004) or in macrophages incubated with SGE of biting midge *Culicoides sonorensis* (Bishop et al., 2006). The presence of sand fly SGE affects the expression of costimulatory molecules on macrophages (Costa et al., 2004; Menezes et al., 2008); similar results were obtained with dendritic cells maturated in the presence of *P. papatasi* SGE (Carregaro et al., 2008) or synthetic maxadilan, a peptide from *L. longipalpis* saliva (Wheat et al., 2008). The ability of macrophages to present antigen to T cells is also decreased in the presence of sand fly SGE (Theodos & Titus, 1993). Cytokine production by LPS- or *L. major*-stimulated macrophages and monocytes is altered in the presence of SGE of sand flies *L. longipalpis*, *L. intermedia*, and *P. papatasi* (Costa et al., 2004; Menezes et al., 2008; Rogers & Titus, 2003). The effect on cytokine production by

macrophages or monocytes is partially retained when using synthetic maxadilan (Soares et al., 1998; Rogers & Titus, 2003). Generally, a tendency to decreased CD80/CD86 expression and Th1 cytokine secretion was observed in these models. In addition, using *L. donovani* promastigotes and murine peritoneal macrophages, Zer et al. observed enhancement of parasite uptake by macrophages in the presence of sand fly SGE (Zer et al., 2001).

Salivary gland material from three different sand fly species was shown to be directly chemotactic for monocytes and macrophages (Anjili et al., 1995; Zer et al., 2001). As for sand flies, it may be concluded that salivary compounds attract host cells for *Leishmania*, enhance promastigote uptake by these cells and at the same time render them incapable of killing the parasites. On the other hand, the chemotactic effect of leukotriene B_4 on neutrophils can be abolished by its preincubation with SGE of the sand fly *L. longipalpis* (Monteiro et al., 2005). Sand fly SGE also affects chemotaxis of immune cells indirectly, by inducing the expression of chemokines, cytokines, or prostaglandins (PG) that eventually results in altered migration patterns of immune cells. Specifically, in a study focused on *L. longipalpis* SGE-induced cellular migration into an air pouch, MCP-1/CCL2 expression was induced by SGE, leading to enhanced macrophage infiltration. The chemotactic effects of SGE and *L. chagasi* promastigotes on macrophages were additive. Eosinophil recruitment in response to SGE was also observed (Teixeira et al., 2005). In other studies using a murine model of ovalbumin (OVA)-induced immune peritonitis, systemic application of SGE from three different sand fly species prior to OVA challenge was able to inhibit OVA-induced neutrophil migration, while enhancing eosinophil migration at the same time (Monteiro et al., 2005; Carregaro et al., 2008). It was suggested that the inhibition of neutrophil migration is dependent on PGE_2 production triggered by systemic SGE application. Sequential production of PGE_2 and IL-10 then suppresses the expression of neutrophil chemotactic mediators, MIP-1α, TNF-α and LTB_4 (Carregaro et al., 2008).

Anopheline saliva contains proteins with chemotactic activity for neutrophils (Owhashi et al., 2001) and eosinophils (Owhashi et al., 2008). In mice bitten by *Anopheles* mosquitoes an indirect effect on cellular migration was also seen. At the site of feeding, skin mast cells degranulate and the released TNF-α acts as a chemoattractive molecule for neutrophils (Demeure et al., 2005).

T cell and B cell functions were also shown to be affected by the interactions of T cells with salivary gland material of bloodsucking insects. Namely, mitogen- as well as antigen-driven proliferation of T cells is suppressed in the presence of sand fly SGE (Theodos & Titus, 1993; Titus, 1998; Rohousova et al., 2005b) or synthetic maxadilan, a peptide from

L. longipalpis saliva (Qureshi et al., 1996). Similar results were reported with SGE of black fly *Simulium vittatum* (Cross et al., 1993), stable fly *Stomoxys calcitrans* (Swist et al., 2002), mosquito *Aedes aegypti* (Cross et al., 1994; Wanasen et al., 2004; Wasserman et al., 2004), biting midge *Culicoides sonorensis* (Bishop et al., 2006), or tsetse fly *Glossina morsitans morsitans* (Caljon et al., 2006a). B cell mitogen- or antigen-driven proliferation was also suppressed in several of the models mentioned above (Wasserman et al., 2004; Cross et al., 1993; Bishop et al., 2006) as well as B cell antibody responses to heterologous antigen (Caljon et al., 2006a). Cytokine production by T cells, or rather spleen cells, *in vitro* is also diminished in the presence of SGE of sand flies (Rohousova et al., 2005b) or *Aedes aegypti* mosquitoes (Wasserman et al., 2004); *in vivo*, enhanced IL-4 secretion after *L. brazilienzis* and *L. longipalpis* SGE coinoculation was ascribed to T cells (Lima & Titus, 1996).

Skin resident mast cells may also be affected by salivary components of bloodfeeding insects. For example, SGE of *Aedes aegypti* mosquito inhibits TNF-α release from peritoneal mast cells (Bissonnette et al., 1993). TNF-α is a potent proinflammatory mediator, and, as noted earlier, after release from mast cells it recruits neutrophils and macrophages to inflamed skin site (Von Stebut et al., 2003). On the other hand, *Anopheles stephensi* mosquito bites result in a local IgE-independent degranulation of murine skin mast cells and a substantial increase of TNF-α concentrations within draining lymph nodes (Demeure et al., 2005). A potential anti-mast cell factor found in saliva of bloodfeeding insects is adenosine deaminase, as adenosine is an inducer of mast cell degranulation. Salivary adenosine deaminase was detected in several insect species, e.g. mosquitoes *Aedes aegypti* and *Culex quinquefasciatus* (Ribeiro et al., 2001), sand flies *Lutzomyia longipalpis* (Charlab et al., 2000) and *Phlebotomus duboscqi* (Kato et al., 2007), and tsetse flies *Glossina morsitans* and *G. palpalis* (Li & Aksoy, 2000). However, the role of adenosine and adenosine deaminase in bloodfeeding is rather puzzling. On the one hand, inosine, the product of adenosine cleavage, has the ability to prevent overproduction of proinflammatory cytokines TNF-α, IL-1, IL-12, IFN-γ, and MIP-1α, while it can enhance the production of IL-10 (Hasko et al., 2000). Although this eventual effect of adenosine deaminase would hardly influence the feeding success of the insect, for the production of cytokines takes some time, it could play a role later, in the development of host immune response to salivary antigens, and possibly the transmitted pathogens as well. Cleaving adenosine would also reduce the induction of mast cell degranulation. On the other hand, adenosine is present in considerable concentration in salivary glands of several sand fly species (Ribeiro et al., 1999; Ribeiro & Modi, 2001), which would implicate that adenosine is

actually a useful compound for a bloodfeeding insect. This interesting topic clearly requires further investigation.

Interestingly, immunomodulation caused by bites of bloodsucking insects was suggested as a mechanism promoting transmission or reactivation of human herpesvirus 8, the causative agent of Kaposi‘s sarcoma. This is a cytokine-driven angioproliferative disorder. The virus is usually transmitted by saliva of infected individuals; the bloodsucking arthropods do not act as biological/mechanical vectors. Rather, the immune reaction at the site of bite was implied in either promoting viral transmission (e.g. when saliva on an infected individual is rubbed into the bitten skin to relieve itching) or reactivation of existing infection at the skin site (Ascoli et al., 2006). Mosquito saliva-induced immunomodulation was also related to viral activation and oncogenesis in natural killer (NK) cell lymphocytosis associated with Epstein–Barr virus (EBV) infection. Mosquito SGE induced NK cell proliferation and markedly increased the expression of EBV oncogene LMP1 in NK cells from infected individuals. As it is known that LMP1 is involved in the oncogenesis of NK cells *in vivo*, the authors of the study conclude that mosquito bites are one of the factors causing NK cell lymphocytosis (Asada et al., 2005).

1.4 Immune response of sand fly-exposed animals

Animals and humans exposed to feeding of bloodsucking arthropods develop immune response to components of saliva injected in their skin by the feeding arthropods. This section focuses on the immune response of hosts bitten by sand flies. Sand flies are generally not the kind of nuisance that leaves their habitat unsuitable for most human activities such as lumbering, agriculture or livestock farming, as might be the case with biting midges, black flies or even mosquitoes (Adler & McCreadie, 2002; Mullen, 2002; Becker et al., 2003). Nevertheless, bites of sand flies can be very irritable and produce marked local reactions. In certain regions, a condition known as „harara“ refers to an urticarial skin reaction resulting from the bites of sand flies (Tesh & Guzman, 1996). However, harara skin reactions have not been the subject of any extensive study, although a mouse model of harara-like DTH reaction was developed (Belkaid et al., 2000b). The authors studied the history of immune reactions to sand fly bites in several volunteers as well and proposed that it was similar to that of individuals repeatedly bitten by mosquitoes. Mellanby (1946) described the course of immune responsiveness to mosquito bites with 4 distinct stages: naive individuals developed no reaction or a DTH reaction on first contact with mosquito bites, evolving gradually during prolonged exposure to combined DTH and

immediate reaction, then to immediate reaction only, and possibly total desensitization. Unfortunately, no similar long-term study was performed using sand flies.

Research on immune response to sand fly saliva is not focused on harara or hypersensitivity reactions as such; rather, the development of anti-saliva immunity is studied with respect to potential application to vaccinology or epidemiology. Firstly, components of arthropod saliva including sand flies are under scrutiny for their potential use in vaccines preventing vector-borne pathogen transmission (reviewed by Titus et al., 2006). Secondly, levels of anti-saliva antibodies can be monitored in epidemiological studies to assess the risk of exposure to vectors or the risk of disease transmission, e.g transmission of leishmaniasis. Results of recent research in both fields are briefly summarized in this section.

1.4.1 Sand fly salivary antigens as potential anti-*Leishmania* vaccine components

The notion that salivary components might become a part of subunit vaccines against leishmaniasis was justified by several studies involving immunization of mice with sand fly SGE, sand fly bites, or individual salivary components. Mice were immunized either by intradermal injection of *P. papatasi* SGE (Belkaid et al., 1998b), partially purified salivary protein SP15 from *P. papatasi* or DNA plasmid coding for the same protein (Valenzuela et al., 2001), *P. papatasi* feeding (Kamhawi et al., 2000), or subcutaneous injection of synthetic maxadilan from *L. longipalpis* (Morris et al., 2001). In all studies with *L. major* partial protection was observed, i.e. lesions were smaller and/or resolved faster. Similar results were obtained in mice intradermally immunized with *L. longipalpis* SGE and challenged with *Leishmania amazonensis* (Thiakaki et al., 2005). Partial protection was achieved in a model of visceral leishmaniasis: vaccination of hamsters with a plasmid encoding the salivary protein LJM19 from *L. longipalpis* prevented the fatal outcome of *L. infantum* infection (Gomes et al., 2008).

Individual salivary components may have, however, quite contrasting effects on anti-*Leishmania* immunity; while vaccination with plasmid encoding SP15 protein from *P. papatasi* is protective against *L. major* infection (Valenzuela et al., 2001; Oliveira et al., 2008), vaccination with PpSP44 plasmid encoding another salivary protein of the same species leads to disease exacerbation despite eliciting a DTH reaction as well (Oliveira et al., 2008).

The protective effect of insect saliva was documented in two other models of insect-borne and insect-associated diseases: malaria and Buruli ulcer, respectively. First, mice pre-exposed to uninfected *Anopheles stephensi* mosquitoes and then infected by bites of *Plasmodium yoelii* sporozoite-carrying mosquitoes harboured significantly lower numbers of parasites in their livers and exhibited lower parasitemia levels (Donovan et al., 2007). Second, prior exposure of mice to

bites of the aquatic insect *Naucoris cimicoides* prevented the mice from developing ulcerative lesions at sites of *Mycobacterium ulcerans*-carrying insect bites (Marsollier et al., 2007).

These results are very promising in terms of development of anti-*Leishmania* vaccines containing sand fly salivary molecules and perhaps vaccines against other vector-borne diseases. On the other hand, before drawing definitive conclusions, several issues have to be addressed. Firstly, it should be noted that the immune response to sand fly salivary antigens was found to be species-specific (Volf & Rohousova, 2001; Rohousova et al., 2005a; Thiakaki et al., 2005). Thus, vaccination with salivary component of a given sand fly species would not prevent disease transmission by sand fly of another genus or even another species of the same genus. Secondly, in other vector-parasite combinations the protective effect of previous exposure to sand fly saliva might not be retained. For example, immunization of mice with SGE of *Lutzomyia intermedia* actually resulted in enhanced *Leishmania braziliensis* infection (de Moura et al., 2007). In addition, immunization by SGE injection or sand fly feeding might not be effective if performed in hosts with life-long exposure to sand flies, as implicated by our recent results (Rohousova et al., unpublished).

It is crucial for further research on vaccines involving saliva-based antigens to characterize the local immune response following sand fly bite and to elucidate the mechanisms of protection against leishmaniasis in sand fly saliva-immunized animals. Repeated exposure to sand fly bites elicits both cellular and humoral immune responses in the host (Barral et al., 2000; Volf & Rohousova, 2001; Gomes et al., 2002; Rohousova et al., 2005a; Silva et al., 2005). Mice exposed to *L. longipalpis* feeding exhibit marked cellular infiltration after needle SGE challenge, consisting of neutrophils, eosinophils and macrophages 2 hours after challenge, persisting for further two weeks (Silva et al., 2005); in ears of mice exposed to *P. papatasi* and challenged by bite of the sand fly there was an infiltrate of eosinophils, macrophages, dendritic cells and $CD4^+$ T cells 24 hours after the bite (Kamhawi et al., 2000). In both cases, the composition of leukocytes in the infiltrate is slightly different from that found in a „typical“ DTH reaction, which is predominantly formed by mononuclear cells.

Currently, cellular immunity and priming of the immune response at the site of the bite towards Th1 response is believed to form the basis of protective immunity in animals immunized by saliva. There are several studies supporting this hypothesis. In particular, the level of protection was the same in wild type and B cell deficient mice immunized with plasmid encoding SP15 protein from *P. papatasi* (Valenzuela et al., 2001), suggesting that the contribution of antibodies to the protective effect was insignificant. Evidence provided by other studies is rather circumstantial, reporting mostly the presence of marked DTH response at the

site of infection in protected immunized mice (Kamhawi et al., 2000; Valenzuela et al., 2001), local production of IFN-γ and IL-12 early after infection (Kamhawi et al., 2000; Oliveira et al., 2008), or up-regulation of production of IFN-γ and NO in lymph nodes draining the infection site (Morris et al., 2001). In some models, DTH response was not monitored in immunized animals challenged with infective inoculum or infected sand fly bites, but rather in immunized animals challenged by uninfected sand flies or SGE injection (Gomes et al., 2008; Oliveira et al., 2008); as in these models immunization lead to protection, the authors assumed that the DTH response plays a role in the establishment of protection. It should be noted that the DTH-eliciting properties of a given salivary component are, by themselves, not a sufficient correlate of protection against *Leishmania* infection. Instead, a good vaccine candidate molecule will not only induce a DTH response, but it shall trigger local production of Th1 cytokines as well; this is best illustrated by the failure of the DTH-inducing protein PpSP44 to trigger IFN-γ and IL-12 expression and to confer protection against *L. major* infection (Oliveira et al., 2008).

As yet, it is unclear what role Th2 cytokines are playing in natural *Leishmania* infections transmitted by sand fly bites. Generally, in non-protected animals there is local production of Th2 cytokines, namely IL-4, after needle inoculation of parasites and SGE (Belkaid et al., 1998b; Oliveira et al., 2008) which is virtually eliminated in mice pre-exposed to SGE and protected against *L. major* (Belkaid et al., 1998b). However, IL-4 has little or no effect on the evolution of infections in C57BL/6 mice after transmission by bite as documented by similar course of lesion development in naturally infected IL-4 deficient and wild type mice (Kamhawi et al., 2000). We should be constantly aware that immune responses elicited after sand fly bite might be different from those elicited by SGE injection.

Another conflicting issue is that of anti-saliva antibodies in protection against leishmaniasis. In some models using immunization with salivary components the production of antibodies was not necessary for the development of protective status (Valenzuela et al., 2001; Gomes et al., 2008). On the contrary, preincubation of *P. papatasi* SGE with serum IgG from sand fly-exposed animals completely neutralized its ability to enhance *L. major* infection (Belkaid et al., 1998b). In conclusion, antibodies can not be entirely ruled out regarding protective immunity in leishmaniasis.

1.4.2 Antibody response to sand fly salivary antigens

The idea of monitoring anti-saliva antibody response for the assessment of risk of disease transmission has only recently come to draw greater attention. It was mentioned above that sand fly bites elicit humoral response in exposed hosts (Barral et al., 2000; Volf &

Rohousova, 2001; Gomes et al., 2002; Silva et al., 2005; Rohousova et al., 2005a). Sand fly bite is the only clinically relevant means of *Leishmania* transmission to humans; therefore, if anti-saliva antibody levels reflected the level of exposure to the vector, they might correlate with the risk of *Leishmania* transmission as well. In an analogous setting, antibodies against salivary antigens of *Ixodes* ticks were found to be significantly elevated in individuals seropositive for Lyme disease (Schwartz et al., 1990; Schwartz et al., 1991). Similarly titres of antibodies against *Anopheles gambiae* salivary antigens were higher in children who developed clinical malaria within the next 3 months than in those who did not (Remoue et al., 2006). Thus, it is conceivable that anti-sand fly saliva antibodies would be detected in animals and humans living in regions where leishmaniasis is endemic.

A confirmation of this theory comes from field studies performed in foci of both visceral and cutaneous leishmaniases. First, children from area endemic for visceral leishmaniasis in Brazil who had a positive leishmanin test (DTH reaction to intradermal inoculation of *L. infantum/chagasi* protein) also had high serum levels of antibodies against *L. longipalpis* salivary antigens (Barral et al., 2000). It was shown that the appearance of anti-saliva humoral response and anti-*Leishmania* DTH is more or less simultaneous (Gomes et al., 2002). Antibodies against *L. longipalpis* salivary antigens were also detected in Brazilian dogs naturally infected with *L. infantum/chagasi* (Bahia et al., 2007), and humans, dogs and wild foxes living in another area endemic for visceral leishmaniasis in Brazil (Gomes et al., 2007). Second, a positive correlation between infection and anti-saliva antibody titres was found in foci of cutaneous leishmaniases caused by *L. tropica* and *L. major* (Rohousova et al., 2005a; Louzir et al., 2005) in Turkey and Tunisia, respectively.

Not all phlebotomine species act as vectors of leishmaniases, and therefore the finding that immune response to sand fly salivary antigens is species-specific has practical implications (Volf & Rohousova, 2001; Rohousova et al., 2005a; Thiakaki et al., 2005). Antibodies raised in individuals exposed to distinct sand fly species thus do not bind to salivary antigens of other phlebotomine species with a different distribution (Rohousova et al., 2005a). In summary, specific antibody response to sand fly saliva could be used for monitoring the exposure of hosts to sand flies and might be used as a marker of risk for *Leishmania* transmission in endemic areas.

Apart from application in epidemiological surveys of leishmaniasis, monitoring of host exposure to sand flies would be a most useful tool for the assessment of efficacy of anti-sand fly campaigns. For example, insecticide-treated bednets for humans or insecticide treatment of dogs, reservoir hosts of *L. infantum/chagasi*, are employed to prevent

transmission of *Leishmania* (Manzillo et al., 2006; Otranto et al., 2007; Courtenay et al., 2007). It remains to be elucidated whether anti-saliva antibody response is sustained even in hosts experiencing long-term or life-long exposure to sand flies. Similarly, kinetics of anti-saliva antibody production must be characterized to allow future monitoring of antibody response in areas where sand fly abundance shows seasonal fluctuations (e.g., de Resende et al., 2006). Finally, for anti-saliva antibody screening to become a routine field test, recombinant antigens have to be developed which would make it possible to avoid the most impractical requirement for sand fly salivary gland homogenates.

2 Objectives

My study was aimed at enzymatic and antigenic properties of sand fly saliva. I was interested in the role of salivary hyaluronidase in *Leishmania* transmission. In certain aspects, I focused specifically on sand fly species *Lutzomyia longipalpis* and *Phlebotomus (Adlerius) arabicus*, vectors of visceral and cutaneous leishmaniases, respectively. I studied the salivary antigens of these species and the immune response in animals exposed to sand fly bites. So far, nothing is known about the composition of saliva in any *Adlerius* species and our colony of *P. arabicus* is at present the only laboratory colony of subgenus *Adlerius* in the world. Therefore I sought to characterise salivary components of this species.

The main objectives of this thesis were as follows:

- to assess the effect of hyaluronidase coinoculation on the size of lesions in BALB/c mice infected with *Leishmania major*
- to determine whether hyaluronidase coinoculation affects the numbers of parasites in lymph nodes draining the site of infection with *L. major*
- to describe the long-term kinetics of anti-saliva antibody response in dogs experimentally exposed to *Lutzomyia longipalpis* females
- to detect and characterise salivary antigens of *L. longipalpis* reacting with antibodies of dogs repeatedly bitten by this sand fly
- to prepare and annotate a cDNA library from *P. arabicus* salivary glands
- to detect and characterise *P. arabicus* salivary antigens reacting with antibodies of mice exposed to *P. arabicus*

3 Publications

- Volf P., **Hostomská J.**, Rohoušová I., 2008. Molecular Crosstalks in *Leishmania*-Sandfly-Host Relationships. Parasite 15 (3)
  accepted for publication 11th April, 2008
- Volfová V., **Hostomská J.**, Černý M., Votýpka J., Volf P. Hyaluronidase of Bloodsucking Insects and its Enhancing Effect on *Leishmania* Infection in Mice. PLoS Neglected Tropical Diseases, in press.
  accepted for publication 25th July, 2008
- **Hostomská J.**, Rohoušová I., Volfová V., Stanneck D., Mencke N., Volf P., 2008. Kinetics of Canine Antibody Response to Saliva of the Sand Fly *Lutzomyia longipalpis*. Vector-Borne and Zoonotic Diseases 8 (4)
  doi:10.1089/vbz.2007.0214
  accepted for publication 30th November, 2007
- **Hostomská J.**, Jochim R.C., Valenzuela J.G., Volf P. Analysis of Salivary Transcripts and Antigens of the Sand Fly *Phlebotomus arabicus.*
  Manuscript ready for submission to BMC Genomics

4 Summary and Conclusions

This thesis sums up the results of three projects I was involved in during my PhD study. Specifically, I adressed the putative effect of sand fly salivary hyaluronidase on transmission and establishment of *Leishmania* infection. The second project was dealing with the kinetics of anti-saliva antibody response in dogs exposed to *Lutzomyia longipalpis* sand flies and with the characterisation of salivary antigens recognized by these dogs. Finally, I constructed and annotated a cDNA library from *Phlebotomus arabicus* and characterised *P. arabicus* salivary antigens reacting with antibodies of mice exposed to this sand fly species. The results of the projects are briefly outlined here.

- We detected hyaluronidase activity in saliva of various bloodsucking Diptera, including sand flies. The role of the enzyme is to cleave the skin of the host, enlarge the feeding lesion, and help to acquire the blood meal. In addition, resulting fragments of extracellular matrix modulate local immune response of the host, which may positively affect transmission of vector-borne diseases, including leishmaniasis. Therefore we studied the enhancing effect of hyaluronidase on *Leishmania* infection. We proved a positive correlation between the size of the cutaneous lesion caused by *Leishmania major* in BALB/c mice and the presence of hyaluronidase in the infective inoculum. In hyaluronidase-coinoculated mice, the lesions were significantly larger from week 3 post infection on when the infection dose was 10^5 parasites and from week 4 post infection on when the infection dose was 10^4 parasites. On the other hand, hyaluronidase did not affect early visceralization of *L. major*. This was documented 24 hrs post infection by similar parasite numbers in draining lymph nodes of mice inoculated with *L. major* only and mice coinoculated with hyaluronidase. Thus, we demonstrated that hyaluronidase promotes *Leishmania* establishment in murine skin and we hypothesise that immunomodulatory effects of hyaluronan fragments generated at infection site are responsible for the effect. We suggest that hyaluronidase is one of the factors responsible for infection-enhancing ability of saliva in New World and Old World sand flies alike. In addition, we propose that salivary hyaluronidase may facilitate the spread of other vector-borne microorganisms.

