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Transmission and epidemiology of visceral leishmaniasis

Přenos a epidemiologie viscerálních leishmanióz

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Ph.D. thesis/Dizertační práce

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I declare that Tatiana Spitzová (Košťálová) substantially contributed to the experimental work in the three projects presented here in her Ph.D. thesis and had a principal role in writing of two of five publications presented.

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CONTENTS

LIST OF ABBREVIATIONS

A.	*Arvicanthis*
CanL	canine leishmaniasis
CL	cutaneous leishmaniasis
DAT	direct agglutination test
DTH	delayed-type hypersensitivity
ELISA	enzyme linked immunosorbent assay
ICT	immunochromatographic test
IFAT	indirect fluorescent antibody test
ITN	insecticide treated bed nets
L.	*Lutzomyia*
Le.	*Leishmania*
LST	*Leishmania* skin test
P.	*Phlebotomus*
PCR	polymerase chain reaction
PKDL	post kala-azar dermal leishmaniasis
SGH	salivary gland homogenate
VL	visceral leishmaniasis

ABSTRACT

Visceral leishmaniasis (VL) is widespread disease caused by protozoa *Leishmania donovani* and *Leishmania infantum*. Human visceral leishmaniasis caused by *Le. donovani* in India is considered an anthroponosis, however in East Africa, the role of animals as reservoirs remains unclear. The first part of this thesis demonstrated natural *Leishmania* infection in wild rodents and bats in Ethiopia. Overall, 8.2% rodents and 4.9% bats were positive for *Leishmania* spp. Subsequent sequencing revealed that 10% of *Leishmania*-positive rodents were infected by parasites from *Le. donovani* complex, on the other hand, no *Le. donovani* DNA was detected in bats. All *Le. donovani*-positive rodents were captured in the localities of southwest Ethiopia where human VL cases have been reported and potential sand fly vectors occur. Our findings indicate that rodents are likely to play a role in VL transmission in Ethiopia.

During blood feeding, sand flies inoculate into the host skin immunogenic salivary proteins which elicit species specific antibody response. Anti-saliva antibodies could be used as a marker of host exposure to sand flies and, in leishmaniasis endemic areas, also as risk markers of *Leishmania* infection. In order to find out if the domestic animals (dog, goat, cow, and donkey) from north and northwest Ethiopia are involved in VL cycle we measured antibodies against saliva of *Phlebotomus orientalis*, the implicated vector of VL in Ethiopia. A total of 23.1% of the animals were seropositive for anti-*P. orientalis* saliva IgG, with the highest seroprevalence observed in dogs (58.8%) and sheep (47.7%) followed by donkeys, goats and cows. These results indicate opportunistic feeding preferences of *P. orientalis* and also the possibility of zoonotic transmission of *Le. donovani.*

The third part of this thesis is focused on the use of recombinant salivary proteins instead of whole saliva in antibody detection assays. We have used, for the first time the *Phlebotomus perniciosus,* salivary recombinant proteins in a longitudinal field study on dogs from *Le. infantum* endemic locality in south Italy. We found a strong correlation between IgG antibodies recognizing 43 kDa recombinant yellow-related protein (rSP03B) and the whole salivary antigen. The kinetics of antibody response had similar pattern for whole saliva and for rSP03B and was clearly related to seasonal activity of *P. perniciosus.* We found association between canine leishmaniasis infection and antibodies against whole saliva but not against rSP03B. Moreover, in cross-sectional study from Portugal and Italy we proposed rSP03B protein as universal marker of canine exposure to *P. perniciosus* within its entire distribution area.

ABSTRACT IN CZECH

Viscerální leishmanióza (VL) je onemocnění působené prvoky *Leishmania donovani* a *Leishmania infantum*. Zatím co v Indii je lidská VL působená *Le. donovani* považována za antroponózu, ve východní Africe se diskutuje o zapojení zvířat jako rezervoárových hostitelů. První část mé dizertační práce je zaměřená na detekci leishmánií v hlodavcích a netopýrech odchycených v různých oblastech Etiopie. Celkově bylo na *Leishmania* spp. pozitivních 8.2 % hlodavců a 4.9 % netopýrů. Následná sekvenace odhalila parazity z komplexu *Le. donovani* u 10 % hlodavců, u netopýrů infekce *Le. donovani* nebyla detekována. Všichni hlodavci pozitivní na *Le. donovani* byli odchyceni v oblastech jihozápadní Etiopie, kde je výskyt lidské VL a jejích potenciálních vektorů známý. Naše výsledky naznačují možnou účast hlodavců v koloběhu VL v Etiopii.

V průběhu sání flebotomové injikují do rány imunogenní slinné proteiny, které vyvolávají druhově specifickou protilátkovou odpověď. Protilátky proti slinám mohou být využity jako ukazatelé expozice hostitele flebotomům a v oblastech s endemickým výskytem leishmanióz jako ukazatelé rizika infekce leishmániemi. S cílem objasnit, zda domácí zvířata (pes, koza, ovce, kráva a osel) ze severní a severozápadní Etiopie mohou hrát úlohu v přenosu VL, jsme měřili hladiny protilátek proti slinám *Phlebotomus orientalis*, předpokládaného vektora *Le. donovani* v Etiopii. Celkově byly protilátky proti slinám *P. orientalis* detekovány u 23.1 % zvířat, nejvyšší seroprevalence byla u psů (58.8 %) a ovcí (47.7 %), následně pak u oslů, koz a krav. Výsledky naznačují oportunistické chování *P. orientalis* při hledání zdroje krve a tedy i možnost zoonotického přenosu *Le. donovani*.

Třetí část mé dizertační práce je zaměřená na použití rekombinantních proteinů namísto celých slinných žláz v sérologických testech. Nejprve byly použity rekombinantní proteiny ze slinných žláz *P. perniciosus* v dlouhodobé studii dělané na psech žijících v oblasti s endemickým výskytem *Le. infantum* v jižní Itálii. Zaznamenali jsme silnou pozitivní korelaci mezi protilátkami proti celým slinným žlázám a 43 kDa rekombinantním yellow proteinem (rSP03B). Kinetika protilátkové odpovědi proti celým slinám i rSP03B proteinu měla stejný průběh a jasně korelovala se sezonním výskytem flebotomů. Popsali jsme také asociaci mezi zvýšenou hladinou protilátek proti celým slinám a leishmániovou infekcí u psů, u protilátek proti rSP03B proteinu však pozorována nebyla. Na základě výsledků z průřezové studie z Itálie a Portugalska jsme navrhli rSP03B protein jako univerzální ukazatatel expozice psů *P. pernicosus* v celém areálu jeho rozšíření.

INTRODUCTION

1 VISCERAL LEISHMANIASIS

Leishmania species (Kinetoplastida: Trypanosomatidae) are the causative agents of leishmaniasis, a neglected disease spread mainly in tropical and subtropical areas. These parasitic protozoans are transmitted by the bite of blood sucking insects, phlebotomine sand flies (Diptera: Psychodidae), to vertebrate host (reviewed in Ready, 2014). Only female phlebotomine sand flies feed on blood and inoculate the promastigote flagellar form of pathogen into the host skin. The parasites are internalized by dendritic cells and macrophages in the dermis and transform into aflagellated amastigotes. They multiply and survive in phagolysosomes (reviewed in Chappuis *et al.*, 2007). Clinical forms of leishmaniasis are diverse and represent a complex of diseases such as visceral leishmaniasis (VL), muco-cutaneous and cutaneous leishmaniasis (CL) (reviewed in Desjeux, 2004).

In this work I focused on the Old World visceral leishmaniasis, caused by *Leishmania donovani* and *Leishmania infantum*. *Leishmania donovani* is found only in the Old World in the northeast of the Indian subcontinent and in East Africa. Most endemic sites are believed to be anthroponotic and *Phlebotomus argentipes* in Indian subcontinent and *Phlebotomus orientalis*, *Phlebotomus martini* and *Phlebotomus celiae* in East Africa served as vectors [reviewed in (Maroli *et al.*, 2013; Ready, 2014)]. Zoonotic transmission of *Le. donovani* has been suspected in mentioned regions, but the role of animals in VL transmission is not still clearly established (Elnaiem *et al.*, 2001; Hassan *et al.*, 2009; Bhattarai *et al.*, 2010; Alam *et al.*, 2011). Visceral leishmaniasis caused by *Le. infantum* is considered to be zoonosis with the canine reservoir host. *Leishmania infantum* is spread not only in the Old World (Mediterranean basin, Middle East and Central Asia to Pakistan and in China) but also in the New World (Central and South America) under the name *Leishmania chagasi* (synonym to *Le. infantum*). The main vector in the New World is *Lutzomyia longipalpis*, in the Old World several species are involved, mainly belonging to the subgenus *Larroussious*. The major proven vectors of *Le. infatum* in Mediterranean area are *Phlebotomus perniciosus*, *Phlebotomus ariasi* and *Phlebotomus neglectus* [reviewed in (Gramiccia & Gradoni, 2005; Maroli *et al.*, 2013)].

The disease tends to be relatively chronic, and children are especially affected. The incubation period ranges from 10 days to over 1 year. Strong inflammatory

reactions develop within the viscera 2-8 months after infection and often without initial skin lesions. Symptoms of VL can take years to appear. The common symptoms are fever, malaise, shivering or chills, weight loss, depressed production of red blood cells, white cells and platelets, splenomegaly, with or without hepatomegaly and lymphadenopathy. If untreated the disease is progressive and a symptomatic infection are often fatal [reviewed in (WHO, 2010; Ready, 2014)].

In areas endemic for VL, approximately 200 000 to 400 000 VL cases appear each year. The mortality estimate is 20 000 to 40 000 deaths per year. More than 90% of global VL cases occur in six countries: India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil (Alvar *et al.*, 2012). The higher incidence of VL is associated with less developed regions (poor housing and peridomestic sanitary conditions, malnutrition, etc.) [reviewed in (Chappuis *et al.*, 2007; WHO, 2010)].

This thesis is comprised from projects dealing with different aspects of VL epidemiology and transmission in East Africa and the Mediterranean region, thus, in the following paragraphs I will focus in detail on the epidemiology of VL and the role of animals in transmission of VL in these endemic localities.

2 EPIDEMIOLOGY OF VISCERAL LEISHMANIASIS IN EAST AFRICA

In East Africa, VL is endemic in the eastern part of Sudan, South Sudan, Somalia, Ethiopia, Kenya and Uganda (reviewed in Ngure *et al.*, 2009). In former studies from Ethiopia and Sudan, the VL strains isolated from human or animal samples were distinguished as three species, *Le. donovani*, *Le. infantum* and *Leishmania archibaldi* (Dereure *et al.*, 2000; Dereure *et al.*, 2003; Alvar *et al.*, 2007). Nowadays, this distinction is considered as controversial and it was suggested that all East African strains should be classified as *Le. donovani* sensu stricto. This taxon includes strains previously classified as *Le. archibaldi* and *Le. infantum*, which are genetically similar to other East African *Le. donovani* strains (Jamjoom *et al.*, 2004; Lukes *et al.*, 2007). Examination of 63 recent *Leishmania* strains isolated from Ethiopian VL patients revealed that all parasites were shown to be *Le. donovani* (Zackay *et al.*, 2013). Moreover, multilocus microsatellite typing divided East African *Le. donovani* strains into two populations, Sudan and north Ethiopia as one population and Kenya and south Ethiopia as another (Gelanew *et al.*, 2010). A similar clustering of Ethiopian *Le. donovani* strains into northern and southern populations was achieved by the difference on the size of k26-PCR (polymerase chain reaction) amplicons (Zackay *et al.*, 2013). Presence of these two populations correlates well with distribution

of the distinctive sand fly species incriminated as vectors of *Le. donovani* in these localities, *P. orientalis* in the north (Elnaiem & Osman, 1998b; Gebresilassie *et al.*, 2015b) and *P. martini* and *P. celiae* in the south (Gebre-Michael & Lane, 1996).

The main incriminated vector of VL in East Africa is *P. orientalis* from subgenus *Larroussius*. This subgenus occurs mainly in Paleartic region and includes most of the VL Old World vectors (reviewed in Lewis *et al.*, 1974). *Phlebotomus orientalis* has been reported to exist in wide areas in the eastern Sudan (Elnaiem *et al.*, 1997; Lambert *et al.*, 2002; Hassan *et al.*, 2004), South Sudan (Schorscher & Goris, 1992), Somali (reviewed in Elnaiem, 2011) and Kenya (Ngumbi *et al.*, 2010). In Ethiopia, *P. orientalis* has been observed from the northern (Gebresilassie *et al.*, 2015c), northwestern (Gebre-Michael *et al.*, 2010; Lemma *et al.*, 2015), northeastern (Gebre-Michae*, et al.*, 2004) and southwestern (Hailu *et al.*, 1995; Balkew *et al.*, 1999) lowlands regions and from the highlands of northwestern region (Gebre-Michael *et al.*, 2007). The distribution of this species seems to be associated by vegetation type, with preference for *Acacia seyal*–*Balanites aegyptiaca* forest and deeply cracking black clay soil (Elnaiem *et al.*, 1997; Elnaiem *et al.*, 1998a; Elnaiem *et al.*, 1999; Lemma *et al.*, 2014b; Moncaz *et al.*, 2014). *Phlebotomus orientalis* have been found infected with *Le. donovani* and implicated as vector of VL in the eastern Sudan (Elnaiem & Osman, 1998b; Elnaiem *et al.*, 2001; Hassan *et al.*, 2004; Hassan *et al.*, 2008) and South Sudan (Schorscher & Goris, 1992) endemic regions. Natural infection of Ethiopian *P. orientalis* was reported only once in 1995 in one specimen from the southwestern region (Hailu *et al.*, 1995). Recently, the natural infection by *Leishmania* promastigotes and detection of *Le. donovani* DNA were both described in a single specimen of *P. orientalis* from the northern Ethiopia (Gebresilassie *et al.*, 2015b). Moreover, the susceptibility of *P. orientalis* to harbour massive late-stage infection of Ethiopian *Le. donovani* strains was proved in laboratory conditions. Experimental infections revealed that infective dose between one and two promastigotes per fly is sufficient for full infection development in *P. orientalis* (Seblova *et al.*, 2013). These results suggest that *P. orientalis* is responsible for the maintenance of *Le. donovani* in VL endemic regions in Ethiopia.

The annual burden of VL incidence in East African region is estimated to be between 29 400 and 56 700 cases. The largest proportions of the cases are reported from Sudan, Ethiopia and South Sudan (Alvar *et al.*, 2012). In Ethiopia, the disease is prevalent mostly in lowland, arid areas with an estimated 2500 to 4000 new cases occurring per year and with the population at risk more than 3.2 million [reviewed in (Leta *et al.*, 2014; Gadisa *et al.*, 2015)].

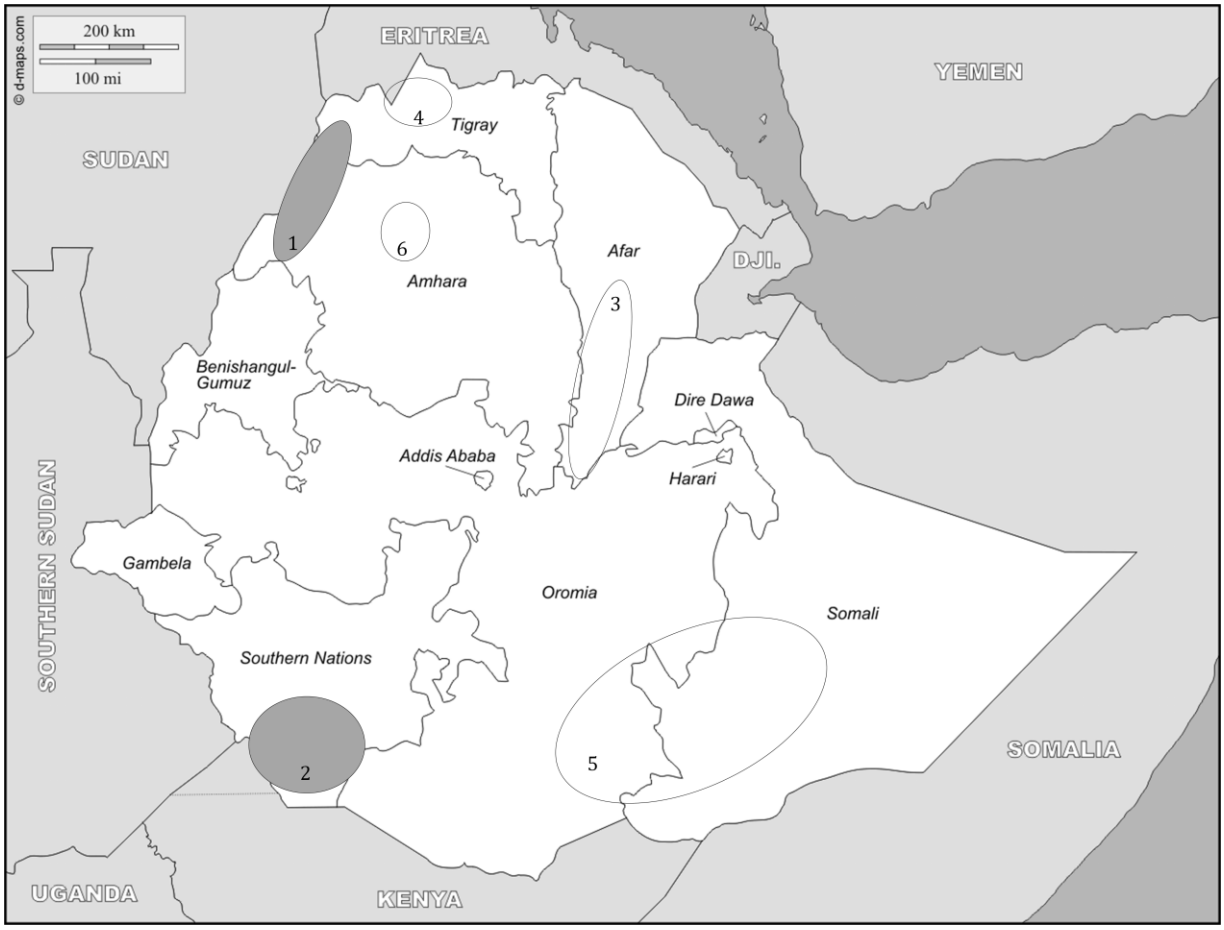

Figure 1. The distribution of VL endemic localities in Ethiopia. Full circles represents traditional VL endemic areas.

The most important traditional endemic foci include Metema and Humera plains (Tigray and Amhara regions) in northwestern lowland focus (Figure 1, area 1) (Yared *et al.*, 2014; Lemma *et al.*, 2015) and Omo plains, Aba-Roba plains and the Segen and Woyto river valleys in southwestern lowland focus ([Southern Nations, Nationalities and Peoples´ Region]; Figure 1, area 2) (Fuller *et al.*, 1979; Ayele & Ali, 1984; Ali & Ashford, 1994). The northwestern focus represent the main VL endemic area in the country with the highest HIV/VL co-infection rate world-wide (reviewed in Gadisa *et al.*, 2015).

Awash valley (Afar region) (Figure 1, area 3) in the northeastern lowland focus represents locality with high positivity reaction to *Leishmania* skin test (LST) but with no confirmed VL cases (Ali *et al.*, 2002; Ali *et al.*, 2004). Despite the presence of the incriminated vector *P. orientalis* (Gebre-Michael *et al.*, 2004), it seems that VL have not been established in this locality.

Climatic and environmental changes, migration of seasonal workers to and from endemic areas and malnutrition resulted in the change of VL epidemiology. Since 2000, VL cases were reported from previously non-endemic localities or from localities with sporadic cases in northern Ethiopia (Tigray region) (Figure 1, area 4) (Abbasi *et al.*, 2013) and southeastern Ethiopia (Somali and Oromia regions) (Figure 1, area 5) (Marlet *et al.*, 2003; Alvar *et al.*, 2012). In May 2005 a new epidemic of VL was reported in the highland district Libo Kemkem (Amhara region) (Figure 1, area 6) in northwest Ethiopia. The outbreak occurred in region, where few cases of the disease had ever been reported before. The VL epidemic expanded rapidly over a several-year period, spread over two district and transformed into sustained endemic situation by 2007 (Alvar *et al.*, 2007; Herrero *et al.*, 2009). The hypothesis behind the highland epidemic is that leishmaniasis was introduced into Libo Kemkem by returning seasonal workers from Humera and Metema lowlands (Alvar *et al.*, 2007). The data of *Leishmania* infection prevalence for Libo Kemkem after the epidemic 2004-2007 showed overall seroprevalence in children 1.02%. This decrease could be a result of establishment of VL treatment centre after the outbreak what contributed to a reduction in the seroprevalence rate for the whole population (Sordo *et al.*, 2012). Despite of this low prevalence, the presence of the vector *P. orientalis* (Gebre-Michael *et al.*, 2007) and the human VL cases support the establishment of new VL endemic focus in Ethiopia.

In East Africa there is no conclusive evidence whether the principal way of transmission of *Leishmania* parasites is zoonotic or anthroponotic [reviewed in (Leta *et al.*, 2014; Gadisa *et al.*, 2015)]. It is supposed that during epidemics, VL is transmitted between humans without the intervention of reservoir host. These epidemics are however, short-lived and must arise from residual foci that have yet to be fully described (reviewed in Ashford, 1996).

During the interepidemic periods with low numbers of VL cases, patients with post kala-azar dermal leishmaniasis (PKDL) may serve as a possible *Le. donovani* reservoir. PKDL is a complication of VL occurring in VL recovered patients characterised by a macular, maculopapular, and nodular rash harbouring *Leishmania* parasites (reviewed in Zijlstra *et al.*, 2003). It is mainly seen in Sudan and India, in Ethiopia the prevalence of PKDL is lower but higher in HIV co-infected patients in comparison with Sudan (reviewed in Desjeux *et al.*, 2013). The possible role of PKDL patients as reservoir is strengthen by the fact, that laboratory-bred females of *P. argentipes* became infected when taking blood meal on PKDL patients from the northeastern Indian subcontinent and developed motile promastigotes in the midgut (Addy & Nandy, 1992). Nevertheless,

the role of PKDL patients in transmission of *Le. donovani* needs further investigations to prove this hypothesis.

In order to determine the role of a given animal host in a reservoir system, it is far from sufficient to simply discover infected individuals. In order to incriminate a reservoir host formally, it is necessary to demonstrate that the parasite population depends on that particular mammal for its long-term maintenance. In general, full and objective incrimination is hardly possible, and any drawn conclusion must depend on an accumulation of evidence based on the following criteria [reviewed in (Ashford, 1996; WHO, 2010)]:

- abundant reservoir host,
- intense host-sand fly contact,
- long-lived reservoir host , at least surviving through any non-transmission season,
- long time infection without acute disease in affected individuals,
- availability of parasites in the skin or the blood in sufficient numbers to be taken up by sand fly
- similar parasite strain in infected reservoir host and in infected human patient.

Host preferences of sand fly vectors represent an important aspect of vector-borne disease dynamics. In experiment involving domestic animals (cow, donkey, sheep, goat) and humans in north Ethiopia, higher numbers of *P. orientalis* females were attracted and engorged on donkey and cow than on other hosts (Gebresilassie *et al.*, 2015d). These animals present favoured bloodmeal sources for *P. orientalis* as demonstrated in direct bloodmeal analysis by enzyme linked immunosorbent assay (ELISA) and PCR (Gebresilassie *et al.*, 2015a). In northwest Ethiopia, the results of blood meal analysis (Gebre-Michael *et al.*, 2010) showed 91.6% of engorged *P. orientalis* positive for bovine blood and only about 2% for human blood and 2.6% for mixed feeding (cattle and human). Another bloodmeal analysis from the same locality (Lemma *et al.*, 2014a) showed that only 8% of engorged *P. orientalis* females fed on cattle. However, 28% bloodmeals were identified as human and 36% as mixed origin (bovine and human). Interestingly, in eastern Sudan, the dog-baited trap significantly attracted the highest number of *P. orientalis* females, followed by the Egyptian mongoose (*Herpestes ichneumon*) baited trap and less frequent by the common genet (*Genetta genetta*) and nile rat (*Arvicanthis niloticus*) baited trap (Hassan *et al.*, 2009). Among smaller wild animals, more numbers of *P. orientalis* females were attracted to ground squirrel (*Xerus rutilus*) followed by hares (*Lepus* sp.), gerbils (*Tatera robusta*) and spiny rat (*Acomys cahirinus*) (Gebresilassie *et al.*, 2015d). The tendency of female *P. orientalis*

to engorge in large numbers on certain species of domestic as well as wild animals strongly indicated that the species is primarily zoophilic with opportunistic feeding preferences depending on the accessibility of bloodmeal host.

Dogs are the most important species among domesticated animals in the VL epidemiology. They play a key role as reservoir hosts in the transmission cycle of the closely related *Le. infantum* in the Mediterranean region and Latin America (reviewed in Ready, 2014). Several studies have reported positivity for *Leishmania* DNA or seropositivity of dogs in VL focus in the eastern Sudan (Dereure *et al.*, 2000; Dereure *et al.*, 2003; Hassan *et al.*, 2009) and the northwestern lowland and highland VL foci in Ethiopia (Alvar *et al.*, 2007; Bashaye *et al.*, 2009; Kalayou *et al.*, 2011; Kenubih *et al.*, 2015). The longitudinal study from a village in eastern Sudan reported seroprevalence rates 72.5%, 74.3% and 42.9% in 1998, 1999 and 2000, respectively (Dereure *et al.*, 2003). On the other hand, a cross-sectional survey conducted in 10 villages in 2002 in eastern Sudan detected anti-*Leishmania* antibodies only in 6.9% of dogs (Hassan *et al.*, 2009). Hassan et al. (2009) hypothesized that the observed differences in canine seropositivity between their study and study of Dereure et al. (2003) may be due to timing of sampling and also due to quite low dog density in eastern Sudan and clear variation in dog ownership by members of different tribes (Hassan *et al.*, 2009).

At the beginning of the VL outbreak in highland area in the northwestern Ethiopia in 2005, 5% of dog cases due to *Le. donovani* complex was reported (Alvar *et al.*, 2007). In 2006, 3.8% of dogs were positive by serology and 2.8% by PCR analysis (Bashaye *et al.*, 2009). Recent study from 2008 and 2009 from the same locality observed occurrence of *Leishmania*-seropositive dogs 39.1%. Authors hypothesized that this difference could be as a result of the time gap between the human outbreak and canine outbreak (Kenubih *et al.*, 2015). Importantly, dog ownership was found to significantly increase the risk of human VL in eastern Sudan (Nackers *et al.*, 2015) and in lowlands (Argaw *et al.*, 2013; Yared *et al.*, 2014) and highlands (Bashaye *et al.*, 2009) of northwestern Ethiopia.

In eastern Sudan screening of domestic animals for the presence of specific *Le. donovani* antibodies revealed 68.7% of seropositive donkeys, 21.4% of seropositive cows and 8.5% of seropositive goats (Mukhtar *et al.*, 2000). Recent study from highland VL focus in the northwestern Ethiopia showed high *Le. donovani* seropositivity in cattle (41.9%) and donkeys (33.3%) and low seropositivity in goats (10%) and sheep (4.8%) (Kenubih *et al.*, 2015). In relation to domestic animals the elevated risk of human VL in northwest Ethiopia was associated with owning sheep or goats (Custodio *et al.*, 2012;

Yared *et al.*, 2014). On the other hand, in eastern Sudan the presence of domestic animals in the yard at night was found protective (Nackers *et al.*, 2015). In the eastern Uganda and western Kenya the application of insecticide to livestock increased the risk for human VL. In turn, sleeping in a compound with animals reduce the likelihood of being infected. This could be explained by the zoophilic behaviour of *P. martini*, the principal VL vector in these regions. The insecticide application on animals indicates the shift in sand fly host seeking behaviour (Kolaczinski *et al.*, 2008).

