Epidemiological Characterization and Control of Old World Cutaneous Leishmaniasis in the Kingdom of Saudi Arabia

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

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June 2015

Abstract

A better understanding of cutaneous leishmaniasis (CL) epidemiology will lead to improved intervention methods to combat CL outbreaks and prevent disease re-emergence. CL is endemic throughout the Kingdom of Saudi Arabia (KSA), and new disease control tools are needed. In this thesis, I studied various aspects essential to the design of a new CL control strategy, including parasite species identification, patient response to anti-leishmanial drug treatment, sandfly species identification, assessment of sandfly biting exposure and risk of disease transmission, implementation of an integrated control strategy, and development of a potential new CL diagnostic tool for use in CL endemic setting.

It was found that *Leishmania major* (responsible for zoonotic CL) and *Leishmania tropica* (responsible for anthroponotic CL) are the main causative agents of CL in KSA, with over 75% of all human cases caused by the former. The current national CL treatment regimen consists of application of topical clotrimazole/fucidine cream followed by 1-2 courses of intralesional sodium stibogluconate; however, treatment efficacy is highly variable and the reasons for this are not well understood. As part of this PhD study, tests to determine the efficacy of this standard CL treatment regime in several endemic regions of KSA were performed. Most *L. major* patients responded favourably to drug treatment, although treatment success varied greatly depending on geographical location. In contrast, 60% of *L. tropica* cases proved completely unresponsive to the same treatment protocol. Furthermore, the development of secondary infections (SI) around or within the CL lesion significantly favoured the treatment response of *L. major* patients, but had no effect on *L. tropica* cases. These findings indicate that there is an urgent need to implement alternative CL treatment protocols based on these parameters, and also indicate a possible increase in drug-resistant parasites.

In this epidemiological study, the potential correlation between the type of immunity generated after exposure to sandfly bites and disease outcome was monitored by measuring anti-SP32 antibody levels. Constant exposure of the local residents to sandflies may help prevent development of severe CL disease outcomes. Notably, comparison of the levels of anti-saliva antibodies of local individuals with migrant labour indicated that the latter had significantly higher levels of anti-saliva marker and also developed a more severe pathology. This implies that the corresponding governmental sectors need to assess all transmission risk areas, as well as greatly improve housing conditions to minimize the risk of non-local construction workers contracting CL. This is essential as CL drug treatment is costly and currently non-local workers comprise around one third of the KSA population.

Additionally, a disease control strategy based on a combination of vector and reservoir control methods was designed to combat zoonotic CL outbreaks. Construction sites at Al-Ahsa, known to be highly endemic for zoonotic CL, were monitored by a vector control team between 2012 and 2014. The control strategy was applied following the outbreak using mechanical, reservoir and vector control methods. No CL cases were reported after the control team's intervention. This case study suggests that implementation of a health impact assessment should be carried out, as well as a control strategy, in areas that share a similar CL ecology and environment in order to minimize incidence cases.

As part of my PhD, I developed a potential new diagnostic tool by using an ELISA assay method to measure the levels of anti-α-galactosyl antibodies in human sera using synthetic neoglycoproteins (NGPs). The assay sensitivity was 96% for *L. major* (95% CI 94%–98%) and 91% for *L. tropica* (95% CI 86%–98%). In addition, the assay had higher sensitivity than microscopy analysis, which only detected 68% and 45% of *L. major* and *L. tropica* infections, respectively. Furthermore, improvement of the selectivity in recognizing different α-galactosylated NGPs suggests that this assay could potentially be developed into a rapid diagnostic test for use in resource-poor settings.

Overall, this thesis should help set a basis for designing a national strategy for CL elimination in KSA.

Acknowledgements

In the name of Allah, the Most Gracious, the Most Merciful, whom I thank for everything in my life. I am indebted to my supervisor Dr. Alvaro Acosta-Serrano for his patience, problem solving, work interactions, direction and guidance. He has advised me during the project, supporting and encouraging me throughout. Also, I would like to thank several other LSTM staff members: Dr. Lee Haines, Dr. Naomi Dyer, Dr. Daniela Ferreira, Dr. Louise Kelly-Hope, Dr. Deirdre Walshe, Dr. Emily Adams, Dr. Emily Adams and Miss Karina Mondragon-Shem for their assistance, patience and problem solving throughout the project. Furthermore I am grateful to Prof. Igor Almeida (UTEP, USA), Dr. Jesus Valenzuela and Dr. Maha Abdeladhim (NIH, USA), and Dr. Essam AL Yamani (KACST, Saudi Arabia) for their invaluable advice and contributions. Also, thanks to my advisory panel, Dr. Nick Beeching and Dr. Mark Paine, for their advice and guidance.

I would like to express my undying gratitude to my wife, Rabab AL-Malki, and my son, Saleh, for their patience, prayers, love and support during my field trips in Saudi Arabia and while living in the UK. My deep thanks to my mother and my mother-in-law for their prayers and for supporting me throughout my life. Also I complete this work in memory of my late father, who was a great source of strength and inspiration to me in starting my research career. I acknowledge my brother-in-law Bader for his prayers, support and encouragment.

My heartfelt appreciation goes to Dr. Turki Bin Saud Al Saud, KACST President for his fully support and encouragement. Also to Dr. Nahar Alazmi, Deputy Minister for Hospitals, Dr. Mansour Alyami, Director of Training and Scholarships Department, and Dr. Mohammed Alzahrani, Director of Saudi Vector Control. Lastly, I thank my sponsor, the Saudi Ministry of Health, for giving me the opportunity to pursue my PhD degree.

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AQP1	Aquaglyceroporin
CL-ELISA	Chemiluminescent enzyme-linked immunsorbent assay
CBAG	Coffee bean α-galactosidase
CCR-1	Chemokine receptor type 1
DDT	Dichlorodiphenyltrichloroethane
EMRO	East Mediterranean Region
FCγR	Fc region of IgG
GPI	Glycosylphosphatidylinositol
GIPLs	Glycoinositol phospholipids
IFN	Interferon
Ig	Immunoglobulin
IRS	Indoor residual spraying
IL	Interleukin
ITS-1	Ribosomal Internal Transcribed Spacer 1
IL Sb	Intralesional Sodium stibogluconate
IM Sb	Intramuscular Sodium stibogluconate
L.	*Leishmania*
LPG	Lipophosphoglycans
M.	*Meriones*
NO	Nitric Oxide
PPGs	Proteophosphoglycans
PSG	Promastigote secretory gel
PCR-RFLP	Polymerase chain reaction- Restriction fragment length polymorphism
PpSP32	Sandfly salivary protein 32
Ph.	*Phlebotomus*
P.	*Psammomys*
rPpSP32	Recombinant form of the *Ph. papatasi* PpSP32 protein
T.	*Trypanosome*

Th	T-helper
ULV	Ultra low volume

Chapter One: Literature Review

1.1 Leishmaniasis

Leishmaniasis is a vector borne disease transmitted by the Phlebotomine female sand fly and caused by parasites of the genus *Leishmania*. Leishmaniasis can be sub-divided into two clinical forms; visceral and cutaneous. The deadliest form is visceral leishmaniasis (VL), which is responsible for 500,000 new cases annually, including 80,000 fatalities (World Health Organization, 2010). 90% of VL occurs in Sudan, Brazil, India, Bangladesh and Nepal. VL can itself be sub-divided into two main forms: anthroponotic visceral leishmaniasis (AVL) which requires sand flies as a vector and humans as a reservoir; and zoonotic visceral leishmaniasis (ZVL), which also requires sand flies as a vector but uses dogs or rodents as a reservoir (although humans may also contract the disease) (Alvar et al., 2012a, World Health Organization, 2010).

The second form, cutaneous leishmaniasis (CL), results in a far greater number of cases, but these cases tend to be less serious and can even be self-healing. Between 1.5 and 2 million new cases of CL are reported annually, 90% occurring in just seven countries (albeit in quite disparate regions): Saudi Arabia, Peru, Iran, Afghanistan, Brazil, Algeria and Syria (Alvar et al., 2012a, World Health Organization, 2010).

1.1.1 The growth and global spread of Leishmaniasis

Leishmaniasis has been found in sporadic cases throughout the world where environmental conditions permit and favour its transmission. Recently, leishmaniasis vectors and reservoirs have spread to new foci throughout the world. For instance, perhaps as one possible result of global warming, new leishmaniasis foci have been found in the southern United States and in northern Mexico (Clarke et al., 2013, Wright et al., 2008). *Leishmania* has been isolated from humans, dogs, rodents and sand flies. (Wright et al., 2008, Clarke et al., 2013). As a result of urbanization, several new foci have also been reported in suburban areas in Venezuela (Aguilar et al., 1998). Soil erosion has been prevented by means of tree plantation in the Kashan region, north of Isfahan, in Iran, but this has created new foci for the *Rhombomys opimus* ZCL reservoir, resulting in a ZCL outbreak with a reported 8-15% incidence within the local population (Neouimine, 1996). *Leishmania tropica* (responsible for CL) cases have tripled in the West Bank and Northern Israel as a result of urbanization, population growth and agriculture (Jacobson et al., 2003). Furthermore, city expansion, urbanisation and agricultural projects are highly correlated with CL outbreaks in Saudi Arabia (Figure 1.1).

In more recent years, Sri Lanka has recorded more than 200 cases annually (Karunaweera and Rajapaksa, 2009) compared to a single case of cutaneous leishmaniasis reported in 1992 (Athukorale et al., 1992b). Several factors correlate with the increase of leishmaniasis cases in Sri Lanka, including population movement, jungle clearing, military activity, large families, human

behavior and low socioeconomics (Ozbel et al., 2011b). In addition, Sri Lanka's cases are correlated with the number of rainy days, style of housing, and literacy rate among males and marginal workers (Sheets et al., 2010).

Figure 1.1 Human made factors are involved in the sustainable CL occurrence in Saudi Arabia. Rubbish accumulation and agricultural projects close to the houses are giving the suitable environment for rodent and sandflies and then higher risk of the disease. Population could be at risk of infection transmission as a consequence of urbanisation and city expansion into transmission risk areas.

Civil war and military activity were the main causes of a visceral leishmaniasis outbreak in Western Upper Nile Province, Southern Sudan in the 1990s. 100,000

cases were reported among a population of just 300,000 (Elnaiem and Osman, 1998, Seaman et al., 1996). The 1990 Gulf War was the most significant factor for the Iraqi leishmaniasis outbreak in 1991. 576 VL cases and 1,799 CL cases were reported in 1990, while 3,713 VL cases and 8,233 CL cases were reported in 1991 (Neouimine, 1996). Furthermore, cutaneous leishmaniasis has become a growing public health problem as a consequence of the conflict in Arab-Spring countries including Syria, Yemen and Iraq. The lack of control measures applied in these countries will have a serious effect on the leishmaniasis situation, as well forced displacement to other countries (Jacobson, 2011, Sharara and Kanj, 2014). These issues have a huge impact on neighboring countries like Lebanon, Jordan and Turkey as a consequence of the establishment of refugee camps in these areas, which have not been previously assessed by a leishmaniasis expert team (Saroufim et al., 2014, Koltas et al., 2014).

CL outbreaks have been reported in several countries in Europe. Recently, CL was reported in the area of Fuenlabrada, close to the Spanish capital, Madrid (Aguado et al., 2013). CL has also been reported in Catania, Southern Italy, where there have been at least 10 confirmed cases (Ragusa et al., 2009). CL cases, caused by *L. tropica* and transmitted by *Ph. similis,* have been increasing recently in Greece (Ntais et al., 2013). *L. donovani* is reported as responsible for CL cases in Cyprus, with *L. tropica* as the main causative parasite for anthroponotic CL in Mediterranean basin countries. (Koliou et al., 2014, Ntais et al., 2013).

1.1.2 Control of leishmaniasis around the world

Some strategies are now widely agreed to offer dependable results in the control of leishmaniasis. Using deltamethrin impregnated dog collars has been shown to reduce sand fly bites (Gavgani et al., 2002). In addition, the setting of deltamethrin impregnated traps on house walls, inside chicken coops and close to sand fly populations has been shown to reduce the density of vectors, while indoor house sprays are very effective to combat indoor biting (Feliciangeli et al., 2003). In addition, an active survey is needed to identify human cases, and a health education campaign should be undertaken in endemic areas. A combination of these strategies should prove particularly effective and will be very important to resolve the on-going problem of ZVL. In addition, sampling should be undertaken for any hepatosplenomegaly patients or febrile patients in endemic areas, and when *Leishmania* infection is detected, treatment should be given promptly. Active case detection in endemic areas is important to identify new cases (World Health Organization, 2010).

Several interventions may be carried out to control ZCL. A combination of reservoir and vector control with activation surveillance through active and passive case detection has been shown to create a dramatic reduction of cases. Certain other measures also give promising results. For example, ecological and environmental intervention was carried out after researchers realised that chenopods are the main food of *Leishmania* reservoir *P. obesus.* Accordingly, they destroyed local chenopods and replaced them with other plants inside the

endemic area to a range of about two kilometres around the city. As a result, rodents migrated to about two kilometres outside town, with the result that the sand flies followed the rodents (Chapter 4) (World Health Organization, 2010). Ultra low volume (ULV) spraying using dichlorodiphenyltrichloroethane (DDT) gives good results to reduce the vector abundance (Killick-Kendrick, 1999). However, *Ph. papatasi,* the main vector of *L. major,* is a peridomestic nuisance. For this reason, relying on ULV fogging as the only intervention does not give good results (Ashford, 1999). Reservoir control gives better results in controlling Old world zoonotic leishmaniasis. *Rhombomys opimus* is the CL reservoir in central Asia, controlled by two strategic steps: firstly, locate the habitat by surveying from the air, and then intervention can be made by deep ploughing or by zinc phosphide poisoning, or by a combination of the two (Ashford, 1999).

On the other hand, strategies to control AVL tend to focus on the behaviour of sand flies and humans. Improvements in building quality help prevent sand fly reproduction in mud cracks, and reduce biting inside houses (Joshi et al., 2009). Additionally, impregnated materials, such as insecticide-treated bed nets (ITNs) and curtains impregnated with insecticide are possible solutions to reduce the abundance and activity of sand flies inside and around residential houses. Lambda-cyhalothrin impregnated bed nets can also reduce the activity of the sand flies (Ritmeijer et al., 2007). In addition, indoor residual spraying (IRS) has been introduced in India and this gives acceptable results since that region has similar vectors of AVL (Chowdhury et al., 2011a, Chowdhury et al., 2011b). In addition,

DDT indoor residual spraying in Bihar has been shown to be successful in controlling AVL (Ostyn et al., 2008). Pyrethrin insecticide-impregnated bed nets were introduced in Aleppo in Syria as one of the interventions to control cutaneous leishmaniasis in urban areas. The efficacy of this intervention was also proven successful (Jalouk et al., 2007, Tayeh et al., 1997). However, in Bihar, India, where the breeding season is much longer than in Aleppo, studies were carried out to measure the efficacy of long-lasting insecticidal nets, and the findings suggest that they made no significant improvement in controlling AVL in that region (Dinesh et al., 2008).

1.2 Life cycle of *Leishmania* parasite

Leishmania is transmitted between mammalian animals by sandflies as shown in figure 1.2. During the first stage, *Leishmania* is taken up by sand flies during blood feeding and then amastigotes transform to procyclic promastigotes from 6 to 12 hours, after which binary fission multiplication occurs (Gossage et al., 2003, Bates and Rogers, 2004). In the second stage, which occurs between two to five days after feeding, the peritrophic matrix ruptures, and the procyclic promastigote attaches to the microvillar lining of the gut. As a result, the procyclic promastigote avoids digestion and then moves to thoracic midgut. After that, procyclic promastigotes transform to leptomonads promastigotes, which will be embedded by a gel-like matrix, which produces by the promastigote secretory gel (PSG) (Rogers et al., 2002, Bates and Rogers, 2004, Rogers et al., 2004).

The third stage begins after blood meal digestion. During oviposition, non-dividing free metacyclic differentiates from leptomondas and then remains behind the stomodeal valve. It then swims to pharynx, cibarium and proboscis. Metacyclic promastigote remains in the proboscis until the next blood feeding when it will transmit during sand fly feeding to humans in the case of ACL or to either humans or animals in the case of ZCL (Gossage et al., 2003, Rogers et al., 2002).

Metacyclic promastigotes invade skin macrophages and skin neutrophils and transform to the amastigote form in the first infection phase (Peters and Sacks, 2009). The *Leishmania* invasion processes in humans undergoes four phases: the “silent phase”; inflammation; the onset of immunity; and finally the chronic or memory phase (von Stebut et al., 2000, Ribeiro-Gomes et al., 2012). In this section, *Leishmania major* is used as a unique parasite to explain this process. The first phase of *Leishmania* infection takes between four and five weeks and begins by deposition of promastigotes by sand fly into the wound. *Leishmania* promastigotes enter to macrophage through CR3 and Fc region of IgG (FCγR) (Polando et al., 2013). Additionally, *Leishmania* Lipophosphoglycans (LPG) work as a barrier to prevent deposition of the C5b-9 membrane-attacking complex on the *Leishmania* promastigote (Hoover et al., 1985, Von Stebut, 2007). Therefore, it is protected from complement lysis. Moreover, IL-12 inhibition occurs in *Leishmania* during this period for avoiding IFN-Ɣ activation. The *Leishmania* parasites are also engulfed by skin macrophages in the first few hours of invasion. In addition, macrophages, neutrophils and dendritic cells are recruited by

Leishmania for skin infection (Woelbing et al., 2006, von Stebut et al., 2000, Von Stebut, 2007, Soong, 2008). Following the *Leishmania* infection, neutrophils are recruited to *Leishmania* infection sites in the skin and as a consequence immune cell activation occurs (Ribeiro-Gomes and Sacks, 2012). In addition, during the first stage *Leishmania* is deposited by sand fly with a mixture of sand fly saliva, and saliva also has another reaction to immunity, as will be discussed later in detail (Rogers et al., 2004).

The second phase occurs when clinical signs in skin are visible and lesions develop and enlarge. IL-12 is inhibited as a consequence of *L. major* infection. Additionally, NO (nitric oxide) which is required for parasite killing, is inhibited by *L. major*. Moreover, infected macrophages recruit other proinflamatory cells including neutrophils, eosinophils and mast cells to the infection site and therefore is involved in granuloma formation and then minimises parasite growth (Soong, 2008, Von Stebut, 2007, von Stebut et al., 2000, Woelbing et al., 2006). *L. major* when deposited attracts PMNs (polymorphs neutrophils) as an early response, and the macrophage migrates to the skin surface to the site of infection (Teixeira et al., 2006).

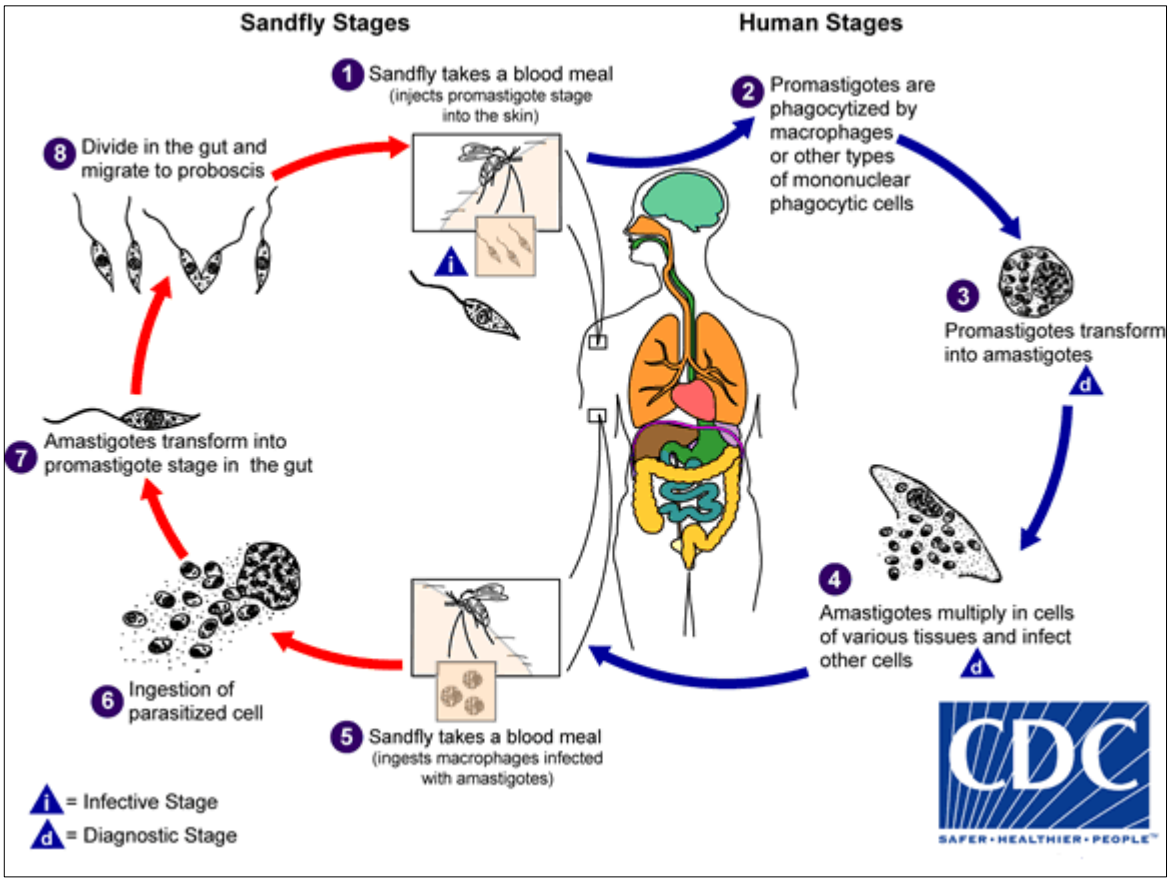


Figure 1.2 *Leishmania* life cycle in human and vertebrate host cited from (CDC website)

The third phase begins at the maximal granuloma size and dendritic cell activation when it is infected by *Leishmania major* at the site of the lesion. In addition, the activation of MC-derived cells, IgG- mediated mechanism, cytokines and chemokines occurs. Moreover, dendritic cells act to induce Th-1, Th-2 and T cytotoxic (Tc) with other interleukin family members including IL-1, IL-12, IL-23 and IL-27. In addition, KC, IL-8 and MIP-2 attract PMNs. MIP-1α, MIP-1β and CCL5 are able to attract monocyte and macrophages. CXCL10 is able to recruit NK cells and dendritic cells to the site of infection. Tc needs IL-12 for IFN-Ɣ activation and then for NO production to kill *Leishmania* organisms. Additionally, CCR1 upregulate Th2 type cytokines (Teixeira et al., 2006). Each immunity

molecule has a different reaction against *Leishmania* parasite and works as a cascade to resolve *Leishmania* infection. The last phase occurs when the lesion is healed and when adaptive immunity begins inducing immunological memory (Von Stebut, 2007, Pakpour et al., 2008).

1.3 Cutaneous leishmaniasis (CL)

Cutaneous leishmaniasis is classified according to its life cycle into two major forms including zoonotic cutaneous leishmaniasis (ZCL), which requires a mammalian reservoir, or another animal such as rodents as a host, and is transmitted by *Ph. papatasi*, *Ph. duboscqi* and other several sand flies in both the old and new worlds. Anthroponotic cutaneous leishmaniasis ACL is another form and is transmitted by *Ph. sergenti,* also requiring humans as a disease reservoir. However other mammals are another possible reservoir in the absence of humans (World Health Organization, 2010).

1.3.1 Old World CL life cycle

Leishmania requires sand flies for disease transmission from human to human in the case of ACL as described in figure 1.3. In addition to the sand fly, humans and several other animals are required to complete the life cycle of ZCL as mentioned on figure 1.3. CL is highly affected by climatic and ecological factors. For instance, relative high humidity and temperature creates conditions for swifter transmission and better feeding activities for sandfly, as mentioned in chapter 4. Moreover, the geographical factors are likely to have strong correlation for the

disease, as sand fly distribution is highly correlated with altitude and humidity levels as a consequence of water reservoir. ZCL is transmitted by *Ph. papatasi* from mammalian reservoirs *i.e Psammomys obesus*, *Meriones libycus*, *Meriones shawi*, and *Rhombomys opimus* to humans as shown in figure 1.2 (Mondragon-Shem et al., 2015, Morsy and al Seghayer, 1992, Akhavan et al., 2010, World Health Organization, 2010).

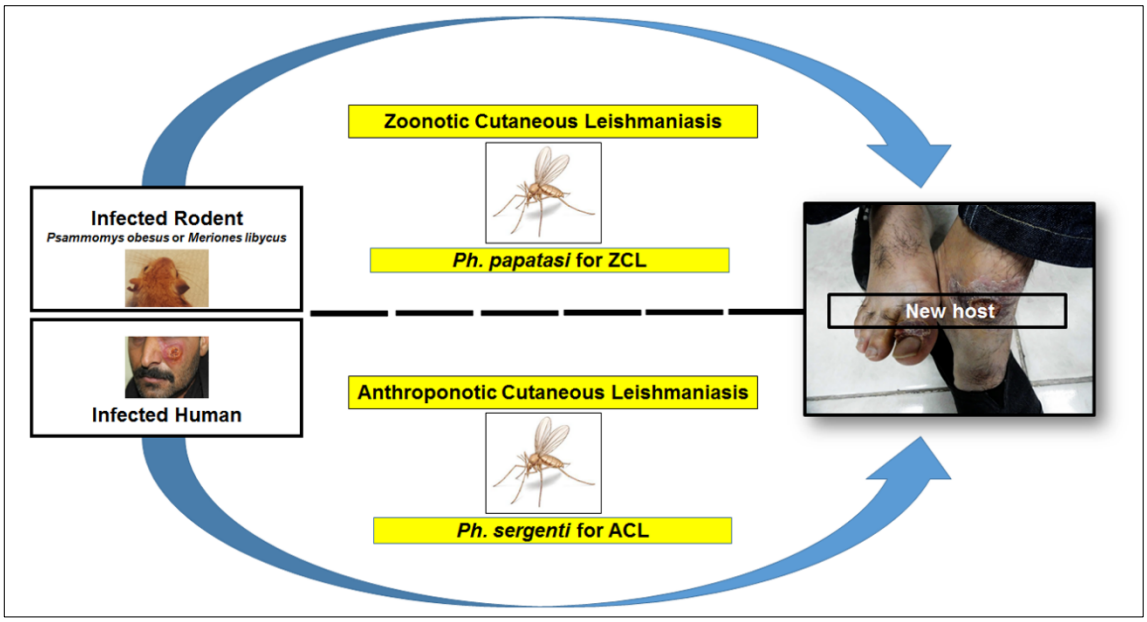


Figure 1.3 CL life cycle. Both zoonotic and anthroponotic cycle are mentioned in the figure. ZCL is required rodent as reservoir in Saudi Arabia. However, ACL require human as disease reservoir.

Furthermore, ZCL is reported in Tunisia and elsewhere as being caused by *L. infantum,* with a dog reservoir which is likely to cause visceral leishmaniasis and is transmitted by either *Ph. perfiliewi* or *Ph. langeroni* (Kallel et al., 2008, Aoun and Bouratbine, 2014b). Additionally, *L. donovani* is well known as a causative agent of visceral leishmaniasis in East Africa and also causes the cutaneous form

in Sri Lanka (Siriwardana et al., 2007). Some other sand fly vectors are reported in the Tehama region of Saudi Arabia as a potential vector for ZCL like *Ph. bergeroti* (Al-Salem, W, unpublished). Moreover, ACL is transmitted by *Ph. sergenti* from human to human, which has been reported in Saudi Arabia (Al-Zahrani et al., 1989a, Mondragon-Shem et al., 2015).

1.3.2 Old World CL distribution and CL causative parasites

Cutaneous leishmaniasis is distributed in the old world and is caused by several parasites. The most significant parasite is *L. major,* the causative agent of ZCL, which is found in Central Asia, Middle East, North and West Africa and Southeast Asia. Two other parasites are able to cause (ZCL) include *L. aethiopica* and *L. killicki*. *L. aethiopica* is reported in Kenya and Ethiopia and *L. killicki* has been identified in North Africa. In addition, anthroponotic leishmaniasis is caused by two parasites, *L*. *tropica* and *L. donovani*. Interestingly, *L. infantum* causes zoonotic cutaneous leishmaniasis in both the Old and New worlds (World Health Organization, 2010, Alvar et al., 2012b).

1.4 cutaneous leishmaniasis in Saudi Arabia

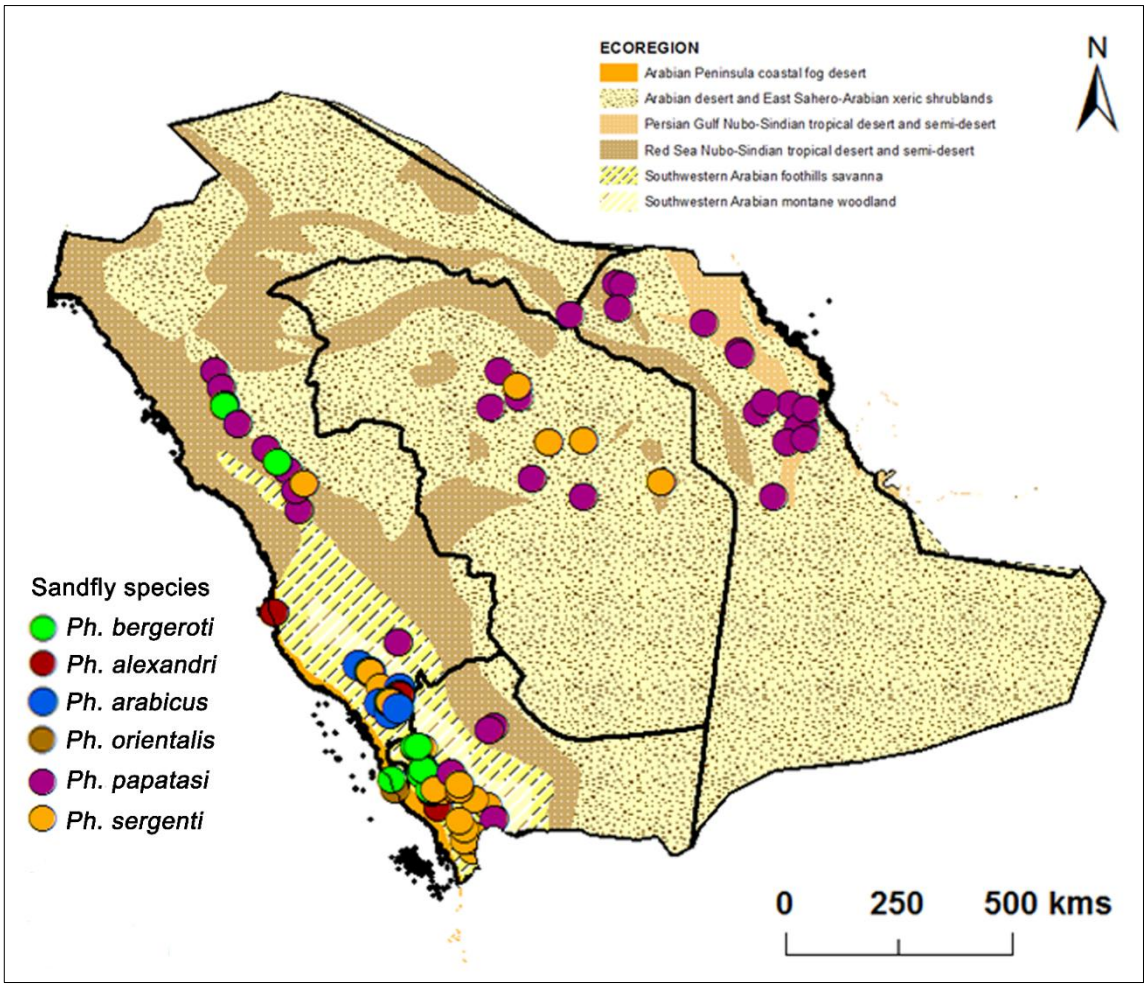


Figure 1.4 Distribution of sand flies across Saudi Arabia. Data were from this PhD project and other resources mentioned in section 1.4.

ZCL accounts for approximately 75% of leishmaniasis human CL cases in KSA (Chapter 2). ZCL is caused by *Leishmania major*, which has been reported in the provinces of Al-Qassim, Riyadh, Al-Ahsa, Hail, Almadinah Almunawarah, Taif and Tabuk (Elbihari et al., 1987, Killick-Kendrick et al., 1985, El-Badry et al., 2008, Al-Cindan et al., 1984, Al-Qurashi et al., 2000, Al-Salem et al., 2014, Büttiker and

Lewis, 1979, Dye et al., 1989, Peters et al., 1985, Khan and Zakai, 2014). The ZCL reservoir species *Meriones libycus* and *Psammomys obesus* are distributed in the Central and Northwest provinces (el-Sibae and Eesa, 1993, el Sibae et al., 1993, El-Badry et al., 2008, Morsy and Shoura, 1976, Uthman et al., 2005) and in Al-Ahsa province (Al-Mohammed, 2010, Elbihari et al., 1987).

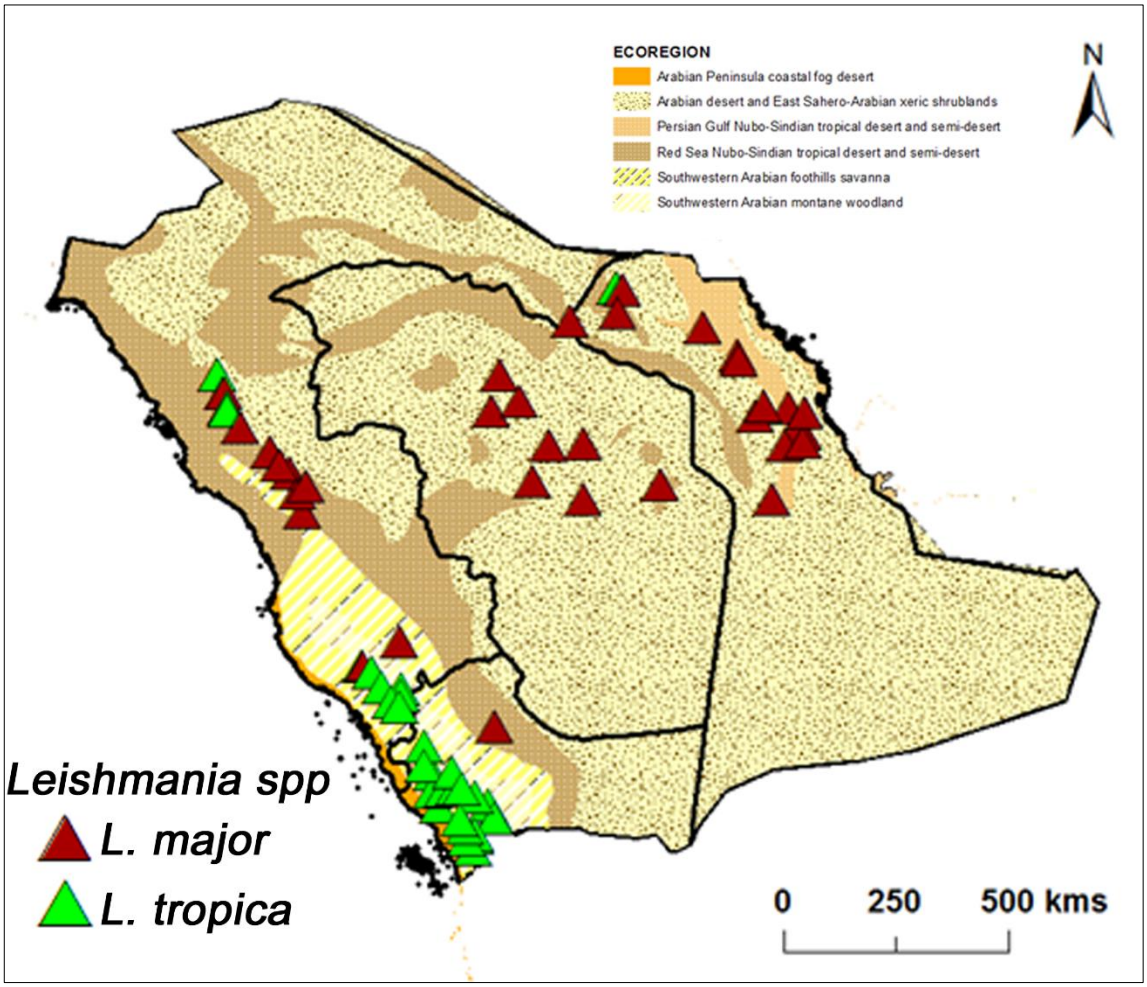


Figure 1.5 Distribution of *Leishmania spp* across Saudi Arabia. Data were from this PhD project and other resources mentioned in section 1.4.