- We studied the antibody response in dogs experimentally exposed to *Lutzomyia longipalpis* females to characterise sand fly salivary antigens recognized by canine

sera and find out whether the level of specific anti-saliva antibodies reflects the intensity of exposure. We showed that dogs experimentally exposed to feeding of *L. longipalpis* sand flies develop specific anti-saliva IgG and IgE antibodies. Sera from repeatedly bitten dogs recognised up to six salivary protein bands with approximate molecular weight of 66, 55, 45, 37-39, 34 and 25 kDa in *L. longipalpis* salivary gland lysate. The levels of anti-saliva IgG, IgG1 and IgG2 were related to numbers of fed *L. longipalpis* females and elevated antibody levels in bitten animals were found throughout the study. Differences in the intensity of antibody response between high-exposed and low-exposed dogs were detected as late as 29 weeks after the last exposure. In contrast, specific IgE response developed in some dogs only and no correlation was observed between its level and the intensity of exposure. Therefore, anti-saliva IgG was found as a useful marker of exposure of dogs to sand flies. Monitoring canine antibody response to sand fly saliva would allow evaluating the effectiveness of anti-vector campaigns. It is likely that a modified antibody detection test will be employed in the field in future. As many endemic regions show marked fluctuations in numbers of sand flies throughout the year, long-term persistence of antibody response is a key prerequisite for the detection of vector exposure in these cases. Our results show that monitoring of canine exposure to sand flies would be feasible even in regions with seasonal variations in sand fly numbers.

- A cDNA library was constructed from salivary glands of *Phlebotomus arabicus* females dissected 1 day after emergence. From this cDNA library, we sequenced 985 randomly selected clones and obtained 395 clusters of related sequences. As in other sand flies studied so far, the most abundant transcripts were those coding for putative secretory proteins; 74 clusters were generated from these sequences, with an average number of 7,65 sequences per cluster. Members of 21 different families were found among putative secretory proteins; most of these proteins have known homologs in other sand fly species. Some of the families contained multiple members, suggesting several gene duplication events. The most abundantly represented families were SP15-like proteins, 27 kDa-like proteins, D7-related proteins, yellow-related proteins, PpSP32-like proteins, antigen 5-related proteins, 34 kDa-like proteins, and the apyrases. Sequences coding for putative secreted enzymes were also found in the cDNA library, including hyaluronidase, endonuclease, pyrophosphatase, amylase and trehalase. From the transcripts that did not match any reported proteins in accessible

databases, none was predicted to be secreted. Eight to ten antigens reacting with sera of mice exposed to *P. arabicus* feeding were detected using different serum samples. The most prominent antigenic bands had apparent molecular weights of 56-58.5, 45, 43, 42, 34.5-36.5 and 30 kDa. Slightly weaker reaction was observed with bands running at 31 and 30.5 kDa. Two very faint bands running at 21 and 16 kDa were recognised by sera of some animals only. In addition, we confirmed our previous findings that the antibody response to sand fly salivary antigens is species-specific. Sera from mice bitten by *P. arabicus* specifically recognized antigens of *P. arabicus* and not those of *P. papatasi*.

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MOLECULAR CROSSTALKS IN *LEISHMANIA*-SANDFLY-HOST RELATIONSHIPS

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Summary:

Sandflies (Diptera: Phlebotominae) are vectors of *Leishmania* parasites, causative agents of important human and animal diseases with diverse manifestations. This review summarizes present knowledge about the vectorial part of *Leishmania* life cycle and parasite transmission to the vertebrate host. Particularly, it focuses on molecules that determine the establishment of parasite infection in sandfly midgut. It describes the concept of specific *versus* permissive sandfly vectors, explains the epidemiological consequences of broad susceptibility of permissive sandflies and demonstrates that genetic exchange may positively affect *Leishmania* fitness in the vector. Last but not least, the review describes recent knowledge about circulating antibodies produced by hosts in response to sandfly bites. Studies on specificity and kinetics of antibody response revealed that anti-saliva IgG could be used as a marker of host exposure to sandflies, i.e. as a useful tool for evaluation of vector control.

KEY WORDS : *Leishmania*, sandfly, host, relationships, molecular crosstalks.

The leishmaniases are parasitic diseases with various clinical signs and symptoms ranging from skin lesions to life-threatening visceral disease. The etiological organisms, parasitic protozoa of the genus *Leishmania*, are transmitted by insect vectors, female phlebotomine sandflies (Diptera: Phlebotominae). During the life cycle, *Leishmania* adapt themselves to varied and heterogeneous environments of the insect vector and vertebrate host that differ in temperature, pH and many other parameters. Recent research revealed that the parasite development is affected by a number of molecules derived from the vector. This short review aims to demonstrate how molecular biology helps us to understand *Leishmania* epidemiology and the circulation of the parasite in nature. The text is divided into three parts. The first briefly summarizes the present knowledge about the life cycle of *Leishmania* in the vector and the other two are deal with selected aspects of the *Leishmania*-sandfly-host interaction about which much new information has been gained during last few years.

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LEISHMANIA LIFE CYCLE IN THE SANDFLY

In the vector, parasites taken up with the blood meal undergo a period of replication and development in the midgut (reviewed by Molyneux & Killick-Kendrick, 1987), after which they differentiate to an infective metacyclic stage adapted for transmission to mammals (reviewed by Killick-Kendrick, 1990; Kamhawi, 2006). Initially, a blood meal containing aflagellated amastigotes is quickly surrounded by peritrophic matrix, a chitinous framework with a protein-carbohydrate matrix that is secreted by epithelial cells of the midgut (Pimenta *et al.*, 1997). Amastigotes divide and then differentiate into flagellated promastigotes, so-called procyclics. During this early stage of development, transforming parasites survive midgut proteases (Schlein & Romano, 1986; Dillon & Lane, 1993; Pimenta *et al.*, 1997; Schlein *et al.*, 1998; Ramalho-Ortigao *et al.*, 2003) and antimicrobial peptides (Boulanger *et al.*, 2004). The peritrophic matrix decays within a few days, the promastigotes escape from the endoperitrophic space and transform to long nectomonad stages. In the subgenus *Leishmania* (Suprapylarian species), promastigotes then attach by their flagella to the microvilli of the sand fly midgut (Killick-Kendrick *et al.*, 1974; Walters *et al.*, 1993; Cihakova & Volf, 1997; Warburg *et al.,* 1989; Sachs *et al.,* 2000). This attachment is an essential part of the *Leishmania* life cycle as it enables the parasite to avoid expulsion from the gut when the remnants of the digested blood meal are defaecated. The nectomonads multiply repeatedly in the abdominal midgut and gradually migrate forwards to the thoracic midgut and the stomodeal valve. These forms are the precursors of metacyclic promastigotes, small, highly motile forms with a long flagellum, which are infective for the vertebrate host (for recent review see Bates, 2007).

The stomodeal valve is situated between the midgut and oesophagus and ensures one-way flow of the food. During the late-stage infection, parasites cause pathological changes in the gut that facilitate the transmission to the vertebrate host (reviewed by Schlein, 1993; Bates, 2007). *Leishmania* block the anterior mid-

gut by the production of a viscous gel-like plug (promastigote-secretory gel-PSG) containing filamentous proteophosphoglycan (Ilg *et al.*, 1996; Stierhof *et al.*, 1999; Rogers *et al.*, 2004). In addition, they attach to the chitinous lining of the stomodeal valve and destroy this lining by chitinase (Schlein *et al.*, 1992, 1993). Unique filamentous structures connecting the lining with the apical end of the epithelial cells are also damaged (Volf *et al.*, 2004), supposedly by proteases. Because of the damaged valve and occlusion of the anterior midgut by PSG, the heavily infected sand fly female finds it difficult to take a blood meal and regurgitates metacyclic promastigotes into the skin of the vertebrate. In addition, infected females probe repeatedly increasing the chance of transmission (Killick-Kendrick *et al.*, 1977).

In various parasite-vector combinations, metacyclic promastigotes were repeatedly found in proboscis (for review see Molyneux & Killick-Kendrick, 1987) and deposition of these parasites in the skin during the next bloodfeeding seems to be the second important mode of *Leishmania* transmission. Moreover, in some parasite-vector combinations, motile metacyclic parasites were observed also in salivary glands (e.g. Killick-Kendrick *et al.*, 1996) and sandfly urine (Sadlova &Volf, 2001) raising questions about alternative ways of transmission (Bates, 2007).

Leishmania are inoculated into the vertebrate host together with saliva of the sandfly. Saliva contains a number of molecules with vasodilatory, anticoagulatory or anti-inflammatory properties. These molecules are important for blood feeding but also play a crucial role in *Leishmania* transmission (Titus & Ribeiro, 1988). Saliva modulates the immune response of a naive host enabling the establishment of *Leishmania* infection (reviewed by Rohousova & Volf 2006). On the other hand, repeated exposure to sandfly bites elicits an immune response that confers protection against naturally transmitted *L. major* Yakimoff & Schokhor in mice (Kamhawi *et al.*, 2000). Two molecules of sand fly saliva, vasodilatory peptide maxadilan of *Lutzomyia longipalpis* Lutz & Neiva and protein SP15 of *Phlebotomus papatasi* Scopoli, have been successfully used in mice as a transmission-blocking vaccines (Morris *et al.*, 2001; Valenzuela *et al.*, 2001). Sand fly species, however, differ in salivary proteins/peptides and their pharmacological and antigenic properties (Warburg *et al.*, 1994; Volf & Rohousova, 2001; Cerna *et al.*, 2002; Anderson *et al.*, 2006) and this variability may have an impact on the clinical manifestations of a *Leishmania* infection. In sibling species of the *Lu. longipalpis* complex, Warburg *et al.* (1994) suggested that the amount of the peptide maxadilan plays a crucial role in visceralization of *L. infantum* Nicolle (syn *L. chagasi* Cunha & Chagas). In addition, due to differences of salivary antigens between various sandfly species, the protective effect they confer is species-specific: mice immunized with *Lu. longipalpis* saliva were more resistant to *Leishmania amazonensis* Lainson & Shaw co-inoculated with saliva of this species but not to those co-inoculated with saliva of *P. papatasi* or *P. sergenti* Parrot (Thiakaki *et al.*, 2005).

MIDGUT MOLECULES DETERMINE THE ESTABLISHMENT OF *LEISHMANIA* INFECTION

Laboratory studies examining the development of different *Leishmania* in a range of vectors showed that sandflies fall into two groups. Several sandfly species are specific vectors as they display remarkable specificity for the *Leishmania* species they transmit. For example, *P. papatasi* supports the development of only *L. major* but not other parasite species tested (Killick-Kendrick *et al.*, 1994; Pimenta *et al.*, 1994). Another example of a specific vector is *P. sergenti*, the vector of *Leishmania tropica* Wright (Killick-Kendrick *et al.*, 1995; Kamhawi *et al.*, 2000). In contrast, most other sandfly species examined to date support the development of a broad range of *Leishmania* species and fall into the second group called permissive vectors (Volf & Myskova, 2007). These include *Phlebotomus* species transmitting parasites of the *L. donovani* complex (Pimenta *et al.*, 1994; Myskova *et al.*, 2007) and *Lu. longipalpis*, the New World vector of *L. infantum* (Walters *et al.*, 1993). Evidently, the parasites are able to develop in any permissive sand fly species, if given the opportunity.

Studies with *L. major* in *P. papatasi* showed that the attachment is controlled by species-specific modifications of the major surface glycoconjugate of *Leishmania* promastigotes, lipophosphoglycan (LPG) (Kamhawi, 2006; Pimenta *et al.*, 1992; Pimenta *et al.*, 1994; Sacks *et al.*, 1995; Butcher *et al.*, 1996) that selectively bind to the midgut galectin receptor PpGalec (Kamhawi *et al.*, 2004; Kamhawi, 2006). In other *Leishmania*-sandfly pairs, the role of LPG in attachment has not been investigated in such detail and mechanisms underlying this broad permissivity of other sandfly species have not been elucidated. A variety of candidate molecules have been proposed to mediate this process, such as a relatively conserved flagellar protein (Warburg *et al.*, 1989).

Recent studies revealed that attachment of *Leishmania* promastigotes in permissive species correlates with the presence of O-glycosylated epitopes on the luminal midgut surface (Myskova *et al.*, 2007; Volf & Myskova, 2007) which may serve as binding sites for lectin-like components found on the surface of parasites. Successful development of LPG-defective mutants in per-

missive sandfly species showed that the establishment of different *Leishmania* in permissive vectors does not arise from interactions of LPG with sandfly lectins. Instead, O-glycoproteins localized on the microvillar border of the midgut are involved in a novel mechanism of attachment as they bind to *Leishmania* promastigotes (Myskova *et al.*, 2007). The "adherence paradigm" described in the *P. papatasi-L. major* combination seems to be inverted in permissive species: N-acetylgalactosamine (GalNAc) containing glycoproteins of the sandfly react with a parasite lectin-like receptor. Potential candidates for this receptor are heparin-binding proteins and lectin-like molecules reported previously in various *Leishmania* species, some of which occur on the cell surface and bind GalNAc (Hernandez *et al.*, 1986; Mukhopadhyay *et al.*, 1989; Kock *et al.*, 1997; Svobodova *et al.*, 1997).

The presence of a conserved sandfly GalNAc ligand-parasite interaction in several permissive sandfly vectors explains successful adaptation of *Leishmania* to sandflies other than the specific ones. An important example is the introduction of *L. infantum* from the Mediterranean to Latin America (Killick-Kendrick *et al.*, 1980; Mauricio *et al.*, 2000). In Southern Europe, this parasite is transmitted to dogs and humans by the permissive vector *P. perniciosus* Newstead and related species of the subgenus *Larroussius* (Killick-Kendrick, 1999). When European colonists arrived with their dogs in Latin America, the parasite was able to switch to a new permissive vector, *Lu. longipalpis* (Killick-Kendrick *et al.*, 1980). Similar phenomena may underlie transmission cycles of atypical *L. tropica* strains. The ecology of emerging cutaneous leishmaniasis caused by *L. tropica* was studied in two adjacent foci in northern Israel (Jacobson *et al.*, 2003; Svobodova *et al.*, 2006). In both foci rock hyraxes (*Procavia capensis*) served as reservoirs. Hyraxes are susceptible to *L. tropica* and experimentally infected hyraxes were shown to be infective to feeding *P. (Adlerius) arabicus* Theodor and *P. sergenti* (Svobodova *et al.*, 2006). However, these two foci in Israel differed with respect to both parasite strains and vector species. The LPG of *L. tropica* from the northern focus was characterized by abundant terminal β-galactose residues on the side chains. On the other hand, LPG side chains of other *L. tropica* isolates are mostly capped with glucose (Soares *et al.*, 2004). Due to LPG modifications, this strain of *L. tropica* is able to develop only in the O-glycosylated midgut of the local permissive vector, *P. arabicus*, but not in the specific vector, *P. sergenti* (Svobodova *et al.*, 2006).

Another recent study that supports the hypothesis about the role of LPG in the attachment of *L. major* to *P. papatasi* midgut was performed on natural genetic hybrids between *L. infantum* and *L. major*. While all *Leishmania* strains tested (*L. major, L. infantum* and two hybrid strains) developed well in the permissive vector *L. longipalpis,* only *L. major* and the hybrid strains produced late-stage infections in *P. papatas*i. In contrast, *L. infantum* was defaecated from the midgut with the blood meal remains and did not develop further (Volf *et al.*, 2007). Indirect immunofluorescence showed that hybrids possess a certain level of *L. major*-type LPG which thus appears to enable the attachment of *P. papatasi* to the midgut (Volf *et al.*, 2007). The finding of the ability of *Leishmania* hybrids to develop in *P. papatasi* may have important epidemiological implications. This sandfly is peridomestic, antropophilic and widespread in much of southern Europe, North Africa and western Asia. The finding that *L. infantum/ L. major* hybrids develop heavy late stage infections in *P. papatasi* suggests that hybrid strains could circulate using this sandfly vector.

In addition, these experiments with hybrids rise questions about genetic exchange in *Leishmania*. Sexuality and genetic exchange remain an elusive point in this parasite. *Leishmania* have developed asexual mechanisms for generating a large repertoire of genotypes and these asexual mechanisms are believed to contribute efficiently to parasite fitness (reviewed by Victoir & Dujardin, 2002) and some authors postulated that parasites succeed better without sex (Ayala, 1998). However, our study showed that fitness of *L. infantum/ L. major* hybrids increased when compared with *L. infantum*. Genetic exchange enabled hybrid parasites to survive in the specific vector *P. papatasi* which means that sex positively affected parasite fitness.

ANTIBODIES AGAINST SANDFLY SALIVA: MARKER OF EXPOSURE TO SANDFLIES AND RISK MARKER OF *LEISHMANIA* TRANSMISSION

Repeated exposure to sandfly bites elicits cellular and humoral immune responses in the host (Barral *et al.*, 2000; Valenzuela *et al.*, 2001; Volf & Rohousova, 2001; Rohousova *et al.*, 2005; Gomes *et al.*, 2006). This immune response to salivary antigens was found to be species-specific. Sera of animals bitten by *P. papatasi* did not cross-react with saliva of other *Phlebotomus* species tested (Volf & Rohousova, 2001). Similarly, sera of humans bitten by *P. papatasi* and *P. sergenti* did not cross-react with *Lu. longipalpis* salivary antigens (Rohousova *et al.*, 2005).

Recent findings have stimulated interest in the use of anti-sandfly saliva antibody diagnostics as a novel tool for measuring exposure risk of *Leishmania* transmission among humans living in endemic regions. In such regions, host exposure to sandflies is an important epidemiological factor which could be used to estimate

the risk of parasite transmission and to measure the effect of insecticide campaigns on sandfly population size. Although methods of measuring the mass effect of control campaigns on sandfly survival and population size have been established (CDC traps, sticky traps) (Lewis & Ward, 1987), these cannot be employed in the field to determine individual protection of the host. Moreover, such a tool will be useful also for epidemiological analysis of risk factors.

To assess the feasibility of employing anti-saliva antibody response in vector exposure screening programmes, we studied the kinetics of anti-saliva antibody response in dogs experimentally exposed to *Lu. longipalpis*. Anti-saliva IgG and its subclasses IgG1 and IgG2 were found as useful markers of exposure to sandflies in experimentally exposed dogs as they reflected the intensity of exposure (numbers of bloodfed *Lu. longipalpis* females). Increased antibody levels and differences between high- and low-exposed dogs were detectable throughout the study, *i.e.* more than six months after the last exposure (Hostomska *et al.*, in press). In many foci of leishmaniasis, sandfly populations show seasonal fluctuations or sandfly-free periods and our results suggest the antibodies can persist until the next sandfly season. Screening of dog sera for specific IgG against salivary antigens of the vector was therefore suggested as a useful epidemiological tool in visceral leishmaniasis foci. Together with infection incidence monitoring, such data would provide valuable information on control programme effectiveness.

There are, however, two main obstacles to routine use of anti-saliva antibody screening . Dissection of large number of salivary glands is time-consuming, laborious, and it requires productive sandfly colonies. In addition, the number of different antigens in whole salivary gland homogenate increases the chance of nonspecific reactions, since some antigens are shared between different sandfly species (Volf & Rohousova, 2001; Rohousova *et al.*, 2005). To address these problems, a robust method for identification of immunodominant salivary proteins and their production as recombinant proteins was described (Valenzuela, 2002). Recently, seven recombinant salivary proteins from *L. longipalpis* were tested with human and fox sera from an area endemic for visceral leishmaniasis and with sera of dogs experimentally exposed to sandflies (Gomes *et al.*, 2007a). Some proteins were recognized by all tested sera which indicates the use of recombinant salivary proteins as potentially feasible epidemiological tool for evaluation of the exposure.

As anti-sandfly saliva antibodies are a good marker of exposure to sandfly bites, their possible use as a marker of risk of *Leishmania* transmission was tested. In an analogous setting, anti-vector saliva antibody response was suggested as a marker of risk of malaria or Lyme disease transmission (Remoue *et al.*, 2006; Schwartz *et al.*, 1991). However, in humans from endemic area for visceral leishmaniasis in Brazil, the presence of antibodies against *Lu. longipalpis* salivary antigens strongly correlated with the development of anti-*Leishmania* delayed type hypersensitivity (a marker of protection) while individuals that converted for anti-*Leishmania* antibodies did not recognize any sandfly salivary proteins (Gomes *et al.*, 2002). On the other hand, a positive correlation between infection and anti-saliva antibody titres was found in foci of cutaneous leishmaniases caused by *L. tropica* and *L. major* (Rohousova *et al.*, 2005; Louzir *et al.*, 2005) in Turkey and Tunisia, respectively. In Turkey, human sera were collected in an endemic focus of *L. tropica* where *P. sergenti* is the vector, while *P. papatasi* is refractory to this *Leishmania* species. In comparison with non-infected individuals from the same place, patients with active lesions had stronger antibody response to saliva of the vector *P. sergenti* while no correlation was found with antibody response to the non-vectorial species *P. papatasi* (Rohousova *et al.*, 2005).

CONCLUSION

In conclusion, two independent studies on cutaneous leishmaniases caused by different *Leishmania* species revealed that antibodies against salivary proteins of the vector could be used as a marker of risk of *Leishmania* transmission. Studies on sandflies gave the first direct evidence that antibodies against saliva of the vector could give a measure of the contact between a vector and a human population in an endemic area and thus may provide an estimate of the risk of transmission of a vector-borne disease.

ACKNOWLEDGEMENTS

We are grateful to Professor R. Killick-Kendrick for valuable comments to the manuscript. Authors are supported by the Ministry of Education (MSM0021620828, LC06009), Czech Science Foundation (206/06/0967, 206/06/P015) and GAUK (139/2006/B-Bio/PrF, 191/2006/B-Bio/PrF).

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Title Page

Hyaluronidase of Bloodsucking Insects and its Enhancing Effect on *Leishmania* Infection in Mice.

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Running Head: Hyaluronidase Effect on Leishmania infection

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ABSTRACT

BACKGROUND: Salivary hyaluronidases have been described in a few blood sucking arthropods. However, very little is known about the presence of this enzyme in various blood-sucking insects and no data are available on its effect on transmitted microorganisms. Here, we studied hyaluronidase activity in thirteen bloodsucking insects belonging to four different orders. In addition, we assessed the effect of hyaluronidase coinoculation on the outcome of *Leishmania major* infection in BALB/c mice.

PRINCIPAL FINDINGS**:** High hyaluronidase activity was detected in several Diptera tested, namely deer fly *Chrysops viduatus*, blackflies *Odagmia ornata* and *Eusimilium latipes*, mosquito *Culex quinquefasciatus*, biting midge *Culicoides kibunensis* and sand fly *Phlebotomus papatasi*. Lower activity was detected in cat flea *Ctenocephalides felis*. No activity was found in kissing bug *Rhodnius prolixus*, mosquitoes *Anopheles stephensi* and *Aedes aegypti*, tse-tse fly *Glossina fuscipes*, stable fly *Stomoxys calcitrans* and human louse *Pediculus humanus*. Hyaluronidases of different insects vary substantially in their molecular weight, the structure of the molecule and the sensitivity to reducing conditions or sodium dodecyl sulphate. Hyaluronidase exacerbates skin lesions caused by *Leishmania major*, more severe lesions developed in mice where *L. major* promastigotes were coinjected with hyaluronidase.