In 1960s in southern Sudan (Hoogstraal & Heyneman, 1969) reported *Leishmania* infection in three rodent species (black rat/*Rattus rattus*, spiny mouse/*Acomys albigena*, Nile grass rat/*Arvicanthis niloticus luctuosus*) and two carnivore species (Senegal geneta/*Genetta genetta senegalensis*, Sudanese serval/*Felis serval phillipsi*). The authors supposed that the infection was caused by *Le. donovani*. However, in all these cases there was no parasite identification (Hoogstraal & Heyneman, 1969). Later, in 1970s the investigation in the northwestern and southwestern lowlands in Ethiopia revealed *Leishmania* infection in spleen and liver of *A. niloticus*. The examination of the isolates showed that the parasites isolated from *A. niloticus* appear to be different from *Le. donovani* isolates (Haile & Lemma, 1977). Recent studies from eastern Sudan reported 0.6% *Le. donovani* DNA positivity and 5.5% *Le. donovani* seropositivity in *A. niloticus* (Mukhtar *et al.*, 2000; Elnaiem *et al.*, 2001) and 13% *Le. donovani* DNA positivity in *Mastomys natalensis* (natal multimammate mouse) (Elnaiem *et al.*, 2001). In eastern Sudan the presence of *Le. donovani* was reported also in 14% of Egyptian mongoose (Elnaiem *et al.*, 2001).

The relative high seroprevalence in dogs (Dereure *et al.*, 2003; Bashaye *et al.*, 2009; Hassan *et al.*, 2009; Kalayou *et al.*, 2011; Kenubih *et al.*, 2015), the long-lasting (Dereure *et al.*, 2003) and the apparently asymptomatic course of the infection (Bashaye *et al.*, 2009; Kalayou *et al.*, 2011) suggest dog as *Le. donovani* reservoir. Moreover, genetically similar canine and human *Le. donovani* strains highlight the role of dogs as important local reservoir (Dereure *et al.*, 2003; Baleela *et al.*, 2014). On the contrary, the failure of isolation of *Le. donovani* from skin, lymph node, liver, spleen and bone marrow of dogs positive by direct agglutination test (DAT) indicates that the involvement of dog as VL reservoir host is still controversial (Kenubih *et al.*, 2015).

The role of other domestic animals in VL transmission is unclear, with only few studies observing the progress of the infection in infected animals. Sheep experimentally infected by *Le. donovani* promastigotes did not show any signs of VL apart from pyrexia. No parasites were isolated from visceral organs throughout the 8 months sampling

period (Anjili *et al.*, 1998). Similarly, the donkeys were able to overcome the experimental infection with *Le. chagasi* and no parasite infection of *L. longipalpis* was observed after the bloodmeal on infected animals (Cerqueira *et al.*, 2003). The results from Nepal indicated that the *Le. donovani* infection could persist for at least several months in goats (Bhattarai *et al.*, 2010). According the results from bloodmeal analyses the most probable involvement of domestic animals in VL transmission is as a source of blood for the sand fly females (Gebre-Michael *et al.*, 2010; Gebresilassie *et al.*, 2015a).

Among wild mammals, the Egyptian mongoose was proposed as a possible reservoir host of *Le. donovani* in eastern Sudan. It is abundant species with gregarious behaviour (Elnaiem *et al.*, 2001) and it is attractive for *P. orientalis* females (Hassan *et al.*, 2009). However, no skin samples gave *Leishmania* specific PCR product (Elnaiem *et al.*, 2001).

Taking together the information about the role of domestic and wild animals in VL transmission are scarce. Therefore, in-depth studies of the reservoir host and transmission dynamics are very crucial for identifying animal reservoir(s), or establishing the absence of zoonosis in East Africa.

3 EPIDEMIOLOGY OF VISCERAL LEISHMANIASIS IN MEDITERRANEAN BASIN

Leishmania infantum is the causative agent of human and canine leishmaniasis (CanL), zoonosis potentially fatal to humans and dogs (reviewed in Solano-Gallego *et al.*, 2011). Around Mediterranean basin, several sand fly species from subgenus *Larroussius* are involved in *Le. infantum* transmission, namely *P. perniciosus*, *P. ariasi*, *P. neglectus* and *Phlebotomus perfiliewi* (reviewed in Killick-Kendrick, 1999). In my thesis I will focused mainly on CanL endemic areas in western and central part of Mediterranean basin with *P. perniciosus* as the major vector. In recent years, the range of this sand fly species is expanding from south Europe foci to northern localities. In Italy, the comparison of recent entomological surveys with historical data showed that this vector evidently increased in density and expanded its geographic range in northern continental Italy (Ferroglio *et al.*, 2005; Maroli *et al.*, 2008; Morosetti *et al.*, 2009). *Phlebotomus perniciosus* was captured also in the Pyrenees Mountains in southern France (Dereure *et al.*, 2009), in northern France, southern Switzerland (ECDC), in the southwestern Germany (Naucke *et al.*, 2008) and Andorra (Ballart *et al.*, 2012b).

Canine leishmaniasis is considered to be traditionally endemic around the Mediterranean countries such as Cyprus, Greece, Albania, Croatia, Italy, Malta, France, Spain and Portugal (Figure 2) (reviewed in Maia & Cardoso, 2015a). According to the database created by Franco *et al.*(2011) from published and unpublished surveys of CanL in southern Europe (Portugal, Spain, France and Italy from 1971 to 2006), the overall CanL seroprevalence in dogs was 23.2%. The median seroprevalence was 10%, with the highest median seroprevalence in Italy (17.7%), followed by France (8%), Portugal (7.3%) and Spain (5.9%) (Franco *et al.*, 2011). The study based on questionnaires send to animal clinics in southwestern European regions over the period 2004-2011 found higher incidence of CanL for Spain, Portugal in comparison with France. Unfortunately, there were no data for Italy (Bourdeau *et al.*, 2014).

Figure 2. Distribution of CanL in Europe. Dark grey colour represent countries or regions where *Le. infantum* is endemic, light grey colour represent potentially endemic regions or countries [modified according (Maia & Cardoso, 2015a)].

Recently the expansion of CanL towards new biotopes at northern latitudes and higher altitudes was observed (Figure 2). Environmental changes, global warming, and growing world trade and animal transportation, including the increased mobility of dogs across borders, have an impact on the geographic distribution of leishmaniases [reviewed in (Antoniou *et al.*, 2013; Maia & Cardoso, 2015a)].

In Italy, the northward spread of CanL has been assessed on retrospective literature analysis of CanL cases and recent analysis of new canine cases (Ferroglio *et al.*, 2005; Maroli *et al.*, 2008). Since 1990s the autochthonous CanL cases were observed in at least 23 foci all over northern regions with seroprevalence ranged from 0.7% to 9.7% (Ferroglio *et al.*, 2005; Maroli *et al.*, 2008).

The Pyrenees Mountains are usually considered to be outside of the endemic range of leishmaniasis in southern France. Nevertheless, in 2007 the seroprevalence of autochthonous *Leishmania*-positive dogs in foothills villages reached 11.32%, in year 1994 it was only 1.43%. This increase could be explained by a rise of 1°C in the mean annual temperature which could affect the density of sand fly vector (Dereure *et al.*, 2009).

In Spain, the new CanL autochthonous foci were reported from the northeastern and northwestern regions, with the seroprevalence 33.1% and 3.7%, respectively (Amusategui *et al.*, 2004; Ballart *et al.*, 2012a). Moreover, study from non-endemic locality in north Spain, where infection in the dog has not been previously reported, provided the first evidence for *Le. infantum* infection in wild carnivores (Del Rio *et al.*, 2014).

Recently, the new *Le. infantum* focus has been described in southwestern Madrid region. Between July 2009 and December 2012 446 human cases were reported, mostly from town Fuenlabrada (366 human cases). Monitoring revealed high densities of the vector *P. perniciosus* (Arce *et al.*, 2013; Jimenez *et al.*, 2013; Jimenez *et al.*, 2014) but no increase in prevalence of CanL (Arce *et al.*, 2013). Monitoring of potential reservoirs showed no role in leishmaniasis outbreak for stray cats (Miro *et al.*, 2014) but revealed high prevalence of *Le. infantum*-positive hares and rabbits (Molina *et al.*, 2012; Jimenez *et al.*, 2014). Establishing of new parks modified the ecology of hares and rabbits, moving them from a woodland cycle to an urban one without any predators. The new VL focus in southwestern Madrid region is thus an example of leishmaniasis emergence due to environmental changes induced by man [(reviewed in Antoniou *et al.*, 2013); Arce *et al.*, 2013].

In European countries, non-endemic for CanL, infection is restricted mainly to dogs that have travelled to and/or from endemic areas of the Mediterranean region as it was documented in CanL cases from Finland (Karkamo *et al.*, 2014), Germany (Menn *et al.*, 2010), Netherlands (Teske *et al.*, 2002) and United Kingdom (Shaw *et al.*, 2009). However, small proportions of autochthonous CanL infections were reported from Hungary (Farkas *et al.*, 2011), Romania (Mircean *et al.*, 2014), Germany (Naucke & Lorentz, 2012) and Finland (Karkamo *et al.*, 2014). It is possible that transmission in some countries (Germany, Hungary) could be due to vectors (Naucke *et al.*, 2008; Farkas *et al.*, 2011). However, vertical or horizontal (via direct blood to blood or sexual contact) transmission of CanL or transmission through bites or wounds was reported in dogs in Germany (Naucke & Lorentz, 2012) and Finland (Karkamo *et al.*, 2014).

Leishmania infantum infection in dogs has an appearance of multisystemic disease with variable clinical signs. It could range from subclinical to fatal illness, any organ, tissue or body fluid could be involved (reviewed in Solano-Gallego *et al.*, 2011). The most common clinical manifestations are lymph nodes enlargement, skin lesions, conjuctivities and weight loss (Ciaramella *et al.*, 1997; Oliva *et al.*, 2006; Otranto *et al.*, 2009; Manzillo *et al.*, 2013; Bourdeau *et al.*, 2014; Perego *et al.*, 2014). Importantly, in CanL it has to be distinguished between clinical leishmaniasis and subclinical leishmaniasis. Dogs with clinical leishmaniasis present clinical signs and/or clinicopathological abnormalities and have a confirmed *Le. infantum* infection. Dogs with subclinical infection or clinical healthy infected dogs present neither clinical signs on physical examination nor clinicopathological abnormalities but have a confirmed *Le. infantum* infection (reviewed in Solano-Gallego *et al.*, 2009). Importantly, the role of clinical healthy but *Leishmania* seropositive dogs in transmission of the disease could be not underestimated. Direct xenodiagnosis confirmed that these dogs are highly competent to infect the vector *P. perniciosus* with *Le. infantum* (Molina *et al.*, 1994; Guarga *et al.*, 2000).

Usually, there is a single primary reservoir host for a given *Leishmania* species in a particular focus. However, other animals may become infected in the same focus and could act as secondary reservoir host or accidental host [reviewed in (Quinnell & Courtenay, 2009; WHO, 2010)]. By definition, primary reservoir host can maintain parasite transmission in the absence of other hosts. On the contrary, secondary reservoir host can transmit infection but cannot maintain parasite transmission in the absence of primary host. Accidental host can be infected but play no role in maintenance of the system. However, differentiating primary and secondary host is difficult, when the

animal species suspected as reservoirs share the same geographic area [reviewed in (Quinnell & Courtenay, 2009; WHO, 2010; Maia & Campino, 2011)]. In case of *Le. infantum*, the well-known primary reservoir host is dog. However, there are other possible reservoir hosts, which could play important role in CanL epidemiology. In this thesis, I highlighted five of them: red fox (*Vulpes vulpes*), rats (*Rattus* sp.), domestic cat (*Felis domesticus*), wild rabbit (*Oryctolagus cuniculus*) and hare (*Lepus* sp.), see below.

Serological and/or direct evidence of infection with *Le. infantum* in south European wildlife carnivores have been reported for many species, e.g. red fox (*Vulpes vulpes*), wolf (*Canis lupus*), Egyptian mongoose, common genet, martens (*Martes* sp.). Nevertheless, red fox achieved most attention, probably due to its taxonomic relationship with dog and that it is the most abundant wild carnivore in Europe (reviewed in Millan *et al.*, 2014a). *Leishmania infantum* DNA in red foxes have been detected in northern, central and southern regions of Spain (Sobrino *et al.*, 2008; Del Rio *et al.*, 2014), in the southern (Dipineto *et al.*, 2007) and central Italy (Verin *et al.*, 2010), southeastern France (Davoust *et al.*, 2014) and in the northern/central Portugal (Cardoso *et al.*, 2015). The prevalence of PCR-positive animals ranged from 2% to 52%, with the lowest prevalence in the northern/central Portugal and with the highest in central Italy (Verin *et al.*, 2010; Cardoso *et al.*, 2015). However, different tissues used for the PCR detection could influence sensitivity of the method (Verin *et al.*, 2010).

Beside wildlife carnivore the *Le. infantum* infection has been detected also in black rat (*Rattus rattus*) and brown rat (*Rattus norvegicus*) (Di Bella *et al.* 2003; Helhazar *et al.*, 2013; Zanet *et al.*, 2014; Navea-Perez *et al.*, 2015). Recently, 15.5% and 33.3% of black rats positive for *Le. infantum* DNA have been recorded in the Montecristo island (Zanet *et al.*, 2014) and in the southeastern Spain (Navea-Perez *et al.*, 2015), respectively. In Portugal, Helhazar *et al.* (2013) reported for the first time natural infection of *Le. infantum* in brown rat.

The most discussed *Le. infantum* reservoir other then dog became Iberian hare (*Lepus granatensis*) and wild rabbit (*Oryctolagus cuniculus*) as a result of community outbreak of leishmaniasis since July 2009 in the southwestern Madrid region Fuenlabrada (Molina *et al.*, 2012; Arce *et al.*, 2013; Jimenez *et al.*, 2014). *Leishmania*-positive hares were found in Madrid region with seropositivity 74% (Moreno *et al.*, 2014) and also in six regions in the northern and southern Spain with *Leishmania* DNA prevalence 43.6% (Ruiz-Fons *et al.*, 2013). Additionally, in the focus of outbreak in the southwestern Madrid it was found high infection rate also in wild rabbits (Garcia *et al.*, 2014; Jimenez *et al.*, 2014; Moreno *et al.*, 2014). In the southeastern regions of Spain the

prevalence of PCR-positive rabbits between October 2009 and March 2010 was 0.6% (Chitimia *et al.*, 2011). On the contrary, study done in another region of southeastern Spain detected sharp increase in infection prevalence of wild rabbits in period from July 2009 to October 2011, from 1.1% to 84.2%. This increase could be more indicated as epidemic outbreak than an endemic steady state (Diaz-Saez *et al.*, 2014).

Domestic cats were usually considered as accidental host for *Le. infantum* infection. However, the development of feline medicine and diagnostic methods led to increased number of feline leishmaniasis case reports (reviewed in Pennisi *et al.*, 2015). Cats positive for *Le. infantum* serologicaly or by molecular methods were found in different areas across the Mediterranean with overall seroprevalence and molecular positivity ranged from 0% to 68.5% and 0% to 60.7%, respectively (Ozon *et al.*, 1998; Poli *et al.*, 2002; Pennisi *et al.*, 2004; Maroli *et al.*, 2007; Martin-Sanchez *et al.*, 2007; Solano-Gallego *et al.*, 2007; Ayllon *et al.*, 2008; Maia *et al.*, 2008b; Maia *et al.*, 2015b). The high variability in antibody or molecular prevalence may be due to different levels of endemicity, characteristics of the population under the study or differences in diagnostic methodologies (reviewed in Pennisi *et al.*, 2015). Interestingly, Miro et al. (2014) studied the role of cats in the recent outbreak of leishmaniasis in southwestern region of Madrid. The seroprevalence of *Le. infantum* infection was 3.2%, with homogenous distribution of infected animals in studied region. According the authors, this data indicate that cats were not playing an important role in *Le. infantum* transmission in this area (Miro *et al.*, 2014).

Detection of *Le. infantum* DNA or antibodies against *Le. infantum* does not necessarily mean active infection and infectiousness to sand flies. Therefore it is important to study the ability of an animal to infect sand flies via xenodiagnoses or detect the presence of the parasites in the skin (reviewed in Millan *et al.*, 2014a). Up to now, the xenodiagnoses of *Le. infantum* infection in hares, wild rabbits (Molina *et al.*, 2012; Jimenez *et al.*, 2014), cats (Maroli *et al.*, 2007) and rats (Pozio *et al.*, 1985) proved that these species are infective to vector *P. perniciosus*. Presence of *Le. infantum* DNA in the host skin was confirmed for rabbits from southeastern Spain (Diaz-Saez *et al.*, 2014) and from natural area of Madrid (Garcia *et al.*, 2014). Information of infectiousness of foxes and rats to sand flies are somehow inconsistent. For example, in south Italy 34% of foxes were PCR-positive when tested for *Leishmania* positivity in skin (Dipineto *et al.*, 2007). On the contrary in central Italy all skin samples from red foxes were PCR-negative (Verin *et al.*, 2010). In rats, Navea-Perez *et al.* (2015) reported one PCR-positive skin

sample from locality in southeastern Spain, no *Leishmania* DNA positive skin samples were found in Lisbon Metropolitan Area in Portugal (Helhazar *et al.*, 2013).

Chronic infection lasting through non-transmission season and non-pathogenic course of disease is another criteria needed to incriminate a species as a reservoir host [reviewed in (WHO, 2010; Millan *et al.*, 2014a)]. Good health status without any signs connected to leishmaniasis (e.g. lesions) in *Leishmania*-positive animals was reported for hares, rabbits, foxes and rats (Sobrino *et al.*, 2008; Verin *et al.*, 2010; Molina *et al.*, 2012; Helhazar *et al.*, 2013; Diaz-Saez *et al.*, 2014; Jimenez *et al.*, 2014; Moreno *et al.*, 2014; Navea-Perez *et al.*, 2015). Interestingly, xenodiagnoses studies carried out during *Le. infantum* non-transmission period proved that rabbits and hares were still infective to *P. perniciosus* proving long-lasting chronic infection (Molina *et al.*, 2012; Jimenez *et al.*, 2014). Study of (Diaz-Saez *et al.*, 2014) indicate that the rabbits can be infected for a long time without any acute symptoms of disease, given that the only clinical symptom was skin lesions in 23.3% of the specimen. The follow-up study of *Leishmania*-positive cats detected that 59.3% of cats revealed clinical signs at the time of the first analysis which, in many cases, were compatible with feline leishmaniasis (Martin-Sanchez *et al.*, 2007). Clinical form of feline leishmaniasis caused by *Le. infantum* demonstrates as a cutaneous form with visceral spread of the parasite (e.g. skin and/or muco-cutaneous lesions, lymph node enlargement) (Pennisi *et al.*, 2004; Martin-Sanchez *et al.*, 2007; Miro *et al.*, 2014). However, it seems that subclinical form of disease is more common than clinical illness (Martin-Sanchez *et al.*, 2007; Maia *et al.*, 2008b).

Species can act as reservoir host if the parasites isolated from the animals are molecularly indistinguishable from those of humans (reviewed in WHO, 2010). The analysis of *Le. infantum* strains in human and dogs from Mediterranean basin revelaled that the predominant *Le. infantum* zymodeme is MON-1. The domestic dog is clearly the reservoir host of this zymodeme in the Mediterranean basin, with prevalence 87.2% (Pratlong *et al.*, 2013). This zymodeme was found also in the rabbits (Diaz-Saez *et al.*, 2014), foxes (Pratlong *et al.*, 2013), cats (Ozon *et al.*, 1998; Maroli *et al.*, 2007; Pratlong *et al.*, 2013; Maia *et al.*, 2015b) and rats (Pratlong *et al.*, 2013) from various Mediterranean countries. Interestingly, the molecular typing of isolates from human samples from outbreak area in southaeastern Madrid showed that the "new" *Le. infatum* strain MHOM/ES/87/Lombardi was responsible of human leishmaniasis cases (Chicharro *et al.*, 2013). This same strain was also isolated from rabbits and hares from the same locality, what support the role of hares and rabbits in the outbreak (Molina *et al.*, 2012; Jimenez *et al.*, 2014).

Additionally, when incriminating species as possible reservoir it is necessary to take into account the presence of another abundant mammalian species. For example, at the Montecristo island, due the complete absence of carnivores, the authors suggested the presence of an autochthonous *Le. infantum* cycle in infected black rats (15.5% PCR-positivity) with *Phlebotomus mascittii* as possible vector. This assumption is based on the fact that from two trapped *P. mascittii* females one was PCR-positive to *Le. infantum* (Zanet *et al.*, 2014). However, Navea-Perez et al. (2015) emphasized that those authors did not take into account the presence of wild rabbits in that island.

Taking together all above mentioned information, it could be assumed that wild rabbits, hares, rats and domestic cats could contribute to maintaining the parasite in a given area. However, there is still need for further studies focused on infectiousness of host to sand flies, parasite isolation, standardization of diagnostic techniques, etc. (reviewed in Millan *et al.*, 2014a).

4 DIAGNOSIS OF VISCERAL LEISHMANIASIS AND SEROLOGICAL MARKERS OF EXPOSURE

The prevalence and incidence of CanL and human VL are important epidemiologic parameters the estimation of which depends on the reliable identification of infection.

Diagnosis of CanL should be based on an integrated approach considering signalment, history, clinical findings, and results of basic laboratory analyses that detect the parasite or evaluate the immune response of the host (reviewed in Paltrinieri *et al.*, 2010). The commonly used diagnostic methods include parasitological detection of the parasite by culture, molecular detection by PCR and serological techniques for detection of antibodies against *Leishmania*, such as indirect fluorescent antibody test (IFAT) and ELISA (Oliva *et al.*, 2006; Otranto *et al.*, 2009; Otranto *et al.*, 2010; Manzillo *et al.*, 2013). Among serological methods, ELISA is a classical method used in detection of antibodies to *Leishmania* and allows screening large number of samples in a short time. Specificity of this method is limited by use of crude antigens, either complete promastigotes or amastigotes or soluble extracts of them (reviewed in Maia & Campino, 2008a). The use of *Leishmania* recombinant proteins and peptides proved to be a sensitive and specific improvement to current serological diagnosis of CanL (Santarem *et al.*, 2010; Costa *et al.*, 2012). Molecular techniques can be easily applied to several biological samples, most sensitive and specific samples are bone marrow, lymph node and spleen, less sensitive is

blood and buffy coat (reviewed in Solano-Gallego *et al.*, 2011). Recently, a noninvasive conjunctival swab sampling coupled with a sensitive and specific PCR analysis, was proposed for the CanL diagnosis (Gramiccia *et al.*, 2010; Di Muccio *et al.*, 2012). This diagnostic method represents very sensitive assay and could be used for diagnosis of CanL in asymptomatic animals and for monitoring relapses in drug-treated dogs (Di Muccio *et al.*, 2012).

The diagnosis of human VL cases is based mainly on presence of clinical symptoms and on serological tests specially developed for field use, DAT and the rapid rK39 based immunochromatographic test (ICT) [reviewed in (Chappuis *et al.*, 2007; Kotb Elmahallawy *et al.*, 2014)]. Definitive VL diagnosis is supported by microscopic observation of parasites in clinical specimens (splenic, bone marrow aspirates) (reviewed in Kotb Elmahallawy *et al.*, 2014). An ICT test using recombinant rK39 protein (39-amino acid repeat that is part of a kinesin-related protein in *Le. chagasi* and which is conserved within the *Le. donovani* complex) has been developed for research purpose in both human and veterinary medicine (Sundar *et al.*, 1998; Otranto *et al.*, 2005). This test was designed based on a high sensitivity and specificity found in previous ELISA results with recombinant K39 protein as antigen (Badaró *et al.*, 1996; Zijlstra *et al.*, 1998). Like the DAT assay, ICT test is positive in a significant proportion of healthy individuals in endemic regions and for long periods after cure, hence these tests cannot discriminate between a case of VL relapse and other pathologies. Antibody based test must therefore always be used in combination with standardized clinical case definition for VL diagnosis [reviewed in (WHO, 2010; Kotb Elmahallawy *et al.*, 2014)]. Molecular techniques such as PCR from spleen or bone marrow samples are more sensitive than microscopic examination, however they remain complex and expensive. Thus in most VL endemic countries they are restricted to a referral hospitals and research centers [reviewed in (Chappuis *et al.*, 2007; Kotb Elmahallawy *et al.*, 2014)].

4.1 ANTIBODIES AGAINST SAND FLY SALIVA AS EPIDEMIOLOGICAL TOOL

During blood feeding sand fly inoculate into the host skin saliva containing a number of pharmacologically active molecules with diverse effects on the host's hemostatic responses. Components of sand fly saliva play a major role driving both the susceptibility to *Leishmania* infection and the disease severity [reviewed in (Andrade & Teixeira, 2012; Gomes & Oliveira, 2012)]. Saliva inoculated by sand fly into host skin also stimulates production of high levels of species-specific anti-saliva antibodies (Volf & Rohousova,

2001). Host specific antibody reaction against sand fly saliva was proposed to be used as marker of exposure to sand flies from both Old and New World (Gomes *et al.*, 2007; Hostomska *et al.*, 2008; Clements *et al.*, 2010; Marzouki *et al.*, 2011; Martin-Martin *et al.*, 2014). This epidemiological tool could be especially appreciated in evaluation of vector control campaigns (Gidwani *et al.*, 2011). Levels of antibodies against sand fly saliva could be also associated with *Leishmania* infection and could be used as risk marker (Barral *et al.*, 2000; Rohousova *et al.*, 2005; Vlkova *et al.*, 2011). The role of these proposed tools will be described in detail below.

The use of antibodies against sand fly saliva as markers of human and animals exposure to sand flies was already shown for Old and New World VL vectors such as *L. longipalpis* (Gomes *et al.*, 2007; Hostomska *et al.*, 2008; Aquino *et al.*, 2010; Soares *et al.*, 2013), *P. argentipes* (Clements *et al.*, 2010) and *P. perniciosus* (Vlkova *et al.*, 2011; Martin-Martin *et al.*, 2014) and for vectors of CL such as *Phlebotomus papatasi* (Rohousova *et al.*, 2005; Marzouki *et al.*, 2011)and *Phlebotomus sergenti* (Rohousova *et al.*, 2005).

In laboratory conditions, animals repeatedly exposed to sand flies revealed increased levels of anti-saliva IgGs. This relationship has been observed for mice, rabbits and dogs experimentally exposed to *P. perniciosus* (Vlkova *et al.*, 2011; Martin-Martin *et al.*, 2015), for mice experimentally bitten by *P. papatasi* (Vlkova *et al.*, 2012) and dogs experimentally exposed to *L. longipalpis* (Hostomska *et al.*, 2008). This association highlights the link between antibody production and the level of exposure. Long-term kinetics of antibodies against sand fly saliva in BALB/c mice experimentally exposed to *P. perniciosus* and *P. papatasi* showed that IgG levels were detectable from the fourth and fifth week, respectively. After the exposure period antibody levels were maintained for the end of the studies (Vlkova *et al.*, 2012; Martin-Martin *et al.*, 2015). To test the presence of memory B cell recall mice were additionally exposed to sand flies after the follow-up period. This exposure elicited in significant increase of anti-saliva antibodies (Vlkova *et al.*, 2012; Martin-Martin *et al.*, 2015). Dogs experimentally bitten by *P. perniciosus* and *L. longipalpis* developed the IgG levels with similar kinetics, gradually increased until week 5 (after the last exposure) and decreased to the end of the study (Hostomska *et al.*, 2008; Vlkova *et al.*, 2011).

In the field, antibodies against sand fly saliva as markers of exposure were applied in dogs from CanL endemic areas with vector *P. perniciosus* (Vlkova *et al.*, 2011; Martin-Martin *et al.*, 2014) or *L. longipalpis* (Gomes *et al.*, 2007). Dogs from mentioned areas revealed higher antibody reactivity against sand fly saliva in comparison with control

canine sera (Gomes *et al.*, 2007; Vlkova *et al.*, 2011; Martin-Martin *et al.*, 2014). Moreover, dogs originated from southwestern region of Madrid with low potential exposure to *P. perniciosus* (young dogs, treated with insecticide or sleeping indoors) revealed lower anti-saliva antibody levels than dogs that were not treated with insecticide and/or slept outdoors (Martin-Martin *et al.*, 2014). Difference in antibody levels between insecticide-treated and non-treated dogs was observed also in dogs experimentally bitten by *P. perniciosus* (Vlkova *et al.*, 2011) and *L. longipalpis* (Hostomska *et al.*, 2008).