The sandfly species *Ph. papatasi* is a vector of ZCL throughout the Central, Eastern and Northern regions of KSA, and it transmits *L. major* (Figure 1.4 and 1.5) (Morsy and al Seghayer, 1992, Elbihari et al., 1987).

ZCL control involves targeting rodents (the disease reservoir) by mechanical means, which in turn requires considerable efforts by large teams of workers. Currently, only Al-Ahsa is applying this intervention, by combining the efforts of the Ministries of Municipality, Agriculture and Health ministries, under the umbrella of the Governorate. Moreover, vector control using thermal fogging is applied at dawn and dusk in areas with a high density of sandflies in ZCL-endemic-regions (chapter 4). The tasks are shared between the three Ministries, although responsibility for each task is not always clear. Consequently, ZCL outbreaks in several regions have gone unnoticed or unreported because of a lack of monitoring when cities are expanded by the Ministry for Municipalities, or when the Ministry of Agriculture introduces a new agricultural project. Additionally, the Ministries of Agriculture and Municipalities are not legally obliged to address anthroponotic and zoonotic CL outbreaks (chapter 4). Anthroponotic CL caused by *Leishmania tropica,* is mainly found in the Western and Southwest provinces, including Jazan, Asir, Al-Baha, Al-Madinah and Taif. ACL is transmitted by *Phlebotomus sergenti* (Chapter 2,3) (Al-Zahrani et al., 1989a, al-Zahrani et al., 1989b, Morsy et al., 1992, Morsy et al., 1991). The removal of general waste and use of indoor residual spraying (IRS), specifically lambda-cyhalothrin, have been prioritised, with the aim of disrupting the anthroponotic cycle of CL in Taif and Al-

Baha. This target has been achieved by general waste elimination minimising the vector breeding sites, and as a consequence there has been a 90% reduction of incidence of reported CL cases between 1990 and 2013. These interventions were accomplished by the Health and Municipality sectors (Chapter 4).

1.4.1 CL distribution in Saudi Arabia and control

Cutaneous leishmaniasis is widespread throughout Saudi Arabia. Environmental and human made factors play a very important role in sustaining the spread of the disease. All Saudi regions have reported CL cases. Approximately 60,000 cases have been reported in the last 17 years in Saudi regions with an average of 3,300 reported cases annually. CL cases are highly correlated with climatic factors, irrigation and urbanization. Therefore, Al Qassim, Al Madinah, Al Ahsa, Hail and Asir are the most regions are affected as these regions have massive urbanization and irrigation development. With very well-designed control programmes, some regions including Al-Ahsa have succeeded in reducing CL cases to a minimum, from above 5,000 in 1987 to fewer than 200 cases in 2014.

Saudi leishmaniasis control initiatives have had a massive success in reducing cutaneous leishmaniasis cases in the endemic regions. The programme was established in 1987 in four regions – Al Ahsa, Asir, Al Madinah and Al-Qassim – and was then expanded to all other regions to include Riyadh, Hail, Tabuk, Jazan, Najran, Taif and Al-Baha. Cyhalothrin is used as IRS in all Saudi regions as well

as the application of both thermal and ULV fogging by Cyphenothrin, Deltamethrin and Cyhalothrin. However, Al Ahsa region has a very organised integrated vector control, alongside *Leishmania* clinics, health education and rodent control. They also have a good connection with other government sectors, including agriculture and the municipality of Al-Ahsa governorate.

The control programme strategy is based on an annual plan to take direct action to control cutaneous leishmaniasis. The annual plan covers four points. Firstly, active and passive case detection is carried out in the endemic areas, and in areas which have a high intensity of active rodent burrows. In addition, active rodents that live close to human settlements are monitored and their burrows are then destroyed. Following this action, the intensity of sand flies is measured and then the sand fly population is controlled. Finally, support is provided for a public health education campaign to improve general awareness of actions that need to be taken (See chapter 4).

Several risk factors are involved in the continuing persistence of the leishmaniasis problem in Saudi Arabia. Social factors include working patterns that involve sleeping rough in distant parts of large farms, or camping in the desert. Illegal immigrants who cross the borders daily become human reservoirs for *L. tropica* throughout the west and southwest regions. Moreover, 50% of the total number of volunteers in the current study are illegal immigrants, which emphasizes how

this factor has become significant. Additionally, unplanned urbanisation in areas such as Al Ahsa or Al Qassim affects people living both in the new settlements and in the surrounding areas (See chapter 4).

1.4.2 Primary health care and reporting system

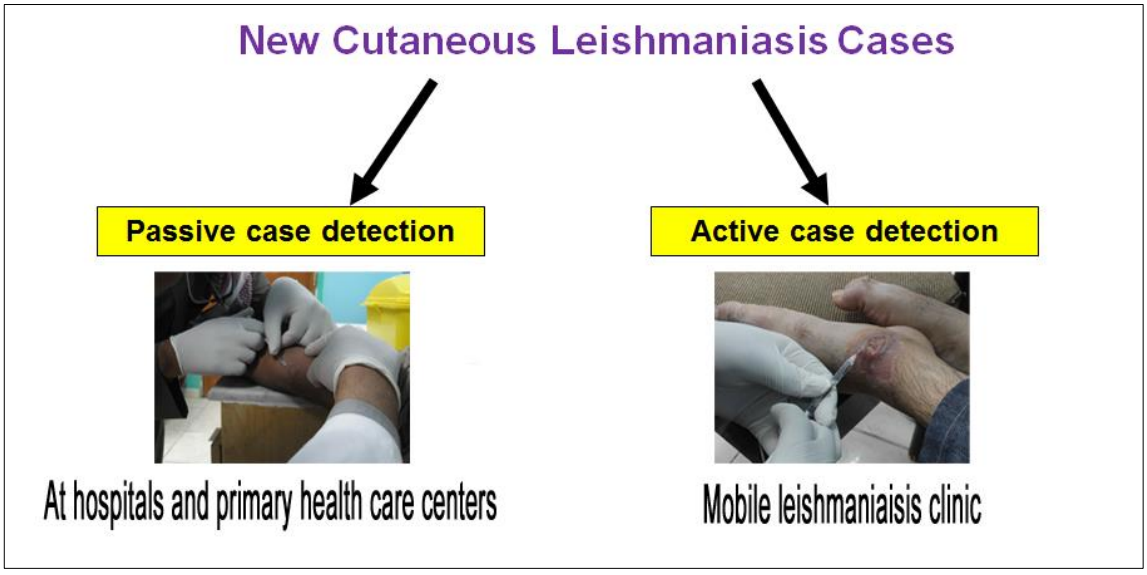


Figure 1.6 CL reporting system in Saudi Arabia. Passive case detection is exclusively performed by personnel from the Saudi MoH. Only patients that are admitted to hospitals with active CL (after assessment by a specialised CL dermatologist) are reported. However, active case detection, which request to seek for CL patients who did not attend to Leishmaniasis clinic is not activated.

The CL reporting system is in need of improvement and standardisation, as each region tends to follow its own system (Figure 1.6). In addition, in KSA CL is only diagnosed at the clinical stage, and no diagnostic methods are available to further confirm *Leishmania* infection apart from the microscopic confirmation tests which are carried out in Asir, Hail and Al Madinah. Case detection is crucial for reporting

cases for those individuals who come to the clinics. The leishmaniasis programme has improved its passive case detection since leishmaniasis clinics were established throughout the country. Each clinic reports cases to the department of vector borne diseases and the leishmaniasis programme.

Al Ahsa for example, which is also reported as a model in the control programme, has designed a referral system for all registered cases, which can be reported to one of three leishmaniasis clinics depending on the distance to the patient's home and the availability of the clinic. Reports about the cases and treatment given and its consequences are then sent to the leishmaniasis control unit. Other regions that have established leishmaniasis clinics within regional hospitals include Riyadh, Al Qassim and Al Madinah (Al-Salem, W., personal observation).

Active case detection is very important to control disease by searching for cases and then diagnosing and treating them as described on figure 1.6. Additionally, to apply this method, the programme can employ house-to-house detection by a medical team experienced in screening individuals. It is also important to establish medical camping to screen and detect individuals who are infected with leishmaniasis. Moreover, in order to enable this kind of detection, it will be necessary to develop a specific and sensitive rapid diagnostic kit.

1.4.3 Leishmaniasis diagnostic tools

Leishmaniasis diagnosis is important for developing a CL programme. As mentioned earlier, the active case detection is not possible where there is a lack of accessible and easy-to-use diagnostic tools in the field. There are different kinds of samples that can be collected from CL patients, including a skin biopsy (punch), skin scraping and skin aspiration (Saab et al., 2015, Al-Salem et al., 2014). The accuracy then depends on whether the test uses microscopy or the molecular method. The skin punch is more accurate than skin scraping compared to microscopy or PCR (Saab et al., 2015). Moreover, skin aspiration could be better than other sample collection methods if the sample is collected properly (Al-Salem et al., 2014) (See chapter 5).

Currently, cutaneous leishmaniasis is diagnosed by highly trained dermatologists. Some regions apply microscopy diagnosis as part of confirmation for clinical diagnosis. However, as mentioned in chapter 5, microscopy sensitivity is not particularly high (68% for *L. major* and 45% for *L. tropica*). Therefore, there is a crucial need to improve the diagnostic tools to assist the CL control programme. New tools have been developed using synthetic BSA with different terminal sugars to determine the antigen and antibody interaction, which is then measured by chemiluminescent assay. These tools are based on human immunity interaction with *Leishmania* parasites (see Chapter 5).

1.4.4 Leishmaniasis treatment

Leishmania parasites are treated by different leishmaniasis drugs according to the treatment response in different geographical areas. Paromomycin, sodium stibogluconate, miltefosine and meglumine antimoniate (glucantime) are all used for leishmaniasis treatment (World Health Organization, 2010). Furthermore, cutaneous leishmaniasis is treated by clotrimazole and miconazole in some regions of the world, such as in Eastern Saudi regions (Larbi et al., 1995). Paromomycin is an aminocyclitol aminoglycoside antibiotic that is used for leishmaniasis treatment. Paromomycin blocks *Leishmania* protein synthesis by binding to ribosomal RNA subunits (Maarouf et al., 1997a, Croft et al., 2006). Additionally, paromomycin has been found to act on a metabolic level upstream of the respiratory chain. Decreased drug uptake is causing drug resistance as a consequence of altered membrane composition (Maarouf et al., 1997b). Both *L. major* and *L. donovani* are developing strains resistant to sodium stibogluconate and glucantime. Aquaglyceroporin (AQP1) is responsible for the transport of antimonate Sb (III) and thus resistance can result from down regulation or abolition of the expression of AQP1 (Uzcategui et al., 2008, Gourbal et al., 2004). More recently, other *Leishmania* genes have also been associated with resistance to antimonial drugs, including heat-shock proteins, several histones and MAP kinases.

1.5 The *Leishmania* surface glycocalyx

The surface of *Leishmania* parasites is covered by stage-specific glycosylated molecules, including glycosylphosphatidylinositol (GPI)-anchored proteins,

transmembrane proteins, secreted glycoproteins and glycolipids of GPI nature. For instance, the promastigote stage expresses Leishmanolysin/gp63/PSP, PSA-2/GP40 and proteophosphoglycans (PPGs) (McConville et al., 2002) and secretes highly glycosylated proteins like acid phosphatase and PPGs, in addition to chitinases (necessary to break the sand fly peritrophic matrix). However, the most abundant molecules on the surface of promastigotes are a family of small glycoinositol phospholipids (GIPLs; also known as free GPIs) and a complex lipopolysaccharide known as lipophosphoglycan (LPG) (McConville et al., 2002). In the amastigote form LPG expression is down-regulated and GIPLs become the main surface glycoconjugate (McConville and Blackwell, 1991). All *Leishmania* surface glycosylated molecules are synthetized in the endoplasmic reticulum (ER) and then processed during their transit through the Golgi apparatus (Figure 1.7).

Regardless of when during the *Leishmania* life cycle a GPI-anchored protein is expressed, their C-terminus end is always attached to the GPI glycan via an ethanolamine phosphate group, which is linked to the terminal α-mannose residue of the conserved glycan backbone Manα1-2Manα1-6Manα1-4GlcNH. The non-acetylated glucosamine of the glycan core is in turn linked to the 6-position of the *myo*-inositol ring of the phosphatidylinositol (PI) moiety (McConville et al., 1993). As described below, only a portion of the glycan core (*i.e.* Manα1-4GlcNH$_2$) is conserved between protein GPIs and GIPLs, and only the latter appear decorated with other immunogenic sugar residues (Fig. 1.8). The best characterized *Leishmania* GPI-anchored protein is the family of GP63 or

Leishmanolysin (see 1.5.3). More details about the structure and function of the *Leishmania* glycosylated surface molecules are given below.

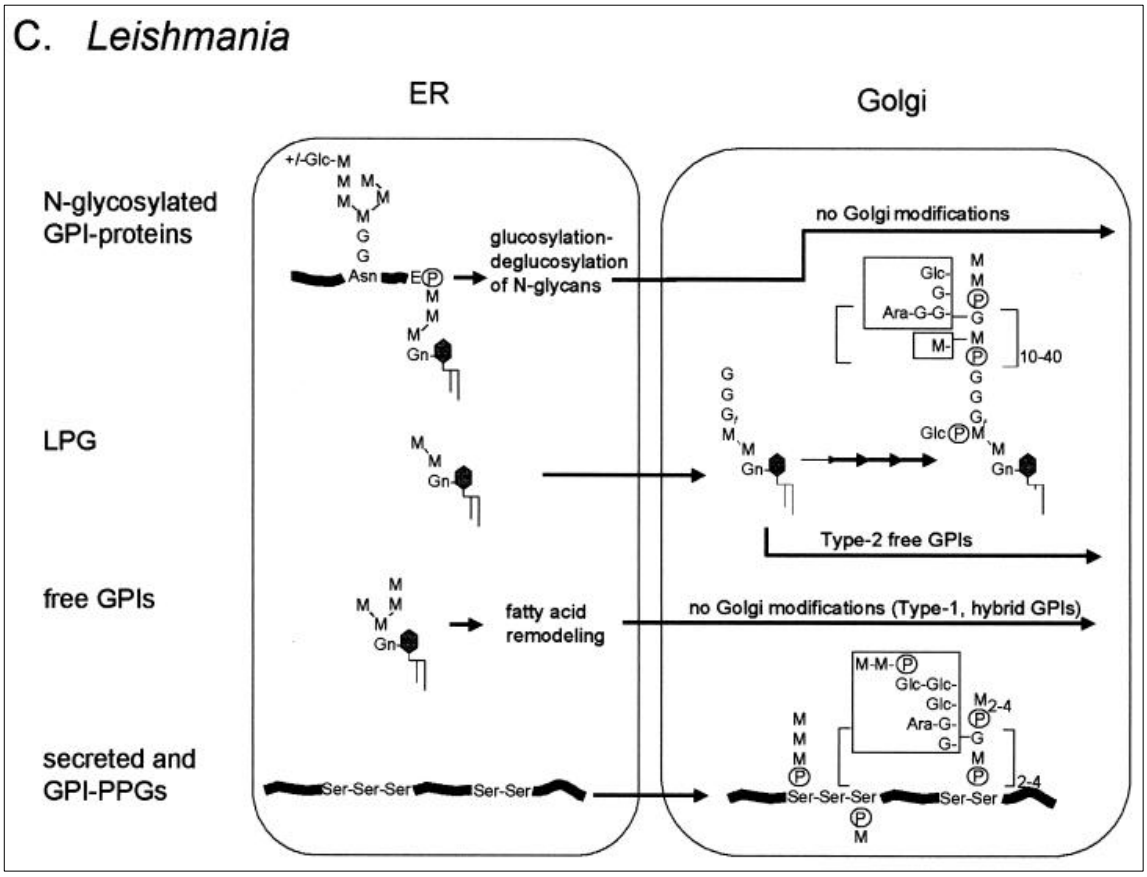


Figure 1.7. *Leishmania* secretory pathway modifications of secreted proteins, GIPLs and GPI-anchored glycoproteins (McConville et al., 2002).

1.5.1 *Leishmania* lipophosphoglycan (LPG)

LPG is a GPI-anchored polysaccharide, which represents the most abundant surface molecule (~5 x 10^6 molecules/cell) expressed by promastigote forms. LPG greatly contributes for the formation of a protective coat that allows the

parasite to survive inside the phagolysosome of infected macrophages and within the sand fly midgut (McConville et al., 1990, Pimenta et al., 1991, Yao et al., 2003). The structural overall organization of *Leishmania* LPG molecules is depicted in Figure. 1.8.

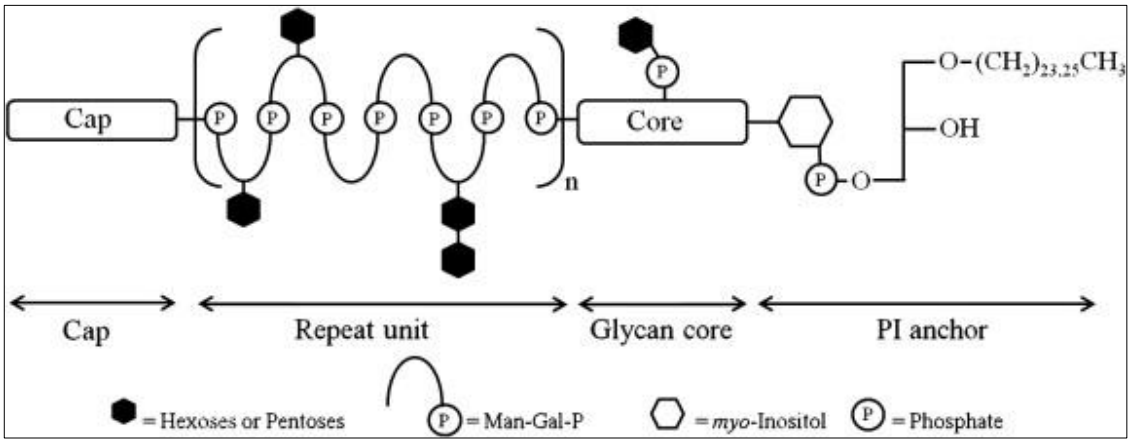


Figure 1.8. Overall structural organization of *Leishmania* LPG. LPG is organised in three distinct domains: one representing the PI moiety consisting of a *lyso*-alkyl$_{(C24:0)}$PI; a glycan core that varies in length depending on the species, but containing at least as the conserved domain Manα1-6Manα1-4GlcN-; a third domain composed of phosphosaccharide repeats (PO_4-Galβ1-4Manα1–), which are conserved in all LPG structures studied so far, but that varies in length and in sugar substitutions. The same repeat sequence can be found as part of *O*-glycans in other *Leishmania* glycoproteins like PPGs and alkaline phosphatase. A fourth (Cap) domain of several αMan residues caps the phosphosaccharide backbone. During metacyclogenesis, this cap structure grows in length and helps the parasite to survive in the mammalian host. Taken from de Assis et al., 2012

Among the LPG functions, it helps metacyclic promastigotes to resist complement lysis by preventing deposition of the C5b-9 membrane-attacking complex of the complement pathway on the parasite surface (Puentes et al., 1990, Forestier et al., 2014). Additionally, LPG has also been implicated in parasite binding and uptake by macrophages, and resistance to oxidative attack during phagocytosis (Brelaz-de-Castro et al., 2012, Chandra and Naik, 2008). Moreover, LPG

regulates Nitric Oxide (NO) synthesis within macrophages by inhibiting NO synthesis. In addition, IL-12 secretion is inhibited by LPG, which impairs development of a protective CD4+ T-cell immune response (Chandra and Naik, 2008).Therefore, the parasite is able to invade and establish an infection within macrophages (Beverley and Turco, 1998, McConville and Ferguson, 1993, McConville et al., 1993). Moreover, in the sand fly vector, *L. major* LPG is essential for transmission because of its binding to galactins expressed by the midgut epithelial cell attachments to *Ph. papatasi* (Lang et al., 1991, Sacks et al., 2000). Parasite binding to the epithelial cells allows time for differentiation into the metacyclic promastigote form and prevents excretion. However, in permissive vectors, LPG does not appear essential for binding to the sand fly gut epithelium and the parasite and sand fly molecules involved in this alternative attachment mechanism are currently unknown (Dostalova and Volf, 2012)

1.5.2 Glycoinositol phospholipids (GIPLs)

GIPLs are short GPI anchor molecules and essential components of the parasite surface glycocalyx. These molecules are synthesized in ER and can be expressed in both the promastigote and amastigote stages, although in the latter are ~10 times more abundant (ca. 10^7 copies/cell). *Leishmania* GIPLs can be classified in three different classes: Type-1, containing an ethanolamine phosphate group at the terminal mannose and involved in protein GPI precursors; Type-2, which are decorated with terminal extra sugar residues like the family of *L. major* galactosylated GIPLs; and hybrid-type, only containing mannosylated

residues (McConville et al., 1990, Novozhilova and Bovin, 2010, McConville et al., 2002) (see Figure 1.9).

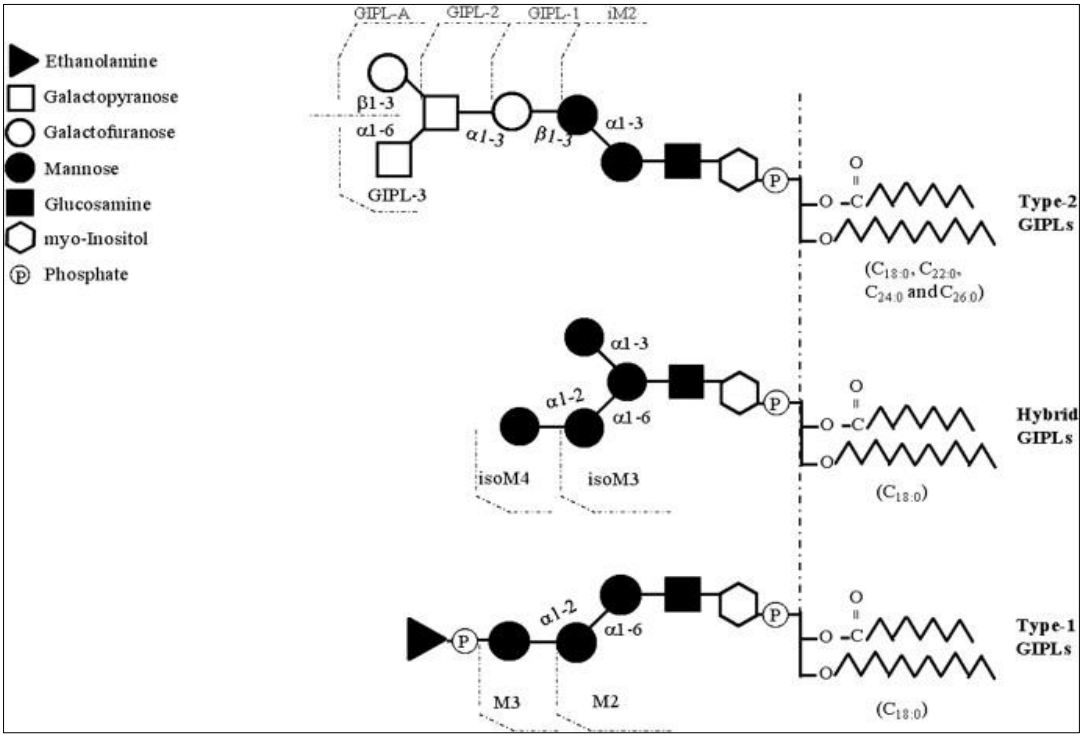


Figure 1.9. Schematic representation of the structure of GIPLs expressed by several *Leishmania* spp. Taken from de Assis et al., 2012. Only Type-2, so far only expressed by *L. major*, can contain α-galactose residues that can be immunogenic in infected humans.

The function of *Leishmania* GIPLs is not clear, but they have been implicated in macrophage invasion by *Leishmania* promastigotes (Assis et al., 2012). In *Leishmania major* promastigotes, GIPLs are involved in inhibiting the NO production and protein kinase C of infected macrophages thus inhibiting its leishmanicidal activity (de Assis et al., 2012). In addition, GIPLs are involved in the protection of amastigotes against macrophage phagolysosomes and therefore they appear important for the survival and replication of the parasite

within the infected cell (Novozhilova and Bovin, 2010). An important aspect of GIPLs is their immunogenicity in both humans (Avila et al., 1991) and animals (Buxbaum, 2013), especially those containing terminal α-galactosylated residues as in the case of some *L. major* GIPLs (McConville and Bacic, 1989).

1.5.3 *Leishmania* surface protease GP63/Leishmanolysin

GP63 is the major surface metalloprotease protease expressed in both *Leishmania* amastigote and promastigote stages. GP63 decreases expression in the amastigote form. However, GP63 is the main protein on the promastigote cell surface. Among its functions, GP63 disrupts the opsonisation component from the vertebrate complement system, hydrolyses extracellular matrix proteins, and has also been implicated in cell adhesions (Olivier et al., 2012, Isnard et al., 2012)

1.5.4 *Leishmania* proteophosphoglycans (PPG)

One of the most abundant *Leishmania* protein families, the proteophosphoglycans (PPGs), is expressed in both the amastigote and promastigote form. However, expression of the different gene products appears to be stage-specific, with aPPG, pPPG, and mPPG, being preferentially expressed in the amastigote, promastigote and metacyclic promastigote stage, respectively (Ilg, 2000). Filamentous PPG (fPPG) is secreted in both amastigotes and the insect stages, whereas SAP, aPPG and pPPG appear localized mainly

in the parasite flagellar pocket. Moreover, mPPG and other cell surface PPGs are found in the cell surface (Novozhilova and Bovin, 2010, Ilg, 2000).

In the sand fly, release of fPPG molecules has a huge impact on parasite transmission (Rogers et al., 2004). The high secretion of the promastigote secretory gel (PSG) and its accumulation around the anterior gut and mouthparts of the fly prevents the normal intake of the blood meal and results in regurgitation of both parasites and the PSG plug (Rogers et al., 2004, Rogers, 2012) . Thus, the feeding phenotype produced as a consequence of the *Leishmania* infection may lead to multiple infectious bites and so an enhancement of the infection (Rogers et al., 2009). This may be particularly relevant for the development of multiple lesions in CL patients.

1.6 Human response to Anti-α-galactosyl (anti-α-Gal) antibodies and relevance during infection by trypanosomatid parasites

Anti-α-galactosyl (anti-α-Gal) antibodies represent 1% of total human IgG (Galili, 1993). In contrast to all other mammals, humans and Old World monkeys do not express terminal, non-reducing α-Gal epitopes freely on their cells. In humans, for instance, the α-Gal epitope is expressed only in a cryptic form in the blood group B glycolipid (Galα1-3[Fucα1-2]Galβ1-4Glc-ceramide). Therefore, terminal non-cryptic α-Gal epitopes are extremely immunogenic to humans, which

produce high levels of natural anti-α-Gal antibodies directed to α-Gal-containing glycans expressed on lipopolysaccharides of Gram-negative bacteria of the gut microbiota (Galili, 1993). In infections with trypanosomatid parasites, high levels of anti-α-Gal have also been reported in both acute and chronic stages of Chagas disease (Avila et al., 1988b, Milani and Travassos, 1988, Gazzinelli et al., 1991, Almeida et al., 1991). In *T. cruzi,* the epitope is mainly expressed on *O*-linked glycans of surface mucin-like glycoproteins (Almeida et al., 1994). Detection of anti-α-Gal levels is also a biomarker for cure in drug-treated patients (Almeida et al., 1993, Andrade et al., 2004, de Andrade et al., 1996, Almeida, 2014) and has been exploited for diagnosis of Chagasic individuals in endemic (Almeida et al., 1997) and non-endemic (Izquierdo et al., 2013) settings.

Anti-α-Gal antibodies are also highly elevated in patients infected with New World *Leishmania* species (*e.g*., *L. mexicana* spp., *L. brasiliensis*) as well as in *L. donovani*-infected patients from Venezuela, (Avila et al., 1988a, Avila et al., 1988b, Avila et al., 1989). However, until now, no studies on the levels of anti-α-Gal (Leish α-Gal) antibodies have been reported in patients suffering from Old World CL, which is the subject of Chapter 5.

1.7 Aims and justification of my thesis

Few studies on the epidemiology and control of CL in KSA have been carried out so far. Furthermore, most of these studies were performed in the 1980's (by Wallace Peters and Robert Killick-Kendrick) and thus it required an update and implementation of modern approaches in order to consider CL as a realistic

elimination target. In this thesis, I characterized both the main parasite (Chapter 2) and sand fly species (Chapter 3) responsible for CL in KSA for a better epidemiological understanding and control planning. The design of new control measures to combat the disease (Chapter 4) was also carried out by strengthening the health system and introducing integrated control measures. Moreover, a potential new diagnostic tool using synthetic alpha-galactosylated glycoproteins that mimic natural immunogenic *Leishmania* surface glycans, was successfully tested as an approach to overcome misdiagnosis with other skin diseases (Chapter 5).

The specific aims of this thesis were to:

1. Update on the CL epidemiology in endemic provinces of KSA, including parasite identification and understanding of clinical features.
2. Re-assess the efficacy of current anti-leishmanial treatment regimes, including understanding the factors influencing a differential response.
3. Determine disease exposure and risk of CL transmission using a specific sand fly saliva marker.
4. Design and test an integrated disease control approach to overcome CL outbreaks in highly endemic regions.
5. Study the basis of the elevated immune response against parasite α-galactosyl epitopes in CL patients and to determine its exploitability to develop novel CL diagnostic tools.

Chapter Two: Molecular epidemiology of cutaneous leishmaniasis in the Kingdom of Saudi Arabia and treatment response

2.1 Background

Old World cutaneous leishmaniasis (CL) is highly endemic throughout the East Mediterranean Region (EMRO) (Postigo, 2010). In 2008, over 100,000 human CL cases were reported in 12 EMRO countries alone (Postigo, 2010, Alvar et al., 2012a). Various factors have contributed to CL spread in this region, including uncontrollable urbanization, irrigation, governmental sector integration, socio-economic factors and lack of health education. War is another important factor responsible for major CL outbreaks in the region, with over 200,000 of people infected during the Afghanistan military conflict (Wallace et al., 2002). A similar number of people have been infected since the start of the Syrian civil war (Alasaad, 2013, Salam et al., 2014, Saroufim et al., 2014, Sharara and Kanj, 2014).

CL is a major vector-borne disease problem in the Kingdom of Saudi Arabia (KSA). Most reported CL cases are concentrated in the regions of Al Ahsa, Al Qassem, Riyadh, Asir, Hail and Al Madinah (Al-Salem et al., 2014, El-Badry et al., 2008, El-Beshbishy et al., 2013a, Dye et al., 1989, Al-Zahrani et al., 1989a, Ibrahim et al., 1994, el Sibae et al., 1993). Due to a well-designed national control programme implemented in the early 1980s, CL cases in the KSA have dropped

in number since 1987, from ~17,000 to ~2,000 cases per year. However a lack of sector integration, amongst other factors, has hampered further reductions in CL case numbers. In addition to other communicable diseases, CL control in the KSA is particularly important because millions of visitors are received annually due to pilgrimage. Furthermore, around 35% of the country's workforce are visiting workers arriving from *Leishmania* endemic countries. Both anthroponotic (*L. tropica*) (Al-Salem et al., 2014, El-Beshbishy et al., 2013b, Al-Zahrani et al., 1989a) and zoonotic (*L. major*) CL have been reported in the KSA (AL-Gindan et al., 1983, El-Beshbishy et al., 2013a, Elbihari et al., 1987, Salam et al., 2014), but very little is known about the national distribution of these parasite species.

Current drug treatment protocols for patients with Old World CL vary between EMRO countries (Ben Salah et al., 2013). Intralesional (IL) Pentostam® (sodium stibogluconate (Sb)) is commonly used in this region, despite its high toxicity and despite the increasing number of people with CL who do not respond to drug treatment. Currently, Sb is the second-line treatment choice for CL in the KSA. In most cases it is administered if the patient does not respond to treatment with topical antifungals, such as miconazole or itraconazole, with antibiotic fucidin cream (fusidic acid) as first-line treatment (Figure 2.1). Recently, topical paromomycin, used either alone or in combination with gentamicin, was found to be effective in treatment of people with *L. major* in Tunis (Ben Salah et al., 2013).

In this thesis chapter, I report an epidemiological map of CL in the KSA, including the clinical features associated with the two main *Leishmania* species (*L. major* and *L. tropica*). Furthermore, I show evidence that the efficacy of the current CL treatment protocol is highly dependent on the *Leishmania* parasite species associated with the infection, the geographical location and the presence of secondary infection (SI) in or around the lesion.

2.2 Methods

2.2.1 Skin aspirate sample collection

Skin aspirate samples were taken from a total of 104 adults clinically confirmed with CL (89% male, 11% female) from several cities or towns. The patient samples were collected as follows: 18 samples from the Central region (15 locals, 3 migrants), 28 samples from the Northwest region (4 locals, 24 migrants), 12 samples from the Southwest region (11 locals, 1 migrant), and 46 samples (15 locals, 31 migrants) from the Eastern region. Overall, this represented around 7% of all reported cases of CL in the KSA in 2012.