CONCLUSIONS**:** High hyaluronidase activities seem to be essential for insects with pool-feeding mode, where they facilitate the enlargement of the feeding lesion and serve as a spreading factor for other pharmacologically active compounds present in saliva. As this enzyme is present in all *Phlebotomus* and *Lutzomyia* species studied to date, it seems to be one of the factors responsible for enhancing activity present in sand fly saliva. We propose that salivary hyaluronidase may facilitate the spread of other vector-borne microorganisms, especially those transmitted by insects with high hyaluronidase activity, namely blackflies (Simuliidae), biting midges (Ceratopogonidae) and horse flies (Tabanidae).

AUTHOR SUMMARY (for general audience)

Hyaluronidases are enzymes degrading the extracellular matrix of vertebrates. Bloodsucking insects use them to cleave the skin of the host, enlarge the feeding lesion and acquire the blood meal. In addition, resulting fragments of extracellular matrix modulate local immune response of the host, which may positively affect transmission of vector-borne diseases,

including leishmaniasis. Leishmaniases are diseases with a wide spectrum of clinical forms, from a relatively mild cutaneous affection to life-threatening visceral disease. Their causative agents, protozoans of the genus *Leishmania,* are transmitted by phlebotomine sand flies. Sand fly saliva was described to enhance *Leishmania* infection, but the information about molecules responsible for this exacerbating effect is still very limited. In the present work we demonstrated hyaluronidase activity in salivary glands of various Diptera and in fleas. In addition, we showed that hyaluronidase exacerbates *Leishmania* lesions in mice and propose that salivary hyaluronidase may facilitate the spread of other vector-borne microorganisms.

INTRODUCTION

Hyaluronidases are a family of enzymes that degrade hyaluronan (HA) and several other glycosaminoglycan constituents of the extracellular matrix of vertebrates [for review see 1]. In insects, hyaluronidases are well-known from venoms of Hymenoptera and represent clinically important allergens of honey-bees, wasps and hornets [2-4]. Hyaluronidases were found also in cDNA libraries of salivary glands (sialomes) of various bloodsucking insects [5-8] and the enzyme activity was found in saliva of three groups of Diptera, namely sand flies, blackflies, and horse flies [9,10]. Salivary hyaluronidases of parasitic insects may have diverse effects on the host. They play an important role in blood meal acquisition; by degrading HA abundant in host skin, hyaluronidases increase tissue permeability for other salivary components that serve as antihaemostatic, vasodilatory or anti-inflammatory agents [5, 9]. This is why hyaluronidases are frequently called “spreading factors“ [11]. The enzyme activity facilitates the enlargement of the feeding lesion and the insect acquires the blood meal more rapidly. In addition, HA fragments were shown to have immunomodulatory properties; they affect maturation and migration of dendritic cells, induction of iNOS and chemokine secretion by macrophages and proliferation of activated T cells [reviewed in 12]. As blood sucking insects represent the most important vectors of infectious diseases, local immunomodulation of the vertebrate host may positively enhance the infection.

Leishmaniasis is one of the most prevalent vector-borne diseases. It is initiated by the intradermal inoculation of *Leishmania* promastigotes during the bite of an infected sand fly (Diptera: Phlebotominae). As shown first by Titus and Ribeiro [13] saliva of the sand fly vector exacerbates the initial phase of *Leishmania* infections in terms of parasite burden and size of the cutaneous lesion. Sand fly saliva was described to contain an array of pharmacologically active compounds affecting host hemostasis and immune mechanisms

[reviewed in 14, 15] but the information about molecules responsible for the exacerbating effect is still very limited. Morris et al. [16] showed that maxadilan, a well-known vasodilator of the New World vector *Lutzomyia longipalpis*, exacerbates *Leishmania* infection to the same degree as whole saliva. Maxadilan inhibits splenocyte proliferation induced *in vitro* and delayed type hypersensitivity in mice [17] and it also has several inhibitory effects on macrophages and monocytes that would support *Leishmania* survival in the host [18]. However, this important peptide was not found in Old World vectors of genus *Phlebotomus* (www.ncbi.nih.gov), including *P. papatasi* where exacerbating effect of saliva was repeatedly demonstrated [19, 20]. The vasodilatory activity of *P. papatasi* was instead ascribed to adenosine and AMP present in saliva of this sand fly [21].

In the present work, we studied hyaluronidase activity in bloodsucking insects of four different orders. In addition, we assessed the effect of hyaluronidase coinoculation on the outcome of *Leishmania major* skin lesions and spreading into draining lymph nodes.

MATERIALS AND METHODS

Insects and preparation of samples. Samples used are summarized in Table 1. The insects originated from laboratory colonies or were collected in the wild. Salivary glands were dissected out in Tris buffer (20 mM Tris, 150 mM NaCl, pH 7.8) and stored in batches (usually 20 glands in 20 μl of Tris buffer) at –70 °C. Where dissection of salivary glands was not feasible, whole bodies (*Ctenocephalides* flea, *Culicoides* midge) or the thoracic parts containing salivary glands (*Pediculus* louse) were used at protein concentration 20 μg/μl. Salivary gland extracts (SGE) or body extracts (BE) were obtained by disruption of tissue by three freeze-thaw cycles in liquid nitrogen, homogenization and centrifugation at 12,000 g for 5 min. Protein concentration was determined by Bradford assay using bovine serum albumin in Tris buffer as a standard.

Detection of hyaluronidase activity. Enzyme activity was detected by the dot method on 10% polyacrylamide gels with copolymerized hyaluronic acid (HA, potassium salt, from human umbilical cord, ICN Pharmaceutical, CA). Gels were prepared using 0.1M acetate, pH 5.5, containing 0.1 M NaCl, 0.05 % Tween-20 and 0.002% HA. This method was previously proved as sensitive and reproducible [10]. Preliminary experiment with selected salivary extracts revealed that *Phlebotomus papatasi* and *Culex pipiens* samples were positive at pH 4.5, 5.5, 6.5 and 7.5 while *Aedes aegypti*, *Anopheles stephensi* and *Glossina fuscipes* samples

were consistently negative (Fig. S1). Therefore pH 5.5 was chosen for this assay as this is about the pH optimum known for salivary hyaluronidases of various Diptera [9, 10]. Insect samples (2 μl volume) were dotted on the gel and sheep testicular hyaluronidase, (Sigma, 1 μg in 1 μl) was used as a control. Incubation was carried out for 24 hrs at 37 °C in a moist chamber. The gels were then washed in water, soaked in 50% formamide for 30 min and stained in Stains-all (Sigma) solution (100 μg/ml in 50% formamide) for 24 hrs in the dark. After a rinse in distilled water the gels were scanned and photographed. To determine whether the enzyme activity was specific for cleaving HA, we tested positive samples also with another component of extracellular matrix, chondroitin sulfate. The method was performed as described above, only HA was replaced by 0.002% chondroitin sulfate (Sigma).

Electrophoretic analysis of hyaluronidase activity. Electrophoresis (SDS PAGE) was carried out on 10% slab gels (0.75 mm thick) using Mini-Protean II apparatus (Biorad) and constant voltage 150 V. Substrate gels were copolymerized with 0.002% HA. As the hyaluronidase activities and band patterns varied among insects, different loads were used per lane in order to obtain bands of equal intensity. Following electrophoresis, gels were rinsed 2×20 min in 0.1 M Tris, pH 7.8, 20 min in 0.1 M acetate buffer, pH 5.5 (both with 1% Triton X-100 to wash out SDS) and then incubated in 0.1 M acetate buffer (without detergent) for 120 min at 37 ºC. After rinsing in water the gels were stained with Stains-all as described above. Hyaluronidase activity was visible as a pink band on a dark blue background.

Hyaluronidase effect on *Leishmania* infection in mice. Experiments on mice were done in accordance with Czech Act No. 246/1992 and approved by IACUC of the Fac. Sci., Charles University in Prague. A mouse ear infection model [19] was used to assess the effect of hyaluronidase coinoculation on the outcome of *Leishmania* infection. *Leishmania major* clone LV561 (MHOM/IL/67/LRC-L137 Jericho II) was cultured on blood agar from defibrinated rabbit blood, supplemented with 50 μg/ml gentamicin. Female BALB/c mice (Charles River Deutschland, Sulzfeld, Germany) were used at the age of 8 weeks. Ether-anaesthetized mice were inoculated in the ear dermis with 10^4 or 10^5 *L. major* stationary-phase promastigotes (subculture 1) in 5 μl sterile saline. The inoculum also contained bovine testicular hyaluronidase (Sigma) in an amount equivalent to 2 or 10 “optimal salivary glands” of *Phlebotomus papatasi* [10], i.e. 0.4 and 2.0 relative turbidity reducing units, respectively. Bovine testicular hyaluronidase belongs to the same enzyme class as the sand fly salivary

hyaluronidases [10] and shares sequence homology with the enzyme of *L. longipalpis* [5]. Control animals were inoculated with parasites in sterile saline only. Sixty mice (10 for each of six groups) were used for Q-PCR and another 48 (8 for each group) for lesion monitoring. The size of skin lesions was measured weekly using a Vernier caliper gauge. Lesions were monitored for 6 weeks post infection: the area was calculated from two perpendicular measurements as an ellipse area, and its appearance (degree of ulceration) was assessed using an arbitrary scale from 1 to 5 (1 - low induration, 2 - high induration, 3 - small ulcer, 4 - large ulcer, 5 - perforated ear pinna). Independently in both parasite doses (10^4 and 10^5), the significance of the hyaluronidase effect was tested using nonparametric Kruskal-Wallis ANOVA and post hoc comparisons of mean ranks using Statistica 7 routines [22]. The tests were performed separately for weeks 3, 4, 5, and 6 post-infection; the size of a lesion was calculated as its area weighted by the degree of ulceration.

Detection and quantification of *Leishmania* parasites in mice. Mice were sacrificed 24 hrs post inoculation (p.i.) as the preliminary experiment revealed that lymph nodes of mice dissected 24 hours p.i. gave more consistent results than those dissected 48 hours p.i. (Fig. S2). Parasite numbers in draining retromaxillar lymph nodes were determined by quantitative PCR (Q-PCR) as described earlier [23]. Briefly, dissected lymph nodes were stored in 10 μl saline at –70 °C. Total DNA was isolated from homogenised samples using High Pure PCR Template Preparation Kit (Roche); kinetoplast DNA was targeted using primers described elsewhere [24]. The relative effectiveness of three hyaluronidase doses (equivalent to 0, 2, and 10 *P. papatasi* salivary glands) with both infection doses (10^4 and 10^5 parasites) was evaluated by analysis of variance (Statistica v. 7.1, factorial and one-way ANOVA).

RESULTS

Detection of hyaluronidase activity. The dot method on gels with copolymerized HA and chondroitin sulfate was used to study the presence of hyaluronidase activity and its substrate specificity. The highest hydrolysis of HA was observed in SGE of deer fly *Chrysops viduatus*. Pronounced hydrolysis was found in SGEs of blackflies *Odagmia ornata* and *Eusimulium latipes*, mosquito *Culex quinquefasciatus*, sand fly *Phlebotomus papatasi* and whole body extract of biting midge *Culicoides kibunensis* (syn. *C. cubitalis*). Lower activity was detected in BE of cat flea *Ctenocephalides felis* (Fig. 1). On the other hand, no detectable hydrolysis of HA occurred in SGEs of kissing bug *Rhodnius prolixus*, mosquitoes *Anopheles*

stephensi and *Aedes aegypti*, tse-tse fly *Glossina fuscipes*, stable fly *Stomoxys calcitrans* and in thoracic extracts of human louse *Pediculus humanus* (Fig. 1). Positive samples were then tested also for chondroitin sulfate hydrolysis (Fig. 2). High activity was observed in *Culex quinquefasciatus* and *Culicoides kibunensis*, in other samples the hydrolysis of chondroitin sulfate was either moderate (*Chrysops viduatus*) or low (*Phlebotomus papatasi*, *Ctenocephalides felis*) (Fig. 2); clearly, HA is the preferred substrate for the enzymes of these three insects.

Electrophoretic analysis of hyaluronidase activity. Seven samples positive in the dot method were analyzed by zymography to reveal the apparent molecular weight (MW) of hyaluronidases. The MW of the enzymes differed among various insects (Figs. 3 and 4). Under nonreducing conditions hyaluronidases were detected as major diffuse bands (Fig. 3). The SGE activity in *Phlebotomus papatasi* had a MW about 70 kDa while those in both blackfly species tested, *Eusimulium latipes*, and *Odagmia ornata*, about 40 kDa. In BE of *Culicoides kibunensis*, the major band of about 35 kDa was accompanied with a minor one of 70 kDa, supposedly a dimer. *Chrysops viduatus* SGE revealed one major band with estimated MW of 50 kDa. In BE of flea *Ctenocephalides felis*, three enzyme bands were detected, the most prominent one of about 52 kDa (Fig. 3). Under reducing conditions, SDS PAGE revealed sharper enzyme bands allowing more precise assignment of corresponding MW (Fig. 4). In sand fly *P. papatasi*, both blackfly species and deer fly *Chrysops viduatus*, hyaluronidase activity was observed within the same MW ranges as under nonreducing conditions (70, 40 kDa, and 50 kDa, respectively). In *Culicoides kibunensis* and *Ctenocephalides felis* hyaluronidase activity was not detectable under reducing conditions (Fig. 4).

No hyaluronidase activity was detected in *Culex quinquefasciatus* SGE under either zymography conditions used, reducing and nonreducing. An additional experiment was performed to explain the contradictory results from the dot method and zymography; SGE of *C. quinquefasciatus* was dotted on the gel with copolymerized HA with and without the presence of SDS. Hydrolysis was observed only in the sample without SDS (Fig. 5).

Hyaluronidase effect on *Leishmania* infection in mice. Next we examined whether hyaluronidase altered the course of *Leishmania major* infection in BALB/c mice. We used intradermal inoculation into the ear and the disease burden was calculated from weekly measuring the lesion size. As shown in Fig. 6, mice coinjected with parasites and

hyaluronidase developed bigger lesions. In all groups of mice, the onset of lesion development was at three weeks p.i. Thereafter, the lesions grew faster in coinoculated groups. The experiment was terminated six weeks post infection when, in some animals, ulcerating lesion spread over the majority of ear pinna. In mice inoculated by higher parasite numbers (10^5), both hyaluronidase treatments produced similar effects (Fig. 6A). In mice with an inoculation dose one order of magnitude lower (10^4), the effect of hyaluronidase was concentration-dependent: lesions were bigger in mice coinoculated with hyaluronidase activity equivalent of 10 *P. papatasi* salivary glands than in those coinoculated with equivalent of 2 glands (Fig. 6B). In both parasite numbers (10^4 and 10^5) over all considered weeks (3 to 6) post-inoculation, Kruskal- Wallis ANOVA showed significant differences among hyaluronidase treatments (p always ≤ 0.025), with only one exception in week 3 of 10^4 parasites treatment ($p = 0.23$). Consequently, the post-hoc comparison of treatments tests confirmed the significant difference between controls (no hyaluronidase) and corresponding inoculated hyaluronidase doses (2 or 10 glands equivalents). We also tested the difference between the 2 and 10 gland equivalent doses: however, despite the common trends apparent in Fig. 6 indicating that there may be a systematic difference between 2 and 10 gland equivalents doses, the post-hoc comparison of treatments test did not prove it in any case but in week 5 of the 10^4 parasites treatment.

We also examined whether hyaluronidase affected *Leishmania major* load in draining lymph nodes of BALB/c mice one day p.i. Using Q-PCR, no significant differences were observed among control and experimental groups of mice at both parasite doses (10^4 or 10^5 *L. major*) tested ($F_{(2, 54)} = 0.043$; $p = 0.96$) (Fig. S3).

DISCUSSION

Parasitic insects utilize two strategies for finding blood: solenophagy (or vessel feeding) and telmophagy (or pool feeding). In solenophagic approach, the feeding fascicle cannulates a blood vessel, while in the pool-feeding mode the mouth part stylets slash through the skin, and the insect sips blood that oozes out from the hemorrhage. In our experiments, pronounced hyaluronidase activity was found in black flies, biting midges, sand flies and deer flies. All these insects belong to parasitic Diptera with pool-feeding mode of blood meal acquisition. The activity was detected also in cat flea (*Ctenocephalides felis*, Siphonaptera) and in *Culex quinquefasciatus* mosquito (Diptera). Although these two species belong to different insect orders, they are both vessel feeders. In contrast, no activity was detected in

other vessel-feeding insects: human lice, kissing bugs, *Anopheles* and *Aedes* mosquitoes, tsetse flies, and stable flies.

Hyaluronidase activity was previously detected in the saliva of various sand fly species [9, 10] as well as in the saliva of the black fly *Simulium vittatum* [9] and horse fly *Tabanus yao* [8]. Sequences predicted to code for hyaluronidases were found in the salivary transcriptomes of the mosquito *Culex quinquefasciatus* [6] and the biting midge *Culicoides sonorensis* [7]. Herein, we demonstrated that *Culex quinquefasciatus* and *Culicoides kibunensis* possess hyaluronidase activity and, in parallel experiments, we detected hyaluronidase activity in saliva of two other species of biting midges *Culicoides sonorensis* and *C. nubeculosus* (Volfova et al., unpublished). Therefore, we showed that in biting midges and in *Culex quinquefasciatus,* the transcripts coding for putative hyaluronidases are translated into functional enzymes.

To determine whether the enzyme activity was specific for cleaving HA, we also tested another component of mammalian extracellular matrix, chondroitin sulfate. All hyaluronidase-positive samples tested cleaved chondroitin sulfate, which would indicate that insect hyaluronidases fall into the same class as mammalian hyaluronidases (E.C. 3.2.1.35 according to IUBMB Enzyme Nomenclature) [25]. Indeed, sequence analysis of transcripts putatively coding for hyaluronidase enzymes reveals their homology to mammalian enzymes [6, 7, 9]. While HA was found as the preferred substrate for most samples tested, very high hydrolysis of chondroitin sulfate was found in *Culex quinquefasciatus* SGE. This mosquito species differs from other samples tested also in other aspects. In zymography assay, salivary hyaluronidase of *Culex quinquefasciatus* was irreversibly sensitive to denaturation effect of SDS while enzymes of other insects tested refolded and regained activity after removal of the denaturating agent. Further work is needed to understand the differences in the molecular structure and substrate specificity of hyaluronidases from *Culex* mosquitoes versus other bloodsucking insects. In addition, in other mosquitoes studied, *Anopheles darlingi* [26], *funestus* [27] and *gambiae* [28] and *Aedes aegypti* [29] and *albopictus* [30], neither hyaluronidase activity nor hyaluronidase gene was found in salivary transcriptomes. Sequences homologous to hyaluronidase were, however, found by genome sequencing in *Anopheles gambiae* [31] and *Aedes aegypti* [32].

As revealed by zymography, hyaluronidases of different insect species tested vary substantially in MW and the structure of the molecule. Putative oligomers were seen in *Culicoides kibunensis*. Oligomeric forms have been found frequently among mammalian hyaluronidases. In sand flies, oligomers or dimers were found in *Lutzomyia longipalpis*,

Phlebotomus papatasi, and *P. sergenti* [10]. Multiple bands observed by zymography in *Ctenocephalides felis* whole body extract could, however, represent multiple hyaluronidase enzymes.

In *Culicoides* and *Ctenocephalides*, reducing conditions affected the stability of the enzymes; 2-mercaptoethanol inhibited hyaluronidase activity. In *Culicoides* midges, the sensitivity of hyaluronidase activity to reducing conditions was confirmed by experiments with pure saliva of laboratory bred *Culicoides sonorensis* and *C. nubeculosus* (Volfova et al., unpublished). This implies that reduction-sensitive residues are either important for the function of the active site of the enzyme, or steric relations in the molecule. On the other hand, hyaluronidases of other insects tested, namely *Phlebotomus papatasi*, *Eusimulium latipes*, *Odagmia ornata*, and *Chrysops viduatus*, remained active under reducing conditions. Addition of 2-mercaptoethanol did not result in differences in the apparent MW, suggesting that the enzymes consist of a single polypeptide chain. These results correspond with previous observations on sand flies; sand fly hyaluronidases strikingly differed in structure and sensitivity to reducing conditions, even among various species of the genus *Phlebotomus* [10].

We showed that hyaluronidase is a common constituent of saliva of bloodsucking insects. It seems to be essential for insects with pool-feeding mode, where it facilitates the enlargement of the feeding lesion, serving as a spreading factor for other pharmacologically active compounds present in saliva. Very little is known, however, about the possible role of salivary gland hyaluronidase in allergic reactions which occur in some patients after repeated bites of bloodsucking Diptera. In Hymenoptera, venom hyaluronidase is largely responsible for the cross-reactivity of venoms with sera of allergic patients [4]. In several patients, coexistent anaphylaxis to Hymenoptera sting and Diptera bite was described [33] and hyaluronidase is a candidate allergen responsible for this type of crossreactions. In experiments of Sabbah et al. [34, 35], IgE of allergic patients recognized shared proteins within MW range 44-50 kDa between wasp venom and total extracts of mosquito and horse fly. Unfortunately, these interesting data are difficult to assess given the incomplete identitification of the mosquito and horse fly species tested.

A mouse ear infection model was used to assess the effect of hyaluronidase coinoculation on the outcome of *Leishmania major* infection. The activity of sand fly enzyme was mimicked by commercially available bovine hyaluronidase. More severe lesions developed in mice where *L. major* promastigotes were coinjected with hyaluronidase. Even the lower dose of the enzyme corresponding with the activity produced by 1–2 sand fly females resulted in significant differences against the control mice where parasites alone were

injected. It would be worth testing if differences observed in lesion size are mainly due to number of parasites or to inflammatory response to coinoculated hyaluronidase. In contrast, there was neither more rapid onset of lesions, nor faster dissemination of *Leishmania* in the lymph node. Parasite numbers in draining lymph nodes collected 24 and 48 hrs p.i. were similar in all experimental groups. Although hyaluronidase activity exacerbated *Leishmania* lesions in the skin, it did not support its visceralization. However, we can not exclude the possibility that consequences of hyaluronidase for parasite visceralization are not immediate and thus could not be detected in the present study.

The way by which hyaluronidase enhances the establishment of *Leishmania* is unknown, but we suggest that it is due to HA fragments generated by hyaluronidase activity in the host skin. HA occurs in two main forms: the high MW (HMW) polymers and the low MW (LMW) fragments. HMW HA is a common component of vertebrate extracellular matrix. LMW HA fragments are generated under inflammatory conditions by endogenous or bacterial hyaluronidases [36], or non-enzymatically by free radicals [37]. HA fragments have diverse immunomodulatory properties; they affect DC maturation, T cell proliferation, cytokine, and chemokine synthesis by lymphocytes and macrophages [reviewed in 12]. Thus, following injury or infection, HA fragments have been implicated as both endogenous and exogenous triggers of repair and/or defense mechanisms [38, 39] and might truly represent a "danger signal" [40]. *Leishmania* parasites, however, may profit from the local increase of HA fragments. Specifically, endothelial cells were shown to respond to LMW HA by IL-8 production [38] that results in neutrophil recruitment. As neutrophil granulocytes were indicated as Trojan horses enabling *Leishmania* silent entry into macrophages [41] their accumulation at the site of sand fly bite might promote infection establishment.

In conclusion, we demonstrated that hyaluronidase promotes *Leishmania* establishment in murine skin. As this enzyme is present in all *Phlebotomus* and *Lutzomyia* species studied to date [10] it seems to be one of the factors responsible for enhancing activity present in saliva of the New-World as well as the Old-World sand flies. We propose that hyaluronidase, in concert with other insect-derived molecules, may facilitate the spread of other vector-borne diseases, especially those transmitted by vectors with high hyaluronidase activity in saliva, namely blackflies, biting midges, deer flies and horse flies.