In field trial in CanL endemic locality in south Italy the prevalence of dogs with anti-*P. perniciosus* IgG and anti-*Le. infantum* IgG increased significantly as the sand fly season progressed. The incidence of *Le. infantum* was very high, 45% of screened dogs were *Leishmania* seropositive. *Leishmania* seropositive and *Leishmania* seronegative dogs did not differ in production of IgG and IgG1 antibodies against sand fly saliva, but *Leishmania* seropositive dogs revealed significantly lower anti-*P. perniciosus* IgG2 antibodies compared to *Leishmania* seronegative dogs. These data may suggest that dogs with low IgG2 levels were at the higher risk of becoming *Leishmania*-infected. This hypothesis is supported by the fact that INF-γ which positively correlates with protective Th1 immune response, was higher in *Leishmania* seronegative dogs than in *Leishmania* seropositive dogs, although it was not significant. Authors proposed that specific IgG2 respone could be used as a risk marker of *Le. infantum* infection for dogs (Vlkova *et al.*, 2011).

Antibodies against sand fly saliva could also serve as markers of exposure in sentinel animals in endemic foci. For example, seroconversion of chicken naturally exposed to *L. longipalpis* was used to monitor the presence of *L. longipalpis* in the peri-domiciliary area in VL endemic regions. This monitoring could be useful tool for detecting areas of sand fly exposure in endemic regions which, ultimately, may help in directing control efforts against VL (Soares *et al.*, 2013).

The measurement of antibody levels against sand fly saliva was used also for the purpose of identification new possible wildlife reservoirs in CanL endemic foci. Anti-*L. longipalpis* saliva IgG antibodies were detected among crab-eating foxes (*Cerdocyon thous*) and dogs from VL endemic locality. The proportion of reactivity was significantly higher among crab-eating foxes than among the local dogs, thus the contact of *L. longipalpis* with vertebrate host may be higher in the wilderness than in domestic setting. These results, together with finding of natural infection of *Le. chagasi* among crab-eating foxes, imply the presence of a sylvatic cycle of VL that is independent of

transmission among dogs and humans (Gomes *et al.*, 2007). Similarly, using the detection of specific IgG antibodies against *P. perniciosus* saliva it was recently demonstrated that wild rabbits and hares captured in new VL focus in southwest Madrid showed high anti-saliva antibody levels (Martin-Martin *et al.*, 2014). The results indicate that hares and rabbit were exposed to *P. perniciosus* and support the hypothesis of existence of the sylvatic cycle (Molina *et al.*, 2012; Jimenez *et al.*, 2014). Monitoring anti-saliva humoral response in experimentally bitten rabbits showed that the IgG antibody levels may persist from the end of the period of *P. perniciosus* activity in Spain (late October) until the following emergence of adults (May) (Martin-Martin *et al.*, 2015). High levels of IgG antibodies against whole saliva were detected in sera from wild hares and rabbits captured during winter season in southwest Madrid region (Martin-Martin *et al.*, 2014). However, anti-saliva IgG antibody levels do not seem to be an accurate marker of exposure in rabbits since no decrease was observed even seven months after experimental exposures. Additionally, as a marker of recent exposure, IgM anti-saliva levels were investigated. No correlation was found between the progress of the immunization process and IgM response in rabbits experimentally exposed to *P. perniciosus* (Martin-Martin *et al.*, 2015).

Human antibody response to sand fly saliva was reported for various sand fly species from CL and VL endemic localities, e.g. *P. argentipes*, *P. papatasi*, *L. longipalpis*. In human populations in VL endemic locality in India, antibody levels against saliva of P. argentipes reflected differences in exposure to this sand fly species. *Leishmania* patients hospitalized and protected from sand fly bite by untreated bed nets showed by an average 15% reduction of IgG antibody titers against *P. argentipes* after 30 days. Upon discharge from hospitals, antibody levels against saliva in these patients increased significantly beyond their original pre-treatment levels after six month re-exposure to bites (Clements *et al.*, 2010). Gidwani et al. (2011) described that using insecticide treated bed nets (ITN) in trial communities over two years reduced the IgG antibody levels to the saliva of *P. argentipes* and *P. papatasi* by 9-12% compared to communities without ITN. The results provide the evidence that ITN have a limited effect on sand fly exposure in VL endemic localities in India and Nepal and support the use of sand fly saliva antibodies as a sensitive marker to evaluate vector control intervention (Gidwani *et al.*, 2011).

In human VL focus in Brazil, individuals with positive anti-*Le. chagasi* delayed-type hypersensitivity (DTH) response (marker of protection against *Le. chagasi*) have increased anti-*L. longipalpis* saliva antibodies while those who seroconverted (marker of

Le. chagasi infection) did not have increased anti-saliva antibody response. These results indicate that induction of immune response against sand fly saliva can facilitate induction of a protective response against leishmaniasis (Barral *et al.*, 2000; Gomes *et al.*, 2002; Aquino *et al.*, 2010). However, Aquino et al. (2010) hypothesized that increased anti-*Leishmania* DTH response in persons reactive to *L. longipalpis* saliva antibodies could quite well reflect increased parasite challenge in individuals who have been bitten more intensively by the sand fly vector.

In CL endemic locality in Turkey, patients with active CL possessed significantly higher anti-*P. sergenti* IgG levels than the healthy individuals from the same place. High levels of anti-saliva antibodies may indicate frequent exposure to sand flies, thus the higher probability of *Le. tropica* transmission (Rohousova *et al.*, 2005). Similarly, in Tunisia, children who developed CL after one sand fly season had significantly higher levels of anti-saliva IgG than children who did not develop CL. Thus, children with high titer of anti-*P. papatasi* antibodies were more likely to develop CL (Marzouki *et al.*, 2011). Higher anti-*Lutzomyia intermedia* saliva immune response was observed in Brazilian CL patients whereas individuals from the endemic area with a positive DTH skin test against *Leishmania* showed a lower IgG response against saliva. These results show that antibody response to saliva of *L. intermedia* is a risk marker of CL (de Moura *et al.*, 2007).

4.2 RECOMBINANT SALIVARY PROTEINS AS REPLACEMENT FOR WHOLE SALIVA

The studies mentioned previously used whole sand fly saliva homogenate (SGH) as antigen in serological tests. While whole salivary extract has advantages as it represents the complete repertoire of crude secreted salivary proteins, it also has several limitations such as necessity of laborious maintenance of sand fly colony and time-consuming dissections of salivary glands (reviewed in Andrade & Teixeira, 2012). Additionally, the use of whole saliva is complicated by potential cross-reactivity with saliva from other sand fly species or from other blood sucking insects (Volf & Rohousova, 2001; Clements *et al.*, 2010). The composition of total salivary proteins depends also on physiological factors such as age and diet (Volf *et al.*, 2000; Prates *et al.*, 2008) and may vary with the geographical origin of the sand fly species (Lanzaro *et al.*, 1999; Volf *et al.*, 2000; Rohousova *et al.*, 2012; Ramalho-Ortigao *et al.*, 2015). These problems could be overcome by using specific immunogenic sand fly salivary recombinant proteins that can

be produced in large quantities. The use of salivary recombinant proteins was already shown for *P. perniciosus* (Drahota *et al.*, 2014; Martin-Martin *et al.*, 2014; Martin-Martin *et al.*, 2015), *P. papatasi* (Marzouki *et al.*, 2012; Vlkova *et al.*, 2012; Marzouki *et al.*, 2015; Mondragon-Shem *et al.*, 2015) and *L. longipalpis* (Souza *et al.*, 2010; Teixeira *et al.*, 2010; Soares *et al.*, 2013).

In *P. perniciosus* the best candidates for evaluation of experimetall exposure of mice and dogs were 43 kDa yellow-related protein (SP03B, GenBank ID: DQ150622) and 35.5 kDa (SP01, GenBank ID: DQ192490) and 35.3 kDa apyrases (SP01B, GenBank ID: DQ192491) (Drahota *et al.*, 2014). Anti-salivary recombinant protein kinetics in sera of experimentally bitten mice highlighted the use of rSP01B (GenBank ID: KF178455) as an epidemiological marker of exposure. Anti-rSP01B IgG antibodies showed similar kinetic to IgG antibodies against whole saliva, gradually increasing during the 12 week-period of exposure to sand fly bites, reaching detectable levels from the fifth week onwards (Martin-Martin *et al.*, 2015). On the other hand, the increase of IgG levels against the D7-related recombinant protein (SP04, GenBank ID: KF178456) in experimentally bitten mice was slower than increase of IgG against whole saliva and peaked at later points, suggesting a delayed immune response to this protein (Martin-Martin *et al.*, 2015). In the field, the rSP03B (GenBank ID: KF257369) showed the best results for dogs, hares and rabbits from the endemic locality in the southwestern Madrid, as it presented greater correlation scores when values were compared with whole saliva in comparison with other recombinant proteins (Martin-Martin *et al.*, 2014).

In *L. longipalpis* recombinant 45 kDa (LJM17, GenBank ID: AF132518) and 43 kDa (LJM11, GenBank ID: AY445935) yellow-related proteins were proposed as suitable replacement for *L. longipalpis* SGH (Teixeira *et al.*, 2010). Recombinant salivary proteins LJM17 and LJM11 were sensitive enough to estimate levels of natural exposure to *L. longipalpis* in chicken (Soares *et al.*, 2013) and human sera (Souza *et al.*, 2010). Moreover, the combination of these two proteins showed higher effectivity than separate recombinants proteins when tested in human sera from locality endemic for VL in Brazil (Souza *et al.*, 2010). Specificity of rLJM11 and rLJM17 was proved by cross-reactivity with *L. intermedia* (sand fly species with rare occurrence in studied locality). Only 2 from 40 individuals who lived in an endemic area for *L. intermedia* recognized the combination of rLJM11+rLJM17, thus the level of cross-reactivity between salivary proteins of *L. longipalpis* and *L. intermedia* was very low (Souza *et al.*, 2010).

In *P. papatasi*, sera of mice experimentally exposed to this sand fly species reacted mainly with recombinant D7-proteins (PpSP30, GenBank ID: AF335489), although, this

protein was not recognize by all sera tested (4 out of 5) (Vlkova *et al.*, 2012). On the other hand, mice immunized with rPpSP30 (GenBank ID: JX411943) did not develop detectable levels of antibodies, while, immunization with rPpSP32 (GenBank ID: JX411944) revealed strong antibody reaction (Marzouki *et al.*, 2012). Recombinant PpSp32 was strongly recognized also by human sera from CL endemic locality in Tunisia (Marzouki *et a.l,* 2012). The applicability of this salivary recombinant protein was also approved on large-scale serological studies done on human sera from Tunisia and Saudi Arabia (Marzouki *et al.*, 2015; Mondragon-Shem *et al.*, 2015). In Saudi Arabia, anti-rPpSP32 IgG antibodies were measured not only in sera from healthy individuals but also from sera of individuals with active and cured CL. Patients with an active infection showed significantly higher levels of anti-rPpSP32 antibodies compared to healthy residents (Mondragon-Shem *et al.*, 2015) as it was already shown for whole *P. papatasi* saliva (Rohousova *et al.*, 2005; Marzouki *et al.*, 2011).

OBJECTIVES

Visceral leishmaniasis is life threatening disease and according WHO belongs to the group of neglected tropical diseases. Despite the extensive research, there are still a lot of unanswered questions aimed on epidemiology of this disease. In this Ph.D. thesis I focused on the role of domestic and wild animals in transmission of VL in endemic localities where the life cycle of the parasite is still not cleared up. On the basis of the fact that *Leishmania* parasites are transmitted by the bite of phlebotomine sand flies we studied if the host antibody response against sand fly saliva could be used as a marker of exposure and risk marker of *Leishmania* infection. We proposed the use of recombinant sand fly salivary proteins which are more suitable antigens in comparison with whole sand fly saliva. Serology test based on recombinant protein could be used as economically sound tool for investigation of host exposure to sand flies in VL endemic localities.

The main objectives of this thesis were:

- detection of natural *Leishmania* spp. infections in wild rodents and bats in Ethiopia using molecular methods in order to estimate the prevalence of *Leishmania* infection in these wild animals

- use of antibody reaction against *P. orientalis* as a marker of exposition in naturally bitten animals from northwestern Ethiopia in order to evaluate the feeding behaviour of *P. orientalis*, the suspected vector of *Le. donovani* in Ethiopia

- evaluate the use of salivary recombinant proteins from *P. perniciosus* as suitable replacement for whole saliva and their use as a risk markers of *Le. infantum* infection in longitudinal canine study from CanL endemic focus in south Italy and the degree of antigenic cross-reactivity between *P. perniciosus* whole saliva and salivary recombinant protein in canine sera originated from distant CanL endemic localities in Italy and Portugal

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1. Kassahun A., Sadlova J., Dvorak V., **Kostalova T.**, Rohousova I., Frynta D., Aghova T., Yasur-Landau D., Lemma W., Hailu A., Baneth G., Warburg A., Volf P. and Votypka J. (2015): Detection of *Leishmania donovani* and *L. tropica* in Ethiopian wild rodents. Acta Tropica 145:39-44

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Detection of *Leishmania donovani* and *L. tropica* in Ethiopian wild rodents

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ABSTRACT

Human visceral (VL, also known as Kala-azar) and cutaneous (CL) leishmaniasis are important infectious diseases affecting countries in East Africa that remain endemic in several regions of Ethiopia. The transmission and epidemiology of the disease is complicated due to the complex life cycle of the parasites and the involvement of various *Leishmania* spp., sand fly vectors, and reservoir animals besides human hosts. Particularly in East Africa, the role of animals as reservoirs for human VL remains unclear. Isolation of *Leishmania donovani* parasites from naturally infected rodents has been reported in several endemic countries; however, the status of rodents as reservoirs in Ethiopia remains unclear. Here, we demonstrated natural *Leishmania* infections in rodents. Animals were trapped in 41 localities of endemic and non-endemic areas in eight geographical regions of Ethiopia and DNA was isolated from spleens of 586 rodents belonging to 21 genera and 38 species. *Leishmania* infection was evaluated by real-time PCR of kinetoplast (k)DNA and confirmed by sequencing of the PCR products. Subsequently, parasite species identification was confirmed by PCR and DNA sequencing of the 18S ribosomal RNA internal transcribed spacer one (ITS1) gene. Out of fifty (8.2%) rodent specimens positive for *Leishmania* kDNA-PCR and sequencing, 10 were subsequently identified by sequencing of the ITS1 showing that five belonged to the *L. donovani* complex and five to *L. tropica*. Forty nine kDNA-positive rodents were found in the endemic localities of southern and eastern Ethiopia while only one was identified from northwestern Ethiopia. Moreover, all the ten ITS1-positive rodents were captured in areas where human leishmaniasis cases have been reported and potential sand fly vectors occur. Our findings suggest the eco-epidemiological importance of rodents in these foci of leishmaniasis and indicate that rodents are likely to play a role in the transmission of leishmaniasis in Ethiopia, possibly as reservoir hosts.

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1. Introduction

Leishmaniasis, a group of diseases ranging from self-healing localized cutaneous (CL) to the life threatening visceral form (VL or Kala-azar), is widely distributed in over 88 countries with up to 1.6 million new cases annually (WHO, 2010). Humans are infected by twenty species of the genus *Leishmania* that are transmitted by the bite of phlebotomine sand fly females. The source of infection for humans and parasite circulation is either anthroponotic (transmitted between humans) or zoonotic, where animals serve as reservoir hosts (Desjeux, 2004).

Leishmania species differ in the degree to which they are associated with different host species and reservoirs, among which rodents are considered to be of most importance. However, their role in the transmission cycle as a reservoir host and source of infection for humans differs significantly. For example *Leishmania turanica* is highly infectious and pathogenic to rodents, but human cases are very rare (Guan et al., 1995). In *L. major*, the parasites circulate under natural conditions in rodent populations; nevertheless, they are equally infective to humans and rodents that represent a natural source (reservoir) for human populations (Ashford, 1996, 2000). Cutaneous leishmaniasis caused by *L. tropica* was generally considered to be anthroponotic; however, in some areas hyraxes and rodents could play a role in zoonotic transmission (Jacobson, 2003; Svobodova et al., 2003; Svobodova et al., 2006).

The etiological agent of human VL in the Old World is represented by two closely related parasite species belonging to the *L. donovani* complex: *L. infantum* which circulates as a zoonosis with domestic dogs and wild canids as the main reservoirs (Baneth and Aroch, 2008; Quinnell and Courtenay, 2009), and *L. donovani*, which is believed to be anthroponotic and mainly transmitted among humans (Chappuis et al., 2007).

Visceral leishmaniasis caused by *L. donovani* has claimed the lives of thousands of people in Ethiopia. The main foci are found in the lowland areas of north, northwestern, and southwestern Ethiopia, with some sporadic cases in the central-east part of the country (Hailu and Formmel, 1993; Hailu et al., 2006a). The main potential vectors of VL include *P. orientalis, P. martini*, and *P. celiae* (Hailu et al., 1995; Gebre-Michael and Lane, 1996). The transmission dynamics of VL in Ethiopia and neighboring East African countries is generally believed to be anthroponotic (Chappuis et al., 2007); however DNA of *L. donovani* complex has recently been detected in both wild and domestic animals (Bashaye et al., 2009) and in certain districts of Sudan, rodents are suspected to be reservoirs of the parasite (Chance et al., 1978; Le Blancq and Peters, 1986; Elnaiem et al., 2001). The closely related species, *L. infantum*, has been detected in rodents in Euro-Asian leishmaniasis foci including Portugal (Helhazar et al., 2013), Italy (Gradoni et al., 1983), Greece (Papadogiannakis et al., 2010), and Iran (Davami et al., 2014). In addition, our recent study demonstrated presence of *L. donovani* complex DNA in blood specimens of various domestic animals in the VL endemic foci of north and northwestern Ethiopia (Rohousova et al., unpublished).

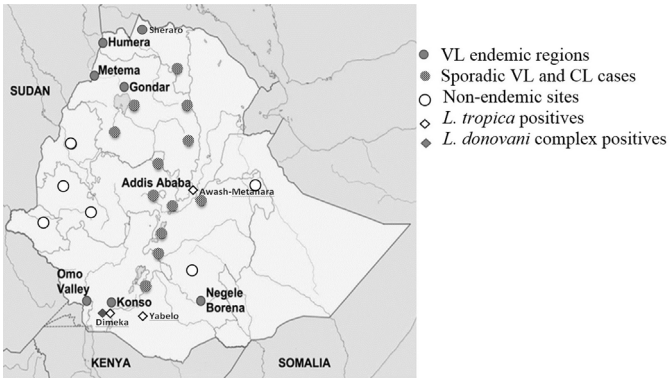

Fig. 1. Rodent trapping sites and their relation to human leishmaniasis foci in Ethiopia. (*Note*: The specific rodent trapping localities were indicated in the supplementary table.)

In Ethiopia, the search for *L. donovani* infection in wild rodents has been going on for many years. Here we focused on the detection of natural *Leishmania* spp. infections in rodents using PCR that targets the kinetoplast (k)DNA and internal transcribed spacer one (ITS1).

2. Materials and methods

2.1. Sample collection

Rodents were trapped in 41 localities (between 2010 and 2013) selected based on altitude, the occurrence of Kala-azar (9 endemic, 18 sporadic and 14 non-endemic), the abundance of sand flies, and the presence of microhabitat features related to *Leishmania* transmission (Fig. 1; Supp. Table S1). Permission to trap rodents was obtained from the Ethiopian Wildlife Conservation Authority (EWCA), Government of Ethiopia.

Supplementary Table S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actatropica.2015.02.006.

Rodents were trapped using Sherman live traps and snap traps baited with a piece of bread with peanut butter or sardine. The traps were placed over-night near houses, animal shelters, around burrows, caves, agricultural fields, termite mounds, under trees, and in other habitats deemed suitable for sand flies. Trapped rodent was photographed and weight, sex, characteristics, and external measurements (lengths of body, tail, hind foot, and ear) were recorded. Rodents captured by live traps were first immobilized in a plastic bag and then humanely euthanized by intra peritoneal injection of ketamine and xylazine, dissected, and a sample of spleen was kept in pure ethanol for subsequent DNA extraction. After removing the viscera, the remaining body was kept in denatured alcohol for further morphological identification.

2.2. DNA extraction

DNA was extracted from spleen samples by QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions or by the guanidine thiocyanate technique (Hoss and Paabo, 1993) with slight modification. Briefly, 10 mg of spleen tissue was homogenized by a grinder in 2 ml eppendorf tube and suspended in 1 ml extraction buffer containing 10 M GuSCN, 0.1 M Tris–HCl (pH 6.4), 0.02 MEDTA (pH 8.0) and 1.3% Triton X-100 and left for overnight agitation in a 56 °C shaker incubator. Then the tissue was boiled for a maximum of 10 min at 94 °C. After centrifugation at 14,000 rpm for 3 min, the supernatant was transferred to a new tube and 1 ml of freshly prepared NaCl solution with 1 μl silica and 1 μl linear acrylamide was added and kept on ice for 1 h with 15 min interval of vortexing. Then the mix was centrifuged at 5000 rpm for 30 s and supernatant discarded. The pellet was washed with washing buffer and then with ethanol and left to air dry. Finally, the pellet was re-suspended in ultra-pure water.

2.3. Host and parasite detection and determination

Confirmation of the morphological identification of hosts was provided by sequencing a fragment of the cytochrome b gene (900 bp). PCR was performed using the following primers: L14723 (forward, 5′-ACC AATGACATGAAAAATCATCGTT-3′) and H15915 (reverse, 5′-TCTCCATTTCTGGTTTACAAGAC-3′) (Lecompte

Table 1
Number of trapped rodents and *Leishmania* infections in different geographical regions of Ethiopia.

Geographic region of Ethiopia	No. of animals sampled	*Leishmania* DNA positive	
		kDNA (%)	ITS1 (*Leishmania* species)
Central	29	–	–
Central-east	53	10 (1.7)	3 (*L. tropica*)
East	72	6 (1.0)	–
North	61	–	–
Northwest	34	1 (0.2)	–
South	97	1 (0.2)	–
Southwest	144	31 (5.3)	7 (5 *L. don.* complex; 2 *L. tropica*)
West	96	1 (0.2)	–
Total	586	50 (8.5)	10 (5 *L. don.* complex; 5 *L. tropica*)

et al., 2002). The PCR product was purified using calf intestine alkaline phosphatase and exonuclease I (New England Biolabs) for sequencing (GATC Biotech company, Germany). All sequences were assigned to genus using BLAST search (https://blast.ncbi.nlm.nih.gov) and species determinations were performed through phylogenetic analysis of our recent unpublished materials.

For the purpose of *Leishmania* detection in the rodent's tissues and species determination, a combination of a mini circle kDNA real time (RT)-PCR and sequencing followed by ITS1-PCR and sequencing was used. PCR targeting fragments of about 116 bp of the kDNA is considered to be highly sensitive due to the presence of thousands of target copies in each parasite cell and has been used for screening of *Leishmania* in various vertebrate hosts (Selvapandiyan et al., 2008; Abbasi et al., 2013). However, as sequencing of kDNA does not identify the *Leishmania* species (Anders et al., 2002; Nicolas et al., 2002; Nasereddin et al., 2008), a more appropriate target, the internal transcribed spacer one (ITS1) gene, was introduced as a specific marker for each species (Schoenian et al., 2003; Talmi-Frank et al., 2010). Primers: JW11 (forward, 5′-CCTATTTTACACCAACCCCCAGT-3′) and JW12 (reverse, 5′-GGGTAGGGGCGTTC TGCGAAA-3′) were used to amplify the mini-circle kDNA of *Leishmania* (Nicolas et al., 2002); while primers ITS-219F (forward, 5′-AGCTGGATCATTTTCCGATG-3′) and ITS-219R (reverse, 5′-ATCGCGACACGTTATGTGAG-3′) amplified a 265 to 288-bp product of the ITS1 region of the *Leishmania* rRNA operon (Talmi-Frank et al., 2010). The RT-PCR conditions for kDNA and ITS1 were as described by Nicolas et al. (2002) and Talmi-Frank et al. (2010). For each set of reactions, a standard positive DNA extracted from 100 μl of *L. infantum* (strain MHOM/TN/1980/IPT1), *L. tropica* (ISER/IL/2002/LRC-L90), and *L. major* (MHOM/TM/1973/5ASKH) promastigote cultures [5×10^2 parasites/μl] and non-template controls (NTC) were used. All *Leishmania* kDNA- and ITS1-PCR positive samples underwent direct sequencing of the target amplicons.

3. Results

During a period of four years, a total of 586 rodents belonging to 17 genera and 34 species were caught from 41 trapping locations grouped in eight geographical regions (Table 1). The following six rodent genera were abundant (each represent at least five percent of the total catches): *Acomys* (24.1%), *Mastomys* (20.0%), *Stenocephalemys* (15.2%), *Lophuromys* (10.6%), *Mus* (8.0%), and *Arvicanthis* (7.8%) (Table 2). Based on cursory inspection of the captured animals, none of the rodents had visible clinical signs that could be attributed to CL. Fifty (8.5%) of the analyzed rodents were kDNA-RT-PCR positive for the presence of *Leishmania* spp. Presence of *Leishmania* DNA was confirmed by subsequent sequencing of the kDNA-RT-PCR amplicon. At least one kDNA *Leishmania*-positive was found in nine rodent genera and in the following five, kDNA-positive samples were detected repeatedly: *Mastomys* (18 kDNA-RT-PCR positive animals out of 117 tested; 15.3%), *Acomys* (14/141; 9.9%), *Arvicanthis* (8/46; 17.4%), *Aethomys* (4/10; 40.0%), and *Gerbilliscus* (2/26; 7.7%). The kDNA-RT-PCR positive rodents were classified generally as "infected with *Leishmania*

Table 2
Total number of trapped rodents according to genera (listed in alphabetical order) and *Leishmania* kDNA (kDNA+) and/or ITS1 (ITS1+) positivity as obtained by (RT)-PCR and subsequent sequencing.

Genus[a]	Number[b] (%)	kDNA+	ITS1+	*Leishmania* species (by ITS1)
Acomys (3)	141 (24.1)	14	3	*L. tropica*
Aethomys (2)	10 (1.7)	4		
Arvicanthis (6)	46 (7.8)	8	2	1 *L. don.* complex and 1 *L. tropica*
Dendromus (1)	5 (0.8)			
Desmomys (1)	3 (0.5)			
Gerbilliscus (4)	26 (4.4)	2	1	*L. donovani* complex
Gerbillus (1)	5 (0.8)	1	1	*L. tropica*
Graphiurus (1)	6 (1.0)	1		
Lophuromys (1)	62 (10.6)			
Mastomys (3)	117 (20.0)	18	3	*L. donovani* complex
Mus (4)	47 (8.0)			
Myomyscus (1)	5 (0.8)	1		
Rattus (1)	19 (3.2)			
Saccostomus (1)	3 (0.5)	1		
Stenocephalemys (1)	89 (15.2)			
Tachyoryctes (1)	1 (0.2)			
Taterillus (1)	1 (0.2)			
Total	586 (100)	50	10	

[a] The number of species per genus is presented in brackets.
[b] Total number and percentage of trapped rodents.

Table 3
Rodent species found ITS1-positive for *Leishmania* parasites and their trapping sites.

Leishmania species	Rodent species	Locality	Geographic region
L. donovani complex	*Arvicanthis* sp.	Alduba	Southwest Ethiopia
	Mastomys erythroleucus	Alduba	
	Mastomys erythroleucus	Dimeka	
	Mastomys erythroleucus	Dimeka	
	Gerbilliscus nigricaudus	Dimeka	
L. tropica	*Acomys* sp.	Sorobo, Konso	South Ethiopia
	Arvicanthis sp.	Derito, Yabelo	
	Acomys cf. *mullah*	Awash-Metahara	Central-east Ethiopia
	Acomys cf. *mullah*		
	Gerbillus nanus		

spp." (Table 2). Only one rodent specimen (*Acomys* sp.) was found positive for *Leishmania* kDNA in the northern part of the country; while the rest were either from the southern or eastern parts of Ethiopia (Table 1).

All of the 50 rodent specimens positive for *Leishmania* spp. by kDNA-RT-PCR and confirmed by sequencing of the kDNA amplicons were further tested and re-screened by amplification of the *Leishmania* ITS1 gene followed by DNA sequencing of the amplicon. A total of ten rodent specimens from the following five genera were positive for ITS1-PCR (Table 2): *Acomys*, *Arvicanthis*, *Gerbilliscus*, *Gerbillus*, and *Mastomys*. The sequencing revealed that five samples belonged to *L. tropica* and five to the *L. donovani* complex. As our sequences of ITS1 are unable to separate *L. donovani* from *L. infantum*, the positive samples of these species are represented here as *L. donovani* complex.