2.2.2 Parasite isolation and *in vitro* culture

Parasite samples were collected by wound aspiration from CL patients after one month of lesion appearance using 200-300 µl of sterile PBS (Phosphate buffered saline). Wound aspirates were performed in triplicate and then were transferred to plastic flasks containing *Leishmania* cultured media, M199 medium (Gibco®) supplemented with 15% heat inactivated FBS (Invitrogen™), 1.5% BME vitamins

(Sigma®) and 25 µg/ml gentamycin sulphate (Sigma®). Parasite cultures were maintained for several days at 27°C. Parasite DNA was extracted from logarithmic phase *Leishmania* cultures using DNeasy Blood and Tissue kit (Qiagen®) according to the manufacturer's instructions. *Leishmania* culture was performed as described on (Dougall et al., 2011).

2.2.3 Identification of *Leishmania* species

Leishmania species were identified by PCR-RFLP (Polymerase chain reaction-Restriction fragment length polymorphism) analysis of the ribosomal Internal Transcribed Spacer 1 (*IST1*), as described previously (el Tai et al., 2000). Primers used were: forward (LITSR): 5′ CTGGATCATTTTCCGATG -3′ and reverse (L5.8S): 5′ TGATACCACTTATCGCACTT -3′. The PCR mixture contained 4.0 mM $MgCl_2$, 200 µM dNTPs, 500 nM primers, 2 U Taq polymerase (KAPA Biosystem) and 1.5 mM $MgCl_2$. After 5 minutes denaturation at 94°C, PCR was performed using 35 cycles of 30 seconds at 94°C, 30 seconds at 53°C, and 60 seconds at 72°C. There was a final extension step of 72°C for 8 mins (el Tai et al., 2000). Subsequently, 10 µL of PCR product was digested with 1 unit of *Hae*III fast digest (Fermentas) for 10 mins at 37°C. Restriction fragments were analysed on 3% (w/v) agarose gels using ethidium bromide. SIs were identified by clinical assessment and confirmed by laboratory analysis, as defined in Section 2.2.4.

2.2.4 Secondary infection culture

Sterile cotton swabs were used for bacterial and fungal isolation. These swabs were then inoculated onto different microbiological agar plates, including nutrient agar, blood agar, MacConkey agar, chocolate agar and Sabouraud dextrose agar. Sabouraud dextrose agar was incubated at 30°C and nutrient agar, blood agar, MacConkey agar and chocolate agar were incubated at 37°C. After microbial growth, one colony from each plate was inoculated onto blood agar and chocolate agar. Similarly, to assess fungal growth, one colony was inoculated onto Sabouraud dextrose agar. All bacteria and fungi species were identified by microscopy and biochemical assays, including catalase, coagulase and DNAse assay. All the laboratory analyses for microbial identification were carried out at the King Abdulaziz Center for Science and Technology (KACST) and King Khalid Hospital, Riyadh, KSA.

2.2.5 Clinical data collection

Case record studies and information sheets were obtained for all patients and re-labelled with the appropriate study code. The recorded information included all relevant clinical data (*i.e.* lesion size, number(s) and location(s) on the body, and clinical features and treatment response), patient age, sex and address. All data were kept in secure computer folders.

2.2.6 Drug treatment

Clinically diagnosed patients were referred for treatment recommended by the current KSA leishmaniasis treatment policy (Figure 2.1). This begins with the

application of topical clotrimazole (1%) and/or fucidin (2%) for the treatment of secondary (bacterial, fungal or both) infections. If healing (re-epithelisation) was not initiated after one week, the patient then received 1 course (of 14 injections each) of IL Sb (20 mg/kg/day). All patients receiving Sb treatment were evaluated before and after treatment for complete blood counts and aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, amylase and gamma glutamyltransferase levels. A few patients (n = 16) that did not respond satisfactorily to IL Sb treatment(*i.e.* those with lesion extension, formation of satellite lesions or recurrence) were referred for a 3rd course of intramuscular (IM) Sb (20 mg/kg/day for 2 weeks) with cryotherapy after clinical assessment *i.e.* blood count, liver and renal function analyses.

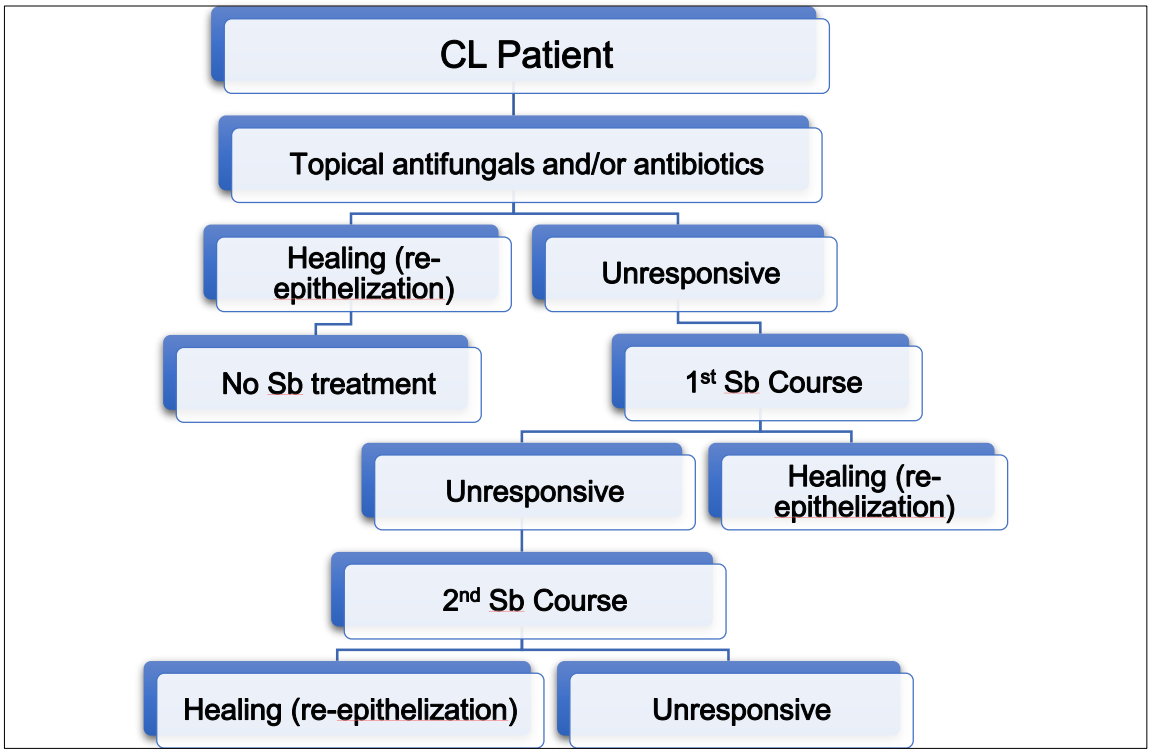


Figure 2.1 Scheme representing the current leishmaniasis treatment policy in the KSA. Clinically diagnosed CL patients were first treated with a topical antibacterial and/or antifungal to eradicate possible SIs. If healing (re-epithelization) was not observed after 1 week of treatment, the patient received a first course of Sb, consisting of 14 IL injections (once every three days). If the patient remained unresponsive, a second course of IL Sb, consisting of 14 doses twice a week, was administered after clinical assessment. In rare occasions, if the patient was still unresponsive after a second course of IL Sb, a third course or IL Sb (14 injections, three times a week) was administered.

2.2.7 Data analysis

All factors affecting the treatment responses were included in our analyses (*i.e.* clinical features, number and sites of the lesions, geographical area and parasite species). Fisher's exact test and Chi^2 test were used to validate the statistical significance of the different factors. IMB SPSS Statistics 21 was employed for all data analyses. Software ArcGIS Desktop 10.0 (ESRI, Redlands, CA) was used to map the distribution of *Leishmania* species and patient response to antileishmanial drug treatment across the regions, and in relation to elevation

based on gridded Global Relief Data (ETOPO2) data from the geographical coordinates (latitude and longitude) of each site. Maps were done in collaboration with Dr. Louise Kelly-Hope.

2.2.8 Ethics

Ethical approval was obtained from both from the Liverpool School of Tropical Medicine (LSTM), UK (12.03RS) and the Saudi Ministry of Health (MoH) Ethical Committees. Written consent was obtained from patients in the ethical approval rubric.

2.3 Results

2.3.1 Identification of *Leishmania* species

In the KSA, Old World CL is only clinically diagnosed. The infective *Leishmania* species is inferred based on the appearance and number of lesions. Thus, *L. major* and *L. tropica* infection prevalence in the main CL-endemic areas of KSA is currently unknown. Using a well-established PCR-RFLP analysis of the *ITS1* region the *Leishmania* spp. present in 104 CL patients was determined (Figure 2.2). All patients were confirmed to be infected with either *L. major* or *L. tropica*. *L. major* was the main species responsible for CL in the KSA, predominately in the regions of Al Ahsa (East), Al Qassem and Riyadh (Central), and Al Madinah (Northwest) (Figure 2.3). However, *L. tropica* was the only species found in the Southwest regions of Asir and Jazan. Interestingly, *L. tropica* was also detected in a few cases from the Al Madinah region, with one particular village reporting the presence of both *L. major* and *L. tropica* infections (Figure 2.3).

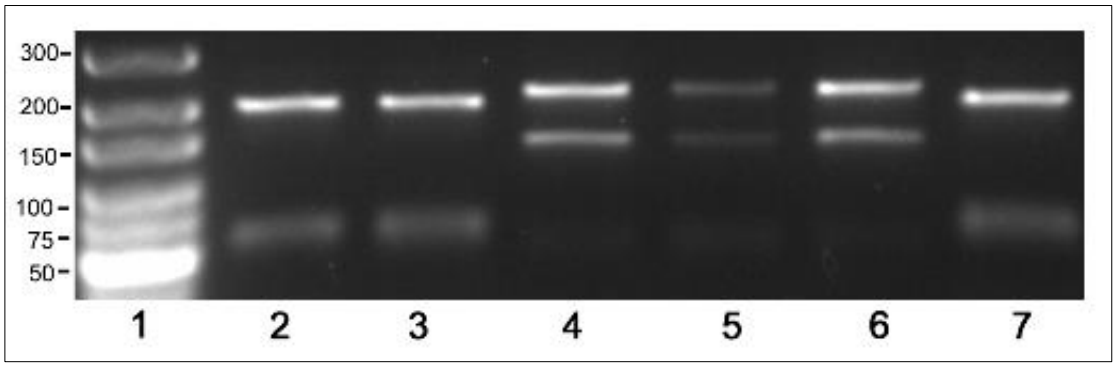


Figure 2.2 *Leishmania* spp. identification by PCR-RFLP analysis of parasite *ITS1* gene. Lane 1: PCR ladder; Lanes 2-5: different examples of *Leishmania* isolates from Aljadaidah, Al Madinah Province; Lane 2 and 3: *L. tropica* samples; Lane 4 and 5: *L. major* samples. Positive controls: Lane 6: *L. major*; Lane 7: *L. tropica*.

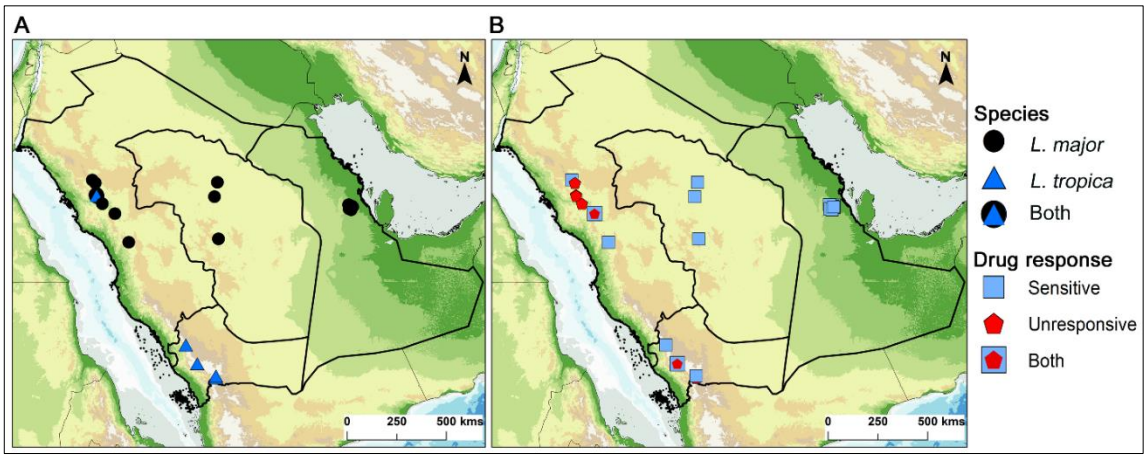


Figure 2.3 Panel (A) Distribution of *Leishmania* species; and Panel (B) Patient response to antileishmanial treatment within the main CL endemic regions of KSA.

2.3.2 Clinical presentation of CL patients varies according to geographical region and *Leishmania* species

The clinical features *i.e.* papular, nodular or ulcerated-nodular lesions of CL-infected patients differed significantly throughout the CL-endemic regions ($p > 0.05$, Fisher's exact test; Table 2.1) (Figure 2.3). Multiple nodular or papular lesions were predominately found on the legs, feet, hand, neck or arms of patients from the Central (Riyadh and Al Qassem) and Eastern (Al Ahsa) regions of the KSA ($p = 0.0006$, Fisher's exact test). Ulcerated nodular lesions were found in more than half the cases from the East and Southwest ($p > 0.05$, Fisher's exact test). However, some cases in Al Madinah also presented papular or dry nodular lesions.

Clinical presentations of CL patients also varied depending on the parasite species that infected the patient (Table 2.1). There was a significant association

between the presence of ulcerated nodular lesions and *L. tropica* infection (p = 0.035, Fisher's exact test). However, papular lesions were more often associated with *L. major* infection, whereas nodular lesions were found in infections by either parasite species. Notably, the appearance of multiple lesions (up to 29 per patient) significantly correlated with *L. major* cases only (p = 0.0006, Fisher's exact test), whereas *L. tropica* patients had no more than three lesions. The mean lesion size of *L. major* or *L. tropica* infected patients differed notably, *i.e.* ~16.5 mm or 22.2 mm (mean overall lesion), respectively (Table 2.2). Furthermore, *L. tropica* patients had significantly more lesions on their arms, face, nose, ears and neck, whereas *L. major* patients had lesions all over the body (p = 0.0005, Fisher's exact test). Over half *L. tropica* cases presented satellite lesions, compared to only 22% of *L. major* patients (p = 0.0006, Fisher's exact test; Table 2.1).

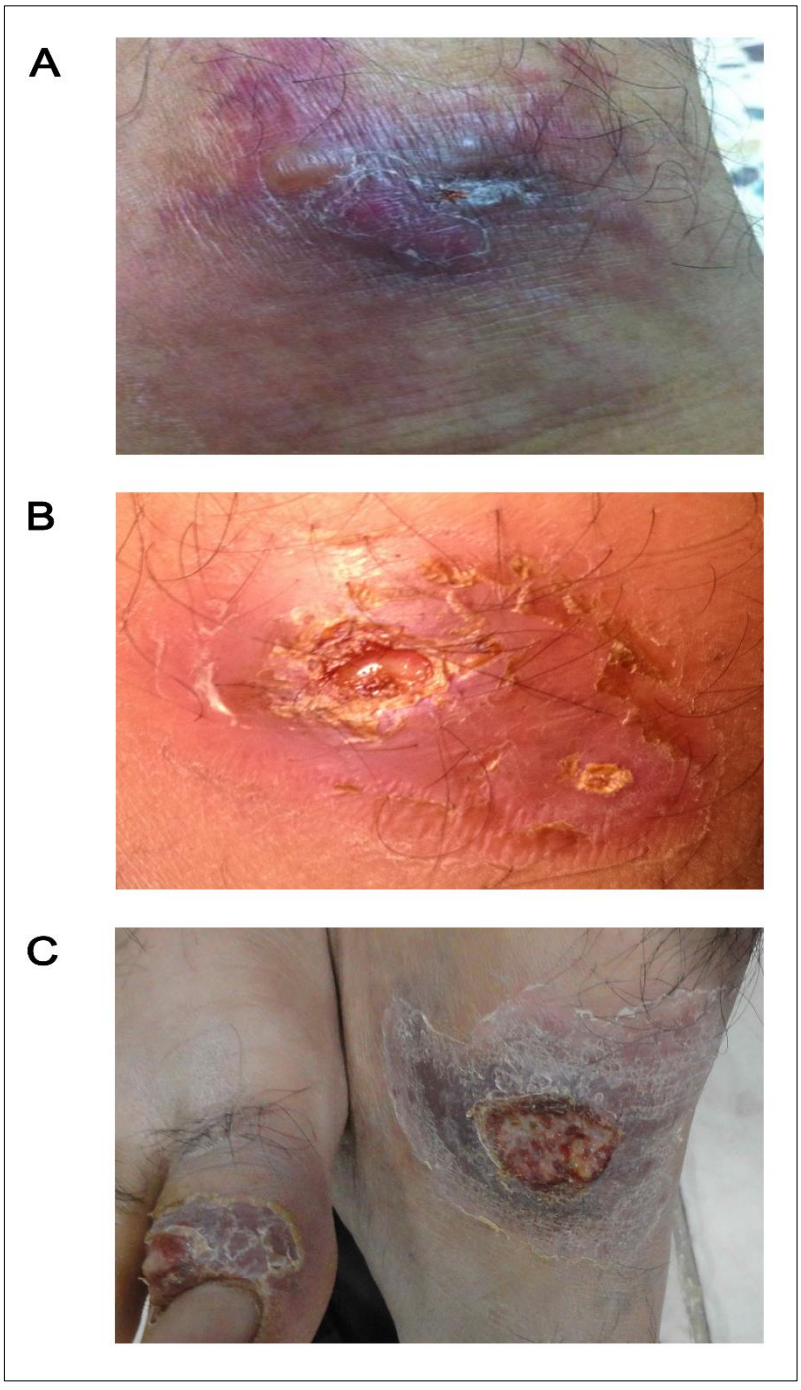


Figure 2.4 CL clinical presentation. Panel (A) Papular lesions with satellite. Panel (B) Nodular lesion. Panel (C) Ulcerated nodular lesions.

Parasite species	Lesion feature p = 0.035 (Fisher's exact test)			Lesion location on patient p = 0.0005 (Fisher's exact test)				
	Papular	Nodular	Ulcerated nodular	Hand, neck or Head	Arms	Trunk, legs or Feet	Face, nose or ear	Whole body
L. major	28	27	30	30	7	19	3	16
L. tropica	0	7	14	2	12	0	7	0

Table 2.1 Clinical presentation of CL lesions according to parasite species.

Parasite species	Lesion numbers per patient p = 0.0006 (Fisher's exact test)						Patients with satellite lesions p = 0.0006 (Fisher's exact test)
	1	2	3	4	5	6-29	
L. major	23	9	5	4	7	27	17
L. tropica	10	3	8	0	0	0	12

Table 2.2 Lesion numbers and satellite lesions of CL patients according to parasite species.

2.3.3 Efficacy of antileishmanial treatment varies depending on parasite species and geographical location

Confirmed (medically diagnosed) CL patients were referred for antileishmanial treatment with topical antifungals alone or in combination with antibiotics (first-line treatment), followed by one or two courses of IL Sb (as described in the Methods section). Overall, 30% of *L. major*-infected patients responded to the first-line treatment alone and 82% after second Sb courses were completed (Figure 2.5). However, no *L. tropica*-infected patients responded to topical azoles/fucidin treatment, and 60% did not respond to treatment even after receiving two IL Sb courses (Figure 2.5).

Interestingly, when the treatment responses of patients from different geographical locations were compared, 90% of patient cases from the Central region (*i.e.* Riyadh; exclusively *L. major*-infected cases) responded favourably (within 1 week) to topical azoles/fucidin alone ($p = 0.001$, Fisher's exact test). However, two-thirds of the cases from the Northwest (Al Madinah; mainly *L. major*-infected cases) and Southwest regions (Asir; exclusively *L. tropica*-infected cases) responded unsatisfactorily to both lines of treatment.

Interestingly, there is a high correlation between lesion location on the body and treatment response. Lesions that developed on the face, nose or ear were likely to be unresponsive to first-line treatment (Table 2.3). Ulcerated *L. tropica* cases with SI reported as highly unresponsive after two courses of the second-line

treatment particularly on the arms and face. (Table 2.3). Approximately half of the cases (39) with multiple lesions caused by *L. major* required more than 2 courses of Sb treatment.

Location of infection	Treatment response			
	1st line	**2nd line 1st course**	**2nd line 2nd course**	**Unresponsive cases**
Head, neck or hand	42%	37%	5%	16%[1]
Arms	31%	0%	23%	46%[2]
Trunk, legs or feet	32%	63%	0%	5%[3]
Face, nose or ear	7%	23%	25%	55%
Disseminated (all body)	13%	31%	13%	43%

Table 2.3 Relationship between location of infection lesion location on the body and treatment response

Footnotes:

[1] Half of cases that did not respond were ulcerated *L. tropica* cases.

[2] Most unresponsive cases that had lesions on their arms were *L. tropica* cases.

[3] Ulcerated *L. tropica* cases with SI.

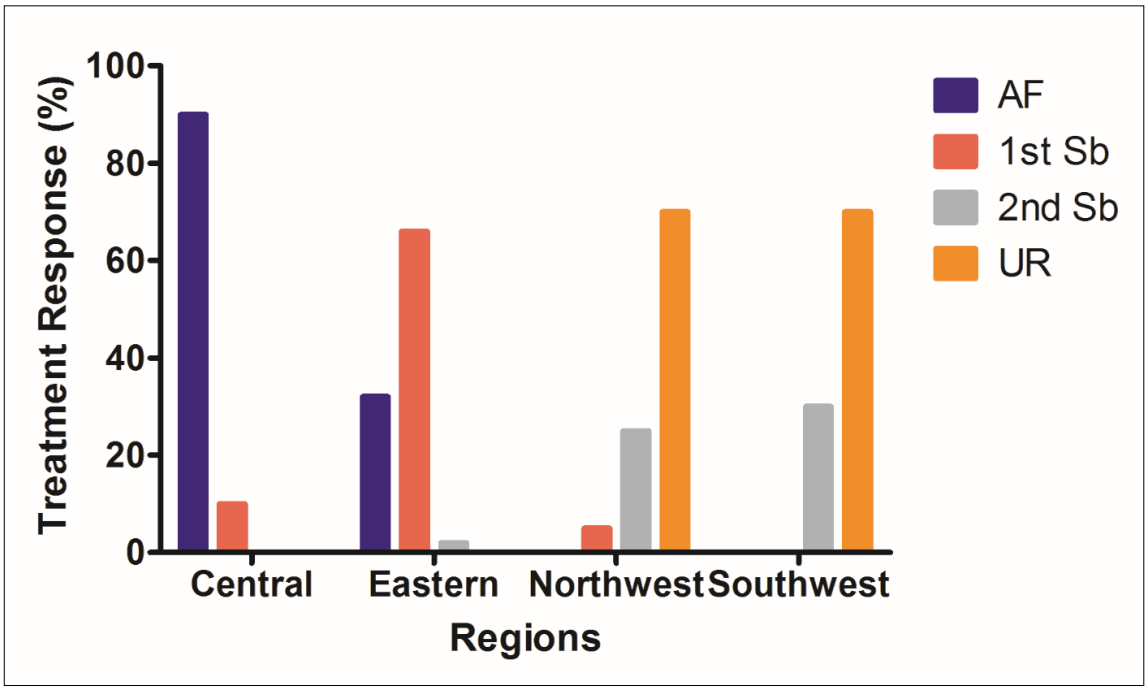


Figure 2.5 Correlation between the responses to drug treatment and CL endemic regions. Patients from the Central region (exclusively infected with *L. major*) had a significantly higher response (healing: p = 0.001, Fisher's exact test) to first-line treatment compared with other CL-endemic regions studied. In addition, patients from the Eastern region responded significantly more favourably (p = 0.001, Fisher's exact test) to the first course of Sb treatment compared with those from Northwest and Southwest regions. Abbreviations: AF: azole/fucidin treatment (first line); 1st and 2nd Sb: first and second sodium IL Sb treatment, respectively; UR: patients "unresponsive" to the sequential antileishmanial treatment regime.

2.3.4 Clinical features and correlation with geographical regions and *Leishmania* spp.

Variation in clinical presentation (*i.e.* popular, nodular or ulcerated-nodular) was also noticed throughout the different endemic regions. Multiple nodular or papular lesions were predominately found in legs, feet, hand, neck or arms in patients from the Central (Riyadh and Al Qassem) and Eastern (Al Ahsa) regions. Interestingly, CL with up to 29 lesions was found in patients from Al Madinah and Al Ahsa. *L. tropica* cases from the Southwest region presented up to three lesions located mainly on the face, ear, nose or hands.Ulcerated nodular lesions were

found in more than half the cases in the Eastern and Southwest regions. However, some cases in Al Madinah presented papular or dry nodular lesions.

2.3.5 Secondary infections and influence on treatment response

SIs were primarily detected in patients from the Eastern (35%; exclusively *L. major*) and Southwest (40%; exclusively *L. tropica*) regions (Figure 2-6). SIs were identified by microscopy and biochemical analysis. *Staphylococcus aureus* bacteria were identified as cluster shaped under the microscope and catalase, coagulase and DNAse positive. Moreover, *Staphylococcus hominis*, *Staphylococcus haemolyticus*, *Staphylococcus epidermidis* and *Micrococcus* spp. were identified with other pathogenic bacteria, such as *Escherichia coli*. Fungal growth was identified under microscopy. *Trichophyton* spp. and *Epidermophyton* from the same sample were the main mould species identified in culture medium.

Interestingly, 52% of *L. major* patients presenting SIs responded favourably (*i.e.* healing was triggered) to treatment with just topical azoles/fucidin ($p = 0.002$, Fisher's exact test; Figure 2.6), with the remaining patients needing just one course of IL Sb to heal. Moreover, the group of *L. major* patients lacking SIs responded poorly to azoles/fucidin treatment and many (~38%) remained unresponsive after a second IL Sb course. In contrast, the development of SIs did not have an effect upon the treatment response of *L. tropica* patients (Figure 2.6).

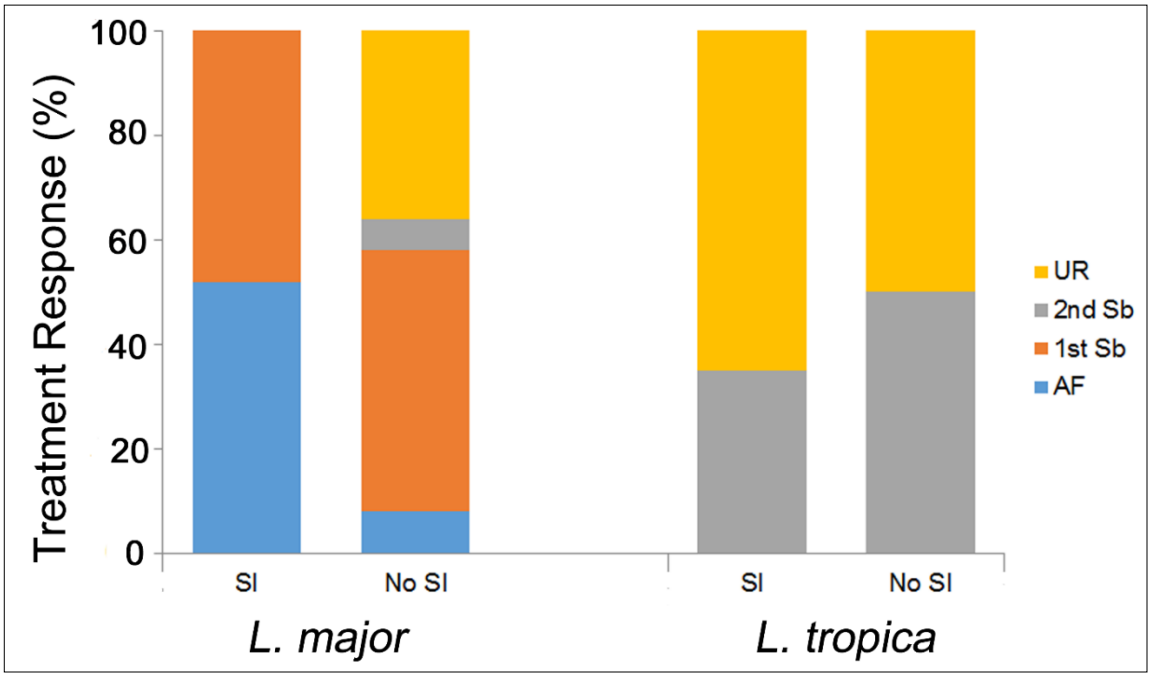


Figure 2.6: Correlation between development of a SI and treatment response in patients with *L. major* or *L. tropica* infection. A significant response to first-line treatment was observed only in *L. major* patients that had developed SIs ($p = 0.0002$, Chi^2 test). However, there were no significant differences in treatment response between *L. tropica* patients with or without SIs. SI and No SI: presence or absence of SIs, respectively; AF: azole/fucidin treatment (first line); 1st and 2nd Sb: first and second sodium IL Sb treatment, respectively; UR: patients "unresponsive" to the sequential antileishmanial treatment regime.

DISCUSSION

Several previous studies have investigated the clinical profiles of CL in KSA (Dye et al., 1989, Al-Zahrani et al., 1989a, Al-Tawfiq and AbuKhamsin, 2004, el-Sibae and Eesa, 1993, El-Beshbishy et al., 2013a). However, this epidemiological investigation is the most comprehensive dataset of CL samples collected patients from across KSA. A total of 104 parasite isolates were collected from infected CL patients in the main endemic regions of KSA. Species identification was performed by PCR. Each patient was medically assessed and referred for

treatment according to the KSA national treatment policy. The findings of this study demonstrate that patient responses to current antileishmanial treatment vary between the different CL endemic areas and are also partially dependent on the development of SIs.

L. major was exclusively found in patients from Al Ahsa, Riyadh, Al Qassim and Al Madinah – all arid or semi-arid areas at a low altitude while *L. tropica* was detected in patients from Asir, Jazan and Al Jadidah (Al Madinah) (high altitude above 1000 m). Interestingly, both *L. major* and *L. tropica* parasites were isolated from patients from Al Jadidah village and from regions of similar altitude levels (~600 m above sea level). It is possible that the *L. tropica*-infected patients found in Al Jadidah may have contracted CL in the Southern region, as the climatic conditions and the ecology of Al Jadidah may not favour transmission by the *L. tropica* vector, *Phlebotomus sergenti*. Furthermore, in collaboration with Karina Mondragon-Shem, sandflies have been recently characterized from the same Al Madinah locations and no evidence of *Ph. sergenti* presence has been reported (Chapter 3).

Clinical presentation of *L. major* patients appear to vary according to the endemic area (Al-Tawfiq and AbuKhamsin, 2004). Although statistically significant differences between the different regions sampled in this study were not found, ulcerated nodular lesions were identified predominantly in cases from Al Ahsa, whereas nodular and papular lesions were common in cases from Al Madinah

and Riyadh. Furthermore, multiple lesions were found in more than half the *L. major* patients from Al Ahsa. This differs from previous reports, where only 5% of the patients (presumably also infected with *L. major*) presented multiple lesions (Al-Qurashi et al., 2000, Al-Tawfiq and AbuKhamsin, 2004). These differences may be due in part to the higher exposure of people in this region to sandfly bites, as most CL patients from Al Ahsa that took part in this study were foreign construction workers.

CL patients presenting ulcerated nodular lesions caused by *L. major* were very sensitive to Sb treatment. In contrast, about half of the patients with nodular lesions caused by *L. major* did not respond to any line of drug treatment. Several factors may account for these differences, including the presence of different drug-resistant *Leishmania* parasite strains, the nutritional and immune status of the patients and their genetic backgrounds. Additionally, sandfly saliva could play an important role in disease outcome as saliva from *Ph. papatasi* (*L. major* vector) has been reported to trigger either a Type I (regarded as protective against a *Leishmania* infection) or Type II delayed-type hypersensitivity in healthy individuals from CL endemic areas as reviewed in (Oliveira et al., 2013b). However, there is lack of knowledge regarding human immune responses to *Ph. sergenti* saliva.

The presence of ulcerated nodular lesions correlated strongly with *L. tropica* infection in patients from all CL endemic regions where this species was found.

Therefore, infection with any of the parasite species appears to favour the development of specific clinical feature(s), which vary depending on geography, and may also impact treatment response. Further investigations are needed to determine if there is a correlation between treatment efficacy and the different clinical presentations of Old World CL patients. Another factor that may contribute to responsiveness to drug treatment is the duration of residency in the region, particularly in migrant workers in Al-Madinah.

Overall, a high proportion of *L. major* lesions were associated with the development of SIs caused by pathogenic opportunistic bacteria or fungi. Preliminary analysis suggests that the main bacteria and fungi species identified in CL lesions from KSA patients were *S. aureus*, *S. hominis*, *S. haemolyticus*, *S. epidermidis*, *Micrococcus* spp. and *E. coli*, *Trichophyton* spp. and *Epidermophyton* spp. Approximately 50% of *L. major* patients (mainly from the Central (Riyadh) or Eastern (Al Ahsa) regions presented ulcerated nodular lesions with detectable SIs. Interestingly, there was a statistically significant correlation with the response to re-epithelization after treatment with azole/fucidin (without further administration of Sb) in patients from the Central (~90%) region compared with patients from other CL endemic areas ($p = 0.0001$, Fisher's exact test; Figure 2.2). This suggests that elimination of the SIs appeared to favour healing of lesions of *L. major* patients from Al Ahsa, but had no effect on patients from Al Madinah.

Like pathogenic fungi, *Leishmania* parasites make ergosterol (Vanden Bossche et al., 1989), an essential membrane lipid, the synthesis of which is inhibited by azoles. Treatment with topical azoles alone may possibly impact directly on *L. major* susceptible strains. Also, elimination of the fungal or bacterial infection may boost the immune system to fight the *L. major* infection in many of the cases, with the exception being those presenting papular lesions from the Al Madinah region. Regarding *L. tropica*-infected patients, most were unresponsive (60%) to antileishmanial treatment and healing only occurred in patients receiving a second (and sometimes a third) course of IL Sb. Unresponsive cases to glucantime have been reported among *L. tropica* patients in neighbouring countries, such as Iran (Hadighi et al., 2006). It remains to be determined whether *L. tropica* strains from the KSA are less sensitive to azoles or, alternatively, the drug is more accessible to the parasite in *L. major* lesions than in *L. tropica* ones.

Some factors that contributed to accelerating treatment response including duration of residency in the endemic areas that lead to have several unresponsive cases of *L. major* in Al-Madinah like those cases in Latin America which described on (Llanos-Cuentas et al., 2008a). Moreover, SIs have an indirect effect on treatment response among *L. major* infected patients. The development of SIs may have influence on the treatment response of *L. major*-infected patients which could result in releasing of bacterial or fungal molecules after application of first-line treatment. This could activate host immunity and contribute to the elimination of *L. major* infection and subsequent activation of wound healing mechanisms.