ACKNOWLEDGEMENTS

We thank Prof. M. Lehane, Prof. P. Billingsley and Dr. N. Mencke who kindly provided parasitic arthropods, Dr. J.M.C. Ribeiro for valuable comments and Dr. P. Cerna for help with statistical evaluation of the experiments. The work was supported by Czech Science Foundation (206/06/0967), Ministry of Education (MSM0021620828, LC06009) and Charles University (GAUK 139/2006/B-BIO/PrF). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

COMPETING INTERESTS. The authors have declared that no competing interests exist.

AUTHOR CONTRIBUTIONS. VV, JH, JV and PV designed and performed the experiments and also wrote the paper. MC analyzed the data and revised the article.

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FIGURE LEGENDS

Fig. 1. Hyaluronidase activity of insects tested by dot method on polyacrylamide gel with incorporated hyaluronan. Protein content per 2 µl dot is indicated in brackets. Tb = Tris buffer, Sh = sheep testicular hyaluronidase (10 µg), Rp = *Rhodnius prolixus* SGE (20 µg), Ph = *Pediculus humanus* BE (20 µg), Cf = *Ctenocephalides felis* BE (20 µg), Pp = *Phlebotomus papatasi* SGE (0.8 µg), Ck = *Culicoides kibunensis* BE (20 µg), As = *Anopheles stephensi* SGE (0.8 µg), Cq = *Culex quinquefasciatus* SGE (0.8 µg), Aa = *Aedes aegypti* SGE (1.3 µg), Oo = *Odagmia ornata* SGE (0.8 µg), El = *Eusimulium latipes* SGE (1.7 µg), Cv = *Chrysops viduatus* SGE (3.5 µg), Gf = *Glossina fuscipes* SGE (14 µg), Sc = *Stomoxys calcitrans* SGE (2.4 µg).

Fig. 2. Substrate specificity of hyaluronidases tested on polyacrylamide gel with incorporated chondroitin sulfate. Protein content per 2 µl dot is indicated in brackets. Sh = sheep testicular hyaluronidase (10 µg), Cf = *Ctenocephalides felis* SGE (20 µg), Cq = *Culex quinquefasciatus* SGE (0.8 µg), Ck = *Culicoides kibunensis* BE (20 µg), Pp = *Phlebotomus papatasi* SGE (0.8 µg), Cv = *Chrysops viduatus* SGE (0.8 µg).

Fig. 3. SDS PAGE zymography under nonreducing conditions on polyacrylamide gel with incorporated hyaluronan. Protein content per lane is given in brackets. Pp = *Phlebotomus papatasi* SGE (0.2 µg), Ck = *Culicoides kibunensis* BE (10 µg), Cq = *Culex quinquefasciatus* SGE (5.6 µg), El = *Eusimulium latipes* SGE (0.4 µg), Oo = *Odagmia ornata* SGE (0.4 µg), Cv = *Chrysops viduatus* SGE (0.2 µg), Cf = *Ctenocephalides felis* BE (15 µg).

Fig. 4. SDS PAGE zymography on the same gel as in Fig. 3. but under reducing conditions. Pp = *Phlebotomus papatasi* SGE (0.2 µg), Ck = *Culicoides kibunensis* BE (20 µg), Cq = *Culex quinquefasciatus* SGE (8 µg), El = *Eusimulium latipes* SGE (3 µg), Oo = *Odagmia ornata* SGE (3 µg), Cv = *Chrysops viduatus* SGE (0.3 µg), Cf = *Ctenocephalides felis* BE (15 µg).

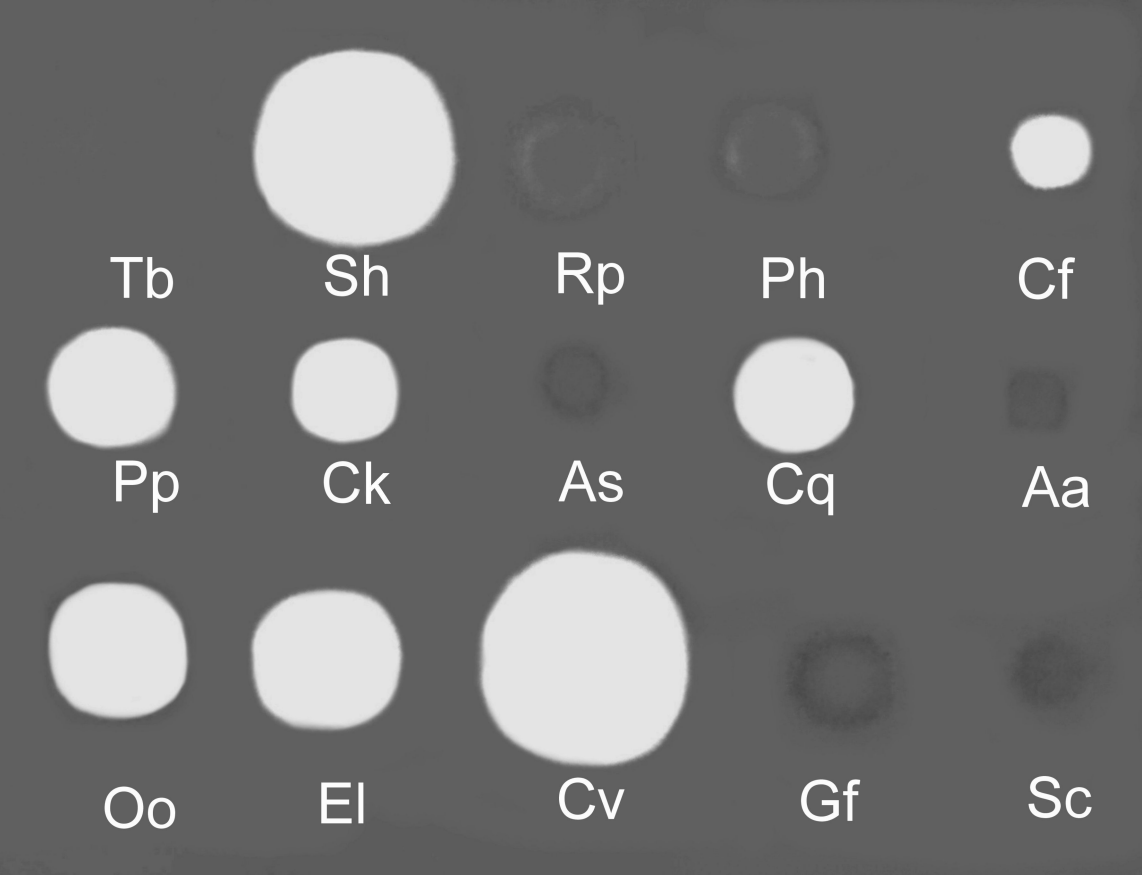
Fig. 5. Effect of SDS on hyaluronidase activity in SGE of *Culex quinquefasciatus*. Activity of hyaluronidase was tested by dot method on polyacrylamide gel with incorporated 0.002% hyaluronan and 0.001% SDS. Protein content per 2 µl dot is indicated in brackets. Sh = sheep testicular hyaluronidase (2 µg), Pp = *Phlebotomus papatasi* SGE (0.8 µg), Cq1 = *Culex quinquefasciatus* SGE (1.6 µg), Cq2 = *Culex quinquefasciatus* SGE (3.2 µg).

Fig. 6. Effect of hyaluronidase on *Leishmania* infection in mice. BALB/c mice were coinoculated intradermally into ear with 10^4 or 10^5 *Leishmania major* and hyaluronidase equivalent to 0, 2 and 10 salivary glands of *Phlebotomus papatasi*. Lesion size, given as a product of its area (mm^2) and the degree of ulceration (1-5), was monitored for 6 weeks post infection. Points (■) = mean values, boxes = 95% confidence intervals, whiskers = min-max values. The *p* values of corresponding Kruskal-Wallis ANOVA are provided.

Table 1. Origin of insects used and the estimated protein content per one salivary gland.

SGE – salivary gland extract, BE – body extract, TE – thoracic extract, nd. – not determined.

Sample	Origin	µg protein per salivary gland
Aedes aegypti SGE	colony, Inst. Parasitol., Ceske Budejovice, CZ	0.67
Anopheles stephensi SGE	colony, University of Aberdeen, UK	0.41
Ctenocephalides felis BE	colony, Bayer CropScience, Germany	nd.
Culicoides kibunensis BE	field, Prague, CZ	nd.
Culicoides pictipennis BE	field, Prague, CZ	nd.
Culex quinquefasciatus SGE	colony, Dept. Parasitol., Charles University, CZ	0.39
Eusimulium latipes SGE	field, Prague, CZ	0.83
Glossina fuscipes SGE	colony, CSIRA, Montpellier, Francie	7
Chrysops viduatus SGE	field, Veseli, CZ	6.9
Odagmia ornata SGE	field, Veseli, CZ	nd.
Pediculus humanus TE	colony, Bayer CropScience, Germany	nd.
Phlebotomus papatasi SGE	colony, Dept. Parasitol., Charles University, CZ	0,4
Rhodnius prolixus SGE	colony, Inst. Parasitol., Ceske Budejovice, CZ	20.4
Stomoxys calcitrans SGE	colony, University of Wales, UK	1.2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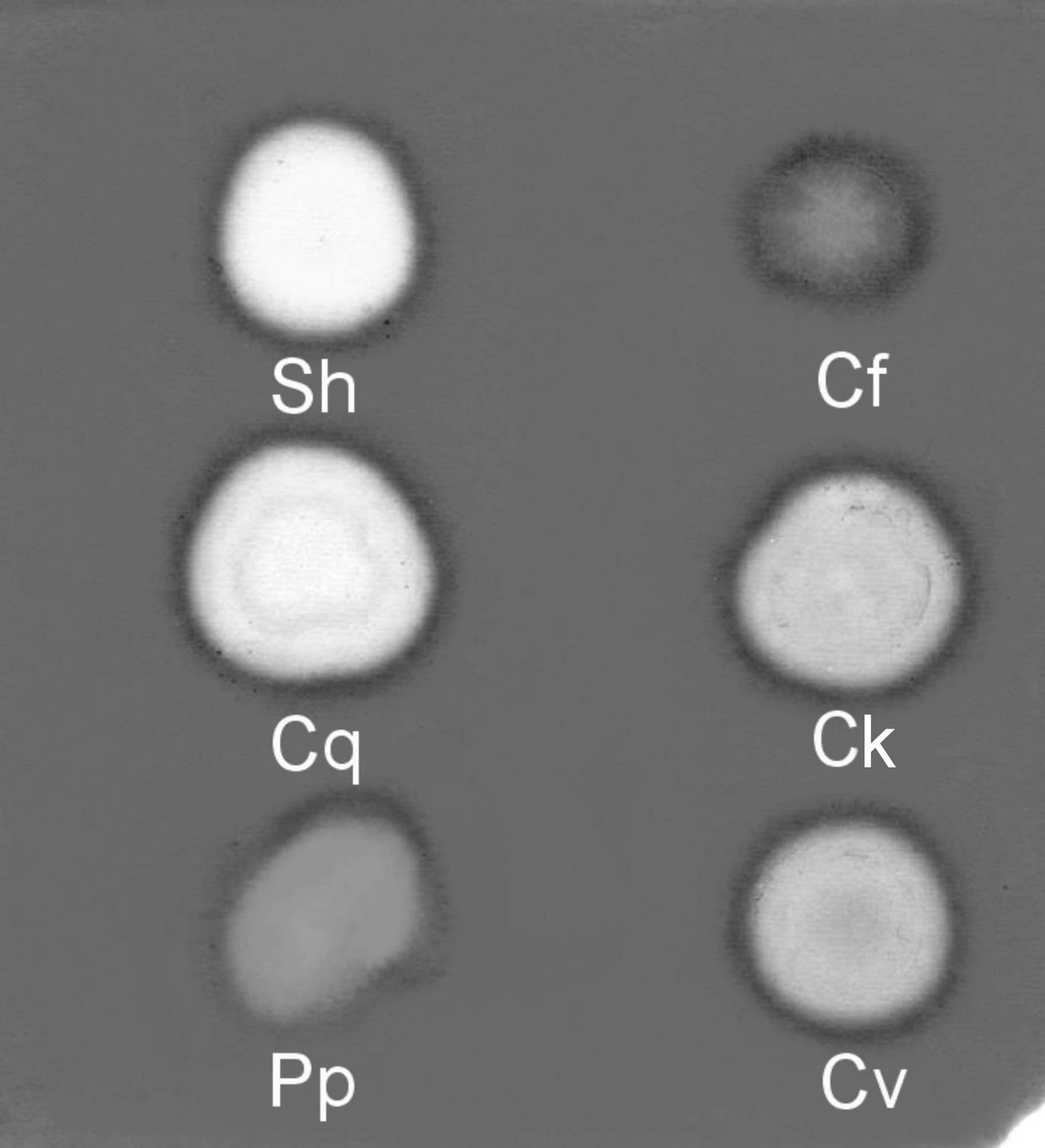
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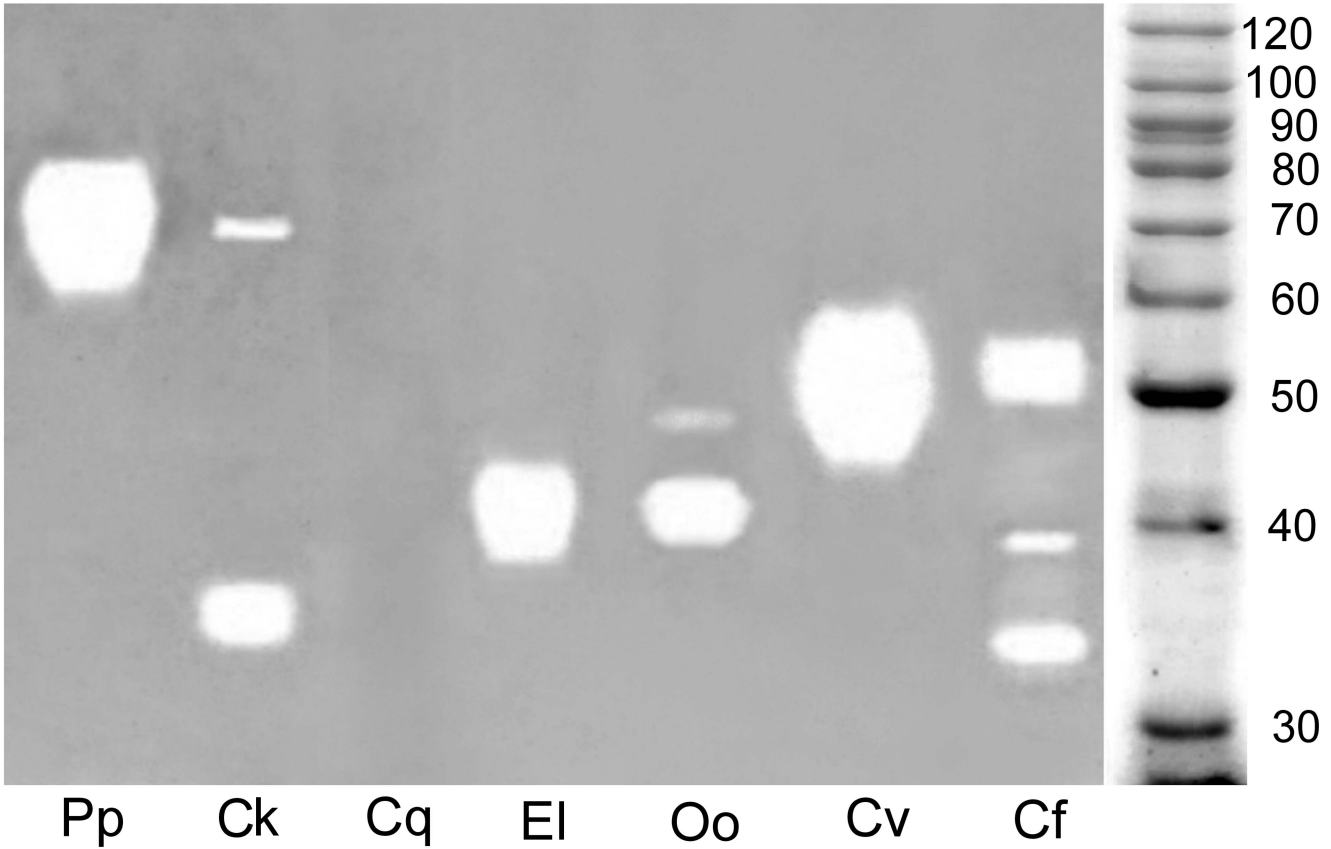
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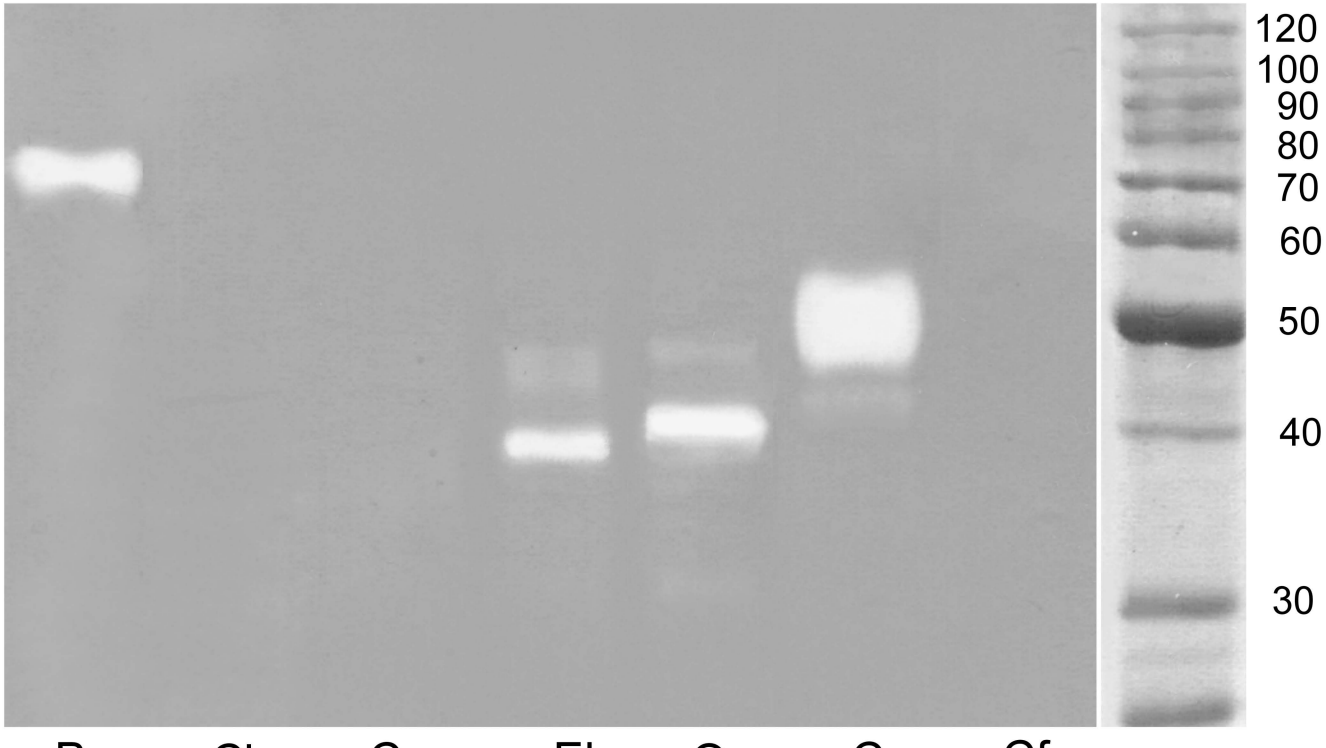
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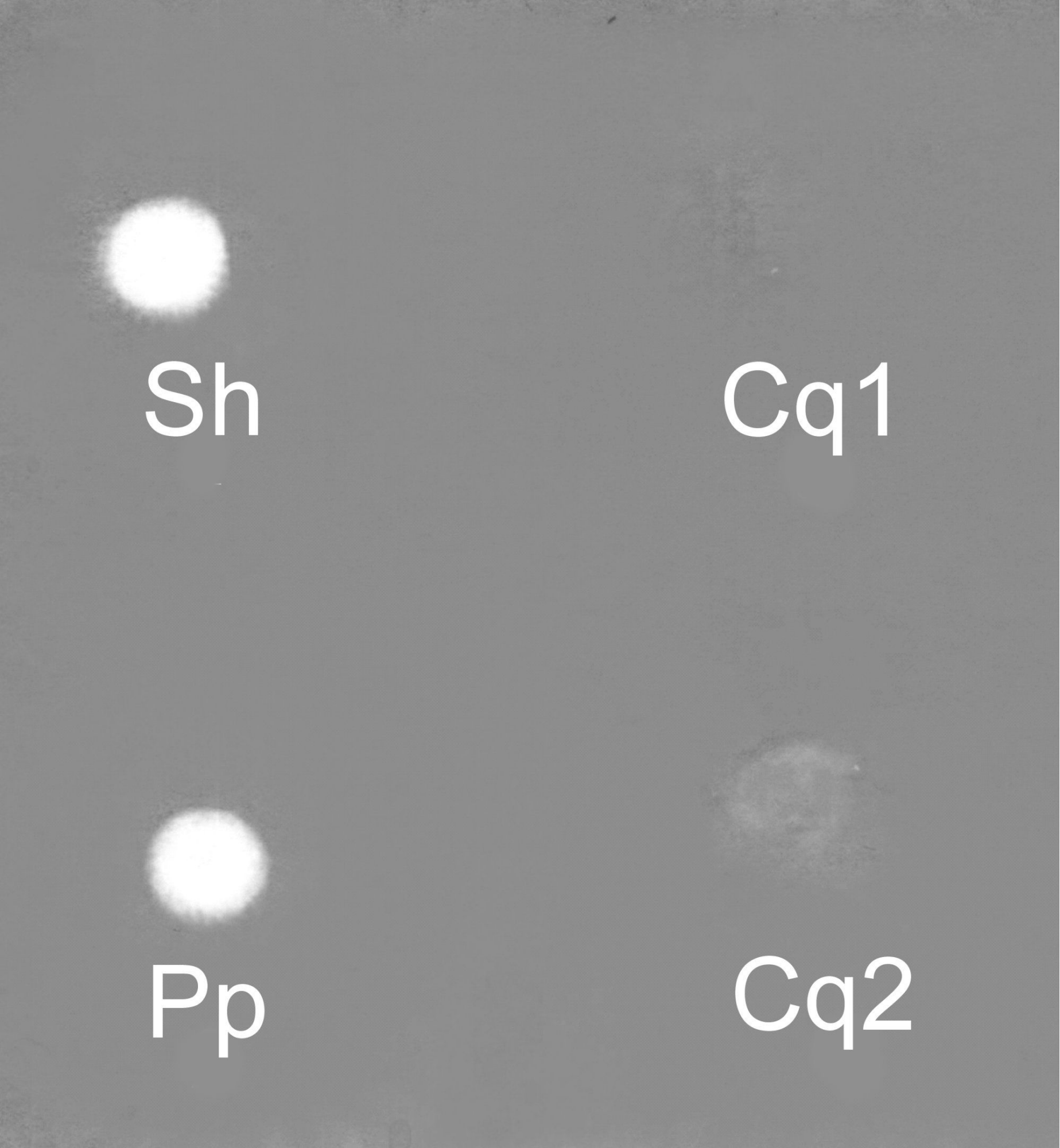
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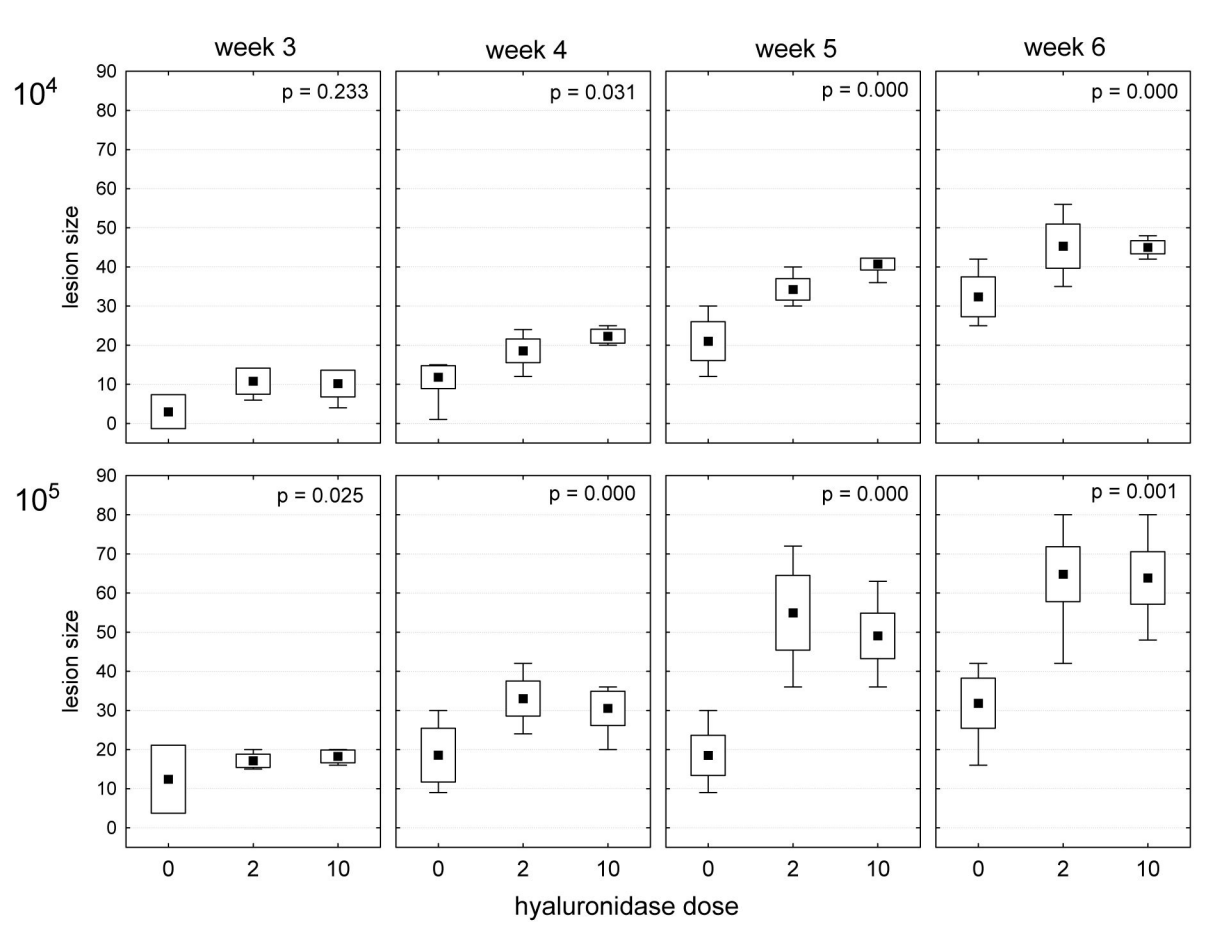
Cq2