The *L. tropica* positive rodents, represented by *Arvicanthis* sp., *Gerbillus nanus*, and three specimens of *Acomys* spp., were caught in Konso and Yabello in southern Ethiopia and in Awash-Metahara in central-east Ethiopia. On the other hand, rodents positive for *L. donovani* complex are represented by *Arvicanthis* sp., *Gerbilliscus nigricaudus*, and three specimens of *Mastomys erythroleucus*, originated from the south western part of Ethiopia in the locality of Dimeka and Alduba (Fig. 1; Table 3).

4. Discussion

Eighty-four different species of rodents have been identified in Ethiopia so far. These include rodents belonging to species in the genera *Acomys*, *Mastomys*, *Arvicanthis*, and *Mus* which are the most common (Bekele and Leirs, 1997). This is in agreement with our collections in which *Acomys* (24.1%) and *Mastomys* (20.0%) are the predominant species. This was probably due to the location of the trapping sites as the majority of our traps were set in domestic and peri-domestic areas and in fields where these rodents are abundant and considered as agricultural pests (Bekele and Leirs, 1997; Chekol et al., 2012).

Correct species identification of Ethiopian rodents remains tricky due to the presence of several cryptic species where identification by morphological parameters alone is not sufficient. The need for molecular identification is crucial; however, the reference species found in Gene Bank or the BOLD (The Barcode of Life Data Systems: http://www.boldsystems.org/) database for analyzing unknown sequences is still limited (Galan et al., 2012). Although we identified all the trapped rodents to the species level; in the present study, we presented the number of rodent species per genus and/or species of ITS1 positive specimens only.

Sharing the same ecological niche and nocturnal activity facilitates the frequent contact between sand flies and rodents and may lead to infection with a *Leishmania* parasite transmitted by a bite. In this study, PCR positive rodents belonged to those genera and species that are common in arable lands and nests in cracks, burrows, or dig holes with multiple entrances (Kingdon et al., 2013; Bekele and Leirs, 1997) which in turn could be resting and breeding sites for sand flies. In addition to this, *Arvicanthis* is one of the rodent genera commonly found around termitaries (Kingdon et al., 2013) and could be a preferred blood source for *P. martini* and *P. celiae*, the two potential vectors of *L. donovani* in southern Ethiopia which are associated with termite mounds (Gebre-Michael and Lane, 1996).

All five rodent specimens infected with *L. donovani* complex were captured in the localities of Dimeka and Alduba, southwestern Ethiopia (Table 2) which is considered an important Kala-azar focus (Hailu et al., 2006b) and where the suspected vector species, *P. orientalis*, *P. martini*, and *P. celiae*, exist sympatrically (Hailu et al., 1995; Gebre-Michael and Lane, 1996). The infected rodents we found belong to *Arvicanthis* sp., *M. erythroleucus*, and *G. nigricaudus*. Natural infections of *Arvicanthis* (*A. niloticus*) and mongoose (*Herpestes ichneumon*) with *L. donovani* were previously reported in the Aethiopian geographical region (Chance et al., 1978; Le Blancq and Peters, 1986; Elnaiem et al., 2001). During our field study we found a fresh body of a white-tailed mongoose (*Ichneumia albicauda*) which was hit by a car in the locality of Alduba and sample taken from this mongoose was positive for *Leishmania* kDNA, and ITS1-PCR revealed *L. donovani* complex (data not shown). Our finding corresponds with the previous ascertainments and therefore could signify the existence of natural infection of wild animals in the whole region.

Three of the rodents infected with *L. tropica* (*G. nanus* and two *Acomys* spp.) were found in the Awash valley, central-east Ethiopia. Previous investigations in this region demonstrated human cases of cutaneous leishmaniasis due to *L. tropica* and sand flies including *P. saevus* and *P. sergenti* were found harboring this parasite (Gebre-Michael et al., 2004; Hailu et al., 2006a). However, no *L. tropica* infections were reported in south and southwestern Ethiopia.

Although leishmaniasis due to *L. tropica* results mainly in cutaneous manifestations in humans, we detected the presence of this parasite in the studied rodents based on PCR of their spleen tissue samples. Experimental infections of rodents demonstrated early dissemination of parasites to internal organs including the spleen (Papadogiannakis et al., 2010). We did not find any visible clinical signs that could be attributed to CL in the *L. tropica*-positive rodents. Although symptomatic cases of disease are the most important in human and veterinary medicine, asymptomatic hosts may be much more abundant and, therefore, crucial sources of infection for sand flies, playing a significant role in the epidemiology and transmission dynamics of the diseases. Asymptomatic and subclinical infections of leishmaniasis have been well documented in humans (Abbasi et al., 2013; Picado et al., 2014), dogs (Baneth et al., 2008; Miro et al., 2008) and rodents (Svobodova et al., 2003). From the epidemiological point of view, asymptomatic hosts contribute to the parasite transmission cycle. Previous studies on subclinical dogs and rodents infected with *L. infantum* and *L. tropica*, respectively, have demonstrated their competence to infect sand fly vectors (Svobodova et al., 2003; Laurenti et al., 2013; Sadlova et al., unpublished).

Only one *Leishmania* kDNA positive rodent was found in the northern part of Ethiopia, in the locality of Mai-Temen, Western Tigray, northwestern Ethiopia, even though we captured almost one hundred rodents in areas with established human VL transmission. The explanation for this finding could be evaluated from different perspectives. Studies on the genetic structure of Ethiopian *L. donovani* isolates have revealed polymorphism with geographical clusters in northern and southern Ethiopian foci (Gelanew et al., 2010; Zackay et al., 2013). Moreover, the fauna of potential sand fly vectors responsible for the transmission of VL in the north and south Ethiopian foci varies: the southern foci are dominated by two proven vectors, *P. martini* and *P. celiae* with sporadic *P. orientalis* while in the north, *P. orientalis* is the sole potential vector and the other two species are not present (Gebre-Michael and Lane, 1996; Hailu et al., 2006b). Thus, our finding could suggest differences in the transmission cycle including vector and reservoir hosts in these two geographical regions exist. Further studies; with special attention to the feeding habits of sand flies particularly on rodents are recommended.

In conclusion, VL caused by *L. donovani* in Eastern Africa is traditionally considered to be anthroponotic. However, our investigations suggest that wild rodents in Ethiopia could play an important epidemiological role in the transmission cycle of two *Leishmania* species, *L. donovani* and *L. tropica*. Further studies focusing on parasite isolation, experimental infection, and xenodiagnosis should be accomplished to prove their epidemiological role.

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Natural infection of bats with *Leishmania* in Ethiopia

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ABSTRACT

The leishmaniases, a group of diseases with a worldwide-distribution, are caused by different species of *Leishmania* parasites. Both cutaneous and visceral leishmaniasis remain important public health problems in Ethiopia. Epidemiological cycles of these protozoans involve various sand fly (Diptera: Psychodidae) vectors and mammalian hosts, including humans. In recent years, *Leishmania* infections in bats have been reported in the New World countries endemic to leishmaniasis. The aim of this study was to survey natural *Leishmania* infection in bats collected from various regions of Ethiopia. Total DNA was isolated from spleens of 163 bats belonging to 23 species and 18 genera. *Leishmania* infection was detected by real-time (RT) PCR targeting a kinetoplast (k) DNA and internal transcribed spacer one (ITS1) gene of the parasite. Detection was confirmed by sequencing of the PCR products. *Leishmania* kDNA was detected in eight (4.9%) bats; four of them had been captured in the Aba-Roba and Awash-Methara regions that are endemic for leishmaniasis, while the other four specimens originated from non-endemic localities of Metu, Bedele and Masha. *Leishmania* isolates from two bats were confirmed by ITS1 PCR to be *Leishmania tropica* and *Leishmania major*, isolated from two individual bats, *Cardioderma cor* and *Nycteris hispida*, respectively. These results represent the first confirmed observation of natural infection of bats with the Old World *Leishmania*. Hence, bats should be considered putative hosts of *Leishmania* spp. affecting humans with a significant role in the transmission

1. Introduction

In Ethiopia, leishmaniases, caused by protozoan parasites of the genus *Leishmania* and transmitted by the bite of female sand flies, are diseases of significant public health importance. The country is endemic for two human disease presentations: cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL, kala-azar). Cutaneous leishmaniasis is widely distributed and usually prevalent in highland areas with occasional reports in the lowland regions of Omo (south) and Awash (central east) (Hailu et al., 2006a). The annual incidence of CL ranges from 20,000 to 50,000 cases, but this is probably an under-estimate (Alvar et al., 2012), with over 28 million people residing in regions with risk of transmission (Seid et al., 2014). The main causative agent of CL in Ethiopia is *Leishmania aethiopica*, however, infections due to *Leishmania tropica* and *Leishmania major* were also reported in the country (Hailu et al., 2006a,b; Abbasi et al., 2013). Visceral leishmaniasis affecting up to 7400 people annually in the country is the most severe form and is fatal, if left untreated. The VL foci lie in the south-west lowland savannah and the north-west semi-arid plains of the country with sporadic cases in highland areas of the Libo Kemkem district (north), the Awash valley (center) and further in the east of the country, bordering Kenya and Somalia (Leta et al., 2014; Hailu et al., 2006a). The causative agent of human VL in Ethiopia is *Leishmania donovani* (Hailu et al., 2006a).

Cutaneous leishmaniasis caused by *L. aethiopica* and *L. major* is commonly zoonotic (Ashford et al., 1973; 2000; Lemma et al., 2009 Lemma et al., 2009). Although being the agent of anthro-

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ponotic CL in urban endemic settings, *L. tropica* has been strongly suspected to be zoonotic in some foci (Sang et al., 1994; Jacobson, 2003; Svobodova et al., 2003). In Ethiopia, rock hyraxes were found infected with *L. aethiopica*, suggesting a zoonotic cycle of the parasite (Ashford et al., 1973; Lemma et al., 2009). Recently, *L. tropica* DNA was detected in spleens of rodents in areas where human cases have been reported (Kassahun et al., 2015). However, no study in Ethiopia demonstrated natural infection in animals by *L. major*.

Most reports agree that like the Indian sub-continent, VL in East Africa is assumed to be anthroponotic (Chappuis et al., 2007). Nevertheless, there is evidence for the possible involvement of zoonotic transmission with uncertain reservoir hosts (Ashford, 2000). Recently, natural infections of dogs (Bashaye et al., 2009), domestic animals (Rohousova et al., 2015) and rodents (Kassahun et al., 2015) with *L. donovani* complex were reported in Ethiopia.

Natural infections by various *Leishmania* species have been repeatedly reported in domestic, peridomestic and wild animals, which dogs and rodents being the most commonly investigated animals and traditionally considered reservoirs (Baneth and Aroch, 2008). However, recent investigations of *Leishmania* parasites in animals including hares (Jimenez et al., 2013), and marsupials (Roque and Jansen, 2014) have diverted attention to other possible sylvatic reservoir hosts in endemic leishmaniasis foci.

Bats ecology and innate behavioral details highlight their prime importance in the reservoir system of infectious diseases such as Ebola virus (Leroy et al., 2005) and various kinetoplastids transmitted by vectors (Lord and Brooks, 2014). Bats were also suggested as possible natural blood source for sand flies after laboratory feeding procedure (Lampo et al., 2000) and known to host several trypanosomes transmitted by sand flies (McConnell and Correa 1964; Williams, 1976). Importantly, being cave-dwelling organisms, bats and sand flies frequently share living habitats where ample opportunity exists for sand flies to feed on bats (Feliciangeli, 2004). Natural *Leishmania* infection in bats has been reported in New World leishmaniasis foci and the findings suggested their possible epidemiological involvement in the transmission cycle (Lima et al., 2008; Savani et al., 2010; Shapiro et al., 2013; Berzunza-Cruz et al., 2015). Despite the attempts else where (Millan et al., 2014; Rotureau et al., 2006; Rajendran et al., 1985; Mutinga, 1975; Morsy et al., 1987), the extent of *Leishmania* natural infection in the Old World bats remains uncertain, and cases of Chiropteran *Leishmania* infections have not been documented in Ethiopia until now. In view of these facts we carried out a *Leishmania* DNA survey in Ethiopian bats.

2. Materials and methods

2.1. Sample collection

Bats were collected as a part of an extensive ecological and faunistic study in Ethiopia. Permission for trapping was obtained from the Ethiopian Wildlife Conservation Authority (EWCA), government of Ethiopia. Here, we reported results for the 163 specimens collected in leishmaniasis endemic (44 bats) and non-endemic (119 bats) areas of Ethiopia (Fig. 1). Bats were captured at presumed flyways using a standard mist-net between 18:00 and 22:00 h. Bats were removed from the net, anesthetized by intra peritoneal injection of ketamine and xylazine. All the necessary external morphological characters including size, color of hair and naked parts, length of forearm, shape of snout, shape of ear and type of membrane concerning the form of tail were recorded and the identification of each particular bat was confirmed based on the keys by Happold and Happold (2013). Then bats were sacrificed and their spleens were removed and kept in ethanol for the subsequent DNA extraction.

2.2. DNA extraction, parasite detection and determination by PCR

All the techniques, materials and procedures: DNA isolation, primers, real time polymerase chain reaction (RT-PCR) procedure, target genes (kinetoplast DNA (kDNA) and 18S rRNA internal transcribed spacer one (ITS1)) and post PCR evaluation and parasite determination, were performed as described in our previous work on rodents (Kassahun et al., 2015). Briefly, for the purpose of *Leishmania* detection and identification, we tested extracted DNA using RT-PCR targeting kDNA of *Leishmania* and positivity was confirmed by direct sequencing of amplicons. Real time PCR targeting kDNA gene is generally considered to be highly sensitive (Selvapandiyan et al., 2008; Selvapandiyan et al., 2008) but sequence does not identify the *Leishmania* species (Nicolas et al., 2002; Nasereddin et al., 2008). Therefore, all the kDNA positive specimens were re-analyzed by RT-PCR of the ITS1 locus and positive samples underwent sequencing of amplicons (Schoenian et al., 2003; Schoenian et al., 2003).

3. Results and discussion

A total of 163 bats, belonging to 25 species of 18 genera (Table 1), were collected. The dominant species in our collection were *Pipistrellus hesperidus* (18%), *Miniopterus africanus* (11%) and *Scotoecus hirundo* (11%).

Amongst the 163 samples, *Leishmania*–kDNA positivity was confirmed by sequencing of a parasite DNA from eight bats belonging to six species. Out of the eight *Leishmania* kDNA PCR positives, the ITS1-PCR and subsequent sequencing revealed infection of *L. tropica* in one specimen of *Cardioderma cor* and *L. major* in one specimen of *Nycteris hispida* (Table 1). We were unable to amplify ITS-1 sequences for the six additional *Leishmania* kDNA positive samples. There was a similar scenario in our previous work (Kassahun et al., 2015). PCR targeting kDNA fragment is considered to be highly sensitive due to the high number of target copies in each parasite cell. Even though ITS-1 based PCR determines the species of the *Leishmania* parasite, the level of sensitivity is lower than that of kDNA PCR (Abbasi et al., 2013) which does not provide sufficient information for species determination.

Leishmaniasis due to *L. tropica* and *L. major* generally cause dermal lesions in humans; however none of the bats had visible dermal signs resembling cutaneous leishmaniasis. It is well know that *Leishmania* species dermotropic for humans could migrate to visceral organs of other animal hosts (Laskay et al., 1995). Moreover, early dissemination of *Leishmania* parasites to the spleen has been reported in asymptomatic animals (Schilling and Glaichenhaus, 2001). Such scenarios may explain our finding of parasite DNA in the spleens of infected bats thus validating our experimental approach for an epidemiological study.

Our finding represents a confirmed first report of natural *Leishmania* infection of bats in the Old World. Previous studies conducted in the Old World (e.g. Spain (Millan et al., 2014), France (Rotureau et al., 2006), India (Rajendran et al., 1985) and Kenya (Mutinga, 1975)) did not yield any positive specimens. Moreover, the attempts in Egypt (Morsy et al., 1987) were using old methods and the detection procedure was speculative with specificity and parasite species characterization. However, bats in the New World were repeatedly investigated and found infected with *Leishmania* species pathogenic to humans. In our study, the prevalence reached 5% (8 out of 163) corresponds with the infection rates of bats recorded in Sao Paulo, Brazil (4%) (Savani et al., 2010); while higher prevalence has been detected in Venezuela (9%) (Lima et al., 2008); Mexico (9.8%) (Berzunza-Cruz et al., 2015) and Mato Grosso do Sul, Brazil (40%) (Shapiro et al., 2013).

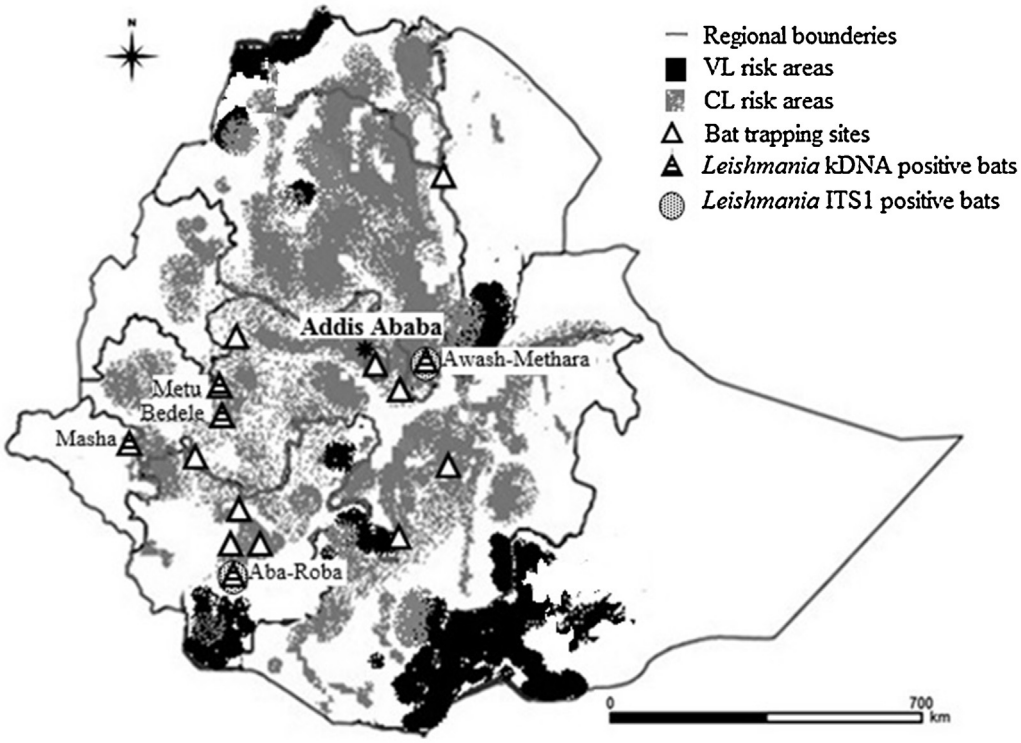

Fig. 1. Map of leishmaniasis distribution in Ethiopia (modified and adapted from Leta et al., 2014; Seid et al., 2014 and unpublished hospital records) and trapping localities with respective *Leishmania* DNA detection results.

Table 1
Bats collected in different trapping localities[a] in Ethiopia and examined for *Leishmania* DNA by RT-PCR. The number of *Leishmania* kDNA positive bats appears in square brackets.

Bat species	BCH	ABR	MSH	BDL	DDS	KNS	TPI	GOB	AMR	ALM	WLT	MTU	SFO	SOR	MNG	Σ (%)
Cardioderma cor	–	–	–	–	–	–	–	–	1 [1][b]	–	–	–	–	–	–	1 (0.6)
Glauconycteris variegata	–	1	–	3[1]	–	–	–	-	–	–	–	4	–	–	–	8 (4.9)
Laephotis wintoni	-	–	–	–	–	–	–	–	–	–	–	–	–	–	2	2 (1.2)
Micropteropus pusillus	-	–	–	2	–	–	–	–	–	–	-	2	–	2	–	6 (3.7)
Miniopterus arenarius	–	–	2[1]	–	–	–	–	–	–	–	–	–	–	–	–	2 (1.2)
Miniopterus africanus	–	–	–	–	–	–	–	–	–	–	–	–	18	–	–	18 (11.0)
Mops condylurus	–	–	–	–	–	–	–	–	–	–	–	9	–	–	–	9 (5.5)
Myotis scotti	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–	2 (1.2)
Myotis tricolor	–	–	–	–	–	–	–	–	–	–	–	–	11	–	–	11 (6.7)
Neoromicia somalica	–	2[2]	–	–	–	2	–	–	–	1	–	–	–	–	1	6 (3.7)
Neoromicia guineensis	–	–	–	3	–	–	–	–	–	–	–	–	–	–	–	3 (1.8)
Neoromicia nana	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	1 (0.6)
Nycteris hispida	–	–	–	–	–	–	–	–	1 [1][c]	–	–	–	–	–	–	1 (0.6)
Nycticeinops schlieffenii	–	3	–	–	–	–	–	–	–	–	–	–	–	–	–	3 (1.8)
Otomops martiensseni	–	–	–	–	–	–	–	–	–	–	–	–	3	–	–	3 (1.8)
Pipistrellus hesperidus	–	–	2	–	–	–	–	4	–	–	–	9	–	2	13	30 (18.4)
Pipistrellus rusticus	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	1 (0.6)
Rhinolophus fumigatus	–	–	–	–	–	–	–	–	–	–	1	–	5	–	–	6 (3.7)
Scotoecus hirundo	–	3	–	1	–	14	–	-	–	–	–	–	–	–	–	18 (11.0)
Scotophilus colias	–	–	–	1	–	–	–	–	–	–	–	11[2]	–	–	-	12 (7.4)
Stenonycteris lanosus	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1 (0.6)
Tadarida sp.	–	–	–	–	8	–	–	–	–	–	–	–	1	–	–	9 (5.5)
Triaenops afer	–	–	–	–	–	–	–	–	–	–	–	–	11	–	–	11(6.7)
Total	1	9	4	12	8	16	1	4	2	1	1	35	49	4	17	163

[a] Abbreviation of localities: BCH-Bechu, ABR-Aba-Roba, MSH-Masha, BDL-Bedele, DDS-Dedesa, KNS-Konso, TPI-Tepi, GOB-Goba, AMR-Awash-Methara, ALM-Alemata, WLT-Welenchiti, MTU-Metu, SFO-Sof Omar caves, SOR-Sorr, MNG-Menagesha.
[b] *L. tropica* positive bats.
[c] *L. major* positive bats.

Four of the positive bats were captured in the Aba-Roba and Awash-Methara leishmaniasis endemic foci while the other four specimens originated from non-endemic localities of Metu, Bedele and Masha (Fig. 1). The Awash-Methara foci are known for *L. tropica* infections in humans (Hailu et al., 2006a), phlebotomine sand flies (Gebre-Michael et al., 2004) and recently rodents (Kassahun et al., 2015). Our results corroborate these findings as one specimen of *C. cor* captured in this area was found infected with *L. tropica*. Although *L. tropica* is regarded to be anthroponotic, infections in dogs (Baneth et al., 2014), golden jackal and red foxes (Talmi-Frank et al., 2010) and rodents (Svobodova et al., 2003; Talmi-Frank et al., 2010) have been well documented generally in zoonotic foci (Sang

et al., 1994). The finding of this parasite both in bats and in our previous study of rodents (Kassahun et al., 2015) points to the possibility of zoonotic transmission in the particular area.

The specimen of *N. hispida* infected with *L. major* was trapped in the same area, Awash-Methara. The findings of *L. major* in Ethiopia are rare but natural infections in humans (Abbasi et al., 2013) and sand flies (Gebre-Michael et al., 1993) were recorded in North and South-west Ethiopia, respectively. No previous *L. major* infection was reported in Awash-Methara region; however our unpublished preliminary entomological survey in this area revealed the presence of *Phlebotomus papatasi* and *Phlebotomus duboscqi*, both being considered as a potential vectors of *L. major* (Dostalova and Volf, 2012).

The finding of four *Leishmania*–kDNA positive bats in the non-endemic localities could be explained by the fact that the geographical distribution of the parasite in Ethiopia is much wider than anticipated. Moreover, bats have a potential to migrate from place to place and we could hardly rule out the possibility that bats from *Leishmania* endemic areas could move to non-endemic areas.

No *L. donovani* complex DNA was detected in our bats sample. It is obvious that *L. donovani* is the sole agent of human VL in Ethiopia with wide geographical areas (Hailu et al., 2006a). The recent finding of DNA in rodents (Kassahun et al., 2015) and domestic animals (Rohousova et al., 2015) could also determine its host range. However, the absence of this species in bats doesn't reflect being refractory or the parasite's specificity.

Generally, to determine the role of a given host in a reservoir system it should fulfill some criteria among others: overlap of geographical distribution of vectors and hosts; forming large biomass, being gregarious and long lived in addition to being found naturally infected and subsequently being infective for transmitting vectors (Ashford, 1996). Some of these conditions work with bats and their ability to fly long distances and colonize places could make them suitable bridge hosts for leishmaniasis. Moreover, most colonies of bats live and rest in caves and cracks that are assumed to provide ambient temperatures and relative humidity suitable for sand fly breeding and diurnal resting (Feliciangeli, 2004). Laboratory feeding experiments on *Lutzomyia longipalpis*, most widely distributed vector of New World VL, was capable of feeding from different families of bats that suggested the importance of bats as a possible natural blood source of sand flies (Lampo et al., 2000). In addition to this, bats are well known hosts of *Trypanosoma* transmitted by sandflies (McConnell and Correa, 1964; Williams, 1976; Lord and Brooks, 2014), which is closely related to the genus *Leishmania*.

In conclusion, bats could have adequate features to be naturally infected by *Leishmania* and could subsequently to play a role in its epidemiological cycle. The present study revealed natural *Leishmania* infections of Old World bats, in areas both endemic and non-endemic for human leishmaniasis. The wide geographical distribution of *Leishmania* parasite in the country could imply the existence of different modes of transmission and our finding might indicate the importance of bats in the disease cycle. However, to play a role in *Leishmania* cycles it is required to investigate the host's pathogenic features and being infectious to vectors; which were not covered in this paper. Thus, further studies on persistence of the *Leishmania* parasite in bats and its interaction with sand fly vectors are recommended for the better understanding of their epidemiological involvement.

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RESEARCH Open Access

Exposure to *Leishmania* spp. and sand flies in domestic animals in northwestern Ethiopia

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Abstract

Background: Human visceral leishmaniasis caused by *Leishmania donovani* is considered an anthroponosis; however, *Leishmania*-infected animals have been increasingly reported in *L. donovani* foci, and the role of these animals as reservoirs for human *L. donovani* infection remains unclear.

Methods: We conducted a study of domestic animals (goats, sheep, cows, dogs, and donkeys) in three *L. donovani* foci in northwestern Ethiopia. Domestic animals were screened for *Leishmania* DNA and for anti-*L. donovani* IgG. Serum anti-sand fly saliva antibodies were used as a marker of exposure to the vector sand fly, *Phlebotomus orientalis*.

Results: Of 546 animals tested, 32 (5.9 %) were positive for *Leishmania* DNA, with positive animals identified among all species studied. Sequencing indicated that the animals were infected with parasites of the *L. donovani* complex but could not distinguish between *L. infantum* and *L. donovani*. A total of 18.9 % of the animals were seropositive for anti-*L. donovani* IgG, and 23.1 % of the animals were seropositive for anti-*P. orientalis* saliva IgG, with the highest seroprevalence observed in dogs and sheep. A positive correlation was found between anti-*P. orientalis* saliva and anti-*L. donovani* IgGs in cows, goats, and sheep.

Conclusions: The detection of *L. donovani* complex DNA in the blood of domestic animals, the reported seroprevalence to the *L. donovani* antigen, and the widespread exposure to sand fly saliva among domestic animals indicate that they are frequently exposed to *Leishmania* infection and are likely to participate in the epidemiology of *Leishmania* infection, either as potential blood sources for sand flies or possibly as parasite hosts.

Keywords: Visceral leishmaniasis, Ethiopia, Domestic animals, Serology, PCR, *Phlebotomus orientalis*, *Leishmania donovani*, Sand fly saliva

Background

Leishmaniasis, a protozoan disease that is transmitted by sand flies (Diptera: Phlebotominae) and caused by parasites of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae), is a neglected tropical and subtropical disease endemic to 98 countries worldwide. In East Africa, life-threatening human visceral leishmaniasis (VL) is caused by *Leishmania donovani* and primarily affects the poor due to the lack of preventive measures and reduced access to health care facilities [1].