These findings could be exploited for a more efficient and a less traumatic drug treatment regime for CL patients, so use of highly toxic and painful drugs, such as Sb, could be minimised. In summary, differences in susceptibility of *Leishmania* strains to either azoles or Sb suggest that current protocols for CL treatment need to consider not only the parasite species, but also the geographical endemicity of CL infections. Further research is required to understand whether treatment unresponsiveness in the KSA is due to the development of drug-resistant *Leishmania* parasites. This information will help to determine whether different antileishmanial treatment protocols need to be considered.

Overall, the results of this investigation have implications for the implementation of differential treatment regimes according to the CL-endemic area, which in turn will ensure appropriate use of financial resources and also ensure patients are treated with the most efficacious antileishmanial therapies. It remains to be determined in CL-endemic areas of other EMRO countries whether such profound differences are observed between the patient treatment response and parasite isolates from different geographical locations.

Chapter Three: CL Disease Outcomes Are Correlated With Sandflies Exposure

3.1 Background

Cutaneous leishmaniasis (CL) is a major public health problem in the Kingdom of Saudi Arabia (KSA). It is transmitted by *Phlebotomus sergenti* and *Phlebotomus papatasi* according to geographical and climatic factors (al-Zahrani et al., 1989b, El-Badry et al., 2008, El-Beshbishy et al., 2013b). Zoonotic CL (ZCL) is widespread in arid and semiarid areas of KSA and is caused by *Leishmania major* and transmitted by the sandfly *Ph. papatasi*. *Leishmania tropica*, on the other hand, is exclusively endemic to the highlands and lowlands in the Southwestern region of KSA (Al-Zahrani et al., 1989a), where it is transmitted by *Ph. sergenti* and causes anthroponotic CL (ACL).

The bite of an infected sandfly results in the injection of a mixture of components in the sandfly saliva through the sandfly's proboscis to the vertebrate host. This saliva contains promastigote secretory gel (PSG) and *Leishmania* parasites (Rogers et al., 2004, Rogers et al., 2009). Importantly, the saliva impairs the haemostatic and inflammatory systems of the vertebrate host, allowing the insect to take a blood meal efficiently (Oliveira et al., 2013a). These salivary components have also been shown to promote or inhibit *Leishmania* development in the vertebrate host depending on the vector species (Gomes and Oliveira, 2012).

Increased sandfly-host contact translates into an increased risk of the vertebrate host being infected with *Leishmania*. Repeated exposure to sandfly bites results in the production of antibodies against salivary components in the vertebrate host. This provides an indirect measure of exposure to sandfly vectors (Andrade and Teixeira, 2012). The presence of IgG antibodies against *Ph. papatasi* saliva has been associated with a higher risk of *L. major* infection (Gomes and Oliveira, 2012, Marzouki et al., 2011). The transient nature of the antibody response to sandfly bites (Marzouki et al., 2011, Clements et al., 2010, Hostomska et al., 2008, Vlkova et al., 2011, Vlkova et al., 2012) allows for the study of temporal changes in transmission risk and the efficacy of vector control programmes (Gidwani et al., 2011).

Biomarkers used to evaluate sandfly exposure need to be species-specific in order to differentiate between antibody responses to vector and non-vector species, or between sandflies and other blood-feeding insects, including mosquitoes. The sandfly salivary protein PpSP32 has been described as a 30 kDa immunodominant target of the host antibody response against *Ph. papatasi* saliva (Rohousova et al., 2005, Marzouki et al., 2012), and was highly specific when tested against people living in a region with a high density of *Ph. perniciosus*. Additionally, PpSP32 transcript expression in the salivary glands is not influenced by the age or diet of the sandfly (Coutinho-Abreu et al., 2010). B-cell epitope prediction analysis showed six epitopes were identical between the Tunisian strain PpSP32 and the PpSP32 protein (Protein accession numbers

(NCBI): *Phlebotomus papatasi* SP32 GI:449060662, *Phlebotomus sergenti*SP44: GI:299829437) deposited in GenBank (Israeli strain), indicating it is a good candidate to assess biting exposure in different ZCL foci (Marzouki et al., 2012). Furthermore, *in vitro* production of the recombinant sandfly salivary protein (rPpSP32), a recombinant form of the *Ph. papatasi* PpSP32 protein, overcomes the difficulty of obtaining large quantities of salivary glands, and facilitates the use of salivary biomarkers for large scale epidemiological studies in CL endemic areas.

To better understand the correlation between sandfly biting exposure and leishmaniasis infection, the level of exposure to *Ph. papatasi* bites in people from several CL endemic areas in the KSA was determined. This was performed by measuring the levels of anti-PpSP32 antibodies in the sera of CL patients and healthy individuals.

3.2 Materials and Methods

3.2.1 Ethics statement

The study was approved by the Liverpool School of Tropical Medicine Ethics Committee UK (12.03RS). All participants provided written informed consent for the collection of blood samples and subsequent analyses. All research was conducted according to Declaration of Helsinki principles.

3.2.2 Study samples

Peripheral blood samples were taken from 411 individuals (106 females and 305 males, aged 18-60 years, median of 36 years). Sample collections were performed in study sites selected according to CL endemicity and areas where people were at higher risk of exposure to sandfly bites. Two areas of high *Ph. papatasi* density (ZCL transmission), including Al-Madinah and Al-Ahsa, and *Ph. sergenti* (ACL transmission) in Asir region (Chapter 2) were selected to test biomarker specificity. Samples were collected from April to December 2012. CL cases were confirmed by microscopic analysis by a trained technician, and the species was confirmed in patients with CL by PCR (Chapter: 2). Moreover, peripheral blood samples were taken from 5 individuals as control for this study to compare sandfly exposure in endemic areas to unexposed individuals from the UK.

3.2.3 Expression and purification of PpSP32 recombinant protein

Mammalian VR-2001 plasmid encoding the PpSP32 protein with a 6 histidine tag was sent to the Protein Expression Laboratory at the Frederick National Laboratory for Cancer Research (Frederick, Maryland). Expression was performed by transfecting HEK-293F cells. The supernatant was collected after 72 hours, filtered and concentrated from 1 litre to 300 ml using an Amicon® concentrator device (Millipore, Billerica, MA, USA) in the presence of NaCl 500mM. The volume was returned to 1 litre at a final concentration of 10 mM Tris,

pH 8.0. The expressed protein was purified by an HPLC system (DIONEX, CA, USA) using two 5 ml HiTrap Chelating HP columns (GE Healthcare, Buckinghamshire, UK) in tandem and charged with 0.1 M NiSO4. The protein was detected at 280 nm and eluted by an imidazole gradient as described by (Teixeira et al., 2010). Eluted proteins were collected every minute in a 96-well microtitre plate using a Foxy 200 fraction collector (Teledyne ISCO, Lincoln, NE, USA). Fractions corresponding to eluted proteins peaks were selected and run on a NuPage Bis-Tris 4–12% Gel (Novex, Life Technologies, Carlsbad, CA, USA) with MES running buffer under reducing conditions, as per manufacturer's instructions. Appropriate fractions, as determined by molecular weight, were pooled and concentrated to 1 ml using an Amicon® Ultra Centrifugal Filter (Millipore, Billerica, MA, USA). Protein concentration was measured using a NanoDrop ND-1000 (Thermo Scientific, Waltham, MA, USA) spectrophotometer at 280 nm and calculated using the extinction coefficient of the protein. Both the expression and purification of PpSP32 were done in collaboration with Dr. Jesus G. Valenzuela (NIH).

3.2.4 Detection of human anti-PpSP32 antibodies

Exposure to sandfly bites was measured through the levels of anti-PpSP32 IgG antibodies in participants' sera. Anti-PpSP32 antibodies were measured by enzyme-linked immunosorbent assay (ELISA), as described by (Marzouki et al., 2012), with some modifications. Briefly, microtitre plates (Thermo-Scientific) were coated overnight with 50 µl rPpSP32 (2 mg/ml = 0.1 mg/well) in 0.1M carbonate

buffer (pH 9.6). Plates were blocked with PBS-BSA at 37°C for one hour and then washed several times with PBS. Diluted sera (1:200) were added to the plates and incubated at 37°C for 2 hours. After washing, plates were incubated with anti-human IgG peroxidase-conjugated antibody (1:10000) (Jackson ImmunoResearch, Suffolk, UK) for one hour at 37°C. Antibody binding was visualized using the substrate 3,3′,5,5′ tetramethylbenzidine (Biolegend, San Diego, CA, USA) and absorbance was read at 450 nm on a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). Each serum was tested in triplicate. Wells without serum were used as negative controls.

3.2.5 Statistical analysis

The Kruskal-Wallis test was used to compare sets of groups. GraphPad Prism Software 5 was used for all data analysis. Statistical significance was considered as $P<0.05$.

3.3 Results

3.3.1 PpSP32 is recognized by sera of people living in CL endemic areas of KSA where *Ph. papatasi* is prevalent

Levels of anti-PpSP32 antibodies in the sera of healthy individuals from KSA were significantly higher ($P\leq0.01$) (Figure 3.1) when compared to unexposed individuals from the UK. This indicates that the biomarker is successfully

recognized by Saudi individuals, and furthermore agrees with the expected level of exposure to sandflies in CL-endemic areas.

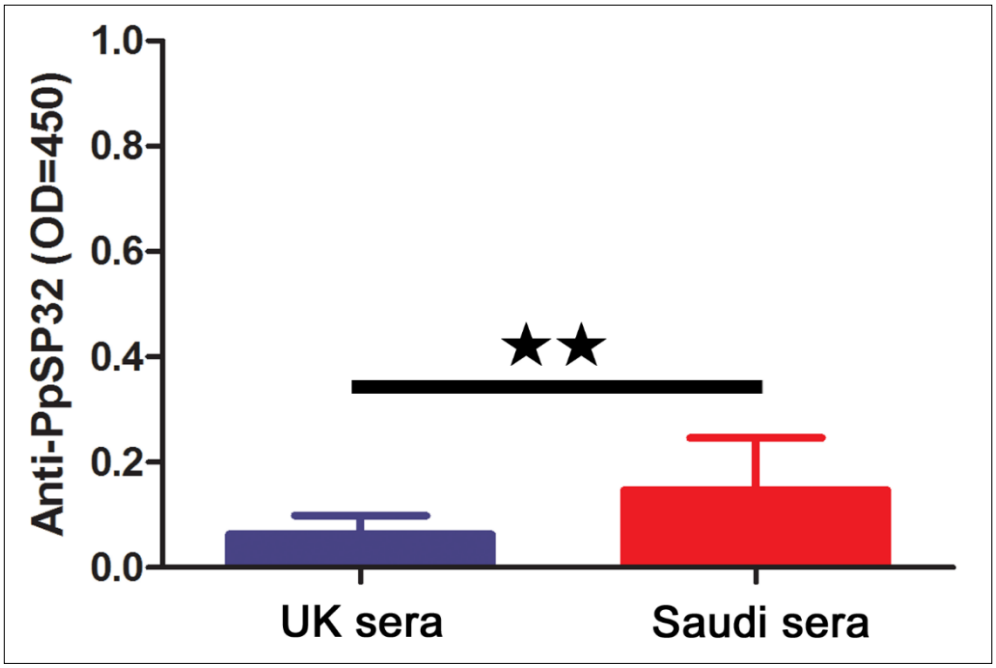


Figure 3.1. Levels of anti-PpSP32 antibodies in the sera of healthy individuals from KSA were significantly higher compared to UK healthy sera (**$P \leq 0.01$; Kruskal-Wallis test).

3.3.2 In Al Ahsa and Al Madinah regions the levels of anti-PpSP32 antibodies are higher in CL patients than healthy individuals

When healthy individuals from the two ZCL endemic regions studied were compared to each other, a significantly higher level of anti-PpSP32 antibodies was found in Al Ahsa compared to Al Madinah (Figure 3.2). Healthy and infected individuals were compared to determine the possibility of the correlation between sandfly bites and CL infection. In both Al Ahsa (Figure 3.3(A)) and Al Madinah (Figure 3.3(B)), patients with an active infection (CL) showed significantly higher levels of anti-PpSP32 antibodies compared to healthy residents ($P<0.001$).

Overall, comparing the groups from both Al Ahsa and Al Madinah, the levels of anti-PpSP32 in Al Ahsa individuals appear to be higher than those from Al Madinah, suggesting that Al Ahsa populations are more exposed to *Ph. papatasi* bites.

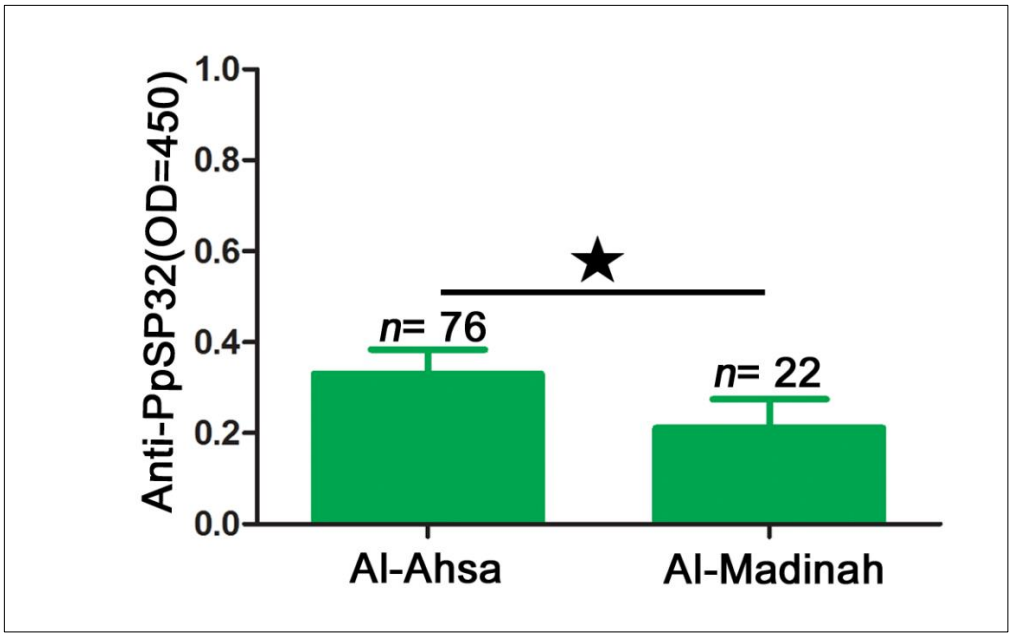


Figure 3.2. Human antibody response to *Phlebotomus papatasi* salivary protein PpSP32. Comparison of anti-PpSP32 antibodies levels in healthy individuals from the ZCL region of Al-Ahsa and Al-Madinah. OD: optical density. *P≤0.05

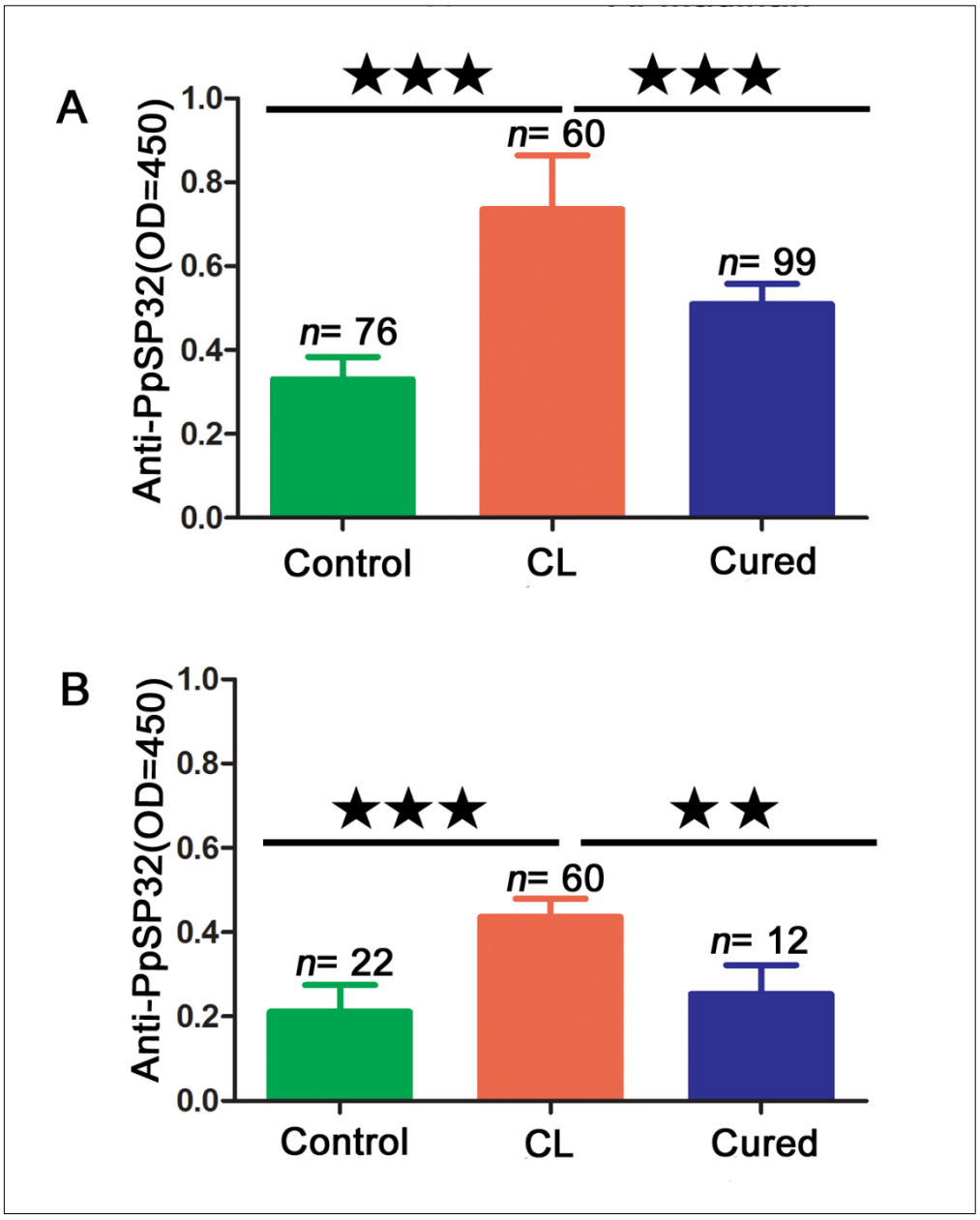


Figure 3.3. Human antibody response to *Phlebotomus papatasi* salivary protein PpSP32. (A) Anti-PpSP32 antibody levels in ZCL patients with active and cured infections from Al-Ahsa. (C) Anti-PpSP32 antibody levels in ZCL patients with active and cured infections from Al-Madinah. Control: healthy individuals; CL: active infection; CR: cured infections; OD: optical density. *P≤0.05; **P≤0.01; ***P≤0.001.

3.3.3 PpSP32 is recognized to a lesser extent by individuals living in areas where *Ph. sergenti* is prevalent.

In individuals from the Asir region (endemic for ACL *L. tropica* infection), both the healthy and cured groups showed very low levels of anti-PpSP32 antibodies (Figure 3.4(A)), which agrees with the near absence of *Ph. papatasi* from this region (Mondragon-Shem et al., 2015). Unexpectedly, the levels of anti-PpSP32 antibodies were significantly higher ($P<0.01$) in individuals with an active *L. tropica* infection, compared to healthy residents and cured patients (Figure 3.4(A)). Sequence alignment of *Ph. papatasi* PpSP32 (Abdeladhim et al., 2012) and the PpSP32-like protein from *Ph. sergenti* (Rohousova et al., 2012) confirmed a significant level of similarity between these homologous proteins (Figure 3.4(B)), suggesting a possible cross-reactivity. Although these patients were Saudi residents and their migration is uncommon, we cannot discard either the possibility that these people may have been exposed to *Ph. papatasi* bites while traveling outside this area or the presence of *Ph. papatasi* in low numbers in Asir region. In both cases, the anti-PpSP32 levels may reflect a low exposure to this sandfly species.

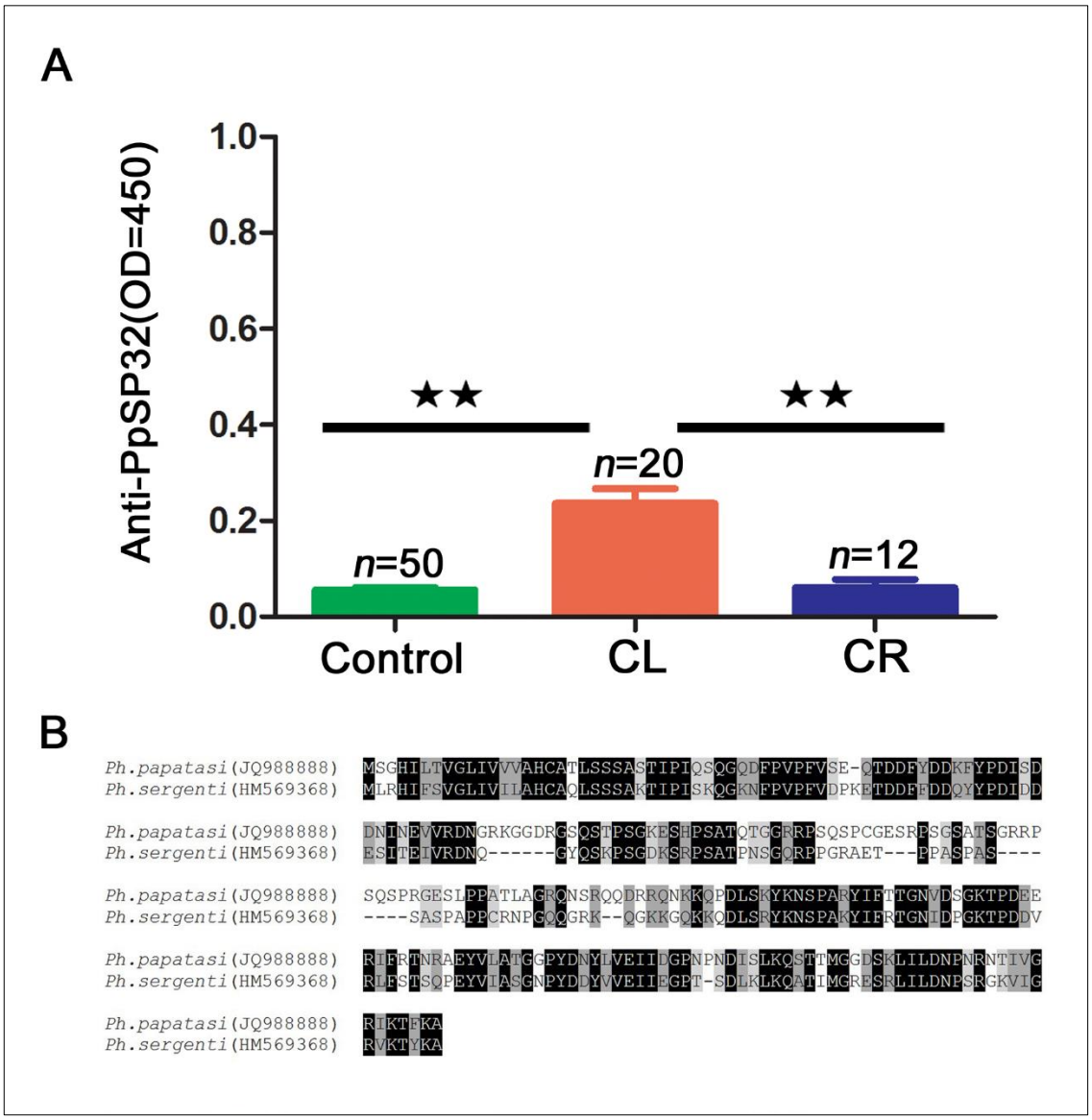


Figure 3.4. (A) Antibody response to PpSP32 from patients in Asir where *L. tropica* is prevalent. Control: healthy individuals; CL: active infection; CR: cured infections; OD: optical density. *P≤0.05; **P≤0.01; ***P≤0.001. (B) Sequence alignment showing similarities between the *Ph. papatasi* and *Ph. sergenti* PpSP32-like proteins. Sequence alignment was carried out using ClustalW and manually annotated. Shading indicates amino acid similarities: Black: fully conserved; dark grey: strongly similar; light grey: weakly similar.

3.3.4 Evidence of an association between the levels of anti-PpSP32 antibodies and ZCL clinical presentation

Levels of anti-PpSP32 in ZCL patients were measured according to the lesion characteristics. Lesion size was classified as either 10-15mm or >15mm in diameter. Patients from Al Ahsa with large lesions >15mm had significantly higher antibody levels (P<0.01) than individuals with lesions between 10-15mm (Figure 3.5(A)). This difference was not observed in Al Madinah. Additionally, when we compared the patients with different lesion numbers (<3 or >3 lesions) (Figure 3.5(B)), no significant differences in antibody levels were found within geographical region. However, anti-PpSP32 levels differed significantly between regions, with higher levels in Al Ahsa than Al Madinah (Figure 3.4(B)).

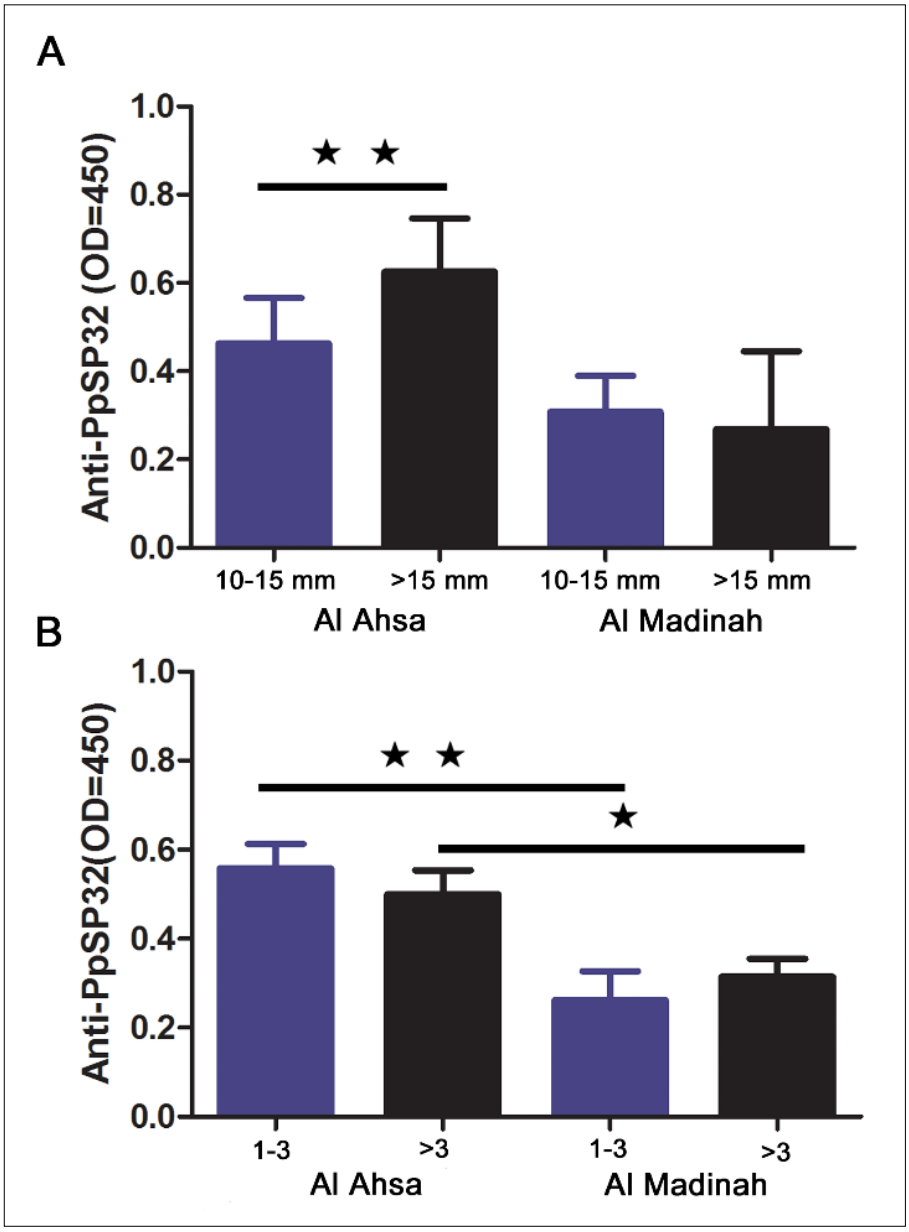


Figure 3.5. Levels of anti-PpSP32 antibodies in patients with active ZCL vary according to the size and number of the lesions. (A) Comparison of antibody levels according to ZCL lesion size in patients from Al Ahsa (** $p \leq 0.01$) and Al Madinah. (B) Antibody levels according to lesion number in Al Ahsa and Al Madinah. Control: healthy individuals; CL: active CL infection; CR: cured CL infection; OD: optical density. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

3.3.5 Visiting labour in the Al Ahsa, KSA exhibit a significantly higher antibody response to PpSP32 compared to residents in Al Ahsa

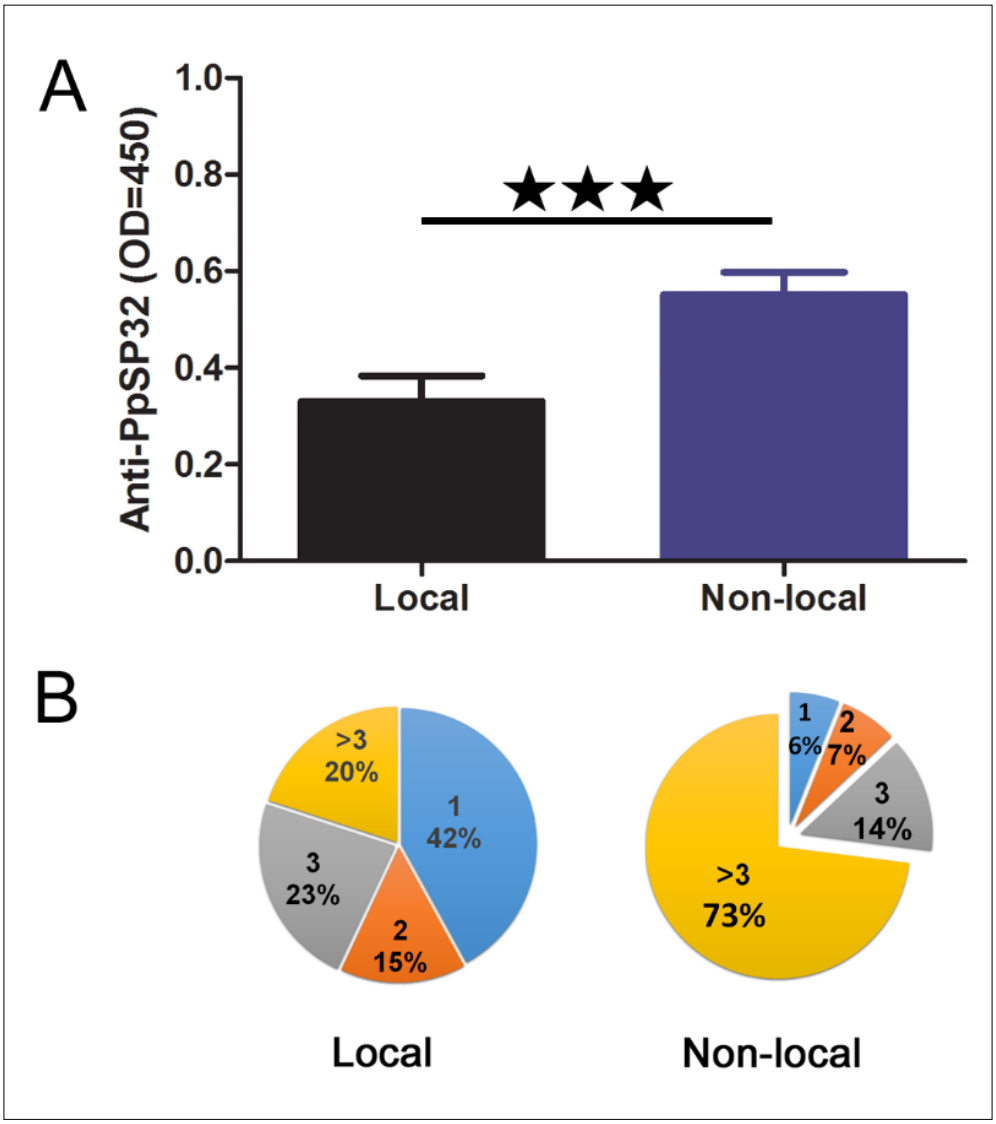


Figure 3.6. Differential antibody response to PpSP32 between local and non-local patients in Al Ahsa. (A) Comparison of anti-PpSP32 antibody levels in local and non-local ZCL patients from Al Ahsa. (B) Comparison of lesion numbers in local residents and non-local ZCL patients in endemic area Al-Ahsa. OD: optical density. *** $P \leq 0.001$.

In Al Ahsa, we found that non-local patients (visiting labour) had significantly higher levels of anti-PpSP32 compared to the local residents (P<0.001; Figure

3.6A). Interestingly, nearly three quarters of the non-local patients developed more than three lesions compared to only 40% in the local group (Figure 3.6(B)). Although such differences did not correlate with the anti-PpSP32 levels, patients from the visiting labour group presented a higher number of lesions in general compared to the residents.

3.4 Discussion

Antibodies to sandfly saliva can be used to indicate disease risk in CL endemic areas (Gomes and Oliveira, 2012, Marzouki et al., 2011, Rohousova et al., 2005, Barral et al., 2000), and the development of biomarkers for this purpose depends on the discovery of highly conserved yet species-specific molecules. SP32-like proteins are unique to sandflies and occur in all species studied to date (Rohousova et al., 2012). Among these, PpSP32 is a highly immunogenic protein isolated from *Ph. papatasi* saliva which serves as a biomarker for vector exposure (Marzouki et al., 2012). Data obtained from a CL-endemic area in Tunisia showed that the human antibody response to PpSP32 is representative of the humoral response against whole salivary gland extract (Marzouki et al., 2011). Here, a recombinant form of this protein was used to evaluate the level of exposure to sandfly saliva in three endemic areas in the KSA. The results show that the severity of human CL pathology appears to be influenced by previous exposure to sandfly bites.

Currently, massive forced displacement in the Middle East as a consequence of civil war in Syria, Libya, Iraq, Lebanon and Yemen (as mentioned in Chapters 4) has resulted in several CL outbreaks in those countries and in countries where the forced migrants have been displaced in refugee camps in Turkey, Jordan and Egypt. Furthermore, migration of non-immune people to leishmaniasis-endemic areas is known to affect groups such as civilian workers and military personnel (Weina et al., 2004, Aagaard-Hansen et al., 2010), resulting in leishmaniasis outbreaks (World Health Organization, 2010).