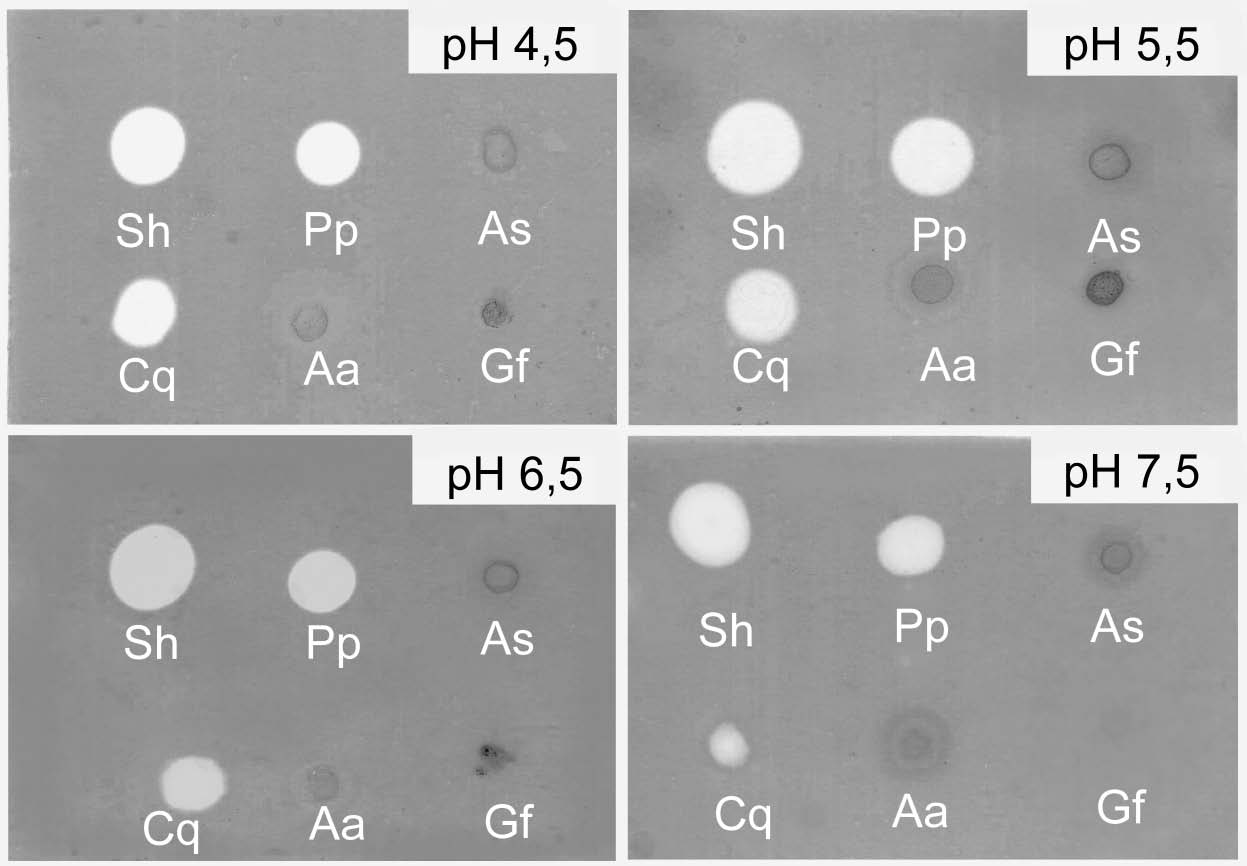

week 3
week 4
week 5
week 6
10^4
10^5
p = 0.233
p = 0.031
p = 0.000
p = 0.000
p = 0.025
p = 0.000
p = 0.000
p = 0.001
lesion size
lesion size
0
10
20
30
40
50
60
70
80
90
0
2
10
hyaluronidase dose

SUPPORTING INFORMATION

Fig. S1. Effect of pH on hyaluronidase activity in SGE of various bloodsucking insects. Activity of hyaluronidase was tested by dot method on polyacrylamide gel with incorporated 0.002% hyaluronan. Four different pH were compared: pH 4.5, pH 5.5 (both 0.1 M acetate buffer), pH 6.5 (0.1 M bis-Tris buffer) and pH 7.5 (0.1 M Tris buffer). Protein content per 2 µl dot is indicated in brackets. Sh = sheep testicular hyaluronidase (2 µg), Pp = *Phlebotomus papatasi* SGE (0.8 µg), As = *Anopheles stephensi* SGE (0.8 µg), Cq = *Culex quinquefasciatus* SGE (0.8 µg), Aa = *Aedes aegypti* SGE (1.3 µg), Gf = *Glossina fuscipes* SGE (14 µg). Samples were incubated for 24 hours at 37 °C.

Fig. S2. Effect of hyaluronidase on *Leishmania major* numbers in draining lymph nodes 48 hrs post infection. BALB/c mice were coinoculated intradermally into ear with 10^4 (A) or 10^5 (B) *Leishmania major* and hyaluronidase equivalent to 0, 2 and 10 salivary glands of *Phlebotomus papatasi*. Points (■) = mean values; vertical bars = 95% confidence intervals.

Fig. S3. Effect of hyaluronidase on *Leishmania major* numbers in draining lymph nodes 24 hrs post inoculation. BALB/c mice were coinoculated intradermally into ear with 10^4 (A) or 10^5 (B) *Leishmania major* and hyaluronidase equivalent to 0, 2 and 10 salivary glands of *Phlebotomus papatasi*. Points (■) = mean values; vertical bars = 95% confidence intervals; **A**: 10^4 parasites, one-way ANOVA $F_{(2, 27)} = 1.989$, $p = 0.16$; **B**: 10^5 parasites, one-way ANOVA $F_{(2, 27)} = 0.145$, $p = 0.87$; **C**: 10^4 and 10^5 parasites combined, factorial ANOVA $F_{(2, 54)} = 0.043$, $p = 0.96$.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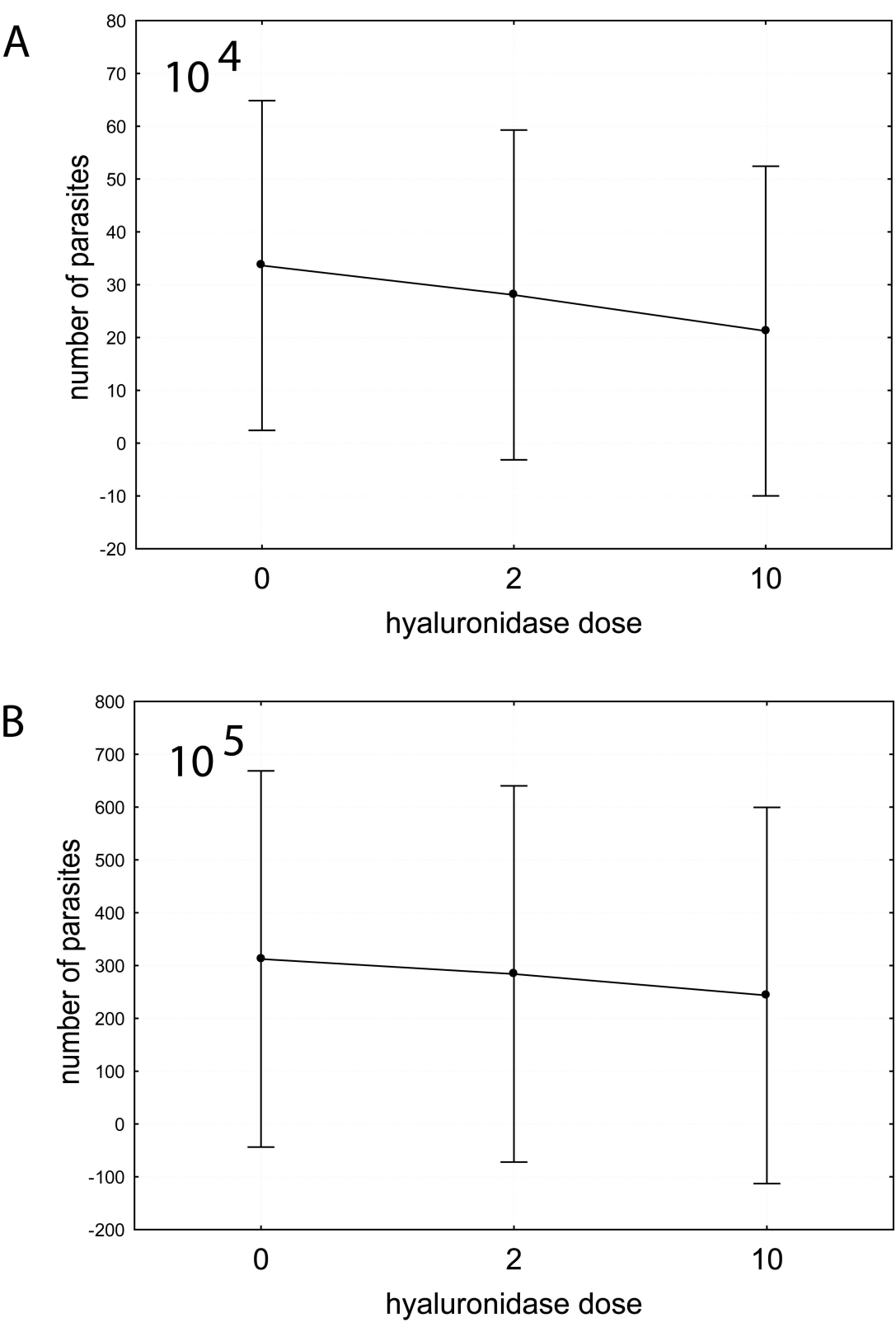
A
10^4
number of parasites
80
70
60
50
40
30
20
10
0
-10
-20
0
2
10
hyaluronidase dose
B
10^5
number of parasites
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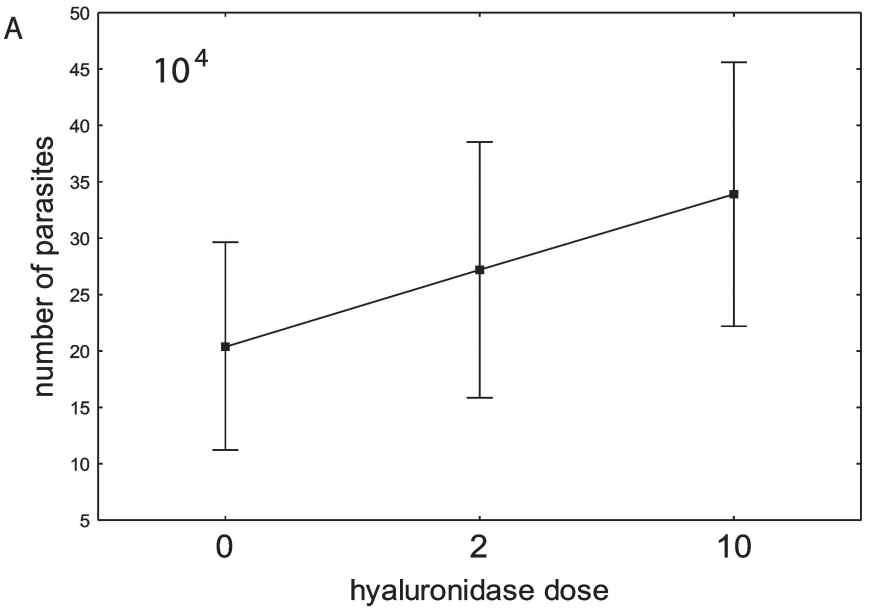
700
600
500
400
300
200
100
0
-100
-200
0
2
10
hyaluronidase dose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number of parasites
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45
40
35
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25
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2
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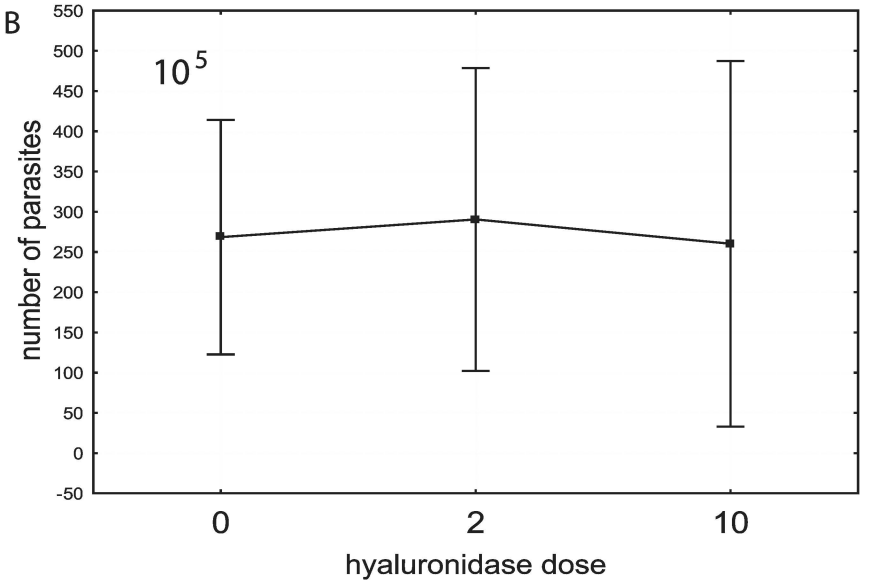
hyaluronidase dose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10^5
number of parasites
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500
450
400
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200
150
100
50
0
-50
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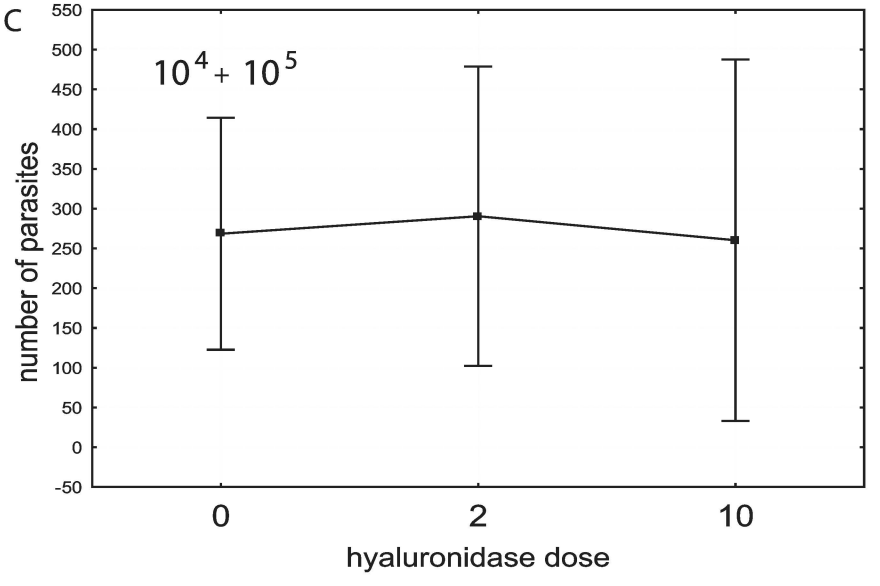
hyaluronidase dose


C
$10^4 + 10^5$
number of parasites
550
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450
400
350
300
250
200
150
100
50
0
-50
0
2
10
hyaluronidase dose

VECTOR-BORNE AND ZOONOTIC DISEASES
Volume 8, Number 1, 2008

DOI: 10.1089/vbz.2007.0214

Kinetics of Canine Antibody Response to Saliva of the Sand Fly *Lutzomyia longipalpis*

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DOROTHEE STANNECK,[2] NORBERT MENCKE,[2] PETR VOLF[1]

ABSTRACT

Zoonotic visceral leishmaniosis (VL) caused by *Leishmania infantum* is transmitted from dogs to humans by sand flies and *Lutzomyia longipalpis* is a major vector of this disease. We studied the antibody response in dogs experimentally exposed to *L. longipalpis* females to characterize sand fly salivary antigens recognized by canine sera and to find out whether the level of specific anti-saliva antibodies reflects the intensity of exposure. Sera from repeatedly bitten dogs revealed up to six salivary protein bands with approximate molecular weight of 66, 55, 45, 37–39, 34, and 25 kDa in *L. longipalpis* salivary gland lysate. Anti-saliva immunoglobin (Ig) G and its subclasses were found to be useful markers of exposure to sand flies. Specific IgG, IgG1, and IgG2 were related to numbers of bloodfed *L. longipalpis* females, and increased antibody levels were detectable throughout the study, i.e. more than 6 months after the last exposure. In contrast, specific IgE response developed in some dogs only, and no correlation was observed between its level and the intensity of exposure. Screening of dog sera for specific IgG against salivary antigens of the vector is suggested as a useful epidemiological tool in VL foci. Monitoring canine antibody response to sand fly saliva also allows evaluation of the effectiveness of anti-vector campaigns.

INTRODUCTION

DOGS AND WILD CANIDS are the natural reservoirs of human visceral leishmaniosis (VL) caused by *Leishmania infantum* (syn. *L. chagasi*). This life-threatening disease occurs in Latin America, Asia, Africa, and Europe. *Leishmania* parasites are transmitted by the bite of an infected sand fly (Diptera: Phlebotominae), and the establishment of infection in naive hosts is promoted by the immunomodulatory effect of sand fly saliva (reviewed by Kamhawi 2000, Rohousova and Volf 2006). At the same time, repeated exposure to sand fly bites elicits an immune response that confers partial protection against naturally transmitted *L. major* in mice (Kamhawi et al. 2000).

Anti-saliva sensitization in hosts bitten by sand flies employs both arms of immunity (Barral et al. 2000). Although cellular rather than humoral immunity is supposed to be responsible for the protective status against leishmanioses (Valenzuela et al. 2001), the study of anti-saliva antibodies is important for characterization of the immune status of the host. Very little is known, however, about the kinetics of anti-saliva immunoglobulin production in hosts bitten by bloodfeeding insects; few data are available from mice experimentally exposed to *Triatoma infestans* (Volf et al. 1993) or humans naturally exposed to mosquitoes for prolonged periods (Peng et al. 2004). To our knowledge, there is no study describing antibody response kinetics in animals exposed to sand flies.

Anti-saliva antibodies developed by hosts

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bitten by sand flies may provide important epidemiological information. Data from humans living in a focus of anthroponotic cutaneous leishmaniosis in Turkey (Rohousova et al. 2005) suggest that the specific antibody response to sand fly saliva, in this case *Phlebotomus* spp., could be used to monitor the exposure of hosts to sand flies and might also be useful as a marker of risk for *Leishmania* transmission in endemic areas. Moreover, control programs for human VL caused by *L. infantum* are targeted at dogs, the main reservoirs. Prevention of sand fly bites by insecticide treatment is the main way to stop the circulation of canine leishmaniosis (reviewed by Gramiccia and Gradoni 2005, Lainson and Rangel 2005); therefore monitoring canine antibody response to vector saliva would allow evaluation of the effectiveness of anti-vector campaigns.

The main objective of the present study was to define the long-term kinetics of anti-saliva antibody response in dogs bitten by *Lutzomyia longipalpis*. This sand fly is the only proven vector of *L. infantum* in Latin America (reviewed by Killick-Kendrick 1999, Lainson and Rangel 2005). Antibody response was monitored in dogs exposed to low or high numbers of sand fly females to determine whether the level of anti-saliva antibodies reflects the intensity of exposure to sand flies. In addition, we sought to determine the number and approximate molecular weight of *L. longipalpis* salivary antigens reacting with dog IgG antibodies.

MATERIALS AND METHODS

Dogs and sand flies

Twelve adult laboratory beagle dogs were housed in the animal facility of Bayer HealthCare AG (Leverkusen, Germany). They were maintained and handled in accordance with European guidelines for ectoparasiticide efficacy studies. Sand flies of *L. longipalpis* colony (originating from Jacobina, Brazil) were reared in standard conditions as described by Benkova and Volf (2007). Salivary glands were dissected from 3–6-day-old females. Groups of 20 glands in 20 μL of 20 mM Tris buffer with 150mM NaCl (TBS) were stored at −70°C. Prior to use, salivary gland lysate (SGL) was prepared by repeated freezing and thawing as described elsewhere (Volf and Rohousova 2001).

Exposure to sand flies and blood collection

Dogs were sedated with medetomidine at a dosage of 1.0 mg active ingredient (a.i.)/m^2 body surface, and diazepan, 5 mg a.i./dog. Dogs were individually exposed to approximately 200 sand fly females as described by Mencke et al. (2003), once a week for a total of five exposures. After exposure, sand fly females were collected and microscopically examined to assess the feeding success of the fly.