The optimal strategy for controlling this disease depends on understanding the epidemiology of VL, including its local transmission cycles. Leishmaniasis caused by *L. donovani* is believed to be an anthroponosis. However, in Latin America and the Mediterranean Basin, the closely related species *L. infantum* causes a zoonosis for which canids are the main reservoirs [2]. Controlling zoonoses involving domestic or sylvatic transmission requires a more complex intervention than would be necessary if humans were the only hosts. Several

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Leishmania-infected animals have been previously reported in *L. donovani* foci, including wild and domestic animals [3–5]. However, the role of these animals as parasite hosts or, possibly, as reservoirs for human *L. donovani* VL remains unclear and requires further examination.

Our study focused on the detection of *Leishmania* infections in domestic animals in three VL foci in northwestern Ethiopia. Domestic animals were screened for *Leishmania* DNA and anti-*L. donovani* IgG in their peripheral blood to detect infection and exposure to *Leishmania*, respectively. Additionally, anti-sand fly saliva antibodies were used as a marker of exposure [6] to *Phlebotomus orientalis*, the suspected vector of *L. donovani* in northwestern Ethiopia [7, 8]. The findings from this study could be used to further study the involvement of domestic animals in the transmission cycle of VL.

Methods

Study sites and sample collection

Animal blood and serum samples were collected in Addis Zemen, Humera, and Sheraro, three localities in northwestern Ethiopia endemic to human VL. In the Humera district (Tigray region), several outbreaks of VL have been recorded since 1970. Addis Zemen (Amhara region) and Sheraro (Tigray region) are sustained VL foci characterized by a local transmission cycle supported by migrant agricultural laborers returning from Humera [1].

Animal surveys were conducted during two field studies. In October 2010, 266 samples were collected in Addis Zemen and Sheraro, and in November 2010, an additional 280 samples were obtained in Humera (Table 1). For DNA extraction, samples of whole blood (with anticoagulant) were transported to the Hebrew University of Jerusalem (Israel), where extraction was performed. For serological testing, serum samples treated with a 1 % azide solution were transported to Charles University in Prague (the Czech Republic) and stored at −70 °C.

Table 1 Serum samples collected from October to November 2010 in Ethiopian VL foci

	Addis Zemen	Sheraro	Humera	Total
Cow	62	26	16	104
Dog	19	7	8	34
Donkey	3	11	6	20
Goat	0	106	133	239
Sheep	27	5	117	149
Total	111	155	280	546

Ethical approval

The study was approved by the Ethiopian National Research Ethics Review Committee (NRERC), under approval no. 3.10/3398/04. Consent was obtained from the owners of the domestic animals for the collection of blood samples by a veterinarian. International animal experimentation guidelines were followed.

DNA extraction and PCR amplification

DNA was extracted from whole blood using the guanidine thiocyanate technique [9]. DNA was tested for *Leishmania* spp. infection via kDNA real-time PCR as previously described [10, 11]. Samples that tested positive were further tested by *Leishmania* internal transcribed spacer 1 (ITS1) real-time PCR and high-resolution melt analysis (ITS1-HRM PCR) [12]. Samples that tested positive by ITS1-HRM PCR were further assessed via conventional PCR to amplify a larger segment of ITS1 [13]. All samples were tested in duplicate, and the results were compared with positive controls: *L. infantum* (MCAN/IL/2002/Skoshi), *L. tropica* (MHOM/IL/2005/ LRC-L1239), and *L. major* (MHOM/TM/1973/5ASKH) promastigotes. The negative controls included blood samples obtained from five Israeli dogs that had tested negative for *Leishmania* by PCR. All positive PCR products were submitted for DNA sequencing to the Center for Genomic Technologies at the Hebrew University of Jerusalem. The derived DNA sequences were compared with sequences in GenBank using the NCBI BLAST program (www.ncbi.nlm.nih.gov/BLAST). The percentage of positive animals for each species was calculated based on positive kDNA PCR results followed by sequencing. Samples were considered positive for *Leishmania* only if their kDNA sequence demonstrated the closest BLAST match to *Leishmania* and was at least 80 % identical. A species was considered to be identified only when its ITS1 sequence shared 99 to 100 % identity with an existing GenBank sequence.

Discrimination between *Leishmania infantum* and *Leishmania donovani*

As ITS1-HRM PCR does not discriminate between *L. infantum* and *L. donovani* infections [12], samples that tested positive for the *L. donovani* complex were further evaluated using conventional PCR to determine the species. Two independent PCR assays were carried out to amplify fragments of the *Leishmania* cysteine protease B (CPB) gene [14, 15]. Furthermore, amplification of the heat shock protein 70 (HSP70) gene, followed by restriction fragment length polymorphism analysis was also attempted for species discrimination [16]. The same positive and negative controls used for ITS1-HRM PCR were employed.

A phylogenetic analysis was carried out using Kalign (www.ebi.ac.uk/tools/msa/kalign/) and BioEdit softwares. Only well-defined ITS sequences that were unambiguously assigned to the species *L. donovani* or *L. infantum* were downloaded from the GenBank database and used in the analysis (Additional file 1). The final alignment included 286 characters and is available upon request. Phylogenetic analyses of the ITS datasets were performed with PhyML for maximum likelihood (ML); the best-fitting model [GTR + *I* + Γ] of sequence evolution was assessed using Modeltest 3.7 software and bootstrapped with 1000 replicates.

Anti-*Leishmania donovani* IgG antibodies

An ELISA was used to measure specific anti-*L. donovani* IgG. Wells (CovaLink NH, Nunc) were coated with *L. donovani* promastigotes (Ethiopian strain MHOM/ET/67/HU3, 10^5 cells per well) in 20 mM carbonate-bicarbonate buffer (pH 9.25) overnight at 4 °C and incubated with 6 % blocking solution for 60 min at 37 °C. Serum samples were diluted in 2 % blocking solution and incubated in duplicate for 60 min at 37 °C. Thereafter, peroxidase-conjugated secondary antibodies were added, followed by 45 min of incubation at 37 °C. For details on the blocking solutions, sample dilutions, and conjugates employed in these assays, see Additional file 2. Absorbance was measured using a Tecan Infinite M200 microplate reader (Schoeller) at 492 nm.

Hyperimmune sera from laboratory-bred mice experimentally infected with *L. donovani* served as positive controls. Negative serum samples were obtained from healthy cattle (n = 33), horses (as controls for the donkeys; n = 9), goats (n = 21), and sheep (n = 32) from the Czech Republic, which is a sand fly- and *Leishmania*-free country. Canine-negative (n = 15) and canine-positive (n = 2) control sera were obtained during a previous study [17] from laboratory-bred beagles with no history of exposure to sand flies or *Leishmania* or from *Leishmania*-positive dogs, respectively.

Anti-sand fly saliva IgG antibodies

To estimate the exposure of domestic animals to *P. orientalis*, anti-saliva IgG antibodies were measured via ELISA. The same protocol applied for anti-*Leishmania donovani* IgG was used, with the following modifications: wells were coated with a salivary gland homogenate (corresponding to 0.2 gland/well, prepared as previously described [18]), and serum samples were incubated in duplicate for 90 min at 37 °C. Hyperimmune sera from laboratory-bred mice exposed solely to *P. orientalis* served as a positive control. The same negative controls employed for the anti-*L. donovani* ELISA were also used here.

To assess the possible cross-reactivity of *P. orientalis* salivary gland homogenate with IgG antibodies against the saliva of other sand fly species, sera from mice and dogs that were experimentally exposed to a single sand fly species were used. Canine sera positive for anti-*P. perniciosus* and anti-*L. longipalpis* IgG antibodies were available from previous experiments in laboratory-bred beagles exposed solely to *P. perniciosus* [17] and *L. longipalpis* [18], respectively, the two proven vectors of *L. infantum*. The ELISA protocol described in Additional file 2 was applied with one modification: the sera were diluted 1:500. For the murine sera, the applied ELISA protocol was modified as follows: low-fat, dry milk (Bio-Rad) was used as a blocking solution and diluent for the serum samples (1:200), and goat anti mouse IgG:HRP (AbD SEROTEC, STAR120P) diluted 1:1000, was used as a secondary antibody. The serum samples were obtained from BALB/c mice subjected to more than ten repeated exposures solely to *P. orientalis* (Ethiopia), *P. papatasi* (Turkey), *P. duboscqi* (Senegal), *P. arabicus* (Israel), or *Sergentomyia schwetzi* (Ethiopia). The experiments were approved by the Committee on the Ethics of Animal Experiments of Charles University in Prague (Permit Number: 24773/2008-10001) and were performed under a Certificate of Competency (Registration Number: CZU 934/05), in accordance with an Examination Order approved by the Central Commission for Animal Welfare of the Czech Republic.

Statistical analysis

For seroprevalence, cut-off values were calculated by the addition of three standard deviations to the mean optical density (OD) of the control sera. The differences in antibody levels between localities were analyzed using the nonparametric Wilcoxon Rank-Sum Test for Differences in Medians. Spearman's rank correlation matrix was used to assess the correlation between the variables. Statistical analyses were performed using NCSS 6.0.21 software, and the p-value was set at 0.05.

Results

Prevalence of *Leishmania* infection

The overall prevalence of *Leishmania* DNA detected via PCR was 5.9 % (32/546) (Table 2, Additional file 3). None of the 546 tested domestic animals presented visible clinical signs associated with leishmaniasis. Of the 32 animals that tested positive by kDNA PCR, nine were also positive for ITS1 PCR (Table 2, Additional file 3). The majority of *Leishmania*-positive animals (30 out of 32) were found in Humera, with the highest prevalence observed in cows (18.8 %). At the other localities, only one donkey in Sheraro and one dog in Addis Zemen were found to be positive for *Leishmania* (Table 2, Additional file 3).

Table 2 *Leishmania* PCR positivity in samples from Ethiopian animals

	Leishmania kDNA positive/total animals sampled (% positive)				*Leishmania* ITS1 positive (% positive)
Species	Addis Zemen	Sheraro	Humera	Total	Total
Cow	0/62	0/26	3/16 (18.8 %)	3/104 (2.9 %)	1 (1 %)
Dog	1/19 (5.3 %)	0/7	1/8 (12.5 %)	2/34 (5.9 %)	1 (2.9 %)
Donkey	0/3	1/11 (9.1 %)	1/6 (16.7 %)	2/20 (10.0 %)	0
Goat	0/0	0/106	16/133 (12.0 %)	16/239 (6.7 %)	3 (1.3 %)
Sheep	0/27	0/5	9/117 (7.7 %)	9/149 (6.0 %)	4 (2.7 %)
Total	1/111 (0.9 %)	1/155 (0.6 %)	30/280 (10.7 %)	32/546 (5.9 %)	9 (1.6 %)

A total of nine ITS1 DNA sequences, 265 bp long and 99 % identical to *L. infantum*/*L. donovani* sequences, were obtained via ITS1-HRM-PCR. None of the animal samples yielded positive PCR results when targeting the CPB and HSP70 genes. A DNA sequence was obtained for only a single longer ITS1 amplicon from one sheep originating in Humera. This sequence (314 bp, [GenBank:KJ010540]) shares 100 % identity with sequences from both *L. infantum* and *L. donovani* with 100 % coverage, and its phylogeny did not permit discrimination between these two closely related species (Fig. 1).

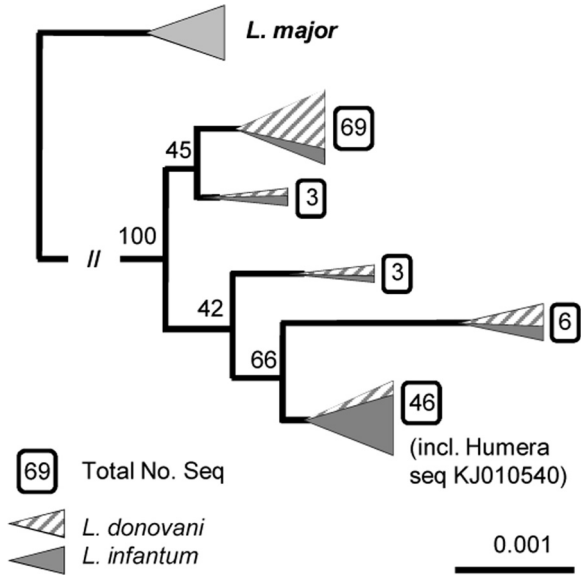

Fig. 1 Phylogenetic analysis of the *Leishmania* ITS1 sequence in a sheep from Humera, Ethiopia. Maximum likelihood (ML) phylogenetic analysis of a 286 bp *Leishmania* sequence amplified from the blood of a sheep from Humera, Ethiopia [GenBank:KJ010540]. Only well-defined ITS sequences that could be unambiguously assigned to a species (*L. donovani* or *L. infantum*) were downloaded from the GenBank database and used for the following analysis (Additional file 1). The ITS sequences were aligned using Kalign (www.ebi.ac.uk/Tools/msa/kalign/), and the resulting alignments were edited manually using the BioEdit software program to remove unambiguous positions. The final alignment included 286 characters and is available upon request. Phylogenetic analyses of the ITS datasets were performed with PhyML for ML (the best-fitting model [GTR + *I* + Γ] of sequence evolution was assessed using Modeltest 3.7 and bootstrapped with 1000 replicates; likelihood: loglk = −404.139)

Anti-*Leishmania donovani* IgG antibodies

Seropositive animals were found for every species tested. The overall seroprevalence of anti-*L. donovani* IgG in the Ethiopian samples was 18.9 % (103/546) (Table 3; Fig. 2). Across all localities tested, the highest seropositivity was observed in dogs (overall 55.9 %) and the lowest in cows and donkeys (Table 3). Of the 32 animals that tested positive for *Leishmania* DNA, 12 animals also demonstrated seropositivity for the *L. donovani* antigen: 1 donkey, 3 goats, and 8 sheep (Additional file 3).

Apart from the cows, all of the Ethiopian animal species exhibited significantly higher levels of anti-*L. donovani* IgG compared with control animals (Fig. 2). Geographically, significantly higher levels of anti-*L. donovani* IgG were observed in all animal species from Humera and in dogs, goats, and sheep from the other localities tested, when compared with control animals (Fig. 2).

Anti-*Phlebotomus orientalis* saliva IgG antibodies

The seroprevalence of anti-*P. orientalis* IgG in Ethiopian animals was 23.1 % (126/546) (Table 4). Seropositive animals were identified for every species and at every locality tested. In Addis Zemen and Sheraro, the highest seroprevalence was observed in dogs (57.9 and 57.1 %, respectively), whereas in Humera, the highest seroprevalence was among donkeys, dogs, and sheep (66.7, 62.5, and 57.3 %, respectively) (Table 4).

Apart from cows, all of the animal species from Ethiopia exhibited significantly ($p < 0.05$) higher anti-*P. orientalis* IgG seroreactivity compared with control animals (Fig. 2). Geographically, elevated levels of anti-*P. orientalis* IgG were observed in dogs, donkeys, and sheep from Humera and in dogs, donkeys, and goats from Sheraro. In Addis Zemen, only dogs exhibited significantly higher seroreactivity than control animals. The seroreactivities in the bovine

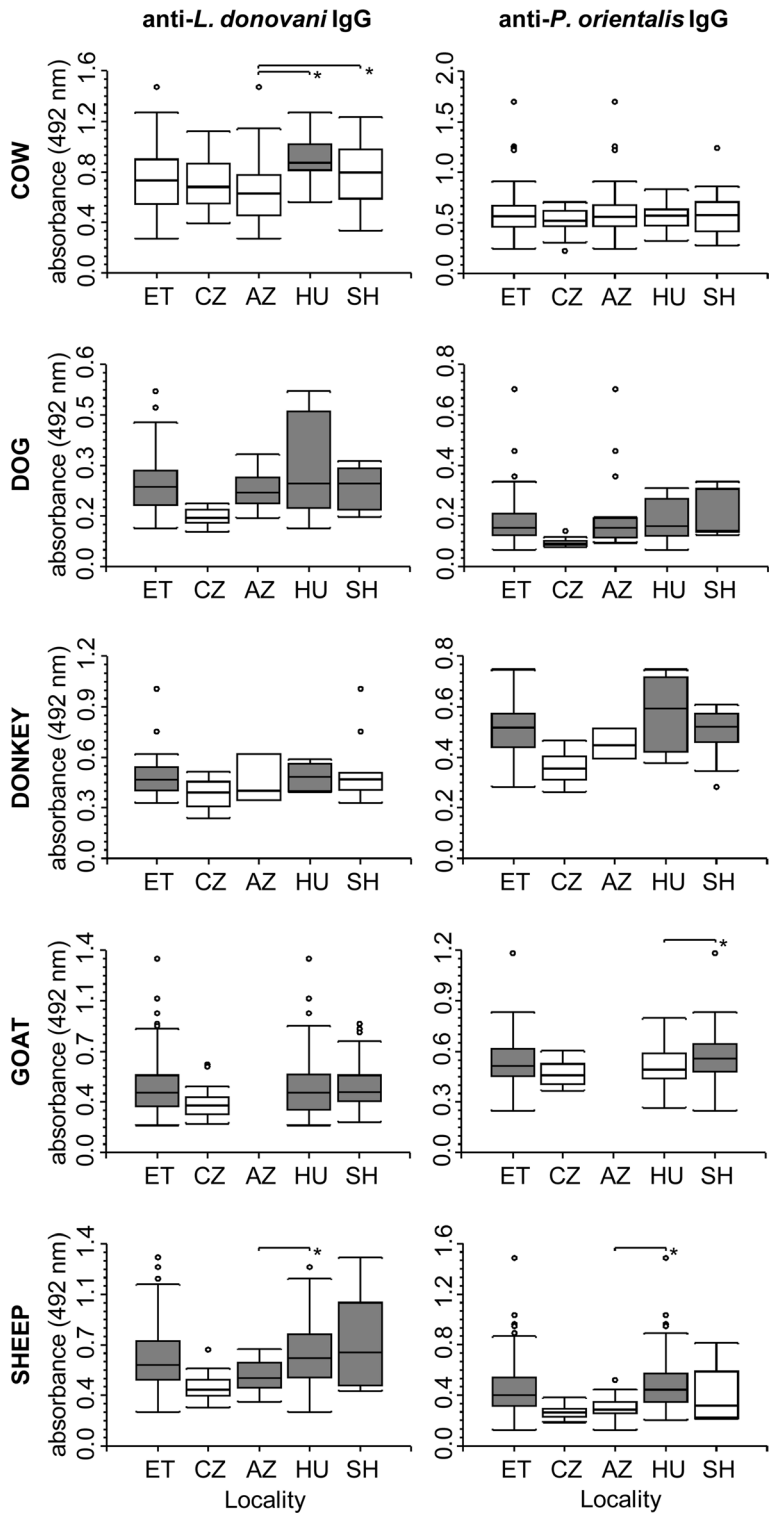

Fig. 2 Serological survey of domestic animals in Ethiopia. IgG antibodies against *Leishmania donovani* promastigotes or *Phlebotomus orientalis* saliva in all serum samples collected from domestic animals in Ethiopia (ET) from Addis Zemen (AZ), Humera (HU), and Sheraro (SH). Significant differences compared with the Czech controls (CZ) are highlighted in gray. The asterisk represents differences between the 3 localities (AZ, HU, and SH) in Ethiopia

Table 3 Seropositivity of Ethiopian animals for *Leishmania donovani* IgG. The cut-off value was calculated as the mean optical density in the control animals plus 3 standard deviations (details provided in the Methods)

		Anti-*L. donovani* IgG positive/total animals sampled (% seropositive)			
Species	Cut-off	Addis Zemen	Sheraro	Humera	Total
Cow	1.298	1/62 (1.6 %)	0/26 (0 %)	0/16 (0 %)	1/104 (1.0 %)
Dog	0.223	9/19 (47.4 %)	5/7 (71.4 %)	5/8 (62.5 %)	19/34 (55.9 %)
Donkey	0.652	0/3 (0 %)	2/11 (18.2 %)	0/6 (0 %)	2/20 (10.0 %)
Goat	0.675		10/106 (9.4 %)	15/133 (11.3 %)	25/239 (10.5 %)
Sheep	0.648	1/27 (3.7 %)	3/5 (60.0 %)	52/117 (44.4 %)	56/149 (37.6 %)
Total		11/111 (9.9 %)	20/155 (12.9 %)	72/280 (25.7 %)	103/546 (18.9 %)

samples were similar to those in control animals, regardless of the locality (Fig. 2).

To verify the specificity of the anti-*P. orientalis* saliva antibodies we used sera from dogs and mice that had been experimentally exposed to a single sand fly species. In dogs, the reactivity of anti-*P. perniciosus* and anti-*Lutzomyia longipalpis* sera against *P. orientalis* salivary gland homogenate (SGH) was similar to that for sera from non-exposed dogs (Fig. 3a). However, all of the selected canine sera of Ethiopian origin reacted strongly to *P. orientalis* SGH (Fig. 3a). In mice, the *P. orientalis* salivary antigen reacted strongly only to the homologous IgGs (Fig. 3b). The reactivities of all heterologous antigen-antibody combinations were similar to those for sera from non-exposed mice (Fig. 3b).

Correlation analysis of serological results

A positive correlation was found between the levels of anti-*P. orientalis* and anti-*L. donovani* IgG in Ethiopian cows ($\rho = 0.37$, $p = 0.0001$), goats ($\rho = 0.37$, $p < 0.0001$), and sheep ($\rho = 0.65$, $p < 0.0001$) (Table 5). This correlation remained significant even when the locality was considered, except for the cows from Humera, for which the correlation was only slightly outside of the level of significance ($\rho = 0.48$, $p = 0.057$). No significant correlation was found for the canine and donkey sera (Table 5).

Discussion

Visceral leishmaniasis is considered to be an anthroponosis in northwestern Ethiopia, but in nearby Sudanese foci, zoonotic transmission has also been suspected, with dogs and mongooses serving as possible reservoirs [3–5, 19]. With regard to domestic animals, sleeping near dogs, cattle, goats, or donkeys has been associated with an increased risk of VL in migrants and residents of Humera [20]. Understanding the mode of disease transmission, whether anthroponotic or zoonotic, is critical for the planning and implementation of effective VL control programs. Thus, one of the main goals of our study was to screen domestic animals for *Leishmania* DNA and discuss their possible involvement in the epidemiology of VL in Ethiopia as possible parasite hosts.

We evaluated two parameters associated with the ability of an animal to be a host for *Leishmania* parasites [21, 22]: (1) exposure to a sand fly vector as a source of blood and (2) the presence of *Leishmania* DNA in the animal's peripheral blood.

In northwestern Ethiopia, the sand fly vector species of *L. donovani* has not yet been identified. However, *Phlebotomus orientalis* is the most probable vector given that it has been found to be infected with *L. donovani* in nearby Sudanese foci [7] and its susceptibility to this *Leishmania* species has been demonstrated experimentally [8]. Exposure to *P. orientalis* was assessed using anti-sand fly saliva antibodies as a marker [6]. Anti-

Table 4 Seropositivity of Ethiopian animals for *Phlebotomus orientalis* saliva IgG. The cut-off value was calculated as the mean optical density in the control animals plus 3 standard deviations (details provided in the Methods)

		Anti-*P. orientalis* IgG positive/total animals sampled (% seropositive)			
Species	Cut-off	Addis Zemen	Sheraro	Humera	Total
Cow	0.876	4/62 (6.5 %)	1/26 (3.8 %)	0/16 (0 %)	5/104 (4.8 %)
Dog	0.143	11/19 (57.9 %)	4/7 (57.1 %)	5/8 (62.5 %)	20/34 (58.8 %)
Donkey	0.550	0/3 (0 %)	3/11 (27.3 %)	4/6 (66.7 %)	7/20 (35.0 %)
Goat	0.685		17/106 (16.0 %)	6/133 (4.5 %)	23/239 (9.6 %)
Sheep	0.410	3/27 (11.1 %)	1/5 (20.0 %)	67/117 (57.3 %)	71/149 (47.7 %)
Total		18/111 (16.2 %)	26/155 (16.8 %)	82/280 (29.3 %)	126/546 (23.1 %)

saliva IgG antibodies were found in all of the animal species tested, which is indicative of the opportunistic feeding behavior of *P. orientalis* [23], thus meeting one criteria for the possible zoonotic transmission of *L. donovani*. Feeding preferences, together with other ecological constraints such as the localization of vector breeding sites [24] or vector susceptibility to harboring *Leishmania* infection [8] may help us to understand the complex picture of the ecology and transmission dynamics of VL in Ethiopia.

The presence of *Leishmania* DNA in animal peripheral blood and *Leishmania* seropositivity serve as reliable epidemiological markers for assessing infection. PCR positivity indicates the presence of the parasite [25, 26]. Although this technique cannot prove the intact integrity of the parasite, viability of the detected *Leishmania* is highly probable given that its DNA degrades shortly after parasite death [27]. Seropositivity, on the other hand, is considered a marker of exposure to *Leishmania* infection [28]. The majority of *Leishmania*-positive animals were found in Humera, indicating dynamic transmission to domestic animals in this well-known active focus. However, many *L. donovani*-seropositive animals were found in all the three surveyed localities, suggesting that exposure to *Leishmania* parasites also occurred in the foci of Addis Zemen and Sheraro.

The fact that only one-third of the PCR-positive animals were positive for both kDNA and ITS1-HRM PCR, is not surprising because the ITS1 region has a considerably lower copy number [11, 12]. Due to the small amount of parasite DNA available in blood samples, distinguishing between the closely related species *L. donovani* and *L. infantum* is notoriously difficult [15]. Moreover, distinction within the *L. donovani* complex in East Africa is controversial; strains that were previously split into *L. donovani*, *L. archibaldi* or *L. infantum* have now been classified into one group: *L. donovani* s.s. [29].

The most suspected animal reservoirs for *L. donovani* are dogs, which are known to play a key role as reservoir hosts in the transmission cycle of the closely related *L. infantum* [2, 30]. Several authors have reported PCR-positivity or seropositivity of dogs in *L. donovani* foci [3, 4, 19, 31–35], including Humera and Addis Zemen in Ethiopia [36–39]. In the present study, dogs demonstrated the highest *Leishmania* seroprevalence out of all the species tested at all study sites, with two PCR-positive dogs identified in Humera and Addis Zemen. As a suspected reservoir species, dogs are also highly attractive to the vector [35], which is supported by our findings that dogs exhibited the highest seroprevalence of anti-*P. orientalis* antibodies among the tested animal species. Most importantly, the same *Leishmania* strains have been recovered from dogs and VL patients [3, 4, 19] and have been shown to persist in dogs for years [19]. Dogs have been recognized as a risk factor for human VL [20, 37, 39], and as the most probable reservoir hosts, their involvement in disease transmission should be addressed in control strategies for VL caused by *L. donovani*.

Almost 38 % of *Leishmania*-positive animals have also been found to be seropositive, indicating these domestic animals (donkeys, goats, sheep) as putative host species in local VL foci. Nevertheless, it is important to mention that neither PCR-positivity nor seropositivity indicates that an animal is able to maintain the parasite for a long period of time. This must be primarily demonstrated by the follow-up of infected animals. Several studies of naturally or experimentally infected non-canine domestic animals have demonstrated their different capabilities to maintain *Leishmania* infection. Cerqueira *et al*. [40] experimentally infected four donkeys with *L. chagasi* (syn. *L. infantum*). These donkeys remained seropositive until the end of the study, which lasted 12 months; however, the donkeys were able to overcome the infection and failed to infect the vector [40]. A PCR survey reported by Bhattarai *et al*. indicated that *Leishmania* infection in goats can persist for at least seven months [41]. On the other hand, *L. donovani* infection in sheep is likely time-limited because only one out of six experimentally infected sheep was shown to develop measurable amounts of anti-*L. donovani* antibodies and the transient presence of amastigotes in sampled tissue in a study that included 244 days of monitoring [42]. Thus, the 37.6 % seropositivity detected in our study may indicate a high infection rate among Ethiopian sheep, further supported by the significantly higher levels of anti-*L. donovani* IgG antibodies among *Leishmania*-positive sheep (Additional files 3 and 4).

The fact that many animals were seropositive for *Leishmania* while PCR-negative in the blood, and, on the other hand, that out of 32 PCR-positive animals, 20 animals were seronegative, could be explained by several possible mechanisms. Seropositivity and PCR-negativity might be attributable to infection in hosts that have resolved the infection but retain high titers of specific antibodies [40, 43]. Another possibility is that seropositive animals might carry the infection in their tissues without parasitemia and are therefore negative according to blood PCR [44]. The reverse situation with PCR-positivity and seronegativity could be attributable to the delayed development of a detectable antibody response in early infection [45], or due to an infection in animals whose B-cells are unresponsive to *Leishmania* antigens, as found in some asymptomatic hosts [2, 43, 44].