Evaluation of biting exposure can be useful for assessing disease risk of such populations in the KSA. The higher serum levels of anti-saliva antibodies in the visiting workers compared with the long-term residents of Al Ahsa suggest the migrant population is highly exposed to sandfly bites and less immune to CL. Residents have a lower (but continuous and long-term) exposure to bites, which might induce desensitisation (tolerance) to sandfly saliva, thus explaining their lower antibody levels compared to the non-locals. This desensitization after long term exposure has been previously observed in mice models (Rohousova et al., 2011). Moreover, the residents seem to suffer less severe leishmaniasis lesions.

Exposure to uninfected bites of *Ph. papatasi* has been shown to be protective against *L. major* in mice (Kamhawi, 2000) and whether the same level of protection is conferred to humans in CL-endemic areas remains to be determined.

Non-locals typically work and dwell closer to sandfly habitats, such as the burrows of rodents (reservoirs of disease), and are consequently plagued by biting sandflies. Previously unexposed to this level of biting, they showed a more intense antibody response over a shorter period of time. The high exposure to sandfly bites might increase susceptibility to infection and severe clinical outcomes as nearly three quarters of them developed multiple lesions. Other factors, such as genetic background, can also influence susceptibility to disease (Sakthianandeswaren et al., 2009); however, this is unlikely in this situation as the visitors originated from eight different countries, mainly from the Middle East, Southern Asia and Africa.

Interestingly, CL patients from both ZCL regions (Al Ahsa and Al Madinah) exhibited even higher levels of anti-PpSP32 antibodies compared to healthy residents from their respective areas. (Marzouki et al., 2011) previously investigated this relationship using whole salivary gland extract and associated the significantly higher antibody levels in ZCL patients with increased risk of developing CL. This difference was also reported for ACL (Rohousova et al., 2005), where exposure to *Ph. sergenti* bites was evaluated in both healthy individuals and patients with *L. tropica*. Similarly, ACL patients produced a significantly higher IgG response compared to healthy people from the same area, likewise supporting the relationship between exposure and leishmaniasis infection.

Sandfly identification was performed for three CL endemic areas; Al Ahsa, Al Madinah and Asir in order to complement the data obtained on bite exposure. In agreement with the anti-PpSP32 levels in patient sera, most sandflies found in Al Ahsa and Al Madinah were identified as *Ph. papatasi.* Other sandfly species identified belong to the *Sergentomyia* genus, whose members rarely bite humans (they are mostly zoophilic) and have been shown to be refractory to *Leishmania* species pathogenic to humans (Sadlova et al., 2013). *Ph. papatasi* accounts for most, if not all, of the bites sustained by individuals in the ZCL areas. This was further supported by finding significant levels of anti-PpSP32 antibodies in healthy donors of these regions compared to UK control sera. However, anti-PpSP32 antibodies were significantly higher in Al Ahsa, suggesting a higher exposure to *Ph. papatasi* in this region.

Unexpectedly, sera from *L. tropica* patients from the Southwest region of Asir (where *Ph. sergenti* is the predominant CL vector) also recognized PpSP32, although levels were much lower compared with ZCL patients. This could be due to a cross reaction with salivary proteins from *Ph. sergenti*. In fact, there is a high degree of similarity (52%) between *Ph. sergenti* SP32-like protein and *Ph. papatasi* SP32. In mice exposed to *Ph. sergenti* bites, a partial cross-reactivity to *Ph. papatasi* whole salivary gland homogenate was reported (Drahota et al., 2009, Rohousova et al., 2005). A similar level of cross-reactivity could also be present between salivary proteins from *Ph. papatasi* and *Ph. bergeroti* (Fryauff and Hanafi, 1991) (the second most abundant species in Asir). Moreover, reports

issued from Asir Regional Health stated that *Ph. bergeroti* is a possible vector of *L. major* in Tehama, Southwest region (Saudi Ministry of Health Report, 2013).

The immune response elicited by sandfly salivary proteins and how it modulates the *Leishmania* infection, varies depending on the vector species and vertebrate host (Ockenfels et al., 2014). Some reports have shown that sandfly saliva is able to preferentially trigger a protective Type I delayed-type hypersensitivity response (Oliveira et al., 2013b, Gomes et al., 2008, Gomes et al., 2012). In animal models a Th1 response to salivary proteins is correlated with protection against CL, and immunization with single proteins from sandfly saliva conferred protection against a *L. major* infection when animals were challenged with infectious *Ph. papatasi* bites (Collin et al., 2009, Zahedifard et al., 2014, Gomes et al., 2012). On the other hand, a Th2 response (and antibodies to salivary proteins) correlates with higher susceptibility and, in some cases, exacerbation of the disease (Oliveira et al., 2008, de Moura et al., 2007). Furthermore, individuals living in a CL endemic region of Tunisia, where the main vector is *Ph. papatasi*, developed a mixed response with a dominance of Type II immunity (Abdeladhim et al., 2011). It may be possible that subjects that develop antibodies (in a Th2 environment) to PpSP32 (and perhaps other salivary proteins) may be more susceptible to CL. It would be relevant to characterize the immune response(s) in individuals with different clinical presentations and from different geographical locations.

In summary, the use of recombinant salivary proteins can help us understand the impact of natural exposure to sandflies in leishmaniasis endemic areas (Oliveira et al., 2013a). Our results provide insights into the relationship between the human antibody response to sandfly saliva and development of CL in different transmission contexts. In addition, they support the use of biomarkers as epidemiological tools to improve the surveillance of human-vector contact and disease transmission. This type of biomarker is needed particularly in areas of conflict, such as the Middle East and North African countries, which are reported to be highly endemic for CL (Jacobson, 2011). Army troops and NGOs deployed to these countries are highly affected by CL due to a lack of immunity of individuals to CL, as mentioned earlier. A biomarker could give an estimation of the exposure level before any CL disease outbreaks could occur.

Chapter Four. Old World CL outbreak management

4.1 Introduction

Cutaneous leishmaniasis (CL) is one of the world's most neglected tropical diseases (Antinori et al., 2012, Salam et al., 2014, Alvar et al., 2012a) and is transmitted by the phlebotomine female sandfly. Between 1.5 and 2 million new CL cases are reported annually. Of these cases, 90% occur in seven countries in quite disparate regions, namely the Kingdom of Saudi Arabia (KSA), Peru, Iran, Afghanistan, Brazil, Algeria and Syria (Alvar et al., 2012a, Organization, 2010).

Recently, leishmaniasis vectors and CL reservoirs have spread to new foci in diverse regions in North Africa and the Middle East. This is as a consequence of civil war and human migration in several nations in the East Mediterranean Region (EMRO), including Afghanistan, Syria, Yemen, Iraq, Libya and Lebanon (Alasaad, 2013, Hotez et al., 2012, Salam et al., 2014, Jacobson, 2011, Saroufim et al., 2014, Sharara and Kanj, 2014, Wallace et al., 2002, van Thiel et al., 2010). The subsequent mass human displacements triggered by these conflicts have been responsible for the current spread of CL to new foci (Koltas et al., 2014, Saroufim et al., 2014). Since the beginning of the civil war in Syria, approximately 2.7 million Syrians have become refugees in Turkey, Lebanon, Jordan, Iraq and Egypt. Moreover, 4.1 million Syrians have been internally displaced within Syria (http://syrianrefugees.eu/). Another consequence of these conflicts is the lack of leishmaniasis control activities, which can further exacerbate CL spread.

Following the construction of the Sidi Saad Dam in Tunisia, a four-fold increase in CL cases occurred. Irrigation may create conditions favourable to the establishment of new CL foci by changing the surrounding environment (Salah et al., 2007, Kamhawi et al., 1993, Ben Salah et al., 2000, Aoun and Bouratbine, 2014b). Soil erosion has been prevented by tree plantation in the Kashan region of Isfahan, Iran. However, this has created new foci for the rodent reservoir, *Rhombomys opimus*, resulting in a CL outbreak with a reported 8-15% incidence within the local population (Neouimine, 1996). *Leishmania tropica* (the causative agent of CL) cases have tripled in the West Bank and Northern Israel as a result of urbanization, population growth and agriculture (Faiman et al., 2013, Jacobson et al., 2003).

In 1992, Sri Lanka reported the first case of CL (Athukorale et al., 1992a). More recently, Sri Lanka reported more than 200 CL cases annually (Karunaweera and Rajapaksa, 2009). Several factors correlate with the increase of CL cases in Sri Lanka, including population movement, jungle clearing, military activity, large families, human behaviour and low socioeconomic levels (Ozbel et al., 2011a, Karunaweera and Rajapaksa, 2009). Notably, foreign troops stationed in the EMRO region tend to be highly affected by CL. This is probably due to a lack of exposure to both the parasite and sandfly vector. In Iraq in 2003, an estimated 2,500 overseas soldiers contracted CL (Faulde et al., 2010, Pages et al., 2010, Coleman et al., 2009). Also the International Security Assistance Force (ISAF) reported 200 CL cases at its military bases in Mazar-e Sharif, Afghanistan in 2005

(Faulde et al., 2008). New control measures, based on a systematic public health approach, are urgently required to minimize the impacts of conflict and mass migration on CL spread. In KSA, uncontrolled urbanization and labour migration have been suggested as the main factors responsible for new CL foci, which are exacerbated by the lack of a health impact assessment for the new settlements.

In the Al-Ahsa Governorate, CL outbreaks are under a very well-designed control programme, which has successfully reduced the number of reported cases from ~5,100 (in 1987) to less than 200 cases (in 2014) (Saudi Ministry of Health Report, 2013). However, outbreaks have been reported in areas that were not covered by the leishmaniasis control programme as a consequence of city expansions and human migration, with the result that half of those infected by CL are migrant workers. Therefore, a disease control strategy is urgently required to address CL outbreaks within migrant worker groups and others affected by urban expansion. In this chapter, a control strategy implemented to control a CL outbreak in Al Ahsa is presented.

4.2 Methodology

4.2.1 Study area

This study was performed in the Al-Ahsa Governorate, the largest oasis in the Arabic Peninsula. It is situated in Eastern KSA and has just over a million inhabitants, comprised of 80% Saudis and 20% non-Saudis. Regarding health infrastructure, the Al Ahsa Governorate currently has 18 hospitals and 68 primary

healthcare centres. There are also three clinics offering CL diagnosis and treatment.

4.2.2 Data and sample collection

This study was undertaken at a new construction site in a remote area of Al-Ahsa, which had not been previously assessed for CL by the local leishmaniasis control team. This region is known for being exclusively endemic for zoonotic CL (ZCL). Prior to the start of the leishmaniasis transmission season (~April 2012), 150 (migrant) construction labourers, with no previous history of CL, arrived at the construction sites. Demographic data was recorded from these recruited workers. On January 2013, ~60% of the construction labourers were reported to have acquired CL. Parasites were isolated from the skin lesions of 30 patients and the *Leishmania* spp identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the ribosomal Internal Transcribed Spacer 1 (*IST1*) as described Chapter 2 (el Tai et al., 2000, El Tai et al., 2001). All cases were referred to the nearest leishmaniasis clinic.

4.2.3 Determination of Sandfly exposure

To determine the risk of disease exposure, we measured the serum levels of anti-PpSP32 (*Phlebotomus papatasi* salivary protein 32) antibodies, a known *Ph. papatasi* saliva marker from either CL patients or control individuals (residents) from Umran and Rumailah in Al-Ahsa. Anti-PpSP32 IgG antibodies were measured by enzyme-linked immunosorbent assay (ELISA) as described in

Chapter 3 (Marzouki et al., 2012, Mondragon-Shem et al., 2015). The Kruskal-Wallis one-way analysis of variance test was used to validate the significance among serum samples. GraphPad Prism 5 (GraphPad Software, USA) was employed for anti-SP32 antibody data analysis.

4.2.4 Sandfly collection and identification

Sandfly vector species were collected using CDC light traps placed from 6:00 pm to 6:00 am around the temporary houses of construction site workers (see example in Figure 4.1). In addition, sticky traps were used to capture sandflies in rodent burrows and close to the labourers' accommodation. Collections were conducted between January and December 2012. Sandflies were preserved in 70% ethanol and species were identified by microscopy. Temperature, relative humidity and rainfall data were collected from the Central Department of Statistic and Information in KSA (http://www.cdsi.gov.sa/english/).

Figure 4.1. An example of sandfly collection from the CL endemic areas, Al Ahsa.

4.2.5 CL outbreak control strategy

The CL outbreak in the studied area was eliminated using an integrated control strategy. Mechanical control involved removal of *Hamada elegans* to eliminate rodent (reservoir) food sources and prevent migration of new animals into this area. Furthermore, rodent burrows within a radius of 500 m around the construction area were destroyed. For sandfly control, thermal fogging using deltamethrin (1/240) and insecticide residual spraying using lambda-cyhalothrin (20 gm/ 10L) were employed (Feliciangeli et al., 2003, Desjeux, 1996, Organization, 2010). Additionally, treatment was applied to all patients after patient referral to the leishmaniasis clinic at Eastern Leishmaniasis and Dermatology Clinic for appropriate treatment. Outbreak management team members from Ministry of Agriculture and the Municipality were observed by the vector control team and managed by the highest authority at Al-Ahsa Mayor (Figure 4.2).

4.2.6 Data analysis

IMB SPSS Statistics 21 was employed for all data analysis. Software ArcGIS 10 (ESRI, Redlands, CA) was used to map both the distribution of *Leishmania* and sandfly species across the construction site and throughout the entire Al-Ahsa governorate. Data were presented in relation to elevation based on gridded Global Relief Data (ETOPO2), which is based on the geographical coordinates (latitude and longitude) of each site.

4.2.7 Ethics

Ethical approval was obtained from both the Liverpool School of Tropical Medicine, UK (12.03RS) and the Saudi Ministry of Health Ethical Committees. Written consent was obtained from patients.

4.3 Results

Over the course of 2013, several governmental sectors of the Al-Ahsa Governorate, including the Ministry of Health, Ministry of Agriculture and the Municipality, designed an integrated control strategy to contain the reported CL outbreak in the study area. The strategy consisted of a combination of rodent and vector control methods, as well as treatment of patients with CL (detailed in Figure 4.2).

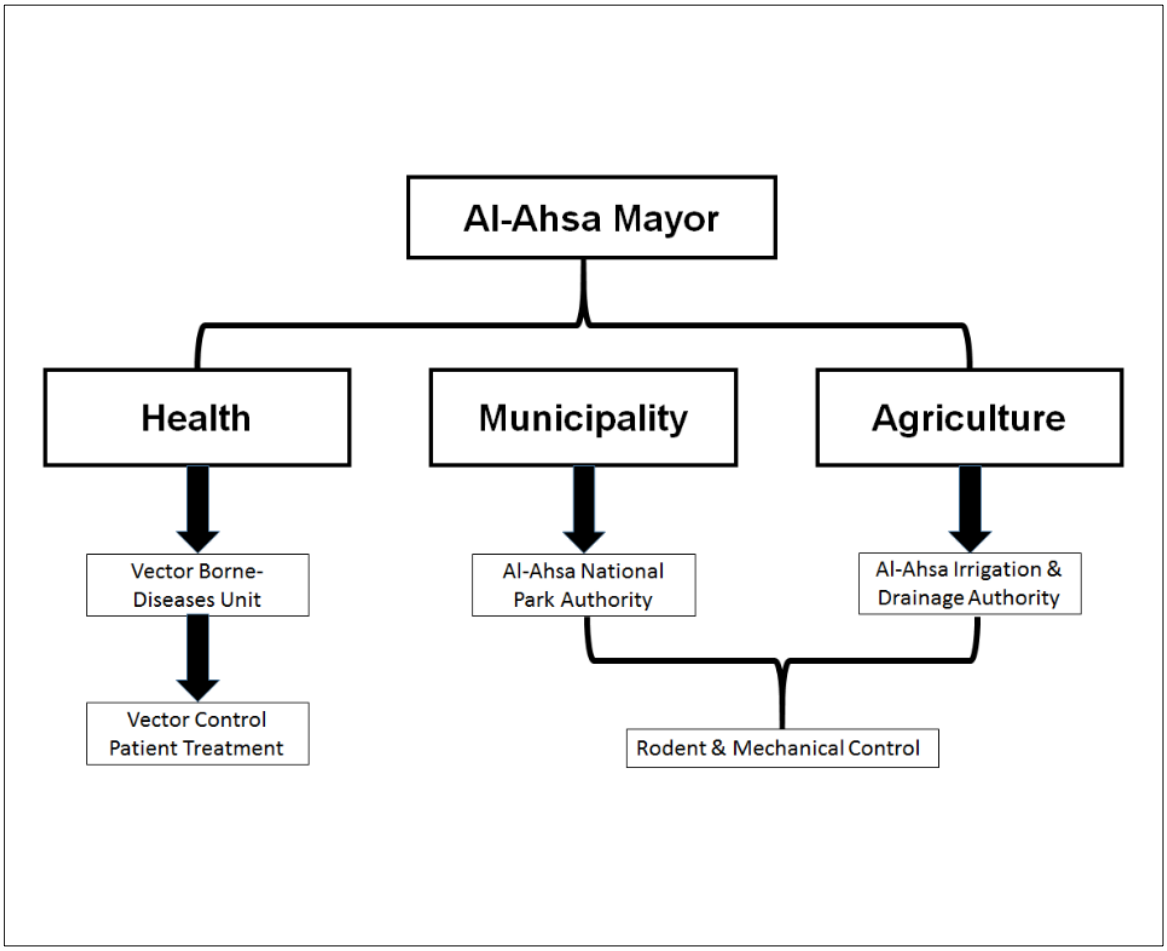


Figure 4.2. Disease control strategy plan under supervision of the governorate mayor. All governmental sectors are integrated in the disease control strategy and committed to this work.

4.3.1 Patient data and *Leishmania* parasite species

Ninety patients with clinically confirmed CL were among the 150 construction workers, and 30 of these patients were recruited for parasite isolation from skin lesion using the skin aspiration method. Using PCR-RFLP it was confirmed that *L. major* was the only parasite responsible for CL cases (Figure 4.3). Multiple nodular lesions were found on more than half the patients at the construction site and also had a developing secondary infection (SI). Satellite lesions were noticed

in more than 30% of the recruited patients. Moreover, recruited patients showed no resistance to sodium stibogluconate (Sb), regardless of whether they had a SI or not.

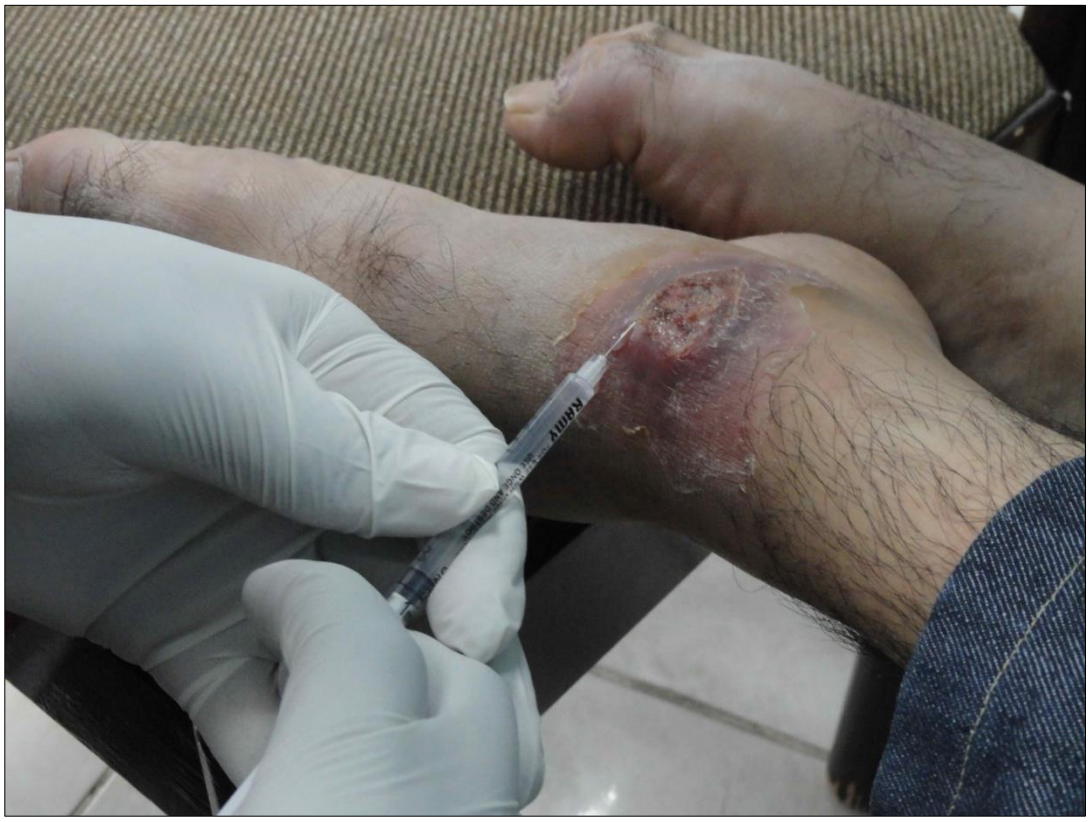
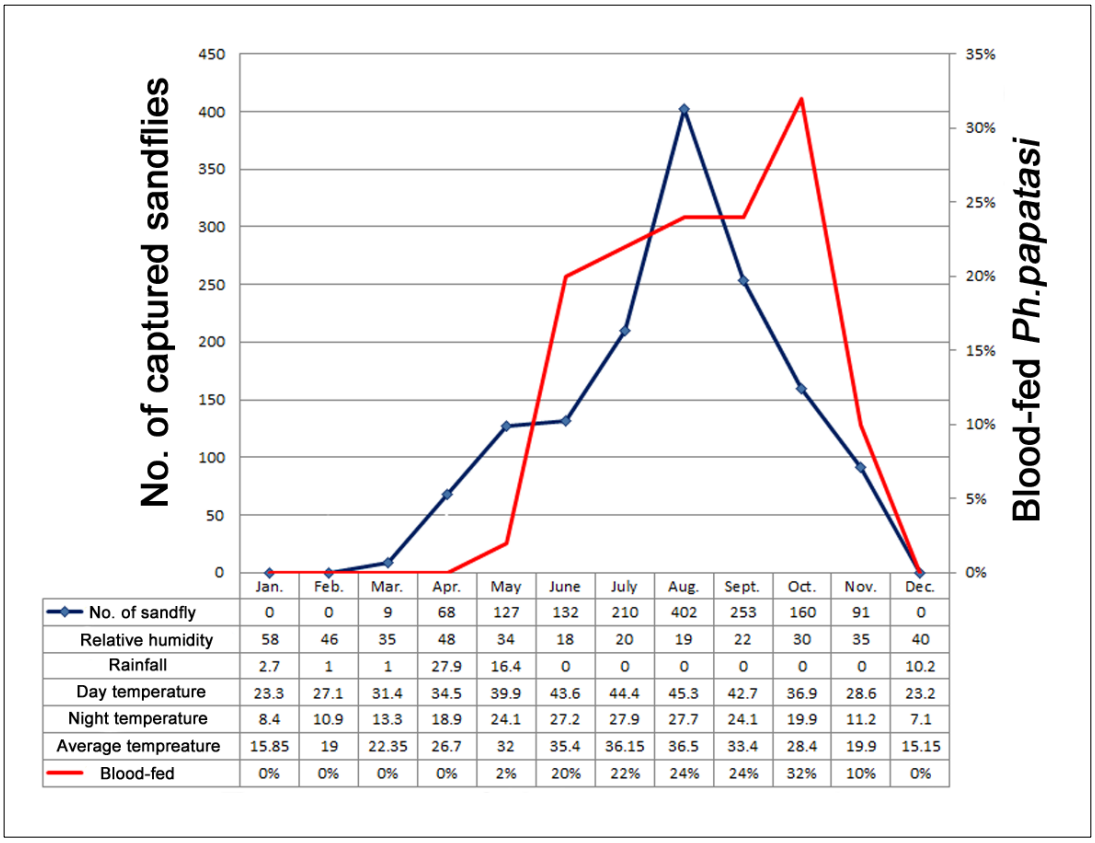


Figure 4.3: Skin aspiration for *Leishmania* isolation and subsequent in vitro culture

4.3.2 Sandfly identification

Ph. papatasi was the only medically important sandfly vector found both indoors and outdoors in Al Ahsa. *Ph. papatasi* were observed between March and October 2012 and at particularly high density between June and September. Blood feeding was reported between May and November, when relative humidity is between 18-35%, and the average temperature is between 22°C and 37°C,

which are very suitable conditions for sandfly feeding and CL transmission (Figure 4.4).



	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
No. of sandfly	0	0	9	68	127	132	210	402	253	160	91	0
Relative humidity	58	46	35	48	34	18	20	19	22	30	35	40
Rainfall	2.7	1	1	27.9	16.4	0	0	0	0	0	0	10.2
Day temperature	23.3	27.1	31.4	34.5	39.9	43.6	44.4	45.3	42.7	36.9	28.6	23.2
Night temperature	8.4	10.9	13.3	18.9	24.1	27.2	27.9	27.7	24.1	19.9	11.2	7.1
Average tempreature	15.85	19	22.35	26.7	32	35.4	36.15	36.5	33.4	28.4	19.9	15.15
Blood-fed	0%	0%	0%	0%	2%	20%	22%	24%	24%	32%	10%	0%

Figure 4.4: Sandfly population seasonality in Omran and Rumailah towns, Al-Ahsa. Sandflies were collected between January and December, and reached highest numbers between June and October. Blood-fed sandflies were collected between May and November.

Sandflies were captured in the construction workers' accommodation and in the area around the construction sites where *Psammomys obesus* burrows were found. The location of the construction site (close to Al-Asfar Lake and less than 1 km from date palm trees) assisted sandfly collection as the required humidity, temperature and sugar meal availability were present.

4.3.3 Disease exposure

The level of anti-SP32 antibodies was measured among 30 construction workers and 30 control individuals from the area where the leishmaniasis strategy was being implemented. The anti-SP32 antibody level of the construction workers at the construction site that has not been assessed by disease control team was between four and five times higher than that of people from the area that applied CL control measures (Figure 4.5).

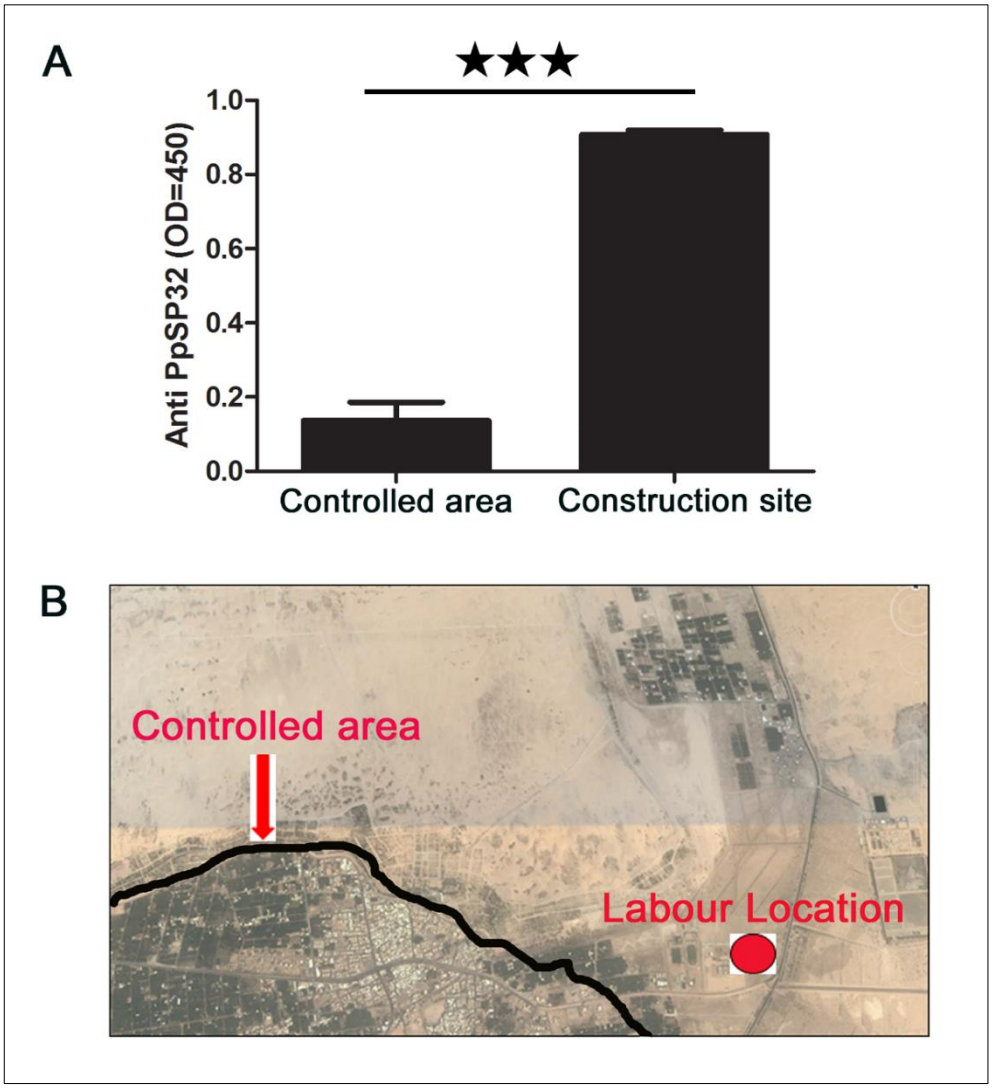


Figure 4.5: Sandfly biting exposure in Omran and Rumailah, Al-Ahsa compared with the labour construction site. (A) Anti-PpSP32 antibody levels of patients from the construction site were compared with those of patients from the controlled area in Omran and Rumailah. Anti-PpSP32 levels were found to be significantly higher in patients at construction site compared with those in areas under control measures (p>0.001). (B) Location of labour construction site and area under leishmaniasis control team assessment.

4.3.4 CL control team intervention

Interventions were introduced after the identification of parasite species as *L. major* and sandfly vector as *Ph. papatasi*, which indicated that the CL type

detected in the construction area was exclusively zoonotic. These interventions required rodent control, vector control and patient referral to a leishmaniasis clinic. These measures resulted in a massive reduction in the number of leishmaniasis cases reported at the construction site from 60% CL infection prevalence to zero cases between October 2013 and April 2014 (Figure 4.6).

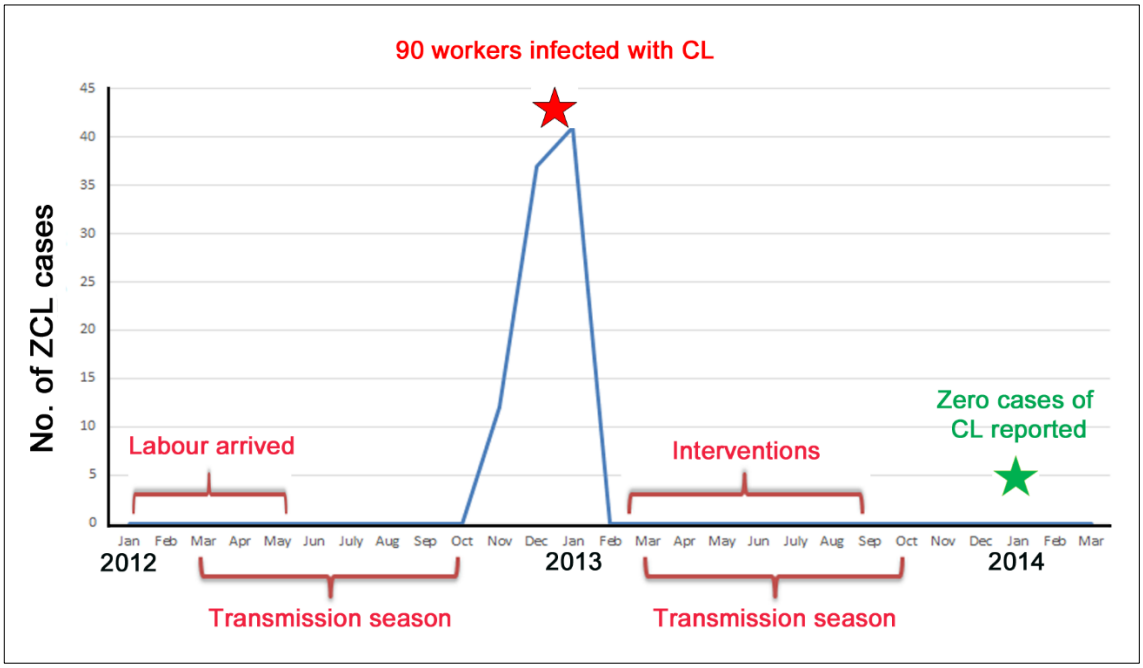


Figure 4.6: Disease outbreak control management timeline. Labour arrived to the construction site between January and March 2012 (i.e. prior to the CL transmission season from March to October). After the transmission season (March to October 2012), CL cases were reported between November 2012 and January 2013. The CL disease control interventions were implemented between March and October, and mechanical, reservoir and sandfly control measures were used. As a result of these interventions and continued control measures, the number of CL cases dropped to zero cases. The red star indicates the time point at which the highest number of CL cases were reported. The green star indicates the time point at which CL control was successful (i.e. the number of reported CL cases was zero).