For the main experiment, two groups of three dogs each (groups 1 and 2) were used. Dogs in group 2 were treated with a repellent prior to first exposure and, as a result, were by far less exposed to sand fly bites than dogs in group 1. Therefore dogs in group 1 are defined as high-exposed; dogs in group 2, as low-exposed. Blood samples were collected from vena cephalica antebrachii and processed to serum. Dogs were sampled according the following schedule: pre-immune serum in week 0, immune serum 6 days after each exposure (weeks 1, 2, 3, 4, and 5) and then weeks 6, 7, 9, 13, 19, and 23.

Because elevated levels of anti-saliva IgG were observed even 19 weeks post-exposure, an additional experiment was designed to follow the tapering of specific antibody response. Six beagle dogs (groups 3 and 4) were immunized according to the same exposure protocol, and dogs in group 3 were untreated (high-exposed) and those in group 4 were treated with a repellent (low-exposed). In this experiment, blood samples were collected in weeks 22, 28 and 33.

Measurement of specific IgG and IgG subclasses

Specific anti-saliva IgG antibodies were measured by enzyme-linked immunosorbent assay (ELISA). Microtiter plate wells (CovaLink™ NH, Nunc) were coated with salivary gland lysate (60 ng of protein per well in 50 mM carbonate–bicarbonate buffer, pH 9.6) overnight at 4°C. The wells were then washed in 20 mM phosphate-buffered saline (150 mM NaCl, pH 7.4) with 0.05% Tween 20 (PBS-Tw) and incubated with 6% horse serum in PBS-Tw for

45 min at 37°C to block free binding sites. Dog sera were diluted 1:500 in 3% horse serum/PBS-Tw and incubated in duplicate for 90 min at 37°C. This step was followed by incubation with peroxidase-conjugated sheep anti-dog IgG (heavy chain specific), goat anti-dog IgG1, or sheep anti-dog IgG2 antibodies (Bethyl Laboratories) at a 1:3,000 dilution for 45 min at 37°C. Orthophenylenediamine and H_2O_2 in McIlwein phosphate-citrate buffer (pH 5.5) were used as a substrate solution. Absorbance was measured with a Multiskan RC ELISA reader (Labsystems) at 492 nm wavelength.

Measurement of specific IgE

Modification of the IgG ELISA protocol was used to measure specific anti–*L. longipalpis* IgE in dog sera. Wells were coated with SGL (180 ng of protein per well), washed in 50 mM Tris buffer (140 mM NaCl, pH 8.0) with 0.05% Tween 20 (TBS-Tw), and blocked with 1% bovine serum albumin (BSA) in TBS-Tw solution for 1 h at room temperature. Dog serum samples were diluted 1:100 in blocking solution and incubated for 1 h at room temperature. The wells were then incubated with goat peroxidase-conjugated anti-dog IgE antibody, ε-chain specific (Bethyl Laboratories), at a 1:1,000 dilution for 1 h at room temperature.

Western blot analysis

Salivary gland lysate proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (2.5% 2-mercaptoethanol in sample buffer) in a Mini-Protean III apparatus (BioRad). In preliminary experiments, SGL proteins were separated on 13% gels and immunoblotted. No serum samples reacted with low molecular weight proteins. Therefore, 10% gel was used in further experiments to achieve better resolution of proteins of interest. Separated proteins were stained by Coomassie blue or were electrotransferred onto a nitrocellulose (NC) membrane by Semi-Phor equipment (Hoefer Scientific Instruments). For Coomassie staining, an equivalent of 10 glands (approximately 2 μg of protein) was loaded per lane. For Western blotting, the protein concentration per lane was slightly lower (approximately 1.5 μg). After transfer, the NC mebrane was cut into strips and blocked by 5% low-fat dried milk in TBS-Tw for 1 h. The strips were incubated with selected dog sera diluted 1:75 in TBS-Tw for 1 h, followed by 1 h incubation with horseradish peroxidase-conjugated sheep anti-dog IgG antibody (Bethyl Laboratories, dilution 1:3,000 in TBS-Tw). The color reaction was developed using Tris buffer with diaminobenzidine and H_2O_2.

Statistical analysis

Data obtained by ELISA were transformed and subjected to GLM ANOVA (analysis of variance) and Scheffe's Multiple Comparison procedure to analyze statistical significance between means of high- and low-exposed groups at all sampling points. The Bonferroni Versus Control Multiple Comparison procedure was used to test for statistically significant differences between mean antibody levels in pre-immune sera versus immune sera in individual groups. The p value was set at 0.05. All statistical analyses were performed with NCSS 6.0.21 software.

RESULTS

Exposure of dogs to sand flies

Beagle dogs (four groups of three dogs each) were individually exposed to approximately 200 *L. longipalpis* females once a week for 5 consecutive weeks. Substantially higher numbers of sand fly females fed on untreated dogs (groups 1, 3) than on repellent-treated dogs (groups 2, 4) (Table 1).

TABLE 1. EXPOSURE OF DOGS TO SAND FLY BITES

Week	*Group 1*	*Group 2*	*Group 3*	*Group 4*
0	150 ± 3	3 ± 2	95 ± 13	1 ± 1
1	212 ± 3	22 ± 2	113 ± 15	1 ± 0
2	141 ± 11	27 ± 8	145 ± 12	10 ± 5
3	125 ± 28	24 ± 7	154 ± 23	7 ± 2
4	142 ± 9	77 ± 5	134 ± 12	51 ± 12

Beagle dogs (four groups of three dogs each) were exposed to *Lutzomyia longipalpis* females weekly five times. Blood-engorged sand fly females were counted after the exposure. Data are represented as mean ± SEM.

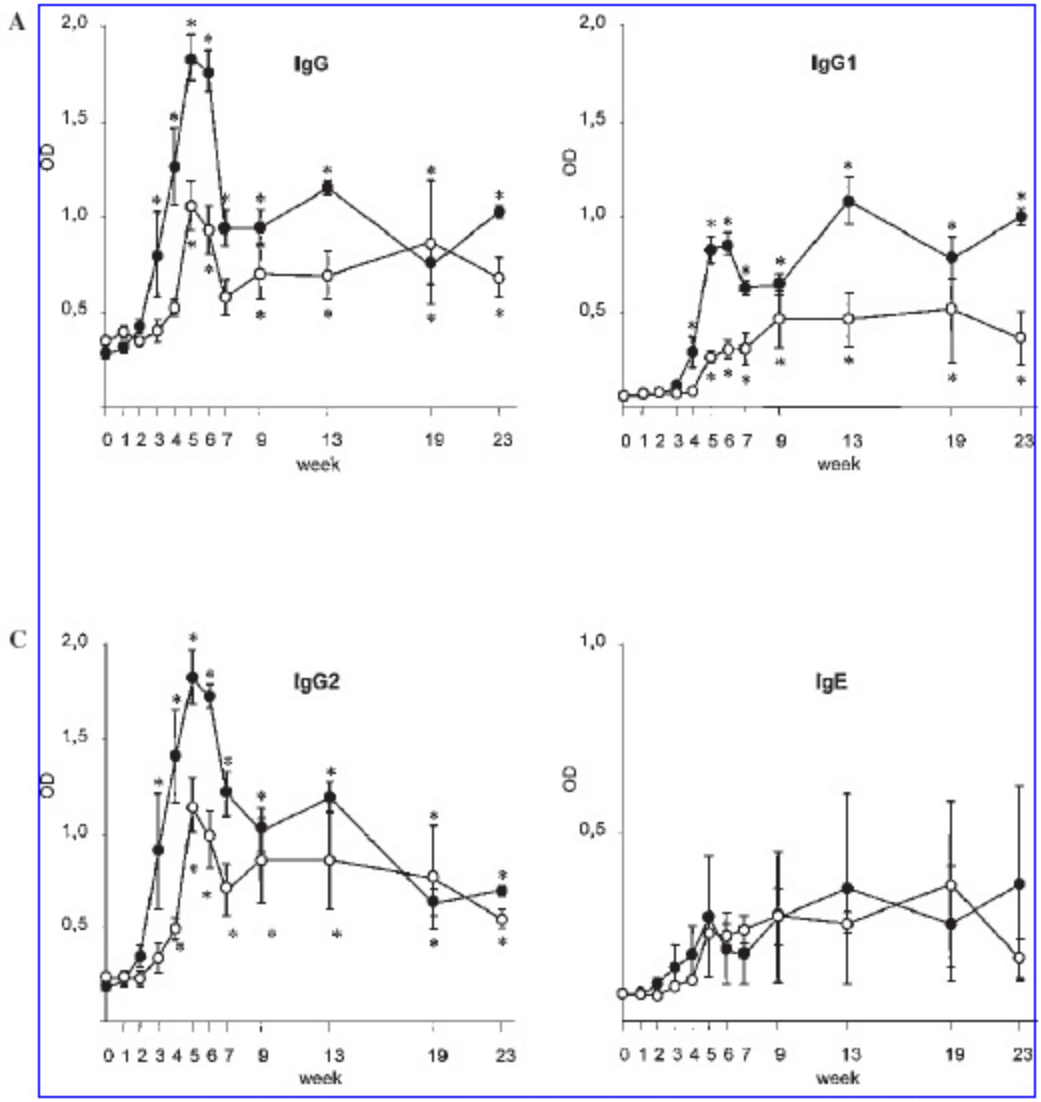

FIG. 1. Anti-*Lutzomyia longipalpis* antibody response in high- and low-exposed dogs. Dogs were immunized by sand fly feeding in weeks 0–4. Specific anti-saliva antibodies were detected by ELISA in pre-immune and immune sera. Full circles, high-exposed dogs (group 1); open circles, low-exposed dogs (group 2); vertical bars, SEM; *Significant difference compared to pre-immune sera ($p < 0.05$)

Kinetics of specific IgG and IgG subclasses

Repeated exposure to *L. longipalpis* bites resulted in high levels of specific IgG to sand fly saliva. No specific antibodies were detected in pre-immune sera of any dog tested.

In three high-exposed dogs (group 1), IgG and IgG2 production showed distinct sharp peaks after the last exposure in weeks 5–6. The trend of IgG1 is different in that, overall, it seems to rise up steadily to week 23. Compared to pre-immune sera, IgG, IgG1, and IgG2 lev-

TABLE 2. SPECIFIC ANTI-*L. LONGIPALIS* SALIVA ANTIBODY RESPONSE IN HIGH- AND LOW-EXPOSED DOGS

	week	*IgG*	*IgG1*	*IgG2*	*IgE*
group 3	22	0.523 ± 0.024*	0.474 ± 0.113	0.478 ± 0.025*	0.049 ± 0.134
	28	0.422 ± 0.036*	0.320 ± 0.075	0.387 ± 0.042*	0.527 ± 0.159
	33	0.471 ± 0.038*	0.390 ± 0.093	0.419 ± 0.058*	0.840 ± 0.396
group 4	22	0.282 ± 0.024	0.161 ± 0.017	0.237 ± 0.035	0.114 ± 0.060
	28	0.192 ± 0.015	0.095 ± 0.011	0.181 ± 0.026	0.092 ± 0.022
	33	0.259 ± 0.019	0.155 ± 0.027	0.233 ± 0.019	0.099 ± 0.031

Immunoglobulin levels were measured by ELISA in serum samples from beagle dogs. The dogs were exposed to high (group 3) or low (group 4) numbers of *L. longipalpis* females in weeks 0, 1, 2, 3, and 4. Serum samples were taken in weeks 22, 28, and 33. Data are represented as mean optical density ± SEM. *Significant difference between group 3 and 4 ($p < 0.05$).

els remained significantly elevated until week 23 (i.e., 19 weeks after the last exposure) (Fig. 1A–C).

In low-exposed dogs (group 2), anti–*L. longipalpis* IgG, IgG1, and IgG2 developed with similar kinetics, with overall lower antibody titers. Significant differences ($p < 0.05$) between IgG responses in the high- and low-exposed groups were detected from week 3 onward, with the exception of week 19. The difference in IgG1 responses was significant from week 4 onward; IgG2 responses differed significantly in weeks 3–7. Importantly, even in low-exposed dogs the levels of IgG and IgG subclasses remained significantly elevated compared to pre-immune sera as late as 19 weeks after the last exposure (Fig. 1A–C).

In an additional experiment aimed at tapering of specific antibody response, six dogs in groups 3 and 4 were blood-sampled in weeks 22, 28, and 33. Three high-exposed dogs (group 3) had significantly stronger anti–*L. longipalpis* IgG and IgG2 response than the three low-exposed dogs at all time points ($p < 0.05$). The variance in IgG1 levels was high among individual dogs in group 3; therefore no statistically significant difference was observed between high- and low-exposed dogs (Table 2).

Kinetics of specific IgE

No specific IgE antibodies were detected in canine pre-immune sera. In sera of immunized dogs, variable levels of anti-saliva IgE were observed that could not be related to sand fly exposure intensity. In high-exposed group 1, only one dog developed specific IgE. In low-exposed group 2, all dogs had detectable anti–*L. longipalpis* IgE, peaking at different time points (Fig. 1D).

Characterization of L. longipalpis salivary antigens

For characterization of *L. longipalpis* salivary antigens recognized by canine sera, serum samples from dogs of group 1 were used. No reaction was observed in control strips where pre-immune sera were used. The antigens recognized by IgG were similar in all dogs, but some differences were observed in time points at which antibodies bound to particular antigens. In all dogs, the reaction was most intense at week 6, i.e., 1 week after the last exposure to sand fly bites. An antigen with a molecular mass of about 66 kDa reacted with sera of all dogs tested; the other bands recognized by sera of exposed dogs had approximate molecular masses of 55, 45, 39, 37, 34, and 25 kDa (Fig. 2).

DISCUSSION

The aim of the present study was to evaluate the kinetics of anti–*L. longipalpis* saliva antibody production in two study groups of low- and high-exposed dogs. The results show that anti-saliva IgG response in dogs bitten by *L. longipalpis* reflects the intensity of exposure. Stronger IgG, IgG1, and IgG2 responses to salivary antigens were observed in high-exposed dogs. The difference in IgG production between high- and low-exposed dogs could be detected as late as 29 weeks after the last exposure to sand flies. This finding may have important consequences for the immune status of

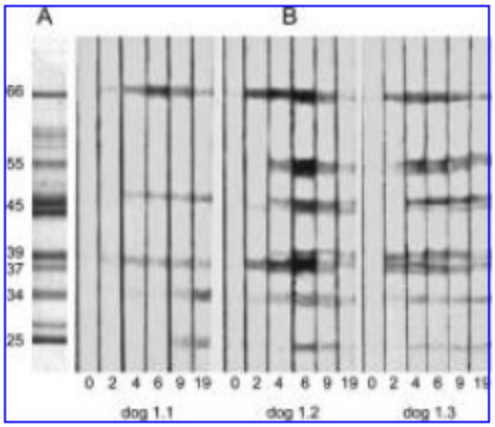

FIG. 2. Characterization of *L. longipalpis* salivary antigens. SGL proteins separated by sodium dodecyl sulfate-polyacrylamid gel electrophoeresis (SDS-PAGE), 10% gel. Approximate molecular masses (kDa) indicated on the left. (A) Total protein profile, Coomassie-stained gel. (B) Western blot analysis, nitrocellulose membrane incubated with sera of three dogs repeatedly bitten by *L. longipalpis*. Dogs of group 1 exposed to sand fly feeding in weeks 0–4; sera collected in week 0, 2, 4, 6, 9, and 19.

hosts in natural conditions. In many foci of leishmanioses, sand fly populations show seasonal fluctuations, and the sand fly-free periods last up to 6 months (Lewis and Ward 1987). For example, a strong *L. longipalpis* abundance fluctuation was observed in a Brazilian focus of VL, with sand flies absent during the dry season (de Resende et al. 2006). Our results suggest that anti-saliva antibodies elicited by sand fly bites can persist until the next sand fly season.

The correlation between intensity of exposure to sand flies and host IgG response has implications for insecticide efficacy assessment. As the importance of asymptomatic dogs in the epidemiology of leishmaniosis has been proved by transmission experiments using *P. perniciosus* and *L. longipalpis* sand flies (Molina et al. 1994, Michalsky et al. 2007), treatment of clinically ill dogs or culling of seropositive animals would not be effective. In contrast, topical insecticide application or insecticide-impregnated collars are a good means of preventing *L. infantum* transmission to dogs (Manzillo et al. 2006, Otranto et al. 2007). Monitoring anti-saliva IgG response in combination with infection incidence would provide valuable information on control program effectiveness.

Canine IgG, like IgG of other mammalian species, exists in subclass variants. Four IgG subclasses found in dogs have not been functionally characterized, although associations of specific subclass responses with canine pathologies have been described (Day 1996, Hou et al. 2006). Data available in the recent literature do not support the view that elevated levels of anti-saliva antibodies of either IgG subclass directly affect *Leishmania* infection. In the sera of healthy dogs, IgG1 and IgG2 are the most abundant subclasses, and they occur in similar concentrations (Mazza et al. 1994). Discussion is ongoing about the use of monoclonal antibodies with better-validated specificity (Day 2007) and commercially available polyclonal antibodies.

In dogs exposed to *L. longipalpis* we observed a significant increase in both IgG1 and IgG2 specific for sand fly salivary antigens. The IgG and IgG2 response kinetics developed with a similar pattern, i.e., with a single peak just after the last exposure. In contrast, IgG1 response peaked several weeks post-exposure. This difference may indicate that specific IgG1 production and IgG2 production are regulated in different ways. Although the exact affinities of polyclonal anti-canine antibody conjugates were not known and individual subclass responses could not be precisely compared, IgG2 response was seemingly stronger than IgG1 response.

In contrast to IgG, the kinetics of IgE response were diverse, and not all dogs in the study had detectable specific anti-saliva IgE antibodies. Thus, no significant differences were found in anti-saliva IgE response between high- and low-exposed groups. Similar results were reported by Gomes et al. (2002) for humans; high variation in anti–*L. longipalpis* IgE was found in children who lived in the study area and who, presumably, were equally exposed to sand flies. Taken together, the level of saliva-specific IgE antibodies is not a good marker of exposure.

Sera of dogs exposed to *L. longipalpis* feeding recognized up to six salivary protein bands.

Molecular weight of some of the antigenic bands corresponds to salivary proteins identified previously (e.g., Valenzuela et al. 2004), and we may thus speculate on their identity. The 45 kDa and 37–39 kDa proteins most likely represent members of the Yellow protein family (NCBI accession numbers AF132518, DQ192488). Presumably, the 34 kDa antigen is the apyrase (NCBI accession number AF131933), and the 25 kDa antigen is the D7-related protein (NCBI accession number AF420274). Homologs of some of these proteins were previously reported as antigens or allergens in saliva of bloodsucking arthropods; D7 proteins were shown to be involved in mosquito hypersensitivity (Peng et al. 1998). Sand fly Yellow protein and D7 protein were identified by mass spectrometry as antigens recognized by sera of dogs naturally infected with *L. infantum* (Bahia et al. 2007). In addition, sand fly Yellow protein, D7 protein, and apyrase were previously proposed as antigens recognized by sera of experimentally bitten mice and naturally exposed humans (Gomes et al. 2002, Rohousova et al. 2005). In agreement with our previous results in mice (Rohousova et al. 2005), no IgG binding to peptides or low molecular weight proteins was observed.

In conclusion, we have shown that dogs experimentally exposed to *L. longipalpis* develop specific IgG and IgE antibodies. The levels of IgG and its subclasses were related to number of fed *L. longipalpis* females, and differences in antibody levels between the high- and low-exposed dogs were detectable up to 29 weeks after the last exposure. The study showed that serum anti-saliva IgG is a useful marker of exposure, and that screening of dogs for antibodies specific to *L. longipalpis* salivary proteins should be of major importance in foci of VL caused by *L. infantum*.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Helena Kulíková and Dusan Eremiás for helpful technical and administrative assistance. The study was partially supported by projects MSM 0021620828, LC 06009 and GAUK 191/2006/B-BIO/PrF.

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TITLE

Analysis of Salivary Transcripts and Antigens of the Sand Fly *Phlebotomus arabicus*

ABSTRACT

Sand fly saliva plays an important role in the transmission of leishmaniases as it increases parasite virulence. On the other hand, immunity to salivary components might hamper the establishment of infection. Therefore, it is most desirable to gain a deeper insight into the composition of saliva in sand fly species which serve as vectors of various forms of leishmaniases. In the present work, we focused on *Phlebotomus (Adlerius) arabicus*, which was recently shown to transmit *Leishmania tropica*, the causative agent of cutaneous leishmaniasis. A cDNA library was constructed from salivary glands of *P. arabicus* females and the sequenced clones were analyzed. The most abundant protein families were SP15-like proteins, 27 kDa-like proteins, D7-related proteins, yellow-related proteins, PpSP32-like proteins, antigen 5-related proteins, and 34 kDa-like proteins. Sequences coding for apyrases and other putative secreted enzymes were also represented, including hyaluronidase, endonuclease, pyrophosphatase, amylase and trehalase. From the transcripts that did not match any reported proteins in accessible databases, none was predicted to be secreted. Proteomic analysis of female salivary proteins was performed and the identity of antigenic bands recognized by sera of mice bitten by *P. arabicus* was suggested. This work brings the first analysis of salivary protein composition of subgenus *Adlerius*.

INTRODUCTION

Phlebotomus (Adlerius) arabicus is distributed in certain parts of the Middle East and Africa. In his revision of subgenus *Adlerius*, Artemiev (1980) lists only the type locality, Yemen; later specimens identified as *P. arabicus* were described from Saudi Arabia (Lewis & Büttiker, 1980), Egypt and Ethiopia (Lane, 1986), Jordan (Kamhawi et al., 1995), and Israel (Jacobson et al., 2003). In Ethiopia, sand flies designated by Ashford (1974) as *P. chinensis* conform with *P. arabicus* description and recently, *P. arabicus* females infected with uncharacterized *Leishmania* sp. were reported (Gebre-Michael et al., 2004). In northern Israel *P. arabicus* is the vector of cutaneous leishmaniasis caused by *L. tropica* (Jacobson et al., 2003; Svobodova et al., 2006).

Leishmaniasis caused by *Leishmania tropica* is found in a vast discontinuous area reaching from the southwestern Mediterranean to Turkey, northwestern India and Subsaharan Africa (Jacobson, 2003). Typically, the disease is cutaneous, although sporadic cases of viscerotropic *L. tropica* have been described (e.g., Mebrahtu et al., 1989; Magill et al., 1993; Sacks et al., 1995). Long supposed to circulate in athroponotic foci exclusively (Ashford, 2000), *L. tropica* was recently shown to occur in zoonotic foci as well (Jacobson et al., 2003; Svobodova et al., 2006).

Transmission of *Leishmania* parasites to mammalian hosts is facilitated by sand fly saliva; at the same time, immune response to salivary components may partially protect the host from *Leishmania* infection (reviewed by Rohousova & Volf, 2006). Therefore, salivary components essential for parasite transmission and/or eliciting protective immune response are sought-after. Salivary compounds from *P. papatasi*, the vector of *Leishmania major*, and *Lutzomyia longipalpis*, the vector of *L. infantum*, have been extensively studied. In addition, cDNA libraries from several other sand fly species were sequenced and annotated.

In the present study, salivary gland transcripts and proteins of *P. (Adlerius) arabicus* were studied by cDNA sequencing and proteomics. Salivary antigens recognized by sera of mice exposed to *P. arabicus* feeding were characterized. As such, it is the first study on salivary repertoire of *L. tropica* vector. In addition, it is the first report on salivary composition in the subgenus *Adlerius.*

MATERIALS AND METHODS

Sand flies and salivary gland dissection

Colonies of *P. arabicus* (origin from Israel) and *P. papatasi* (origin from Turkey) were reared in the insectary of Charles University in Prague in standard conditions as described by Benkova and Volf (2007). For mRNA extraction salivary glands of *P. arabicus* females were dissected in RNA later (Ambion) from 1-day-old females and for analysis of N-terminal protein sequences in NuPAGE LDS sample buffer (Invitrogen) from 5- to 7-day-old females. Salivary glands from 5- to 7-day-old *P. arabicus* or *P. papatasi* females dissected in Tris buffer (20 mM Tris, 150 mM NaCl, pH 7.5) were used for Western blotting.