The role of other domestic animals as hosts or potential reservoirs for *L. donovani* is still unclear. The

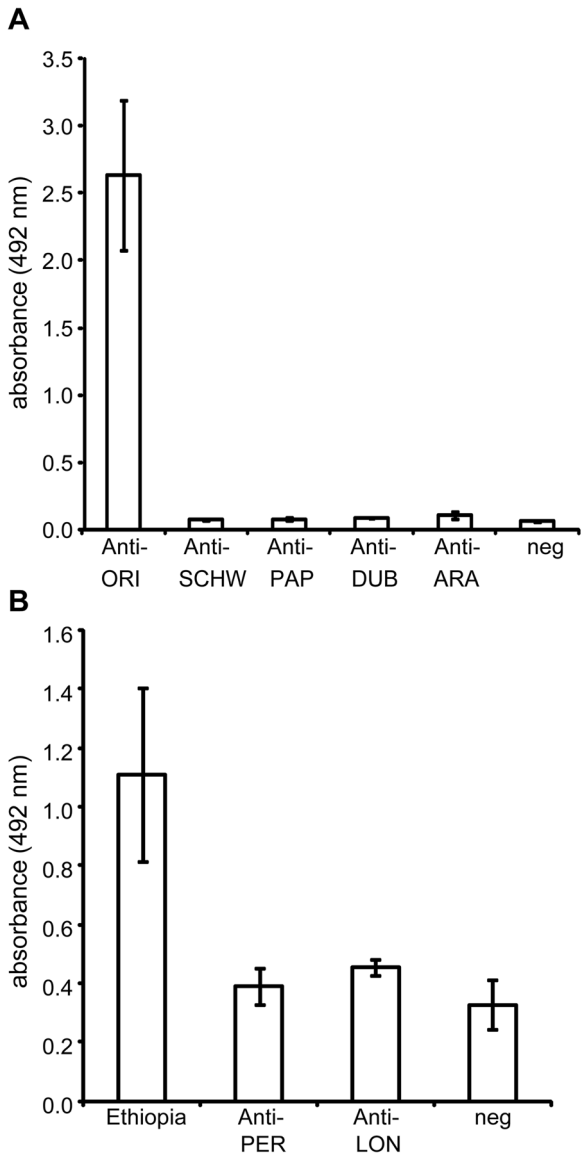

Fig. 3 Specificity of the anti-sand fly saliva IgG antibody response. The reactivity of *Phlebotomus orientalis* salivary gland homogenates (SGH) with sera from mice (**a**) and dogs (**b**) repeatedly exposed to a single sand fly species was measured via ELISA. In graph A, SGH was incubated with sera from mice exposed to *P. orientalis* (Anti-ORI), *Sergentomyia schwetzi* (Anti-SCHW), *P. papatasi* (Anti-PAP), *P. duboscqi* (Anti-DUB), or *P. arabicus* (Anti-ARA). Each bar represents the mean for two serum samples ± the standard error. The values for the positive controls (the sera of mice incubated with homologous antigen) were as follows: *S. schwetzi* = 1.48 ± 0.43, *P. papatasi* = 2.38 ± 0.32, *P. duboscqi* = 2.87 ± 0.60, and *P. arabicus* = 1.86 ± 0.24. In graph B, the SGH was incubated with the sera from dogs exposed to *P. perniciosus* (Anti-PER) or *Lutzomyia longipalpis* (Anti-LON). Seropositive Ethiopian dogs (Ethiopia) and dogs that had never been exposed to sand flies (neg) were used as positive and negative controls, respectively. Each bar represents the mean of five serum samples ± the standard error. The absorbencies of the sera incubated with the homologous antigen were 2.42 ± 0.06 for *L. longipalpis* and 1.73 ± 0.13 for *P. perniciosus*

Table 5 Correlation analysis of serological results

Species		Ethiopia	Addis Zemen	Sheraro	Humera
Cow	ρ	0.37***	0.38**	0.43*	0.48
	n	104	62	26	16
Dog	ρ	0.12	0.15	−0.46	0.36
	n	34	19	7	8
Donkey	ρ	0.31	0.50	0.52	−0.03
	n	20	3	11	6
Goat	ρ	0.37***		0.36***	0.37***
	n	239		106	133
Sheep	ρ	0.65***	0.67***	1.00***	0.61***
	n	149	27	5	117

Results from the Spearman-Rank Correlation Matrix test for anti-*Leishmania donovani* IgG and anti-*Phlebotomus orientalis* saliva IgG
ρ correlation coefficient, *n* number of serum samples tested
Asterisk (*) indicate significant correlations: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

present study is the first to report PCR-positive cattle, donkeys, goats, and sheep in Ethiopia. These animals, especially cattle, serve as sources of blood for *L. donovani* vectors [23, 46]. Even if these species do not serve as reservoir hosts for the parasite, they still attract large numbers of blood-questing female sand flies and may, therefore, act as a protective barrier in the case of resistant or refractory mammal species or as a risk factor in the case of susceptible species [30, 37, 47]. Prediction of the role of domestic animals in the amplification or dilution of VL risk might be possible using a recently described mathematical model for multi-host infectious diseases by applying relevant data [48].

In addition to the maintenance of persistent infection, the transmissibility competence, e.g. infectivity for the sand fly vector, is an important prerequisite for any mammal to serve as a *Leishmania* reservoir [28, 49]. These two criteria, among other aspects, can distinguish between a reservoir host and an incidental host that is not capable of infecting the vector [25]. Validation of these prerequisites for domestic animals in northwest Ethiopia, however, requires further investigation.

Conclusions

In conclusion, leishmaniasis caused by *L. donovani* is traditionally considered to be an anthroponosis in East Africa. However, the present study revealed widespread exposure to *L. donovani* and sand fly vector bites among domestic animals. The possible involvement of domestic animals as sources of blood for vector sand flies should therefore be considered in VL control strategies. However, the direct involvement of domestic animals in the transmission cycle of *L. donovani* warrants further

investigation, most importantly by xenodiagnosis to determine their transmissibility competence.

Additional files

Additional file 1: Accession numbers for *Leishmania* ITS sequences downloaded from the GenBank database and used for the phylogenetic analysis presented in Fig. 1.

Additional file 2: Details of the ELISA methods.

Additional file 3: Detailed list of Ethiopian animals positive for *Leishmania* DNA.

Additional file 4: Differences in the levels of anti-*Leishmania donovani* IgG and anti-*Phlebotomus orientalis* saliva IgG between *Leishmania*-positive (full circle) and *Leishmania*-negative (open circle) animals in the Humera region (the majority of PCR-positive animals are from this locality: 30 out of 32). Significant differences are marked by the probability level on the X-axis.

Abbreviations

ELISA: Enzyme-linked immunosorbent assay; ITS1: Internal transcribed spacer 1; kDNA: Kinetoplast deoxyribonucleic acid; *L.*: *Leishmania* or *Lutzomyia*; OD: Optical density; *P.*: *Phlebotomus*; PBS: Phosphate-buffered saline; PBS-Tw: Phosphate-buffered saline with Tween; PCR: Polymerase chain reaction; SGH: Salivary gland homogenate; VL: Visceral leishmaniasis.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

IR participated in the study design and field sample collection, carried out *Leishmania* serology, performed the statistical analysis, and drafted and finalized the manuscript. DTF and DYL carried out the *Leishmania* PCR and sequencing. TK, NP, and TL performed sand fly serology. AK, CM, RK, CLJ, and AW participated in field sample collection. JV carried out the sequence alignment and phylogenetic analysis. JV, AW, AH, and PV participated in the study design and coordination. GB conceived and designed the study, coordinated and participated in field work, and drafted and finalized the manuscript. DTF and TK contributed equally to the paper. All authors read and approved the final manuscript.

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RESEARCH ARTICLE

Canine Antibodies against Salivary Recombinant Proteins of *Phlebotomus perniciosus*: A Longitudinal Study in an Endemic Focus of Canine Leishmaniasis

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Abstract

Background

Phlebotomine sand flies are vectors of *Leishmania* parasites. During blood feeding, sand flies deposit into the host skin immunogenic salivary proteins which elicit specific antibody responses. These anti-saliva antibodies enable an estimate of the host exposure to sand flies and, in leishmaniasis endemic areas, also the risk for *Leishmania* infections. However, the use of whole salivary gland homogenates as antigen has several limitations, and therefore, recombinant salivary proteins have been tested to replace them in antibody detection assays. In this study, we have used for the first time sand fly salivary recombinant proteins in a longitudinal field study on dogs.

Methodology/Principal Findings

Sera from dogs naturally exposed to *P. perniciosus* bites over two consecutive transmission seasons in a site endemic for canine leishmaniasis (CanL) were tested at different time points by ELISA for the antibodies recognizing whole saliva, single salivary 43 kDa yellow-related recombinant protein (rSP03B), and a combination of two salivary recombinant proteins, 43 kDa yellow-related protein and 35.5 kDa apyrase (rSP01). Dogs were also tested for *Leishmania infantum* positivity by serology, culture, and PCR and the infection status was evaluated prospectively. We found a significant association between active CanL infection and the amount of anti-*P. perniciosus* saliva antibodies. Importantly, we detected a high correlation between IgG antibodies recognizing rSP03B protein and the whole salivary antigen. The kinetics of antibody response showed for both a whole saliva and rSP03B a similar pattern that was clearly related to the seasonal abundance of *P. perniciosus*.

Competing Interests: The authors have declared that no competing interests exist.

Conclusions

These results suggest that *P. perniciosus* rSP03B protein is a valid alternative to whole saliva and could be used in large-scale serological studies. This novel method could be a practical and economically-sound tool to detect the host exposure to sand fly bites in CanL endemic areas.

Author Summary

Canine leishmaniasis (CanL) is a widespread zoonosis caused by protozoan parasite *Leishmania infantum*. CanL is endemic in more than 70 countries, including regions of south-western Europe, where the main vector is *Phlebotomus perniciosus*. Saliva inoculated into the host during blood feeding provoke species-specific antibody response. They could be used as a serological marker of exposure to sand flies and, in leishmaniasis endemic areas, also as an indication of *Leishmania* infection risk. However, in case of large-scale serological studies, it is difficult to obtain appropriate amounts of crude salivary antigen from laboratory-reared sand flies. Therefore, we studied if specific salivary recombinant proteins could be suitable to replace whole saliva as an antigen for serological assays. Antibodies recognizing recombinant salivary protein in naturally bitten dogs were highly correlated with antibody response against whole saliva. Seasonal exposure of dogs to sand flies led to antibody response fluctuations related to the period of activity and abundance of vectors. In a context of high CanL endemicity, we found a significant association between progressive CanL infections and antibodies against sand fly saliva. Thus, salivary recombinant proteins could be used as suitable tool for investigations host exposure to sand flies in endemic settings.

Introduction

Canine leishmaniasis (CanL), caused by protozoan parasite *Leishmania infantum*, is a systemic and potentially fatal disease [reviewed in [1, 2]]. It may affect any organ or body fluid [reviewed in [1]] and can manifest variable clinical signs [reviewed in [2, 3]]. However, the majority of infected dogs do not develop any clinical signs. Importantly, *L. infantum* is also a causative agent of human visceral leishmaniasis and both symptomatic and asymptomatic dogs have a crucial role in the epidemiology of this disease, serving as reservoirs [reviewed in [2]]. CanL is endemic in many regions of southern Europe and Latin America, however, climate changes and socioeconomic factors, particularly increased travelling of dogs between endemic and non-endemic areas, led to changes in the distribution of CanL in both continents [reviewed in [1, 2, 4]].

Diagnosis of CanL should be based on an integrated approach considering signalment, history, clinical findings, and results of basic laboratory analyses that detect the parasite or evaluate the immune response in the host [reviewed in [3]]. The commonly used diagnostic methods include direct detection of the parasite by culture or polymerase chain reaction (PCR) and indirect techniques for detection of antibodies against *Leishmania*, such as immunofluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) [5–8]. However, there is still lack of practical methods for detection of the risk of *Leishmania* transmission according to the exposure of dogs to sand fly vectors.

Results from studies on human and canine antibody response against sand fly saliva revealed high immunogenicity of sand fly salivary proteins as well as high specificity of anti-

saliva IgG [9–12]. Studies based on dynamics of IgG response in dogs experimentally exposed to the main *L. infantum* vectors, *Lutzomyia longipalpis* and *Phlebotomus perniciosus* [12, 13], suggest that monitoring canine antibody response to sand fly saliva could be a useful epidemiological tool in CanL foci. However, the use of whole sand fly saliva in such studies is limited by time-consuming salivary gland dissection and complicated by potential cross-reactivity with saliva from sand fly species with no role in *Leishmania* transmission [11] or from other blood sucking insects [14]. These problems could be overcome by using specific immunogenic sand fly salivary recombinant proteins [reviewed in [15]]. The use of recombinant proteins was already shown for *P. papatasi* [16, 17], *Lu. longipalpis* [18, 19] and *P. perniciosus* [20, 21]. Although these studies confirmed the advantages of salivary recombinant proteins, most of them were tested on small sets of samples and never in association with naturally transmitted *Leishmania* infections.

Previous studies on different hosts (dogs, foxes, and humans) bitten by *P. perniciosus* or *Lu. longipalpis* revealed high antigenicity of salivary yellow-related proteins and apyrases [12, 13, 19]. Apyrases are enzymes with potent anti-hemostatic activity hydrolyzing the platelet activator ADP [22]. The role of yellow-related proteins is less clear [23] but most probably they act as a histamin-binding molecules [24]. Recent work using a low number of sera from dogs experimentally exposed to *P. perniciosus* showed that recombinant forms of these proteins could be used as potential candidates for markers of canine exposure to sand flies [20]. Therefore, herein we used the recombinant 43 kDa yellow-related protein (rSP03B) from *P. perniciosus* and its combination with recombinant *P. perniciosus* 35.5 kDa apyrase (rSP01) to study the specific antibody response in a large number of dogs naturally exposed to *P. perniciosus* over two years in a focus endemic for *L. infantum*.

Methods

Ethical statement

The technical protocol for the investigation of natural canine *Leishmania* infections performed in the frame of experimental drug trials was approved by the Veterinary Board of the Italian Ministry of Health (authorization no.4051/P) following the European Directive 86/609/EEC, adopted by the Italian Government with the Law 116/1992.

Experimental design and background

A longitudinal study including two sand fly seasons was performed on 56 Beagle dogs housed in the same open-air kennel sited in a rural municipality of the Naples province (Campania region, southern Italy) where both human visceral leishmaniasis and CanL are highly endemic. Here, *P. perniciosus* is the only *Leishmania* vector, whose activity period ranges from the end of May through late October [25]. *Leishmania infantum* infection rates in dissected sand flies were found to range from 2.8% to 6.2% in places not far from the kennel [26]. In cohorts of naïve dogs previously exposed in the same kennel, the annual incidence of CanL infection and clinical disease was reported to average about 40% and 20%, respectively [8]. The dogs included in our study were part of experimental trials of pharmacological products against CanL. The animals were born in a breeding facility sited in a non-endemic area of northern Europe, and were confirmed to be CanL-free at the time they were moved to the study site when they were about 6 months old. Once housed in the open-air kennel, the use of topical or environmental insecticides was avoided to allow natural exposure of dogs to sand fly bites in the warm season.

Antibodies against sand fly salivary proteins were analyzed retrospectively in dog sera taken for routine clinical examination and periodical CanL serology, and that were stored frozen. Available samples included sera obtained shortly before the dogs were transferred to the

endemic area in July (first year), and they served as pre-immune sera (n = 56). Responses to salivary antigens were then analyzed on selected samples collected at four-time points in the first year [August (n = 53), September (n = 54), October (n = 34), December (n = 54)] and at five occasions during the second year [January (n = 56), March (n = 56), July (n = 56), August (n = 56), September (n = 54)]. As per established protocols [5], follow-up analyses for the detection and classification of natural CanL infections were performed at the beginning of the study in the first year in July (n = 56), twice in the second year [March (n = 56) and July (n = 56)] and once at the beginning of the third year [March (n = 53)].

Analysis of *Leishmania* infection

Dogs were examined by serology, culture, and PCR. Detection of anti-*Leishmania* IgG antibodies was performed by an in-house IFAT assay using *L*. *infantum* promastigotes as antigen and following the protocol recommended by the Office International des Epizooties [27]. The cut-off dilution for *Leishmania* exposure was set at 1:40 (i.e. the upper part of the so-called "IFAT grey zone") [3]. Bone-marrow aspirate material was examined by nested-PCR assay as previously described [28]. Lymph-node aspirate material was cultured from each popliteal node in Evans' Modified Tobie's medium and cultures were periodically examined for promastigotes growth during one month.

At each assessment, the dogs were classified as follows with regards to the infection status: i) "*Leishmania* negative"if found negative by all assays, ii)"*Leishmania* exposed"if tested positive by IFAT at low titers and negative by other tests, iii) having a"subpatent *Leishmania* infection" if bone-marrow PCR tested positive, IFAT was either negative or positive at low titers, and lymph node culture was negative, iv) having an"active *Leishmania* infection" if both bone-marrow PCR and lymph node culture were tested positive. In this infection stage, IFAT can be found negative or positive at low titer initially, but converts shortly to very elevated titers. Once established, active infections do not regress spontaneously towards negative or subpatent conditions and dogs invariably progress to clinical disease [8].

Sand flies and salivary proteins

A colony of *P*. *perniciosus* was reared under standard conditions as described in [29]. Salivary glands, dissected from 4–6 day old female sand flies, were pooled in 20 mM Tris buffer with 150 mM NaCl and stored at -20°C. Recombinant salivary proteins from *P*. *perniciosus*, 35.5 kDa apyrase (rSP01, Genbank accn. DQ192490) and 43 kDa yellow-related protein (rSP03B, Genbank accn. DQ150622) were obtained from Apronex s.r.o. (Prague) as mentioned in [20]. The concentrations of these proteins were quantified by the Lowry method (Bio-Rad) following the manufacturer´s protocol.

Detection of anti- *P*. *perniciosus* IgG

Anti-*P*. *perniciosus* IgG were measured by enzyme-linked immunosorbent assay (ELISA) as described in [12] with minor modifications. Briefly, microtiter plates were coated either with salivary gland homogenate (SGH) (0.2 salivary gland per well) or with rSP03B (5μg/ml) or with rSP03B+rSP01 (5μg/ml of each protein) in 20 mM carbonate-bicarbonate buffer (pH 9.5) overnight at 4°C. The plates were incubated with blocking solution, 6% (w/v) low fat dry milk in PBS with 0.05% Tween 20 (PBS-Tw). Canine sera were diluted 1:200 for SGH and 1:100 for recombinant proteins in 2% (w/v) low fat dry milk/PBS-Tw. Secondary antibodies (anti-dog IgG, Bethyl laboratories) were diluted 1:9000 in PBS-Tw. Absorbance was measured at 492 nm using a Tecan Infinite M200 microplate reader (Schoeller). Each serum was tested in duplicate and the experiment was repeated twice.

Statistical analysis

Antibodies against sand fly saliva are reported as optical densities (OD) with subtracted blanks and multiplied by 100 for easier readability. In case of linear mixed models, ODs were log transformed (OD value+1).

Statistical analyses were carried out using R software (http://cran.r-project.org/). Repeatability defined as the degree of agreement between repeated OD measures on the same samples was estimated within and between plates by calculating the concordance correlation coefficient (CCC) that ranges from 0 (no concordance) to 1 (perfect concordance) [30].

Antibodies against sand fly saliva and *Leishmania* infection status frequency distributions were analysed over time. Proportions and medians between time points were compared using McNemar's chi-square test for paired data and Wilcoxon signed rank sum test, respectively. Correlations were analysed using Spearman rank correlation test [31].

The "nlme" package [32] was then used to develop multilevel linear regression models to investigate the relationship between IgG anti-saliva antibodies as continuous dependent variable, and sampling months and *L. infantum* infection status (as described above) included as categorical predictor variables, taking into account the correlation between repeated measures of the same dogs over time. Two hierarchical levels were considered in the analysis with repeated measures within individual dogs as the level-1 units and individual dogs as the level-2 units. The random variation at the dog level was examined at both the intercept and at the slopes and significance was assessed testing the -2 log likelihood ratios between the model with and the model without the random effect [33]. The correlation between level 1 units was considered as having a compound-symmetry (CS) or autoregressive with a lag of 1 (AR-1) structure or as unstructured (UN) and Akaikes's Information Criterion (AIC) was used to compare the goodness of fit of models with different correlation structures, selecting those with the smallest values of these statistics [33, 34]. Parameter estimates for fixed effects were estimated using restricted maximum likelihood estimation (REML) and significance was assessed using conditional t-tests and F-tests, and alpha was taken at the 5% ($p<0.05$) level for a two-tailed test.

Results

Dynamics of *L. infantum* infection status

The percentage of dogs classified as negative, *Leishmania* exposed, subpatently and actively infected is presented in Fig 1.

At the beginning of the study (July in the first year) all dogs were negative by all tests, thereafter, the proportion of *L. infantum* infected dogs increased throughout the trial ($p<0.05$) (Fig 1). In the following sampling, in March, 32% of dogs were classified as *Leishmania* exposed, while subpatent and active infections were found in 2% and 4% of dogs, respectively. The percentage of dogs in each category was similar 3 months later in July (Fig 1). In contrast, by the end of the study in March of the third year, the percentage of subpatently and actively infected dogs sharply increased to 17% and 13%, respectively. By this time, 36% of dogs were classified as *Leishmania* exposed and 34% as negative ($p<0.05$) (Fig 1).

Distribution and dynamics of antibody response against salivary proteins using SGH, rSP03B, and rSP03B+rSP01 as antigens

The overall median values of ELISA ODx100 using SGH, rSP03B, and rSP03B+rSP01 antigens were 10 (range: 2–194), 24 (1–234) and 37 (11–189), respectively ($p<0.05$). However, the median increased significantly with time, following a similar pattern for all three antigens

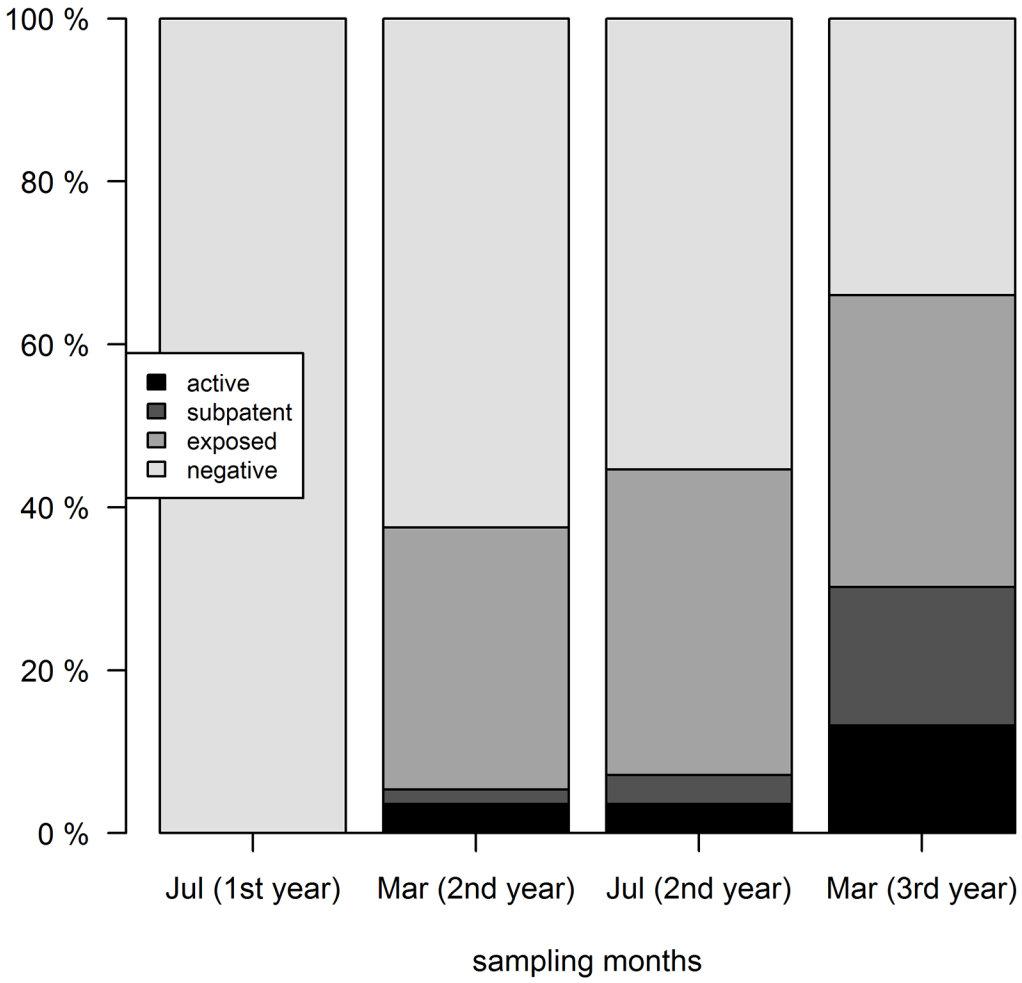

Fig 1. Dynamics of *L. infantum* infection statuses in dogs from a CanL endemic site in southern Italy. Dogs were screened for *L. infantum* infection on four occasions, 56 dogs in July first year, 56 dogs in March the second year, 56 dogs in July the second year and 53 dogs in March the third year.

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tested, most significantly for SGH and rSP03B (Fig 2). For example, the median ODx100 for SGH was 5 at the baseline in July of the first year, increased significantly to 11 through October, decreased thereafter to 8 in January, increased again up to a peak of 24 detected in July of the second year, and remained similar until September (Table 1). The sharp OD increase observed in summer of the second year for SGH and rSP03B was less pronounced for rSP03B+rSP01 (Table 1). Moreover, correlation of antibody response between SGH and rSP03B was stronger (r = 0.77) than between rSP03B+rSP01 and SGH (r = 0.65) (Fig 3). In addition, we detected high correlation between antibodies recognizing rSP03B and antibodies recognizing combination of rSP03B+rSP01 (r = 0.75) (Fig 3).

Within plate repeatability was high for each antigen and the CCC varied from 0.94 (95% CI: 0.93–0.95) to 0.99 (95% CI: 0.98–0.99), depending on the antigen. However, the CCC between plate was moderately high for whole SGH (0.77, 95% CI: 0.73–0.8) and rSP03B (0.88, 95% CI: 0.87–0.9) and low for the combination of rSP03B+ rSP01 (0.53, 95% CI: 0.47–0.59).