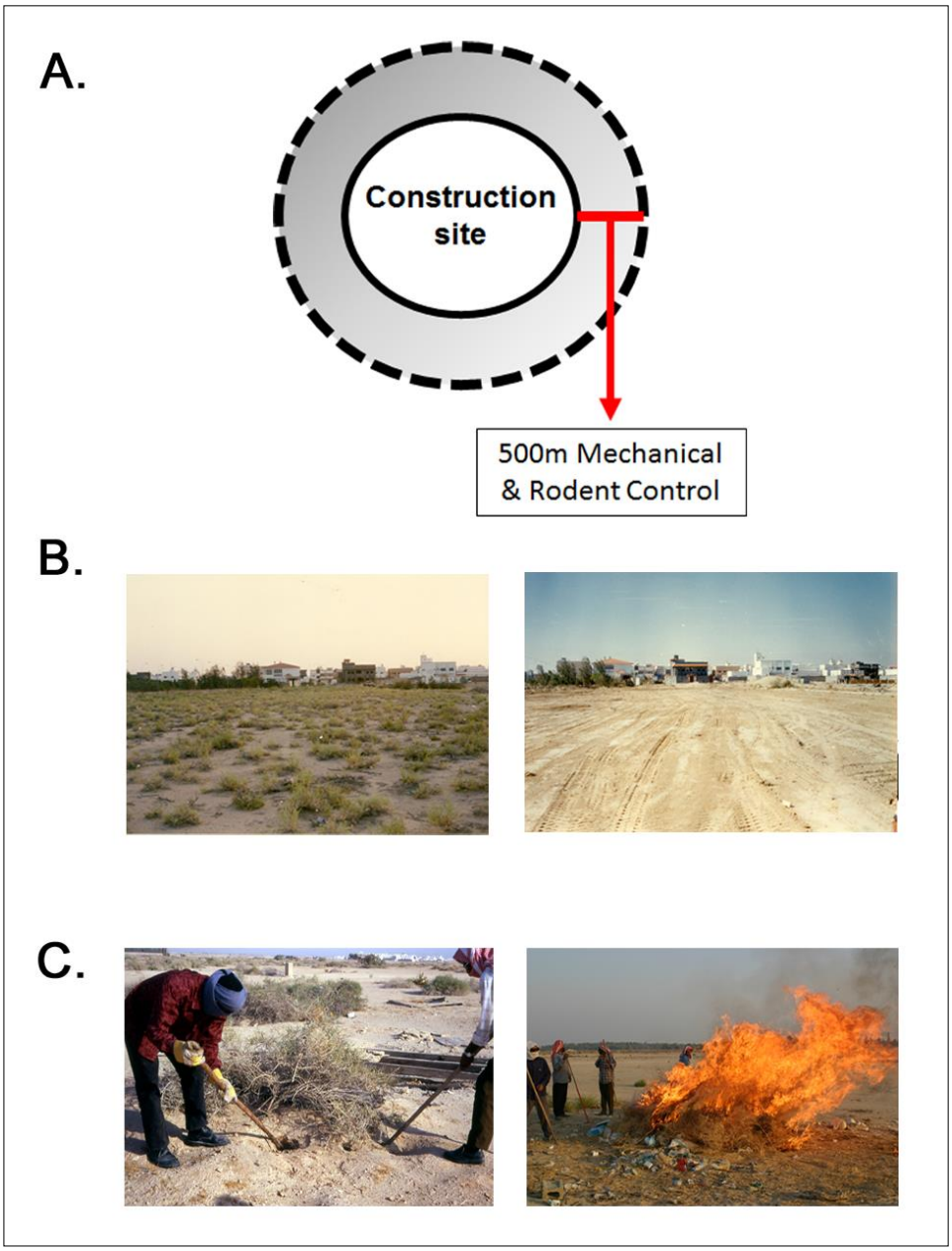


Figure 4.7: Mechanical control of the CL reservoir**.** (A) Distance targeted for mechanical control. Mechanical control was performed by destroying rodent burrows and removing *Hammada elegans*. (B) Area before (left panel) and after (right panel) *Hammada elegans* removal and burrow destruction. (C) Burning of *Hammada elegans* by seasonal leishmaniasis control team workers.

4.4 Discussion

As a consequence of various factors, including urbanisation, agricultural projects, deployment of troops throughout the Middle East and Central Asia, civil war and low socioeconomic levels, CL has spread to new foci and has expanded to new countries with no previous experience of the disease (Jacobson, 2011, Saroufim et al., 2014, Sharara and Kanj, 2014). Due to the lack of a protective vaccine, the current high toxicity of anti-*Leishmania* drugs and an increase in the number of cases unresponsive to CL drug treatment at several locations, a new strategy for short-term action is urgently required to minimise the impact of CL outbreaks that have resulted from a lack of health impact assessment and human movement.

Several vector control measures are applied to combat CL in the Middle East. Setting of deltamethrin impregnated curtains on house walls, traps inside chicken coops, and areas close to sandfly populations reduce vector density, while indoor house sprays are effective in preventing indoor biting, and prevention of anthroponotic CL in particular (Desjeux, 1996, Feliciangeli et al., 2003, Noazin et al., 2013, Moosa-Kazemi et al., 2007, Orshan et al., 2006). In this case study, lambda-cyhalothrin was applied as an insecticide residual spray in construction site areas. Deltamethrin has been heavily used for thermal fogging of areas of high rodent infestation and within palm date agricultural projects in combination with mechanical control.

Vector control is one measure that is crucial for CL control (Organization, 2010, Davies et al., 2000, World Health Organization, 2010). Active and passive surveillance is needed to identify human CL cases in peripheral areas that are newly settled at Al-Ahsa, as well as new construction sites. All new CL cases can then be referred to leishmaniasis clinics for treatment. Vector and reservoir surveillance is important to prevent a future CL outbreak incident (Kassi et al., 2008). Mechanical control by eliminating the food source (*Hammada elegans*) of *Psammomys obesus* and burrow destruction are essential measures to combat ZCL in Al-Ahsa (Figure 4.7). A combination of these strategies should prove particularly effective and will be very important to resolve the ongoing problem of ZCL (Organization, 2010, Elbihari et al., 1987, Dye et al., 1989, Al-Mohammed, 2010).

CL outbreaks resulting from labour migration, agricultural projects and city expansion still require further attention. Our case study offers one short-term resolution. There is an urgent requirement for insecticide resistance assessment to develop vector control and also to assess treatment efficacy in KSA. Moreover, carrying out a health impact assessment prior to any city expansion projects will help to minimise risk of CL outbreaks. Additionally, the involvement of decision-makers is crucial to organise CL control. In our case study the sectors of the Ministry of Agriculture, Ministry of Health and the Municipality worked together to control the CL outbreak at Al-Ahsa.

It is also necessary to re-assess and evaluate the leishmaniasis control programme from the perspective of the health system. For instance, the reporting system in most provinces needs improvement. In Al-Ahsa, where this case study took place, reorganisation of the CL control programme is required in terms of the reporting system. Also, linking of municipality and agricultural projects to the vector-borne disease unit is needed to assess these projects prior to establishing any new construction work. Among KSA agricultural provinces where CL outbreaks have occurred, integration of control strategy can minimise outbreaks in agricultural projects and city expansions.

The recommendations from our case study could be implemented in areas that share a similar CL ecology and environment, and also contain *Psammomys obesus* as a host species. Importantly, several outbreaks have recently occurred in the EMRO region, specifically at refugee camps within Syria and in neighboring countries as a consequence of a lack of health impact assessment. This has resulted in some camps being established in ZCL areas. In 2013, over 100,000 cases were reported in a range of Syrian provinces (Saroufim et al., 2014, Sharara and Kanj, 2014, Hotez et al., 2012). Moreover, several CL outbreaks were reported in neighboring countries, including Lebanon, Turkey, Jordan, Iraq and Afghanistan (Bailey et al., 2012, van Thiel et al., 2010, Faulde et al., 2010, Pages et al., 2010).

Additionally, troops deployed throughout the Middle East and Central Asia, where similar situations might arise, could benefit from this case study. This case study recommends that a health impact assessment be carried out, followed by rodent control and vector assessment within 500m, prior to the establishment of troop camps. Recently, Al-Ahsa Governorate reported the lowest number of CL cases since the CL control programme was established in 1987 (Saudi Ministry of Health Report, 2013). Only 196 cases were reported in 2014 compared with over 5000 reported cases in 1987. The new strategy could assist populations at risk of CL and located at the periphery of Al-Ahsa Governorate. Also it will reduce the number of CL cases among new construction and agricultural projects.

Chapter Five: Developing a rapid diagnostic test for Old World cutaneous leishmaniasis based on sugar coat

5.1 Background

The surface of the *Leishmania* parasite is composed of abundant glycoconjugates of different natures. The structure and properties of these molecules are described in Chapter 1. Some of these glycoconjugates are highly immunogenic to the human immune system because they contain terminal alpha-gal residues (see 1.6 for the relevance of this sugar epitope in humans). During an infection with *L. major* (McConville et al., 2002) and several other New World *Leishmania* species (Avila et al., 1991, Avila et al., 1989), parasite specialized glycolipids called GIPLs (see 1.5.2) appear to be the main surface galactosylated molecules and thus partially responsible for the strong anti-α-Gal response in infected patients. The immune response to the α-gal epitopes can be exploited to design novel diagnostic tools for the detection of Old World CL infections. It can also present an alternative way to generate a novel glycovaccine against these diseases.

In this chapter, I describe the development of a diagnostic test using a chemiluminescent enzyme-linked immunsorbent assay (CL-ELISA) and report that patients infected with either *L. major* or *L. tropica*, from different endemic regions of KSA, have elevated levels of serum Leish α-Gal antibodies. The CL-ELISA assay is highly sensitive and specific for CL diagnosis. In addition, I discuss

the possible implementation of this assay in a CL elimination setting, such as KSA.

The diagnosis of CL in the KSA relies solely on examination of the patient's skin. This method fails to detect the disease in a considerable number of patients and does not permit identification of the causative parasite species. Therefore, sensitive and specific molecular and serological approaches are needed. The CL-ELISA assay could be very useful for both active and passive surveillance in a CL elimination setting and, importantly, potentially adapted as rapid diagnostic test for its implementation in poor resource settings.

5.2 Materials and Methods

5.2.1 Ethical approval

Ethical approval was obtained from both from the Liverpool School of Tropical Medicine (12.06R) and the Saudi Ministry of Health Ethical Committees. Written consent was obtained from patients in the ethical approval rubric.

5.2.2 Sample collection and sites

Sera samples (n = 346) were collected in between 2011 and 2012 from five CL endemic regions: Al Qassim, Al Ahsa, Riyadh, Asir and Al Madinah. The cohort was divided into three groups: healthy individuals from endemic regions (HIER; n = 150); patients with active CL lesions (ACLL; n = 100); and cured CL patients (CR; n = 100), including both drug-treated (n = 95) and self-cured (n = 5) patients.

Cure or healing was defined as lesion size reduction, and induration or crusting. Samples were taken at either the leishmaniasis clinics or in the field with the help of Ministry of Health leishmaniasis control team members. The arms, feet, legs, hands, neck, and face of HIER volunteers were medically examined to ensure there was no previous CL infection. In 2013, samples were collected from an additional 104 patients from the same endemic regions as above and used for skin biopsy, skin aspiration and serological assays. This latter cohort was characterized in detail in another study (see Chapter 2).

5.2.3 Parasite collection and *Leishmania spp*. identification

Leishmania species were identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the ribosomal Internal Transcribed Spacer 1 (*ITS1*) as described previously (el Tai et al., 2000). A detailed description of these analyses is presented in Chapter 2.

5.2.4 Microscopy analysis

Using a punch biopsy tool, samples for parasitological diagnosis were obtained from the edge of the lesion. The tissue sample was then transferred onto a clean microscope slide fixed in 10% buffered formaldehyde and processed for Giemsa staining. In addition, skin aspiration samples were taken and then cultured in M199 media containing Hanks medium (Life Technologies), 1% BME vitamins (Sigma-Aldrich), 20% heat-inactivated foetal bovine serum (HI-FBS) (PAA) and (1/400) gentamycin sulphate (Sigma-Aldrich) (Dougall et al., 2011). Growing

parasites were then isolated using DNeasy Blood and Tissue kit (Qiagen) and identified by PCR-RFLP (el Tai et al., 2000). Skin biopsies were collected for microscopy examination after Giemsa staining.

5.2.5 CL-ELISA for IgG antibody detection

To determine levels of *Leishmania* anti-α-Gal antibodies in human sera, 96-well microplates for CL-ELISA (Nunc) were coated overnight at 4°C with 50 µl of 5 µg/ml neoglycoprotein (NGP) Galα1-3Galβ1-4GlcNAc-BSA (Galα1-3LacNAc-BSA) (V-Labs), diluted in carbonate-bicarbonate buffer, pH 9.6. After washing several times with 200 µl PBS-0.05% Tween 20 (PBS-T) per well, wells were blocked with phosphate buffered saline (PBS)-1% BSA for 1 h at 37ºC. After washing, human serum samples (diluted 1/400 in PBS-1% BSA) were added and incubated at 37 ºC for 1 h. After three washes with 200 µl PBS-T, 50 µl goat anti-human IgG was added (1 µg/ml; Thermo Scientific), followed by 50 µl donkey anti-goat IgG-biotin conjugate (1 µg/ml; eBioscience). Streptavidin-horseradish peroxidase (1:2,000; Zymed), diluted in PBS-BSA 1%, was added for 1 h at 37°C, and after three washes with PBS-T, the reaction was developed by adding 50 µl Super-Signal (Pierce, Thermo) diluted 1:5 in 100 mM carbonate-bicarbonate buffer, pH 9.6. Plates were read in a Fluorostar Omega microplate reader (BMG Labtech) and values expressed as relative luminescent units (RLUs).

5.2.6 Coffee bean α-galactosidase (CBAG) treatment

For anti-α-Gal specificity (Figure 5.1(B)), 5 1 µg/ml of NGP Galα1-3LacNAc-BSA were treated overnight at 28 °C with 0.1 U per well of CBAG (Sigma). After incubation, the plates were washed several times with PBS-T and the CL-ELISA was performed as described above. A pool of 10 sera (diluted 1/400 in PBS-1% BSA) from HIEA, CL, CR and people with Chagas disease was used as primary antibodies.

5.2.7 CL-ELISA for IgM and IgA antibody detection

To determine the levels of anti-α-Gal IgM and IgA antibodies in human serum, samples were processed and analysed as indicated in Section 5.2.6, except that 50 µL (1 µg/ml) of biotin-conjugated goat anti-human IgM and 50 µL (1 µg/ml) (ABD Serotec) of biotin-conjugated goat anti-human IgA (Abcam) were used as secondary antibodies per well. Plates were read in a Fluorostar Omega microplate reader (BMG Labtech) and values expressed as RLUs. To minimize interplate variation, values were normalized by dividing the mean value of the triplicate of each serum sample by the mean value of the positive control (CR) triplicate in each plate.

5.2.8 Chemiluminescent (CL)-ELISA for IgG subclasses antibody detection

To determine levels of Leish anti-α-Gal IgG 1, 2, 3 and 4 antibodies in human sera, four 96-well microplates for CL-ELISA (Nunc) were coated overnight at 4°C with 50 µl of 5 µg/ml neoglycoprotein (NGP) Galα1-3Galβ1-4GlcNAc-BSA (Galα1-3LacNAc-BSA) (V-Labs) per well, diluted in carbonate-bicarbonate buffer, pH 9.6.

After washing several times with 200 µl PBS-0.05% Tween 20 (PBS-T), wells were blocked with phosphate buffered saline (PBS)-1% BSA for 1 h at 37ºC. After washing, human serum samples (diluted 1/300 in PBS-1% BSA) were added and incubated at 37ºC for 1 h. After three washes with 200 µl PBS-T, 50 µl HRP anti-human IgG1 (1 µg/ml; Biolegend), IgG4 (1 µg/ml; Biolegend) IgG2 (1 µg/ml Abcam) and IgG3 (1 µg/ml Abcam) were added in separate plates, followed by streptavidin-horseradish peroxidase (1:2,000; Zymed) diluted in PBS-1% BSA for 1 h at 37°C. After three washes with PBS-T, the reaction was developed by adding 50 µl Super-Signal (Pierce, Thermo) diluted 1:5 in 100 mM carbonate-bicarbonate buffer, pH 9.6. Plates were read in a Fluorostar Omega microplate reader (BMG Labtech) and values expressed as RLUs.

5.2.9 Neoglycoprotein screening methods

Neoglycoproteins were a kind donation of Prof Katja Michael (University of Texas at El Paso, USA). These compounds are a non-commercial synthetic library of BSA-based neoglycoproteins that contain different terminal sugar epitopes (Ashmus et al., 2013), and were used for measuring the specificity of the sera from infected, cured and healthy individuals from CL endemic regions. Sera from patients with active CL and cured individuals are able to recognise α-Gal epitopes on different oligosaccharidic structures. The CL-ELISA was used as described in Section 5.2.5, except that pools of sera (of randomly selected 10 patients, for each group) were used.

5.2.10 Parasite isolation and RFLP-PCR identification

Full description on the isolation and identification of parasites and patients' details are given in Chapter 2.

5.2.11 Statistical analysis

The Kruskal-Wallis one-way analysis of variance test was used to validate statistical significance among serum samples. GraphPad Prism 5 was employed for all data analyses.

5.3 Results

5.3.1 CL diagnostic tools comparisons

Serum, skin aspiration, and biopsy samples were collected from five CL endemic regions of KSA in two different cohorts. Identification of parasite species by RFLP-PCR analysis of the *ITS1* gene (Chapter 2) resulted in the detection of 92% and 91% of *L. major* and *L. tropica* cases, respectively. Compared to the results obtained by RFLP-PCR, we found that 95% and 91% of the *L. major* (95% CI; 88-94%) and *L. tropica* (95% CI; 92-97%) patients, respectively, had significantly high levels of *Leishmania* anti-α-Gal antibodies compared to HIEA (Table 5.1).

Sample	CL-ELISA	Microscopy	RFLP-PCR
L. major [A]	96%	68%	92%
L. tropica [B]	91%	45%	91%
Cured [C]	96%	NA [E]	NA
Healthy individuals[D]	4%	NA	NA

Table 5.1 Comparison of the results of CL-ELISA of anti-alphagalactosyl antibodies, microscopy and RFLP-PCR for detection of leishmania infection. A. *Leishmania* major-infected patients. B. *Leishmania tropica*-infected patients C. Cured individuals from Al Qassem, Al Madinah and Al Ahsa regions. D. Healthy individuals from a CL-endemic region (Al Ahsa). E. Not applicable.

Moreover, the CL-ELISA test had a high sensitivity than microscopy analysis of biopsy samples taken from the same cohort of patients, which only detected 68% and 45% of *L. major* and *L. tropica* infections, respectively (Table 5.1). Therefore, the CL-ELISA using Galα1-3LacNAc-BSA as antigen for detection of Leish anti-α-Gal IgG antibodies proved to be more sensitive than RFLP-PCR and microscopy analysis of biopsy samples. However, currently it is unable to discriminate between *L. major* and *L. tropica* infection.

5.3.2 CL-ELISA with different groups of individuals

CL-ELISA was performed using the NGP Galα1-3LacNAc-BSA as antigen. The levels of anti-α-Gal antibodies in patients infected with either *L. major* or *L. tropica* were determined. The mean anti-α-Gal IgG titres of individuals infected with either *L. major or L. tropica* were significantly ($p<0.005$) higher than those of HIEA

(Figure 5.1A and Table 5.1). Patients infected with *L. major* showed a 9-fold increase in antibody titres and patients with *L. tropica* had about an 8-fold increase in the mean *Leishmania* anti-α-Gal IgG titres when compared to HIEA. Strikingly, patients who responded to treatment and were considered CR (or self-healed) showed a 31-fold increase ($p \leq 0.001$) in the mean titres of anti-α-Gal antibodies (Figure 5.1A and Table 5-1). The CL-ELISA was specific for terminal αGal residues as treatment with coffee bean alpha-galactosidase, which cleaves all types of terminal α-galactosyl linkages, abolished recognition of the resulting NGP, LacNAc-BSA (Figure 5.1(B), (C)).

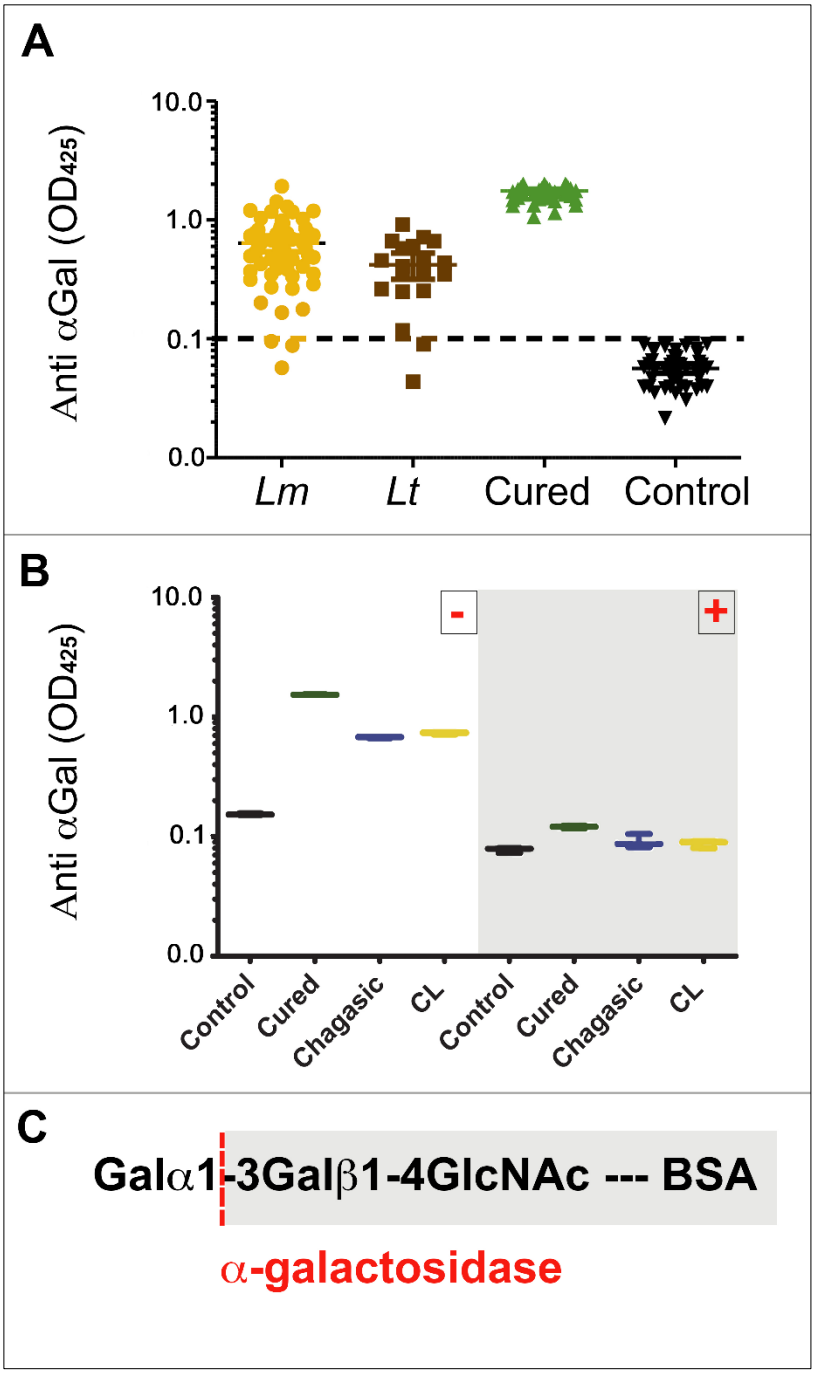


Figure 5.1 High levels of anti-α-Gal IgG in CL patients from KSA. (A) CL-ELISA comparing the levels of anti- α-Gal in healthy individuals (HIEA), *L. major*-infected patients (LM); *L. tropica*-infected patients (LT) and cured individuals (CR). P< 0·001 (***). (B) Pretreatment of NGP Galα1-3Galβ1-4GlcNAc-BSA with CBAG abolishes recognition by anti-α-Gal antibodies in pools of sera from different groups. CH, Chagasic patients. (C) Cleavage specificity of CBAG on an α-galactosylated NGP antigen.

5.3.3 IgA and IgM antibody levels

The anti-α-Gal IgM titres were found to be significantly higher (p≤0.01) in patients with active CL compared to either the control or CR patients (Figure 5.2(A)). Moreover, the levels of anti-α-Gal IgA antibodies were slightly higher in CL-infected individuals compared to either control or CR individuals (Figure 5.2(B)).

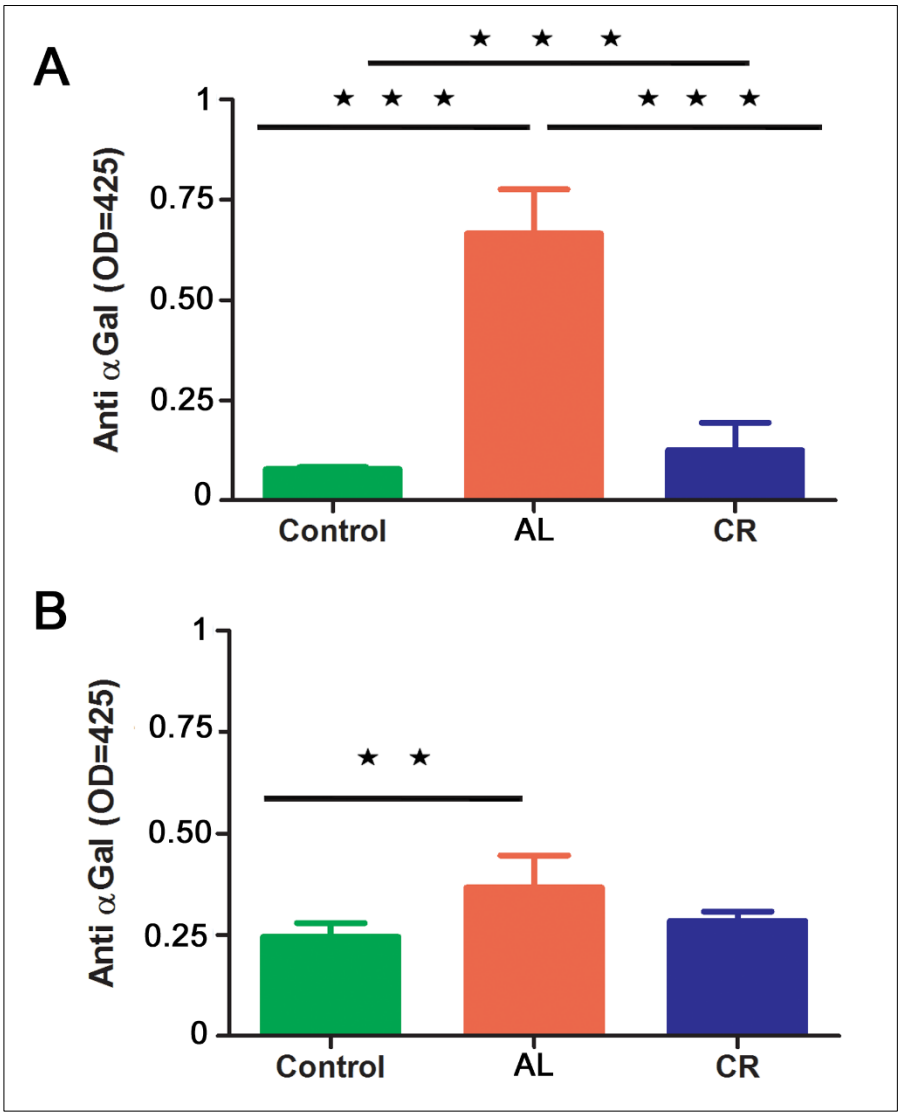


Figure 5.2 Levels of anti-α-Gal IgM and IgA using ELISA assay. IgM (Panel A) and IgA levels (Panel B) in CL patients from KSA. Control is healthy individuals from endemic areas; AL, *Leishmania*-infected patients; CR, CL cured individuals. P< 0·001 (***); P< 0·01 (**).

5.3.4 IgG subclass switching in *L. major* patients and leishmaniasis cured individuals

The IgG (IgG1-4) anti-α-Gal subclasses for *L. major* were determined by CL-ELISA. The anti-α-Gal subclasses of *L. major* patients switched from IgG1 and IgG3 with active lesions to IgG2 and IgG4 in patients cured of CL. Anti-IgG1 antibodies were significantly higher in patients with active CL infection compared to cure as shown in Figure 5.3 ($p \leq 0.01$). However, anti IgG2 and IgG4 antibodies are significantly higher within cured more than active lesion, as shown in Figure 5.3 ($p \leq 0.01$).

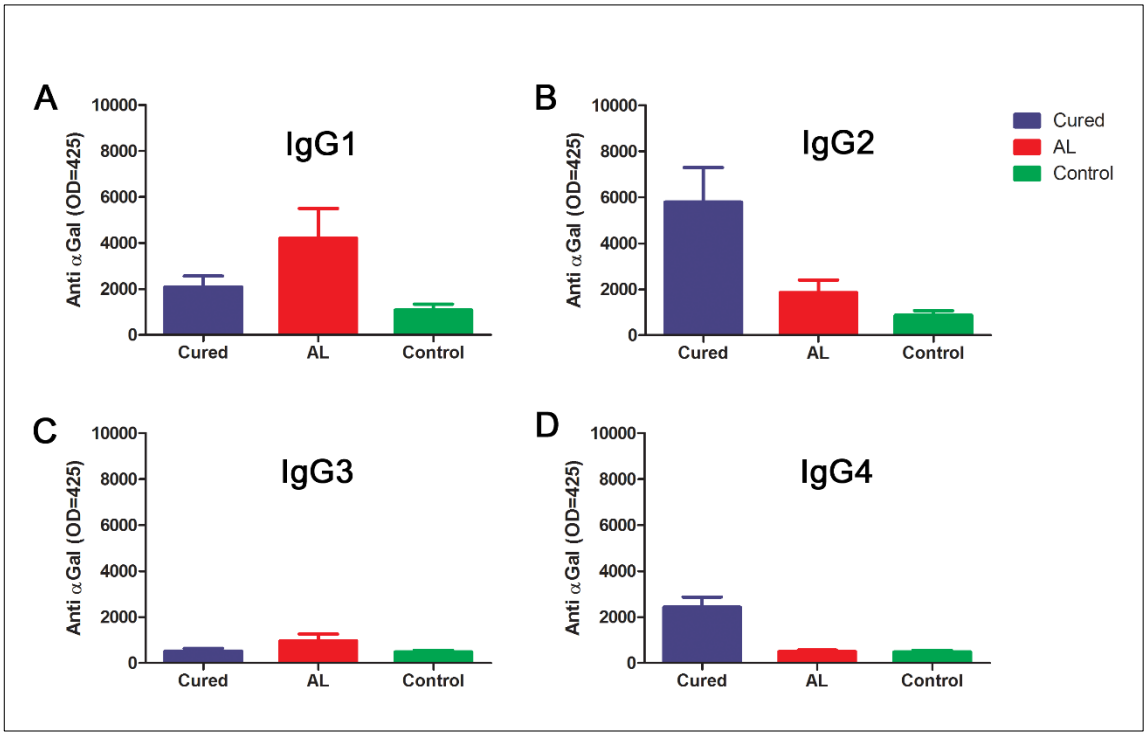


Figure 5.3 IgG subclasses for *L. major* patients, cured and healthy individuals in leishmaniasis endemic areas of KSA. Cured (n = 25); control is healthy individuals (n = 25); (AL) infected with *L. major* (n = 25). (A) The anti-IgG1 level was significantly higher in the AL group compared with cured and control groups ($p \leq 0.01$). (B) The anti-IgG2 level was significantly higher in the cured group compare with the AL and control groups ($p \leq 0.01$). (C) The anti-IgG3 level was notably higher in people with active infection (AL) compared to patients cured of CL. D) The anti-IgG4 level was significantly higher on cured compare to CL patients ($p \leq 0.01$).

5.3.5 Notable level of IgG (1-3) expression level in *L. tropica* patients

The expression of anti-α-Gal IgG isotypes was also determined in *L. tropica* infected patients and cured patients were assessed by CL-ELISA *L. tropica* patients showed high expression levels of anti-IgG (1-3) during active lesions. Anti-IgG (1-4) expression levels were very low in patients cured of *L. tropica*. However, anti-IgG4 antibody levels were slightly higher compared to IgG (1-3) within leishmaniasis cured compared to active lesion as shown in Figure 5.4.

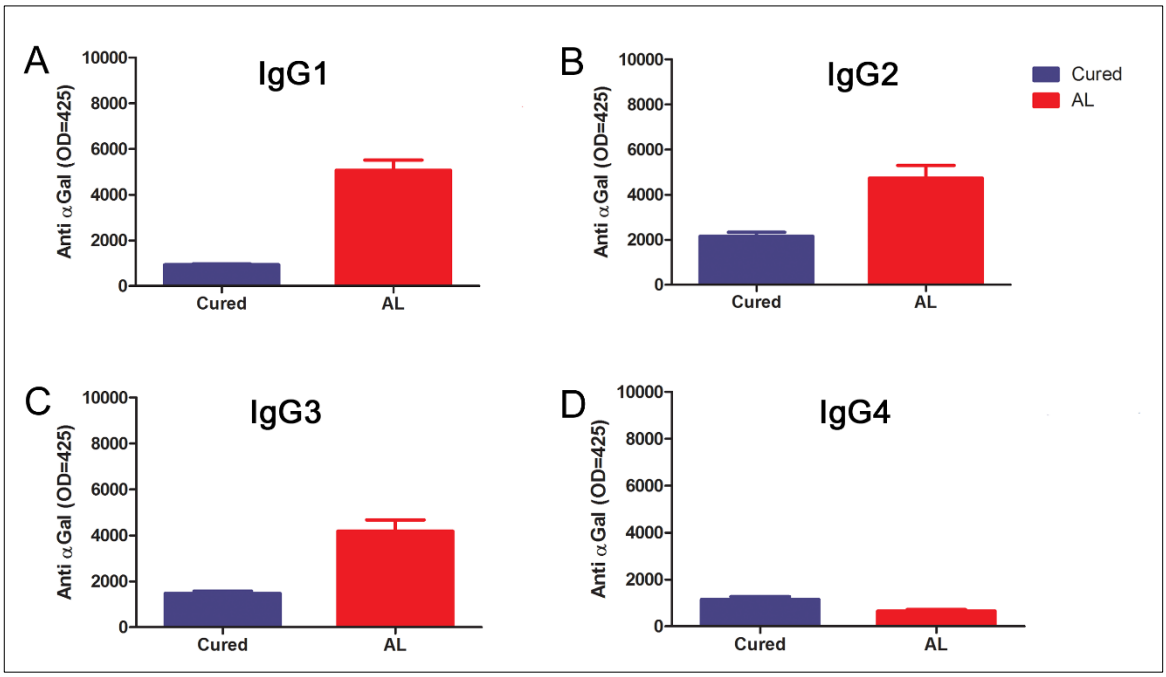


Figure 5.4 IgG subclasses for *L. tropica* patients and patients cured of CL. Cured (n = 5); (AL) infected with *L. tropica* (n = 15). (A) Anti-IgG1 levels were notably higher in AL patients compared to patients cured of CL. (B) Anti-IgG2 levels were notably higher in individuals with active CL lesions compare those cured of CL. (C) Anti-IgG3 levels were notably higher in people with active infection compared to those cured of CL. (D) Anti-IgG4 levels were slightly higher in cured compared to CL patients.

Unfortunately for these particular experiments there was no available serum from control (healthy) individuals from *L. tropica* endemic areas, although based on the results obtained with *L. major* patients (Fig 5.3), their anti-α-Gal levels were probably comparable to the cure group.

5.3.6 Refining anti-α-Gal specificity: screening of a novel library of α-galactosylated neoglycoproteins

The overlap in the high anti-Gal titre found between CL infected patients and cured individuals using the commercial Galα1-3LacNAc-BSA from V-Labs, could be a barrier for discriminating the infection between the two species. In addition, it could prevent its exploitation as potential biomarker for cure. Therefore, we contacted Prof Igor Almeida and Prof Katja Michael (UTEP, USA) who synthesized a non-commercial synthetic library of BSA-based neoglycoproteins (NGPs) that contain different terminal sugar epitopes (Ashmus et al., 2013). This library was successfully used to compare the specificity the Chagasic anti-Gal versus that of healthy individuals (Ashmus et al., 2013).

As shown in figure 5.5, the alpha-galactosylated NGP library enabled differentiation of sera from patients with active CL from sera of cured individuals, as well as to discriminate between *L. major* and *L. tropica* patients (Figure 5.5). NGPs with oligosaccharides terminating in Galα(1-3)Galα- and Galα(1-4)Galβ- were found to be good candidates for discriminating *L. major* infection from *L. tropica* infection, and also *L. major* infected from cured infections (Figure 5.6).