Construction of salivary gland cDNA library

Salivary gland mRNA was isolated from 30 pairs of glands using Micro-FastTrack mRNA isolation kit (Invitrogen). PCR-based cDNA library was made following the manufacturer's instructions for SMART^{TM} cDNA library Construction Kit (BD Clontech) with some modifications described by Chmelar et al. (2008). The cDNA library was fractionated into three sets of cDNAs containing large, medium and small fragments. Gigapack® III Gold Packaging Extract (Stratagene) was used for packaging the phage particles. The libraries were plated by infecting log phase XL-1 blue cells (Clontech). Several plaques from each plate were selected and a PCR with vector primers flanking the inserted cDNA was performed. The presence of recombinants was checked by visualisation the PCR products on 1,1% agarose gel with ethidium bromide.

Sequencing of Selected cDNA Clones

Plaques were randomly selected from the plated libraries and transferred to 96-well polypropylene plate containing 75 μl of water per well. The PCR reaction amplifying randomly selected cDNAs was performed using FastStart PCR Master mix (Roche), 3 μl of the phage sample as a template and primers described elsewhere (Oliveira et al., 2006). Amplification conditions were as follows: 1 hold of 75°C for 3 min, 1 hold of 94°C for 2 min and 34 cycles of 94°C for 1 min, 49°C for 1 min and 72°C for 2 min. Final elongation step lasted for 10 min at 72°C. Reaction products were cleaned using ExcelaPure 96-Well UF PCR Purification Plates (EdgeBio) and used as templates for cycle-sequencing reaction using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and a forward primer described elsewhere (Oliveira et al., 2006). Cycle-sequencing reaction products were cleaned using sephadex

and MultiScreen HV Plates (Millipore), dried and stored at -20°C. Sequencing was performed on an ABI 96 capillary DNA sequencer (Applied Biosystems).

Bioinformatics

Detailed description of the bioinformatic treatment of the data can be found elsewhere (Valenzuela et al., 2002b; Chmelar et al., 2008). Briefly, EST trace files were analyzed using a customized program based on the Phred algorithm (Ewing et al., 1998; Ewing & Green, 1998). Sequences with Phred quality scores lower than 20 were removed, as well as primer and vector sequences. Resulting sequences were grouped into clusters using a customized program based on identity (95% identity, 64 word size) and aligned into contiguous sequences (contigs) using the CAP3 sequence assembly program (Huang & Madan, 1999). BlastX, BlastN or RPS Blast programs (Altschul et al., 1997) were used to compare contigs and singletons (contigs with a single sequence) to the non-redundant (NR) protein database of the NCBI, the gene ontology (GO) fasta subset (Ashburner et al., 2000), to the Conserved Domains database (CDD) of NCBI (Marchler-Bauer et al., 2002) which contains KOG (Tatusov et al., 2003) , Pfam (Bateman et al., 2000) and Smart databases (Schultz et al., 2000), and to mitochondrial and rRNA nucleotide sequences available from NCBI. The three frame translations of each dataset were submitted to the SignalP server (Bendtsen et al., 2004) to detect signal peptides. The grouped and assembled sequences, Blast results and SignalP results were combined in an Excel spreadsheet and manually verified and annotated. N-glycosylation site prediction was performed for selected sequences using NetNGlyc 1.0 software (www.cbs.dtu.dk/services/NetNGlyc).

Phylogenetic analysis

Protein sequences of the members of identified protein families were compared with related sequences of other sand fly species obtained from GenBank. Sequences were aligned using ClustalW (version 1.4; Higgins & Sharp, 1988) running under BioEdit sequence-editing software, version 7, and manually refined in BioEdit. For each alignment, best substitution matrix was determined by ProtTest software, version 1.4 (Abascal et al., 2005). This matrix was then used by TREE-PUZZLE 5.2 (Schmidt et al., 2002) to reconstruct phylogenetic trees from the protein alignments by maximum likelihood. TREE-PUZZLE implements quartet puzzling (QP) tree search; at the same time, the algorithm estimates support values for each internal branch. The number of puzzling steps was 1000 in each phylogenetic analysis. Resulting trees were viewed in MEGA 4 (Tamura et al., 2007).

SDS-PAGE and aminoterminal sequencing of proteins

Phlebotomus arabicus salivary glands were electrophoretically separated on 1 mm thick 4-20% NuPAGE Novex Bis-Tris gels using MES SDS buffer (Invitrogen). A sample containing 30 glands was reduced with NuPAGE Sample Reducing Agent (Invitrogen) and run in parallel with non-reduced samples (50 glands) on the same gel. Wet blotting on a PVDF membrane was performed using XCell II^{TM} Blot Module (Invitrogen). SeeBlue® Pre-Stained Standards (Invitrogen) were used to estimate molecular weight (M_w) of separated proteins and assess transfer efficiency. The membrane was stained with 0.025% Coomassie blue without acetic acid. Stained bands were cut and subjected to Edman degradation using a Procise sequencer (Perkin-Elmer). cDNA sequences corresponding to obtained N-terminal aminoacid sequences of salivary proteins were identified using an in-house search program (Valenzuela et al., 2002b). This program compared three possible translations of each cDNA sequence obtained in the *P. arabicus* cDNA sequencing project with the aminoacid sequences.

Immunization of mice

Experiments on mice were done in accordance with Czech Act No. 246/1992 and approved by IACUC of the Faculty of Science, Charles University in Prague. Female BALB/c mice, 8 weeks old (Charles River Deutschland, Sulzfeld, Germany) were used for the exposure to *P. arabicus* sand flies. In biweekly intervals, the mice were anaesthetized with ketamin (1.5 mg / 10 g body weight) and xylazin (0.15 mg / 10 g body weight) and exposed to sand fly females. Each time, an average of 60 females fed on each mouse (SE = 14.9). Ten days after the sixth exposure the mice were bled and obtained sera were kept at -20°C.

Western blotting

Phlebotomus arabicus and *P. papatasi* salivary gland proteins were disrupted by three freeze-thaw cycles in liquid nitrogen and separated by SDS-PAGE on 12% polyacrylamide gel, 0.75 mm thick using Mini-Protean III apparatus (BioRad). As a reducing agent, 2.5% 2-mercaptoethanol was used in sample buffer. Biotinylated low range standards (BioRad) were run on the same gel. Separated proteins were electrotransferred onto a nitrocellulose membrane by iBlot^{TM} Gel Transfer Device (Invitrogen). After transfer, the mebrane was cut into strips corresponding to sample load of 5 salivary glands per strip. These strips carrying salivary gland samples were either stained with amidoblack (Merck; 0.1% solution in 25% isopropanol and 10% acetic acid) or blocked with 5% BSA in phosphate-buffered saline, pH 7.4, with 0.05%

Tween-20 (PBS-Tw) overnight. The sample-carrying strips were incubated for 1 hour with pre-immune and immune sera of BALB/c mice exposed to *P. arabicus* feeding diluted 1:250 in PBS-Tw, and then with horseradish peroxidase-conjugated swine anti-mouse antibody (Sevapharma, 1:1000 in PBS-Tw) for 45 minutes. Streptavidin-conjugated peroxidase (Sigma, 1μg/ml in PBS-Tw) was used for the biotinylated standards. The color reaction was developed using H_2O_2 and diaminobenzidine in PBS. Washes between individual steps were done with PBS-Tw.

RESULTS AND DISCUSSION

Western blotting

Western blots were performed to detect salivary gland antigens of *P. arabicus* recognized by sera of BALB/c mice exposed to *P. arabicus* feeding. The most prominent antigenic bands recognized by sera of all bitten mice had apparent molecular weights of 56-58.5 kDa, 45 kDa, 43 kDa (a double band), 42 kDa, 34.5-36.5 kDa and 30 kDa. Slightly weaker reaction was observed with bands running at 31 kDa and 30.5 kDa. In addition, sera of some animals recognized two very faint bands, running at 21 kDa and 16 kDa (Figure 1). The identity of individual antigenic bands was estimated based on predicted M_w and is discussed in the following sections. Sera from mice bitten by *P. arabicus* did not recognize *P. papatasi* antigens.

Sequencing of salivary gland cDNA library

A cDNA library was constructed from salivary glands of *Phlebotomus arabicus* females dissected 1 day after emergence. From this cDNA library, 1152 random plaques were selected and 985 clones were successfully sequenced. Related sequences were clustered in 107 clusters and 288 sequences were assessed as singletons, i.e. clusters containig only one sequence. Similarly to other sand flies studied so far, the most abundant transcripts were those coding for putative secretory proteins; 74 clusters were generated from these sequences, with an average number of 7,65 sequences per cluster. Predicted proteins containing retention signals for endoplasmic reticulum and/or transmembrane domains were not treated as putatively secreted.

Members of 21 different families were found among putative secretory proteins. Most of these proteins have known homologs in other sand fly species. Overall, high similarity was observed namely with salivary proteins of *L. infantum* vectors *P. (Larroussius) ariasi* and *P. (L.) perniciosus*, which is fully in concert with the close evolutionary relationship of *Larroussius* and *Adlerius* subgenera reported by Aransay et

al. (2000). To a lesser extent, similarity with salivary proteins of *P. (Euphlebotomus) argentipes*, the vector of *L. donovani* in India, was observed.

Some of the protein families contained multiple members. The observed variability among individual protein family members might be explained by the intraspecific polymorphism, as the tested sample was heterogeneous (a pool of salivary glands from about 40 sand fly females). Nevertheless, analysis of genetic variation of SP15 salivary protein in *P. papatasi* brought strong evidence that SP-15 is a multicopy gene (Elnaiem et al., 2005). While individual intraspecific variability of sand fly salivary proteins awaits broader analysis, we suppose that the variability among individual protein family members which we observed in *P. arabicus* salivary gland cDNA library rather reflects multiple gene duplication events.

Full-length sequences were obtained for most clusters coding for putatively secreted proteins. Only sequences containing a signal peptide and a polyA tail in the coding cDNA were considered full-length. Table 1 lists clusters for which full length sequences were obtained, including the name of the sequence, the best match to NCBI NR database, predicted site of signal peptide cleavage, and predicted M_w and pI of the mature protein.

Biochemical and antigenic characteristics of putative secreted proteins

SP15-like proteins

Thus far, SP15-like proteins were only reported in sand flies and their function is unknown. It was suggested that SP15-like proteins were derived from an ancestral odorant-binding protein and were closely related to short D7 proteins (Anderson et al., 2006). Immunization of mice with SP15 confered partial protection against *L. major* infection (Valenzuela et al., 2001a). Two different aminoterminal sequences of SP15-like proteins were found in *P. arabicus* proteome. Transcripts coding for these proteins represented the most abundant family in *P. arabicus* salivary gland cDNA library and clustered into ten separate groups. The transcripts are translated into 8 different aminoacid sequences of mature proteins which share 22.9% aminoacid identity and 23.7% aminoacid similarity. When SP15-like proteins from other sand flies were added in the analysis, only the six conserved cysteines and three other aminoacids were conserved in the sequence of 124 aminoacids (Figure 2A), reflecting the previously reported divergence among SP15-like proteins in sand flies (Anderson et al., 2006). While in *L. longipalpis* a single SP15-like protein was found, SL1, in *P. arabicus* and other *Phlebotomus spp.* studied so far multiple members of SP15 family are present. A

phylogenetic analysis revealed three separate groups of *P. arabicus* SP15-like proteins, showing close relationships to *P. ariasi* proteins ParSP03, ParSP06 and Par08, respectively (Figure 2B). The predicted pI of all *P. arabicus* SP15-like variants is highly basic (average pI = 9.18), corresponding to the fact that most sand fly salivary proteins have very high predicted pI values. Although immunization with SP15 DNA or protein results in antibody production in mice (Valenzuela et al., 2001a), using sera of mice exposed to *P. arabicus* feeding we did not detect any bands running at corresponding M_w in Western blots.

27 kDa and 25 kDa proteins

Six clusters coding for proteins related to *P. ariasi* 27 kDa salivary protein (ParSP25) and *P. perniciosus* 29 kDa salivary protein (PpeSP08) were found on *P. arabicus* salivary gland cDNA library. N-terminal aminoacid sequences of these proteins were found in salivary proteome. There are no other homologs of these proteins in accessible databases, no conserved domains were found in the translated sequences, and no function has been assigned to these proteins. However, in this cDNA library they represent second most abundant protein family. Transcripts coding for ParSP25-like proteins occured in long and short forms, with very little variability among individual clusters. The mature proteins coded by these transcripts had 90.5% identical aminoacids and 0.9% similar aminoacids. The predicted pI of the proteins is acidic (average pI = 5.03), unlike most sand fly salivary proteins predicted so far. No glycosylation sites were predicted for ParSP25-like proteins and most likely none of these proteins corresponds to any of the bands observed in Western blots.

D7-related proteins

D7 proteins are well known from the saliva of mosquitoes, sand flies, and biting midges (James et al., 1991; Valenzuela et al., 2002a; Campbell et al., 2005). While the structure of anopheline D7 proteins allows binding of biogenic amines and components of the contact activation system of coagulation (Calvo et al., 2006; Isawa et al., 2007), related proteins in sand flies lack conserved residues responsible for stabilizing bound ligands (Mans et al., 2007). Thus, they can hardly interfere with host hemostasis and their function is unknown. Seven clusters of sequences coding for D7-related proteins were found on *P. arabicus* salivary gland cDNA library; predicted mature proteins have M_w of 26-28 kDa and an average basic pI of 9.24. The protein sequences of mature proteins were 20.3% identical and 14.6% similar (Figure 3A). The phylogenetic analysis showed four distinct clades among *P. arabicus* D7 proteins, all of them bearing

high similarity to *P. ariasi* and *P. perniciosus* proteins (Figure 3B). D7 proteins are involved in hypersensitivity to mosquito bites (Peng et al., 1998) and antibodies against D7 proteins were found in dogs naturally exposed to *L. longipalpis* (Bahia et al., 2007). Sera from experimentally exposed mice and dogs and naturally exposed humans also recognize an antigen with an apparent M_w corresponding to D7 protein (Rohousova et al., 2005; Hostomska et al., 2008; Gomes et al., 2002). Although in our experiments no bands running at 26-28 kDa were detected on Western blots, it should be noted that two of the seven clusters have potential N-glycosylation sites and the apparent M_w of *P. arabicus* D7 proteins could easily be around 30 kDa, corresponding to the antigenic band running at 30 kDa.

Yellow-related proteins

Yellow-related proteins are common in insects. In bloodsucking Diptera, yellow-related proteins were described from mosquitoes and sand flies. Dopachrome-converting enzyme (DCE) activity is associated with *Aedes aegypti* yellow proteins (Johnson et al., 2001) and, according to Li et al. (1994), it might play a role in melanotic encapsulation of parasites in the hemocoel. Yellow protein of *Phlebotomus duboscqi* was detected in midgut and salivary glands and shown to have lectin properties (Volf et al., 2002); however, DCE activity could not be detected in sand fly salivary gland samples (Hostomska, unpublished observations). In *P. arabicus* salivary gland cDNA library two variants of yellow-related proteins were found. The predicted M_w of the proteins is 42.9 kDa, average pI 8.5. No glycosylation sites were predicted in the protein sequence. Sand fly yellow proteins were previously proposed as antigens recognized by sera of experimentally bitten mice and dogs, and naturally exposed humans (Rohousova et al., 2005; Hostomska et al., 2008; Gomes et al., 2002). Using sera of dogs naturally exposed to *L. longipalpis*, the antigenicity of *L. longipalpis* yellow protein was confirmed by mass spectrometry (Bahia et al., 2007). In our experiments, the band running at 43 kDa was recognized by immune murine sera and supposedly represents *P. arabicus* yellow protein.

PpSP32-like proteins

PpSP32-like proteins have only been found in sand flies; their function is unknown. In *P. perniciosus* they possess a collagen-related internal sequence (Anderson et al., 2006). In *P. arabicus*, however, these proteins bear no significant similarity with collagen; this feature is shared with PpSP32-like proteins of *P. papatasi*, *P. ariasi* or *P. argentipes*. Similarly to other protein families analyzed, the phylogenetic

position of *P. arabicus* PpSP32-like proteins is close to that of *P. ariasi* and *P. perniciosus* homologs (Figure 4B). Three different clusters were found on *P. arabicus* salivary gland cDNA library, the mature proteins being 88.1% identical (Figure 4A). The variable length of the middle part of the protein sequence results in three different variants of mature proteins; the predicted M_w of the three variants are 25, 26.3 and 27.8 kDa. All three proteins have a remarkably basic pI (10.37 on average). No N-glycosylation sites were predicted for these proteins and thus none of the bands observed in Western blots is likely to represent PpSP32-like proteins.

Antigen 5-related proteins

Antigen 5 (Ag5) protein is present in vespid venom (Fang et al., 1988) and related proteins were reported in saliva of bloodsucking insects (e.g., Charlab et al., 1999; Valenzuela et al., 2002b). Similarly to most other sand fly species studied so far, only one cluster coding for Ag5-related protein (PabSP4) was found on *P. arabicus* cDNA library (Charlab et al., 1999; Anderson et al., 2006; Oliveira et al., 2006). Mature Ag5-related proteins of sand flies are 45.6% identical and 14.5% similar. The phylogenetic analysis shows close relationship of *P. arabicus* Ag5-related protein to *P. perniciosus* and *P. ariasi*, much in the same way as in other salivary protein families. The predicted M_w of the mature protein is 31.1 kDa and the pI very basic (9.27). In the immunoblot, the band running at 31 kDa might represent Ag5-related protein.

Apyrase

Apyrases are widespread in saliva of bloodsucking insects; for a great part, the antihemostatic effects of saliva are due to apyrase anti-platelet activity (Champagne, 2005). Sand fly apyrases belong to the *Cimex* apyrase family (Valenzuela et al., 2001b). Three very similar apyrase clusters coding for apyrases were found on *P. arabicus* cDNA library (98.4% identity). The predicted average pI of *P. arabicus* apyrases is 8.85 and the predicted M_w is 35.3 kDa. Apyrases were previously suggested as antigens recognized by sera of mice and dogs experimentally exposed to sand flies or people living in areas endemic for leishmaniases (Rohousova et al., 2005; Hostomska et al., 2008; Gomes et al., 2002). It is likely that apyrase proteins are present within the broad band of 34.3-36.3 kDa observed in our immunoblot experiments.

Endonuclease

One cluster (PabSP49) coding for putative endonuclease was idintified on *P. arabicus* cDNA library. The sequence has a match with NUC domain in Conserved Domains database, which is typical for DNA/RNA non-specific endonucleases. Since

all residues composing the active site, the substrate binding site and the Mg^{2+} binding site are conserved in this cluster, we suggest that PabSP49 might possess endonuclease activity. The predicted pI of the mature protein is very high (9.45); it should however be noted that endonucleases with a highly basic pI are not exceptional. Predicted M_w of the mature protein is 40.5 kDa; as two N-glycosylation sites were predicted for this protein, we assume that the apparent M_w of PabSP49 protein may correspond to the band in the immunoblot running at 42 kDa.

Hyaluronidase and other putative enzymes

Putative hyaluronidase is coded by cluster PabSP72. Hyaluronidase activity was detected in several species of bloodsucking insects including sand flies (Cerna et al., 2002; Volfova et al., 2008). Accessible cDNA data suggest that hyaluronidase transcripts are absent from salivary transcriptomes of *P. papatasi* and *P. duboscqi.* Nonetheless, the enzyme activity was detected in these species (Cerna et al., 2002), which probably reflects the scarcity of both hyaluronidase transcript and hyaluronidase protein in sand fly salivary glands, and at the same time underlines the remarkably high specific activity of the enzyme. The predicted pI for mature PabSP72 is 9.07 and the M_w is 53 kDa; however, as 9 N-glycosylation sites were predicted in the protein sequence, it is very likely that the actual M_w is much higher. Two arguments support our assumption that the 56-58.5 kDa band observed in immunoblots represents hyaluronidase. Firstly, hyaluronidase is a well-known allergen of hymenopteran venoms (King et al., 1996); secondly, no predicted secreted proteins from *P. arabicus* have M_w higher than PabSP72. However, we cannot exclude the possibility that the 56-58.5 kDa band corresponds to a dimer of lower-M_w salivary proteins.

Sequences coding for other putative enzymes could not be obtained as full-length clones. These included a pyrophosphatase and enzymes involved in sugar digestion (amylase, trehalase).

Other putative secreted proteins

Several other proteins predicted to be secreted were found, most of them with unknown function. *P. arabicus* salivary transcripts code for two different variants of ParSP09-like proteins with a predicted M_w of 33 and 34 kDa, respectively. Apart from 4 sand fly species (*P. ariasi*, *P. perniciosus*, *P. argentipes* and *L. longipalpis*), no other related proteins from any organism were reported. Homologs of 30 kDa proteins and the extremely basic *P. arabicus* 2.7 kDa protein were only found in *P. ariasi* and *P. perniciosus* (Anderson et al., 2006; Oliveira et al., 2006); our finding contradicts the

suggestion that 2.7 kDa proteins are specific for the subgenus *Larroussius* (Anderson et al., 2006). Likewise, homologs of the acidic PabSP75 protein were found exclusively in *P. ariasi* (ParSP13; Oliveira et al., 2006) and homologs of acidic 16 kDa salivary proteins PabSP63 and PabSP64 only in *P. argentipes* (PagSP73; Anderson et al., 2006). Two sequence clusters coding for putatively secreted proteins show no similarity with known sand fly sequences. First, cluster PabSP107 is related to *Aedes aegypti* salivary secreted mucin. Second, cluster PabSP126 is a homolog of conserved hypothetical proteins of culicine as well as anopheline mosquitoes. Several sequences with no matches to accessible databases were found on *P. arabicus* cDNA library. However, none of these was predicted to code for a secreted protein.

Concluding remarks

Analysis of salivary transcripts of *P. arabicus* showed that most sequences were closely related to those of *Larroussius* species, confirming current data on sand fly phylogenetics. Most of the sequences present in *P. arabicus* proteome have homologs in other sand fly species studied so far; however, some of them were only reported from members of the subgenus *Larroussius*. We suggest the identity of all antigenic bands observed in immunoblots of *P. arabicus* salivary glands with sera of mice bitten by *P. arabicus*; these results should be confirmed by mass spectrometry analysis in future.

ACKNOWLEDGEMENTS

We thank Dr. Helena Kulíková and Dušan Eremiáš for helpful technical and administrative assistance. The study was partially supported by projects MSM 0021620828, LC 06009 and GAUK 139/2006/B-BIO/PrF.