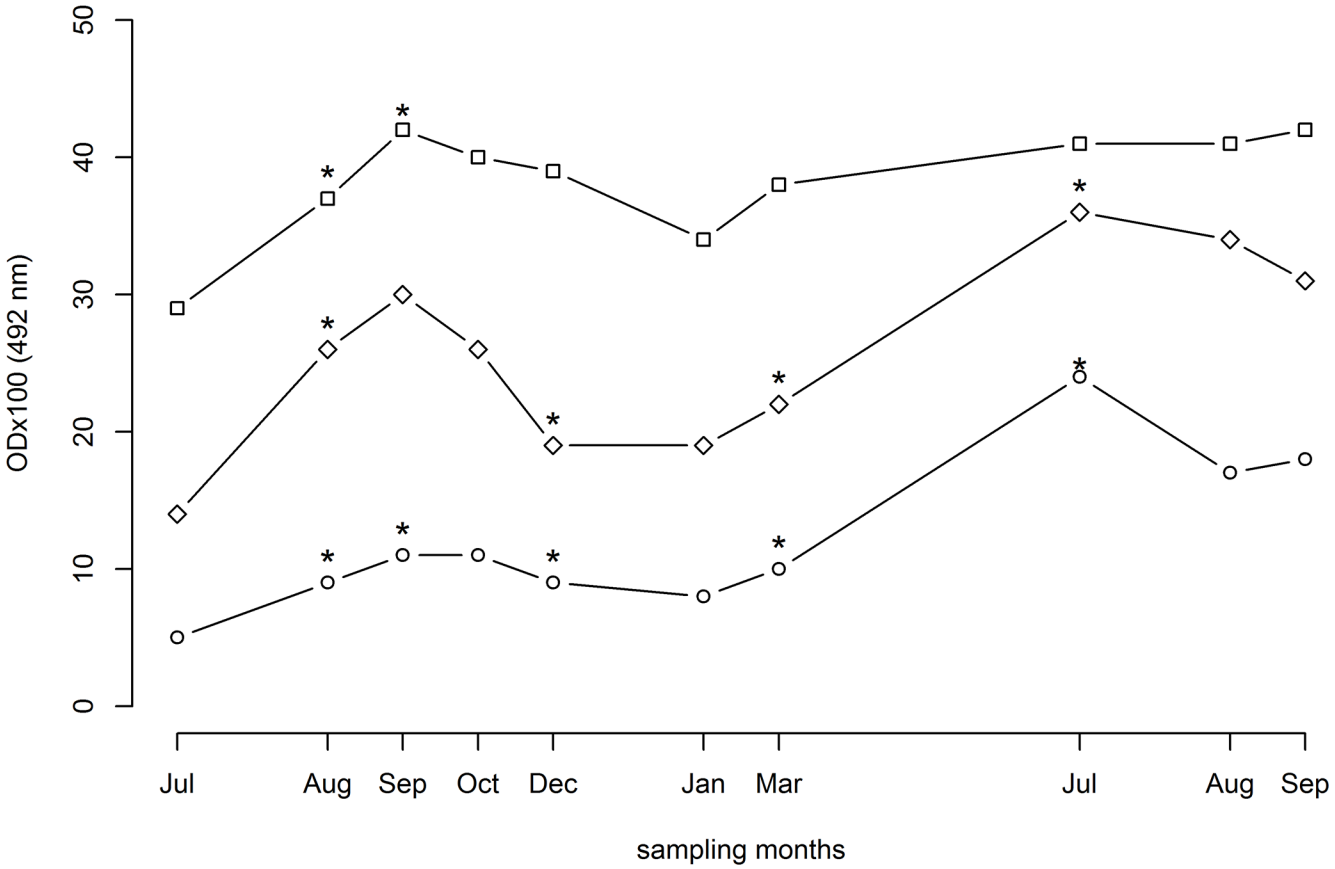

Fig 2. Dynamics of IgG antibody response against sand fly salivary proteins in dogs naturally exposed to *P. perniciosus* over two years in endemic foci. Canine sera were tested by ELISA for the antibodies recognizing SGH (open circle), rSP03B protein (open diamond) and combination of rSP03B+rSP01 proteins (open square). Data are presented as median values for each sampling month. Asterisk represents significant change in the median compared to previous sampling. ODx100 = optical density multiplied by 100.

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Table 1. Median and range antibody OD values (multiplied by 100) for each used antigen according sampling month.

Study year	Sampling month		SGH	rSP03B	rSP03B+rSP01
		n	Median(range)	Median(range)	Median(range)
First	July	56	5 (3–12)	14 (7–36)	29 (19–49)
	August	53	9 (4–42)*	26 (14–75) *	37 (16–71) *
	September	54	11 (4–56) *	30 (11–165)	42 (19–116)*
	October	34	11 (3–59)	26 (11–93)	40 (17–85)
	December	54	9 (4–50) *	19 (10–81) *	39 (21–100)
Second	January	56	8 (2–49)	19 (1–69)	34 (19–93)
	March	56	10 (4–62)*	22 (9–89)*	38 (11–117)
	July	56	24 (6–161) *	36 (9–223) *	41 (17–177)
	August	56	17 (5–129)	34 (11–154)	41 (18–157)
	September	54	18 (6–194)	31 (11–234)	42 (18–189)
	Total		10 (2–194)	24 (1–234)	37 (11–189)

n = number of dogs

* significant change in the median compared to previous sampling

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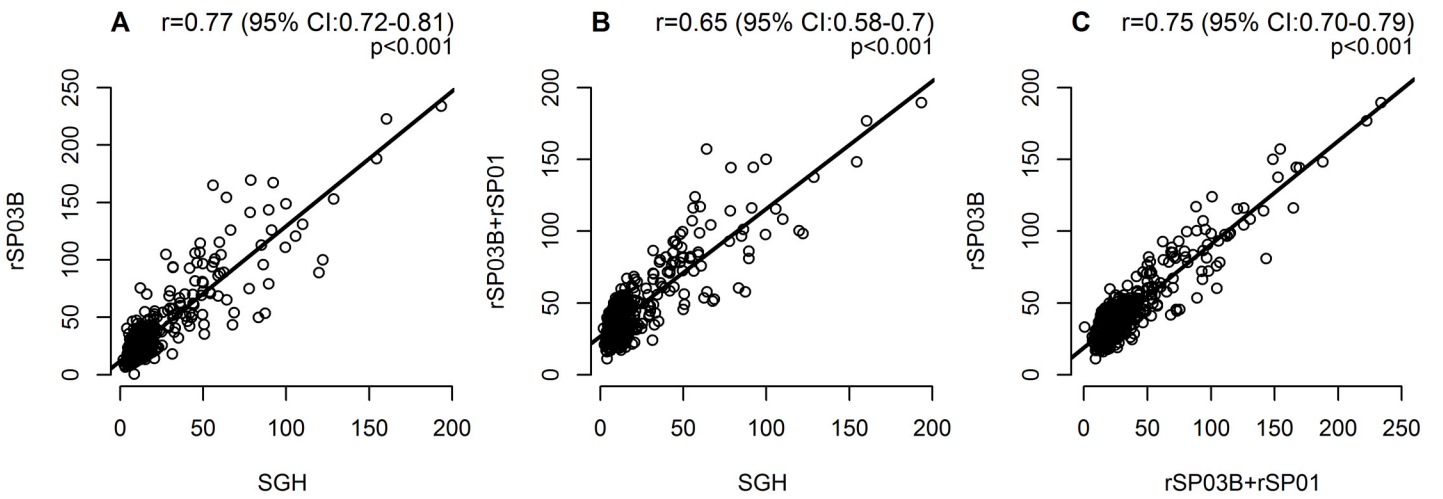

Fig 3. Correlations between IgG antibodies recognizing SGH and recombinant salivary proteins in dogs naturally exposed to sand flies. Correlation between SGH and rSP03B protein (A), between SGH and combination of rSP03B+rSP01proteins (B) and between rSP03B protein and combination of rSP03B+rSP01proteins (C) was performed using Spearman rank correlation. OD values were multiplied by 100. r = correlation index, CI = confidence interval.

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Multivariable relationship between salivary antibodies, season, and *L. infantum* infection

Multilevel models confirmed the strong association between antibodies against SGH and recombinant proteins and sampling month, and between SGH and active *L. infantum* infection.

Tables 2 and 3 present parameters estimates for models including only sampling date (model a) and both sampling date and *L. infantum* infection status (model b) for SGH and rSP03B, respectively (for combination of rSP03B+rSP01 data are showed in S1 Table). Estimates for sampling months alone reflect a significant increase in log OD values by September in comparison to July of the first year, when the study started, then they started to decrease in October, and raised again in March and especially in July of the second year, with the highest log OD estimate detected in the following month of September ($p<0.05$) (Tables 2 and 3, model a). Including dog as a random effect significantly improved the model and the variance estimate indicated that for SGH it was 33% [(10.06/(10.06+20.35))*100] of the variation in log OD dog related. For rSP03B it was 38% [(17.33/(17.33+28.67))*100]. This can be appreciated in Fig 4A and 4B representing the sampling month-specific ODs for the 56 study dogs. Finally, model b shows the sharp increase in log OD between March and July of the second year (Tables 2 and 3) and highlights the strong association between the amount of antibodies against whole saliva and dogs with active *L. infantum* infection ($p<0.05$) (Table 2). The association between active infection and anti-rSP03B antibodies (Table 3) and combination of rSP03B+rSP01did not reach statistical significance. The model also shows that the greatest variation in log ODs between dogs was observed in July of the second year (Tables 2 and 3). There was no evidence of correlation between repeated measures in any of the above models.

Discussion

This paper describes for the first time the dynamics and diagnostic potential of antibodies recognizing *P. perniciosus* salivary recombinant proteins in dogs following natural exposure to the sand flies over two years.

Table 2. Estimates of the multilevel linear regression model of the relationship between log transformed SGH OD values (multiplied by 100) and sampling time (model a), and *Leishmania* status and sampling time (model b).

Variable	Levels	Estimate	SE	P value
a) sampling month only				
Intercept		4.33	0.74	<0.001
Fixed effects				
Sampling month	July (first year)	0.00		
	August (first year)	1.56	0.87	0.073
	September (first year)	2.85	0.86	0.001
	October (first year)	2.49	0.99	0.012
	December (first year)	1.60	0.86	0.064
	January (second year)	1.55	0.85	0.069
	March (second year)	2.36	0.85	0.006
	July (second year)	9.21	0.85	<0.001
	August (second year)	7.84	0.85	<0.001
	September (second year)	9.62	0.86	<0.001
Random effects	**Variance**			
Dog	10.06			
Residual	20.35			
b) sampling month and *Leishmania* infection status	Levels	Estimate	SE	P value
Intercept		4.33	0.11	<0.001
Fixed effects				
Sampling month	July (first year)	0.00		
	March (second year)	1.71	0.56	0.003
	July (second year)	8.46	1.2	<0.001
Leishmania status	Negative	0.00		
	Exposed	1.39	0.90	0.13
	Subpatent	1.28	2.96	0.67
	Active	5.11	2.38	0.03
Random effects	**Variance**			
Dog	0.11			
March (second year)	10.42			
July (second year)	71.52			
Residual	0.51			

SE = standard error

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The study area is considered traditionally endemic for *L. infantum* [35]. The rate of dogs with active infection was increasing over the two-year period, and this pattern was in accordance with previous studies carried out in the area [5, 8]. Our results indicate that there is a significant positive association between anti-*P. perniciosus* saliva antibodies and active *L. infantum* infection. This finding could reflect that these dogs had a greater sand fly challenge compared to other dogs and therefore developed a stronger humoral response against salivary proteins, thus had bigger chance to become infected. Alternatively, because active *L. infantum* infection in dogs is characterized by a mixed Th1/Th2 response associated with marked antibody production [reviewed in [36]], it is possible that our *Leishmania* infected dogs were more sensitive to develop a humoral antibody response against sand fly salivary gland proteins. So far, there is only one report describing an association between CanL and positivity for anti-sand fly saliva antibodies. The study, performed in a *L. infantum* focus in Apulia region of

Table 3. Estimates of the multilevel linear regression model of the relationship between log transformed rSP03B OD values (multiplied by 100) and sampling time (model a), and *Leishmania* status and sampling time (model b).

Variable	Levels	Estimate	SE	P value
a) sampling month only				
Intercept		8.18	0.91	<0.001
Fixed effects				
Sampling month	July (first year)	0.00		
	August (first year)	4.15	1.03	<0.001
	September (first year)	5.87	1.02	<0.001
	October (first year)	3.61	1.18	0.002
	December (first year)	2.14	1.02	0.037
	January (second year)	1.53	1.01	0.131
	March (second year)	2.88	1.01	0.005
	July (second year)	10.81	1.01	<0.001
	August (second year)	8.50	1.01	<0.001
	September (second year)	9.67	1.02	<0.001
Random effects	**Variance**			
Dog	17.33			
Residual	28.67			
b) sampling date and *Leishmania* infection status	Levels	Estimate	SE	P value
Intercept		8.18	0.32	<0.001
Fixed effects				
Sampling month	July (first year)	0.00		
	March (second year)	2.26	0.74	0.003
	July (second year)	10.13	1.49	<0.001
Leishmania status	Negative	0.00		
	Exposed	1.47	1.11	0.188
	Subpatent	-1.11	3.59	0.758
	Active	4.68	3.14	0.139
Random effects	**Variance**			
Dog	2.39			
March (second year)	15.34			
July (second year)	104.54			
Residual	3.31			

SE = standard error

doi:10.1371/journal.pntd.0003855.t003

Italy, showed that *Leishmania* positive and negative dogs did not differ in IgG and IgG1 production against the whole *P. perniciosus* salivary lysates but the *Leishmania* positive ones had significantly decreased levels of IgG2 antibodies [12]. However, we did not find any association between antibodies against rSP03B protein and active CanL infection. Although, the use of rSP03B protein as a marker of exposure to sand flies is promising, use of recombinant proteins as risk markers for infection need more investigation.

In the present study *P. perniciosus* salivary recombinant proteins, rSP03B (yellow- related protein) and its combination with rSP01 (apyrase) were used. For rSP03B, the repeatability of ELISA test was even higher than for SGH, proving this recombinant protein to be better antigen for large scale studies. The achieved high correlation score between SGH and rSP03B was in agreement with results from previous studies [20, 21]. However, the combination of two recombinant proteins (rSP03B and rSP01) did not show better performance than a single

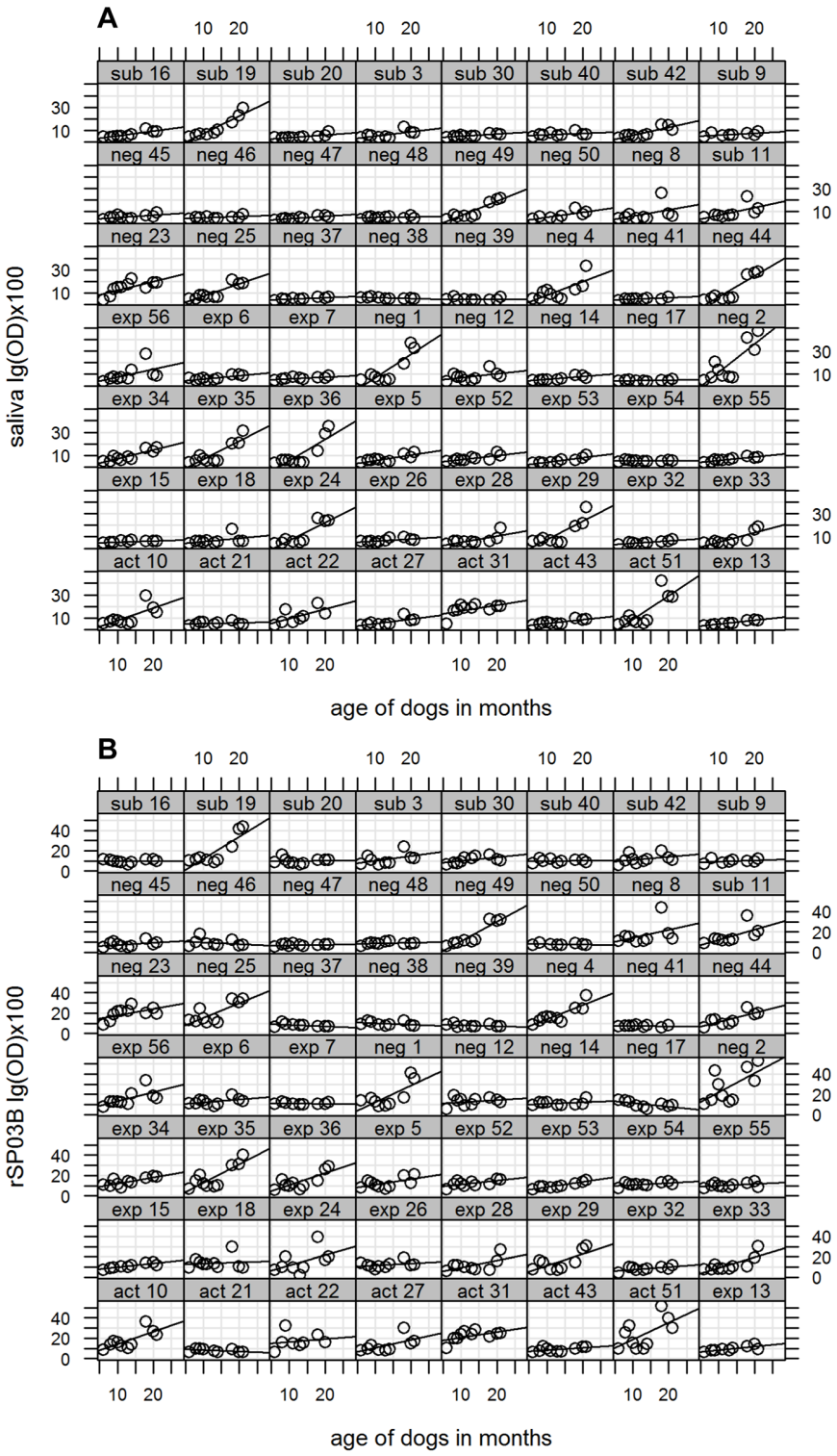

Fig 4. Dynamics of IgG antibodies recognizing SGH (A) and rSP03B protein (B) in individual dogs from CanL endemic locality. *Leishmania* infection status was assigned for 53 dogs according their infection status at the end of the study in March third year and for 3 dogs according their infection status in July second year (dog number 7, 8, 49). OD values were log transformed and multiplied by 100. OD = optical density, neg = negative, exp = exposed, sub = subpatent, act = active.

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SP03B. Repeated experiments suggest that this could be due to instability of rSP01 protein and its higher susceptibility to repeated thawing and freezing. Previously, better results for recombinant proteins combination were obtained for detection of anti-*Lu. longipalpis* antibodies when two recombinant yellow-related proteins were combined together [18].

Kinetics of anti-SGH and anti-rSP03B IgG antibodies developed with similar pattern and were clearly seasonal: rising during summer months when sand fly density is the highest and decreasing during winter months when sand flies are not active. This positive correlation between antibodies against salivary proteins and seasonal abundance of blood feeding insects has been previously reported in mosquitoes [37, 38]. The smaller increase of antibodies against *P. perniciosus* salivary proteins during the first transmission season was probably due to the fact that dogs were exposed to the vector for the first time and were moved to an endemic locality in the middle of the transmission season (July). In central and southern peninsular Italy, the sand fly season usually lasts from late May to late October, with two density peaks [25, 39]. The significant increase of IgG antibodies for SGH and rSP03B protein in March compared to January in the second year (Fig 2) remains unexplained, since it cannot be attributed to such an early activity of sand flies. Even in the southernmost region of Italy (Sicily) the earliest collection of *P. perniciosus* was recently reported to be in April [40]. The marked increase in antibody levels in the second transmission season is much probably caused by re-exposure of dogs to sand flies following antigenic priming in the previous season. Similar antibody responses to sand fly saliva were observed in mice re-exposed to *P. papatasi* [17] and in humans re-exposed to *P. argentipes* [11]. Moreover, the vector population density could vary between years due to the different climatic conditions [39] and therefore, the dogs could have been exposed to higher sand fly challenge during the second transmission season. However, as shown by the multilevel models, there was significant variation in the amount of anti-saliva antibodies between dogs, particularly during July of the second year, the reason of which remains unclear. It could be associated with innate differences in their antibody responses to sand fly salivary antigens or in attractiveness to sand fly bites, or with other unknown factors that need to be investigated. Interestingly, it was recently reported that Beagle dogs may exhibit markedly different attractiveness to *P. perniciosus* under experimental exposure [41].

In conclusion, the dynamics of antibody response against *P. perniciosus* salivary proteins clearly showed seasonal changes due to the expected sand fly abundance. Our study confirmed that recombinant yellow-related protein rSP03B of *P. perniciosus* is a valid alternative to whole sand fly saliva as marker of sand fly exposure. Serology tests based on this recombinant protein could be a practical and economically-sound tool for investigations dog exposure to sand flies in endemic settings of zoonotic visceral leishmaniasis.

Supporting Information

S1 Table. Estimates of the multilevel linear regression model of the relationship between log transformed rSP03B+rSP01 OD values (multiplied by 100) and sampling time (model a), and *Leishmania* status and sampling time (model b).
(DOCX)

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Author Contributions

Conceived and designed the experiments: PV IR LG MG. Performed the experiments: GO AS LG MG EF TK TL PS MV. Analyzed the data: TK EB. Contributed reagents/materials/analysis tools: PV LG MG. Wrote the paper: TK PV LG MG EB.

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The recombinant protein rSP03B is a valid antigen for screening dog exposure to *Phlebotomus perniciosus* across foci of canine leishmaniasis

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The recombinant protein rSP03B is a valid antigen for screening dog exposure to *Phlebotomus perniciosus* across foci of canine leishmaniasis

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Short title: Recombinant protein as exposure marker

Abstract

Frequency of sand fly-host contacts can be measured by host antibody levels against sand fly salivary proteins. Recombinant salivary proteins are suggested as valid replacement for salivary gland homogenate (SGH), however, it is necessary to prove that such antigens are recognized by antibodies against various populations of the same species. *Phlebotomus perniciosus* (Diptera: Psychodidae) is the main vector of *Leishmania infantum* (Trypanosomatida: Trypanosomatidae) in south-western Europe, being widespread from Portugal through Italy. In this study, sera were sampled from naturally exposed dogs from distant regions, Campania (south Italy), Umbria

(central Italy) and Metropolitan Lisbon region (Portugal), where *P. perniciosus* is the unique or principal vector species. Sera were screened for anti-*P. perniciosus* antibodies using SGH and 43 kDa yellow-related recombinant protein (rSP03B). Robust correlation between antibodies recognizing SGH and rSP03B was detected in all regions, suggesting substantial antigenic cross-reactivity among different *P. perniciosus* populations. No significant differences in this relationship were detected between regions. Moreover, we showed that rSP03B and the native yellow-related protein share similar antigenic epitopes, as canine IgG binding to the native protein was inhibited by pre-incubation with recombinant form. We propose rSP03B protein as universal marker of sand fly exposure throughout geographical distribution of *P. perniciosus*.

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Key words: *Leishmania infantum*, antibody response, dog, markers of exposure, Mediterranean region, salivary proteins, sand flies

Leishmaniasis is a widely distributed disease, caused by *Leishmania* protozoans and transmitted by phlebotomine sand fly vectors. During blood-feeding, sand flies inoculate saliva into the host. Bitten hosts then develop species-specific antibody response against salivary antigens that reflects the intensity of sand fly exposure and thus provides a useful marker of exposure to generate epidemiological data (Vlkova *et al.*, 2011; Martín-Martín *et al.*, 2014; Kostalova *et al.*, 2015).

Large-scale serological studies using total sand fly salivary gland homogenate (SGH) are currently impractical due to the difficulty of dissecting large numbers of sand flies to obtain sufficient amount of SGH. Another potential complication is variability in protein composition of the sand fly saliva that was found to fluctuate depending on physiological factors such as sand fly age and diet (Volf *et al.*, 2000; Prates *et al.*, 2008). Studies on Old World sand fly species also revealed a certain degree of intra- and inter-population variability in protein and mRNA levels e.g. (Rohousova *et al.*, 2012; Ramalho-Ortigão *et al.*, 2015). Therefore, salivary recombinant proteins were suggested to replace the whole salivary gland protein cocktail, and some have

already been validated in the field e.g. (Martín-Martín *et al.*, 2014; Kostalova *et al.*, 2015). The use of specific recombinant salivary antigen circumvents the necessity for laborious maintenance of sand fly colonies, and potentially refined to minimise antigenic cross-reactivity with taxonomically close sand fly relatives. A useful recombinant salivary protein would demonstrate comparable antigenicity to SGH and share similar antigenic epitopes with the native proteins, and demonstrate similar antigenic patterns throughout the geographical distribution of a particular sand fly vector.

In this study, we focused on *Phlebotomus perniciosus,* a sand fly species widely distributed in south-western Europe where it acts as a proven vector of *Leishmania infantum*, the causative agent of canine leishmaniasis (CanL), human visceral leishmaniasis and sporadic cutaneous leishmaniasis in Portugal, Spain, Malta, France and Italy (Maroli *et al.*, 2013). We tested (i) the levels of individual canine antigenic responses to *P. perniciosus* 43 kDa yellow-related recombinant protein (rSP03B) compared to *P. perniciosus* SGH; and (ii) the degree of similarity in these antigenic associations, across endemic canine populations, in Portuguese and Italian foci where *P. pernicious* is the unique or principal phlebotomine vector, in order to assess its universal use as a marker of natural sand fly exposure. Additionally, we studied the antigenic similarity of rSP03B to its native form.

Canine sera originated from three regions: (i) Campania (n=118), a traditional high risk area for CanL in southern continental Italy (Oliva *et al.*, 2006); (ii) Umbria (n=96), an inland area of central Italy recently recorded as medium-high risk area of CanL (Di Muccio *et al.*, 2012); and (iii) the Metropolitan Lisbon region (n=341), which is a well-known CanL endemic locality in west-coast Portugal (Cortes *et al.*, 2012). In all three areas, *P. perniciosus* is the unique or principal vector of CanL. Single sera samples from Campania and Umbria were purposely selected from archived samples collected in 2007-2013 to represent the period from July (i.e. at least two months after the beginning of the sand fly season) through to October (i.e. the end of the sand fly season).Single sera samples from Metropolitan Lisbon region were randomly collected from dogs at the beginning of sand fly season in May 2012.

Samples from Campania consisted of stored sera sent by veterinary clinics to Istituto Superiore di Sanità for routine serological diagnosis of suspected CanL in owned dogs. Sera from Umbria were collected from healthy dogs that were enrolled on a voluntary basis in the Perugia University CanL surveillance program. Blood sampling were performed in accordance with the

Italian guidelines for animal welfare, following owners' consent, and did not include additional or unnecessary invasive procedures. Collection of sera in Metropolitan Lisbon region was ethically approved by the board of the Institute of Hygiene and Tropical Medicine (IHMTUNL) (authorization no.8 2011- PI) complying with the Portuguese legislation for the protection of animals (Law 113/2013).

Detection of anti-*Leishmania* IgG in canine sera from Campania and Umbria was performed by an in-house IFAT assay using *L. infantum* promastigotes as antigen, as described in Gradoni & Gramiccia (2008). Samples showing an IFAT titre equal or above 1:40 were considered as indicative of exposure to *Leishmania*. Detection of IgG antibodies against *Leishmania* in canine sera from the Metropolitan Lisbon region was performed by enzyme-linked immunosorbent assay (ELISA) kit (Bordier Affinity Products SA, Switzerland) according to manufacturer guidelines (Maia *et al.*, 2010). The result was considered positive when the absorbance of the analyzed sample was higher than the absorbance of the weak positive control serum provided with the kit.

Long term established laboratory colony of *P. perniciosus* originating from Spain (Murcia) was reared under standard conditions as described in Volf & Volfova (2011). Salivary glands and rSP03B from *P. perniciosus* (Genbank accn. DQ 150622) were obtained for this study as previously described (Kostalova *et al.*, 2015) and used as antigens for testing the canine sera.

Antibodies against *P. perniciosus* SGH and rSP03B protein were measured by ELISA as described by Kostalova, *et al.*(2015). Each serum was tested in duplicate. Test absorbance values were reported as optical densities with subtracted blanks (the ELISA plate background mean absorbance value measured in control wells).

Western blot analysis was used to confirm the similarity of antigenic epitopes between the native yellow-related protein found in *P. perniciosus* SGH and the corresponding recombinant protein rSP03B. SDS-PAGE of SGH (equivalent of 4 μg total salivary proteins per lane) and rSP03B (1 μg per lane) was run on a 12% gel and blotted onto the nitrocellulose membrane using the iBLOT instrument (Invitrogen). Membrane with separated proteins was cut into strips and blocked in 5% milk diluted in Tris-buffered saline with 0.05% Tween 20 (Tris-Tw) overnight at 4°C. For the inhibition test, three Italian canine sera possessing high levels of anti-*P. perniciosus* IgG against SGH and rSP03B, were pooled. The positive serum pool was diluted 1:50 in Tris-Tw and split into halves. The first half was incubated for 2 hours on a shaker with rSP03B (10

µg/ml), the latter half was incubated without rSP03B. Negative control sera (canine sera from non-endemic locality) were diluted 1:50 in Tris-Tw and incubated without rSP03B on a shaker for 2 hours. In the next step, a part of the positive serum pool, either incubated with or without rSP03B protein, and part of the negative control sera, were incubated with strips of separated *P. perniciosus* SGH. The same procedure was repeated for strips containing rSP03B, except that sera were diluted 1:100 in Tris-Tw. After 1 hour, all strips were rinsed in Tris-Tw and subsequently incubated for 1 hour with peroxidase-conjugated anti-dog IgG (1:3000, Bethyl laboratories). The colour reaction was developed by substrate solution containing 3, 3'-diaminobenzidine (Sigma).