Moreover, the terminal branch Galα(1-6)[Galα(1-2)]Galβ- or single Galα- were good candidates for differentiating *L. tropica* infection from the other groups (Figure 5.6).

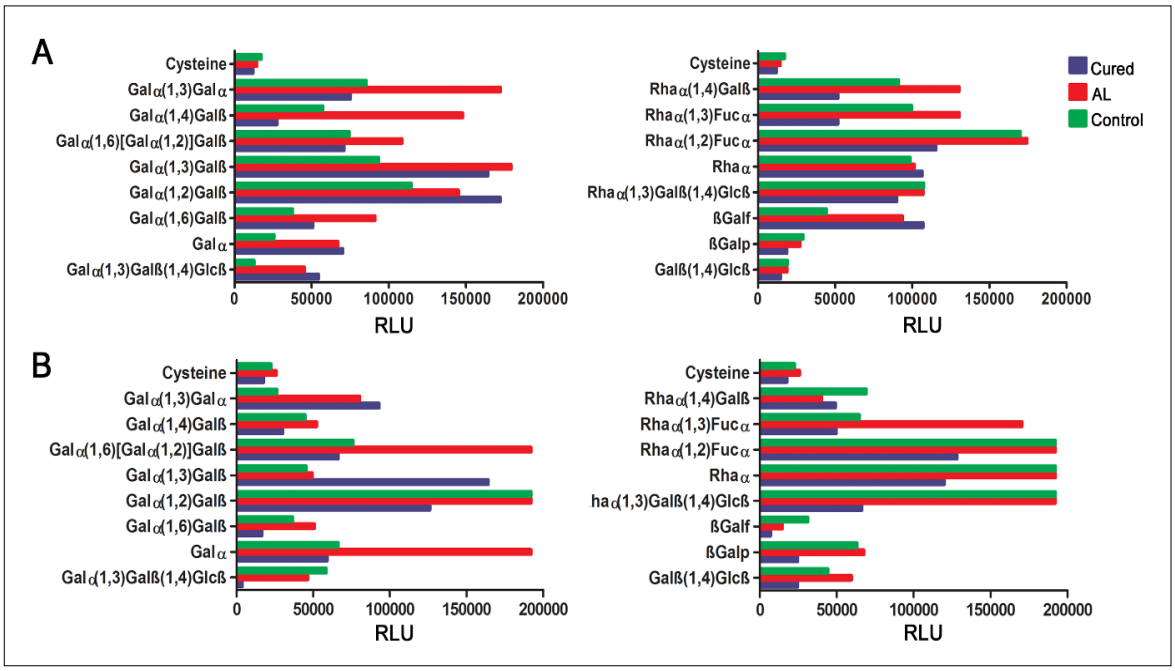


Figure 5.5. Screening of an alpha-galactosylated neoglycoproteins library with human sera from different groups. 17 neoglycoproteins were used to differentiate between **(A)** *L. major* patients **(B)** *L. tropica* patients. Cured (blue); active infection (red); control (green) in leishmaniasis endemic regions. (A) Cured, infected with *L. major* or healthy in *L. major* endemic areas. (B) Cured, infected with *L. tropica* or healthy in *L. tropica* endemic areas.

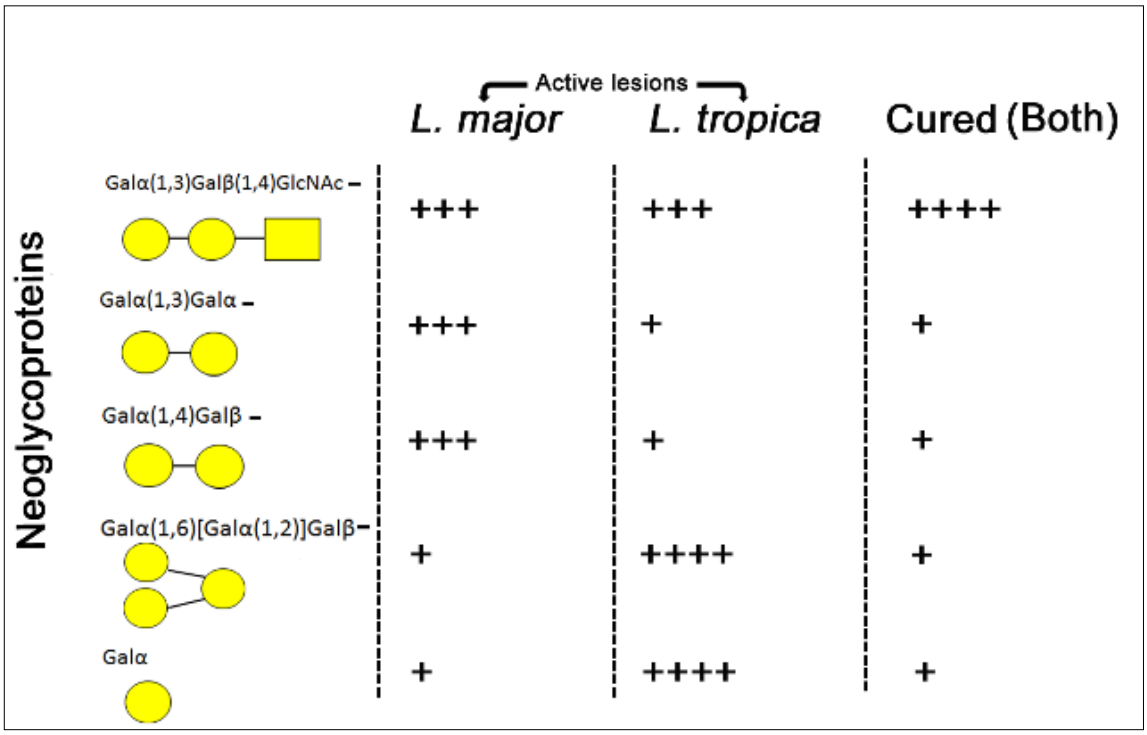


Figure 5.6 Summary of the best neoglycoproteins candidates that are selectively recognized by anti-Gal antibodies from CL infected patients and cured individuals. Glycans that could be suitable to apply as diagnostic tools for discriminating between cured CL infection and active CL infection with either *L. major* or *L. tropica*.

5.4 Discussion

Elevated levels of anti-α-Gal IgG levels have previously been detected in cases of infection with other kinetoplastid diseases, particularly in patients with Chagas disease (Avila et al., 1988b, Milani and Travassos, 1988, Gazzinelli et al., 1991, Almeida et al., 1991), or infection with either CL or VL in Latin America (Avila et al., 1988b, Avila et al., 1988a). Whereas the main target of the Chagasic anti α-Gal antibodies is a large family of surface mucin-like molecules (Almeida et al., 1994), *Leishmania* anti-α-Gal antibodies are believed to be produced by the expression of GIPLs containing terminal α-Gal residues. This is the case of *L.*

major GIPL2 and GIPL3, which contain oligosaccharides terminating in Galα1-3Gal*f*- and Galα1- 6Galα1-3Gal*f*-, respectively (McConville et al., 1990). However, it is intriguing that although *L. tropica* patients show high levels of anti-α-Gal antibodies, the same species is reported to express only mannosylated type-1 GIPLs, which contain no terminal α-Gal residues (Schneider et al., 1994).

It is possible that this particular isolate does not express α-galactosylated GIPLs or, alternatively, that terminal α-Gal epitopes are present on glycoproteins. Moreover, it remains to be determined why cured individuals showed even higher expression levels of anti-α-Gal antibodies compared with patients with active CL, although it is possible that complete parasite clearance takes several years or is never accomplished. However, it may be possible that upon lysis of infected cells following chemotherapy, more α-Gal epitopes on intracellular amastigotes could be exposed to the host immune system, leading to the activation of new or existing B-cell clones, resulting in a considerable increase in anti-α-Gal antibody titres. However, upon elimination of the glycan stimulus, the titres of anti-α-Gal antibodies should eventually decrease considerably in cured CL patients, as previously observed in patients with chronic Chagas disease subjected to chemotherapy (Andrade et al., 2004, de Andrade et al., 1996).

Leishmania parasite and host interactions vary according to the *Leishmania* species. Higher expression levels of total IgG (IgG1-3) have been reported in localized CL infections in Latin America (Rodriguez et al., 1996). Moreover, IgG1

has been reported to be highly expressed in patients with mucocutaneous leishmaniasis, whereas IgG4 levels increase with diffuse CL (Rodriguez et al., 1996). Moreover, significant expression of IgG 1, 2 and 4 has been shown among patients with diffuse CL (Ulrich et al., 1995). An increase in IgG4 levels correlates with a Th2 response. IgG4 expression is reported to increase in the individuals with multiple CL lesions (Ulrich et al., 1995, Rodriguez et al., 1996). Th1 activity, on the other hand, is correlated with an increase in IgG1, IgG2 and IgG3 isotypes in people infected with either mucocutaneous or localized CL in Latin America (Rodriguez et al., 1996). However, mixed Th1 and Th2 activities have been reported in people infected with mucocutaneous leishmaniasis (Rodriguez et al., 1996). High IgG1 levels have also been reported among Old World CL patients in Turkey (Ozbilge et al., 2006). Furthermore, slightly higher levels of expression of IgG2 and IgG3 have been reported in patients with Old World CL compared with healthy individuals in the same CL endemic regions (Ozbilge et al., 2006).

In this work, *L. major* patients were shown to have high levels of anti-α-Gal IgG1 and anti-α-Gal IgG3 antibodies during an active leishmaniasis infection. These results could be interpreted as IgG 1 and IgG3 resulting from the Th1 activation, as described by (Rodriguez et al., 1996) as well as secretion of cytokines IL-4 and IL-10 (Brelaz-de-Castro et al., 2012). In contrast, the expression anti-α-Gal IgG1, IgG2 and IgG3 subtypes was higher in *L. tropica* infected patients. These findings may correlate with a Th1 response, which is proposed to have the main role in *L.*

tropica infection. It would be interesting to measure the pattern of cytokines of patients of these patients in relation to the expression of anti-α-Gal antibodies.

Regarding the anti-α-Gal IgG subtypes in cured individuals (previously infected with *L. major*) a switch in IgG2 to IgG4 expression was significantly higher in 16 out of 25 individuals. IgG2 has been recently identified as involved in anti-carbohydrate responses (Vidarsson et al., 2014), which matches its specificity for a sugar antigen . Moreover, an increase in IgG4 expression appears to correlate with Th2 activation, and thus IFN-γ and TNF-α expression (Cardoso et al., 2015). In fact, *L. major* patients show high levels of total IgG4, which could come from the multiple lesions, although this requires further investigation (Rodriguez et al., 1996). This is in contrast with the obseration in individuals clinically cured from a *L. tropica* infection, which showed very low levels of expression of all four IgG isotypes compared with patients with active *L. tropica* infections (Figure. 5.4). A bigger cohort of patients infected with *L. tropica* and also cured (in addition to healthy individuals from *L. tropica* endemic areas) is needed to further corroborate these findings.

Higher levels of IgG expression in CL cured (i.e. undergoing re-epithelisation, crusting or scar formation) has been previously reported in Sudan in patients cured of *L. major* infection (Abushama et al., 2014). Here, significantly higher levels of anti-α-Gal IgG expression in cured individuals was reported, even after months of healing. Based on the investigations of this chapter, it was rare to

identify cases of recurrent infection of leishmaniasis, particularly in *L. major* patients after healing, with the minor exception of diabetic patients. Whether this kind of protection is related with a persistent expression of anti-α-Gal antibodies expression levels.

Prior to this study, no obvious biomarker to differentiate individuals with an active *Leishmania* infection compared to cured ones has been identified. Therefore, an increase in the levels of anti α-Gal could be exploited as a potential biomarker for CL cure. Further studies are required to address issues such as false positives (as shown in Table 5.1), possibly caused by the presence of some other diseases. For instance, an increase in the expression of anti-α-Gal IgG antibodies can be found in some autoimmune diseases like rheumatoid arthritis (Malaise *et al*, 1986), Henoch-Schonlein (Davin *et al*, 1987), autoimmune thyroiditis and thyroid-associated ophthalmopathy (TAO) (Winand et al, 1995), and Graves' disease (Galili, 1999). Alternatively, an alteration in gut microbiota during a leishmaniasis infection (or even as result of the anti-leishmanial drug treatment), may also lead to an increase in the level of anti-α-Gal as reported individuals with either Crohn's disease or ulcerative colitis (D'Alessandro *et al*, 2002). Moreover, gut microbiota species such as *Salmonella minnesota*, *S. typhimurium*, *S. milwaukee*, *Klebsiella* genus and *E. coli* can be involved in this alteration. Early invasive cervical carcinoma and squamous cell carcinoma are involved in the elevation of α-gal IgG expression. Additionally, wounds and burns may contribute to an increase in

α-gal IgG expression (Towbin *et al*, 1987; Farzan *et al*, 2013; Iannacone *et al*, 2012, Knobel *et al*; 1999; 1997; D'Alessandro *et al*, 2002).

According to the Saudi Leishmaniasis Programme, CL diagnosis relies upon well-trained dermatologists. In this chapter, it was confirmed that microscopy is not the best confirmation test to use for diagnosis of CL. The recommendation from this study was for molecular tools to be used that could be easily implemented in the KSA. All confirmed cases received treatment as recommended by the Ministry of Health, which focuses on the elimination of secondary infections by both antibiotic and antifungal treatment, followed by one or two courses of sodium stibogluconate. The treatment costs are around 200 $US per patient. Disease diagnosis requires further expense.

Diagnostic tools have been found to be effective in curtailing the spread of neglected tropical disease (NTDs) when used in parallel with other interventions (Pelletreau *et al*, 2011). CL elimination requires a range of interventions, which include vector control, reservoir control, improving treatment policy, active and passive case detection, health education and interaction with all governmental sectors. KSA is no longer a highly CL endemic country as it used to be three decades ago, and the massive reduction in total CL cases is remarkable. However, further reduction of CL cases is necessary and will require implementation of cheap and reliable diagnostic tools and preferentially as rapid diagnostic tests (RDTs). Some RDT models have been recently applied for VL,

but so far no RDTs for CL diagnosis have been developed and introduced in the field (Cunningham et al, 2012).

Chapter 6: General discussions and conclusions

CL is currently widespread throughout all Saudi regions. A range of recent human-made factors such as migration, irrigation and urbanization have combined with prevailing environmental and climatic factors (both of which have also been exacerbated by human activity) to compound the problem of disease spreading. The correlation of these factors with CL transmission is certain, because the disease cycle depends upon mammalian animals and sand fly vectors. Despite concerted efforts to combat CL, the disease has been reported in new foci throughout KSA as a consequence of the lack of regular surveillance; the absence of active case detection, reliance on passive case detection has proven to be a very weak system. Additionally, the general lack of research into this area has created an absence of disease control strategy, vector control, available treatment, and reservoir studies, as well as prevented the development of a better national health system.

CL outbreaks have also been reported recently in neighboring countries including Iraq, Lebanon, Jordan, Syria and Yemen (Jacobson, 2011, Sharara and Kanj, 2014). Moreover, outbreaks were reported across a range of Saudi Arabian regions. Approximately, 200 cases have been reported at one construction site alone in the Eastern region of Saudi Arabia (personal observation, 2015). Given the lack of an active reporting system, it is likely that the current total of reported cases does not represent the reality of the CL situation in this country. Therefore,

it is essential that an improved CL programme be established in Saudi Arabia to resolve all problems related to treatment, disease diagnosis, the reporting system and disease control.

The development of an active reporting system is particularly urgent, with a view of identifying current unknown cases and thus creating a more accurate figure to determine the size and nature of the problem. This will entail allocating an appropriate budget for the CL programme in order to recruit seasonal workers, epidemiologists, medical statisticians, nurses and laboratory specialists. This recruitment could also help to minimize outbreak issues and to resolve cases by referring them to nearest leishmaniasis clinic, if there is one nearby, or to a primary health care centre.

Diagnostic tools are important for both active and passive case detection. The development of such tools, as described in chapter 5, will be a vital component of an effective strategy. Currently, CL diagnosis in most Saudi regions is undertaken by well-trained dermatologists. Microscopic diagnosis is conducted in just four regions: Hail, Asir, Al-Ahsa and Al-Madinah. Furthermore, 68% sensitivity for *L. major* cases and 45% for *L. tropica* cases represents very low sensitivity and thus is not very reliable. Molecular tools could be used to confirm clinical diagnosis using lesion swabs as described (Adams et al., 2014). Skin aspiration is another method used to perform for disease diagnosis as described in Chapter 5 (Al-

Salem et al., 2014). The new tools that I have developed to diagnose leishmaniasis in Saudi Arabia using synthetic neoglycoproteins and measured by chemiluminescent ELISA offer very promising results, with 91% and 92% sensitivity for *L. major* and *L. tropica* respectively. However, these tools still need to be standardized in blind clinical trials in endemic CL areas.

Developing a CL diagnostic kit will be an important step forward, enabling active surveillance in the field, given that all current methods require a suitably equipped laboratory. The reporting system and diagnostic tools are correlated. Moreover, without the creation of new diagnostic tools then active case detection will not be possible; the creation of such a toolkit would be a starting point for active case detection, similar to the advancements made with VL. Indeed, rK39 which is used as a diagnostic test for VL diagnosis can be used in the field and something similar should be implemented for the detection of CL.

Another problem that requires attention is the treatment protocol currently applied in Saudi Arabia. In this thesis, I clearly report that the efficacy of the current anti-CL treatment protocol varied according to the *Leishmania* species and geographical location (Chapter 2). For instance, *L. major* infections reported in the Central, Northwest and East regions of the country, are likely to be highly sensitive to SSG treatment. However, in the Southwest and some Northwest regions where *L. tropica* is found, most cases were unresponsive to the same

drug treatment. These results suggest that a new treatment protocol should be developed after a trial program has been undertaken. Treatment failure has been reported for SSG (Llanos-Cuentas et al., 2008b). Instead of using unpleasant treatments such as SSG, topical creams such as paromomycin could be tested, as it has been reported to efficiently cure *L. major* infections in Tunis (Ben Salah et al., 2013). It remains to be determined whether this drug will also be effective against *L. tropica* infections.

Disease control strategies require a tried and tested treatment, very sensitive diagnostic tools and an efficient case reporting system in order to achieve the best available interventions at the right time. These interventions are also required to determine the parasite species, sandfly vector and reservoir in the area of the disease, so as to determine the best intervention to be implemented. The emergence and re-emergence of the disease usually occurs in the absence of reservoir screening, and these diseases spread rapidly as a consequence of poor integration between governmental sectors. Nevertheless, such outbreaks could be prevented if the government establishes a health warning system and if intervention is introduced at an earlier stage, with a view to preventing such outbreaks. Alternatively a National Coordinating body should be established with authority and with sufficient resources to ensure that the respective Ministerial roles are fulfilled in a timely way in response to epidemics, as well as to ensure ongoing surveillance. Remote as the West African Ebola problem is from KSA,

the lessons learned – in terms of the need to deploy resources rapidly to tackle the potential emergence of CL – are clear.

As mentioned in Chapter 4, ZCL control is regulated by the Ministries of Health and Agriculture and Municipality, and this has proved a massive success in reducing cases, as rodents are the disease reservoir in Al-Ahsa and the control cover of both the sandfly vector and the reservoir is combined with attention to the treatment of patients. However, the lack of disease reservoir studies in some Saudi provinces and shortage of experts in this field precludes effective disease control measures from being deployed. Moreover, governmental sector integration is present in only a few provinces of KSA. Therefore, the suggested integration of the sectors could help organise and develop effective leishmaniasis control measures.

The current situation of the disease expansion, with outbreaks in Middle Eastern and North African countries, requires a control model that could be applied first in peaceful countries such as Saudi Arabia and then implemented in countries where there is conflict (Salam et al., 2014, Jacobson, 2011) (see appendix 1). A combination of successful vector and reservoir control, after parasite and sandfly species determination, should be conducted in endemic sites. Furthermore, the establishment of a very well designed surveillance system will lead to minimizing the CL problem in endemic areas as discussed in appendix 2.

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Appendix: Leishmaniasis and conflict in the Middle East

A.1 Background

Old World cutaneous leishmaniasis (CL) is one of the most prevalent vector borne diseases in the East Mediterranean Region (EMRO) (Aoun and Bouratbine, 2014a, Postigo, 2010, Salam et al., 2014, Alvar et al., 2012a, Jacobson, 2011). Zoonotic CL, caused by *Leishmania major,* and anthroponotic CL, caused by *L. tropica,* have been reported across the region, including Saudi Arabia, Iran, Jordan, Syria, Egypt, Lebanon, Iraq and Israel (Alvar et al., 2012a, Jacobson, 2011). A diversity of disease reservoirs have been implicated in the zoonotic transmission cycles, including *Psammomys obesus*, *Meriones libycus*, *Meriones shawi* and *Rhombomys opimus* (2010). While *Phlebotomus papatasi* has been incriminated as the main vector for ZCL in the Middle East, *L. tropica* is typically transmitted between humans by *Ph. sergenti* (Postigo, 2010, Salam et al., 2014).

In total, more than 100 000 CL cases have been reported in the last two years in Iraq, Syria, Lebanon and Yemen (Saroufim et al., 2014, Alasaad, 2013, Hotez et al., 2012, Koltas et al., 2014, Sharara and Kanj, 2014, Jacobson, 2011). Civil unrest and armed conflict have triggered mass human displacements which are responsible for the current spread of CL to new foci as a consequence of the migrants' carrying the disease with them or moving to where a ZCL outbreak is occurring. Approximately 4.1 million Syrians have been forced to move within their country whilst a further 2.7 million have fled to nearby countries. Meanwhile,

routine leishmaniasis control activities have been halted, further exacerbating CL spread (Sharara and Kanj, 2014).

With increasing violence in Syria and counter-terrorist military activities in northern and western Iraq regions, combined with increasing numbers of refugees, the number of CL cases is on the rise. This is a consequence both of the unrest and also because of the lack of health impact assessments within new refugee and deployed troops camps. There continues to be significant underreporting in these areas both because the unrest is affecting routine surveillance and also because there are no specific systems in place in the camps to record this information. As a result, leishmaniasis cases have emerged in places where displaced people and the disease reservoir *Psammomys obesus* co-occur – such as in the arid and semi-arid regions of Jordan and Syria. *Phlebotomus papatasi* and *L. major* (Arbaji et al., 1993, Kamhawi et al., 1995, Jumaian et al., 1998, Mosleh et al., 2008, Saliba et al., 1993) have been reported in several areas close to the Al-Furat River, including Diyar Al-Zour (Theodor, 1929, Sukariah, 2012) in Syria and Al-Anbar (Shaker, 2011, Pringle, 1953) in Iraq, as well as on the Jordan-Syria border (Kamhawi et al., 1995), where the Zaatari refugee camp has been established. This is an area at high risk of cutaneous leishmaniasis, due to the presence of infected reservoir and vector species. Over the last two years, approximately 400 cases of leishmaniasis have been reported in Jordan, and a further 1400 cases were detected in Lebanon as a consequence of forced displacement (Saroufim et al., 2014). The refugee camp at Nizip in Turkey has also reported several hundred cases (Sharara and Kanj, 2014).

Ph. sergenti is the anthroponotic vector for *L. tropica,* and has been reported in several places in Iraq, Jordan, Syria and Lebanon (Alasaad, 2013, Alvar et al., 2012a, Hotez et al., 2012, Jalouk et al., 2007, Postigo, 2010, Salam et al., 2014, Theodor, 1929, Stoops et al., 2013, Pringle, 1953). The disease has also been reported in towns and cities on rivers and lakes, such as in the towns of central and northern Iraq, and also in Aleppo and Idlib in Syria, as well as in the mountains both of northern Jordan and of Lebanon (Knio et al., 2000, Salam et al., 2014, Sharara and Kanj, 2014, Theodor, 1929, Postigo, 2010, Jacobson, 2011). Aleppo and Idlib have each reported several thousand cases of leishmaniasis, probably as a consequence of rubbish accumulation and the building of new displaced camps close to areas where sandflies breed, such as open sewers. In addition, visceral leishmaniasis has been reported in Southern Iraq (Pigott et al., 2014), where *Phlebotomus alexandri* is a known vector of the disease (Pringle, 1953, Stoops et al., 2013, Coleman et al., 2009).

A.2 Materials and methods:

Previous literature searches (Pigott et al., 2014, Coleman et al., 2009, Hotez et al., 2012, Postigo, 2010, Pringle, 1953, Salam et al., 2014, Stoops et al., 2013, Haddad et al., 2003, Knio et al., 2000, Nuwayri-Salti et al., 1994, Nuwayri-Salti et al., 1998, Saroufim et al., 2014, Sharara and Kanj, 2014, Alasaad, 2013, Koltas et al., 2014, Theodor, 1929, Sukariah, 2012, Arbaji et al., 1993, Dweik et al., 2007, Jumaian et al., 1998, Kamhawi et al., 1995, Kamhawi et al., 1993, Mosleh et al.,

2008, Mosleh et al., 2009, Nimri et al., 2002, Saliba et al., 1993, Saliba et al., 1994, Saliba et al., 1988, Saliba et al., 2004, Saliba et al., 1997, Yuval, 1991) were supplemented by specific searches of literature in PubMed and Arabic literature published in Syrian and Iraqi journals. Geographical coordinates were recorded for the occurrence of one or more leishmaniasis cases or sandfly presence (Wan and Li, 1997, Tatem et al., 2004, DAAC, 2000, Trabucco, 2009). Similarly, reported infections in reservoir species were noted. Elevation, aridity and enhanced vegetation index layers were provided for comparison. The current research is tracking leishmaniasis species, areas of displacement and conflict using Software ArcGIS 10 (ESRI, Redlands CA) to map the distribution of *Leishmania* spp.

A.3 Results:

A.3.1: Visceral and cutaneous leishmaniasis distribution:

Both visceral and cutaneous leishmaniasis are highly distributed throughout East-Mediterranean regions. As shown in Figure A-1, CL is found in most of the inhabited areas. Obviously, Syria, Jordan, the Palestinian territories and Israel are at higher risk of the disease compared to Iraq. However, in the case of visceral leishmaniasis, Iraq reports higher risk than countries that are situated on the Mediterranean coast, as shown in Figure A-2. Occurrences of cutaneous and visceral leishmaniasis derived from (Pigott et al., 2014). Specific settlements reporting cases are identified as point occurrences; administrative units are highlighted where leishmaniasis cases are known to occur.

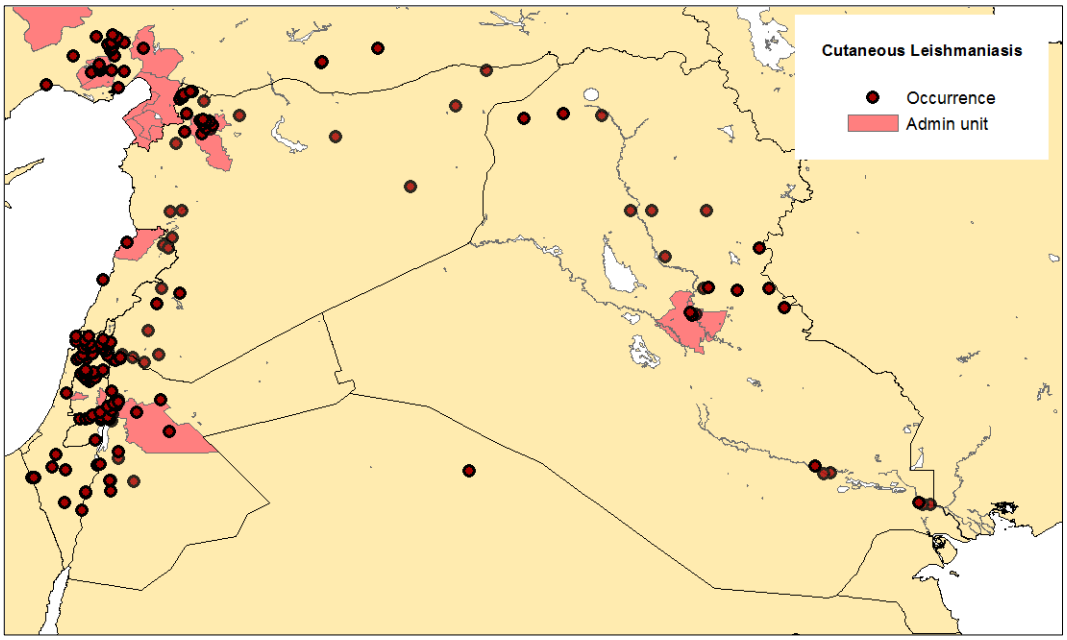

Figure A.1. Cutaneous leishmaniasis distribution across the countries of the East-Mediterranean regions; Iraq, Syria, Lebanon, Jordan, Palestinian territories, Israel and Sothern Turkey.

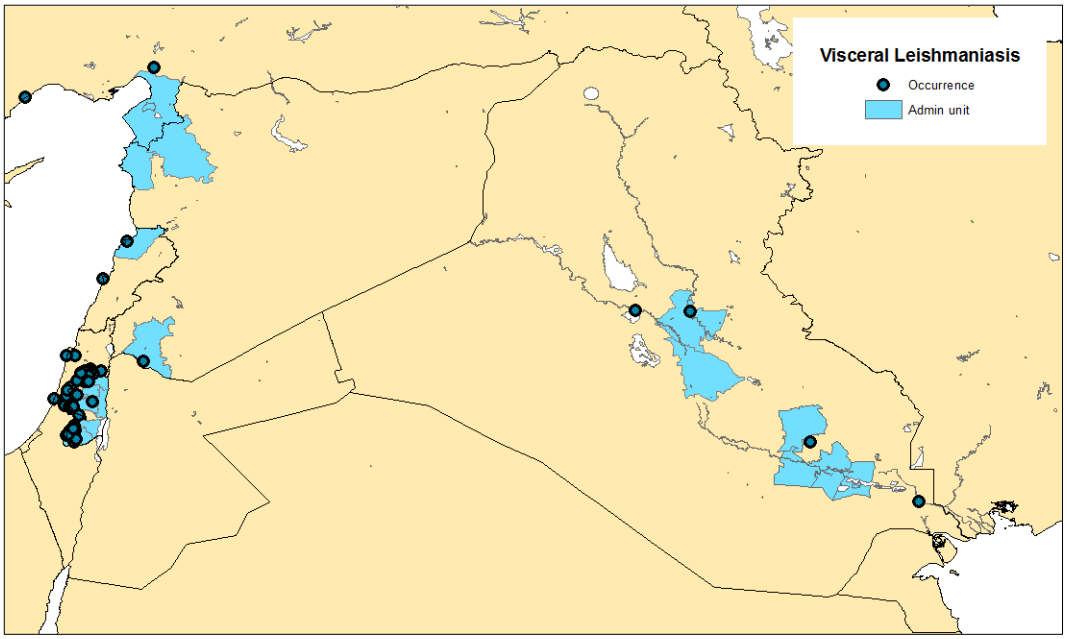

Figure A.2. Visceral leishmaniasis distribution across the countries of the East-Mediterranean regions; Iraq, Syria, Lebanon, Jordan, Palestinian territories, Israel and Sothern Turkey.

A.3.2 Disease reservoir distribution:

Psammomys obesus (Figure A-3) is reported in areas with low and medium vegetation and also in arid and semi-arid areas on the Jordan-Syrian border, and in the east of Syria around Al-Furat River, such as in the regions of Diyar Al-Zour (Sukariah, 2012, Theodor, 1929), Ar-Raqqah and Hasakah.

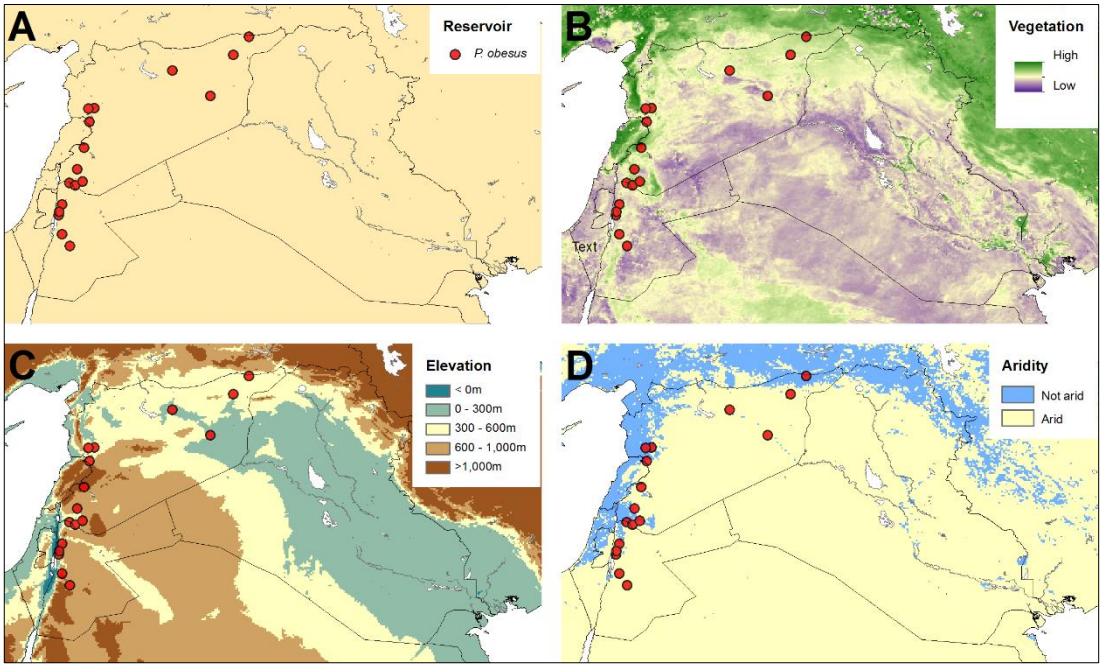

Figure A.3: ZCL disease reservoir A) shows the distribution of reported infection in *Psammomys obesus* rodents. **B)** Vegetation and disease reservoir **C)** Elevation and disease reservoir**. D)** Aridity and disease reservoir.

A.3.3 Sandfly distribution:

Sandfly distributions tend to have a different relationship with the environment (Figure A-4). *Ph. papatasi* is reported in Jordan-Syrian border and around Al-Furat Rivers in Ramadi Fallujah, Diyar Al-Zour, Ar-Raqqah and Hasakah and Northwest Iraq in Tikrit, Kirkuk, Diyala, Slahaddin and Kurdistan Iraq (Jabir A. Kadhim, 2014, Shaker, 2011, Stoops et al., 2013, Pringle, 1953). *Ph. sergenti* is related to high vegetation, water, higher altitude and with foci that include

Baghdad and Tikrit in Iraq, and Aleppo and Idlib in Northwest Syria (Theodor, 1929). It is also reported in the Lebanon Mountains (Haddad et al., 2003, Nuwayri-Salti et al., 1994, Nuwayri-Salti et al., 1998). *Ph. alexandri* has been reported in low altitude regions of Iraq as a visceral leishmaniasis vector (Pringle, 1953). However, *Ph. tobbi* is reported at high altitude and in areas of high vegetation such as the Lebanon mountains, where it can act as a vector for visceral leishmaniasis (Haddad et al., 2003).