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Table 1

Cluster number	Sequence name	Present in proteome	Number of sequences in cluster	Best match to NR protein database	E value	Signal peptide cleavage site	pI	M_w
1	SP15-related salivary protein	Y	31	gi|61817267, 14 kDa salivary pr	1E-062	20/21	8.98	14.3
2	SP15-related salivary protein	Y	70	gi|61817267, 14 kDa salivary pr	5E-062	20/21	9.1	14.2
3	SP15-related salivary protein	Y	26	gi|61817267, 14 kDa salivary pr	1E-061	20/21	9.21	14.2
4	antigen 5-related protein		28	gi|61373238, salivary protein	1E-133	18/19	9.27	31.1
6	SP15-related salivary protein	Y	1	gi|61817267, 14 kDa salivary pr	3E-062	20/21	8.98	14.3
7	SP15-related salivary protein	Y	1	gi|61817267, 14 kDa salivary pr	2E-061	20/21	9.1	14.2
8	SP15-related salivary protein	Y	1	gi|61817267, 14 kDa salivary pr	1E-060	20/21	9.21	14.2
11	27 kDa salivary protein	Y	3	gi|61744161, 27 kDa salivary pr	2E-090	21/22	4.97	26.6
12	25 kDa salivary protein	Y	3	gi|61744161, 27 kDa salivary pr	9E-090	21/22	5.04	24.8
13	25 kDa salivary protein	Y	5	gi|61744161, 27 kDa salivary pr	6E-091	21/22	5.04	24.9
14	25 kDa salivary protein	Y	18	gi|61744161, 27 kDa salivary pr	3E-090	21/22	5.04	24.8
15	27 kDa salivary protein	Y	49	gi|61744161, 27 kDa salivary pr	9E-092	21/22	5.04	26.7
16	25 kDa salivary protein	Y	6	gi|61744161, 27 kDa salivary pr	1E-089	21/22	5.03	24.8
20	D7-related salivary protein	Y	50	gi|74099915, 27 kDa salivary pr	1E-108	19/20	9.22	26.9
21	D7-related salivary protein	Y	2	gi|76446595, 27 kDa D7-related	1E-104	19/20	9.29	26.1
22	D7-related salivary protein	Y	4	gi|74099915, 27 kDa salivary pr	1E-109	19/20	9.26	27.0
26	yellow-related salivary protein	Y	29	gi|61373243, salivary protein	0.0	18/19	8.40	42.9
27	yellow-related salivary protein	Y	5	gi|61373243, salivary protein	0.0	18/19	8.57	42.9
29	PpSP32-like salivary protein		8	gi|61817261, 30.5 kDa salivary..	4E-069	18/19	10.52	27.8
30	PpSP32-like salivary protein		12	gi|61817261, 30.5 kDa salivary..	1E-068	18/19	10.32	26.3
31	PpSP32-like salivary protein		14	gi|61817261, 30.5 kDa salivary..	7E-066	18/19	10.27	25.0
32	34 kDa-like salivary protein		8	gi|61807168, 34 kDa salivary pr	1E-160	21/22	8.89	34.0
33	34 kDa-like salivary protein		2	gi|61807168, 34 kDa salivary pr	1E-139	21/22	9.15	32.8
34	34 kDa-like salivary protein		12	gi|61807168, 34 kDa salivary pr	1E-139	21/22	8.89	34.1
35	34 kDa-like salivary protein		5	gi|61807168, 34 kDa salivary pr	1E-151	21/22	9.10	33.0
39	apyrase	Y	11	gi|61817259, 35.5 kDa salivary..	1E-167	20/21	8.89	35.3
40	apyrase	Y	3	gi|61817259, 35.5 kDa salivary..	1E-168	20/21	8.77	35.3
41	apyrase	Y	4	gi|61817259, 35.5 kDa salivary..	1E-168	20/21	8.89	35.3
43	SP15-related salivary protein	Y	2	gi|61807162, 14 kDa salivary pr	2E-059	19/20	9.33	14.2
44	SP15-related salivary protein	Y	2	gi|61807162, 14 kDa salivary pr	2E-059	19/20	9.33	14.2
45	SP15-related salivary protein	Y	12	gi|61807162, 14 kDa salivary pr	8E-060	19/20	9.33	14.2
49	putative endonuclease	Y	14	gi|61807170, 41 kDa salivary pr	0.0	23/24	9.45	40.5
52	30 kDa-like salivary protein		14	gi|76446615, 30 kDa salivary pr	1E-150	29/30	8.76	29.8
53	46 kDa salivary protein		12	gi|76446617, 46 kDa salivary pr	1E-172	20/21	5.97	46.4
54	D7-related salivary protein	Y	7	gi|61744153, 27 kDa salivary pr	1E-103	19/20	9.11	26.7
55	D7-related salivary protein	Y	3	gi|76446593, 24.5 kDa D7-relate	1E-103	19/20	9.04	26.7
57	2.7 kDa salivary protein		9	gi|61744159, 2 kDa salivary pro	0.015	23/24	12.31	2.7
59	D7-related salivary protein		7	gi|61807164, 27 kDa salivary pr	1E-102	19/20	9.42	27.4
63	16 kDa salivary protein		4	gi|74486577, 16 kDa salivary pr	4E-032	21/22	5.36	15.9
64	16 kDa salivary protein		3	gi|74486577, 16 kDa salivary pr	6E-033	21/22	5.18	16.0
72	putative salivary hyaluronidase		4	gi|4887110, putative hyaluronida	2E-096	19/20	9.07	53.0
75	10 kDa salivary protein		4	gi|61744147, 12 kDa salivary pr	2E-032	21/22	4.56	10.3
84	D7-related salivary protein		3	gi|74099915, 27 kDa salivary pr	1E-053	19/20	9.34	28.1
93	SP15-related salivary protein		3	gi|61807166, 14 kDa salivary pr	4E-055	20/21	9.22	14.1
107	putative salivary secreted mucin 3		2	gi|94468382, putative salivary...	1E-27	20/21	4.37	22.2
126	conserved hypothetical protein		2	gi|108871689, conserved...	5E-049	22/23	5.34	17.2

FIGURE LEGENDS

1. Characterization of *P. arabicus* salivary antigens. Salivary proteins separated by SDS-PAGE, 12% gel, and blotted onto nitrocellulose membrane. Position of molecular weight markers (kDa) indicated on the left. 1: total protein profile, amidoblack staining. 2-5: Western blot incubated with sera of naive mouse (2), mice repeatedly bitten by *P. arabicus* (3,4), or mouse repeatedly bitten by *P. papatasi* (5).

2. Analysis of SP15 family of sand fly salivary proteins. (A) Multiple sequence alignment of SP15 and SP15-like salivary proteins from *Phlebotomus arabicus* (Pab), *P. ariasi* (Par), *P. argentipes* (Pag), *P. perniciosus* (Ppe), *P. papatasi* (Pp), *P. duboscqi* (Pdu), and *Lutzomyia longipalpis* (Lulo). Sequences were aligned using ClustalW and manually refined using BioEdit. (B) Phylogenetic tree analysis of sequences shown in (A). Phylogenetic analysis was performed in TreePuzzle 5.2 by maximum likelihood using quartet puzzling, automatically estimating internal branch node support (1000 replications). *Drosophila melanogaster* NP_651713 was set as outgroup for the reconstruction of the phylogenetic tree.

3. Analysis of D7 family of sand fly salivary proteins. (A) Multiple sequence alignment of D7-related salivary proteins from *Phlebotomus arabicus* (Pab). Sequences were aligned using ClustalW and manually refined using BioEdit. (B) Phylogenetic tree analysis of D7-related salivary proteins from *P. arabicus* (Pab), *P. ariasi* (Par), *P. argentipes* (Pag), *P. perniciosus* (Ppe), *P. papatasi* (Pp), *P. duboscqi* (Pdu), *Lutzomyia longipalpis* (Lulo), *Culex quinquefasciatus* (clu1), and *Anopheles stephensi* (clu2). Phylogenetic analysis was performed in TreePuzzle 5.2 by maximum likelihood using quartet puzzling, automatically estimating internal branch node support (1000 replications). *Drosophila melanogaster* NP_651713 was set as outgroup for the reconstruction of the phylogenetic tree.

4. Analysis of PpSP32 family of sand fly salivary proteins. (A) Multiple sequence alignment of PpSP32-like salivary proteins from *Phlebotomus arabicus* (Pab). Sequences were aligned using ClustalW and manually refined using BioEdit. (B) Phylogenetic tree analysis of PpSP32-like salivary proteins from *P. arabicus* (Pab), *P. ariasi* (Par), *P. argentipes* (Pag), *P. perniciosus* (Ppe), *P. papatasi* (Pp), *P. duboscqi* (Pdu), and *Lutzomyia longipalpis* (LJL). Phylogenetic analysis was performed in TreePuzzle 5.2 by maximum likelihood using quartet puzzling, automatically estimating internal branch node support (1000 replications). *Corynebacterium diphteriae* NP_939825 was set as outgroup for the reconstruction of the phylogenetic tree.

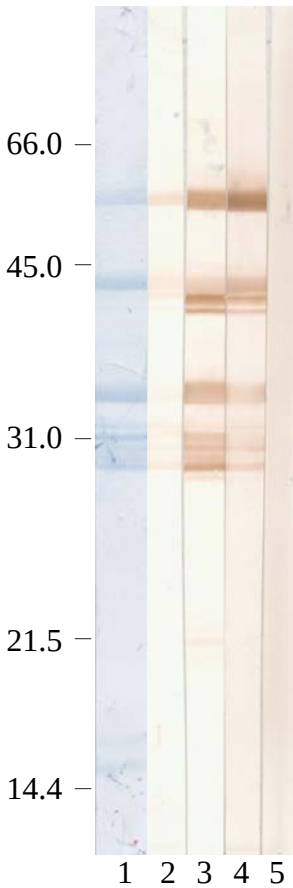

Figure 1

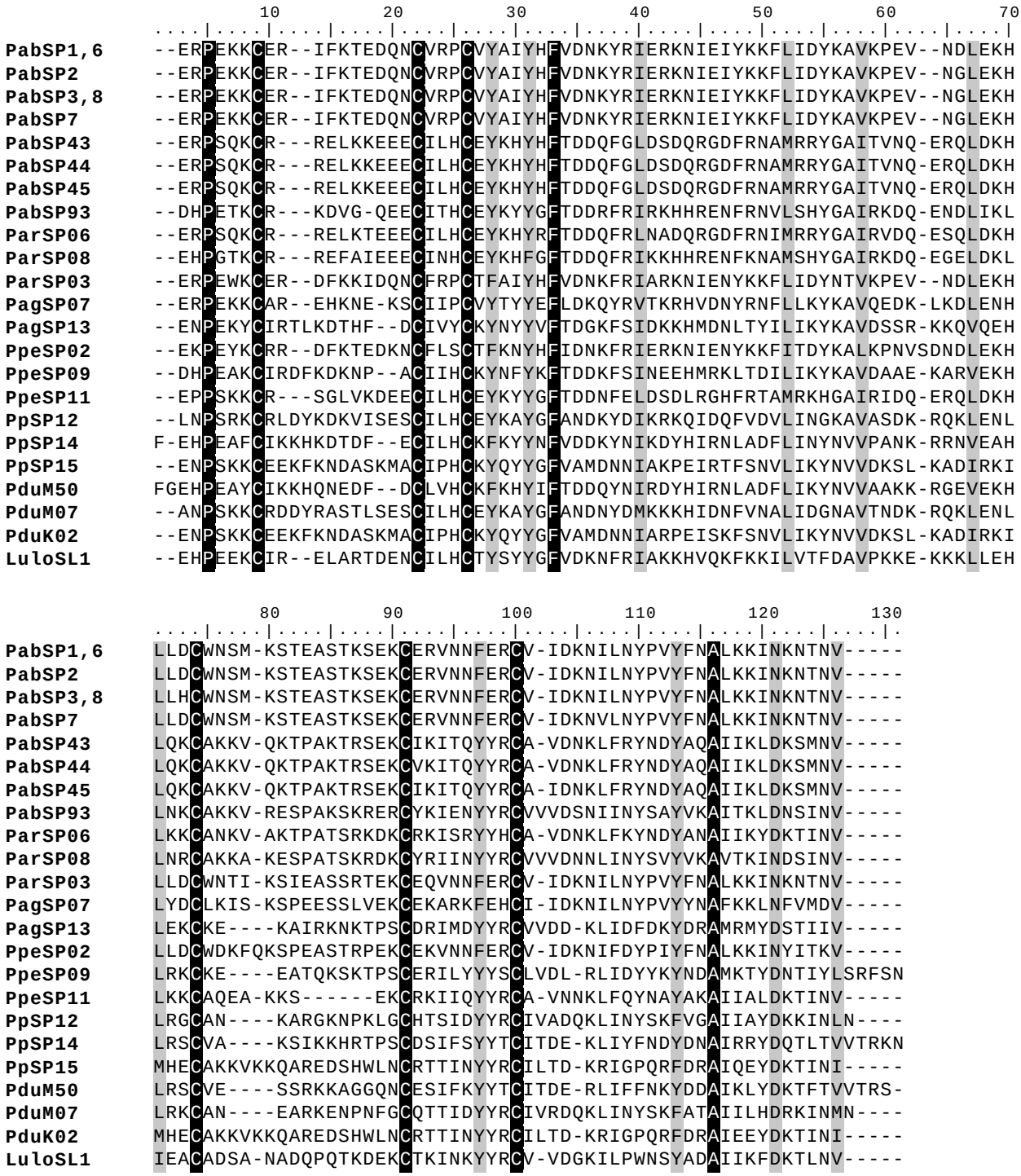
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Figure 2A

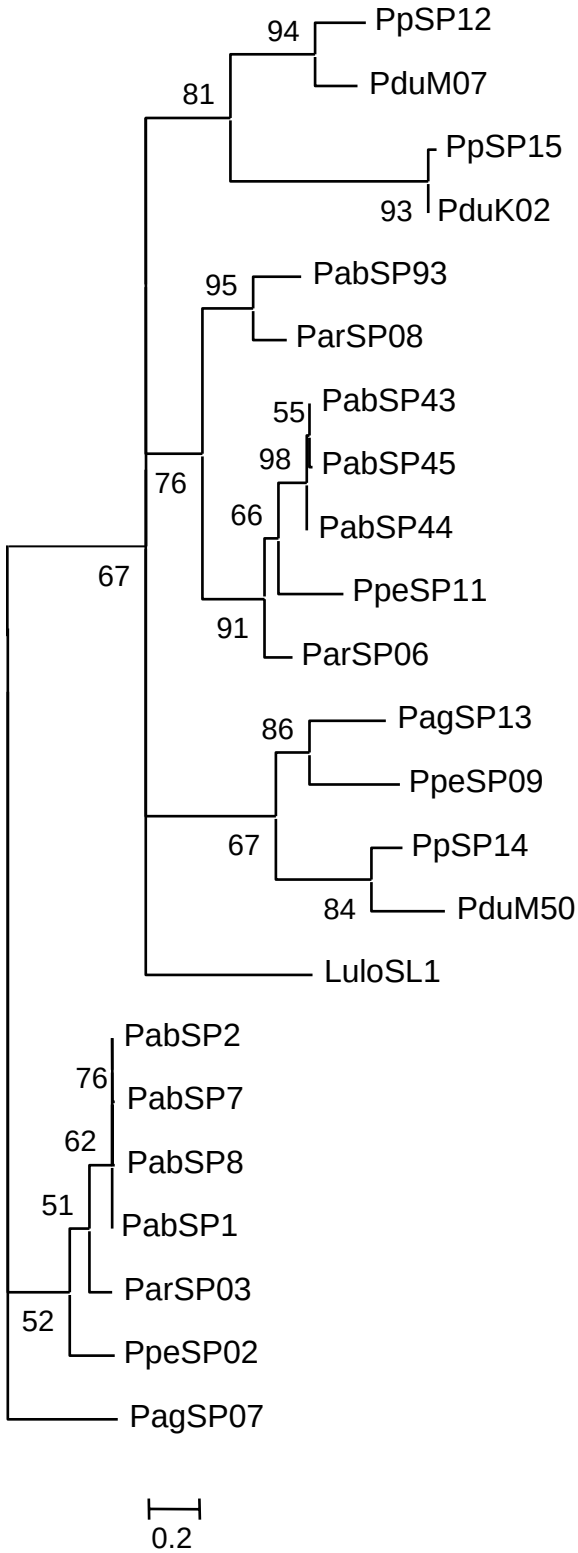
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Figure 2B

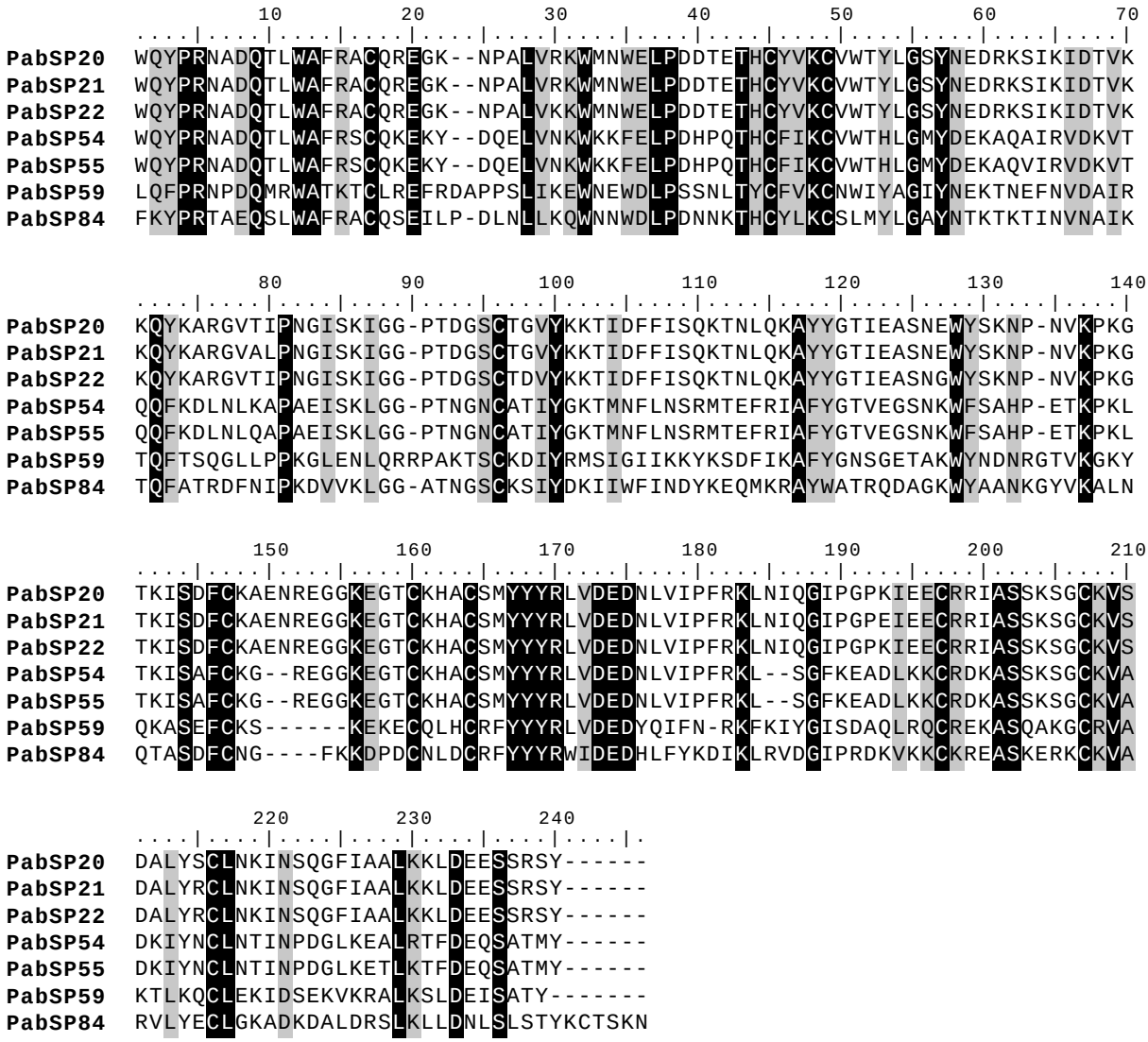
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Figure 3A

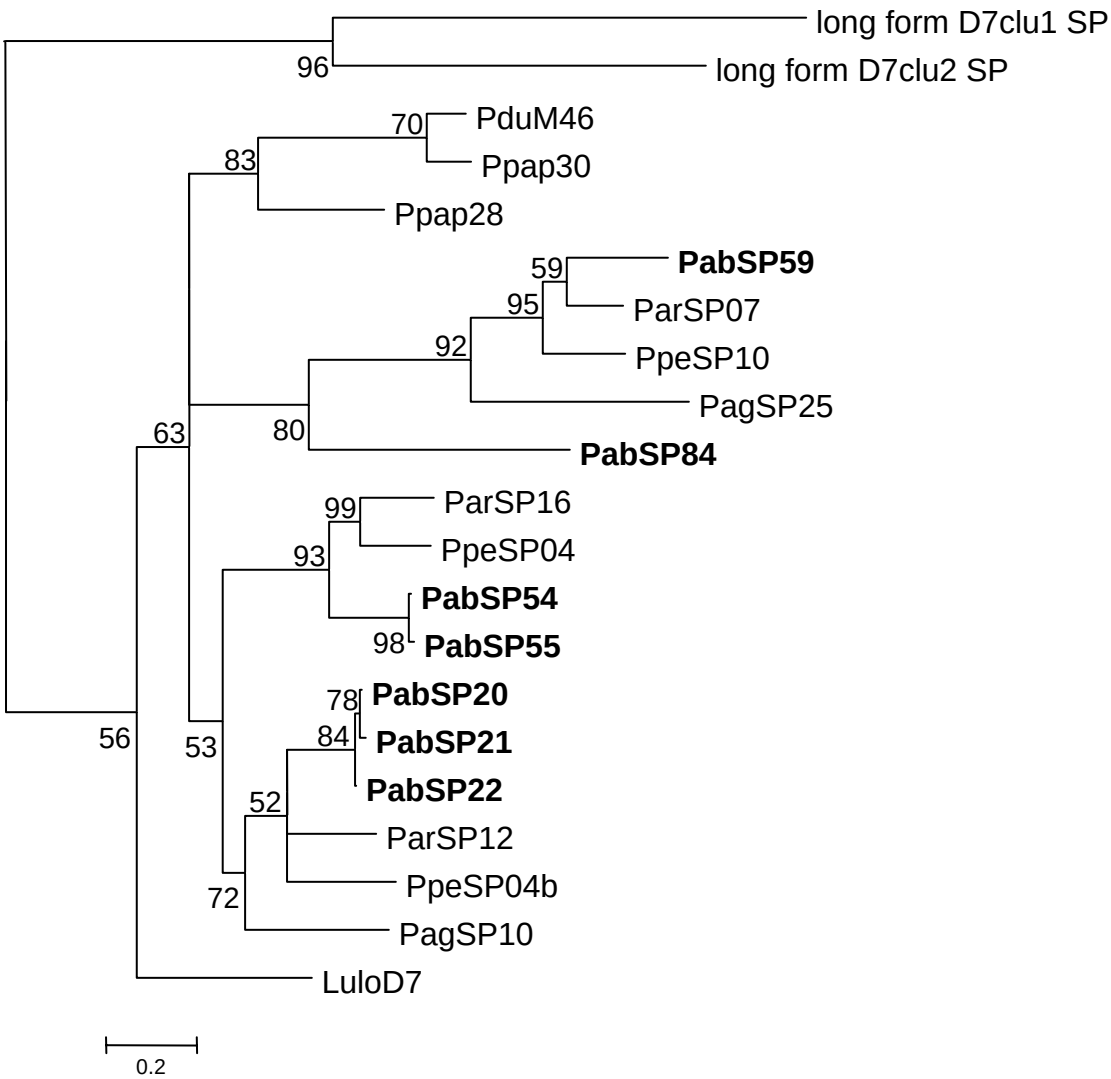
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Figure 3B

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Figure 4A

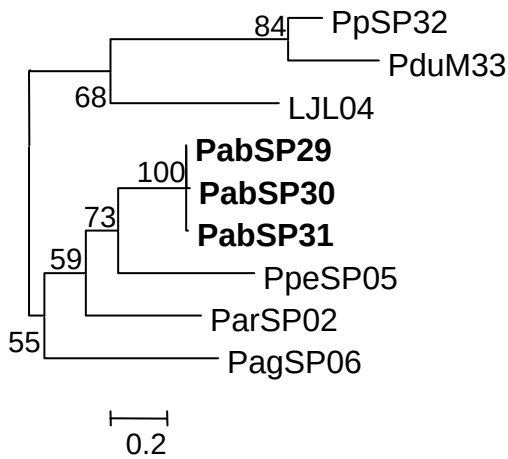

Figure 4B