Statistical analyses were carried out using R software (http://cran.r-project.org/) and Stata v. 13. 1 software (Stata Corporation, College Station, Texas, USA). Correlations were analysed using Spearman rank correlation test and medians compared between groups using a Wilcoxon rank sum test. In Figure 1 the OD values were logarithmised (natural logarithm) for better readability. Statistical analyses of the relationships between SGH and rSP03B OD values between the canine populations were statistically tested by fitting general linearised (GLM) Poisson models with a ln link function, having shown that the right skewed frequency distributions did not follow a negative binomial distribution (Deviance goodness-of-fit chi2>56.2; P=1, D.F=549, for each antibody). The full GLM Poisson models included interaction terms to test differences between the regions, both in terms of baseline anti-rSP03B value (intercept where anti-SGH equals 0) and the relationship between antibodies against SGH and rSP03B (slopes). Statistical significance was considered when the P-value was below 0.05.

We tested the use of *P. perniciosus* rSP03B as an epidemiological tool for investigations of canine exposure to sand fly bites in geographically distinct localities where *P. perniciosus* is the prevalent phlebotomine vector. The recombinant protein rSP03B used in this study, was obtained from the salivary glands of *P. perniciosus* laboratory-reared colony originated from Murcia in Spain and was used as antigen for serology of dogs living in Campania and Umbria regions in Italy and in Metropolitan Lisbon region in Portugal.

Levels of canine IgG antibodies reacting with SGH and rSP03B were measured by ELISA. We observed positive but variable correlations between antibody responses to SGH and rSP03B antigens in all three localities (Campania: r=0.73, 95% CI: 0.62-0.82, P<0.001; Umbria: r=0.56, 95% CI: 0.38-0.71, P<0.001; Metropolitan Lisbon region: r=0.81, 95% CI: 0.76-0.84,

P<0.001) (Figure 1). Table 1 summarises the OD values for each region indicating that the OD frequency distributions were over-dispersed. To query possible differences in the relationships between SGH and rSP03B antibody responses between geographical regions, we tested the equality of the population specific regression slopes by fitting a Poisson model. No significant differences were detected (population*antigen interaction terms: z>-0.85, P>0.365). Relative to the Metropolitan Lisbon region, both Campania and Umbria populations tended to produce higher baseline antibody responses against rSP03B, though this failed to reach significance at the 5% level (Campania: z=1.66, P=0.097; Umbria: z=1.95, P=0.051). One plausible explanation for the putative difference in baseline rSP03B antibody levels between populations is that the populations differ in their condition or past history of infections affecting their general immunological responses to certain antigens, and/or the sand fly biting pressure in these populations differed. Age is a frequent covariate of cumulative exposure used to model cross-sectional age-prevalence data of *Leishmania* infection (Courtenay *et al.*, 1994), where the average older dog is expected to experience more sand fly seasons. Canine age was not collected systematically in this study hence we could not adjust the analyses by this likely confounder. Despite this short-coming, the results nonetheless indicate substantial salivary antigen cross-reactivity amongst *P. perniciosus* populations from Campania, Umbria and the Metropolitan Lisbon region. The strong antigenic cross-reactivity between populations of the same sand fly species was similarly observed between two geographically distant colonies of *Phlebotomus orientalis* in Ethiopia (Vlkova *et al.*, 2014), and among colonies of *Phlebotomus sergenti* originating from Israel and Turkey (Rohousova *et al.*, 2012).

The similarity of antigenic epitopes between native yellow-related proteins in Spanish *P. perniciosus* SGH and rSP03B was demonstrated by an inhibition test (Figure 2). For this analysis, sera of dogs from Campania and Umbria with high levels of specific antibodies were selected and pooled. The inhibition test showed that all IgG antibodies specific for the native yellow-related protein bind to the recombinant form during pre-incubation of the sera, which resulted in complete disappearance of the corresponding band on western blot (Figure 2). Thus, we demonstrated that rSP03B shares antigenic epitopes with the native yellow-related proteins contained within *P. perniciosus* saliva and we assume that it identifies proportion of bitten dogs in a similar way as using SGH.

Italy and Portugal are generally assumed to be under CanL endemic transmission (Oliva *et al.*, 2006; Cortes *et al.*, 2012; Di Muccio *et al.*, 2012). In this study, CanL seropositivity ranged from 5% to 30%, with the lowest prevalence in Umbria and the highest in Campania (Table 2). The use of antibodies against sand fly salivary proteins as risk markers of *L. infantum* infection was already proposed not only for SGH (Vlkova *et al.*, 2011) but also for salivary recombinant proteins, among which rSP03B proved to be a powerful marker (Kostalova *et al.*, 2015). Therefore, we analysed the relationship between anti-*P. perniciosus* antibodies and *Leishmania* serological status. Using rSP03B antigen, significantly higher levels of specific IgG in *Leishmania* seropositive dogs (median [interquartile range]=0.346 [0.257-0.536]) than in *Leishmania* seronegative dogs (median [interquartile range]=0.32 [0.229-0.422]) was found only in Metropolitan Lisbon region (Wilcoxon rank sum test, W=5391.5, P=0.025). In Campania, the difference in antibodies against rSP03B between *Leishmania* seropositive (median [interquartile range]=0.457[0.357-0.55]) and *Leishmania* seronegative (median [interquartile range]=0.379[0.303-0.499]) dogs were marginally significant (Wilcoxon rank sum test ,W=1123.5, P=0.053). Previous studies of the relationship between anti-*P. perniciosus* antibodies and seropositivity to *L. infantum* show variable correlations. In Kostalova *et al.* (2015), a positive association was observed between levels of canine IgG antibodies against sand fly saliva and active CanL infection in dogs sampled longitudinally over two years. In contrast, the study by Vlkova *et al.* (2011) described a negative correlation between levels of specific IgG2 and the risk of *Leishmania* infection. Comparisons between studies are difficult following observations that anti-saliva antibodies wax and wane with sand fly exposure and seasonality (Kostalova *et al.*, 2015), whereas, at least in dogs actively infected, anti-*Leishmania* antibodies tend to persist after an initial increase whereas in exposed resistant animals they tend to fluctuate or convert to negative (Oliva *et al.*, 2006). In the current study, the canine samples were collected at variable periods relative to the sand fly season, hence we could not control for this potential confounder in our analyses. As studies tend to be cross-sectional, and use different approaches to determine the *Leishmania* infection status, makes cross-study comparisons difficult. Certainly, longitudinal studies demonstrate the potential usefulness of sand fly saliva antigenic responses in dogs as a marker for *Leishmania* infection (Kostalova *et al.*, 2015; Quinnell, 2016, personal communication). The possibility of using sand fly salivary recombinant proteins in a similar way in cross-sectional surveys still needs to be validated.

In conclusion, this study showed that *P. perniciosus* rSP03B, the 43 kDa yellow-related recombinant protein, possesses the same antigenic epitopes as its native form in salivary glands, and that it binds similarly in canine sera from Italy and Portugal foci. Therefore it could serve as a universal marker of sand fly exposurein dogs across the *P. perniciosus* geographical distribution.

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284

285 **Table 1.** Summary statistics of OD values recorded by ELISA using *Phlebotomus perniciosus*
286 salivary antigens

Antigen	Region	N	Median (IQR)	Min-Max
SGH	Campania*****	118	0.131 (0.073-0.241)	0.011-0.1.899
	Umbria******	96	0.218 (0.133-0.409)	0.005-1.652
	Lisbon*******	341	0.221(0.165-0.311)	0.081-1.39
rSP03B	Campania*****	118	0.407 (0.311-0.516)	0.091-1.761
	Umbria******	96	0.495 (0.386-0.649)	0.026-1.925
	Lisbon*******	341	0.323 (0.234-0.436)	0.092-1.766

287 N=number of dogs
288 IQR=interquartile range
289 *****Southern Italy
290 ******Central Italy
291 *******Metropolitan Lisbon region (Portugal)
292

293 **Table 2.** Proportion of *Leishmania* seropositive and seronegative dogs from different regions

		anti-*L. infantum* IgG positive / total animals sampled (%)		
Diagnostic method	Serological status*****	Campania	Umbria	Lisbon
IFAT	Positive	35/118 (30%)	5/96 (5%)	-
	Negative	83/118 (70%)	91/96 (95%)	-
ELISA	Positive	-	-	46/341 (13%)
	Negative	-	-	295/341 (87%)

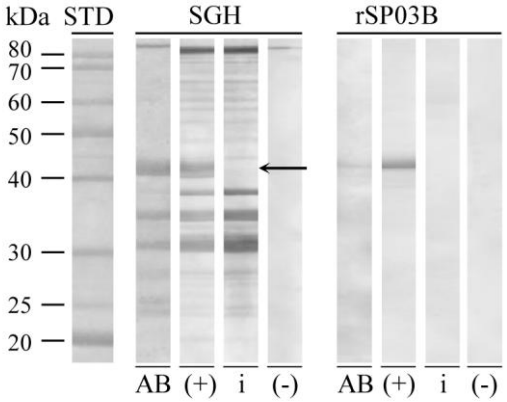
294 *****As determined by the IFAT titre or the ELISA cut-off

295

Figure legends

Fig. 1. Correlation between antibodies recognizing SGH and rSP03B in naturally bitten dogs from different localities of *P. perniciosus* occurrence. Correlation between SGH and rSP03B in dogs from Campania (A), Umbria (B) and Metropolitan Lisbon region (C) was performed by Spearman rank correlation. r=correlation index, CI=confidence interval.

Fig. 2. Western blot analysis of SGH and rSP03B and inhibition test. A mixture of canine sera positive to *P. perniciosus* SGH was pre-incubated with rSP03B and then tested in Western blot against SGH. The arrow indicates the emplacement of rSP03B. STD=standard, AB=strip stained by amidoblack, i=inhibition strip, (+)=positive control strip, (-)=negative control strip.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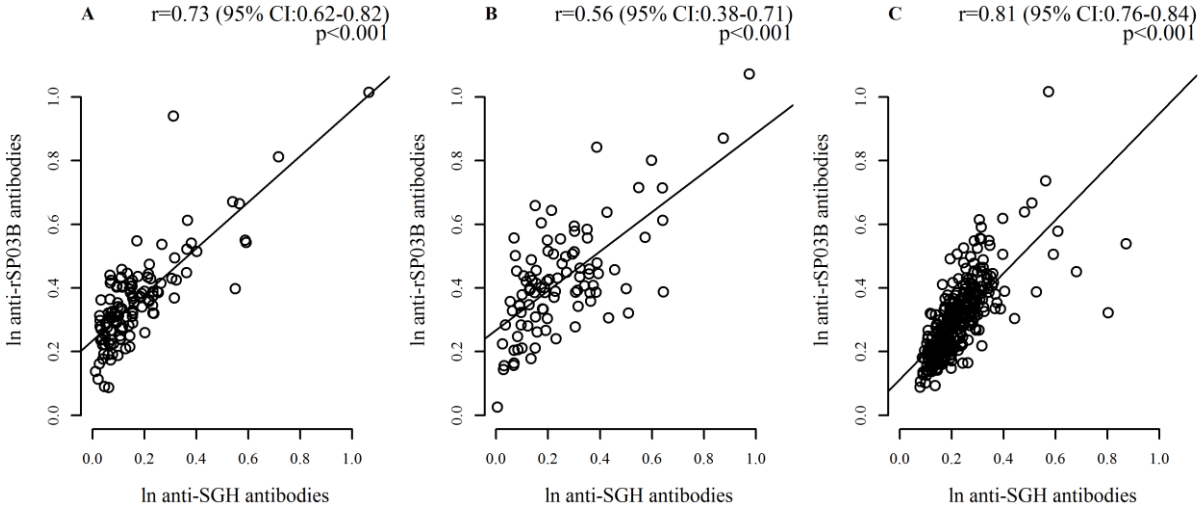
A
r=0.73 (95% CI:0.62-0.82)
p<0.001
B
r=0.56 (95% CI:0.38-0.71)
p<0.001
C
r=0.81 (95% CI:0.76-0.84)
p<0.001
ln anti-rSP03B antibodies
ln anti-SGH antibodies
0.0
0.2
0.4
0.6
0.8
1.0

SUMMARY AND CONCLUSIONS

This Ph.D. thesis constitutes of the results from three projects. The results of these projects emphasized the role of wild and domestic animals in transmission of VL and use of host antibody reaction against sand fly salivary proteins as a useful marker of exposition in endemic localities.

Leishmania donovani is the causative agent of VL in Ethiopia (Zackay *et al.*, 2013). The transmission is generally assumed to be anthroponotic, however zoonotic transmission is also possible (reviewed in Gadisa *et al.*, 2015).

In order to determine the role of wild animals in VL cycle we analyzed spleen samples from rodents and bats from both endemic and non-endemic Ethiopian VL localities. A total of 586 rodents belonging to 17 genera and 34 species were caught in 8 geographical regions throughout a period of 4 years. *Leishmania* DNA was detected in 8.5% (50/586) of spleens of analyzed rodents, the highest positivity rate was observed for genus *Aethomys* (40%; 4/10), *Arvicanthis* (17.4%; 8/46) and *Mastomys* (15.3%; 18/117). Only in 20% (10/50) of *Leishmania* spp.-positive rodents was possible subsequently identified infection by *Le. donovani* complex and by *Leishmania tropica*. Infections by *Le. donovani* complex were found in one species of *Arvicanthis* sp. and *Gerbilliscus nigricaudus* (black-tailed gerbil) and in three species of *Mastomys erythroleucus* (Guinea multimammate mouse), all these species were captured in the localities of southwestern Ethiopia (Southern Nations, Nationalities and Peoples´ Region). The southwestern lowland focus belongs to one of the stable VL areas (Fuller *et al.*, 1979; Ayele & Ali, 1984; Ali & Ashford 1994) with incriminated sand fly vectors *P. martinii*, *P. celiae* and *P. orientalis* (Balkew *et al.*, 1999; Hailu *et al.*, 1995; Gebre-Michael & Lane, 1996). Only one *Leishmania* spp.-positive rodent was found in the north Ethiopia (Tigray region). This locality is considered as new VL focus (Abbasi *et al.*, 2013).

The low *Le. donovani*-positivity in genus *Arvicanthis* is in accordance with previous studies from Sudan, where only small proportion of tested animals was *Le. donovani* PCR-positive or seropositive (Mukhtar *et al.*, 2000; Elnaiem *et al.*, 2001). On the top of that, (Hassan *et al.*, 2009) reported low attractivity of *A. niloticus* as a blood source for *P. orientalis*. Authors suggested that rodent species *A. niloticus* serves probably as accidental hosts, whereas Egyptian mongoose represents more suitable *Le. donovani* reservoir host (Elnaiem *et al.*, 2001; Hassan *et al.*, 2009). This finding corresponds with

our detection of *Le. donovani* complex infection in white-tailed mongoose from southwestern Ethiopia (*Ichneumia albicauda*).

Bats were suggested as *Leishmania* reservoirs due the fact, that sand flies breeding sites are frequently found in caves inhabited by bats (Feliciangeli, 2004). The ability of bats to serve as important source of blood meal for sand flies was proven in laboratory feeding experiments on *L. longipalpis* (Lampo *et al.*, 2000). In our study, a total of 163 bats, belonging to 25 species of 18 genera were collected in west, southwest, south and central-east Ethiopia. The dominant species were *Pipistrellus hesperidus* (dusky pipistrelle) (18.4%; 30/163), *Miniopterus africanus* (African-long-fingered bat) (11%; 18/163) and *Scotoecus hirundo* (dark-winged lesser house bat) (11%; 18/163). The prevalence of *Leishmania* sp. PCR-positive spleen samples was 4.9% (8/163). Subsequent sequencing revealed infection of *Le. tropica* in one species of *Cardioderma cor* (heart-nosed bat) and *Leishmania major* infection in one species of *Nycteris hispida* (hairy slit-faced bat). These findings represent a first report of natural *Leishmania* infection in bats in the Old World.

Previous studies from Spain (Millán *et al.*, 2014b) and Kenya (Mutinga, 1975) did not report any *Leishmania* spp. infection in bats. Study from Egypt reported detection of *Leishmania* antibodies in few bats, however, the used technique had a speculative sensitivity (Morsy *et al.*, 1987). The absence of *Le. donovani* infection in bats from Old World is in contrast with findings from New World. Studies from Venezuela and Brazil reported *Le. chagasi* infection rate to be from 0.46% up to 17.4% (Savani *et al.*, 2010; De Lima *et al.*, 2008; de Oliveira *et al.*, 2015). In CL endemic localities in Mexico and Brazil bats were found to be infected by *Leishmania mexicana* and *Leishmania braziliensis,* respectively (Shapiro *et al.*, 2013; Berzunza *et al.*, 2015). On the other hand, no *Leishmania*-positive bats were detected in CL endemic focus in French Guiana (Rotureaur *et al.*, 2006).

The main causative agent of CL in Ethiopia is zoonotic species *Leishmania aethiopica*, classical parasite of the *Hyracoidea* (reviewed in Maroli *et al.*, 2013); infections due to *Le. tropica* and *Le. major* are believed to be less common (Hailu *et al.*, 2006; Abbasi *et al.*, 2013). *Leishmania tropica* has predominantly anthroponotic transmission pattern, however, the zoonotic cycle has been indicated in some endemic localities (Svobodova *et al.*, 2006). In our study, *Le. tropica*-positive rodents were caught in the southern Ethiopia (Southern Nations, Nationalities and Peoples´ Region and Oromia region) and in Awash-Metahara in the central-east Ethiopia (Oromia region), where *Le. tropica*-positive bat was also trapped. Previous investigations in Awash valley

area reported *Le. tropica* infection in human and also in sand flies (*P. sergenti*, *Phlebotomus seavus*) (Gebre-Michael *et al.*, 2004; Hailu *et al.*, 2006), however, no *Le. tropica* infections were reported from south Ethiopia. The single bat infected with *Le. major*, the zoonotic agent of CL, was trapped in Awash valley. This is the first *Le. major* infection in central east region, albeit the presence of vector *Phlebotomus duboscqi* was already report in this area (Gebre-Michael *et al.*, 2004).

Our results suggest that wild rodents and bats could play a certain epidemiological role in transmission cycle of visceral and cutaneous leishmaniasis in Ethiopia. Moreover, we reported first natural *Leishmania* spp. infections in bats from the Old World. In order to asses more knowledge there is need for further studies with special attention on xenodiagnosis and feeding habits of sand flies to prove epidemiological role of wild animals in VL cycle.

The second project of my Ph.D. thesis was focused on evaluation of parameters associated with the ability of a domestic animal (cow, dog, donkey, goat, sheep) from VL endemic locality in the northern (Tigray region) and northwestern Ethiopia (Amhara region) to be a host for *Le. donovani*. One of the possibilities how to find out if animal is involved in the transmission cycle is to measure its attractivity to sand fly vector. The host antibody response against injected salivary proteins was already used as epidemiological marker of exposure to sand fly vectors of *Le. infantum* in Spain (Martin-Martin *et al.*, 2014), Italy (Vlkova *et al.*, 2011) and Brazil (Gomes *et al.*, 2007) and *Le. donovani* in India (Clements *et al.*, 2010). In northern parts of Ethiopia *P. orientalis* was implicated as vector of *Le. donovani* (Seblova *et al.*, 2013; Gebresilassie *et al.*, 2015b), however, there is still a lot of ambiguities about the behaviour of this sand fly vector. Therefore we decided to measure levels of antibody response of domestic animal to *P. orientalis* and evaluated the attractivity of animals to female sand flies in endemic localities.

The total of 546 sera was collected from two localities in the northwestern Ethiopia, lowland area of Humera and highland area of Addis Zemen, and one locality in the northern Ethiopia, the Sheraro focus. The overall seroprevalence in Ethiopian domestic animals was 23.1% (126/546). The highest seropositivity was found in dogs (58.8%; 20/34), sheep (47.7%; 71/149) and donkeys (35%; 7/20). The highest seropositivity for *P. orientalis* was detected in Humera (29.3%; 82/280), in a well known active VL focus (Yared *et al.*, 2014; Lemma *et al.*, 2015). The highest levels of antibodies against saliva of *P. orientalis* in this locality were detected in donkeys (66.7%; 4/6), dogs

(62.5%; 5/8) and sheep (57.3%; 67/117). The overall seropositivity rates in Addis Zemen and Sheraro were almost similar, 16.2% (18/111) and 16.8% (26/155), respectively. In both localities, canines represented the majority of seropositive animals [57.9% (11/19) in Addis Zemen and 57.1% (4/7) in Sheraro] followed by donkeys, sheep, goats and cows.

The recent study from the northern Ethiopia of host feeding preference of *P. orientalis* females showed that percentage of attracted and engorged *P. orientalis* was the highest in donkey- and cow-baited traps (Gebresilassie *et al.*, 2015d). Direct analysis of the bloodmeal source of trapped *P. orientalis* from the same locality in the northern Ethiopia revealed predominant preference to bovines (Gebresilassie *et al.*, 2015a). In both studies canines represented one of the least attractive sources of blood (Gebresilassie *et al.*, 2015a; Gebresilassie *et al.*, 2015d). However, in dog bitted traps in the eastern Sudan, dogs were highly attractive to vectors (Hassan *et al.*, 2009). Interestingly, in our study seropositivity rates of cows were the lowest in every locality tested. This is in contrary with findings from the northern and northwestern Ethiopia, where cows belonged to ones of the most attractive animals for *P. orientalis* (Gebre-Michael *et al.*, 2010; Gebresilassie *et al.*, 2015a; Gebresilassie *et al.*, 2015d). On the other hand, recent study from Humera-Metema lowlands revealed that only 8% of blood-feed sand flies took the blood meal on cow, in the majority (36%) of blood-fed sand flies the blood was mixed origin (human and cow) (Lemma *et al.*, 2014a).

In conclusion, we found anti-*P. orientalis* antibodies in all animals species tested. Our results are in accordance with recent findings, that the feeding behaviour of *P. orientalis* is opportunistic, thus fulfilling one of the criteria for zoonotic transmission of *Le. donovani*. Even if domestic animals do not play role as reservoir host, they attract large number of blood-questing female sand flies, thus domestic animals could represent the risk factor (Custodio *et al.*, 2012; Yared *et al.*, 2014) or the protective barrier of human VL (Kolaczinski *et al.*, 2008; Nackers *et al.*, 2015).

In the third project we focused on the possibility to replace whole *P. perniciosus* saliva by individual salivary recombinant proteins. Previous studies on laboratory- or naturally-exposed animals (dogs, mice, hares and rabbits) to *P. perniciosus* bites showed high antigenicity of 43 kDa yellow-related recombinant protein (rSP03B) and 35.5 kDa recombinant apyrase (rSP01) (Drahota *et al.*, 2014; Martin-Martin *et al.*, 2014). Although these studies reported that *P. perniciosus* salivary recombinant proteins are suitable replacement for whole saliva, they were tested on small sets of samples. Our present

study is therefore the first one where we tested the dynamics and diagnostics potential of antibodies recognizing *P. perniciosus* salivary recombinant proteins in large number of canine sera. Tested dogs were naturally exposed to *P. pernicious* over two years in endemic CanL locality in south Italy (Campania region). As antigens we used SGH, rSP03B protein (GenBank ID: DQ150622) and combination of rSP03B+rSP01 (GenBank ID: DQ192490).

We observed that kinetics of anti-SGH and anti-rSP03B IgG antibodies developed in a similar pattern and were clearly seasonal, rising during summer and decreasing during winter months. This result was in accordance with the sand fly season in central and southern Italy, which usually begins in the late May and last to the late October (Rossi *et al.*, 2008). In another field trial from CanL endemic locality in south Italy (Apulia region), dog screened at the end of the transmission season had significantly higher levels of IgG antibodies against *P. perniciosus* than at the beginning of the transmission season (Vlkova *et al.*, 2011). This positive correlation between the seasonal abundance of blood feeding insect and antibodies against sand fly saliva has been previously reported in mosquitoes (Palosuo *et al.*, 1997; Fontaine *et al.*, 2011). When we compared in our longitudinal study the both transmission seasons, the antibody response against *P. perniciosus* salivary proteins was lower in first one than in second one. This could be due the fact, that dogs were exposed to the vector for the first time in July, in the middle of the transmission season, as until that time they lived in non-endemic region. In the second season, the re-exposure of dogs to *P. perniciosus* following the antigenic priming in the previous season could lead to higher antibody levels against SGH and also rSP03B protein. Similar results were obtained in mice experimentally re-exposed to *P. papatasi* (Vlkova *et al.*, 2012) and in humans naturally re-exposed to *P. argentipes* (Clements *et al.*, 2010).

The correlation we found between antibody response to SGH and rSP03B was stronger than between SGH and combination rSP03B+rSP01. Reproducibility of ELISA test was even higher for rSP03B than for SGH. These results proved that rSP03B protein showed very good results not only on small subset of samples as shown by Drahota et al. (2014) and by Martin-Martin et al. (2014) but also on samples from large scale study.

In addition, we tested the use of rSP03B as a universal marker of sand fly exposure for naturally bitten dogs from localities on boundaries of *P. perniciosus* occurrence, particularly Campania and Umbria in Italy and Metropolitan Lisbon region in Portugal. In this cross-sectional study we detected substantial antigenic cross-reactivity between populations of the same sand fly species, similarly as it was shown for whole

saliva of the two geographically distant colonies of *P. orientalis* from Ethiopia (Vlkova *et al.*, 2014) and two colonies of *P. sergenti* from Israel and Turkey (Rohousova *et al.*, 2012).

The low correlation coefficient between anti-SGH and anti-rSP03B+rSP01 antibodies, the low repeatability of ELISA test and the impaired course of anti-rSP03B+rSP01 kinetics suggest that the combination of these two recombinant proteins is not suitable for further studies as its performance is worse than an single rSP03B. In conclusion, the *P. perniciosus* salivary rSP03B protein represents a valid alternative to whole sand fly saliva as a marker of exposure to sand flies. Moreover, it is applicable in wide range of *P. perniciosus* occurrence thus 43 kDa yellow-related salivary protein could be used as suitable tool for investigations host exposure to sand flies in endemic setting.

The study area in south Italy (Campania region) is considered as traditional CanL endemic locality (Oliva *et al.*, 2006; Manzillo *et al.*, 2013). Thus, dogs from longitudinal study were also tested for *Leishmania* infection by serology, culture, and PCR. At the end of the study 13% of dogs were classified as having an active *Le. infantum* infection with positive bone-marrow PCR and lymph-node culture and with IFAT negative or positive at low titre initially, but shortly converted to elevated titre. We found strong positive association between the levels of antibodies against whole saliva and dogs with active *Le. infantum* infection status. We did not find any relationship between antibodies against rSP03B protein or its combination with rSP01 and active CanL infection. This result suggests that dogs with active *Leishmania* infetion had a greater sand fly challenge, thus had bigger chance to become infected. Alternatively, *Le. infantum* infection is associated with a mixed Th1/Th2 response (Carrillo & Moreno, 2009), thus *Leishmania*-infected dogs could develop antibody response against sand fly saliva with higher probability than *Leishmania*-negative dogs.

In the cross-sectional study from Campania and Umbria regions in Italy and from Metropolitan Lisbon region in Portugal, the dogs were screened for antibodies against *Le. infantum* by serological methods. The analysis of relationship between anti-*P. perniciosus* antibodies and *Leishmania*-seropositivity and seronegativity revealed that only in Metropolitan Lisbon region *Leishmania*-seropositive dogs had significantly higher levels of specific anti-rSP03B IgG than *Leishmania* seronegative dogs.

Up to date, there is only a single study describing the association between antibodies against *P. perniciosus* whole saliva and *Leishmania*-seropositive dogs. Dogs naturally exposed to *P. perniciosus* in CanL endemic region in south Italy (Apulia region) with positive *Leishmania* serology had significantly lower levels of IgG2 antibodies than

Leishmania seronegative dogs. This result imply that dogs with lower IgG2 levels are at higher risk of becoming *Leishmania* infected (Vlkova *et al.*, 2011).

Comparison between three mentioned studies is difficult, due the different approaches to determine the *Leishmania* infection status. However, the longitudinal study demonstrates the potential use of sand fly saliva as risk marker for *Leishmania* infection. The use of sand fly salivary proteins as *Leishmania* risk marker in cross-sectional studies needs to be further validated.

In conclusion, we showed that antibody response against sand fly saliva is useful epidemiological tool applicable in *Leishmania* endemic localities. The use of recombinant proteins enables the wider use of serological methods and it is practical and economically sound tool for investigation of host exposure. In future we plan to develop strip test with fixed recombinant proteins. This test will be suitable for field use when measuring vector-exposure in larger subset of samples. Although this test will be not as precise as ELISA, it should be able to distinguish between low- and high-exposition of host to sand flies. This test will not require any laboratory equipment and will be simple to evaluate, thus, it will simplify the whole process of samples analyzing.

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