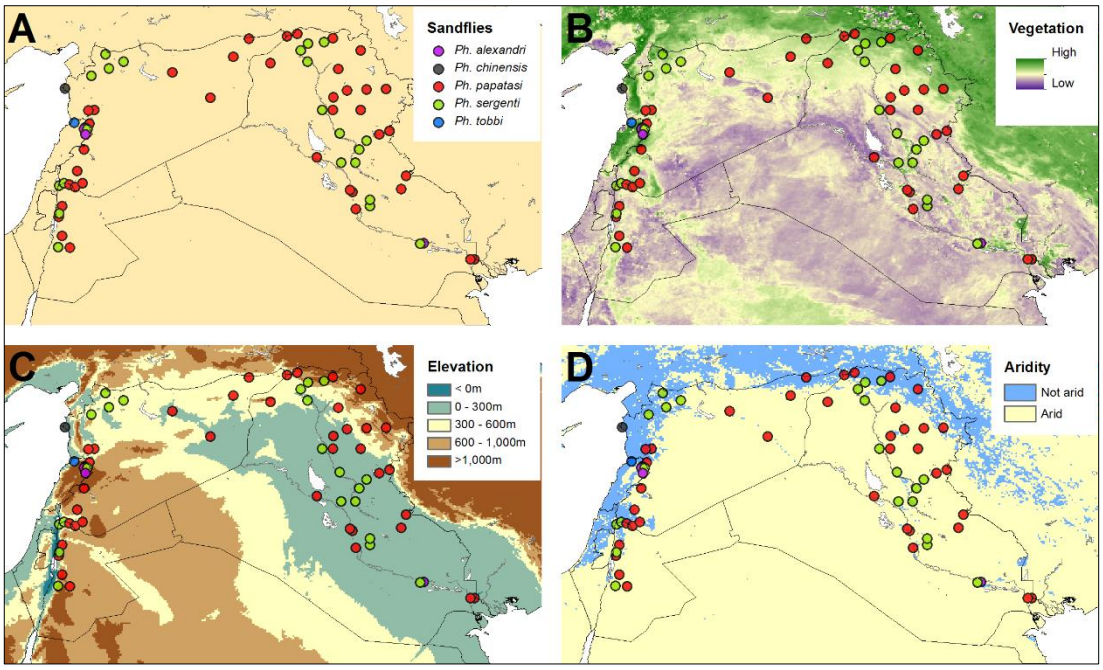

Figure A.4. A) Shows the distribution of reported sandflies, divided by species. **B)** Vegetation and sandflies **C)** Elevation and sandflies**. D)** Aridity and sandflies.

A.3.3 Parasite species distribution:

The distribution of *Leishmania* parasites is influenced by the sandfly vector and reservoir species. Therefore *L. tropica,* which is responsible for the anthroponotic cycle, is found with *Ph. sergenti* at high altitude, in cities and towns, high vegetation and water (Figure A-5). *L. major* is correlated with *Ph. papatasi* and is

found in low vegetation and semi-arid and arid areas. Thus the zoonotic cycle is always correlated with remote areas and the outskirts of towns. Visceral leishmaniasis is caused by both *L. donovani* and *L. infantum* and is transmitted by *Ph. alexandri* in Iraq and *Ph. tobbi* in Lebanon and onSyria's east coast (Theodor, 1929).

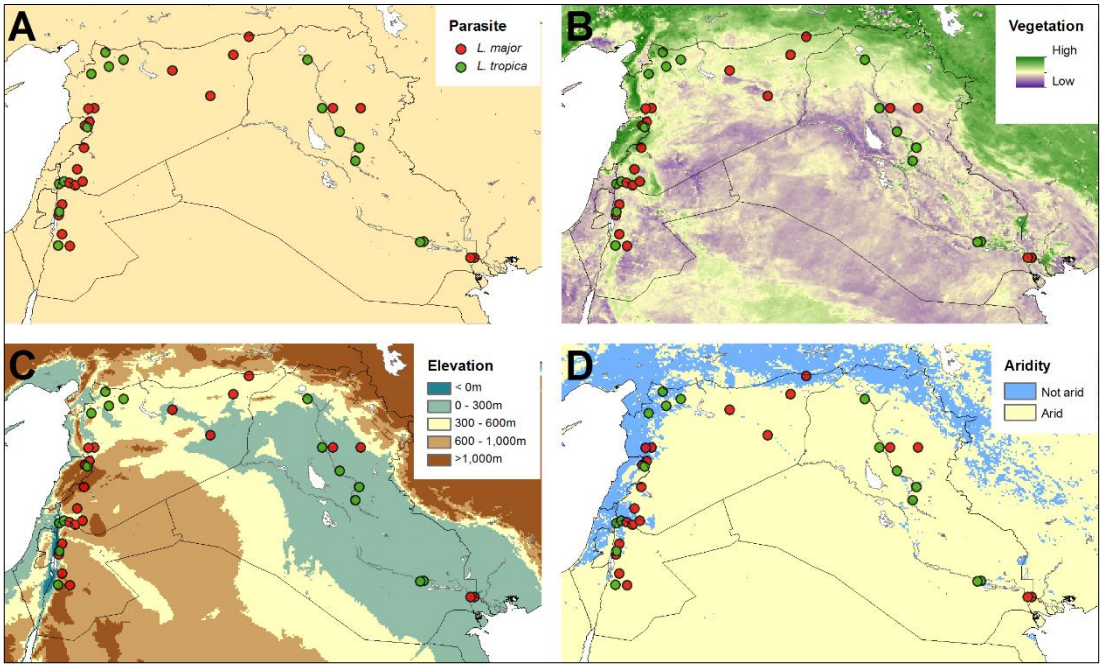

Figure A.5. A) Shows the distribution of *Leishmania* species causing cutaneous leishmaniasis. **B)** Vegetation and parasite species **C)** Elevation and parasite species**. D)** Aridity and parasite species.

A.4. Discussion and recommendations

These new foci of leishmaniasis follow the migration of susceptible individuals into areas of disease transmission as a consequence of civil war, or where there are deployed troops or urban expansion. The link between conflict and leishmaniasis has already been identified: following the civil war in South Sudan, 100 000 deaths occurred from visceral leishmaniasis outbreak (Zijlstra and el-Hassan, 2001, Postigo, 2010); 17% of British soldiers deployed in a military camp near Mazar-e-

Sharif contracted ZCL caused by *L. major* (Bailey et al., 2012, Jacobson, 2011) and approximately 2,500 foreign troops developed CL in Iraq after their tour of duty (Pages et al., 2010, van Thiel et al., 2011, van Thiel et al., 2010). Urban expansions such as those seen in Saudi Arabia and Israel have similarly experienced several outbreaks of cutaneous leishmaniasis (Faiman et al., 2013, Jumaian et al., 1998, Svobodova et al., 2006, Talmi-Frank et al., 2010, Vinitsky et al., 2010, Yuval, 1991, Al-Salem et al., 2014, Jacobson, 2011).

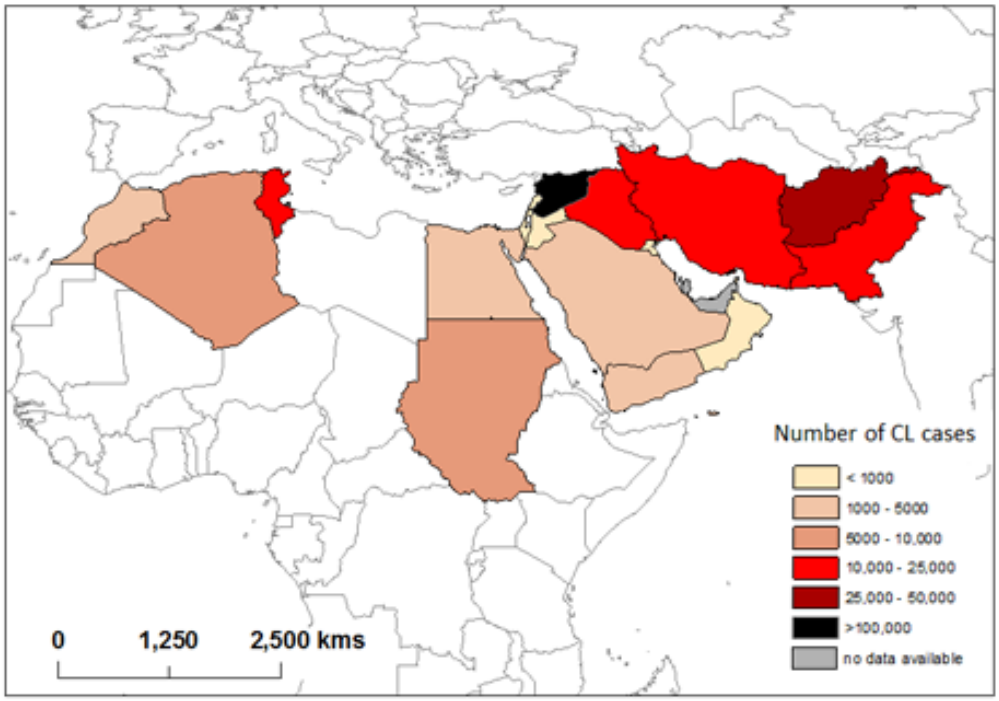

Figure A.6. Cutaneous leishmaniasis distribution in North Africa and Middle East

As is evident from the maps, leishmaniasis is highly endemic in all areas of conflict. Moreover, the leishmaniasis parasite, vectors and reservoirs are widely spread throughout Syria, Iraq, Lebanon and Jordan as shown in figure A.6 (Theodor, 1929, Pringle, 1953, Haddad et al., 2003, Jacobson, 2011). The forced displacement of new refugees to areas with poor healthcare infrastructure has

allowed leishmaniasis outbreaks to occur unchecked. High abundance of vectors and reservoirs, as well as the lack of immunity to leishmaniasis in these refugee populations, has exacerbated this situation. Therefore, prospective surveillance prior to mass movement of people should be conducted, and suitable locations should be selected for refugee camps, while avoiding open sewage and refuse build-up to prevent them from developing into vector breeding grounds, and thus to break the anthroponotic cycle that has been noted in areas such as the refugee camps at Southern Turkey and Aleppo (Koltas et al., 2014, Jacobson, 2011, Salam et al., 2014, Saroufim et al., 2014).

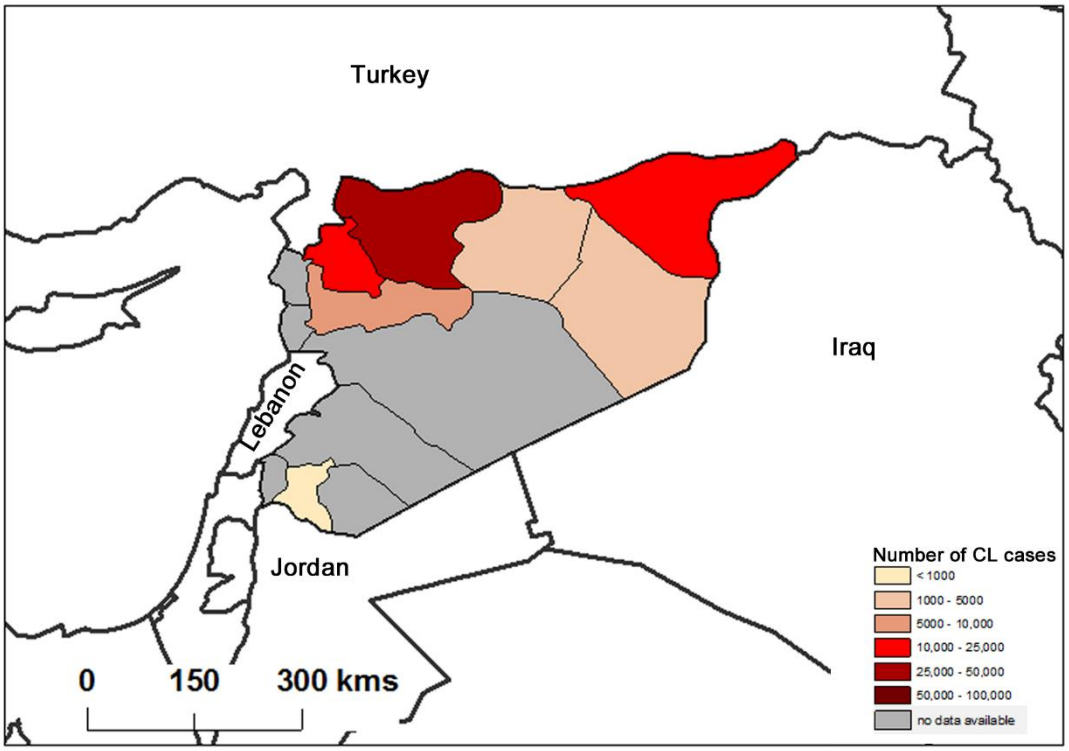

Figure A.7. Cutaneous leishmaniasis distribution across Syrian regions. Approximately 2000 CL cases were reported among Syrian refugees in Lebanon, and more than 2000 CL cases have been reported among Syrian refugees in Turkey. Also, 500 CL cases were reported among Syrian refugees in Jordan. Few cases were reported from Syrian refugees in Dahuk, Iraq.

Similarly, areas with high abundance of both vectors and reservoirs are especially vulnerable to zoonotic CL as in Zaatari in Jordan. When making provision for leishmaniasis treatment and control efforts, these newly at-risk populations must be considered as a priority. In addition, effective treatment should be provided to all leishmaniasis patients and effective control should be provided to minimise the disease impact. The maps above show the distribution of cutaneous and visceral leishmaniasis in Iraq, Syria, and neighbourhing countries where refugees have gathered in large numbers. Avoiding the build-up of refugees in these areas, following prior health impact assessment, could have a huge impact in minimising disease outbreaks.

Appendix 2: Endemic neglected vector-borne diseases in Saudi Arabia required revised control measures and research priority

A.2.1 Background

A number of neglected tropical diseases (NTDs) transmitted by vectors are currently is given the high levels of endemicity of these diseases, there continues to be a lack of integration between relevant governmental sectors in their approaches to combating diseases such as dengue, the leishmaniasis (both cutaneous and visceral) and alkhumra haemorrhagic fever. This situation prevails despite the fact that an unlimited budget has been allocated to the control of these diseases under programmes supervised by the Ministries of Health, Municipalities and Agriculture. There is an urgent need for a reappraisal of the current structures and strategies for the control and treatment of these diseases given KSA welcomes 12 million visitors, including 2.5 million Muslim pilgrims on the hajj, each year.

The role of reservoir hosts in cutaneous leishmaniasis (CL) has made elimination complicated, despite strenuous efforts by the Ministry of Health to tackle the disease over several decades (Ministry of Health report, 2014). Incidence of dengue haemorrhagic fever is correlated with low income populations and related socioeconomic factors. Breeding sites of the major vector of dengue *Aedes aegypti* are widespread in urban and periurban settings due to the number of water containers in the vicinity of low-income households and the absence of appropriate measures to dispose of breeding materials or the application of rigid policies to deter creation of human made breeding sites. Disease control

strategies for (Neglected vector-borne disease) NVBDs always face serious challenges and require the combined efforts of three sectors: health, municipalities and agriculture. Here we report on the current state of NVBDs in KSA and make recommendations as to how the situation can be improved.

Zoonotic cutaneous leishmaniasis (ZCL) accounts for approximately 75% of leishmaniasis human CL cases in KSA (Alsalem *et al*, submitted). ZCL is caused by *Leishmania major*, which has been reported in the provinces of Al-Qassim, Riyadh, Al-Ahsa, Hail, Almadinah Almunawarah and Tabuk (Elbihari et al., 1987, Killick-Kendrick et al., 1985, El-Badry et al., 2008, Al-Cindan et al., 1984, Al-Qurashi et al., 2000, Al-Salem et al., 2014, Büttiker and Lewis, 1979, Dye et al., 1989, Peters et al., 1985). The ZCL reservoir species *Meriones libycus* and *Psammomys obesus* are distributed in Central and Northwest provinces (el-Sibae and Eesa, 1993, el Sibae et al., 1993, El-Badry et al., 2008, Morsy and Shoura, 1976, Uthman et al., 2005) and in Al-Ahsa province (Al-Mohammed, 2010, Elbihari et al., 1987). The sandfly species *Phlebotomus papatasi* is a vector of ZCL throughout the Central, Eastern and Northern regions of KSA, and it transmits *L. major* (Morsy and al Seghayer, 1992, Elbihari et al., 1987) . ZCL control involves targeting rodents (the disease reservoir) by mechanical means, which requires considerable efforts by large teams of workers. Currently, only Al-Ahsa is applying this intervention by combining the efforts of the Ministries of Municipality, Agriculture and Health ministries, under the umbrella of the Governorate. Moreover, vector control using thermal fogging is applied at dawn and dusk in areas with a high density of sandflies in ZCL-endemic-regions

(Figure.A.2.1). The tasks are shared between the three Ministries, although responsibility for each task is not always clear. Consequently, ZCL outbreaks in several regions have gone unnoticed and unreported because of a lack of monitoring when cities are expanded by the Ministry for Municipalities, or when the Ministry of Agriculture introduces a new agricultural project. Additionally, the Ministries of Agriculture and Municipalities are not legally obliged to address both of anthroponotic and zoonotic CL outbreaks. However, anthroponotic CL caused by *Leishmania tropica,* is mainly found in the Western and Southwester provinces, including Jazan, Asir, Al-Baha, Al-Madinah and Taif. ACL is transmitted by *Phlebotomus sergenti* (Al-Salem et al., submitted), 2014; (Al-Zahrani et al., 1989a, al-Zahrani et al., 1989b, Morsy et al., 1992, Morsy et al., 1991). The removal of general waste and use of indoor residual spraying (IRS), specifically lambda-cyhalothrin, have been prioritised, with the aim of disrupting the anthroponotic cycle of CL in Taif. The target has been achieved by general waste elimination minimising the vector breeding sites and as a consequence there has been a 90% reduction of incidence of reported CL cases between 1990 and 2013. These interventions were accomplished by the Health and Municipality sectors (Al-Salem et al., in preparation & Chapter 4).

Figure.A.2.1. Zoonotic cutaneous leishmaniasis caused by *L. major* and transmitted by sandfly. Control strategy is suitable when *Psamommys obesus* is the reported as a disease resirvoir.

VL caused by both *L. donovani* and *L. infantum,* have been identified specifically in the Southwestern regions of Asir and Jazan. *Phlebotomus sergenti* is a possible vector for VL (Al-Orainey et al., 1994, Ibrahim et al., 1995, Ibrahim et al., 1992, al-Zahrani et al., 1988, Al-Salem et al., 2014). There has been a 96% case reduction in the past three decades. Use of IRS after sandfly surveillance has

been the main intervention to control VL. Moreover, ultra-low-volume (ULV) spray has also been introduced where VL cases have been identified. Additionally, long-lasting insecticide treated bed-nets (LLINs) are distributed where VL cases have been reported. Active case detection is employed using rapid diagnostic test (RDTs) in schools and villages with VL reported history report, and for the migrant population. Currently, VL cases are exclusively found in Samta, Alaydabi, Harooub, Al-Ardah and Al-Khoubah, with a few cases emerging in Taif in January 2014 (MoH report, 2014), although no further investigations were performed.

Additionally, dengue has recently been reported as endemic in Makkah and Jeddah in the KSA. New reports state that the foci have recently emerged in the provinces of Al-Madinah, Jazan and Asir (MoH report 2013). A recent outbreak of 4411 cases across the country was reported in 2013 (Aziz et al., 2014), highlighting the need for a reappraisal of the current control strategy to combat the disease. Disease control in KSA relies almost entirely on raising public awareness, under the auspices of the Ministry of Health, and chemical controls which are applied intensively by Municipalities using pyrethroids such as cyfluthrin, deltamethrin, lambdacyhalothrin and permethrin, which have been identified as effective chemical controls in KSA(Aziz et al., 2014). This complex control strategy involves both the Municipalities and Health sectors and requires reorganisation. The lack of vector control expertise and inappropriate strategies introduced by the Ministry of Municipalities has resulted in the heavy use of pyrethroids with potential for the development of resistance. The technical skills

to monitor insecticide resistance are not available whilst there is no consideration of an environmental, health education or legal enforcement approaches such water container removal or legislative action to reduce the number of breeding sites around households which would help to prevent the problem from growing.

Furthermore, the alkhurma haemorrhagic fever virus (AHFV) is transmitted by soft ticks (*Ornithodoros savignyi*) and hard ticks (*Hyalomma dromedarii*). Disease transmission can also occur mechanically through contact with infected blood and animals products (Figure.A.2.2). AHFV emerged in Jeddah in 1995, and subsequent cases were reported in Taif, Jazan, Makkah and Najran. The current AHFV disease control strategy relies on the efforts of the Ministries of Health and Agriculture. Suspected cases are admitted to the molecular diagnostic laboratory test to confirm AHFV infection. In addition epidemiological investigations are carried out in and around the suspected patient's home location. Once AHFV infection is confirmed, the Ministry of Health authority reports this to the agricultural authority to control tick populations.

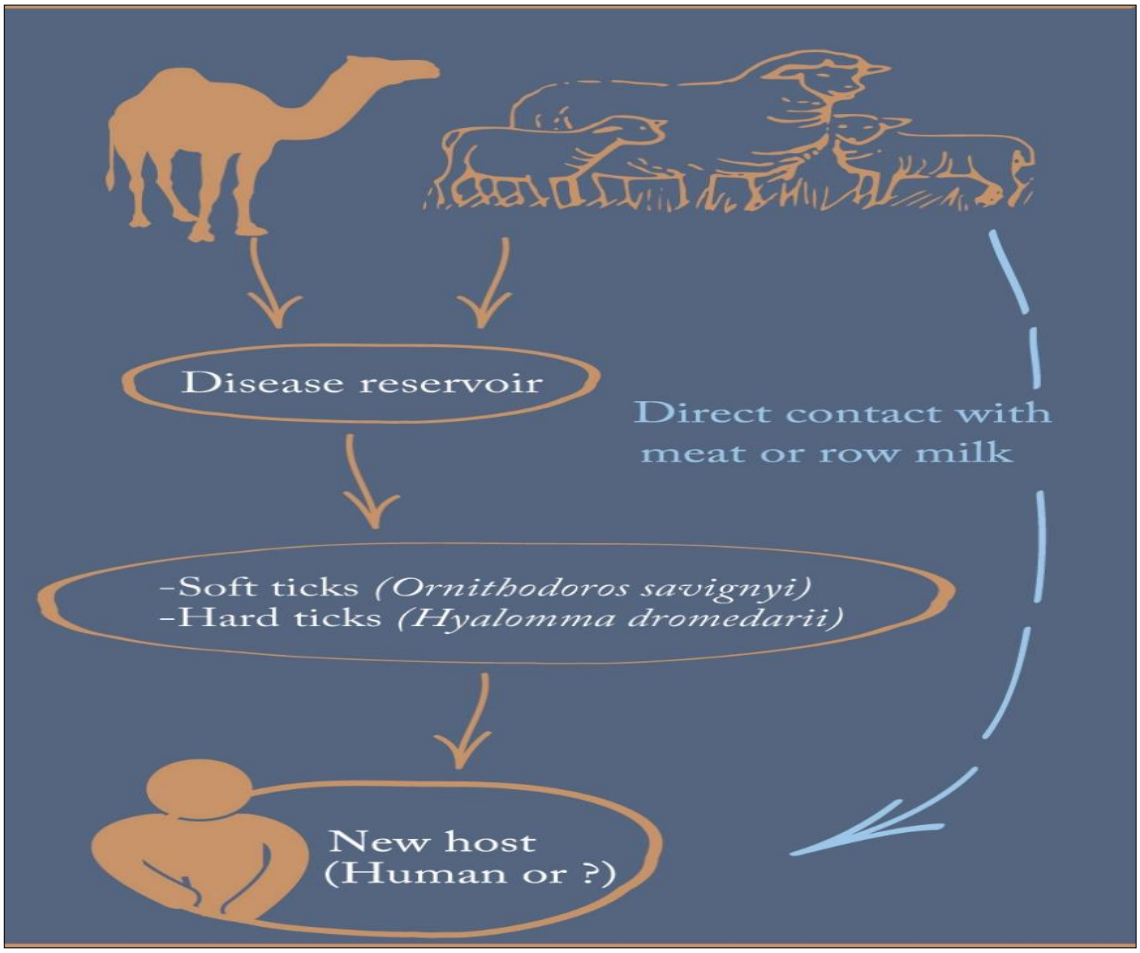

Figure A.2.2. Alkhurma haemorrhagic fever virus (AHFV) disease cycle in Saudi Arabia

Findings

According to the vector-borne disease and health cities units that are distributed throughout Saudi Arabia, NVBDs continue to present a huge challenge that calls for the active engagement of three governmental sectors: Health, Agriculture and Municipalities (Figure.A.2.3).

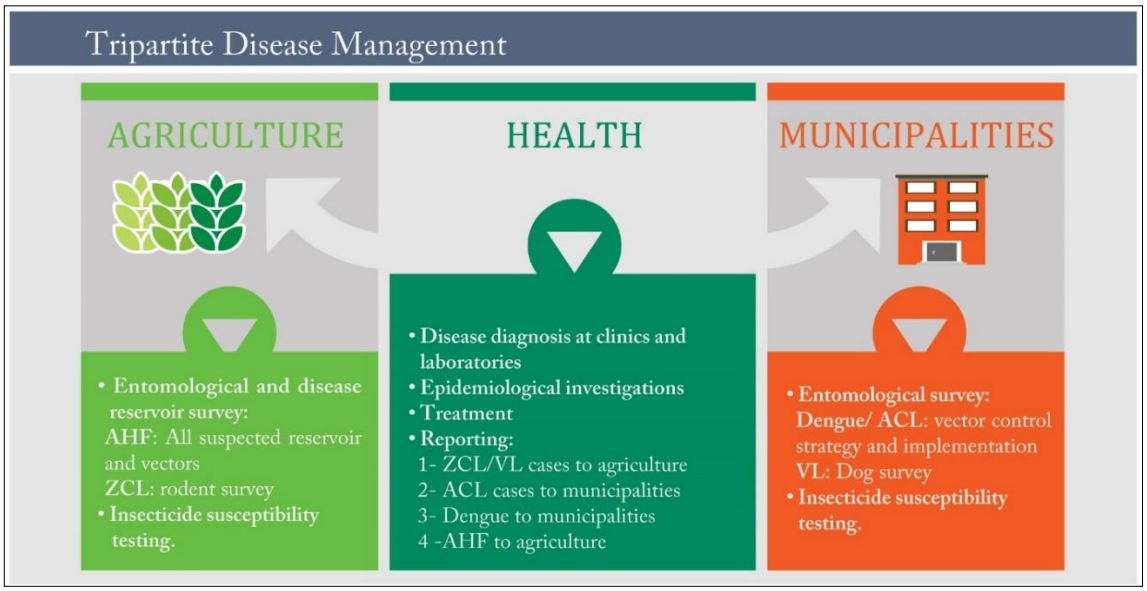

Figure A.2.3. Engagement of three governmental sectors: Health, Agriculture and Municipalities

There is a real need to establish a national protocol to combat NVBDs, which will cover elements such as establishing endemic disease centres to coordinate control strategies; identifying research priorities and developing a protocol for insecticide use; funding diagnostic and research laboratories; preparing a case reporting system and allocating financial support based on unit needs. For the current research, 32 health workers of vector-borne disease units and health cities programme were interviewed about the vector-borne diseases reported in their provinces. Vector borne diseases were reported in all provinces. Moreover, it became clear from the interviews that the direct and coordinated involvement of the ministries of Municipalities and Agriculture is a fundamental requirement for success in tackling such diseases. Accordingly, the questionnaire explores the required levels of integration between the sectors of Health, Municipalities and Agriculture within the provinces to combat NVBDs. VBD and health cities Unit

Heads and Deputies are particularly concerned about the collaboration of Health and Municipalities, particularly with regard to combating leishmaniasis through control of the sandfly vector in Taif, Al-Baha, Al-Ahsa, Hail, and Al-Madinah. Moreover, integration with Municipalities is required for dengue control in Jeddah, Makkah and Al-Madinah. In these districts, a lack of integration creates delays in dealing with the dengue source, and as a consequence the disease re-emerges. Most provincial officials believe either that there is no integration between the Health and Agriculture ministries, or that this integration is very poor. Alkhumra is a disease which requires more attention from the Ministry of Agriculture, since camels and sheep are involved in the disease cycle. Consequently, screening of the reservoir population should be undertaken regularly. However, the need for integration is particularly acute given the expansion of the disease from Jeddah to Najran, Makkah and Taif.

The elimination of *Hamada elegans* (a source of rodent food) from areas close to human residences would be an essential intervention to minimise the risk of zoonotic cutaneous leishmaniasis. Such an intervention is a task for the authorities responsible for the Agriculture sector. In regions where there is no integration with Agriculture, no such intervention will take place, and leaving the health sector to face the complicated task alone. The re-emergence of infections usually occurs in the absence of reservoir screening, and these diseases then spread rapidly as a consequence of poor integration between governmental sectors. Nevertheless, such outbreaks could be prevented if government

establishes a health warning system and if intervention is introduced at an earlier stage, with a view to preventing such outbreaks. Alternatively a National Coordinating body should be established with authority and resources to ensure the respective Ministerial roles are fulfilled in a timely way in response to epidemics but also to ensure ongoing surveillance. Remote as the Ebola problem is in West Africa are from KSA the lessons learned in the need to rapidly deploy resources to tackle potential emerging NVBDs is clear.

Discussion and recommendations:

We here attempt to assess the health system issue regarding sectoral integration between governmental bodies. As previously mention, ZCL control is regulated by the Ministries of Health and Agriculture as rodents are the disease reservoir. However, the lack of disease reservoir studies in some provinces and shortage of experts in this field precludes effective disease control measures being deployed. Moreover, governmental sector integration is present in only a few provinces of KSA. Therefore, suggested integration sectors are shown in Figure 3, which could organise and develop the leishmaniasis control measures.

The problem of dengue infection has expanded over recent years as a consequence of poor control measures applied at the country level and due to water supply difficulties. People with low income areas and areas with shortages of water supply are at the highest risk of dengue(Khormi and Kumar, 2011). Disease control engagement of some governmental sectors, such as the National

Water Company, is essential to minimise disease risk. Moreover, municipality experience regarding vector control is limited. As a consequence, heavy spraying of insecticide is implemented and no testing of insecticide susceptibility of vector populations is introduced. Moreover, vector surveillance after spraying is not performed. Thus, development of insecticide resistance among vector populations in KSA could occur. My suggestion is to reorganise the control structure by supervising dengue control through the Ministry of Health, as all the expertise to control dengue is found in this governmental body. Moreover, Ministry of Municipalities and the National Water Company should be responsible for supplying water and elimination of water containers which are habitats of immature *Aedes aegypti* populations.

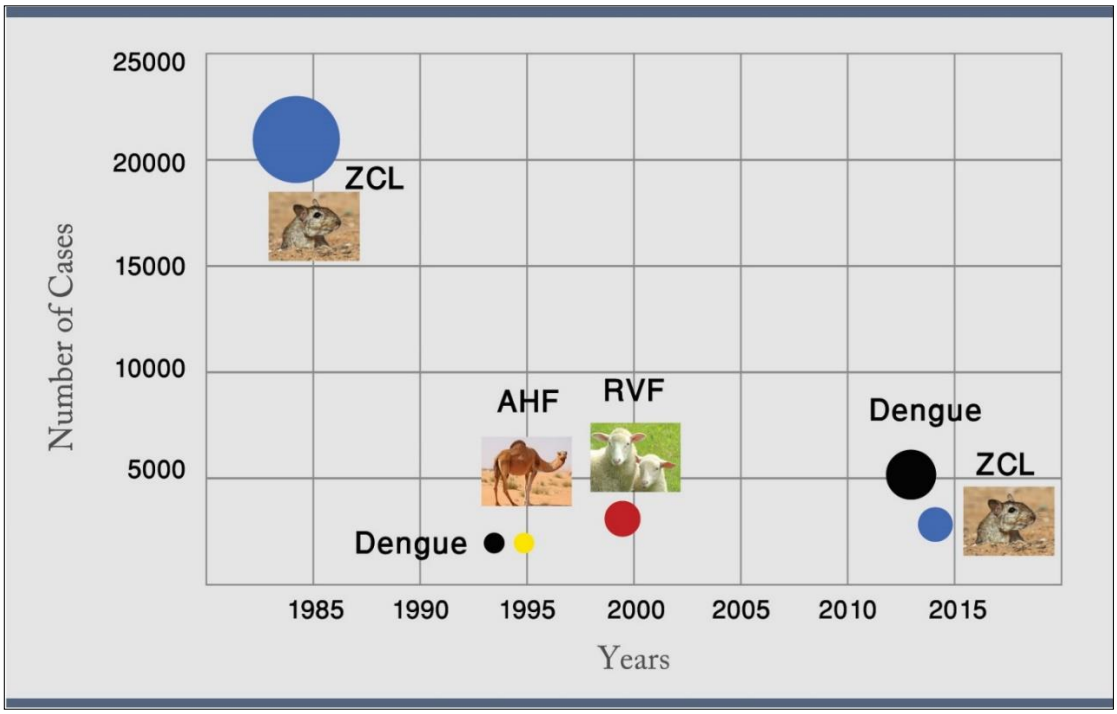

Figure A.2.4. Emerging and re-emerging of NTD's in Saudi Arabia time line. Between 20 and 25 thousands reported ZCL cases in 80's. However, less than 3000 cases were reported in 2014. First case of dengue was reported in 1994 and problem have been increasing to report more than 4400 cases in 2013. RFV outbreak reported between 1999 and 2000 in Southwest region and Yemen as first report in Arabian Peninsula. AHF emerged in Makkah Province in 1995 as hemorrhagic fever caused by both soft and hard tick and spread to South and Southwest regions.

Camel and sheep are found involved on AHF cycle and studies suggest that mechanical transmission through direct contact with meat and raw milk particularly with housewives and home maids. Additionally, soft and hard ticks have been reported as disease vectors (Figure A.2.2). Therefore, the Ministry of Agriculture should set up an initiative to screen the source of infection and the mode of transmission. Additionally, more clinical and entomological studies are required to understand nature of the disease.

National and regional laboratories are urgently required to assess transmission of NVBDs and of other zoonotic and vector-borne diseases. Moreover, implementation of disease research and management centres could have a huge impact as the unlimited budget is allocated to these diseases. However, poor achievements as a consequence of disorganised work have resulted in NVBD spread and sudden outbreaks. NVBDs are only an example of how the interaction between governmental sectors is crucial for any step forward. Moreover, several other neglected endemic diseases are facing similar control problems, including brucellosis, Middle East respiratory syndrome coronavirus (MERS-CoV), Rift Valley fever (RVF) and helminths infections (Figure.A.2.4).